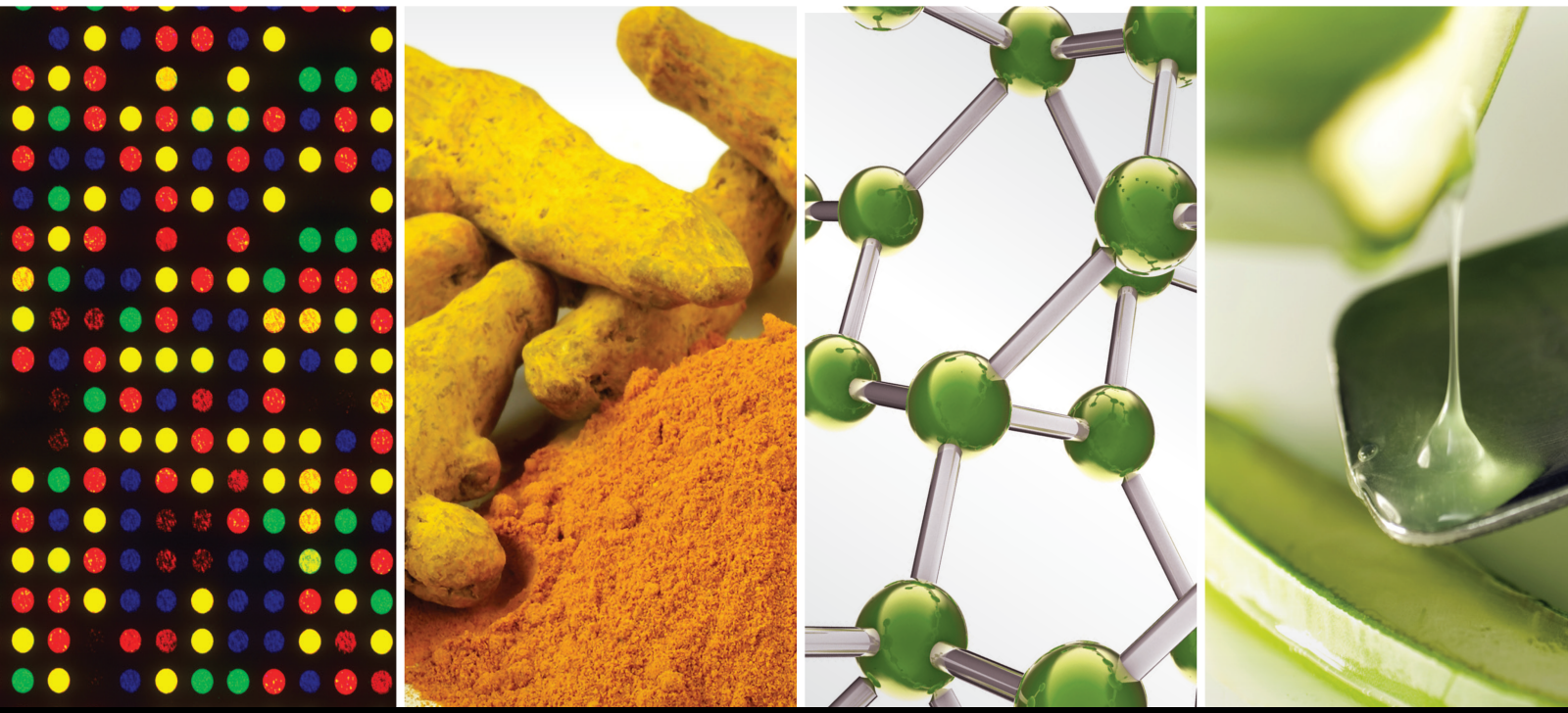
Evidence-Based Complementary and Alternative Medicine

Ethnopharmacological Studies for the Development of New Drugs 2019

Lead Guest Editor: José C. T. Carvalho
Guest Editors: Caio P. Fernandes, Jesus R. R. Amado, Andrés Navarrete, and Lucindo Q. Júnior



Ethnopharmacological Studies for the Development of New Drugs 2019

Evidence-Based Complementary and Alternative Medicine

Ethnopharmacological Studies for the Development of New Drugs 2019

Lead Guest Editor: José C. T. Carvalho
Guest Editors: Caio P. Fernandes, Jesus R. R. Amado, Andrés Navarrete, and Lucindo Q. Júnior

Chief Editor

Associate Editors

Academic Editors

Contents

Contents

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2020, Article ID 8463858, 23 pages
https://doi.org/10.1155/2020/8463858

Research Article

RNA-Seq Analysis of the Liver Transcriptome Reveals the Networks Regulating Treatment of Sitagliptin Phosphate plus Fuzhujiangtang Granule in the Zucker Diabetic Fatty Rats

Xuan Guo,[1,2] Wen Sun,[2,3] Guangyuan Xu,[4] Dan Hou,[2] Zhuo Zhang,[2] Lili Wu,[2,3] and Tonghua Liu[1]

[1]*Dongfang Hospital of Beijing University of Chinese Medicine, Beijing 100078, China*
[2]*Key Laboratory of Health Cultivation of the Ministry of Education, Beijing University of Chinese Medicine, Beijing 100029, China*
[3]*Beijing Key Laboratory of Health Cultivation, Beijing University of Chinese Medicine, Beijing 100029, China*
[4]*Department of Traditional Chinese Medicine, Fu Xing Hospital of Capital Medical University, Beijing 100045, China*

Correspondence should be addressed to Lili Wu; qingniao_566@163.com and Tonghua Liu; thliu@vip.163.com

Received 22 February 2019; Accepted 12 March 2020; Published 13 April 2020

Guest Editor: Caio P. Fernandes

Diabetes is one of the most serious chronic diseases. Numerous drugs including oral agents and traditional Chinese medicines, such as sitagliptin phosphate (SP) and Fuzhujiangtang granules (FJG), have been discovered to treat diabetes and used in combination in clinical practice. However, the exact effect and underlying mechanism of using combined medicine is not clear. In this study, we compared the antidiabetic effect of SP, FJG, and SP plus FJG (SP-FJG) using forty 8-week-old Zucker diabetic fatty (ZDF) rats and 10 age-matched Zucker lean rats as the normal control group. ZDF rats were treated with different therapies, respectively, for 6 weeks. The study showed that the fast blood glucose, random blood glucose (RBG), oral glucose tolerance test (OGTT), insulin tolerance test (ITT), homeostasis model of assessment-insulin resistance index, triglyceride (TC), superoxide dismutase, and malondialdehyde of each treatment group were improved when compared with the diabetes mellitus (DM) control group. Using SP-FJG in combination had better improvements in OGTT, fast serum insulin levels, TNF-α, and IL-6 compared with using SP individually. Besides, the increased LDL and TC caused by using SP was attenuated by using FJG in combination. Meanwhile, compared with the DM group, 1781 differentially expressed genes (DEGs) (including 1248 mRNA, 211 ncRNA, 202 cirRNA, and 120 miRNA) were enriched in 58 pathways. Through analysis of ceRNA networks, we found that *rno-miR-326-3p*, *rno-miR-423-5p*, *rno-miR-15b-5p*, *rno-let-7c-5p*, and *rno-let-7b-5p* were related to pharmacodynamics in different groups. By analyzing the protein-protein interaction (PPI) and coexpression networks of the transcriptomes of different groups, it is inferred that *Lrrk2* and *Irak3* may be pharmacodynamic genes for type 2 diabetes mellitus (T2DM). Our research compared the treatment of SP, FJG, and SP-FJG and acquainted the PPI network, coexpression network, mutations, and pharmacodynamics genes, which reveals the new mechanisms of pathogenesis of T2DM.

1. Introduction

Worldwide, the prevalence of chronic noncommunicable diseases is growing at a phenomenal rate [1]. A growing number of people get diabetes as a result of population growth, aging, urbanization, and increasing prevalence of obesity and lack of exercises [2]. Throughout the world, the number of people with diabetes is estimated to increase from 171 million in 2000 to 366 million by 2030. Furthermore, this increase will be most evidenced in developing countries, where the number of people with diabetes is expected to increase from 84 million to 228 million. Similarly, diabetes is becoming more and more serious in China, and the study of the prevalence of diabetes showed that the total proportion

of diabetes patients was 9.7% (10.6 percent for men and 8.8 percent for women, 50.2 million men and 42.2 million women) [3].

Now, some oral agents such as sulfonylureas, metformin, sitagliptin phosphate (SP), and injection of insulin are extensively used in the treatment of type 2 diabetes mellitus (T2DM). In the United States, five classes of oral agents (sulfonylureas, metformin, acarbose, troglitazone, and repaglinide) with different mechanism of action are currently available to improve glycemic control in patients with T2DM [4]. Besides, four classes of new agents are available on glycemic in T2DM, including the glucagon-like peptide-1 (GLP-1) analogue exenatide, dipeptidyl peptidase-4 (DPP-4) inhibitors sitagliptin and vildagliptin, and the long-acting insulin analogues, glargine and detemir [5]. In addition, many traditional Chinese medicines are also been reported to be used in the treatment of T2DM. Buddleia flower (Mi-Meng-Hua in Chinese) [6], Szechuan Lovage Rhizome (Chuan-Xiong in Chinese) [7], *Rehmannia* (Di-Huang in Chinese) [8], and *Coptis* (Huang-Lian in Chinese) [9] were studied that confirmed these herbs can be used alone or in conjunction with other herbs to enhance the therapeutic effects. Furthermore, the GC-TOF/MS analysis and sequencing technology were used to study the therapeutic action of MDG-1, a water-soluble β-d-fructan polysaccharide from *O. japonicus* [10], and Tangnaikang [11] in T2DM.

Numerous research studies of noninsulin-dependent diabetes mellitus have been described over the years [12, 13]. In the past several years, the Zucker diabetic fatty (ZDF) rat provides a model for human T2DM. The ZDF rat carries a spontaneous mutation in the leptin receptor (*fa* gene) which was originally derived from the Zucker fatty rat [14].

Recently, a certain amount of research studies were performed to explore the expression profiles of lncRNAs (noncoding RNA range from 200 nt to 100 kb) in different diseases besides T2DM which enriched the raw data in studying its primary functions [13–15]. The regulatory effect of lncRNAs is realized by a large complex network that involves mRNAs, miRNAs, and proteins rather than solitary [16]. Circular RNAs (circRNAs) is another group of non-coding RNAs that are widely distributed in animal cells. It is similar to lncRNAs, and studies showed the expression of circRNAs in different cell types with different parameters indicating its possible regulatory function [16, 17]. To date, there are few studies focusing on the role of lncRNA and circRNAs in treating T2DM with combination of Western medicine and traditional herbal medicine.

In the present study, we performed microarray analysis on the expression profiles of lncRNAs, mRNAs, circRNAs, and miRNAs using ZDF rats, a model of T2DM which was given different treatment. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were done based on the function of mRNAs that their expression levels changed with lncRNAs's expression in a positive or negative correlation. The coexpression network was constructed according to the sequencing results and bioinformatics predictions, which was to reflect the potential targeting relationship.

2. Materials and Methods

2.1. Animals. Eight-week-old ZDF rats were feeding rodent chow (Purina #5008, Harlan Teklad, Indianapolis, IN) for 4 weeks. 40 rats with blood glucose >11.1 mM were identified as successful diabetes mellitus models. A normal control group (NC) of age-matched Zucker lean control rats was also included in the study and fed a standard laboratory chow diet throughout the study. All rats were housed under controlled conditions (12 : 12-h light-dark cycle, 24°C, and 50% relative humidity) with free access to water and food according to a protocol approved by the Beijing University of Medicine Animal Care Committee.

2.2. Herbs and Reagents. Fuzhujiangtang granule (FJG) was made of *Momordica charantia* (Ku-Gua in Chinese), *Polygonatum odoratum* (Yu-Zhu in Chinese), *Morus alba L.*(Sang-Ye in Chinese), *Panax notoginseng* (San-Qi in Chinese), and *Cinnamomum cassia Presl* (Rou-Gui in Chinese) with a ratio 1 : 1.33 : 1.07 : 0.27 : 0.07. The *Momordica charantia* granule was produced by Tian Yi Biopharmaceutical Co. Ltd. Other granules were produced by Sun Ten pharmaceutical Co. Ltd. (purchased from the Dongfang Hospital of Beijing University of Chinese Medicine). The granules were mixed into deionized water and stored at 4°C before use. SP (MSD, USA) tablets were dissolved in the deionized water and stored at 4°C before use.

2.3. Experimental Design. 40 successful diabetes mellitus model rats were divided into the following groups and treated as indicated: diabetes mellitus group (DM) group (deionized water), SP group (SP [9 mg/kg·d^{-1}]), FJG group (FJG [0.64 g/kg.d^{-1}]), and SP-FJG group (SP [9 mg/kg·d^{-1}] and FJG [0.64 g/kg·d^{-1}]). Besides, the NC group was intragastrically administered with the same volume of deionized water. All treatments were given via oral gavage once a day, while the SP-FJG group was given FJG at 9am and SP at 9pm a day. The drugs were orally administered for 6 weeks in different groups.

2.4. Measurement of Body Weight, Blood Glucose, Oral Glucose Tolerance Test (OGTT), and Insulin Tolerance Test (ITT). The activity, diet, and body posture of rats were recorded. Weekly body weight monitoring, FBG (fasting blood glucose), and RBG (random blood glucose) were measured at 7am to 9 am.

OGTT was measured at the sixth week of administration, and the area under the curve (AUC) was calculated. The rats were fasted for 12 h, and 50% glucose solution was used for intragastric administration according to 2 g/kg criteria. Blood glucose value was measured with the intragastric administration before (0 min) and 30, 60, and 120 min after. The formula for AUG calculation for blood glucose (BG) levels observed during the OGTT is as follows: $\mathrm{AUC} = 0.5 \times (\mathrm{BG}\ 0\ \mathrm{min} + \mathrm{BG}\ \ 30\ \mathrm{min})/2 + 0.5 \times (\mathrm{BG}\ \ 30\ \mathrm{min} + \mathrm{BG}\ \ 60\ \mathrm{min})/2 + 1 \times (\mathrm{BG}\ 60\ \mathrm{min} + \mathrm{BG}\ 120\ \mathrm{min})/2$.

Rats were given the ITT test on the last day of 6 weeks of administration, and 2 U/kg insulin (Humulin R, Novo Nordisk, Denmark) was subcutaneously injected in rats, and hypoglycemic effect was observed at 0 min, 30 min, 60 min, and 120 min. Also AUC was calculated according to the above formula.

2.5. Blood Biochemical Index. The fast serum insulin levels (FINS, mIU/L) were measured with an enzyme-linked immune sorbent assay (ELISA) using a rat insulin ELISA kit (Alpco, USA). Tumor necrosis factor-α (TNF-α, pg/ml) and interleukin-6 (IL-6 pg/ml) were determined using ELISA kits for rat, respectively (SINO-UK Institute of Bio-Tech, China). High-density lipoprotein (HDL, mmol/L), low-density lipoprotein (LDL, mmol/L), total cholesterol (TC, mmol/L), and triglyceride (TG, mmol/L) were measured by specific kits (Zhong Sheng Bei Kong, China), and superoxide dismutase (SOD, U/ml) and malondialdehyde (MDA, nmol/ml) were analyzed by kits (Nanjing Jiancheng, China). All assays were performed according to the manufacturers' recommendations. Insulin sensitivity was assessed using the homeostasis model of assessment-insulin resistance index (HOMA-IR), which was calculated using the following equation: HOMA-IR = FBG (mmol/L) × FINS (mIU/L)/22.5.

2.6. Sample Preparation. Total RNA was extracted from the liver by the Trizol reagent (Invitrogen) separately. The RNA quality was checked by Bioanalyzer 2200 (Aligent) and kept at −80°C. The RNA with RNA integrity number (RIN) > 8.0 is right for rRNA depletion. The RNA with RIN >8.0 is right for miRNA purification. The miRNA was purified by miRNeasy Mini Kit (Qiagen), and the purification result was validated by gel electrophoresis.

2.7. cDNA Library Construction. The cDNA libraries were constructed for each pooled RNA sample using the VAHTSTM Total RNA-seq according to the manufacturer's instructions. The tagged cDNA libraries were pooled in equal ratio and used for 150 bp paired-end sequencing in a single lane of the Illumina HiSeqTM 2500 with 51 plus 7 cycles by NovelBio Corp. Laboratory, Shanghai.

2.8. miRNA Library Construction and RNA Sequencing. The complementary DNA (cDNA) libraries for single-end sequencing were prepared using Ion Total RNA-Seq Kit v2.0 (Life Technologies) according to the manufacturer's instructions. After enrichment, the mixed template-positive Ion PITM Ion SphereTM Particles of samples was loaded on to 1 P1v2 Proton Chip (Life Technologies) and sequenced on Proton Sequencers according to Ion PI Sequencing 200 Kit v2.0 (Life Technologies) by NovelBio Corp. Laboratory, Shanghai.

2.9. Prediction of circRNA. We use the special splicing form of circRNA in the expression process to forecast the sequencing reads and to find such a read: covering two exons and the direction is opposite to the linear RNA, that is, the possible circRNA in the sequencing sample is obtained.

2.10. Differential Expression Analysis. We analyzed the differentially expressed circRNA, mRNA, and miRNA based on the data obtained by sequencing. The different mRNA, circRNA, and miRNA were selected by an international universal differential screening algorithm EB. The difference screening criteria were fold change >1.5, fold change <0.667, and FDR < 0.05. Differential mRNA and differential ncRNA were identified according to the gene type annotation provided by NCBI. The rno-miRNA in the rat miRbase and the predicted miRNA were performed, respectively, to screen differential expression and subsequent analysis.

2.11. Prediction of Target Genes. The mature miRNA is composed of longer primary transcripts by a series of nucleases cleaved and then assembled into the RNA-induced silencing complex, by means of complementary base pairing to identify target genes and the degree of complementarity of different guiding silencing complex degradation of target mRNA or inhibit translation of the target mRNA. Based on the trend of mRNA, circRNA, and ncRNA, we used the internationally recognized miRNA target gene prediction algorithm Miranda to predict the negative correlation trend.

2.12. Series Cluster. We obtained the union of all differentially expressed RNA (including mRNA, circRNA, ncRNA, and lncRNA) of the DM group vs. NC group, treatment groups vs. NC group, and treatment groups vs. DM groups. Then, according to the measured signal value of sequencing to analyze the state trend on the set of RNA showed that the minimum correlation coefficient is 0.85. The significant state trend expression profiles of the set of RNA were identified and focused on the RNA associated with these trends. Drug-related expression trends is that the gene expression levels between the DM group vs. NC group and the treatment group vs. DM group show the opposite trend, and the expression level in the NC group is consistent with the treatment group.

2.13. Analysis of Gene Function. We analyzed the acquired genes by GO and pathway enrichment based on the DAVID database to obtain all GO and pathway of related genes. Fisher test was used to calculate the significant level of each GO and pathway (P value). The results of multiple hypothesis test are corrected, and the misjudgment rate (FDR) is obtained. If a test function value P <0.05, then the function of this gene is significantly enriched.

2.14. Construction of Functional Regulatory Network. GO-Tree is constructed based on the gene ontology directed acyclic graph to provide user friendly data navigation and visualization. We selected the significant GO-Term (P value <0.01) in GO Analysis based on the up and down differentially expressed genes to construct the GO-Tree to

summarize the function affected in the experiment. Taking the genes under the trend, we focused to do GO-Analysis, and the significant GO-Term (P value <0.05) is used as the research object to perform functional regulation analysis and construct a regulatory network. The picture deletes which part of the two terms has a hierarchical subordinate relationship and no relation to other term, but are not deleted in the list of relationships. Pathway analysis was used to find out the significant pathway of the differential genes according to the KEGG database. We turn to Fisher's exact test to select the significant pathway, and the threshold of significance was defined by$P$ value and FDR. We picked the genes in the enriched biological pathway and used Cytoscape for graphical representations of pathways.

2.15. ceRNA Network. miRNA can cause degradation and hinder the protein translation of mRNA and influence the function of important proteins by influencing the structure of mRNA and protein. Therefore, the changes in the expression of miRNA and mRNA should be negatively correlated. At the same time, miRNA will combine with circRNA and ncRNA, thereby affecting the regulatory role of the same miRNA on mRNA. Extracting miRNA that regulates both mRNA and circRNA simultaneously shows there is a positive correlation between the trend expression of mRNA and circRNA. Other ncRNA was analyzed as above. Select the mRNA contained in the significant entries of the GO pathway analysis based on the results of miRNA, and GO pathway significant genes are intersected. Based on the list of ceRNA relationships of intersection genes, circRNA-miRNA-mRNA and ncRNA-miRNA-mRNA network diagrams are drawn.

2.16. Weighted Gene Coexpression Network Analysis. Coexpression means that the expression patterns of two genes have high similarity in a set of samples. The basic mechanism of the coexpression network is to extract the signal value of each gene, calculate the Pearson correlation between two genes, and set threshold. When the Pearson correlation exceeds the threshold between the two genes, there is a coexpression relationship between the two genes. Finally, we construct a network with all coexpression genes, which is a coexpression network. The WGCNA algorithm is a typical system biology algorithm for constructing a gene coexpression network, which is based on high-throughput gene mRNA expression data and is widely used in the field of international biomedicine. Taking trend mRNA and ncRNA and trend mRNA and circRNA as the research object, the weight coexpression analysis was carried out using the WGCNA algorithm.

2.17. Statistical Analysis of Biological Experimental Results. Biological experimental results were performed using IBM SPSS statistics 20.0. ALL biological experimental results were presented as mean ± standard deviation (SD). Comparisons among groups were performed using one-way ANOVA. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Comparison of Body Weight, FBG, and RBG in SP, FJG, and SP-FJG Groups. As shown in Table 1, compared with the NC group, the body weight of all ZDF (fa/fa) rats was increased significantly since the first week ($P < 0.01$); compared with the DM group, body weight of the SP group significantly decreased at the fourth week ($P < 0.05$). The SP-FJG group body weight decreased significantly from the beginning of the fourth week ($P < 0.05$). It shows that the SP group and the SP-FJG group have the effect of reducing body weight.

Compared with the NC group, the FBG and RBG of the DM group increased significantly from the first week ($P < 0.01$). In comparison with the DM group, FBG decreased significantly from the beginning of the third week in the SP group (all $P < 0.01$) and RBG decreased significantly at the third, fifth, and sixth week ($P < 0.05$). As for the FJG group, FBG decreased at the beginning of the fourth week (all $P < 0.01$), while RBG decreased at the fourth, fifth, and sixth week ($P < 0.05$). In the SP-FJG group, FBG decreased significantly at the fourth, fifth, and sixth week ($P < 0.05$) and RBG decreased significantly from the beginning of the second week to the sixth week ($P < 0.05$). In addition, the sitagliptin phosphate (SP group) and combined medication (SP-FJG group) had significant effect of reducing FBG, and the effect of combined medication on reducing RBG was better than monotherapy (Table 1).

3.2. FJG Combined with SP Improved Glucose Tolerance and Insulin Tolerance Better than Individual Treatment. In the OGTT, the blood glucose values of DM rats compared with the NC group were significantly increased ($P < 0.01$), and the AUC was significantly increased ($P < 0.01$) as well. Compared with the DM group, the blood glucose values of all three treatment groups decreased at 0 min and 60 min ($P < 0.05$), and the value of blood glucose of 30 min and 120 min in the SP-FJG group was significantly decreased ($P < 0.05$). The AUC of all three treatment groups was decreased significantly ($P < 0.05$), while AUC of the SP-FJG group was significantly downregulated compared with the SP group. These data indicated that the effects of combination of drugs on improving glucose tolerance were better than that of the individual drugs (Table 2).

We performed ITT, and the blood glucose values and AUC of the DM group compared with the NC group were similar with OGTT experiments. Compared with the DM group, the blood glucose of SP, FJG, and SP-FJG groups was significantly decreased ($P < 0.05$) as well as AUC ($P < 0.01$). In the SP-FJG group, the blood glucose values of 30 min, 60 min, and 120 min and AUC were the lowest of all the three treatment, indicating that both SP as the monotherapy group (SP group) and the adjunctive therapy group (SP-FJG group) have the effect of increasing insulin sensitivity, but the combination of drugs was better (Table 2).

3.3. FJG Combined with SP Decreased FINS and Improved Insulin Sensitivity. Compared with the NC group, FINS and HOMA-IR in the DM group were notably increased at the

Table 1: Measure of body weight, FBG, and RBG of rats in NC, DM, SP, FJG, and SP-FJG groups.

	Group	1st week	2nd week	3rd week	4th week	5th week	6th week
Body weight (g)	NC	284.3 ± 11.82**	309.3 ± 44.57**	297.6 ± 11.78**	310.1 ± 10.76**	322.8 ± 9.93**	316.2 ± 9.66**
	DM	331.2 ± 18.09	352.4 ± 13.07	363.15 ± 23.32	375.13 ± 29.89	374.4 ± 29.08	378.6 ± 52.48
	SP	320.8 ± 16.6	342.37 ± 22.5	346.46 ± 14.33	344.4 ± 17.95*	357.7 ± 21.14	343.94 ± 25.17
	FJG	331.3 ± 18.05	337.8 ± 17.77	345.8 ± 19.28	353.1 ± 26.55	356.6 ± 22.71	342.8 ± 26.75
	SP-FJG	341.6 ± 21.60	353.1 ± 16.86	354.9 ± 22.03	346.64 ± 21.3*	344.5 ± 16.73*	324.09 ± 18.95**
FBG (mmol/L)	NC	3.71 ± 0.39**	3.59 ± 0.21**	3.43 ± 0.18**	3.41 ± 0.17**	4.03 ± 0.29**	3.7 ± 0.39**
	DM	4.68 ± 0.93	5.6 ± 0.99	7.79 ± 1.79	8.38 ± 2.12	11.53 ± 2.63	12.65 ± 3.31
	SP	4.64 ± 0.86	4.96 ± 0.86	5.68 ± 1.26**	5.36 ± 0.92**	5.43 ± 1**	7.31 ± 2.49**
	FJG	4.55 ± 0.69	5.11 ± 0.63	6.45 ± 1.73	5.47 ± 1.29**	6.12 ± 1.07**	6.9 ± 0.77**
	SP-FJG	4.7 ± 0.51	4.8 ± 0.72	6.69 ± 2.45	5.32 ± 1.19**	5.45 ± 1.27**	7.44 ± 1.12**
RBG (mmol/L)	NC	5.04 ± 0.26**	4.21 ± 0.23**	4.15 ± 0.24**	3.66 ± 0.14**	3.48 ± 0.27v	3.61 ± 0.12**
	DM	18.58 ± 3.24	20.07 ± 3.71	19.88 ± 2.8	22.25 ± 2.85	23.03 ± 4.85	24.84 ± 4.29
	SP	17.6 ± 4.75	17.08 ± 3.8	18.75 ± 3.36*	19.45 ± 3.54	17.06 ± 2.04**	18.4 ± 2.31**
	FJG	19.08 ± 4.55	16.87 ± 3.2	18.2 ± 3.56	18.07 ± 4.3*	17.08 ± 2.96**	19.27 ± 2.25**
	SP-FJG	18.44 ± 4.24	16.34 ± 3.76*	15.85 ± 4.17*	16.92 ± 2.1**	14.92 ± 4.9**	16.65 ± 3.18**

All values represent the means ± SD ($n = 10$). *Significant difference with the DM group designated as $P < 0.05$. **Significant difference with the DM group designated as $P < 0.01$. #Significant difference with the SP group designated as $P < 0.05$. ##Significant difference with the SP group designated as $P < 0.01$.

Table 2: Measure of OGTT and ITT of rats in NC, DM, SP, FJG, and SP-FJG groups.

	Group	Blood glucose (mmol/L)				AUC
		0 min	30 min	60 min	120 min	
OGTT	NC	3.72 ± 0.47**	6.24 ± 2**	4.46 ± 0.48**	3.42 ± 0.37**	9.11 ± 1.04**
	DM	11.24 ± 2.06	23.48 ± 3.37	27.04 ± 3.95	22.64 ± 5.73	46.15 ± 5.74
	SP	8.4 ± 1.08*	20.08 ± 1.63	21.56 ± 2.17*	17.68 ± 2.02	37.15 ± 1.54*
	FJG	7.84 ± 1.39*	21.1 ± 2.83	20.52 ± 2.15*	17.94 ± 4.29	36.87 ± 2.77*
	SP-FJG	7.94 ± 1.43*	17.98 ± 2.17*	19.04 ± 3.1*	14.62 ± 1.67*#	32.57 ± 3.15**#
ITT	NC	3.02 ± 0.17**	1.88 ± 0.44**	1.66 ± 0.41	2.04 ± 0.6	3.96 ± 0.45
	DM	16.7 ± 2.77	12.34 ± 1.69	8.64 ± 1.21	10.46 ± 1.81	22.06 ± 2.17
	SP	7.8 ± 0.66**	4.68 ± 0.61**	4.36 ± 0.85**	5.3 ± 0.87**	10.21 ± 1.02**
	FJG	10.1 ± 1.34**	7.08 ± 0.83**	6.26 ± 1.8*	6.08 ± 0.63**	13.8 ± 1.56**
	SP-FJG	9.76 ± 2.25**	3.88 ± 0.42**	4 ± 0.71**	4.38 ± 0.64**	9.57 ± 1.08**

All values represent the means ± SD ($n = 5$). *Significant difference with the DM group designated as $P < 0.05$. **Significant difference with the DM group designated as $P < 0.01$. #Significant difference with the SP group designated as $P < 0.05$. ##Significant difference with the SP group designated as $P < 0.01$.

sixth week ($P < 0.01$). Compared with DM, there was no significant change in FINS of SP and FJG groups ($P > 0.05$), while FINS in the SP-FJG group was dramatically lowered ($P < 0.01$). In addition, compared to DM, the HOMA-IR of the SP group decreased greatly ($P < 0.05$) and for FJG and SP-FJG groups ($P < 0.01$); the SP-FJG group was lower than the SP group but not significant (Table 3).

Table 3: FINS and HOMA-IR of rats in NC, DM, SP, FJG, and SP-FJG groups.

Group	FINS (uIU/ml)	HOMA-IR
NC	13.00 ± 0.40**	2.14 ± 0.25**
DM	21.48 ± 2.22	12.19 ± 4.47
SP	23.84 ± 5.62	7.98 ± 3.37*
FJG	24.21 ± 3.26	7.37 ± 1.21**
SP-FJG	17.32 ± 1.55**	5.69 ± 1.30**

All values represent the means ± SD ($n = 7$). *Significant difference with the DM group designated as $P < 0.05$. **Significant difference with the DM group designated as $P < 0.01$. #Significant difference with the SP group designated as $P < 0.05$. ##Significant difference with the SP group designated as $P < 0.01$.

3.4. Comparison of Serum Detection in SP, FJG, and SP-FJG Groups. Compared with the NC group, TNF-α and IL-6 of the DM group were significantly increased ($P < 0.01$). Compared with the DM group, only in the SP-FJG group TNF- α and IL-6 were significantly decreased ($P < 0.05$), indicating that SP-FJG combination therapy reduced the inflammatory state in rats better than that of monotherapy (Table 4).

Compared with the NC group, HDL was significantly decreased ($P < 0.01$) while LDL, TC, and TG raised significantly ($P < 0.01$) in the DM group; compared with the DM group, HDL, LDL, and TC of the SP group increased ($P < 0.05$) and TG decreased ($P < 0.01$). In the FJG group, TC and TG were decreased ($P < 0.05$), and LDL distinctly decreased. In the SP-FJG group, TG was significantly lowered ($P < 0.01$). Notably, the increased LDL and TC in the SP group were attenuated with the combination group, indicating that combination therapy had better effect in lipid metabolism than monotherapy (Table 4).

Compared with the NC group, SOD of the DM group significantly decreased while MDA increased significantly ($P < 0.01$). Compared to the DM group, SOD in the SP

Table 4: Measure of serum targets in NC, DM, SP, FJG, and SP-FJG groups.

Group	TNF-α (pg/ml)	IL-6 (pg/ml)	HDL (mmol/L)	LDL (mmol/L)	TC (mmol/L)	TG (mmol/L)	SOD (U/ml)	MDA (nmol/ml)
NC	$56.13 \pm 3.82^{**}$	$107.88 \pm 10.53^{**}$	$1.71 \pm 0.17^{**}$	$0.29 \pm 0.08^{**}$	$1.99 \pm 0.21^{**}$	$0.31 \pm 0.11^{**}$	$80.39 \pm 4.25^{**}$	$4.50 \pm 0.58^{**}$
DM	74.37 ± 3.79	151.73 ± 5.62	1.42 ± 0.24	1.58 ± 0.19	2.92 ± 0.29	2.86 ± 0.11	71.64 ± 2.73	6.91 ± 0.69
SP	75.58 ± 5.30	153.08 ± 9.63	$1.94 \pm 0.20^{**}$	$1.80 \pm 0.26^{*}$	$3.20 \pm 0.22^{*}$	$2.31 \pm 0.47^{**}$	$87.90 \pm 3.59^{**}$	$5.07 \pm 0.95^{**}$
FJG	78.89 ± 6.32	159.44 ± 17.62	1.44 ± 0.20	1.40 ± 0.27	$2.56 \pm 0.37^{**}$	$2.46 \pm 0.48^{*}$	$100.07 \pm 9.60^{**}$	$4.72 \pm 0.41^{**}$
SP-FJG	$69.01 \pm 7.18^{*\#}$	$133.02 \pm 17.92^{**\#\#}$	1.59 ± 0.12	1.44 ± 0.15	2.79 ± 0.13	$2.39 \pm 0.35^{**}$	$89.11 \pm 6.69^{**}$	$4.82 \pm 0.41^{**}$

All values represent the means ± SD ($n = 7$). *Significant difference with the DM group designated as $P < 0.05$. **Significant difference with the DM group designated as $P < 0.01$. #Significant difference with the SP group designated as $P < 0.05$. ##Significant difference with the SP group designated as $P < 0.01$.

group, the FJG group, and the SP-FJG group increased significantly while MDA decreased significantly ($P < 0.01$). It shows that combination therapy and individual treatments can improve the state of oxidative stress (Table 4).

3.5. Differential Expression Analysis in SP, FJG, and SP-FJG Groups. According to the expression of RNA, the differentially expressed RNAs were screened by the algorithm EB and conformed to the standard of $|\log 2(\text{Fold Change})| > 0.667$ and the FDR < 0.05. If $\log^2 \text{FC} > 0.667$, the RNA is up while $\log^2 \text{FC} < -0.667$ is down. In different cases, the number of differentially expressed RNAs is shown in Table 5. Correspondingly, 134, 1026, and 621 differentially expressed genes (DEGs) were detected in the liver for SP vs. DM, FJG vs. DM, and SP-FJG vs. DM, details are given in Table 5.

3.6. Expression Trend Analysis in SP, FJG, and SP-FJG Groups. We conducted a trend analysis of the differential expression of RNA in the SP group, the FJG group, and the SP-FJG group according to the RNA category (Figure 1). In view of the trend of expression, trend 2 and 5 are related to the effects of drugs. And then, we made a statistical analysis of the two trends of RNA, as shown in Figure 2.

Table 5: All the DEGs in different comparisons.

	mRNA		ncRNA		cirRNA		miRNA		Total
	UP	Down	Up	Down	Up	Down	Up	Down	
DM vs. NC	513	572	73	45	49	29	29	29	1339
SP vs. DM	41	21	7	2	19	30	1	13	134
SP vs. NC	435	463	80	29	24	29	39	36	1135
FJG vs. DM	516	259	102	23	28	41	27	30	1026
FJG vs. NC	1000	784	194	58	40	46	49	60	2231
SP-FJG vs. DM	269	142	58	19	32	52	23	26	621
SP-FJG vs. NC	732	621	164	34	43	45	50	48	1737

3.7. Gene Function Analysis of the Relationship between Pharmacodynamics Trend of RNA in SP, FJG, and SP-FJG Groups. Biological function of drug effect trend gene in different therapeutic effects was analyzed. Functional enrichment analysis of the genes in trend 2 and 5 was performed. The significant enrichment gene ontology terms in biological process (GO BP) and pathways in different treatments are shown in Figures 3(a) and 3(b), respectively. The trend genes of FJG are mainly associated with sulfation, cellular detoxification of nitrogen compound, etc. The efficacy genes of the SP group mainly focus on the biological process, for example, purine nucleotide biosynthetic process, innate immune response, and cellular response to jasmonic acid stimulus. The trend genes of the SP-FJG group were mainly involved in biological processes as flavonoid glucuronidation, xenobiotic glucuronidation, and cellular response to organic cyclic compound. Effect genes of both SP group and SP-FJG group regulate the process in response to nutrient. The biological processes of the fructose transport is regulated by both the SP-FJG drugs and FJG. According to the biological pathway of pharmacophore regulation, the pathway of TNF signaling, and hematopoietic cell lineage, the pharmacophores are regulated by both FJG and SP. The trend genes of the SP-FJG group and the FJG group both regulated the metabolism of xenobiotics by cytochrome P450, metabolic pathways, maturity onset diabetes of the young, drug metabolism cytochrome P450, and chemical carcinogenesis pathway. The pharmacophore of SP can specially regulate the toll-like receptor signaling pathway and other pathways. Pharmacophore of FJG specifically regulates alanine aspartate and glutamate metabolism. Apoptosis pathway, ascorbate and aldarate metabolism, and other pathways are regulated by the pharmacophore of the SP-FJG (Figure 3(b)).

Then, the GO BP and pathway functions of circRNA were enriched. The biological process enrichment (Figure 4(a)) analysis shows that circRNA efficacy trends of three administration are focused on vitamin transport response to nutrient control, oxidation-reduction, process, and long-chain fatty acid metabolic process (Figure 4(a)). From the enriched pathway (Figure 4(b)), the treatment groups are mainly involved in metabolic pathways, pathway

Class	FJG group				SP group				SP-FJG group			
	mRNA	ncRNA	circRNA	miRNA	mRNA	ncRNA	circRNA	miRNA	mRNA	ncRNA	circRNA	miRNA
	2e − 17	0.9	3e − 12	0.3	1.0	1.0	0.5	0.2	2e − 3	1.0	8e − 2	0.8
1	4e − 6	1.0	0.7	0.1	3e − 114	3e − 3	0.7	8e − 4	2e − 21	1.0	0.7	0.6
2	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0	1.0	1.0	1.0	0.9
3	1.0	1.0	5e − 2	1e − 2	1.0	1.0	0.5	0.9	1.0	1.0	0.5	0.1
4	4e − 3	2e − 10	0.8	0.3	1.0	0.6	1.0	1.0	0.9	6e − 12	0.5	0.3
5	1.0	1.0	0.2	1.0	1.0	1.0	8e − 3	1.0	1.0	1.0	4e − 2	1.0
6	2e − 18	3e − 5	0.5	0.2	1e − 91	1e − 9	0.1	2e − 2	2e − 29	7e − 4	0.1	0.6
7	3e − 28	1e − 16	0.9	0.2	4e − 3	1e − 9	0.9	7e − 4	3e − 31	1e − 18	0.9	1e − 5

Figure 1: Trend analysis of differential expression of RNA.

valine, *Staphylococcus aureus* infection, protein processing in endoplasmic reticulum, PPAR signaling pathway, etc.

3.8. Construction of Functional Regulatory Network in SP, FJG, and SP-FJG Groups. To study the function of regulatory networks of genes involved in different treatment, we constructed a pathway function control network (Figure 5).There are many common pathways in the FJG group and the SP-FJG group, which contain metabolic pathways, drug metabolism cytochrome P450, N-glycan biosynthesis, protein processing in the endoplasmic reticulum, and apoptosis. The apoptosis pathway affects pathway pertussis, herpes simplex infection, and toll-like receptor signaling of SP (Figure 5).

3.9. Construction of ceRNA Network in SP, FJG, and SP-FJG Groups. The miRNAs of different administration trends were obtained, and then the trends of miRNA negatively regulated ncRNA, circRNA, and mRNA were predicted. ceRNA networks of three different treatments were constructed. Then, the topological properties of the network were analyzed and the RNAs of top 5 were obtained. As seen from Table 6, *rno-miR-326-3p, rno-miR-423-5p, rno-miR-15b-5p, rno-let-7c-5p,* and *rno-let-7b-5p* are the top 5 miRNAs. These miRNAs are involved in the three treatments, and rno-miR-326-3p acts on multiple RNAs (Figure 6).

3.10. Weighted Coexpression Network Relationship of ncRNA and mRNA. Significant modules were screened to perform the coexpression analysis of ncRNA and mRNA in different treatments. Functional modules are obtained from the above significant modules (Figure 7). The results showed that the SP-FJG group was significantly correlated with red modules, the SP group was significant in the green modules, and the FJG group was also significantly in the green module, and then the three significant modules were analyzed by pathway enrichment (Figure 8).

Pathway analysis showed that the main regulatory pathways in the SP group were chemical carcinogenesis, metabolism of xenobiotics by cytochrome P450, etc. In the FJG group, that were herpes simplex infection, measles, and so on. The SP-FJG group mainly affected the adipocytokine signaling pathway, T-cell receptor signaling pathway, and RIG-I-like receptor signaling pathway. In addition, the significantly expressed RNA value of three kinds of processing module was extracted, and then a heatmap was generated (Figure 9).

3.11. Weighted Correlation Network Analysis of circRNA. Significant modules were screened to perform the coexpression analysis of circRNA and mRNA in different treatments. Functional modules are obtained from the above significant modules (Figure 10). The results showed that the SP-FJG group was significantly correlated with red modules, the SP group and the FJG group were significant in the green modules, and then the three significant modules were analyzed by pathway enrichment to select significantly enriched pathways (P value <0.05) (Figure 11).

FJG module mainly regulates butanoate metabolism, osteoclast differentiation, cytokine-cytokine receptor

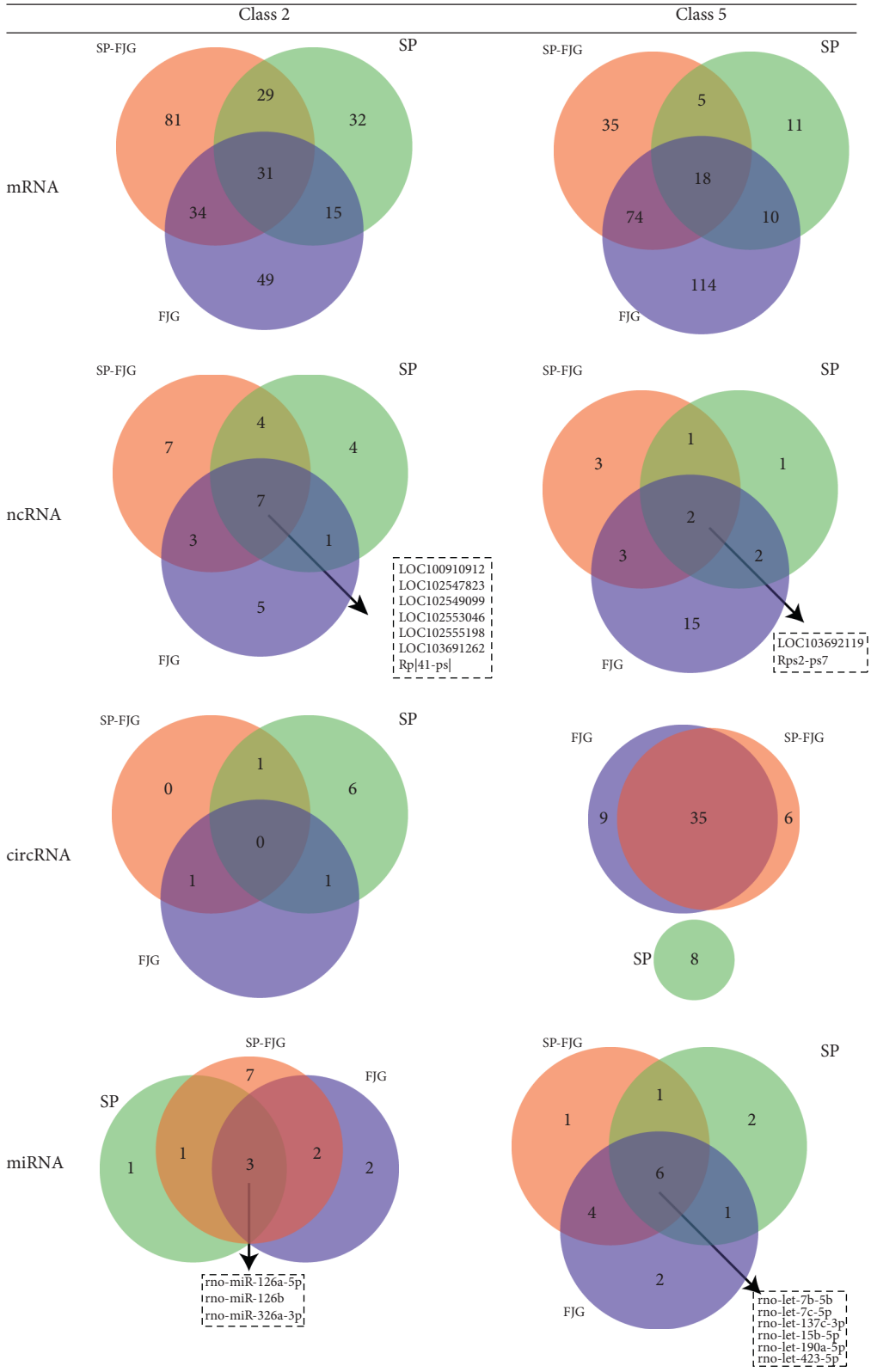
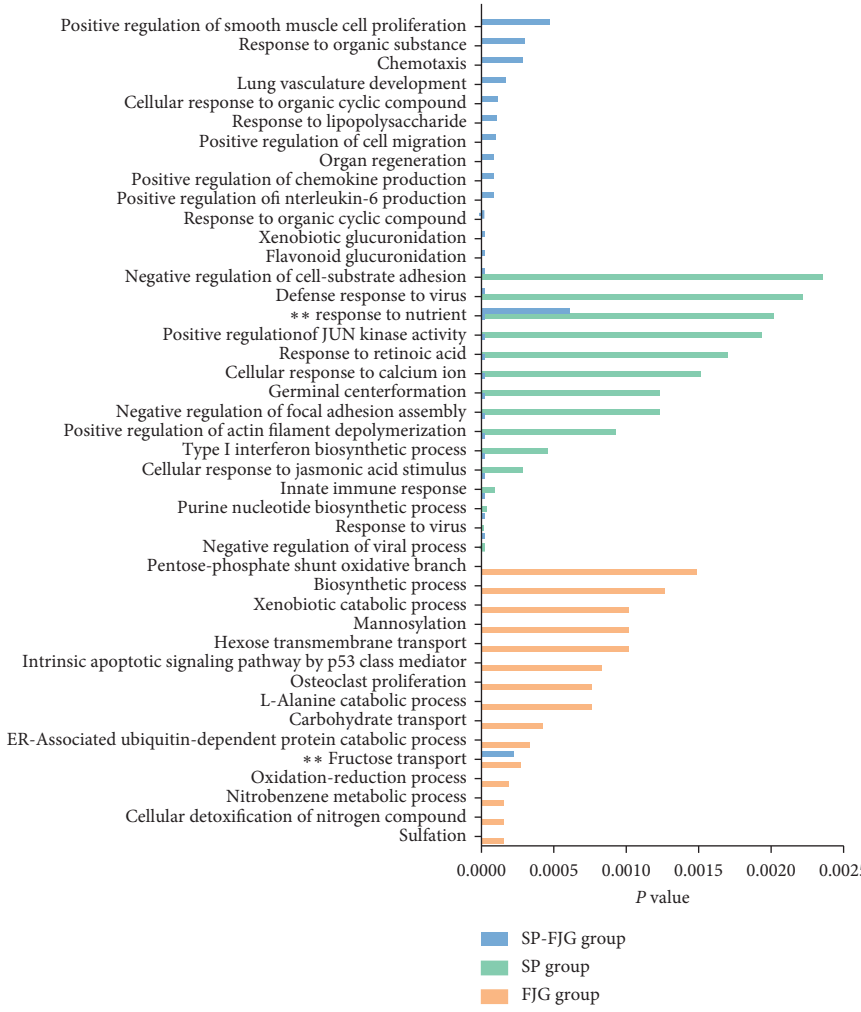


Figure 2: The relationship between the pharmacodynamic trends of RNA in different treatment groups. The left is the four RNA; the corresponding RNA is in the Wayne map of the three treatments, and the box in the Wayne map indicates the RNA expressed in all of the three treatments.



(a)

Figure 3: Continued.

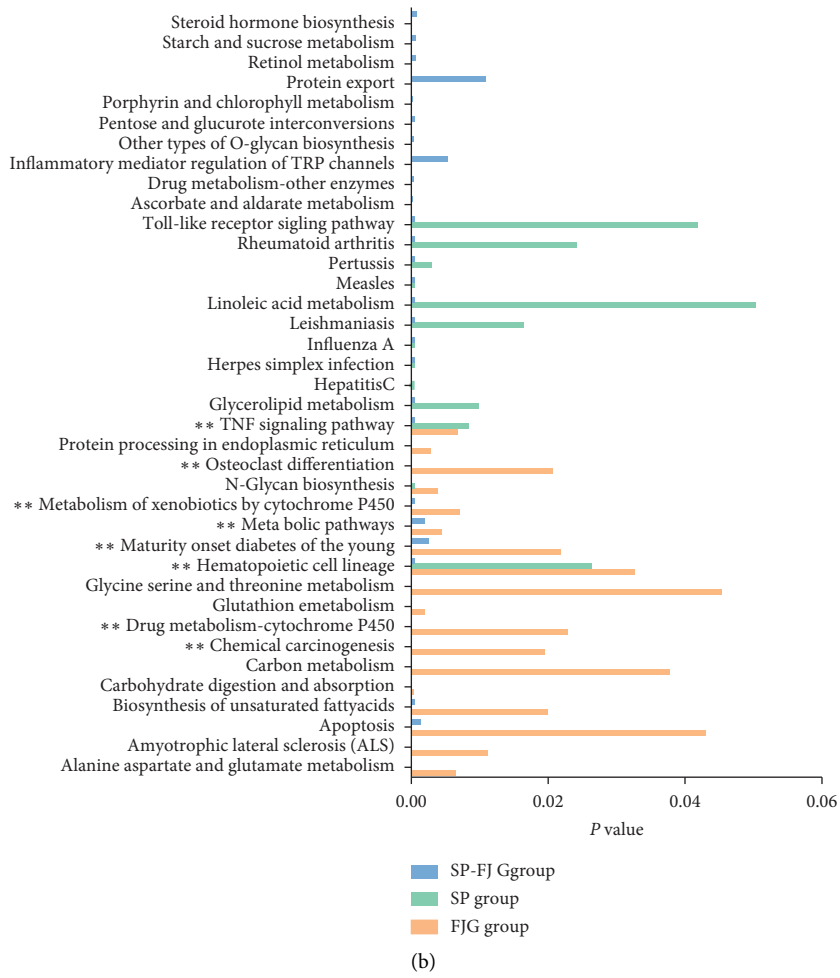


Figure 3: Significant enrichment of GO BP and pathway in different treatment groups. (a) Significantly enriched GO BP; (b) significantly enriched pathway.

interaction, and other pathways; the SP module mainly controls amyotrophic lateral sclerosis (ALS), p53 signaling pathway, apoptosis, and biosynthesis of unsaturated fatty acids, etc. SP-FJG chiefly controls autoimmune thyroid diseases, type 1 diabetes mellitus, etc. FJG and SP-FJG coregulation pathway is the herpes simplex infection. SP-FJG and SP coregulated pathways including chemical carcinogenesis, steroid hormone biosynthesis, and metabolism of xenobiotics by cytochrome P450.

The significantly enriched pathways were obtained. The expression value of RNA of the green module of the SP group and FJG group and the red module of the SP-FJG group were extracted, and then a heatmap was generated (Figure 12).

3.12. ceRNA Network Analysis of Significant Modules. To detect clusters of highly interconnected genes and explore the function of ceRNAs on the basis of protein-coding genes, we performed weighted correlation network analysis (WGCNA). From the analysis, we obtained the significant modules of ncRNA and mRNA and circRNA and mRNA. In ncRNA and mRNA, the significantly enriched pathway of the green module of the FJG group, the green module of the SP group, and the red module of the SP-FJG group were obtained. While in circRNA and mRNA, the obviously enriched pathway of the green module of the FJG group, the green module of the SP group, and the blue module of the SP-FJG group were obtained. The ncRNA, mRNA, and circRNA of class 2 and class 5 in the significant modules were extracted, and then the ceRNA networks were constructed (Figure 13).

The ceRNA network analysis showed the top 5 miRNAs in the global ceRNA network which played an important role in the ceRNA of the significant modules. The miRNA *Il1a, Rif1*, and *Leng8* [18] are regulated by *rno-let-7c-5p* as well as *rno-let-7b-5p*, and *Il1a* is a pharmacophore in the SP group and *Rif1* is the effective gene of FJG treatment. *Leng8* is an ncRNA, which have pharmacodynamic function in FJG and SP treatment. The miRNA *rno-let-7c-5p* and *rno-let-7b-5p* had efficacy trends in the three treatments.

3.13. Building PPI Network of SP, FJG, and SP-FJG Groups. In order to study the role of pharmacophore obtained from different treatments in the biological networks, we obtain the

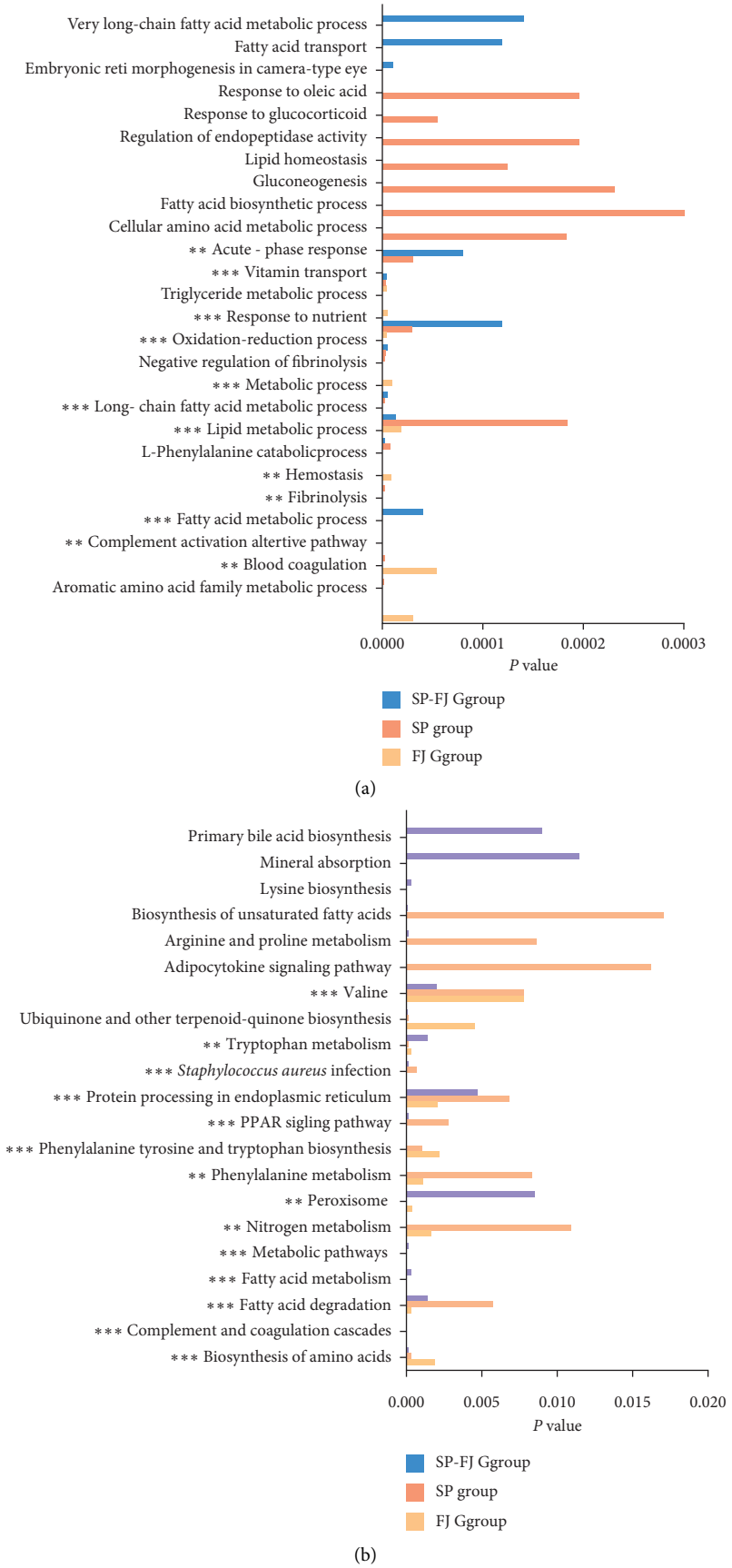


Figure 4: Significant enrichment of GO BP and pathway of circRNA in different treatment groups. (a) Significantly enriched GO BP; (b) significantly enriched pathway.

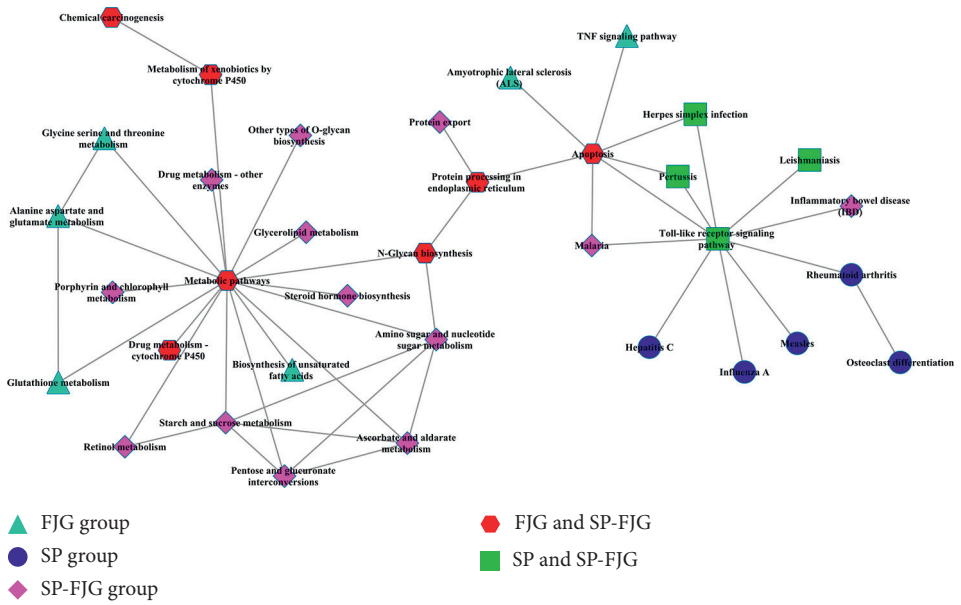


Figure 5: Functional regulatory network of the pathway.

Table 6: Topological features for top 5 RNAs in the ceRNA network.

Name	Degree	Average shortest path length	Betweenness centrality	Closeness centrality	Neighborhood connectivity	Radiality	Topological coefficient
rno-miR-326-3p	61	2.0423	0.5409	0.4897	2.4098	0.8263	0.1007
rno-miR-423-5p	37	2.3643	0.3499	0.4230	2.3243	0.7726	0.0883
rno-miR-15b-5p	37	2.4214	0.3353	0.4130	2.4324	0.7631	0.1102
rno-let-7c-5p	35	2.5643	0.1614	0.3900	3.3429	0.7393	0.2130
rno-let-7b-5p	35	2.6214	0.1533	0.3815	3.2000	0.7298	0.2444

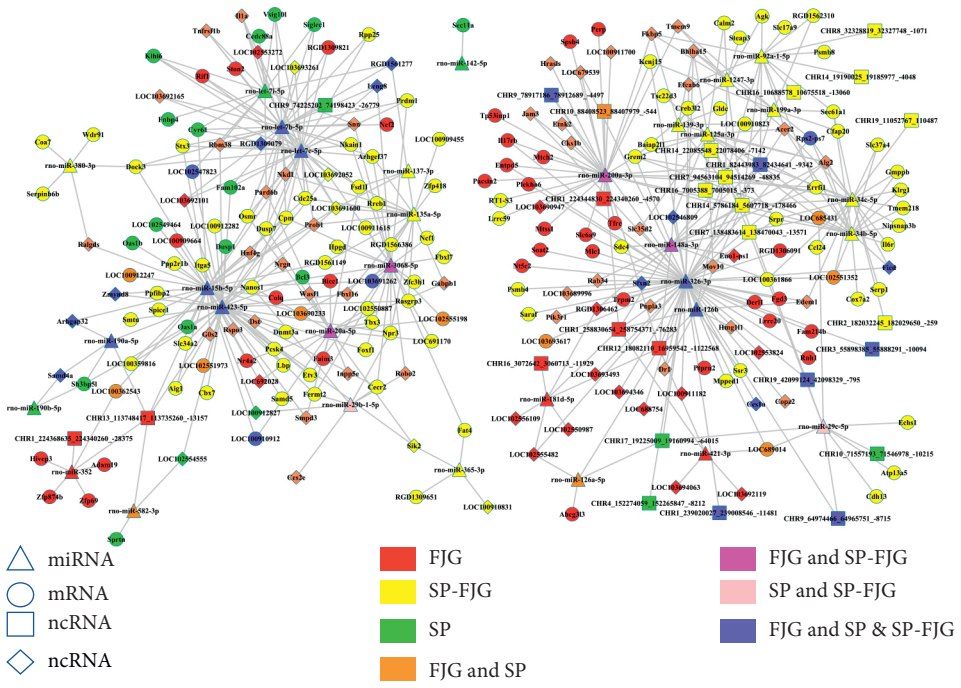
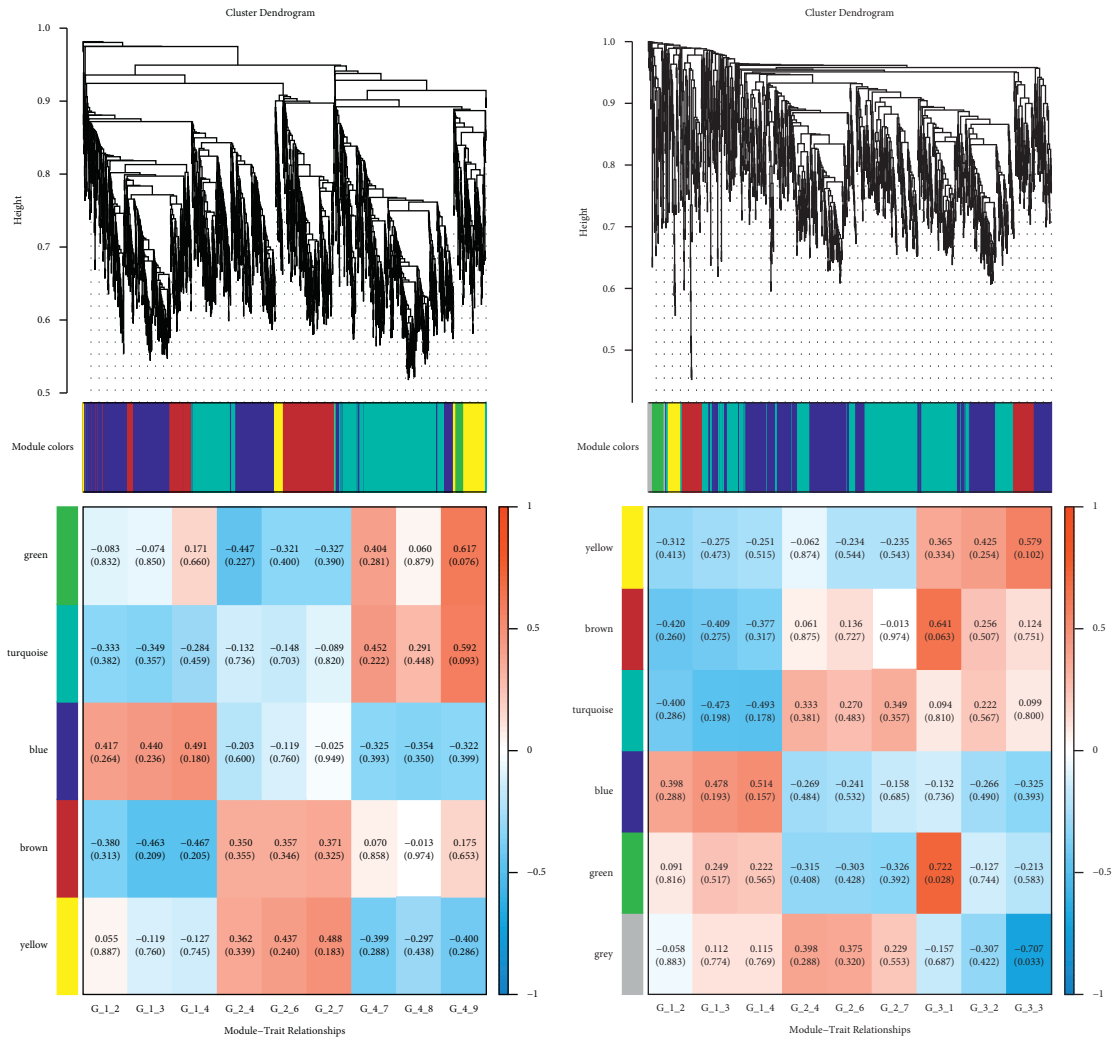
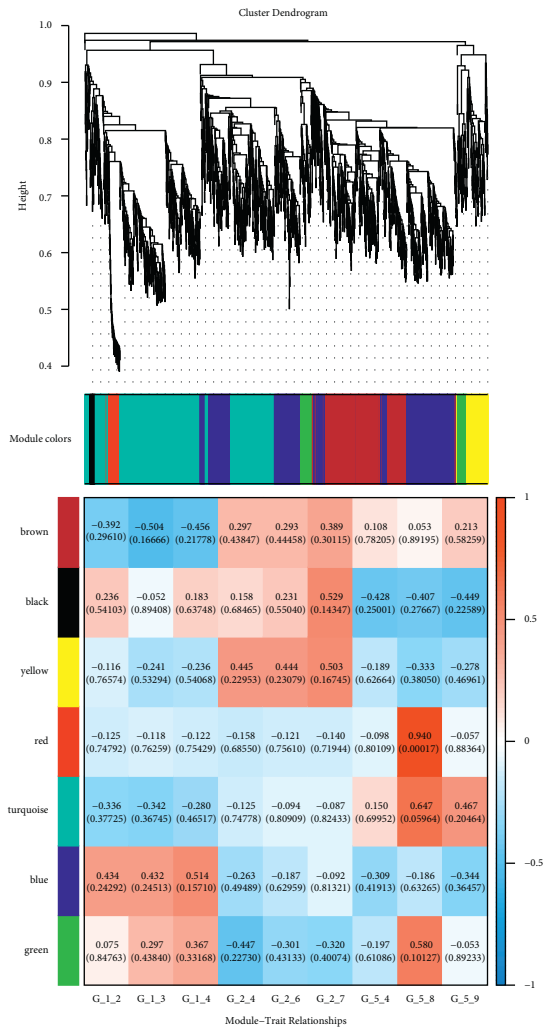


Figure 6: ceRNAs network of different treatments.



(a) (b)

Figure 7: Continued.



(c)

Figure 7: ncRNA and mRNA coexpression analysis of different treatments. (a) WGCNA analysis of the FJG group; (b) WGCNA analysis of the SP group; (c) WGCNA analysis of the SP-FJG group.

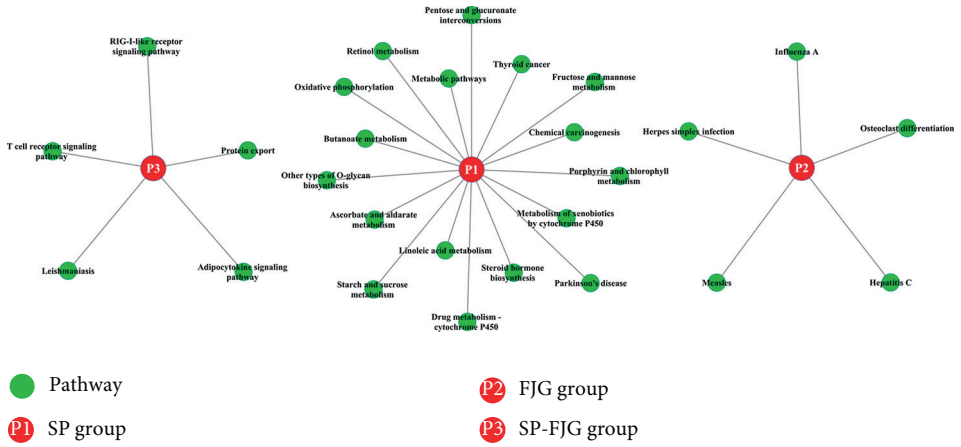
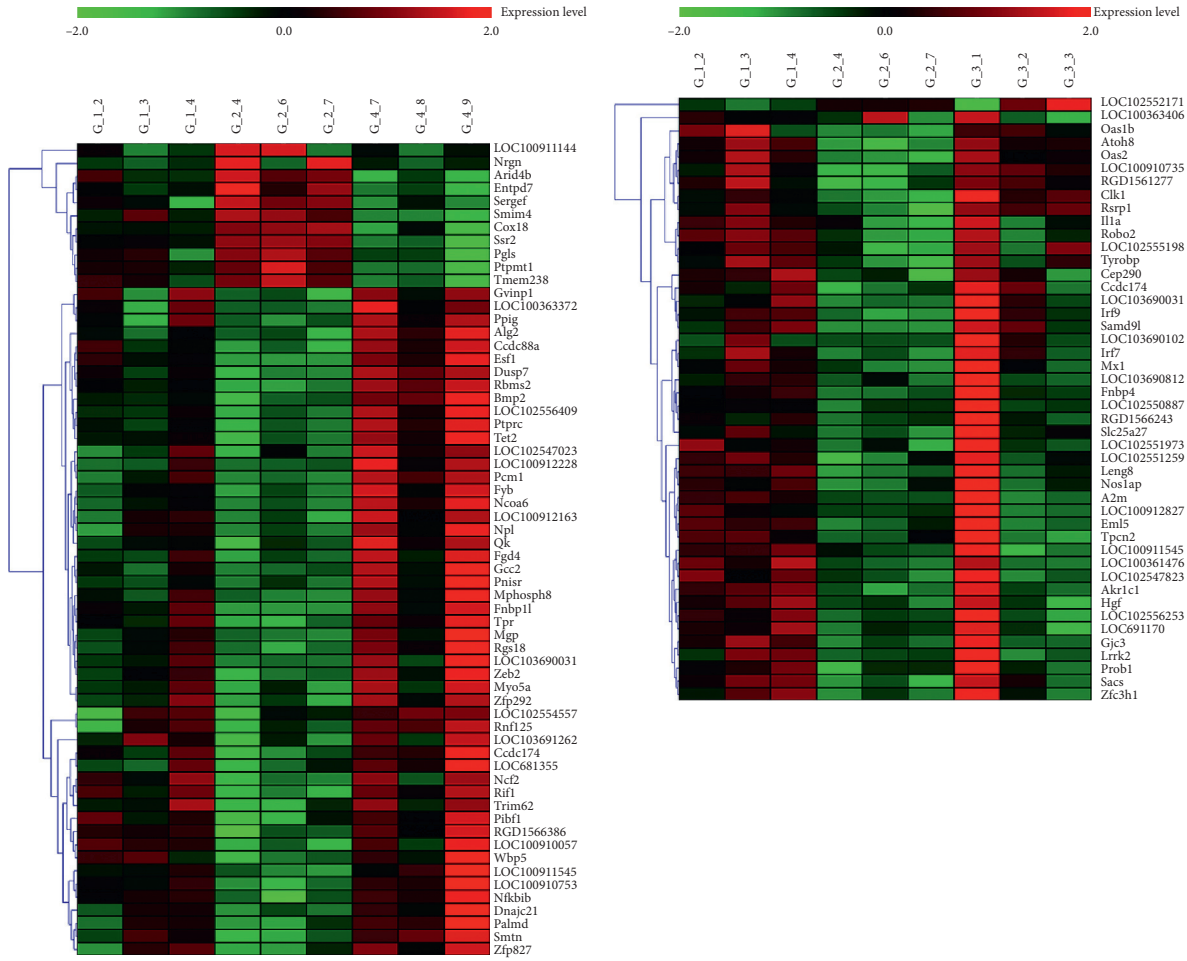
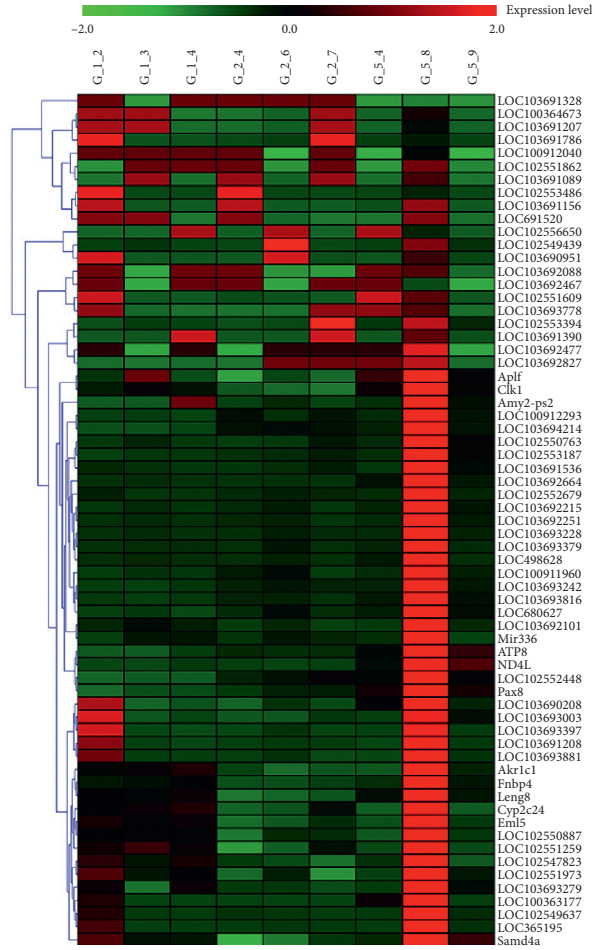


Figure 8: Pathway enrichment analysis of the significant modules. P1 represents SP, P2 represents FJG, and P3 is a combination (SP-FJG). The significant pathway is a green circle, and the significant pathway is $P < 0.05$.



(a) (b)

Figure 9: Continued.



(c)

Figure 9: The heatmap of the significant module in different administration. (a) FJG group, (b) SP group, and (c) SP-FGJ group.

rat protein interaction data from the string database. The data of drug effect trend genes in the three administration groups were obtained, and then the PPI network was constructed (Figure 14).

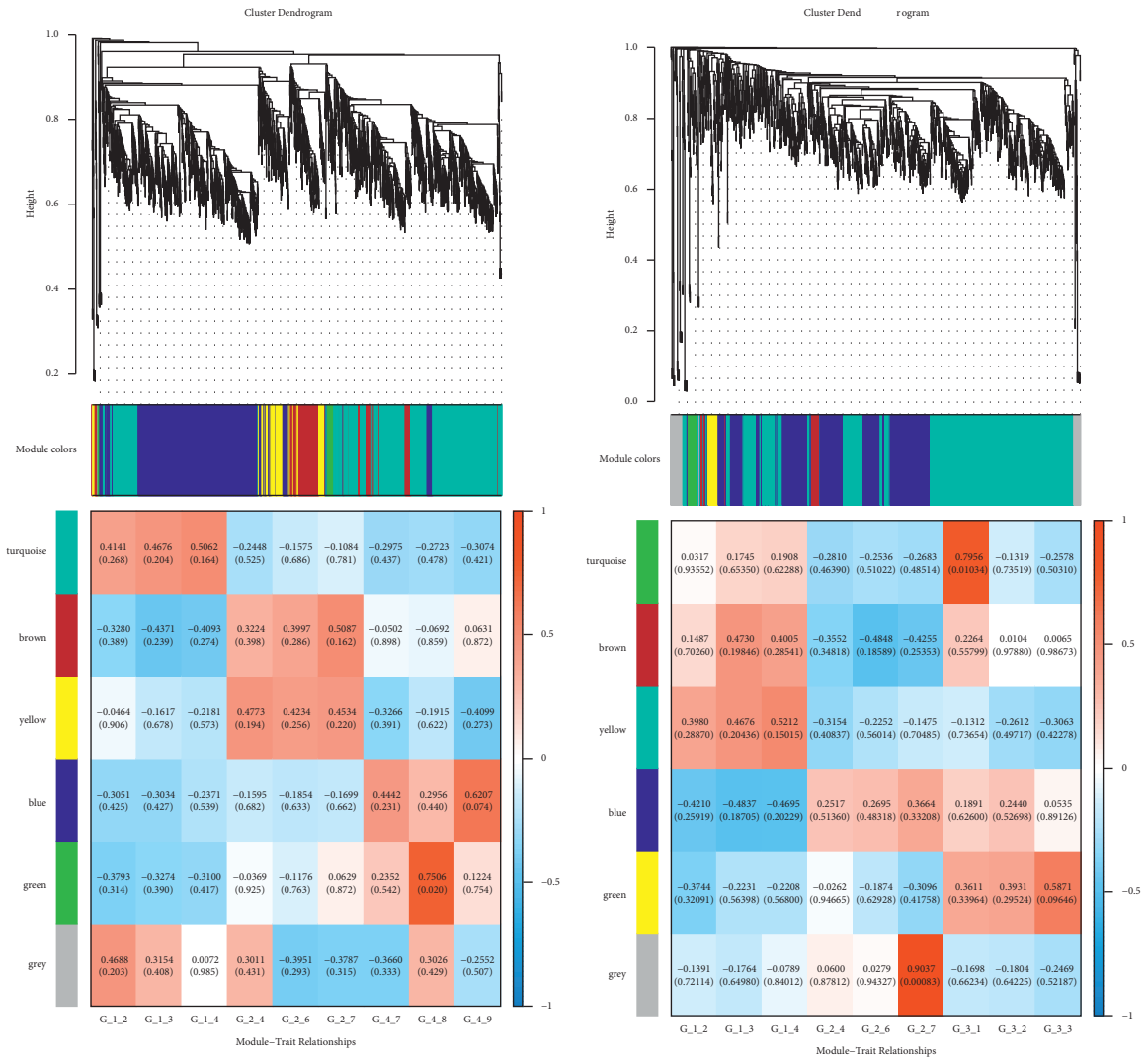
By analyzing the topological properties of the network, the nodes were arranged in the descending order of degree in topological properties, showing the gene of top 20 (Table 7). *Lrrk2*, a pharmacodynamic gene under the treatment of traditional FJG and SP, got the highest degree in the PPI network and played the most important role in the network. *Irak3* was differentially expressed in the three different treatment groups and was related to drug efficacy, which had a great influence on the network. It can be concluded that *Irak3* is a potential target in drug treatment of disease.

4. Discussion

This study is a report of the mechanisms of SP plus FJG in the treatment of diabetes mellitus. Transcriptome and miRNA sequencing were performed to comprehensively elucidate the mechanisms at the genetic level.

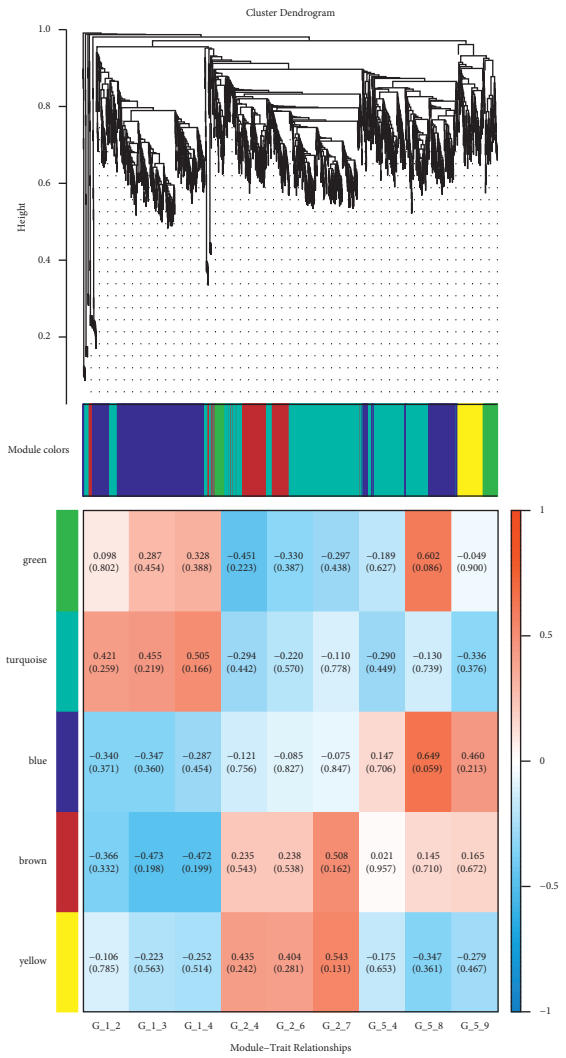
In 2007, Aschner et al reported that SP, the first dipeptidyl peptidase 4 (DPP-4) inhibitor, provided a new treatment for patients with type 2 diabetes [19]. SP was proved to be a safe antibiotic medicine that could reduce glycosylated hemoglobin significantly ($P < 0.001$ vs. placebo). FBG, body weight, and systolic blood pressure values were also significantly reduced at week 26 and 52 in the sitagliptin 100 mg group patients compared with placebo ($P < 0.001$). [19, 20]. From the in vivo observation of oral glucose tolerance test by SP, blood glucose level decreased (22.22%) significantly [22].

In recent years, TCMs have been extensively studied in the treatment of T2DM. Xu's et al. study found that compared with the control group, the blood glucose of administration the MDG-1 (300 mg/kg) group decreased by 30% [23]. One study found that administration with high-dose Tangnaikang (TNK) (3.24 g/kg) in SHR/cp rats for 3 weeks, the body weight, and fat mass of SHR/cp rats significantly reduced without affecting food consumption. FBG and FINS in the TNK-treated groups decreased after 6 weeks of treatment. Furthermore, TNK-treated rats exhibited obvious improvements in glucose intolerance and insulin



(a) (b)

Figure 10: Continued.



(c)

Figure 10: Coexpression of circRNA and mRNA in different administration. (a) WGCNA analysis of the FJG group; (b) WGCNA analysis of the SP group; (c) WGCNA analysis of the SP-FJG group.

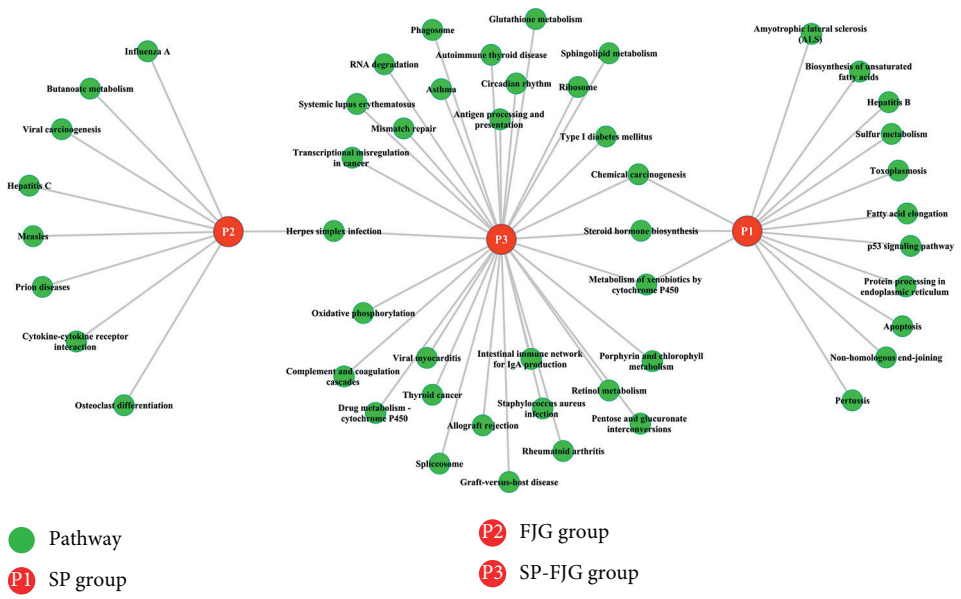


Figure 11: Pathway analysis of significant modules. The SP group is expressed in P1, P2 is the FJG group, and P3 is the SP-FJG group. The significant path is the green round.

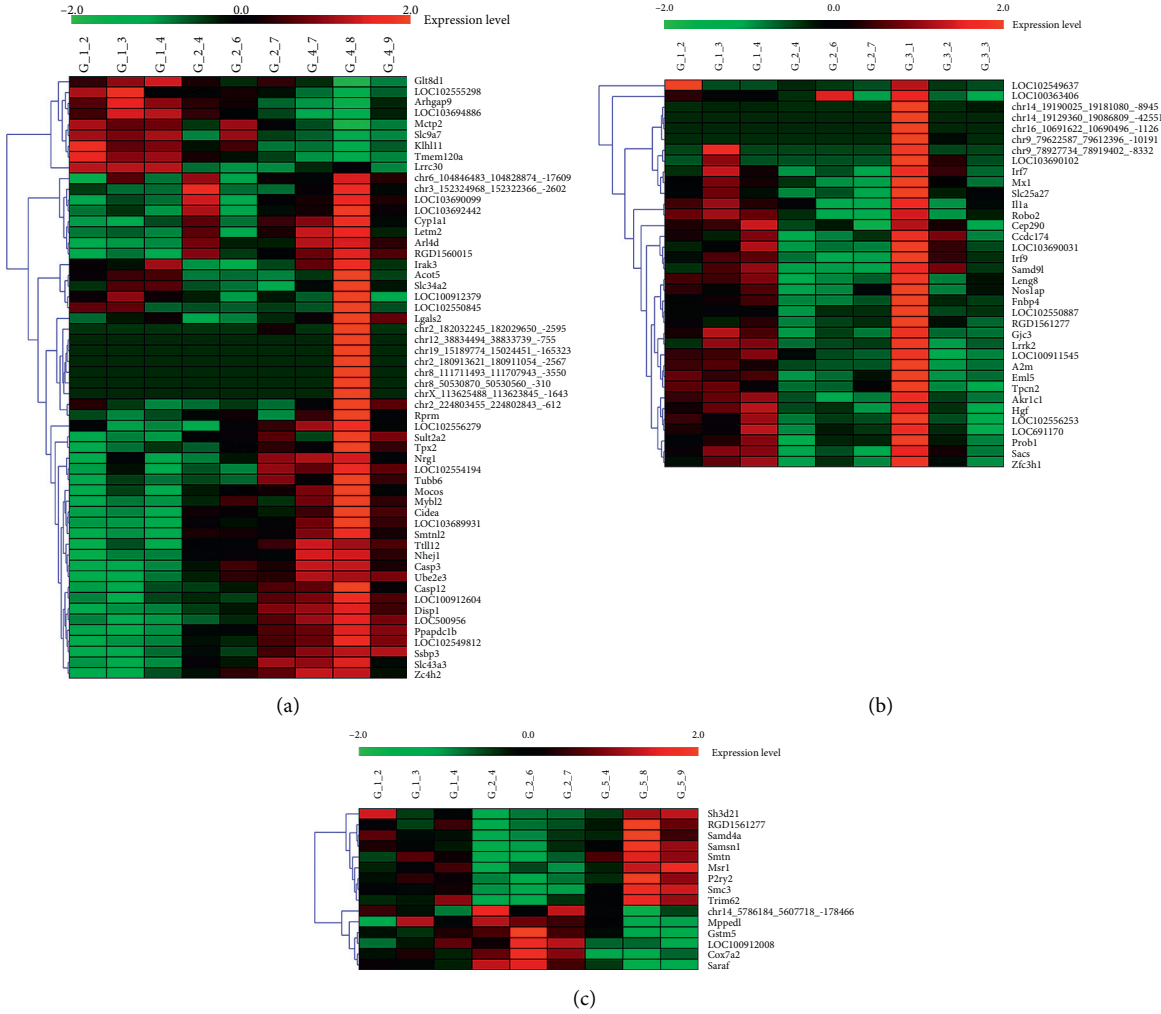


Figure 12: The heatmap of significant module in different administration. (a) FJG group, (b) SP group, and (c) SP-FGJ group.

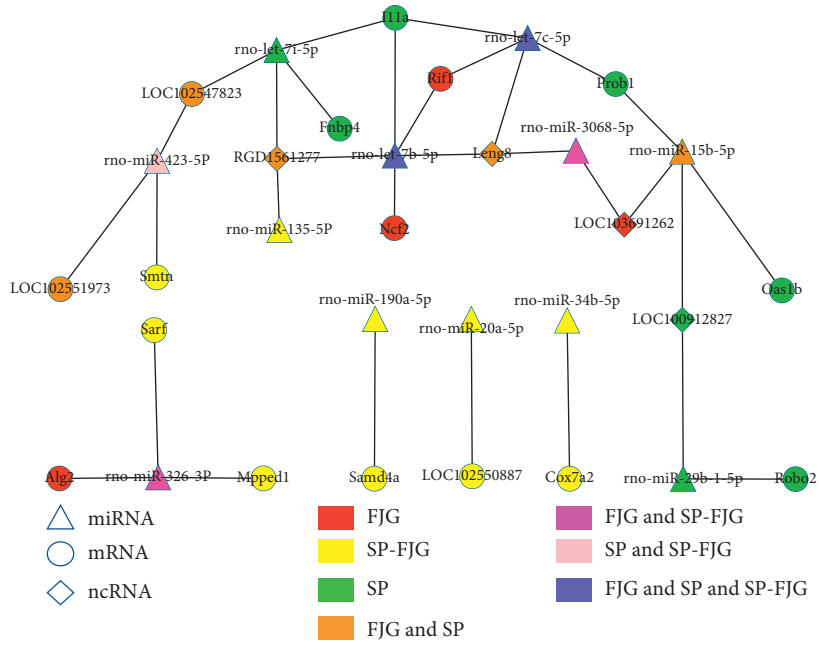


Figure 13: ceRNA network of the significant module in different administration.

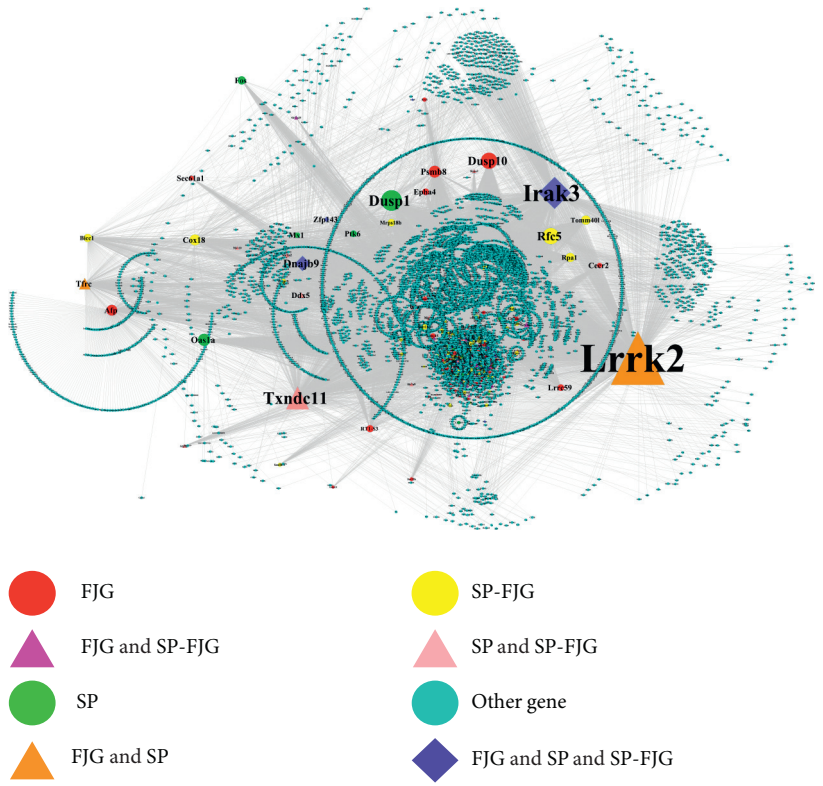


Figure 14: PPI network of the three different administration.

Table 7: Topological features for top 20 genes in the PPI network.

Name	Degree	Average shortest path length	Betweenness centrality	Closeness centrality	Clustering coefficient	Topological coefficient
Lrrk2	2413	1.9294	0.3861	0.5183	0.0022	0.0026
Irak3	1387	2.1311	0.1271	0.4692	0.0039	0.0032
Txndc11	968	2.2135	0.1126	0.4518	0.0046	0.0038
Rfc5	652	2.6483	0.0679	0.3776	0.0022	0.0066
Dusp1	848	2.3084	0.0644	0.4332	0.0066	0.0044
Afp	393	2.7355	0.0565	0.3656	0.0023	0.0069
Tfrc	416	2.6417	0.0563	0.3785	0.0027	0.0066
Dnajb9	592	2.3182	0.0549	0.4314	0.0062	0.0044
Psmb8	443	2.7090	0.0510	0.3691	0.0024	0.0077
Oas1a	452	2.1887	0.0461	0.4569	0.0067	0.0077
Dusp10	651	2.3613	0.0320	0.4235	0.0089	0.0051
Cox18	398	2.6361	0.0315	0.3793	0.0047	0.0064
Bicc1	267	2.8185	0.0308	0.3548	0.0015	0.0113
Tomm40l	321	2.7891	0.0288	0.3585	0.0031	0.0096
RT1-S3	217	2.7531	0.0259	0.3632	0.0052	0.0093
Mrps18b	231	2.9355	0.0258	0.3407	0.0011	0.0137
Mtx2	195	2.9554	0.0256	0.3384	0.0024	0.0123
Rpa1	316	2.7432	0.0254	0.3645	0.0053	0.0081
Ddo	215	2.8658	0.0249	0.3489	0.0020	0.0120
Sult1b1	136	2.9094	0.0238	0.3437	0.0021	0.0110

resistance [11]. Another research of *Jiangtang Xiaozhi*, which comprised six commonly used herbs, found that 16 weeks of *Jiangtang Xiaozhi* treatment did not lower fasting blood glucose, but it improved FINS and HDL cholesterol in a Western population with prediabetes or controlled diabetes [24].

In our study, after treatment for 6 weeks, the improvement of FBG, RBG, OGTT, ITT, HOMA-IR, TC, SOD,

and MDA were presented by FJG and SP. Combining SP and FJG, not only OGTT, FINS, TNF-α, and IL-6 were better improved compared with monotherapy but increased LDL and TC by SP were attenuated when using FJG in combination. Besides, only the combined medication group decreased FINS, TNF-α, and IL-6.

In addition, we performed miRNA and transcriptome sequencing of the liver tissue of ZDF rats with different drug treatment. Compared with the DM group, 134, 1026, and 621 DEGs were found in the SP/FJG/SP-FJG group, respectively. According to the type of RNA count, 1248 mRNA, 211 ncRNA, 202 cirRNA, and 120 miRNA were differentially expressed in different groups. In subsequent series cluster analysis, we identified profile 2 and 5 having important biological significance, and these two gene expression trends were related to the drug treatment process. KEGG analysis of the weighted coexpression networks of ncRNA and mRNA and ceRNA indicated that 22 pathways were significantly enriched. PPAR pathway [11], fatty acid metabolism [25], and nitrogen metabolism [26] have been reported to be related to T2DM. Usually, a certain miRNA may have multiple different mRNA targets, whereas a given target gene may also be targeted by multiple miRNAs [27]. In this thesis, by the analysis of the ceRNA network, we found that *rno-miR-326-3p* [28], *rno-miR-423-5p* [29], *rno-miR-15b-5p*, *rno-let-7c-5p*, and *rno-let-7b-5p* were related to pharmacodynamics in different groups.

Wang Y's study confirmed that miR-326-3p targeted on FcγRIII and inhibited its expression under the condition of high glucose, which were associated with glomerular sclerosis of diabetic kidney disease [28]. Another study found that under the condition of obesity, activation of the hepatic NFE2/miR-423-5p axis plays important roles in the progression of type 2 diabetes and NAFLD by repressing the FAM3A-ATP-Akt signaling pathway [29]. Besides, a study found the elevated serum miR-423-5p combined with oxidized lipoprotein can be used as novel biomarkers for potential auxiliary diagnosis of T2DM patients and T2DM patients with microvascular complications [30]. *MiR-15b* maybe a potential therapeutic target for therapeutics for the diabetic retina. It plays a major role in the inhibition of insulin resistance via reduced *TNFα* and *SOCS3* signaling and increased IGFBP-3 levels, resulting in REC protection from hyperglycemia-induced apoptosis [31].

By using the PPI and coexpression networks of the transcriptomes in different treatment groups, we generated highly connected modules which were used to enrich the gene mutations in ZDG rats using the WGCNA algorithm. Using the string database, PPI network analysis found *Lrrk2, Irak3,* and other 20 genes, and these genes may be pharmacodynamic genes. The 3′UTR of the *Lrrk2* gene would have been a target of *miR-712* and then dampens the phosphorylation of p38 and ERK1/2 kinases. However, *miR-712* restored insulin-stimulated glucose uptake by myoblasts through downregulating macrophage-mediated inflammatory response [32]. A study in 123 patients and 46 age-matched controls reported the addition of reactive oxygen species, obesity, low adiponectin, and high glucose and interleukin-6 as cause of the abatement in *Irak3* in THP-1 cells in vitro [33]. *Irak3* is a crucial inhibitor of inflammation, obesity, and metabolic syndrome. And chronic low-grade inflammation is now considered to have a vital role in the development of obesity and related metabolic diseases such as T2DM, insulin resistance, and the metabolic syndrome and cardiovascular disease [34, 35].

5. Conclusion

In conclusion, the effect of SP-FJG in regulating glucose tolerance, TNF-α, IL-6, FINS, and hyperlipidemia was better than using SP individually in ZDF rats. We conducted a comprehensive comparison of gene expression treated with SP, FJG, and SP-FJG. We identified that many genes may be responsible for T2DM by transcriptome and miRNA sequencing. In combination with the sequencing results, we speculated that 1248 mRNAs, 211 ncRNA, 202 cirRNA, and 120 miRNA were related to the treatment of SP and FJG for diabetic. By ceRNA, *rno-miR-326-3p*, *rno-miR-423-5p*, *rno-miR-15b-5p*, *rno-let-7c-5p*, and *rno-let-7b-5p* were connected with pharmacodynamics in different groups. PPI network analysis discovered that *Lrrk2, Irak3,* and other 18 genes may be pharmacodynamic genes. This study provides the basis for functional study of diabetes-related genes and the molecular mechanism of T2DM.

Data Availability

All data generated or analyzed during this study are included in this published article. The datasets supporting the conclusions of this article are available in the NCBI's Sequence Read Archive repository (PRJNA523690: https://www.ncbi.nlm.nih.gov/bioproject/523690).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xuan Guo and Tonghua Liu proposed the idea and designed the study. Wen Sun, Guangyuan Xu, Dan Hou, and Zhuo Zhang performed the study. Xuan Guo, Wen Sun, and Guangyuan Xu participated in data analysis. Xuan Guo, Lili Wu, and Tonghua Liu wrote and improved the manuscript. All authors read and approved the final manuscript. Xuan Guo and Wen Sun contributed equally to this work.

Acknowledgments

This study was supported by the Common Construction Project of Beijing Municipal Education Commission (no. 100006250488/001), 111 Project (no. B07007), International Joint Research Center of Prevention and Treatment of Diabetes with Traditional Chinese Medicine by Ministry of Science and Technology of China, Key Laboratory of Health Cultivation of the Ministry of Education, Key Laboratory of Health Cultivation of Beijing (BZ0259), and Beijing International Scientific and Technological Cooperation Base for

the Prevention and Treatment of Diabetes with Traditional Chinese Medicine.

References

[1] P. Hossain, B. Kawar, and M. El Nahas, "Obesity and diabetes in the developing world - a growing challenge," *New England Journal of Medicine*, vol. 356, no. 3, pp. 213–215, 2007.

[2] S. Wild, G. Roglic, A. Green, R. Sicree, and H. King, "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030," *Diabetes Care*, vol. 27, no. 5, pp. 1047–1053, 2004.

[3] S. H. Yang, K. F. Dou, and W. J. Song, "Prevalence of diabetes among men and women in China," *New England Journal of Medicine*, vol. 362, no. 25, pp. 2425-2426, 2010.

[4] R. A. DeFronzo, "Pharmacologic therapy for type 2 diabetes mellitus," *Annals of Internal Medicine*, vol. 133, no. 1, pp. 73-74, 2000.

[5] R. E. Amori, J. Lau, and A. G. Pittas, "Efficacy and safety of incretin therapy in type 2 diabetes," *JAMA*, vol. 298, no. 2, pp. 194–206, 2007.

[6] J. Yan, J. S. Gao, C. H. Jie, J. T. Song, Z. Z. Wu, and X. L. Guo, "Effects of Mimenghua formula on TCM symptoms of early diabetic retinopathy and safety," *Journal of Beijing University of Traditional Chinese Medicine*, vol. 33, pp. 773–776, 2010.

[7] Z. Luan, G. Jianshene, and C. Jie, "Effects of Buddleia decoction on the proliferation of HUVEC and the expression of HIF-1α in HUVEC in hypoxia state," *China Journal of Chinese Ophthalmology*, vol. 21, pp. 4–7, 2011.

[8] R. X. Zhang, Z. P. Jia, M. X. Li, J. Wang, and J. G. Qiu, "Effects of rehmannia glutinosa oligosaccharideon peripheral Acta Universitatis Medictnae Tangji hemogram hormone levels and pancreatic island pathology in type 2 diabeticrats," *Medical Journal of National Defending Forces in Northwest China*, vol. 30, pp. 161–164, 2009.

[9] F. Lu, S. Leng, Q. Tu, L. Xu, M. Yang, and K. Wang, "Comparative study on the effectsof huanglianjiedu decoction and berberine on glucose and lipid metabolisms in type 2 diabetic rats," *Acta Universitatis Medictnae Tangji*, vol. 31, no. 6, pp. 662–665, 2002.

[10] Y. Zhu, W. Cong, L. Shen et al., "Fecal metabonomic study of a polysaccharide, MDG-1 from *Ophiopogon japonicus* on diabetic mice based on gas chromatography/time-of-flight mass spectrometry (GC TOF/MS)," *Molecular Bio Systems*, vol. 10, no. 2, pp. 304–312, 2014.

[11] L. Li, H. Yoshitomi, Y. Wei et al., "Tang-Nai-Kang alleviates pre-diabetes and metabolic disorders and induces a gene expression switch toward fatty acid oxidation in SHR.Cg-Leprcp/NDmcr rats," *PLoS One*, vol. 10, no. 4, Article ID e0122024, 2015.

[12] R. G. Peterson, W. N. Shaw, M.-A. Neel, L. A. Little, and J. Eichberg, "Zucker diabetic fatty rat as a model for non-insulin-dependent diabetes mellitus," *Ilar Journal*, vol. 32, no. 3, pp. 16–19, 1990.

[13] J. B. Clark, C. J. Palmer, and W. N. Shaw, "The diabetic Zucker fatty rat," *Experimental Biology and Medicine*, vol. 173, no. 1, pp. 68–75, 1983.

[14] S. C. Griffen, J. Wang, and M. S. German, "A genetic defect in -cell gene expression segregates independently from the fa locus in the ZDF rat," *Diabetes*, vol. 50, no. 1, pp. 63–68, 2001.

[15] J.-Y. Liu, J. Yao, X.-M. Li et al., "Pathogenic role of lncRNA-MALAT1 in endothelial cell dysfunction in diabetes mellitus," *Cell Death & Disease*, vol. 5, no. 10, p. e1506, 2014.

[16] J. Salzman, "Cell-type specific features of circular RNA expression," *PLoS Genetics*, vol. 9, no. 9, Article ID e1003777, 2013.

[17] C. Dou, Z. Cao, B. Yang et al., "Changing expression profiles of lncRNAs, mRNAs, circRNAs and miRNAs during osteoclastogenesis," *Scientific Reports*, vol. 6, p. 21499, 2016.

[18] P. Arora, B. Garcia-Bailo, Z. Dastani et al., "Genetic polymorphisms of innate immunity-related inflammatory pathways and their association with factors related to type 2 diabetes," *BMC Medical Genetics*, vol. 12, p. 95, 2011.

[19] P. Aschner, M. S. Kipnes, J. K. Lunceford et al., "Effect of the dipeptidyl peptidase-4 inhibitor sitagliptin as monotherapy on glycemic control in patients with type 2 diabetes," *Diabetes Care*, vol. 29, no. 12, pp. 2632–2637, 2006.

[20] F. J. Lavalle-González, A. Januszewicz, J. Davidson et al., "Efficacy and safety of canagliflozin compared with placebo and sitagliptin in patients with type 2 diabetes on background metformin monotherapy: a randomised trial," *Diabetologia*, vol. 56, no. 12, pp. 2582–2592, 2013.

[21] T. Zerilli and E. Pyon, "Sitagliptin phosphate: a DPP-4 inhibitor for the treatment of type 2 diabetes mellitus," *Clinical Therapeutics*, vol. 29, no. 12, pp. 2614–2634, 2007.

[22] M. M. Hossen, "In vitro and in vivo drug-drug interaction between sitagliptin phosphate and atenolol," *International Journal of Pharmacy*, vol. 6, no. 2, pp. 283–291, 2016.

[23] J. Xu, Y. Wang, D.-S. Xu, K.-F. Ruan, Y. Feng, and S. Wang, "Hypoglycemic effects of MDG-1, a polysaccharide derived from *Ophiopogon japonicas*, in the ob/ob mouse model of type 2 diabetes mellitus," *International Journal of Biological Macromolecules*, vol. 49, no. 4, pp. 657–662, 2011.

[24] S. J. Grant, "Chinese herbal medicine for impaired glucose tolerance: a randomized placebo controlled trial," *BMC Complementary and Alternative Medicine*, vol. 13, no. 1, p. 104, 2013.

[25] E. E. Blaak, "Basic disturbances in skeletal muscle fatty acid metabolism in obesity and type 2 diabetes mellitus," *Proceedings of the Nutrition Society*, vol. 63, no. 2, pp. 323–330, 2004.

[26] N. V. Zharkova, P. P. Potapov, and A. Y. Stelmach, "Nitrogen metabolism in rats with experimental diabetes during acute alcohol intoxication," *Bulletin of Experimental Biology and Medicine*, vol. 152, no. 1, pp. 32–34, 2011.

[27] A. Krek, D. Grün, M. N. Poy et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[28] Y. Wang, R. Zhang, J. Zhang, and F. Liu, "MicroRNA-326-3p ameliorates high glucose and Ox-LDL-IC- induced fibrotic injury in renal mesangial cells by targeting FcgammaRIII," *Nephrology*, vol. 23, no. 11, pp. 1031–1038, 2017.

[29] W. Yang, J. Wang, Z. Chen et al., "NFE2 induces miR-423-5p to promote gluconeogenesis and hyperglycemia by repressing the hepatic FAM3A-ATP-Akt pathway," *Diabetes*, vol. 66, no. 7, pp. 1819–1832, 2017.

[30] J. Cai, C. Wang, D. Niu et al., "Study on levels of serum miR-342-5p and miR-423-5p in type 2 diabetes patients with or without microvascular complications and their clinical significance," *Chinese Journal of Clinical Laboratory Science*, vol. 33, no. 1, pp. 24–28, 2015.

[31] E.-A. Ye and J. J. Steinle, "miR-15b/16 protects primary human retinal microvascular endothelial cells against hyperglycemia-induced increases in tumor necrosis factor alpha and suppressor of cytokine signaling 3," *Journal of Neuroinflammation*, vol. 12, no. 1, p. 44, 2015.

[32] M. Talari, T. K. S. Nayak, V. Kain, P. P. Babu, P. Misra, and K. V. L. Parsa, "MicroRNA-712 restrains macrophage pro-inflammatory responses by targeting LRRK2 leading to restoration of insulin stimulated glucose uptake by myoblasts," *Molecular Immunology*, vol. 82, pp. 1–9, 2017.

[33] M. Hulsmans, "Interleukin-1 receptor-associated kinase-3 is a key inhibitor of inflammation in obesity and metabolic syndrome," *PLoS One*, vol. 7, no. 1, Article ID e30414, 2012.

[34] M. Navab, N. Gharavi, and A. D. Watson, "Inflammation and metabolic disorders," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 11, no. 4, pp. 459–464, 2008.

[35] P. Holvoet, "Relations between metabolic syndrome, oxidative stress and inflammation and cardiovascular disease," *Verhandelingen—Koninklijke Academie Voor Geneeskunde Van Belgie*, vol. 70, no. 70, pp. 193–219, 2008.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 3239649, 8 pages
https://doi.org/10.1155/2019/3239649

Research Article

Therapeutic Effect of *Scutellaria baicalensis* on L-Thyroxine-Induced Hyperthyroidism Rats

Mia Kim[1] **and Byung-Cheol Lee**[2]

[1]*Department of Cardiovascular and Neurologic Disease (Stroke Center), College of Korean Medicine, Kyung Hee University, 23 Kyungheedae-ro, Dongdaemun, Seoul 02447, Republic of Korea*
[2]*Department of Clinical Korean Medicine, Graduate School, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea*

Correspondence should be addressed to Byung-Cheol Lee; hydrolee@khu.ac.kr

Received 21 February 2019; Revised 24 July 2019; Accepted 31 July 2019; Published 15 September 2019

Academic Editor: Wen-yi Kang

Background. This study was performed to evaluate the anti-hyperthyroidal effects and action mechanism of *Scutellaria baicalensis* Georgi (SB), a medicinal herb, on levothyroxine (LT4)-induced hyperthyroidal rats. *Methods*. Male Wistar rats were divided into five groups, namely, euthyroidal normal group (Normal), hyperthyroidism control group (Control), hyperthyroidism plus PTU-treated group (PTU) as a positive control, hyperthyroidism plus 400 mg/kg SB-treated group (SB400), and hyperthyroidism plus 800 mg/kg SB-treated group (SB800). The rats in groups other than Normal were injected with LT4 for 2 weeks to induce hyperthyroidism and then were administrated each treatment for 2 weeks. Clinical symptoms and biomarkers related to hyperthyroidism were examined, and the gene expressions related to the regulation of thyroid hormone were determined. *Results*. Compared with the Control group, pulse rate, serum T3, T4, triglyceride, thyroid follicle size, and the deiodinase 1 (Dio1) gene expression were significantly reduced in the SB and PTU groups. Serum TSH and the thyroxine-binding globulin (Tbg) gene expression were significantly increased in the SB and PTU groups. *Conclusions*. These results suggest that SB might suppress T3, T4, and adrenergic activity by modulating Dio1 and Tbg expression, and therefore, SB could be an alternative therapy for hyperthyroidism.

1. Introduction

Hyperthyroidism is a hypermetabolic condition of thyrotoxicosis resulting from an overproduction of thyroid hormone in the thyroid gland [1]. Graves' disease, the most common cause of hyperthyroidism, manifests the clinical symptoms of goiter, palpitation, sweating, weight loss, and ophthalmopathy and laboratory findings of increased levels of T3, T4, and TSH receptor antibody and decreased TSH [2]. The cause of Graves' disease is that when MHC class II is demonstrated in the epithelial cell of the thyroid, T cells recognize the thyroid-stimulating hormone receptor (TSHR) as an extrinsic antigen and create autoantibodies from the B cell [3]. This stimulates the thyroid receptor resulting in a hypersynthesis and secretion of the thyroid hormone [4]. Thus, for a complete fundamental treatment, the production of autoantibody which stimulates TSH receptors should be suppressed, but this is unavailable in current clinical trials [5]. Instead, methods of destroying or removing thyroid tissue by radioactive iodine or surgery, or using a drug that restrains the production and distribution of thyroid hormones are used to maintain normal thyroid function [6]. However, it is difficult to expect a clinically complete remission of Graves' disease with such treatments [5]. Frequent relapses and side effects often follow drug treatments, while radioactive iodine therapy and thyroidectomy may lead to hypothyroidism [7]. Especially in countries like Korea and Japan with high reliance upon antithyroid drugs, where the proportion of drug treatment accounted for 80 to 88 percent of the treatments,

development of a treatment to supplement or replace the use of antithyroid drugs is desperately needed [8]. Today, due to such problems of the commonly used treatments, resistance to antithyroid drugs, and the tedious clinical improvement of these treatments, more patients are willing to cure their disease through herbal remedies in clinical practice [9]. Ahnjeonbaekho-tang (AJBHT) has been used as an herbal remedy for Graves' disease [10]. Among the components of AJBHT, daidzein and baicalein are known to have antithyroid effects [11]. Clinical studies showed that AJBHT reduced the level of thyroid hormone in Graves' disease patients who were resistant to methimazole (MMI) [12]. In in vitro study, AJBHT was suggested to have a different mechanism of modulating cyclic AMP and Tbg expression [13]. In contrast, MMI reduced the synthesis of thyroid hormone by inhibiting thyroid peroxidase (TPO) activity [14]. In this study, we evaluate the anti-hyperthyroidal effects and action mechanism of the *Scutellaria baicalensis* Georgi (SB), a main herb of AJBHT, on levothyroxine (LT4)-induced hyperthyroidal rats.

2. Materials and Methods

2.1. Preparation of SB. SB was purchased from the Department of Pharmaceutical Preparation of the Hospital of Korean Medicine, Kyung Hee University (Seoul, Korea). One thousand grams of SB was boiled with 1,500 mL of 80% ethanol using a heating mantle for 2 hours. The extract was transferred to a 500 mL flask by an applicator and filtered. The filtrate was concentrated with a rotary evaporator (Model NE-1, EYELA Co., Japan). The extract was freeze-dried and stored at room temperature. The final extraction yield of SB was 33%.

2.2. Animal Model and Treatment. Six-week-old male Wistar rats were purchased from the Central Lab. Animal Inc. (Seoul, Korea). They were in a moisture-controlled room (40–70%) with a 12-hour light-dark cycle and allowed access to water and diet *ad libitum*. After 1-week period of acclimation, every rat except the Normal group was daily subcutaneously injected with levothyroxine (LT4) (Sigma, MO, USA) at a dose of 0.3 mg/kg for 2 weeks for inducing hyperthyroidism. The rats were divided into five groups: euthyroidal normal group (Normal, $n = 6$), LT4-injected hyperthyroidal control group (Control, $n = 6$), LT4 plus 10 mg/kg propylthiouracil-treated group (Sigma, MO, USA) (PTU, $n = 6$) as a positive control, LT4 plus 400 mg/kg SB-treated group (SB400, $n = 6$), and LT4 plus 800 mg/kg SB-treated group (SB800, $n = 6$). The dosages of SB extracts and PTU used in this study were selected based on the previous report. SB extract was orally administered once a day for 14 days from 15th LT4 treatment, and PTU was intraperitoneally injected. The body weight of each rat was measured at the beginning and before the final sampling. The total amount of food consumption was recorded every day. To assess the food intake, the total consumption of food during a day was measured in every cage. Then the 1-day consumption of each rat was calculated by dividing into the number of the rats in each cage. At week 4, the rats were sacrificed and the weights of thyroid and livers were measured. This study was approved by the Institutional Animal Care and Use Committee of Kyung Hee University, Seoul, Korea.

2.3. Measurement of Pulse Rate and Oxygen Saturation. The pulse rate and oxygen saturation of rats were measured using a stand-alone pulse oximeter (Med Associates Inc., VT). Each rat was placed at the center of the fixing apparatus (Kenis, Japan) and was kept for 5 minutes until motionless, and then the wrap sensor was securely held in the tail. Once a stable signal was acquired, the data were recorded using the pulse oximeter software (Med Associates Inc., VT) for analysis.

2.4. Measurement of Serum Thyroid Hormones. Blood samples were collected, and serum was separated by centrifugation at 3000 rpm for 10 min at 4°C. Serum levels of triiodothyronine (T3), T4, and thyroid-stimulating hormone (TSH) were analyzed by colorimetric competitive enzyme immunoassay using individual ELISA Kit (Cusabio, China). In detail, microtiter wells coated with antibody were prepared and 100 μl of samples and standard T3, T4, or TSH solution were applied, then followed by 50 μl of HRP conjugate, 50 μl of color solution, and 50 μl of stop solution. The absorbance was measured by an ELISA reader at 450 nm.

2.5. Biochemical Assays. At the end of the experiment, after 14-hour fasting, serum total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride (TG), aspartate transaminase (AST), alanine aminotransferase (ALT), and creatinine levels were measured.

2.6. RNA Extraction and Analysis of Gene Expression. At week 4, the rats were sacrificed and the livers were dissected. RNA extraction was performed using a Mini RNA Isolation IITM (Zymo Research Corp, CA, USA). RNA was extracted using TRIzol reagent. To evaluate gene expression including deiodinase 1 (Dio1), thyroid hormone responsive spot 14 (Thrsp or Spot14), and thyroxine-binding globulin (Tbg), quantitative real-time polymerase chain reaction (qRT-PCR) was performed. Prior to qRT-PCR, the complementary DNA (cDNA) was synthesized using an Advantage RT for PCR Kit (Clontech, USA). To the cDNA obtained through reverse transcription PCR, 2x SYBR reaction buffer, primers, and dH_2O were added, and qRT-PCR was carried out using 7900HT Fast Real-Time PCR System (Applied Biosystems®, USA). The primer sequencing is as follows: *Dio1*, 5′-TTTAAGAACA-ACGTGGACATCAGG-3′ and 5′-GGTTTACCCTTG-TAGCAGATCCT-3′; *Spot14*, 5′-CTTACCCACCTGA-CCCAGAA-3′ and 5′-CATCGTCTTCCCTCTCGTGT-3′; *Tbg*, 5′-GCTGCTTTAGCCATGCTTTC-3′ and 5′-AAACTGCATTTCCCATCTGC-3′; and *GAPDH*,

5′-GTCGGTGTCAACGGATTTG-3′ and 5′-AGCTTCCCATTCTCAGCC-3′. For gene expression analysis, the threshold cycle for each gene, obtained with SDS Software 2.4 (Applied Biosystems®, USA), was converted to relative quantitation based on GAPDH, and the fold change was calculated. The fold change value of each experimental group was normalized according to the Normal group, which was defined as 1.

2.7. Histomorphometric Analyses of Thyroid. Obtained thyroid samples were fixed in 10% neutral buffered formalin and embedded in paraffin to make paraffin blocks. Each block was sliced into 4 μm thick sections with a microtome and attached to a gelatin-coated slide. Two sections per animal were stained with hematoxylin and eosin, and digital images were obtained using a high-resolution camera-mounted optical microscope (Olympus BX-50, Olympus Optical, Tokyo, Japan) connected to a computer. Using ImageJ, the thyroid follicular lumen area in thyroid tissue was measured.

2.8. Statistical Analysis. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). Statistical comparisons between the groups were performed with one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The data are presented as mean ± SEM. A two-tailed P value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of SB on Body Weight and Thyroid and Liver Weight in Hyperthyroidal Rats. The body weight of the hyperthyroidal control group increased to 389.57 ± 9.19 g, while that of the Normal group was 358.5 ± 9.18 g ($P = 0.05$). However, the body weight of the SB and PTU groups was no different compared to the Control group (Figure 1(a)). The amount of food intake was also examined to determine if the weight loss was due to a decrease in intake. Compared with the Normal group, there was a significant increase in food intake in the hyperthyroidal control group ($P < 0.01$) and SB400 group ($P < 0.05$) but not in the SB800 and PTU groups (Figure 1(b)). The weight of thyroid and liver in the hyperthyroidal control group did not show the differences compared to the Normal group and SB and PTU groups (Figures 1(c) and 1(d)).

3.2. Effects of SB on Thyroid Hormones in Hyperthyroidal Rats. As expectation, serum T4 levels in the hyperthyroidal control group (12.90 ± 0.71 ng/ml) were significantly elevated compared to the Normal group (6.01 ± 0.29 ng/ml, $P < 0.001$). SB significantly reduced T4 level in the SB400 group (10.62 ± 0.64 ng/ml, $P < 0.05$) and in the SB800 group (0.66 ± 0.31 ng/ml, $P < 0.01$) compared to the Control group, which showed similar effect to the PTU group (10.27 ± 0.40 ng/ml, $P < 0.001$) (Figure 2(b)). As a consequence of T4 elevation, serum T3 levels in the hyperthyroidal control group (0.99 ± 0.02 ng/ml) were also significantly elevated compared to the Normal group (0.84 ± 0.04 ng/ml, $P < 0.01$). SB significantly reduced T3 level in both the SB400 group (0.87 ± 0.04 ng/ml, $P < 0.05$) and SB800 group (0.83 ± 0.03 ng/ml, $P < 0.01$) compared to the Control group, and the PTU group showed a great reduction in T3 level (0.63 ± 0.01 ng/ml, $P < 0.001$) (Figure 2(a)). Serum TSH levels in the hyperthyroidal control group (0.62 ± 0.13 ng/ml) were significantly decreased compared to the Normal group (1.75 ± 0.34 μIU/ml, $P < 0.001$). SB significantly improved TSH level in the SB800 group (1.86 ± 0.41 μIU/ml, $P < 0.01$) compared to the Control group, and TSH level in the PTU group (3.91 ± 0.87 μIU/ml) increased higher than that of the Control and Normal groups ($P < 0.001$, $P < 0.001$, respectively) (Figure 2(c)).

3.3. Effects of SB on Pulse Rate and Oxygen Saturation in Hyperthyroidal Rats. The injection of LT4 significantly raised pulse rate in the hyperthyroidal control group (412.69 ± 10.84 bpm) compared to the Normal group (365.21 ± 9.54 bpm, $P < 0.001$), and SB400 (372.52 ± 6.36, $P < 0.01$), SB800 (350.88 ± 14.78, $P < 0.01$), and PTU (339.29 ± 10.25 bpm, $P < 0.001$) treatment decreased (Figure 2(d)). The oxygen saturation was slightly increased in the LT4-injected hyperthyroidal control group without significance (Figure 2(e)).

3.4. Effects of SB on Lipid Profile and Other Biochemical Profiles in Hyperthyroidal Rats. The injection of LT4 increased food intake and body weight, which induced increase in total cholesterol (Figure 3(a)), HDL cholesterol (Figure 3(b)), and triglyceride (Figure 3(d)) in the hyperthyroidal control group compared to the Normal group ($P < 0.05$, $P < 0.05$, $P < 0.05$, respectively), and SB400 decreased TG ($P < 0.05$), SB800 decreased total cholesterol ($P < 0.05$), HDL cholesterol ($P < 0.05$), and TG ($P < 0.05$), and PTU decreased TG ($P < 0.01$). The serum glucose (Figure 3(e)), AST (Figure 3(f)), and ALT (Figure 3(g)) did not show differences among the Normal, hyperthyroidal control, and SB-treated groups, but PTU significantly increased glucose compared to the Control ($P < 0.01$) and Normal groups ($P < 0.05$) and decreased AST compared to the Control group ($P < 0.05$). There was no difference among all groups in creatinine (Figure 3(h)).

3.5. Effects of SB on the Thyroid Follicular Lumen Area in Hyperthyroidal Rats. Because LT4 injection which suppresses pituitary TSH synthesis induces the decrease of thyroid cell proliferation [15], we analyzed the thyroid follicular lumen area followed by H&E stain. While marked increases in colloid space due to atrophic changes of follicular cells were observed in the hyperthyroidal control group, SB and PTU treatment restored it (Figure 1(d)). In the histomorphometric analysis, the thyroid follicular lumen area in the hyperthyroidal control group (21429.65 ± 5098.02 μm^2) was significantly increased compared to the Normal group (4648.60 ± 1077.72 μm^2, $P < 0.001$). SB significantly reduced the thyroid follicular lumen area in the

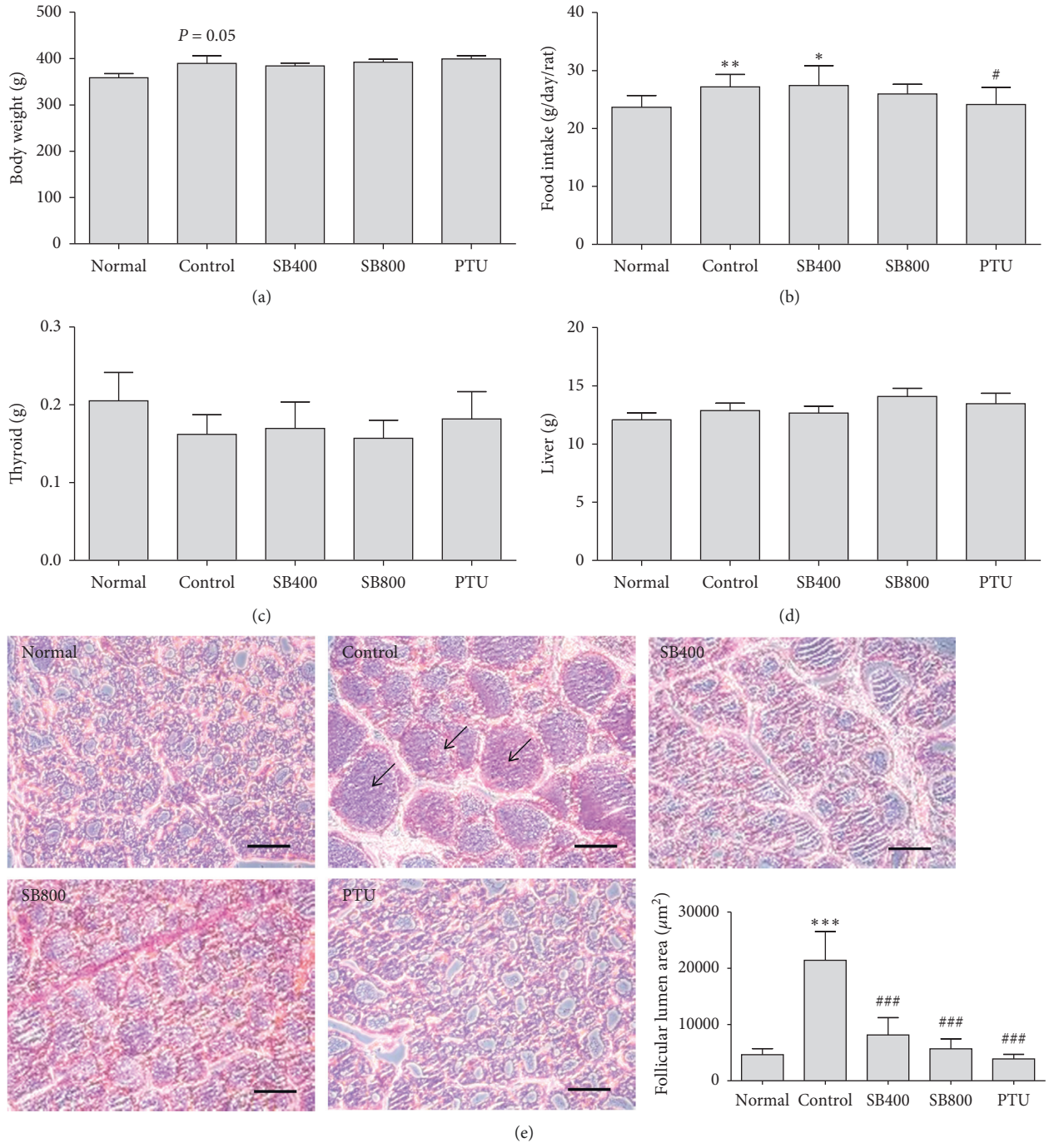


Figure 1: The changes by SB on body weight (a), food intake (b), thyroid weight (c), liver weight (d), and histological changes in thyroid (e). Representative histological images were assessed by hematoxylin and eosin (H&E) staining, scale bar indicates 100 μm, and arrow indicates thyroid follicular lumen (e). $N = 6$ in each group. Data shown as mean ± standard error of the mean (SEM). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Normal group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus Control group.

SB400 group ($8185.35 \pm 3049.92\,\mu m^2$, $P < 0.001$) and in the SB800 group ($5691.67 \pm 1764.79\,\mu m^2$, $P < 0.001$) compared to the Control group, and PTU group showed a great decrease in the thyroid follicular lumen area ($3901.213 \pm 822.24\,\mu m^2$, $P < 0.001$) (Figure 1(d)).

3.6. Effects of SB on Thyroid Hormone-Regulated Gene Expression of Liver in Hyperthyroidal Rats. The LT4 injection changes the expression of thyroid hormone-regulated genes in the liver, so we analyzed the thyroid hormone-regulated gene expression including *Dio1*, *Spot14*, and *Tbg* in liver tissue.

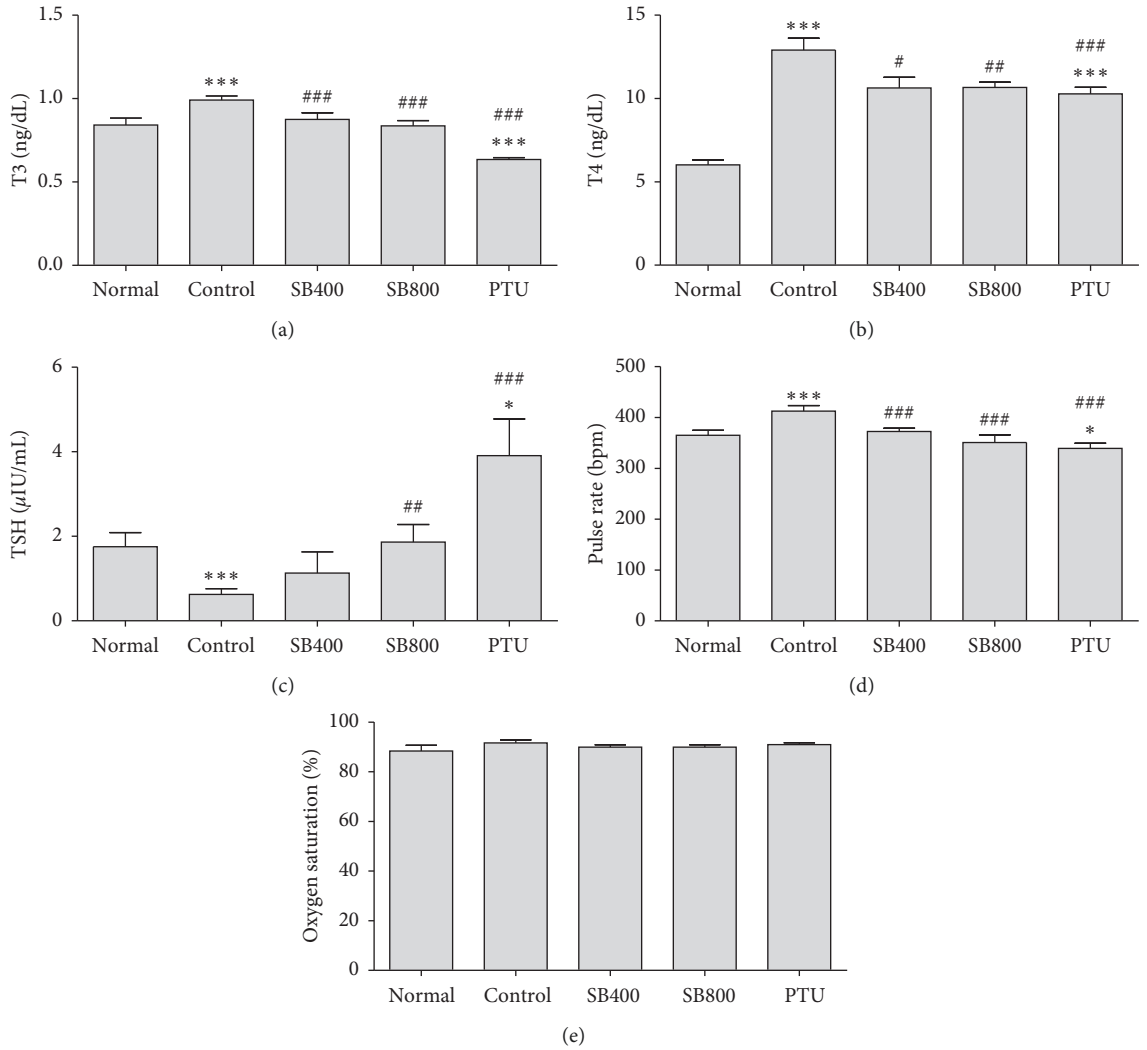


Figure 2: The changes by SB on of triiodothyronine (T3) (a), thyroxine (T4) (b), thyroid-stimulating hormone (TSH) (c), pulse rate (d), and oxygen saturation (e). Blood samples were obtained at week 4. $N = 6$ in each group. Data shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Control group.

The *Dio1* and *Spot14* expression levels were significantly increased in the hyperthyroidal control group relative to the Normal group (*Dio1,* $P < 0.001$; *Spot14,* $P < 0.01$), and SB significantly decrease the *Dio1* levels in both SB400 ($P < 0.05$) and SB800 groups ($P < 0.05$), and PTU also decreased level ($P < 0.001$) compared to the Control group (Figure 4(a)), but in case of *Spot14* expression, only PTU significantly decreased the level ($P < 0.05$) (Figure 4(b)). The expression level of *Tbg* in the hyperthyroidal control group was significantly downregulated compared to the Normal group ($P < 0.001$). SB significantly upregulated the expression level in the SB400 group ($P < 0.05$) and in the SB800 group ($P < 0.05$) compared to the Control group, and PTU group showed great upregulation of *Tbg* level ($P < 0.01$) (Figure 4(c)).

4. Discussion

Hyperthyroidism is characterized by palpitation, weight loss, increased appetite, and anxiety, which is similar to a state of increased adrenergic activity [16]. In thyrotoxicosis, plasma catecholamines are unchanged, and the beta-adrenergic receptor density is altered in a time- and tissue-dependent manner, resulting in increased tissue sensitivity to catecholamines [17]. In this study, we directly injected levothyroxine (LT4) at a dose of 0.3 mg/kg to rats for 2 weeks, causing thyrotoxic state, and we examined body weight, appetite, heart rate, and the level of thyroid hormone. Food intake significantly increased in the hyperthyroidal control group and SB400 group but not in the SB800 and PTU groups. Body weight actually increased in the hyperthyroidal control group. This result seems to be due to the short duration of hypermetabolic state, along with the increase in food intake of the rats in the Control group. In terms of the thyroid hormone, SB significantly reduced T3 and T4 levels in the SB400 group and in the SB800 group compared to the Control group, which showed similar effects to the PTU. With decreased T3 and T4, the TSH level increased in the SB800 and PTU groups. The Control group in a thyrotoxic

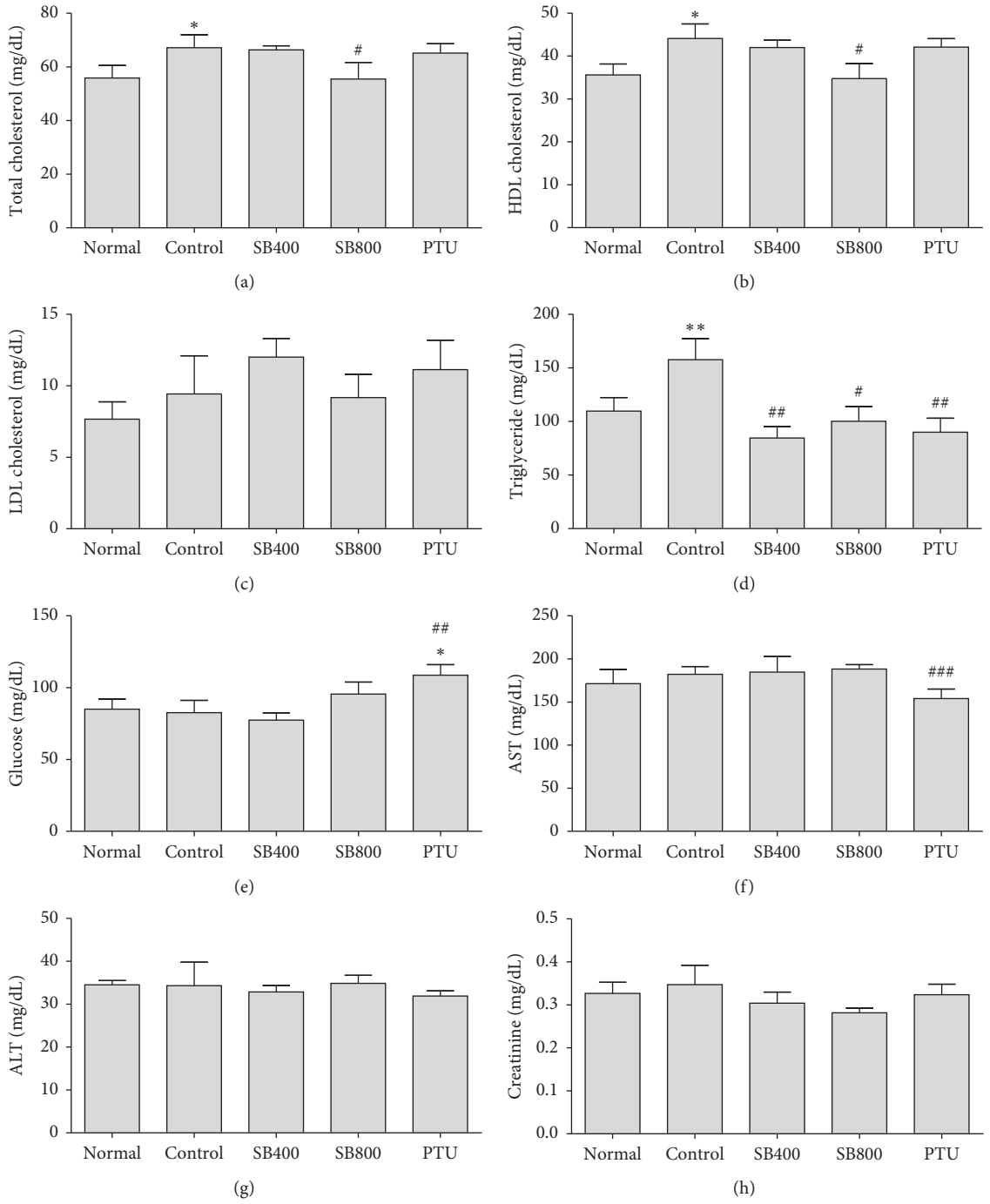


Figure 3: Effects of SB on total cholesterol (a), HDL cholesterol (b), LDL cholesterol (c), triglyceride (d), glucose (e), aspartate transaminase (AST) (f), alanine aminotransferase (ALT) (g), and creatinine (h). Blood samples were obtained at week 4. $N = 6$ in each group. Data shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Control group.

state showed increased heart rate, but the heart rate decreased in the SB400, SB800, and PTU groups. The heart relies mainly on the action of T3, since T3 is transported into the myocyte [18]. Most of T4, acting mostly as a prohormone, is converted to biologically active T3 through the removal of iodide by deiodinases [19]. Type 1 deiodinase (Dio1) activates the thyroid hormone by converting T4 to active T3, and it deactivates the thyroid hormone by converting T4 to inactive reverse T3 (rT3) or to T2 [20]. Both T4 and T3 circulate in the blood almost entirely bound to thyroxine-binding globulin [21]. The remaining unbound T3 is transported through a variety of membrane transport proteins and subsequently to the cell nucleus to regulate the expression of selected genes. Therefore, deiodinases are critical for biological effects mediated by thyroid hormone [22]. In this study, we found decreased expression of *Dio1*

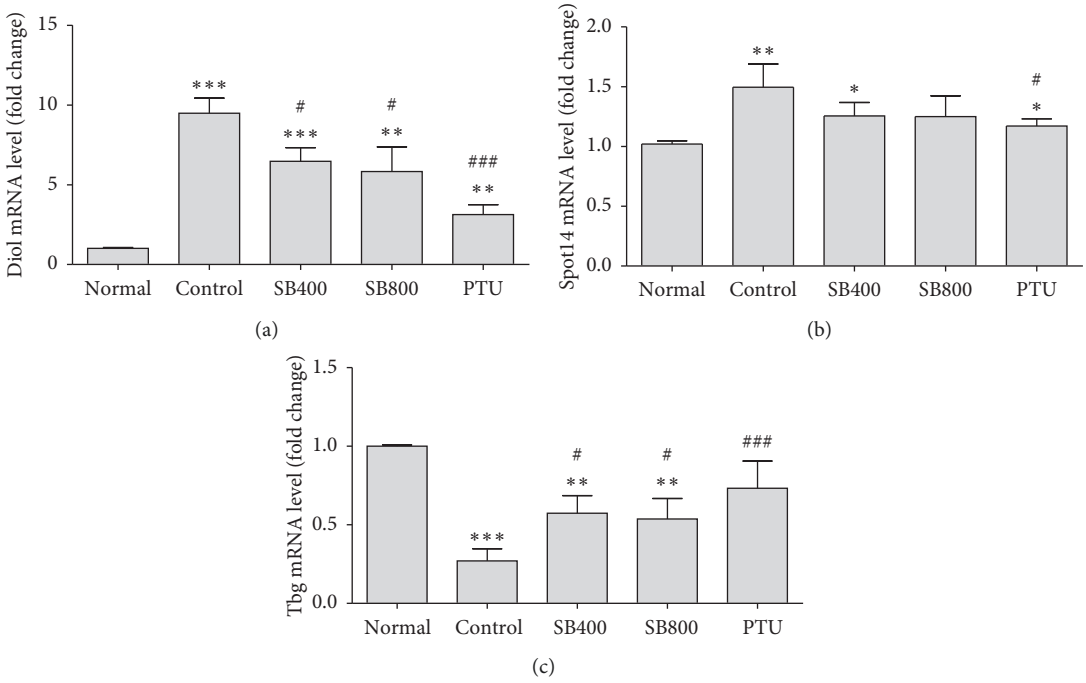


Figure 4: The changes by SB on thyroid hormone-regulated genes including deiodinase 1 (Dio1) (a), thyroid hormone responsive spot 14 (Thrsp or Spot14) (b), and thyroxine-binding globulin (Tbg) (c) expression in liver tissue. Liver tissue was obtained at week 4, and quantitative RT-PCR was used to measure. Gene expression was normalized to that of GAPDH. $N = 6$ in each group. Data shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Normal group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Control group.

and upregulated level of Tbg in the SB400, SB800, and PTU groups. In contrast, hyperthyroidal control group revealed increased expression level of *Dio1* and downregulated level of Tbg. SB and PTU suppressed the action of Dio1, therefore inhibiting the conversion of T4 into an active unbounded T3. These results suggest that SB might suppress T3, T4, and adrenergic activity by modulating Dio1 and Tbg expression. In Asia, SB has been widely used for treating cardiovascular disease and anxiety disorder [23], and SB seems to be helpful for treating these diseases by regulating thyroid hormone.

5. Conclusions

Based on these results, we conclude that SB improved thyroid hormones, adrenergic activity, and lipid metabolism in LT4-induced hyperthyrodism rats. Our findings suggest that these antithyroidal, antiadrenergic, and antilipid effects of SB could be mediated by the modulation of thyroid hormone-regulated gene expression. For the clinical use of SB, further clinical research and mechanism study on other related factors is needed.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The authors are responsible for the writing and contents of the paper.

Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgments

This study was supported by the Traditional Korean Medicine R&D program funded by the Ministry of Health & Welfare through the Korean Health Industry Development Institute (KHIDI) (HI18C0902).

References

[1] S. De Leo, S. Y. Lee, and L. E. Braverman, "Hyperthyroidism," *The Lancet*, vol. 388, no. 10047, pp. 906–918, 2016.

[2] J. Ginsberg, "Diagnosis and management of Graves' disease," *CMAJ*, vol. 168, no. 5, pp. 575–585, 2003.

[3] T. J. Smith and L. Hegedus, "Graves' disease," *New England Journal of Medicine*, vol. 375, no. 16, pp. 1552–1565, 2016.

[4] A. Antonelli, S. M. Ferrari, A. Corrado, A. Di Domenicantonio, and P. Fallahi, "Autoimmune thyroid disorders," *Autoimmunity Review*, vol. 14, no. 2, pp. 174–180, 2015.

[5] R. S. Bahn, "Autoimmunity and Graves' disease," *Clinical Pharmacology and Therapeutics*, vol. 91, no. 4, pp. 577–579, 2012.

[6] I. Klein, D. V. Becker, and G. S. Levey, "Treatment of hyperthyroid disease," *Annals of Internal Medicine*, vol. 121, no. 4, pp. 281–288, 1994.

[7] D. S. Cooper, "Antithyroid drugs," *New England Journal of Medicine*, vol. 352, no. 9, pp. 905–917, 2005.

[8] L. Wartofsky, D. Glinoer, B. Solomon et al., "Differences and similarities in the diagnosis and treatment of Graves' disease

in Europe, Japan, and the United States," *Thyroid*, vol. 1, no. 2, pp. 129–135, 1991.

[9] X. X. Zen, Y. Yuan, Y. Liu, T. X. Wu, and S. Han, "Chinese herbal medicines for hyperthyroidism," *Cochrane Database System Reviews*, vol. 2, article CD005450, 2007.

[10] K. Soon-Il, K. Ki-Hoon, K. Young-Seok et al., "The clinical effects of Ahnjeonbaekho-tang (AJBHT) on Graves' disease," *The Journal of Korean Medicine*, vol. 26, no. 4, pp. 122–129, 2005.

[11] A. C. Ferreira, P. C. Lisboa, K. J. Oliveira, L. P. Lima, I. A. Barros, and D. P. Carvalho, "Inhibition of thyroid type 1 deiodinase activity by flavonoids," *Food and Chemical Toxicology*, vol. 40, no. 7, pp. 913–917, 2002.

[12] H. Li, J. Okuda, T. Akamizu, and T. Mori, "A hyperthyroid patient with Graves' disease who was strongly resistant to methimazole: investigation on possible mechanisms of the resistance," *Endocrine Journal*, vol. 42, no. 5, pp. 697–704, 1995.

[13] B. C. Lee, S. I. Kang, Y. M. Ahn, H. K. Doo, and S. Y. Ahn, "An alternative therapy for Graves' disease: clinical effects and mechanisms of an herbal remedy," *Biological & Pharmaceutical Bulletin*, vol. 31, no. 4, pp. 583–587, 2008.

[14] G. Roy and G. Mugesh, "Anti-thyroid drugs and thyroid hormone synthesis: effect of methimazole derivatives on peroxidase-catalyzed reactions," *Journal of the American Chemical Society*, vol. 127, no. 43, pp. 15207–15217, 2005.

[15] A. Hood, Y. P. Liu, V. H. Gattone II, and C. D. Klaassen, "Sensitivity of thyroid gland growth to thyroid stimulating hormone (TSH) in rats treated with antithyroid drugs," *Toxicol Sciences*, vol. 49, no. 2, pp. 263–271, 1999.

[16] S. Ertek and A. F. Cicero, "Hyperthyroidism and cardiovascular complications: a narrative review on the basis of pathophysiology," *Archives of Medical Science*, vol. 9, no. 5, pp. 944–952, 2013.

[17] H. Vargas-Uricoechea, A. Bonelo-Perdomo, and C. H. Sierra-Torres, "Effects of thyroid hormones on the heart," *Clínica e Investigación en Arteriosclerosis*, vol. 26, no. 6, pp. 296–309, 2014.

[18] I. Klein and S. Danzi, "Thyroid disease and the heart," *Circulation*, vol. 116, no. 15, pp. 1725–1735, 2007.

[19] S. Danzi and I. Klein, "Thyroid disease and the cardiovascular system," *Endocrinology and Metabolism Clinics of North America*, vol. 43, no. 2, pp. 517–528, 2014.

[20] A. C. Bianco, D. Salvatore, B. Gereben, M. J. Berry, and P. R. Larsen, "Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases," *Endocrine Reviews*, vol. 23, no. 1, pp. 38–89, 2002.

[21] G. C. Schussler, "The thyroxine-binding proteins," *Thyroid*, vol. 10, no. 2, pp. 141–149, 2002.

[22] G. A. Brent, "Mechanisms of thyroid hormone action," *Journal of Clinical Investigation*, vol. 122, no. 9, pp. 3035–3043, 2012.

[23] C.-Z. Wang, S. R. Mehendale, T. Calway, and C.-S. Yuan, "Botanical flavonoids on coronary heart disease," *The American Journal of Chinese Medicine*, vol. 39, no. 4, pp. 661–671, 2011.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 2107231, 8 pages
https://doi.org/10.1155/2019/2107231

Research Article

Salix babylonica L. as a Natural Anticoccidial Alternative in Growing Rabbits

Nallely Rivero-Perez,[1] **Jerelly L. Hernández-Alvarado**,[1] **Benjamín Valladares-Carranza**,[2] **Lucía Delgadillo-Ruiz**,[3] **Deyanira Ojeda-Ramírez**,[1] **Carolina G. Sosa-Gutiérrez**,[1] **Ana L. Morales-Ubaldo**,[1] **Vicente Vega-Sanchez**,[1] **and Adrian Zaragoza-Bastida**[1]

[1]*Área Académica de Medicina Veterinaria y Zootecnia, Instituto de Ciencias Agropecuaria, Universidad Autónoma del Estado de Hidalgo, Av. Universidad Km 1, Ex-Hda. de Aquetzalpa, C.P. 43600 Tulancingo, Hidalgo, Mexico*
[2]*Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Km 15.5 Carretera Panamericana Toluca-Atlacomulco, C.P. 50200 Toluca, Estado de México, Mexico*
[3]*Unidad Académica de Ciencias Biológicas, Universidad Autónoma de Zacatecas, Zacatecas, Mexico*

Correspondence should be addressed to Nallely Rivero-Perez; nallely_rivero@uaeh.edu.mx and Adrian Zaragoza-Bastida; adrian_zaragoza@uaeh.edu.mx

Received 23 February 2019; Revised 29 July 2019; Accepted 16 August 2019; Published 29 August 2019

Guest Editor: Caio P. Fernandes

Coccidiosis in rabbit production is responsible for high morbidity, mortality, and economic losses. The use of natural antimicrobial substances in rabbits represents a promising way to improve their health and production. The aim of the present study was to assess the activity of *Salix babylonica* hydroalcoholic extract (SBHE) on the elimination of *Eimeria* spp. in rabbits. The phytochemical compounds and chemical composition of SBHE were determined. The cytotoxicity of SBHE was determined by a microwell assay using *Artemia salina*. Twenty-five New Zealand rabbits, 28 days old and 872 ± 171 g body weight (BW), were used in a completely randomized design. The rabbits were assigned to five groups of five rabbits, control group (A) received only basal diet (BD), B group received BD + 25 mg/kg BW of SBHE, C group received BD + 50 mg/kg BW of SBHE, D group received BD + 100 mg/kg BW of SBHE, and E group received BD + coccidiostat Baycox® (75 mg/kg body weight) for 28 days. Feces samples were collected at days 0, 7, 14, 21, and 28; morphological and morphometric identifications of *Eimeria* were carried out by the flotation technique and counting of oocysts by the McMaster technique. The rabbits were found naturally infected with *Eimeria* spp. The SBHE present phytochemicals with anticoccidial activity, and the cytotoxicity test indicate that this extract is nontoxic. This study demonstrates that oral administration of SBHE at 25 and 50 mg/kg BW reduced the release of oocysts per gram of feces. This effect was observed at day 14 and had the most significant effect at day 28 for both concentrations. The results indicate that SBHE could be a natural alternative for the control of coccidiosis in rabbit production.

1. Introduction

Rabbits are potential livestock commodities for alternative meat supplies as well as pets and laboratory animals. Rabbit meat contains high protein content, low fat, and cholesterol and is rich in calcium and phosphorus as well as high linoleic acid [1, 2]. Rabbit production is affected by different factors including viral, bacterial, and parasitic diseases. Coccidiosis in domestic rabbits (*Oryctolagus cuniculus*) is a parasitic disease caused by different species of genus *Eimeria* [3]. Eleven different species of *Eimeria* have been discovered in domestic rabbits; ten of these species colonize the intestinal tract and *Eimeria stiedae* infects the bile ducts [3, 4]. These species of *Eimeria* affect rabbits in different ways and intensities, according to their degree of pathogenicity, which can result in stunted growth and death, especially in young animals [3, 5].

The transmission of coccidiosis is due to the intake of food contaminated with feces containing the sporulated oocysts which develop within the digestive system of rabbits, where they reproduce causing lesions and are excreted again in stool to reinitiate the infection cycle [6]. Coccidiosis in rabbit production might be responsible for high morbidity, high mortality, and economic losses. Clinical signs of coccidiosis in rabbits are diarrhea, appetite loss, weight loss, dehydration, secondary sepsis, and death. However, it is common that rabbits present subclinical coccidiosis, characterized by reduced feed intake and higher feed conversion ratio [7, 8].

Coccidiosis is mainly controlled with in-feed anticoccidial drugs which have been proven effective in preventing coccidiosis. Current treatment of coccidiosis consists of sulfonamides, salinomycin, and robenidine, which may become toxic to young rabbits and pregnant females; however, few anticoccidial drugs exist for commercial use and those that are available are prescribed mainly for poultry [2, 9, 10]. Few effective products for the control and treatment of coccidiosis are available; *Eimeria* spp. can generate resistance against these anticoccidial drugs. Previous suggestions included an increase in the use and production of organic food, along with extracts of medicinal plants. There are reports that support the effect that plant extracts have on the elimination of oocysts of *Eimeria* spp. in rabbits, such as garlic (*Allium sativum*) and oregano (*Origanum vulgare*) extracts [11, 12]. However, data concerning the effects of medicinal plant extracts on the coccidiosis in rabbits are scarce. Thus, it is important to find alternatives for the treatment of coccidiosis.

Salix babylonica extract has been widely used in ruminants to improve health and productive parameters, with excellent results and without showing negative effects on animal health [13, 14]. The anthelmintic effect of *Salix babylonica* extract on gastrointestinal parasites in sheep and goats has been reported including those of the genus *Eimeria* [15, 16]. For these reasons, *Salix babylonica* hydroalcoholic extract may be an alternative treatment for coccidiosis in rabbit production.

Given the above, the aim of the present study was to assess the activity of *Salix babylonica* hydroalcoholic extract on *Eimeria* spp. elimination in rabbits.

2. Materials and Methods

2.1. Preparation of the Hydroalcoholic Extract. The leaves of *Salix babylonica* were harvested from Tulancingo de Bravo, State of Hidalgo, Mexico, during the months of June–August. For plant identification, the Herbarium of UNAM (Universidad Nacional Autonoma de Mexico) was consulted, and the vegetal specimen was identified as *Salix babylonica* L. (IBUNAM: MEXU: 9744).

The hydroalcoholic extract was prepared according to the methodology described by Rivero et al. [13] with some modifications. The fresh leaves were collected randomly from several young and mature trees, washed, and then dried at room temperature in the dark. The dried *Salix babylonica* L leaves (70 kg) were macerated using a hydroalcoholic solution of water: ethanol (30 : 70 v/v) in the proportion of 1 kg leaf per 8000 ml of solvent at room temperature for 48 h to obtain of the extract. The extract was filtered using gauze and Whatman filter paper (Whatman® 42). The solvent was eliminated using a rotary evaporator (Büchi R-300, Suiza) to obtain a semisolid extract, and this extract was lyophilized (LABCONCO®) and finally freeze-dried and stored at −4°C, until the phytochemical analysis and experimentation.

2.2. Qualitative Tests of Chemical Profile of Extract. The chemical profile of hydroalcoholic extract of *Salix babylonica* was made according to the procedure described by Bañuelos-Valenzuela et al. [17] with some modifications [9], and the chemical tests performed are shown in Table 1.

2.3. Chemical Composition of the Salix babylonica Hydroalcoholic Extract by Gas Chromatography. The chemical composition was determined by gas chromatograph (GC: Agilent Technologies series 6890N, USA), with polar column DB_WAXetr, at 250°C, 12.13 psi, and flow of 36.5 mL of He min^{-1}. Conditions for the column were as follows: initial temperature 50°C, from 0 to 2 min, increase of 10 in 10°C up to 250°C, constant for 5 min, reduction to 50°C for 2 min with flow of 1.6 mL of He min^{-1} at 12.13 psi, and average velocity of 25 cm s^{-1}. The flame ionization detectorionizing (FID) was used at 210°C with flow of 40 mL of H_2 min^{-1} and a flow of 450 mL of air min^{-1}. The standards (Sigma-Aldrich) were used in various concentrations (Table 2).

2.4. Brine Shrimp Lethality Test. The cytotoxicity of *Salix babylonica* hydroalcoholic extract was determined by a microwell assay using *Artemia salina* (brine shrimp), according with the procedure described by Solis et al. [18], with some modifications.

Brine shrimp eggs of *Artemia salina* were hatched in artificial sea water prepared from sea salt (38 gr/L) supplemented with 6 mg/L dried yeast and oxygenated with an aquarium pump. After 48 hours incubation in a warm room (29°C), nauplii were collected with a Pasteur pipette.

The hydroalcoholic extract was diluted with artificial seawater, serial dilutions were made in 96-well microplates, and the concentrations evaluated were 125 to 0.12 mg/mL. Each concentration was evaluated in triplicate. Tween® 80 (SIGMA P1754) was used a positive control. A suspension of nauplii containing 10–15 organisms (200 μl) and a concentration to evaluate was added to each well. The covered plate was incubated at 29°C for 24 hours. Plates were examined under a stereo microscope, and the numbers of dead (nonmotile) nauplii in each well were counted.

The statistical analysis was carried out following the methodology described by Syahmi et al. [19]; based on the percentage of the mortality, the concentration that led to 50% lethality (LC50) of the nauplii was determined by using the graph of mean percentage mortality versus the log of concentration using Microsoft Excel, which also formulated

Table 1: Qualitative tests to determine the chemical profile of *Salix babylonica* hydroalcoholic extract.

Qualitative tests	Sample processing
Test with $KMnO_4$ to detect unsaturation	2 mg of sample was resuspended in 1 mL of methanol, and $KMnO_4$ at 2% was added drop by drop in water. The test was positive when there was discoloration or formation of brown precipitate.
Test with $FeCl_3$ to detect phenolic oxydrils (vegetable tannins)	2 mg of sample was resuspended in 1 mL of water, and some drops of $FeCl_3$ (III) at 12.5% in water were added. The test was positive when red, blue-violet, or green precipitate was formed.
Liebermann–Bouchard test to detect sterols and triterpenes	The reactive prepared by mixing 1 mL of acetic acid and 1 mL of chloroform, cooled to 0°C, with sulfuric acid added drop by drop until there was no chemical reaction, and added drop by drop to the sample. The test was positive when blue, green, red, or orange colors were developed during that time.
Salkowski test to detect sterols and triterpenes	2 mg of sample was dissolved in NaOH at 10%. The test was positive when it developed yellow coloration which was eliminated by acidulation of the mixture.
Test of coumarins	2 mg of sample was dissolved in 10% NaOH; if a yellow coloration appears, which disappears when the test is acidulate, the test is positive.
Baljet test to detect sesquiterpenlactones	2 mg of the extract was mixed with 3 or 4 drops of the mixture solution (acid picric and NaOH). The test was positive when the coloration changed from orange to dark red.
Test of H_2SO_4 to detect flavonoids	2 mg of the sample was dissolved in H_2SO_4. Yellow coloration indicated the presence of flavonoids, orange-maroon that of flavons, bluish-red that of chalcons, and reddish-purple that of quinones.
Shinoda test for flavonoids	2 mg of sample and 1 mL of ethanol were placed in a test tube, magnesium filings (0.5 g) and three drops of concentrated HCl were added. The presence of flavonoids was confirmed when orange, red, pink, and violet coloration developed.
Dragendorff test to detect alkanoids	Two or three drops of the A (bismuth nitrate and glacial acetic acid) and B (potassium iodate) reactive were added in 2 mg of sample. Orange to red coloring was considered positive.
Tannin test	1 mL of the sample and 20 mL of H_2O were boiled in a test tube, and 3 drops of 0.1% $FeCl_3$ were added. The positive test is considered if it appears green or blue-black color.
Phlorotannins test	1 mL of the sample was boiled with 20 mL of 1% HCl. The test is considered positive if there is a presence of a red precipitate.
Steroid test	2 mL of acetic acid was placed with 0.5 mL of the extract sample and 2 mL of H_2SO_4 in a test tube. The appearance of a blue-violet-green color is considered positive.
Sodium bicarbonate test	2 mg of the sample was dissolved in water, 3 drops of sulfuric acid were added, and 3 drops of a solution of sodium bicarbonate (10%) were added. The test is considered positive with the appearance of bubbles and its permanence for more than 1 minute indicating the presence of saponins.
Salkowski test for saponins	2 mg of sample was dissolved in 1 mL of chloroform, and 1 mL of sulfuric acid was added. The test is considered positive with the appearance of a red color.

Table 2: Concentrations of standards ($mg \cdot mL^{-1}$) to determine the chemical composition of *Salix babylonica* hydroalcoholic extract of by gas chromatograph.

	Terpenes ($mg \cdot ml^{-1}$)				
Standard	Thymol	Carvacrol	Linalol	Terpinene	Limonene
1	10.373	8.284	7.744	7.154	8.496
2	5.186	4.142	3.872	3.577	4.248
3	2.593	2.071	1.936	1.789	2.124
4	1.297	1.035	0.968	0.894	1.062
5	0.648	0.518	0.484	0.447	0.531
6	0.324	0.259	0.242	0.224	0.265

the regression equations. These equations were later used to calculate LC50 values for the samples tested with consideration of value greater than 1.0 mg/mL, suggesting that the extract is nontoxic.

2.5. Animals and Management. The experiments were performed at the experimental farm of Academic Area of Veterinary Medicine and Zootechnics of the Autonomous University of Hidalgo State. Twenty-five New Zealand white rabbits, 28 days old (newly weaning), with 872 ± 171 gr of body weight were used, and the rabbits were naturally infected and belonged to a farm with a history of intestinal coccidiosis. Rabbits were kept in individual galvanized cages of size 80 cm wide × 50 cm long × 40 cm high and fed with a basal diet based on alfalfa hay, ground corn, canola paste, soybean paste, ground sorghum, molasses, soybean husk, wheat bran, and mineral premix. The diet contained 16% crude protein, 13.2% raw fiber, and 2.5 $Mcal \cdot kg^{-1}$ of metabolizable energy. The general conditions regarding hygiene and equipment were typical of this type of production, and the handling of animals was according to international bioethical standards and NOM-062-ZOO-1999 [20].

2.6. Experimental Design and Sampling. Before the beginning the experiment, the *Eimeria* species were identified using a flotation technique and the oocysts per gram of feces (OPG) were quantified with the technique of McMaster in order to confirm the natural infection by *Eimeria* spp.

The rabbits were randomly assigned to five groups with five rabbits (A, B, C, D, and E). The control group (A) received only basal diet (BD), B group received BD + 25 mg/kg body weight (BW) of *Salix babylonica* hydroalcoholic extract (SBHE) (22.5 mg in 100 gr of basal diet), C group received BD + 50 mg/kg BW of SBHE (45 mg in 100 gr of basal diet), D group received BD + 100 mg/kg BW of SBHE (90 mg in 100 gr of basal diet), E group received BD + coccidiostat Baycox® in the water for 7 consecutive days (75 mg/kg body weight). The rabbits consumed fresh water and food *ad libitum* during the 28 days of experimentation.

2.7. Evaluation of Anticoccidial Activity. Feces samples were collected with gauze placed under the cages and then placed in polyethylene bags and transferred to the laboratory at 4°C. This methodology was performed at 0, 7, 14, 21, and 28 days of experimentation. A morphological and morphometric identification of *Eimeria* species present in the experimental groups was made using the flotation technique [6, 21]. The oocyst count per gram of the feces (OPG) was quantified according to McMaster techniques with four repetitions per group [22]. The data were analyzed using PROC MIXED procedure of SAS (2002) with repeated measures; significant differences between treatment means and time were assessed using the Tukey procedure at $P < 0.05$ level.

3. Results

3.1. Chemical Composition of Hydroalcoholic Extract of S. babylonica. The SBHE exhibited unsaturation, phenolic oxidrils, coumarins, lactones, sterols, triterpenes, flavonoles, flavonoids, sesquiterpene lactone, saponins, and floratanins. Gas chromatograph determined that SBHE contained terpinene (0.3050 mg/mL), linalol (0.3901 mg/mL), thymol (0.5319 mg/mL), and carvacrol (0.4158 mg/mL), without detecting the presence of limonene (Table 3).

3.2. Brine Shrimp Lethality Test. The *Salix babylonica* hydroalcoholic extract showed positive results, indicating that the samples are biologically active. The extract resulting in LC50 values of less than 1 mg/mL is considered as significantly active which suggests that the SBHE, with LC50 values of 2.3 mg/mL at 24 hours, has a very low toxicity. Plotting of mortality percentage versus log of concentration of SBHE for all tests demonstrates an approximate linear correlation (Figure 1). Furthermore, there is a direct proportional relationship between the concentration of the extracts and the degree of lethality. This is shown by the fact the maximum mortalities occurred at a concentration of 125 mg/mL whilst a concentration of 0.12 mg/mL only caused minor mortalities.

Table 3: Phytochemical compounds of *Salix babylonica* hydroalcoholic extract.

Qualitative tests of chemical profile		Chemical composition by gas chromatography	
Compounds	SBHE	Compounds	SBHE (mg/mL)
Unsaturation	+	Terpinene	0.3050
Phenolic oxidrils	+	Limonene	0
Coumarins	+	Linalol	0.3901
Lactones	+	Thymol	0.4721
Sterols	−	Carvacrol	0.3616
Triterpenes	+		
Flavonoles	−		
Flavonoids	+		
Chalcones	−		
Quinones	−		
Sesquiterpene lactone	−		
Saponins	+		
Aromaticity	−		
Triterpenes	−		
Tannins	−		
Floratanins	+		
Steroids	−		

Note: + = detected; − = not detected; SBHE = *Salix babylonica* hydroalcoholic extract.

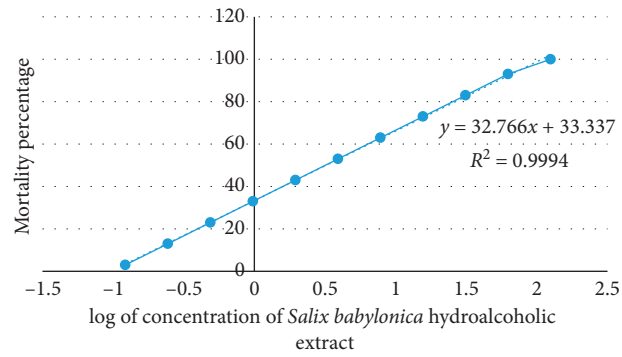


Figure 1: Brine shrimp lethality of *Salix babylonica* hydroalcoholic extract at 24 h.

3.3. Evaluation of Anticoccidial Activity. The multivariate analysis showed a significant interaction ($P < 0.001$) between time and treatment. Despite trying to form homogeneous groups, on day 0 of the experiment, there were statistical differences, treatments B, C, and D showed no significant differences ($P > 0.05$) as well as groups A and E as shown in the Table 4. Through morphological and morphometric examination, *E. stiedae*, *E. magna*, *E. coecicola*, *E. media*, *E. perforans*, and *E. exigua* were identified and all of them were distributed evenly in the experimental groups (Figure 2).

At day 7, significant statistical differences ($P < 0.001$) were observed in the average of oocysts per gram of feces (OPG), between the treatment E (Baycox®) and treatments A, B, C, and D; in these groups, the OPG were increased, without significant statistical differences between them ($P > 0.05$) (Table 4, Figure 3), with the exception of the E group (Baycox®) in which the OPG decreased 98% ($P < 0.001$) with respect to day 0 (Table 5, Figure 4).

Table 4: Efficacy of *Salix babylonica* hydroalcoholic extract on the elimination of *Eimeria* spp. oocysts in rabbits.

	Average of oocysts g^{-1} (time ± SD)				
Group	0	7	14	21	28
A	12868 ± 564^{cB}	32496 ± 156^{aB}	17962 ± 151^{bC}	7825 ± 322^{dB}	5631.3 ± 177^{eC}
B	17318 ± 597^{bA}	40318 ± 108^{aA}	17834 ± 100^{bC}	7231 ± 307^{cB}	$443.8 \pm 16^{dE*}$
C	18093 ± 371^{cA}	40062 ± 161^{aA}	37312 ± 338^{bB}	6875 ± 322^{dB}	4437 ± 161^{eD}
D	17525 ± 277^{eA}	32562 ± 161^{dB}	53343 ± 363^{bA}	60062 ± 161^{cA}	64450 ± 322^{aA}
E	12325 ± 322^{aB}	$200.0 \pm 10^{dC*}$	$487.5 \pm 32^{dD*}$	3368.8 ± 48^{cC}	9231.3 ± 306^{bB}

[abc]Different letters within a row indicate significant statistical differences in the time ($P < 0.05$). [ABC]Different letters within a column indicate significant statistical differences in the treatment ($P < 0.05$). *No statistical differences between treatments over time ($P > 0.05$).

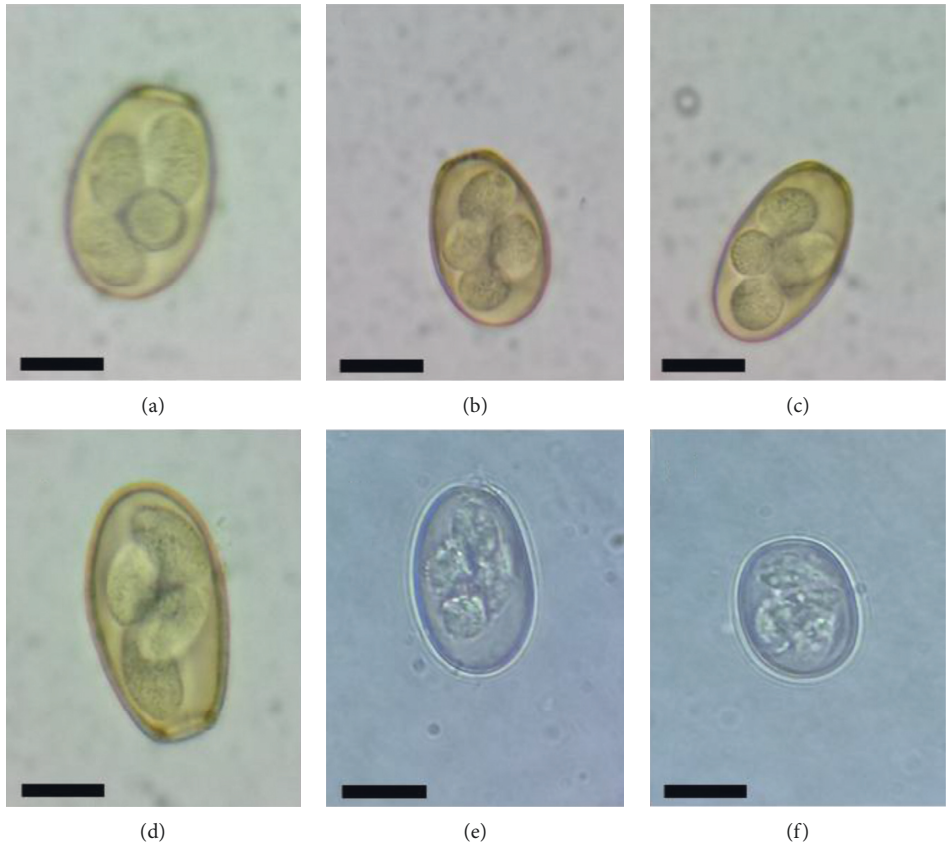


Figure 2: Light micrographs of oocysts of the six species of *Eimeria* collected from naturally infecting domestic rabbits. Scale bar = 10 μm. (a) *E. stiedae*, (b) *E. magna*, (c) *E. coecicola*, (d) *E. media*, (e) *E. perforans*, and (f) *E. exigua*.

In the next sampling (day 14), significant statistical differences were observed ($P < 0.001$) in the average of OPG. In the A and C groups, the average OPG decreased but unlike values of day 0, showing significant statistical differences ($P < 0.05$) with respect to day 0 and unlike group B in which a decrease in the average OPG was determined, without presence of significant statistical differences ($P > 0.05$) between days 0 and 14. The D group did not present a reduction of OPG on day 14. Group E (Baycox®) did not exhibit significant statistical differences ($P > 0.05$) in the average of OPG with respect to day 7 and with a reduction of 96% in the average of OPG with respect to day 0 (Tables 4 and 5; Figures 3 and 4).

Samples observed on the day 21 had significant statistical differences ($P < 0.001$) in the average of OPG, in the A, B, and C groups; the average of OPG decreased to 37, 58, and 66%, respectively, with significant statistical differences between them ($P < 0.001$); group D did not present a reduction in the release of OPG. In group E, statistically significant differences ($P < 0.001$) were observed with respect to the percentage of OPG (73%) on days 7 (98%) and 14 (96%) (Table 5).

The final day of sampling (day 28) in the experimental groups indicated statistically significant differences ($P < 0.001$) in the average of OPG (Table 4). The A, B, and C groups presented a reduction of 56, 97, and 78%, respectively, with significant statistical differences between them ($P < 0.05$) (Table 4); the percentage of reduction of OPG of group B at day 28 did not show differences on days 7 and 14 of group E (Baycox®) (Tables 4 and 5). A linear

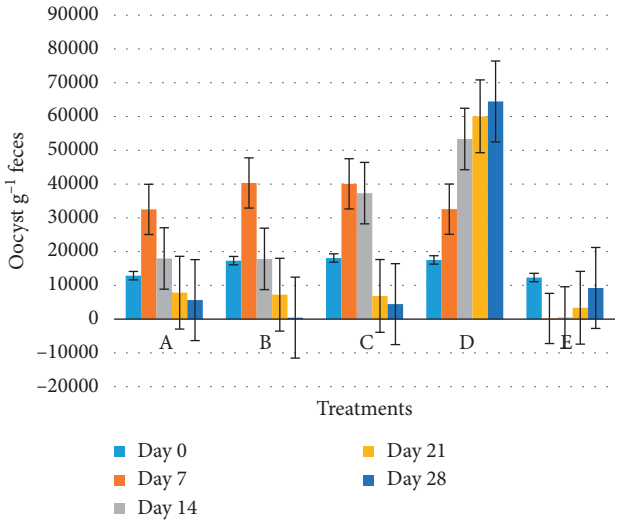


Figure 3: Efficacy of *Salix babylonica* hydroalcoholic extract on elimination of *Eimeria* spp. oocysts per group.

Table 5: Effect of *Salix babylonica* hydroalcoholic extract on the reduction percentage of *Eimeria* spp. oocysts in rabbits with respect to day 0 of the experiment.

	Day			
Group	7 (%)	14 (%)	21 (%)	28 (%)
A	0	0	37 ± 5.29^{d}	56 ± 3.31^{c}
B	0	0	58 ± 0.331^{c}	$97 \pm 0.1^{a*}$
C	0	0	66 ± 2.24^{b}	78 ± 1.2^{b}
D	0	0	0	0
E	$98 \pm 0.04^{A*}$	$96 \pm 0.36^{A*}$	73 ± 0.08^{aB}	25 ± 4.45^{dC}

^{abc}Different letters within a column indicate significant statistical differences ($P < 0.05$). ^{ABC}Different letters within a row indicate significant statistical differences ($P < 0.05$). *No statistical differences between treatments over time ($P < 0.05$).

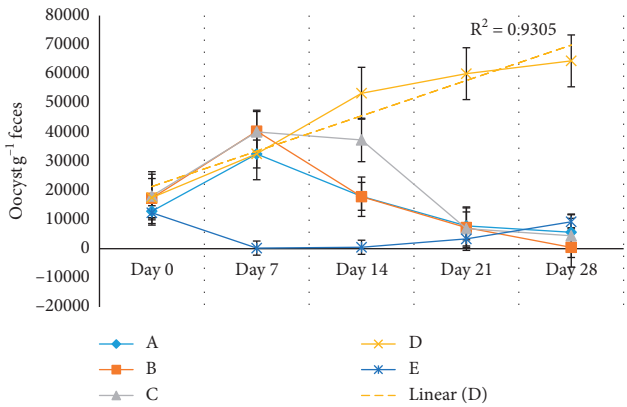


Figure 4: Efficacy of *Salix babylonica* hydroalcoholic extract on the elimination of *Eimeria* spp. oocysts the days 7, 14, 21, and 28.

tendency ($R = 0.9305$) was observed in group D in the release of oocysts per gram of feces (Figure 4). It is important to highlight that from day 21, the rabbits in the E group showed a reinfestation, because the average of OPG increased from 487.5 (day 14) to 3368.8 (day 21) until 9231.3 (day 28) (Table 4).

4. Discussion

Around the world, coccidiosis is a serious health and economic problem in rabbits, affecting mainly young rabbits after weaning [23]. The use of natural antimicrobial compounds is a promising way to improve health and commercial rabbit production. In the current work, the anticoccidial mechanism induced by *Salix babylonica* hydroalcoholic extract was not studied; however, previous studies demonstrated that phytochemical compounds of plants can suppress coccidiosis by intervention with the developmental stages of life cycle in *Eimeria* species [24]. The phytochemicals present in *Salix babylonica* hydroalcoholic extract with reports of the anticoccidial activity are coumarins, triterpenes, flavonoids, sesquiterpene lactone, saponins, terpinene, linalol, thymol, and carvacrol.

Studies conducted by Michels et al. [25] demonstrated the efficacy of coumestans (coumarin) from *Eclipta alba* against avian coccidiosis. Pop et al. [26] demonstrated the efficacy of artemisinin, a sesquiterpene lactone derived from *Artemisia annua*, against *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* in poultry. De Pablos et al. [27] demonstrated the efficacy of maslinic acid (triterpene), from leaves and fruit of olive tree (*Olea europaea* L.), against *Eimeria tenella*. Ademola et al. [28] determined the activity of *Pleurotus ostreatus* extract (with saponins, flavonoids, anthraquinones, and alkaloids) against *Eimeria* spp. In avian in vivo studies realized by Remmal et al. [29], they demonstrated the efficacy of essential oil components (terpinene, linalol, thymol, and carvacrol) against chicken *Eimeria* oocysts. According to Muthamilselvan et al. [24], the flavonoids interfere with the life cycle of *Eimeria* species through oxidative stress and the saponins, terpinene, linalol, thymol, and carvacrol by destruction of oocysts and parasites.

The LC50 value of *Salix babylonica* hydroalcoholic was determined using the brine shrimp lethality test. According to Meyer et al. [30], extracts derived from natural products which have LC50 ≤ 1.0 mg/mL are known to possess toxic effects. In this study, the LC50 value of the crude extract is 2.3 mg/mL at 24 hours. These results prove that the *Salix babylonica* hydroalcoholic extracts are nontoxic.

The animals used in the present experiment were found naturally infected with multiple species of *Eimeria*; the morphological and morphometric examination allowed species identification, as follows: *E. stiedae*, *E. magna*, *E. coecicola*, *E. media*, *E. perforans*, and *E. exigua*; Heker et al. [7] identified ten species of *Eimeria* in Brazilian rabbit farms, *E. coecicola*, *E. flavescens*, *E. intestinalis*, *E. irresidua*, *E. magna*, *E. media*, *E. perforans*, *E. vejdovskyi*, *E. media*, and *E. stiedae*; on the other hand, García-Rubio et al. [31] identified *E. magna*, *E. media*, *and E. perforans* associated with enteric problems in rabbits from the State of Mexico, Mexico. The species identified in the present investigation are associated with high morbidity, mortality, and economic losses in commercial rabbit farms [23].

The results indicate that the oral administration of *Salix babylonica* hydroalcoholic extract has an effect on the release of OPG in rabbits naturally infected. The SBHE to 25 and

50 mg/kg of BW decreased the release of OPG; however, at the threshold, 100 mg had a negative effect and presented a linear increase in the release of OPG over time. Michels et al. [25] evaluated the efficacy of a food formulation with two different doses of coumestans from *Eclipta alba* (120 and 180 ppm) against avian coccidiosis; they determined that the food formulation containing the lower dose (120 ppm) showed a therapeutic effect on *Eimeria alba*, while the higher dose of coumestan proved to be inefficient as a therapeutic agent against avian coccidiosis, and severe destruction of the cecal lining was found in the intestinal tract of broilers fed with the product containing the higher dose (180 ppm).

In this same sense, Khalafalla et al. [32] evaluated the effects of curcumin (diferuloylmethane) on *Eimeria tenella* sporozoites in vitro and they determined that sporozoite infectivity was reduced at curcumin concentrations of 100 and 200 μM by 41.6% and 72.8%, respectively, without observing negative effects of curcumin on Madin–Darby bovine kidney (MDBK) cells at these concentrations; however, curcumin at concentrations of 1800, 600, and 400 μM was toxic to MDBK cells and affected cell proliferation. According to the studies conducted, the negative effect of SBHE may be due to the toxic effect that the extract has when the concentration of it is increased as shown in Figure 1.

The best results of *Salix babylonica* hydroalcoholic extract on the release to OPG were observed at 25 and 50 mg/kg of BW; however, statistical differences ($P < 0.05$) between the groups was determined; this effect was observed since day 14 and had the most favorable outcomes on day 28 for both concentrations. Cervantes-Valencia et al. [33] evaluated the hydroalcoholic extract of *Curcuma longa* in rabbits naturally infected with *Eimeria* spp. and determined that at doses of 25 and 40 mg/kg BW, the OPG of *Eimeria* spp. decreased within 24.2 and 80.1%, respectively, on day 28 [16], while the *Salix babylonica* hydroalcoholic extract decreased the release the OPG up to 78% (Group C) and 97% (group B) on day 28 of experiment.

The results of the present study showed that rabbits treated with *Salix babylonica* hydroalcoholic extract at 25 mg/kg of BW (Group B) had a reduction of OPG, starting 14 days after the ingestion of the extract, and on day 28, a reduction of 97% was observed without observing any statistical differences on days 7 and 14 of the group treated with Baycox® (Group E). This result coincides with that reported by Simonová et al. [34] who observed a reduction in the release of OPG in rabbits naturally infected with *Eimeria* spp., on day 21 of the administration of chamomile essential oil as well as that published by Indrasanti et al. [1] who observed the same result when administering garlic extract in infected rabbits with *Eimeria stiedai*.

The group of rabbits treated with Baycox® (Group E) exhibited a reduction of the OPG observed on days 7 (98%) and 14 (96%) as expected; nevertheless, from day 21, reinfection was evident, a situation that did not occur in groups B and C. This outcome coincides with the results published by Nosal et al. [11] who observed a reinfection in rabbits by *Eimeria* spp., five weeks after treatment with Baycox®.

5. Conclusions

Salix babylonica hydroalcoholic extract decreased the release of OPG in rabbits that were naturally infected with the *Eimeria* spp. This activity is due to its content of phytochemicals with anticoccidial properties such as coumarins, triterpenes, flavonoids, sesquiterpene lactone, saponins, terpinene, linalol, thymol, and carvacrol. The best results on the reduction of OPG were observed at 25 and 50 mg/kg of BW. This effect was observed since day 14 and had the most favorable effect on day 28 for both concentrations. *Salix babylonica* hydroalcoholic extract at 25 mg/kg of BW had a reduction of OPG of 97% on day 28 of the experimentation without observing any statistical differences on days 7 and 14 of the group treated with Baycox®. In fact, reinfection was observed in those groups on day 21. Results of the cytotoxicity test showed that *Salix babylonica* hydroalcoholic extract is nontoxic. *Salix babylonica* hydroalcoholic extract could be a natural alternative for the control of the coccidiosis in rabbit production.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors would like to acknowledge the financial assistance from the Secretary of Research of Universidad Autónoma del Estado de Hidalgo (UAEH).

References

[1] D. Indrasanti, M. Indradji, S. Hastuti, E. Aprilliyani, F. Fatikha, and K. A. Rosyadi, "The administration of garlic extract on *Eimeria stiedai* oocysts and the hematological profile of the coccidia infected rabbits," *Media Peternakan*, vol. 40, no. 3, pp. 158–164, 2017.

[2] M. Pakandl, "Coccidia of rabbit: a review," *Folia Parasitologica*, vol. 56, no. 3, pp. 153–166, 2009.

[3] J. DeCubellis and J. Graham, "Gastrointestinal disease in Guinea pigs and rabbits," *Veterinary Clinics of North America: Exotic Animal Practice*, vol. 16, no. 2, pp. 421–435, 2013.

[4] U. C. Oliveira, J. S. Fraga, D. Licois, M. Pakandl, and A. Gruber, "Development of molecular assays for the identification of the 11 *Eimeria* species of the domestic rabbit (*Oryctolagus cuniculus*)," *Veterinary Parasitology*, vol. 176, no. 2-3, pp. 275–280, 2011.

[5] J. Kvičerová, M. Pakandl, and V. hypša, "Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features," *Parasitology*, vol. 135, no. 4, pp. 443–452, 2008.

[6] M. C. Campillo and F. A. Rojo, *Parasitologia Veterinaria. Parasitos Del Conejo*, MacGraw-Hill Interamericana, New York, NY, USA, 1999.

[7] M. M. Heker, A. A. Nakamura, B. N. Santana, and M. V. Meireles, "Etiological aspects of Eimeria spp. infection in Brazilian rabbit (Oryctolagus cuniculus) farms," *Veterinary Parasitology: Regional Studies and Reports*, vol. 8, pp. 78–81, 2017.

[8] S. Maziz-Bettahar, M. Aissi, H. Ainbaziz, M. S. Bachene, S. Zenia, and F. Ghisani, "Prevalence of coccidian infection in rabbit farms in north Algeria," *Veterinary World*, vol. 11, no. 11, pp. 1569–1573, 2018.

[9] T. K. Bhat, K. P. Jlthendran, and N. P. Kurade, "Rabbit coccidiosis and its control: a review," *World Rabbit Science*, vol. 4, no. 1, p. 1, 1996.

[10] K. O. Ogolla, P. K. Gathumbi, R. M. Waruiru, P. O. Okumu, J. Chebet, and P. M. Kitala, "Efficacy of sulphachloropyrazine, amprolium hydrochloride, trimethoprim-sulphamethoxazole, and diclazuril against experimental and natural rabbit coccidiosis," *Journal of Veterinary Medicine*, vol. 2018, Article ID 5402469, 11 pages, 2018.

[11] P. Nosal, D. Kowalska, P. Bielański, J. Kowal, and S. Kornas, "Herbal formulations as feed additives in the course of rabbit subclinical coccidiosis," *Annals of Parasitology*, vol. 60, no. 1, pp. 65–69, 2014.

[12] R. Szabóová, A. Lauková, Ľ. Chrastinová et al., "Benefficial effect of plant extracts in rabbit husbandry," *Acta Veterinaria Brno*, vol. 81, no. 3, pp. 245–250, 2012.

[13] N. Rivero, A. Salem, H. Gado et al., "Effect of exogenous enzymes and *Salix babylonica* extract or their combination on haematological parameters in growing lambs," *Journal of Animal and Feed Sciences*, vol. 21, no. 4, pp. 577–586, 2012.

[14] J. Cedillo, J. F. Vázquez-Armijo, A. González-Reyna et al., "Effects of different doses of *Salix babylonica* extract on growth performance and diet in vitro gas production in pelibuey growing lambs," *Italian Journal of Animal Science*, vol. 13, no. 3, p. 3165, 2014.

[15] A. Z. M. Salem, M. M. Y. Elghandour, A. E. Kholif et al., "Tree leaves of *Salix babylonica* extract as a natural anthelmintic for small-ruminant farms in a semiarid region in Mexico," *Agroforestry Systems*, vol. 91, no. 1, pp. 111–122, 2017.

[16] P. M. Hernandez, A. Z. M. Salem, M. M. M. Y. Elghandour, M. Cipriano-Salazar, B. Cruz-Lagunas, and L. M. Camacho, "Anthelmintic effects of *Salix babylonica* L. and *Leucaena leucocephala* Lam. extracts in growing lambs," *Tropical Animal Health and Production*, vol. 46, no. 1, pp. 173–178, 2014.

[17] R. Bañuelos-Valenzuela, L. Delgadillo-Ruiz, F. Echavarría-Cháirez, O. Delgadillo-Ruiz, and C. Meza-López, "Chemical composition and ftir of ethane extracts of *Larrea tridentata*, *Origanum vulgare*, *Artemisa ludoviciana* and *Ruta graveolens*," *Agrociencia*, vol. 52, pp. 309–321, 2018.

[18] P. N. Solis, C. W. Wright, M. M. Anderson, M. P. Gupta, and J. D. Phillipson, "A microwell cytotoxicity assay using Artemia salina (brine shrimp)," *Planta Medica*, vol. 59, no. 03, pp. 250–252, 1993.

[19] A. R. M. Syahmi, S. Vijayarathna, S. Sasidharan et al., "Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* jacq., (oil palm leaf) methanol extract," *Molecules*, vol. 15, no. 11, pp. 8111–8121, 2010.

[20] Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA), *Norma Oficial Mexicana NOM-062-ZOO-1999. Especificaciones Técnicas Para la Producción, Cuidado y Uso De Los Animales De Laboratorio*, Diario Oficial de la Federación, Mexico City, Mexico, 1996.

[21] J. Karamon, I. Ziomko, T. Cencek, and J. Sroka, "Modified flotation method with the use of percoll for the detection of isospora suis oocysts in suckling piglet faeces," *Veterinary Parasitology*, vol. 156, no. 3-4, pp. 324–328, 2008.

[22] C. Bortoluzzi, K. L. Paras, T. J. Applegate, and G. G. Verocai, "Comparison between Mcmaster and mini-FLOTAC methods for the enumeration of *Eimeria maxima* oocysts in poultry excreta," *Veterinary Parasitology*, vol. 254, pp. 21–25, 2018.

[23] C. Papeschi, G. Fichi, and S. Perrucci, "Oocyst excretion pattern of three intestinal *Eimeria* species in female rabbits," *World Rabbit Science*, vol. 21, no. 2, pp. 77–83, 2013.

[24] T. Muthamilselvan, T.-F. Kuo, Y.-C. Wu, and W.-C. Yang, "Herbal remedies for coccidiosis control: a review of plants, compounds, and anticoccidial actions," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 2657981, 19 pages, 2016.

[25] M. G. Michels, L. C. T. Bertolini, A. F. Esteves, P. Moreira, and S. C. Franca, "Anticoccidial effects of coumestans from Eclipta alba for sustainable control of *Eimeria tenella* parasitosis in poultry production," *Veterinary Parasitology*, vol. 177, no. 1-2, pp. 55–60, 2011.

[26] L. Pop, A. Györke, A. F. Tăbăran et al., "Effects of artemisinin in broiler chickens challenged with *Eimeria acervulina, E. maxima* and *E. tenella* in battery trials," *Veterinary Parasitology*, vol. 214, no. 3-4, pp. 264–271, 2015.

[27] L. M. De Pablos, M. F. B. dos Santos, E. Montero, A. Garcia-Granados, A. Parra, and A. Osuna, "Anticoccidial activity of maslinic acid against infection with *Eimeria tenella* in chickens," *Parasitology Research*, vol. 107, no. 3, pp. 601–604, 2010.

[28] I. O. Ademola, P. O. Ojo, and P. O. Odeniran, "*Pleurotus ostreatus* extract inhibits *Eimeria* species development in naturally infected broiler chickens," *Tropical Animal Health and Production*, vol. 51, no. 1, pp. 109–117, 2019.

[29] A. Remmal, S. Achahbar, L. Bouddine, F. Chami, and N. Chami, "Oocysticidal effect of essential oil components against chicken *Eimeria* oocysts," *International Journal of Veterinary Medicine: Research & Reports*, vol. 2013, Article ID 599816, 2013.

[30] B. Meyer, N. Ferrigni, J. Putnam, L. Jacobsen, D. Nichols, and J. McLaughlin, "Brine shrimp: a convenient general bioassay for active plant constituents," *Planta Medica*, vol. 45, no. 05, pp. 31–34, 1982.

[31] V. G. García-Rubio, L. G. Bautista-Gómez, J. S. Martínez-Castañeda, and C. Romero-Núñez, "Multicausal etiology of the enteric syndrome in rabbits from Mexico," *Revista Argentina de Microbiología*, vol. 49, no. 2, pp. 132–138, 2017.

[32] R. E. Khalafalla, U. Müller, M. Shahiduzzaman et al., "Effects of curcumin (diferuloylmethane) on *Eimeria tenella* sporozoites in vitro," *Parasitology Research*, vol. 108, no. 4, pp. 879–886, 2011.

[33] M. E. Cervantes-Valencia, Y. Alcala-Canto, A. Z. M. Salem et al., "Influence of curcumin (*Curcuma longa*) as a natural anticoccidial alternative in adult rabbits: first results," *Italian Journal of Animal Science*, vol. 14, no. 3, p. 3838, 2015.

[34] M. Simonová, V. Strompfová, M. Marciňáková et al., "Chamomile essential oil and its experimental application in rabbits," *Acta Horticulturae*, vol. 749, no. 749, pp. 197–201, 2007.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 1524195, 8 pages
https://doi.org/10.1155/2019/1524195

Research Article

Evaluation of Alternative Methods to Assess the Biological Properties of Propolis on Metabolic Activity and Biofilm Formation in *Streptococcus mutans*

Jorge Jesús Veloz,[1] **Marysol Alvear**,[2,3] **and Luis A. Salazar**[3]

[1]*Departamento de Ciencias Biológicas y Químicas, Facultad de Medicina y Ciencia, Universidad San Sebastián, Campus Los Leones, Lota 2465, 7510157 Providencia, Santiago, Chile*
[2]*Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Avenida Francisco Salazar 01145, 4811230 Temuco, Chile*
[3]*Center of Molecular Biology and Pharmacogenetics, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Avenida Francisco Salazar 01145, 4811230 Temuco, Chile*

Correspondence should be addressed to Luis A. Salazar; luis.salazar@ufrontera.cl

Received 17 February 2019; Revised 29 June 2019; Accepted 17 July 2019; Published 18 August 2019

Academic Editor: Lucindo Q. Júnior

Several biological activities have been reported for the Chilean propolis, among their antimicrobial and antibiofilm properties, due to its high polyphenol content. In this study, we evaluate alternative methods to assess the effect of Chilean propolis on biofilm formation and metabolic activity of *Streptococcus mutans* (*S. mutans*), a major cariogenic agent in oral cavity. Biofilm formation was studied by using crystal violet and by confocal microscopy. The metabolic activity of biofilm was evaluated by MTT and by flow cytometry analysis. The results show that propolis reduces biofilm formation and biofilm metabolic activity in *S. mutans*. When the variability of the methods to measure biofilm formation was compared, the coefficient of variation (CV) fluctuated between 12.8 and 23.1% when using crystal violet methodology. On the other hand, the CV ranged between 2.2 and 3.3% with confocal microscopy analysis. The CV for biofilm's metabolic activity measured by MTT methodology ranged between 5.0 and 11.6%, in comparison with 1.9 to 3.2% when flow cytometry analysis was used. Besides, it is possible to conclude that the methods based on colored compounds presented lower precision to study the effect of propolis on biofilm properties. Therefore, we recommend the use of flow cytometry and confocal microscopy in *S. mutans* biofilm analysis.

1. Introduction

Propolis is a product collected by honeybees (*Apis mellifera*) and formed by the resinous excretions of buds and bark of trees and shrubs [1]. Propolis is widely used for its medicinal properties. Several biological activities have been reported for propolis including anti-inflammatory, antibacterial, antifungal and/or antiviral, immunomodulatory properties, suppression of HIV-1 replication and immunoregulatory effect, cytotoxicity, hepatoprotection, and free-radical scavenging activity [2–5].

Similarly, the Chilean propolis has showed different biological properties, including antiangiogenic [6–8], anti-atherosclerotic [7, 9], antifungal [10], antidiabetic [11], antimicrobial [12–16], hepatoprotective [17], anti-proliferative [18], and antioxidant [18] activities, due to its high content of polyphenols, mainly pinocembrin [13].

In relation to antimicrobial activity, our group has demonstrated specifically the antibiofilm properties of the Chilean propolis against *Streptococcus mutans* (*S. mutans*) [14–16], a major cariogenic agent in oral cavity. Biofilms are clusters of single or multiple species of bacteria encased in a matrix composed of polysaccharides, proteins, and DNA that protect the bacteria from environmental pressures. There are many protocols that have been proposed to analyze relative biofilm formation [19]. However, some assays cannot usually distinguish between planktonic killing by the antibiotic and specific antibiofilm effects since bacteria are

exposed to the compound of interest before they have a chance to adhere [20]. Similarly, assaying residual bound bacteria using crystal violet, the most widely used method to evaluate biofilm activity, has issues since crystal violet stains biomass rather than living bacteria, and thus, dead bound bacteria will still be stained [19, 20]. Thus, it is important to assess the best method to monitor and to analyze biofilm growth in the presence of antibiofilm/antimicrobial agents. Here, we compared various methods for quantifying the antibiofilm activity of Chilean propolis on *S. mutans*, including crystal violet staining, metabolic dyes, flow cytometry, and confocal microscopy.

2. Materials and Methods

2.1. Preparation of Polyphenol-Rich Extract of Propolis (EP). To evaluate the effect of polyphenols from EP in antimicrobial activity and biofilm formation of *S. mutans*, propolis was collected during the Spring of 2008 from the La Araucanía region (Chile). A crude propolis sample was kept frozen (−20°C) and later crushed in cold, and 30 grams was dissolved in 100 mL of ethanol (70%) and macerated for 7 days at room temperature. The ethanolic extract of propolis (EEP) was filtered with a Whatman 2.0 paper and centrifuged at 327 g, during 20 minutes at 5°C. Finally, the solvent was evaporated at a temperature of 40°C, for 2 hours in a rotavaporator (Buchi, R-210, Germany) and dissolved for 24 h with sterile DMSO (0.01%) to obtain polyphenol-rich extract of propolis (EP).

2.2. Determination of Total Phenolic Content in EP. The content of total polyphenols in EP was quantified by Folin–Ciocalteu reaction by a modification of Popova and collaborator's methodology [21]. For this assay, 100 μL of EP was mixed with 100 μL of distilled water and 2 mL of Folin–Ciocalteu reagent (Merck, Germany). The resulting solution was incubated for 8 minutes, and finally, 3 mL of sodium carbonate (20%) (w/v) was added. The absorbance of this solution was measured at 760 nm after 2 hours of incubation at room temperature. The concentration of polyphenols was calculated from a calibration curve and was expressed in mg·mL^{-1} equivalent to the pinocembrin-galangin standard mixture (1 : 1).

2.3. Bacteria Culture Conditions and Inoculum. Bacteria were obtained from clinical isolates from children with tooth decay. *S. mutans* was identified using the methodology proposed by Salazar et al. [22]. The cultures were made in Petri plates with Columbia agar (Becton Dickinson and Co., NY, and USA) supplied with sucrose (1%) in an anaerobic container (GasPak EZ. Becton Dickinson and Co., NY, USA) and it was incubated at 37°C and 5% of CO_2, for 24 hours. The inoculum was adjusted using optical density comparison from 1.0 to 550 nm which corresponds to 2×10^8 CFU·mL^{-1} (stock suspension).

2.4. Determination of Antibacterial Activity. Minimum inhibitory concentration (MIC) was determined by the serial dilution method following the NCCLS guidelines [23]. The suspension of 5×10^5 CFU·mL^{-1} was inoculated in 96-well microplates, containing 100 μL of sterile trypticase soy broth (Becton Dickinson and Co., NY, USA) with sucrose 1% and with different concentrations of polyphenol-rich extract of propolis (EP) (from 0.1 to 1.96 μg·mL^{-1}). Chlorhexidine digluconate (0.2%) was used as positive control, and cultures in DMSO (0.01%) without propolis were used as a negative control. The assay was performed in triplicate and incubated for 48 hours.

2.5. Biofilm Formation and S. mutans Adherence. Biofilm growth was quantified and indirectly assessed by crystal violet staining assay. The *S. mutans* attachment cells were grown in microplates with sterile trypticase soy broth (TSB) and sucrose (1%). *S. mutans* cultures were supplied with concentrations of EP, between 0.1 and 1.96 μg·mL^{-1}, under anaerobic conditions 37°C and 5% of CO_2, for 48 hours. First, the broth was removed, the plates were washed three times to eliminate nonadherent bacteria with PBS, and the plates were dried at 60°C for 45 minutes. After that, each well was stained with 100 μL of crystal violet 1% (w/v) solution, incubated for 15 minutes, and washed again with sterile PBS. Biofilm formation was determined by adding 125 μL of ethanol 95% per well and then transferred to a new plate to measure the optical density (OD) at 590 nm in a microplate reader in comparison with the control biofilm (without EP) [24].

2.6. Biofilm Growth for Morphology Analysis. For biofilm generation, samples were prepared in FluoroDish plates (World Precision Instrument Inc., China) that contained 3 mL of sterile tripticase soy broth supplied with sucrose, 1%. The plates were prepared with 10 μL of bacterial inoculum (5×10^5 UFC·mL^{-1}) for incubation at 5% of atmosphere CO_2, at 37°C for 48 hours. Biofilm was generated in a liquid medium, and different concentrations of EP were added (0.1 to 1.96 μg·mL^{-1}).

2.7. Fluorescent Labeling. Biofilms were stained with calcein Biofilm Tracer™ (Invitrogen, the USA). First, the plates were incubated with 50 μL of calcein probe and were incubated for 1 hour. After incubation, a medium was removed and, then, the plates were washed three times using PBS to eliminate unabsorbed tracer [22].

2.8. Microscopic Analysis of Biofilm Thickness. A 60×0.21 NA objective lens was used to visualize bacterial plaque by means of the confocal scanning laser microscope Olympus Fluoview 100. For imaging antimicrobial effect, the 480 nm laser was used for excitation and the fluorescent signal was detected in a green channel. All images were captured by directed acquisition by *Z*-step, ranging to take a series of time-lapse image scans (512×512 pixels) at intervals of 15 seconds and 0.5 μm for each confocal plane. Data were analyzed by ImageJ Mac Biophotonic software.

2.9. Analyses of Biofilm Metabolic Activity in 96-Well Microplates. EP cytotoxicity was performed by the

modified reduction assay of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium (MTT) bromide (Sigma-Aldrich, France). Because of this, the reagent was dissolved in PBS, taking it to a concentration of 5 mg·mL^{-1}, and added in microplate wells containing 90 μL of trypticase soy broth, plus sucrose (1%). Besides, 10 μL of a bacterial suspension equivalent to 5×10^4 CFU·mL^{-1} was added and supplied with concentrations of EP (0.1 to 1.6 μg·mL^{-1}) and 200 μL of MTT to obtain formazan. The plates were incubated at 37°C and 5% CO_2 for 48 hours. The absorbance of obtained solutions was quantified at 490 nm. Cytotoxicity was expressed in percentages of inhibition in the cellular viability in cultures with less than 50% growth (IC50%) and compared to the color developed by untreated cells. The controls included DMSO at 0.01% as vehicle control, and untreated cells (negative control) and chlorhexidine digluconate at 0.2% as positive control. All the tests were carried out in triplicate.

The inhibition percentage of biofilm viability was calculated using the following formula:

$$\text{inhibition percentage} = \frac{\text{control OD570} - \text{treated OD570}}{\text{control OD570}} * 100. \quad (1)$$

2.10. Biofilm Viability by Flow Cytometry. A biofilm was formed in glass tubes at anaerobial conditions at 37°C and for 48 hours using sterile trypticase soy broth (TSB), sucrose (1%), and EP (0.1 to 1.96 μg·mL^{-1}) to a final volume of 10.000 μL. After incubation, an aliquot of 100 μL of biofilm suspension was transferred to an eppendorf and was stained with Live/Dead® BacLight Bacterial Viability Kit. First, 10 μL of Syto9 (20 μM) was added for 15 minutes, and later, 10 μL of PI (800 μM) was added for 5 minutes; the tubes were washed twice with sterile PBS and centrifuged for 1 minute at 5000 g. Finally, cells were resuspended in sterile PBS, and they were analyzed by flow cytometry (FACS CantoII™, BD Biosytem). The sample with two stain components was excited at 488 nm, and the emission was registered using the FITC channel for Syto 9 (530/30) and PerCP channel (670/LP) for propidium iodide. Suspension containing 5×10^6 cell·mL^{-1} were aspirated with a flow rate 12 μL·min^{-1}. The results of biofilm cell viability were expressed in percentage in relation with untreated control cells [24].

2.11. Statistical Analysis. Statistical analyses were performed using the computational Statistical Program R, version 3.5.1. The D'Shapiro–Wilson test was applied to determine the results' normal distribution. Afterwards, the values were analyzed using the nonparametric Wilcoxon test for related samples. The values are expressed as median ± SD. The variability of the evaluated methods was determined by calculating the coefficient of variation (CV). Significant differences were considered at $p < 0.05$.

3. Results

3.1. Total Polyphenol Content in Chilean Propolis. The content of polyphenols in EP in equivalence of the pinocembrin-galangin mixture was quantified by Folin–Ciocalteu reaction, and it was 137.7 ± 0.7 mg·g^{-1}. Previous studies of our group described the chemical composition of the EP. The main flavonoids identified in the Chilean propolis by means of the HPLC-DAD as quercetin, apigenin, pinocembrin, and caffeic acid phenethyl ester (CAPE) [13, 16].

3.2. Biofilm Inhibition Assessed by Crystal Violet Staining. The percentage of biofilm inhibition in cultures was calculated considering untreated cells as 100% of biofilm growth (control). When EP was added at 1.96 μg·mL^{-1} and 0.8 μg·mL^{-1}, the values of biofilm reduction were 53.4 ± 2.0% and 47.7 ± 2.0%, respectively ($p < 0.01$). Similarly, when we used EP at a concentration of 0.4 μg·mL^{-1}, the bacterial pellicle decreased in 43.5 ± 3.3% ($p < 0.01$). Also, when we used EP at 0.2 and 0.1 μg·mL^{-1}, these concentrations prevented the biofilm growth in 24.9 ± 4.0% and 19.2 ± 3.3%, respectively ($p < 0.05$). Finally, chlorhexidine and DMSO reduced the biofilm formation in 18.8 ± 2.9% and 8.8 ± 2.1%, respectively (Figure 1).

3.3. Biofilm Formation Analysis by Confocal Microscopy. The extracellular matrix (biofilm) obtained from *S. mutans* cultures treated with EP at a concentration of 0.8 μg·mL^{-1} shows a size of 7.3 ± 0.2 μm in comparison to the control biofilm (20.8 ± 0.3 μm); when 0.4 μg·mL^{-1} of polyphenols was applied, the obtained biofilm was 9.3 ± 0.2 μm; other concentrations such as 0.2 μg·mL^{-1} and 0.1 μg·mL^{-1} generated greater biofilms, with 10.7 ± 0.2 μm and 15.0 ± 0.2 μm. Although the effect of chlorhexidine was higher than these low concentrations, the synthetic compound allowed sizes of 13.9 ± 0.6 μm. This may probably be because excipients contained in their formulation may be affecting this result. Figure 2 shows the effect of different polyphenol concentrations in biofilm thickness.

3.4. Analyses of Biofilm's Metabolic Activity by MTT. The MTT method showed significant reduction values in a percentage of cellular viability for concentrations lower than MIC. For the EP concentration at 0.8 μg·mL^{-1}, the reduction was 58.2 ± 3.5%; for EP at 0.4 μg·mL^{-1} was 69.2 ± 8.6%; for EP at 0.2 μg·mL^{-1} was 82.1 ± 8.8%, and for EP at 0.1 μg·mL^{-1} was 85.0 ± 7.2%. The reduction for chlorhexidine was 83.0 ± 7.0%, with significant statistical differences. Cellular viability at different EP concentrations is shown in Figure 3.

3.5. Biofilm Metabolic Activity of S. mutans by Flow Cytometry. Figure 4 shows individual dot plots of the *S. mutans* biofilm analyzed by flow cytometry; the assays to measure metabolic activity in the *S. mutans* biofilm generated for 48 hours facilitated the differentiation of live and dead cell populations performed with excitation/emission fluorescence Syto 9 and propidium iodide stains. Some EP concentrations such as 0.8 μg·mL^{-1} (0.04 ± 0.005% of live cells) and 0.4 μg·mL^{-1} (0.08 ± 0.004% of live cells) had a higher effect than chlorexidine in the reduction of live cells detected; the value for chlorexidine was 0.95 ± 0.003%. For the EP at 0.2 μg·mL^{-1} and EP at 0.1 μg·mL^{-1}, a low number of detected viable cells but less than chlorhexidine was observed (1.41 ± 0.004 and 4.1 ± 0.008 of live cells; Figure 5).

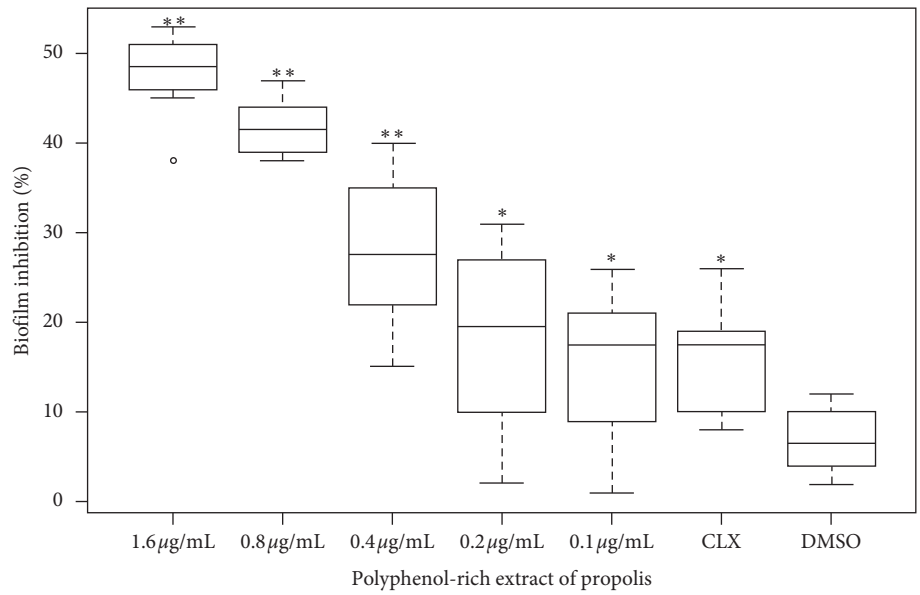


Figure 1: Biofilm inhibition evaluated by crystal violet staining. *S. mutans* cultures were treated with different polyphenol-rich extract of propolis concentrations (0.1 to 1.6 $\mu g \cdot mL^{-1}$). CLX, chlorhexidine gluconate 0.2% (positive control); DMSO (vehicle control). $^{*}p < 0.05$ or $^{**}p < 0.01$ from the nonparametric Wilcoxon test when compared to untreated cells (100% of growth biofilm). Values are expressed as median ± SD.

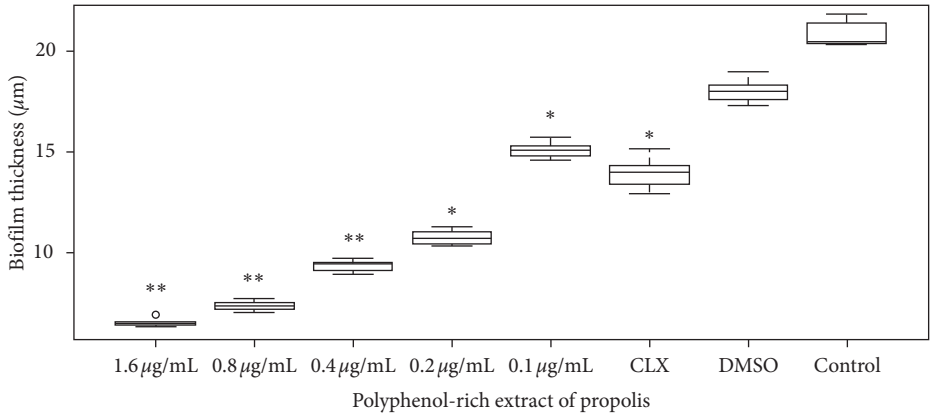


Figure 2: Effect of different polyphenol-rich extract of propolis concentrations in biofilm formation by confocal microscopy. Antibiofilm activity was expressed as thickness (median ± SD) in *S. mutans* cultures treated with different polyphenol-rich extract of propolis concentrations. $^{*}p < 0.05$ and $^{**}p < 0.01$ from the nonparametric Wilcoxon test when compared to untreated cells.

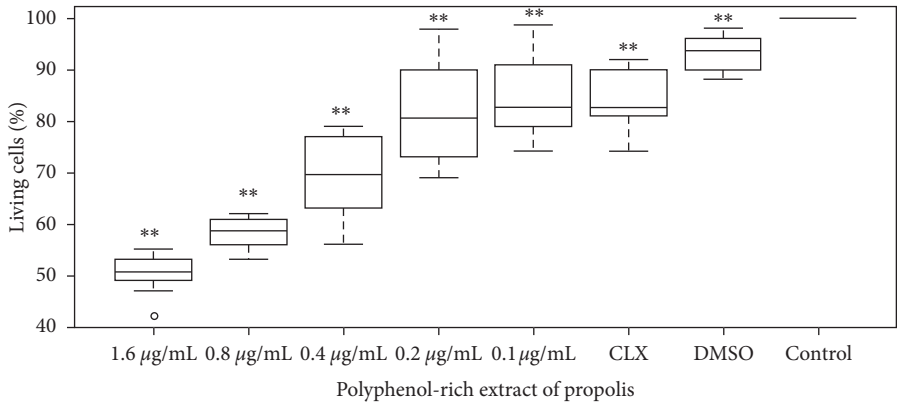


Figure 3: Biofilm metabolic activity determined by the MTT method. The effect of polyphenol-rich extract of propolis in *S. mutans* cultures was quantified in 96-well microplates. The percentage of living biofilm cells was expressed as mean ± standard deviation. CLX, chlorhexidine digluconate 0.2% (positive control), DMSO (vehicle control). $^{**}p < 0.01$ from the nonparametric Wilcoxon test when compared to untreated cells.

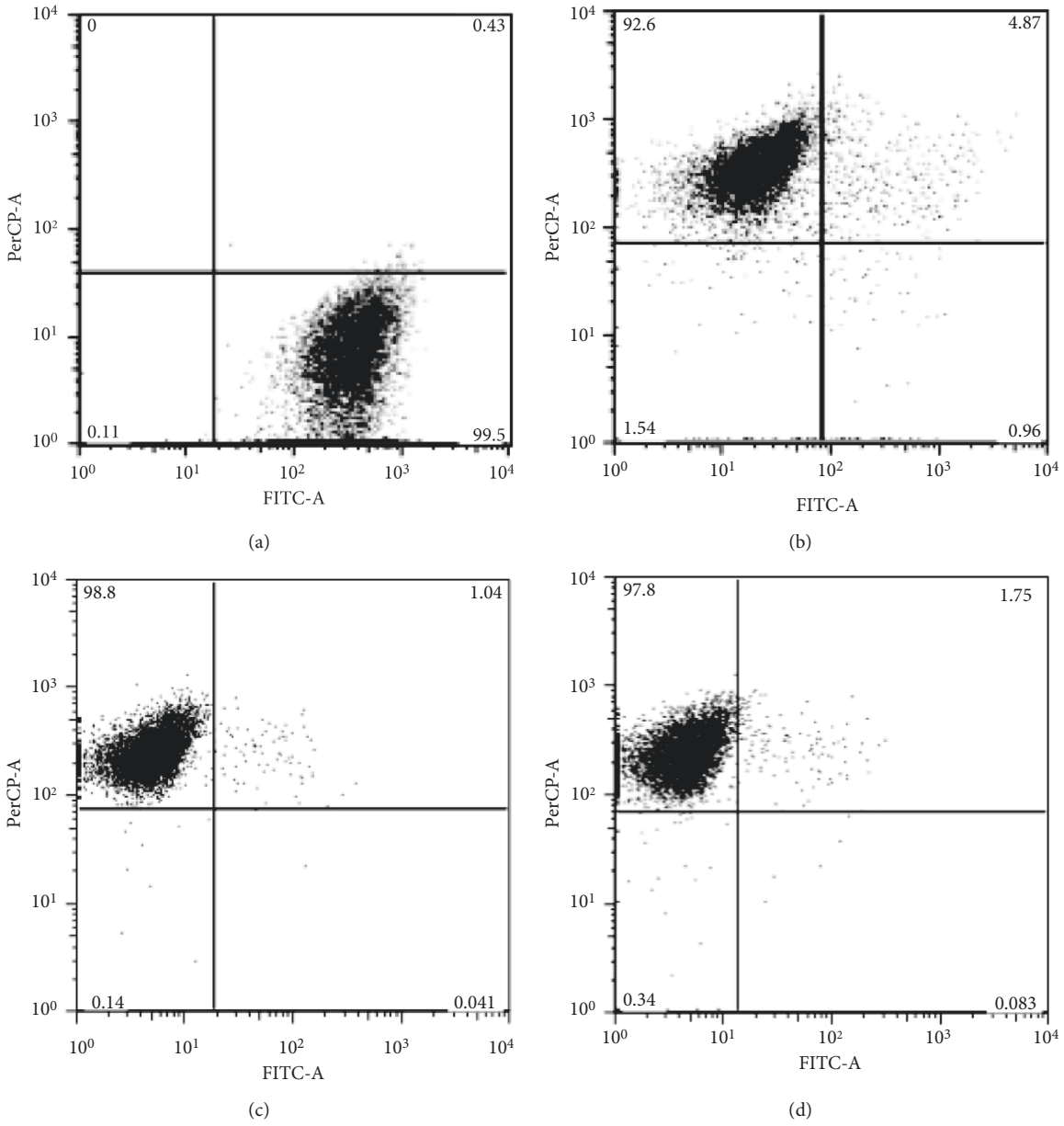


Figure 4: Continued.

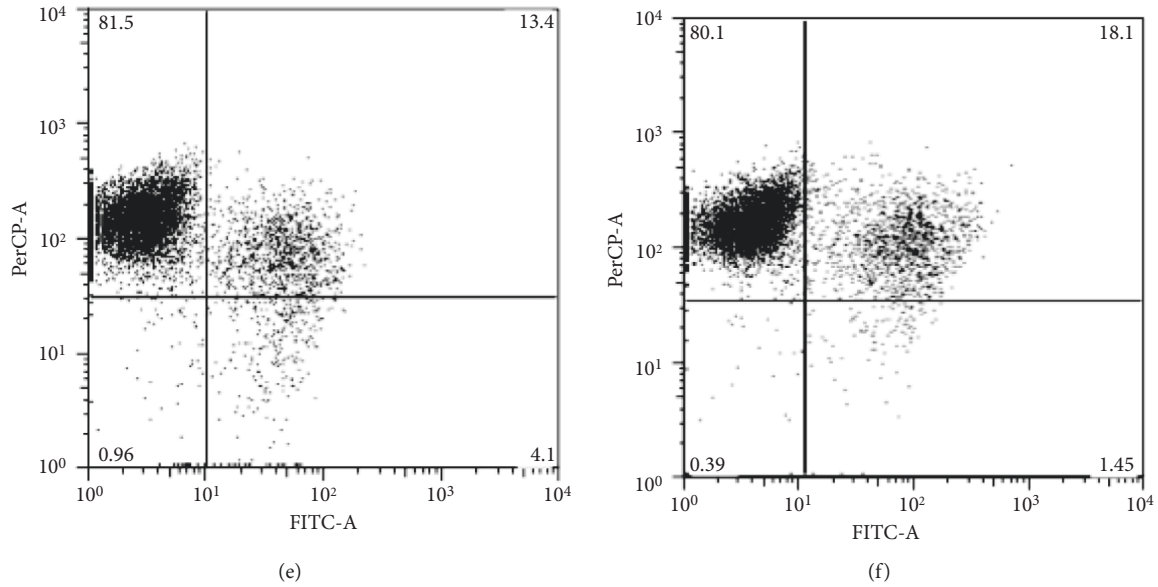


Figure 4: Metabolic activity in *S. mutans* biofilm measured by flow cytometry. The biofilm cellular suspension was excited at 488 nm, and emission was registered with FITC channel for Syto 9 (530/30) and PerCP channel (670/LP) for propidium iodide. (a) Untreated cells. (b) Chlorhexidine. (c) EP at 0.8 μg·mL^{-1}. (d) EP at 0.4 μg·mL^{-1}. (e) EP at 0.2 μg·mL^{-1}. (f) EP at 0.1 μg·mL^{-1}. EP, polyphenol-rich extract of propolis.

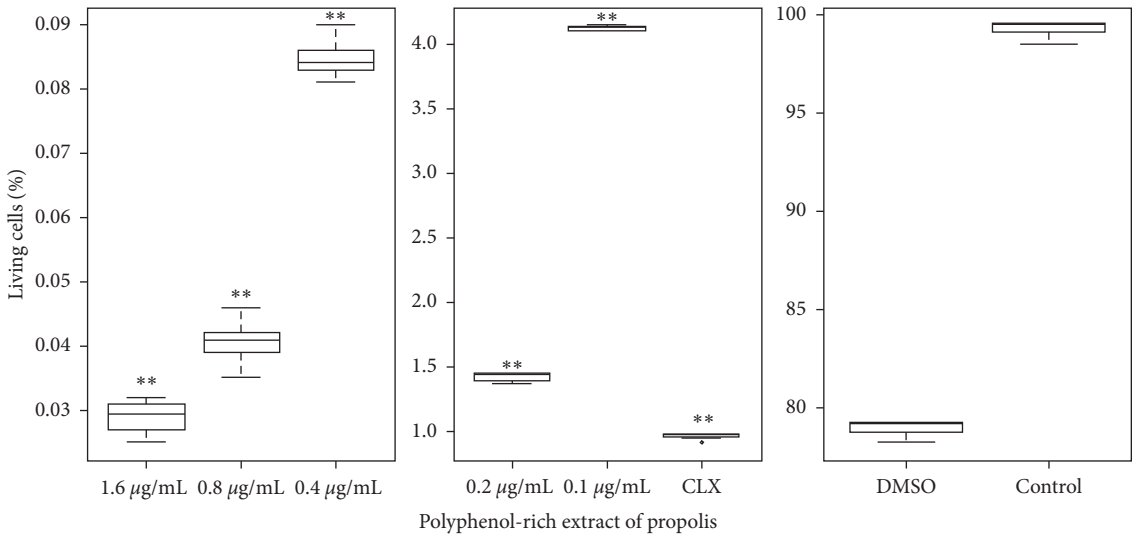


Figure 5: Effect of different concentrations of polyphenol-rich extract of propolis (EP) on biofilm metabolic activity, determined by flow cytometry in *S. mutans*. The percentage of biofilm living cells was expressed as median ± SD. ** $p < 0.01$ from the nonparametric Wilcoxon test when compared to untreated cells (control).

We found higher statistical differences in comparison with untreated cells in all treatments ($p < 0.01$).

4. Discussion

The antimicrobial agents in propolis produce changes in biofilm structure and in cellular aggregation due to fluctuations in the levels of protein and enzymatic expression [25]. In this study, we evaluated several methods to assess the antibiofilm activity of the Chilean propolis. When we compared the variability of the methods to measure biofilm formation, the coefficient of variation (CV) fluctuated between 12.8 and 23.1% after using crystal violet methodology. However, the CV ranged between 2.2 and 3.3% when we used confocal microscopy. The high degree of dispersion for crystal violet staining is probably due to the interference by color development. The other factor that explained the differences is that the crystal violet stains biomass rather than living bacteria, and thus, dead bound bacteria will still be stained. In spite of its popularity, crystal violet has certain weaknesses, including nonspecific binding to anionic proteins and other negatively charged molecules, like capsules,

lipopolysaccharides, and DNA/nucleic acids, leading to an inability to distinguish between live and dead bacterial populations [19, 20]. These problems contribute to a large variability among samples that may complicate the interpretation of biofilm screening results.

Colorimetric methods such as MTT have been used to quantify metabolic activity in cultures. The basic principle is the conversion by cellular metabolic activity of the substrate into a colored formazan from tetrazolium, later measurable with a spectrophotometer. Our results show that the CV for biofilm's metabolic activity measured by MTT ranged between 5.0 and 11.6%, in comparison with 1.9 to 3.2% when using flow cytometry analysis. The differences between these methodologies can be explained considering that the flow cytometry used propidium iodide costained with Syto9 (LIVE/DEAD staining) as an indicator for cellular membrane integrity. This combination on stable biofilm with calcein probe alone improves the discrimination between live and dead cells [26]. These results confirm that the techniques involving probes or excitation by laser allowed acquire more accurate information.

Nevertheless, it is necessary to establish a gold standard method to validate the accuracy of the evaluated methods. In addition, it is necessary to eliminate interferences that could appear on having used these methodologies, implementing some validation, for example, using culture-based methods as a reference, to assess metabolic activity (MTT methodology) in parallel to DNA staining or minimizing extracellular matrix coharvesting, if harvested cell viability is to be assessed by staining [19].

5. Conclusions

These results suggest that the staining methods presented a large variability to evaluate the effect of propolis on biofilm formation and metabolic activity. Flow cytometry and confocal microscopy allowed more accurate results when compared with traditional methodologies. Thus, we recommend the use of flow cytometry and confocal microscopy to evaluate the antimicrobial properties of propolis in *Streptococcus mutans*.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All authors have read the manuscript and agreed with the content. JJV and LAS conceived and designed the study. JJV performed the experiments. JJV, MA, and LAS analyzed the data. MA and LAS contributed reagents/materials and analysis tools. JJV wrote the paper. LAS reviewed and edited the manuscript.

Acknowledgments

The authors would like to express their gratitude to CONICYT Scholarship and the financial support of DIUFRO Project (#DI10-0031).

References

[1] P. G. Pietta, C. Gardana, and A. M. Pietta, "Analytical methods for quality control of propolis," *Fitoterapia*, vol. 73, no. 1, pp. S7–S20, 2002.

[2] V. R. Pasupuleti, L. Sammugam, N. Ramesh, and S. H. Gan, "Honey, propolis, and royal jelly: a comprehensive review of their biological actions and health benefits," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 1259510, 21 pages, 2017.

[3] H. Koo, P. L. Rosalen, J. A. Cury, Y. K. Park, and W. H. Bowen, "Effects of compounds found in propolis on *Streptococcus mutans* growth and on glucosyltransferase activity," *Antimicrobial Agents and Chemotherapy*, vol. 46, no. 5, pp. 1302–1309, 2002.

[4] S. A. Libério, A. L. A. Pereira, M. J. A. M. Araújo et al., "The potential use of propolis as a cariostatic agent and its actions on mutans group streptococci," *Journal of Ethnopharmacology*, vol. 125, no. 1, pp. 1–9, 2009.

[5] L. Netikova, P. Bogusch, and P. Heneberg, "Czech ethanol-free propolis extract displays inhibitory activity against a broad spectrum of bacterial and fungal pathogens," *Journal of Food Science*, vol. 78, no. 9, pp. M1421–M1429, 2013.

[6] A. Cuevas, N. Saavedra, M. F. Cavalcate, L. A. Salazar, and D. S. P. Abdalla, "Identification of microRNAs involved in the modulation of pro-angiogenic factors in atherosclerosis by a polyphenol-rich extract from propolis," *Archives of Biochemistry and Biophysics*, vol. 557, pp. 28–35, 2014.

[7] J. B. Daleprane, V. da Silva Freitas, A. Pacheco et al., "Anti-atherogenic and anti-angiogenic activities of polyphenols from propolis," *The Journal of Nutritional Biochemistry*, vol. 23, no. 6, pp. 557–566, 2012.

[8] A. Cuevas, N. Saavedra, M. Rudnicki, D. S. P. Abdalla, and L. A. Salazar, "ERK1/2 and HIF1α are involved in anti-angiogenic effect of polyphenols-enriched fraction from Chilean propolis," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 187575, 2015.

[9] N. Saavedra, A. Cuevas, M. F. Cavalcante et al., "Polyphenols from Chilean propolis and pinocembrim reduce MMP-9 gene expression and activity in activated macrophages," *BioMed Research International*, vol. 2016, Article ID 6505383, 8 pages, 2016.

[10] C. L. Herrera, M. Alvear, L. Barrientos, G. Montenegro, and L. A. Salazar, "The antifungal effect of six commercial extracts of Chilean propolis on *Candida* spp.," *Ciencia e Investigación Agraria*, vol. 37, no. 1, pp. 75–84, 2010.

[11] A. Pacheco, J. B. Daleprane, V. S. Freitas et al., "Effect of Chilean propolis on glucose metabolism in diabetic mice," *International Journal of Morphology*, vol. 29, no. 3, pp. 754–761, 2011.

[12] N. Saavedra, L. Barrientos, C. L. Herrera, M. Alvear, G. Montenegro, and L. A. Salazar, "Effect of Chilean propolis on cariogenic bacteria *Lactobacillus fermentum*," *Ciencia e Investigacion Agraria*, vol. 3, pp. 117–125, 2011.

[13] L. Barrientos, C. L. Herrera, G. Montenegro et al., "Chemical and botanical characterization of Chilean propolis and biological activity on cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*," *Brazilian Journal of Microbiology*, vol. 44, no. 2, pp. 577–585, 2013.

[14] J. J. Veloz, N. Saavedra, M. Alvear, T. Zambrano, L. Barrientos, and L. A. Salazar, "Polyphenol-rich extract from propolis reduces the expression and activity of *Streptococcus mutans* glucosyltransferases at subinhibitory concentrations," *BioMed Research International*, vol. 2016, Article ID 4302706, 7 pages, 2016.

[15] J. J. Veloz, N. Saavedra, A. Lillo, M. Alvear, L. Barrientos, and L. A. Salazar, "Antibiofilm activity of Chilean propolis on *Streptococcus mutans* is influenced by the year of collection," *BioMed Research International*, vol. 2015, Article ID 291351, 6 pages, 2015.

[16] J. J. Veloz, M. Alvear, and L. A. Salazar, "Antimicrobial and antibiofilm activity against *Streptococcus mutans* of individual and mixtures of the main polyphenolic compounds found in Chilean propolis," *BioMed Research International*, vol. 2019, Article ID 7602343, 7 pages, 2019.

[17] C. L. Herrera, O. Fritz, G. Montenegro, M. Alvear, M. del Sol, and L. A. Salazar, "El propóleos reduce la esteatosis hepática inducida por dieta en ratones," *International Journal of Morphology*, vol. 28, no. 1, pp. 75–77, 2010.

[18] A. Russo, V. Cardile, F. Sanchez, N. Troncoso, A. Vanella, and J. A. Garbarino, "Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines," *Life Sciences*, vol. 76, no. 5, pp. 545–558, 2004.

[19] J. Azeredo, N. F. Azevedo, R. Briandet et al., "Critical review on biofilm methods," *Critical Reviews in Microbiology*, vol. 43, no. 3, pp. 313–351, 2017.

[20] E. Haney, M. Trimble, J. Cheng, Q. Vallé, and R. Hancock, "Critical assessment of methods to quantify biofilm growth and evaluate antibiofilm activity of host defence peptides," *Biomolecules*, vol. 8, no. 2, p. 29, 2018.

[21] M. Popova, V. Bankova, D. Butovska et al., "Validated methods for the quantification of biologically active constituents of poplar-type propolis," *Phytochemical Analysis*, vol. 15, no. 4, pp. 235–240, 2004.

[22] L. A. Salazar, C. Vásquez, A. Almuna et al., "Molecular detection of cariogenic streptococci in saliva," *International Journal of Morphology*, vol. 26, no. 4, pp. 951–958, 2008.

[23] D. W. Hecht, D. M. Citron, M. Cox et al., *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard*, Clinical and Laboratory Standards Institute (CLSI), Wayne, PA, USA, 2007.

[24] B. Kouidhi, T. Zmantar, and A. Bakhrouf, "Anti-cariogenic and anti-biofilms activity of *Tunisian propolis* extract and its potential protective effect against cancer cells proliferation," *Anaerobe*, vol. 16, no. 6, pp. 566–571, 2010.

[25] J. Abranches, L. Zeng, J. K. Kajfasz et al., "Biology of oral streptococci," *Microbiology Spectrum*, vol. 6, no. 5, 2018.

[26] P. N. Tawakoli, A. Al-Ahmad, W. Hoth-Hannig, M. Hannig, and C. Hannig, "Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm," *Clinical Oral Investigations*, vol. 17, no. 3, pp. 841–850, 2013.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7693010, 10 pages
https://doi.org/10.1155/2019/7693010

Research Article

Evaluation of Male Fertility-Enhancing Activities of Water Seed Extract of *Hunteria umbellata* in Wistar Rats

Adejuwon Adewale Adeneye,[1] **Joseph Abayomi Olagunju**,[2] **and Babatunde Adekunle Murtala**[3]

[1]*Department of Pharmacology, Therapeutics & Toxicology, Faculty of Basic Clinical Sciences, Lagos State University College of Medicine, 1-5 Oba Akinjobi Way, G.R.A, Ikeja 100001, Lagos, Nigeria*
[2]*Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, 1-5 Oba Akinjobi Way, G.R.A, Ikeja 100001, Lagos, Nigeria*
[3]*Department of Physiology, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, 1-5 Oba Akinjobi Way, G.R.A, Ikeja 100001, Lagos, Nigeria*

Correspondence should be addressed to Adejuwon Adewale Adeneye; adeneye2001@yahoo.com

Received 31 December 2018; Revised 15 April 2019; Accepted 14 July 2019; Published 14 August 2019

Guest Editor: José C. T. Carvalho

Background. In this study, the male fertility-enhancing activity of 100, 200, and 400 mg/kg/day of *Hunteria umbellata* water seed extract (*HU*) in Wistar rats was studied for 60 days. In doing this, effect of repeated doses of *HU* was studied on the weight gain pattern, gonadosomatic index (GSI), serum follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone (TS), prolactin (PRL), and estradiol (ES)} as well as testicular antioxidant status of the treated rats as a way of elucidating the mechanism(s) of action of *HU*. *Method.* Thirty-six (36) male Wistar rats were randomly divided into six groups (I-VI) of six rats per group. Group I rats were gavaged with 10 ml/kg/day of distilled water and served as an untreated control; Group II rats were gavaged with 0.3 mg/kg/day of clomiphene in distilled water; Groups III-V rats received 100 mg/kg/day, 200 mg/kg/day, and 400 mg/kg/day of *HU*, respectively, and Group VI rats received 20 mg/kg/day of Vitamin C all in distilled water. All treatments were for 60 days after which the treated rats were humanely sacrificed. Sera of blood samples were processed for the above stated hormonal profile. Similarly, testicular tissues obtained were processed for semen analysis and complete antioxidant profile of the *HU*-treated testicles by assaying for superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), glutathione reductase (GSR), glutathione peroxidase (GSH-Px), and Thiobarbituric Reactive Species (TBARS). *Results*. Prolonged treatments with 100 mg/kg/day, 200 mg/kg/day, and 400 mg/kg/day of *HU* for 60 days induced dose dependent reductions in weight gain pattern with the most significant ($p<0.001$) effect recorded with the highest dose of *HU*. Conversely, significant ($p<0.001$) increase was recorded for GSI at the same *HU* dose. Clomiphene and *HU* also induced significant ($p<0.01$, $p<0.001$) dose dependent increases in the total sperm count, %live sperm, but reverse effects on %dead sperm and %abnormal sperm. On the hormonal profile, oral treatment with 100 mg/kg/day, 200 mg/kg/day, and 400 mg/kg/day of the extract induced profound ($p<0.05$, $p<0.01$, and $p<0.001$) dose related increases in the sera TS, LH, and FSH while it caused reverse effect on serum PRL but caused no significant alterations in the serum ES levels. Similarly, oral treatment with vitamin C and 100-400 mg/kg/day of *HU* induced profound ($p<0.05$, $p<0.01$, and $p<0.001$) increases in the antioxidant enzyme activities. *Conclusion.* Overall, prolonged oral treatment with 100-400 mg/kg body weight of *HU* for 60 days significantly improved sperm function which was mediated via enhanced spermatogenesis, steroidogenesis, and antioxidant mechanisms.

1. Background

Infertility is a major public health issue affecting one out of five every married couple worldwide, with approximately 30% of the condition attributable to male factors [1]. It is on records that several factors can interfere with the process of spermatogenesis and reduce sperm quantity and quality with some of the identifiable causal factors being ischemic heart disease, diabetes mellitus, chronic liver diseases, cigarette smoking, agrochemical run-offs, air pollutants, and

hypovitaminosis [2]. However, previous study has reported that regular intake of antioxidants and vitamins such as vitamins A, B, C, and E improves testicular blood barrier stability and protects sperm DNA damage from endogenous oxidative stress resulting from the activities of highly reactive free radicals generated within the body [3].

Male infertility can manifest in the form of premature ejaculation, hypoactive sexuality, erectile dysfunction, oligospermia, azoospermia, *etc*., but its most common manifestation form is as oligospermia. In treating this condition, testosterone and other forms of hormone replacement therapy are often clinically employed due to their ability to stimulate/enhance sexual appetite in hypogonadal male patients [4, 5]. However, despite the proven efficacy of this replacement therapy in the management of hypoactive sexual desire [6], many patients still prefer to use natural plants because of the attendant undesirable side-effects associated with these hormonal therapies. Two examples of popular male fertility-promoting herbs are *Panax* spp. (ginseng) and *Lepidium meyenii* (Maca) which are reputed for their supposed aphrodisiac- and spermatogenesis-enhancing effects [7–9].

Hunteria umbellata (K. Schum.) Hallier f., belonging to Apocynaceae family, is a tropical rainforest tree that is commonly used in the African folkloric medicine to treat human diseases such as blood deficiencies, infections, swellings, diabetes mellitus, and obesity [10–12]. It is known as "Demouain" (in French) and "Abeere" (in Yoruba dialect) (Southwest Nigeria) [11–13]. In Southwest region of Nigeria, water infusion of *Hunteria umbellata* dried seed is reputably used in the local management of diabetes mellitus and obesity [12, 14]. *Hunteria umbellata* water seed extract (*HU*) of the plant has been reportedly used to effectively control blood glucose and glycosylated hemoglobin concentration in types 1 and 2 models of drug-induced hyperglycemic Wistar rats [12, 14] with its alkaloid content implicated for the observed biological effect [15]. The same plant seed extract was also reported to possess antiobesity and antihyperlipidemic effects in experimental models of hyperlipidaemia which was mediated via *de novo* inhibition of cholesterol and triglyceride biosynthesis [16]. In addition, the acute, chronic, and reverse oral toxicity studies of *HU* have shown it to be relatively safe [12, 17].

Recent ethnobotanical survey conducted among selected Ijebu herbal practitioners in Ogun State (Southwest Nigeria) revealed that *HU* also has a wide application in the indigenous management of infertility (unpublished data). Despite this folkloric use, there are no scientific reports to either validate or refute this folkloric claim. More so, chronic oral toxicity and toxicity reversibility studies of *HU* have shown it to induce profound proliferation of spermatogenic primordial, Sertoli and Leydig's cells in the testicular tissues of extract-treated rats [17]. Similarly, *HU* has been reported to possess antioxidant and free radical scavenging properties mediated primarily by its alkaloid content [18]. Thus, this study was designed at evaluating the effects of chronic oral treatment with 100, 200, and 400 mg/kg of *HU* on body weight, gonadosomatic index, and semen parameters of male Wistar rats treated for 60 days. In addition, effects of HU oral treatment on the complete antioxidant enzymes system [superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), glutathione reductase (GSR), glutathione peroxidase (GSH-Px), and Thiobarbituric Reactive Species (TBARS)] of treated rat testicles as well as its effect on serum testosterone (TS), luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), and estradiol (ES) were evaluated.

2. Methods

2.1. Plant Material Collection and Identification. After plant identification and deposit of voucher specimen were done as earlier described by Adeneye and Adeyemi [12], eight (8) ripe *Hunteria umbellata* fruits were freshly harvested from the deciduous forest of Odorasanyin District of Ijebu-Igbo in Ogun State, Nigeria, in the months of July-August, 2016. Collected fruits were cut into pieces and their fresh seeds were rinsed generously under a running tap water and air-dried at room temperature (25 ± 1°C) for 1 month, protected from direct heat and sunlight. The dried seeds were milled using Laboratory Hammer Mill in the Pharmacognosy Department, Faculty of Pharmacy, University of Lagos, Idi-Araba, Lagos. The milled sample was preserved in a water- and air-proof container and stored at 4°C.

2.2. Aqueous Extraction Process. 50 g of milled seeds of *Hunteria umbellata* was soaked in 500 ml of distilled water and kept in the refrigerator for 72 hours. The solution was repeatedly stirred using magnetic stirrer for 6 hours before it was filtered with sterilized white handkerchief-packed filter funnel. The filtrate obtained was then completely air-dried in aerated oven preset at 40°C resulting in a deep brown, sweet-smelling solid residue (*HU*). This procedure was repeated 9 more times and the residues were pooled into a water- and air-proof container and stored in the freezer at -4°C to prevent HU from decomposing.

2.3. Experimental Animals. After an Institutional Ethical Approval on the Use of Experimental Animals was obtained, thirty six 12-14-week-old male Wistar rats weighing between 220 g and 250 g were obtained from Bayo Farms, Sango-Otta, Ogun State, Nigeria, and housed in the Animal House of Lagos State University College of Medicine for acclimatization for 14 days. While being acclimatized, rats were fed with standard rat chow and tap water *ad libitum* and maintained at standard laboratory conditions (12/12 hour light-dark periodicity, temperature: 23-26°C and 40-50% relative humidity) as prescribed by the United States National Institute for Health [19]. Two days to the commencement of the animal experiment, rats were randomly allotted into 6 groups of 6 rats per treatment group such that the weight differences within and between treatment groups do not exceed ±20% of the average weight of the rat sample population, respectively.

2.4. Treatment of Rats. Thirty six (36) male Wistar rats of proven fertility were divided randomly into 6 groups of 6

animals each and their oral treatments per group were as follows:

Group I: 10 ml/kg of distilled water/day for 60 days.

Group II: 0.3 mg/kg/day clomiphene in distilled water for 60 days.

Group III: 100 mg/kg/day *HU* in distilled water for 60 days.

Group IV: 200 mg/kg/day *HU* in distilled water for 60 days.

Group V: 400 mg/kg/day *HU* in distilled water for 60 days.

Group VI: 20 mg/kg/day of vitamin C in distilled water for 60 days.

2.5. Body Weight Measurement. Rat body weights were measured using digital Mettler weighing balance and values obtained recorded a day prior to commencement of the experiment, biweekly during the treatment period, and on the last day of the experiment.

2.6. Calculation of Gonadosomatic Index (GSI). The testes and their cauda epididymis were identified, removed, and weighed and immediately fixed in Bouin's solution for morphometric study. The gonadosomatic indices were calculated as follows:

{testes weight ÷ body weight} x 100 [20]

2.7. Hormonal Assays. The testosterone level in the serum was estimated by radioimmunoassay (RIA). The assays were performed using commercially available kits (Diagnostic Products Company, Los Angeles, CA, USA). All samples were run in the same assay period. The within assay variation was 5·5% while the sensitivities of the testosterone assay was 8 ng/ml. Sera FSH, LH, PRL, and ES levels were measured by immunoradiometric assay (IRMA) in solid phase also using commercially available kits (Diagnostic Products Company, Los Angeles, CA, USA).

2.8. Preparation of Semen Sample for Analysis. On day 61, the rats were euthanized with inhaled diethyl ether and a longitudinal surgical incision along the scrotal raphe and scrotal septum was made to expose the testes and its epididymis. The epididymis was freed from the adhering fat and connective tissues. The left epididymis was collected, weighed, and cut at the distal end using a clean surgical blade. Cauda epididymis (100 mg) was gently minced with glass rod without damaging the tissue into 5 ml of 0.9% NaCl [21].

2.9. Semen Analysis for Motility, Count, and Morphology. Complete semen analysis to determine progressive motility, count, and morphology was done in clean Neubauer's haemocytometer counting chamber under its cover-slip using the method of Amman [21].

Number of sperms per cauda epididymis was calculated as follows:

{Mean count x 50} ÷ {0.01 x 0.01}

2.10. Estimation of Testicular Reduced Glutathione Levels. In determining the testicular GSH level, testicular tissue homogenate in 0.1M phosphate buffer at pH of 7.4 was processed through the procedure described by Shaik and Mehvar [22].

2.11. Estimation of Testicular Tissue SOD and CAT Activities. Testicular tissue SOD and CAT activities were estimated using the method of Zhang *et al.* [23] and Iwase *et al.* [24], respectively.

2.12. Data Analysis. Data obtained were presented as mean ± S.E.M. of six observations. Data were analyzed statistically using One-way analysis of variance on SYSTAT 10.6. *Post hoc* test was done using Student's t-test and levels of significance were considered at $p<0.05$, $p<0.01$, and $p<0.001$.

3. Results

3.1. Water Extraction. Water extraction of milled *Hunteria umbellata* dried seeds produced a deep brown, sweet-smelling solid residue weighing an average of 7.33 ± 0.32 g with a %yield of 14.66 ± 0.65%.

3.2. Effects of Oral Treatments with Clomiphene, Vitamin C, and Hunteria umbellata Water Seed Extract on Rat Body Weights. Repeated oral treatments with 100-400 mg/kg/day of *HU* induced significant ($p<0.05$, $p<0.01$, and $p<0.001$) dose related decreases in the weight gain pattern of treated rats effective from the 30th day to the 60th day of oral treatment when compared to the weight gain pattern of Vitamin C- and clomiphene-treated rats over the same treatment period. The most significant ($p<0.001$) weight reduction was observed at 400 mg/kg/day *HU* on the 60th day (Table 1). However, daily oral treatment with 0.3 mg/kg/day of clomiphene for 60 days caused steady and consistent body weight increases of treated rats with the most significant ($p<0.001$) increase recorded on day 60 (Table 1). Similar effect was reported for 20 mg/kg/day of Vitamin C (Table 1).

3.3. Effect of Oral Treatment with 100-400 mg/kg/day of Hunteria umbellata Water Seed Extract on Rat Testicular Weight (TW) and Gonadosomatic Indices (GSI). Repeated oral treatment with 100, 200, and 400 mg/kg *HU* induced dose related increases in the testicular weight and GSI of treated rats with significant increases ($p<0.001$) recorded for the 400 mg/kg/day *HU*-treated rats (Table 2). The same effects were also recorded in clomiphene- and vitamin C-treated rats (Table 2).

3.4. Effect of Oral Treatment with 100-400 mg/kg/day of Hunteria umbellata Water Seed Extract on Rat Semen Parameters. Repeated daily oral treatment with 100 mg/kg/day, 200

Table 1: Effect of repeated daily oral treatments with 100-400 mg/kg/day of *Hunteria umbellata* aqueous seed extract on the weight gain pattern of treated rats.

Groups	Average body weight (g) on the following:				
	Day 1	Day 15	Day 30	Day 45	Day 60
I	227.80±12.95	229.80±11.58	240.70±9.11	260.70±8.54	290.50±11.90
II	229.70±13.37	239.20±16.90	256.20±13.72	$276.7\pm12.47^{a+}$	$315.30\pm13.76^{c+}$
III	226.80±12.16	231.80±10.94	241.00±9.80	256.30±5.13	280.00±9.42
IV	230.20±13.56	236.00±13.61	254.50±11.29	264.80±13.08	279.20±14.95
V	225.00±14.52	226.70±12.45	$234.00\pm13.45^{a-}$	$242.80\pm12.95^{b-}$	$243.70\pm11.74^{c-}$
VI	225.00±15.67	240.50±13.32	$259.50\pm9.94^{a+}$	$282.20\pm6.71^{b+}$	$307.80\pm5.78^{c+}$

$^{a+,b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to Group I values, while $^{a-,b-}$ and $^{c-}$ represent significant decreases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to Group II values.
I = 10 ml/kg/day of distilled water.
II = 0.3 mg/kg/day of clomiphene dissolved in distilled water.
III = 100 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
IV = 200 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
V = 400 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
VI = 20 mg/kg/day of Vitamin C dissolved in distilled water.

Table 2: Effect of repeated daily oral treatments with 100-400 mg/kg/day of *Hunteria umbellata* aqueous seed extract on the average testicular weight (TW) and gonadosomatic indices (GSI) of treated rats.

Groups	Average rat weight on day 60 (g)	TW (g)	SGI ($x10^{-2}$)
I	290.50 ± 11.90	03.51 ± 0.32	12.07 ± 0.66
II	$315.30 \pm 13.76^{c+}$	$04.27 \pm 0.43^{c+}$	$13.50 \pm 0.81^{b+}$
III	280.00 ± 09.42	03.37 ± 0.20	12.01 ± 0.32
IV	279.20 ± 14.95	03.36 ± 0.27	12.04 ± 0.34
V	243.70 ± 11.74	03.94 ± 0.03	$16.21 \pm 0.88^{c+}$
VI	307.80 ± 5.78	$04.04 \pm 0.16^{b+}$	$13.05 \pm 0.38^{a+}$

$^{a+,b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to Group I values.
I = 10 ml/kg/day of distilled water.
II = 0.3 mg/kg/day of clomiphene dissolved in distilled water.
III = 100 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
IV = 200 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
V = 400 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
VI = 20 mg/kg/day of Vitamin C dissolved in distilled water.

mg/kg/day, and 400 mg/kg/day *HU* for 60 days on semen analysis showed significant ($p<0.05$, $p<0.01$, and $p<0.001$) dose-related increases in the total sperm count of treated rats with the most significant ($p<0.001$) boost recorded in rats treated with the highest dose (400 mg/kg/day) of the extract which was comparable with what was recorded for the standard drug (0.3 mg/kg/day of clomiphene) (Table 3). Similar pattern was also recorded for % motile sperm counts (Table 3). However, *HU* had a reverse effect on the % dead sperm and % abnormal sperm counts (Table 3) while 0.3 mg/kg/day of clomiphene caused a significant ($p<0.001$) increase in the %abnormal sperm count when compared to untreated control (Group I) rats (Table 3).

3.5. Effect of Oral Treatment with 0.3 mg/kg/day of Clomiphene and 100-400 mg/kg/day of Hunteria umbellata Water Seed Extract on Rat's Sera TS, LH, FSH, PRL, and ES. Repeated oral treatment with 0.3 mg/kg/day of clomiphene resulted in significant ($p<0.001$) increases in the sera TS, LH, and FSH levels when compared to the untreated control (Group I) values (Table 4). However, 0.3 mg/kg/day clomiphene significantly ($p<0.05$) reduced circulating serum ES level when compared to Group I values (Table 4). Similarly, repeated treatments with graded oral doses of *HU* for 60 days resulted in significant ($p<0.05$, $p<0.01$, and $p<0.001$) dose related increases in the circulating serum TS, LH, and FSH levels while resulting in significant ($p<0.05$) reductions in serum PRL when compared to untreated control (Group I) values (Table 4). However, there were no significant alterations in the serum ES levels between the treatment groups and the untreated control group (Table 4).

3.6. Effect of Oral Treatment with 0.3 mg/kg/day of Clomiphene and 100-400 mg/kg/day of Hunteria umbellata Water Seed Extract on Rat Testicular Tissue SOD, CAT, and TBARS. Repeated oral treatments with 0.3 mg/kg/day of clomiphene did not significantly ($p>0.05$) alter the testicular tissue activities of SOD, CAT, and TBARS compared to those of untreated control (Group I) values (Table 5). However, repeated daily oral treatment with 100-400 mg/kg/day of

Table 3: Effect of repeated daily oral treatments with 100-400 mg/kg/day of *Hunteriaumbellata* aqueous seed extract on semen parameters of treated rats.

Groups	Total sperm/g cauda epi (x10^7)	%motile sperm	%dead sperm	%abnormal sperms
I	29.58 ± 0.55	77.03 ± 0.56	22.97 ± 0.56	08.97 ± 0.41
II	50.85 ± 1.01	87.23 ± 1.44^{c+}	12.77 ± 1.44^{f}	14.03 ± 0.87^{c+}
III	31.68 ± 0.55	78.55 ± 0.40	21.45 ± 0.40	19.12 ± 0.19^{c+}
IV	37.93 ± 0.71^{b+}	79.27 ± 0.62	20.73 ± 0.62	12.17 ± 0.62^{c+}
V	47.38 ± 0.84^{c+}	87.17 ± 1.32^{c+}	16.83 ± 1.32^{f}	07.67 ± 0.91^{f}
VI	31.70 ± 1.03	78.22 ± 0.38	21.78 ± 0.38	10.86 ± 0.48^{e}

$^{a+,b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to Group I values, while ^{e} and ^{f} represent significant decreases at $p<0.05$ and $p<0.001$, respectively, when compared to Group I values.
I = 10 ml/kg/day of distilled water.
II = 0.3 mg/kg/day of clomiphene dissolved in distilled water.
III = 100 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
IV = 200 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
V = 400 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
VI = 20 mg/kg/day of Vitamin C dissolved in distilled water.

Table 4: Effect of repeated daily oral treatments with 100-400 mg/kg/day of *Hunteria umbellata* aqueous seed extract on serum testosterone (TS), luteinizing hormone LH), follicle stimulating hormone (FSH), prolactin (PRL), and estradiol (ES) of treated rats.

Groups	TS (ng/ml)	LH (ng/ml)	FSH (ng/ml)	PRL (ng/ml)	ES (ng/ml)
I	02.53 ± 0.04	0.66 ± 0.03	02.32 ± 0.04	03.14 ± 0.19	2.40 ± 0.06
II	05.87 ± 0.68^{c+}	01.47 ± 0.44^{c+}	03.06 ± 0.09^{c+}	02.86 ± 0.11	2.13 ± 0.07
III	04.04 ± 0.16^{a+}	00.83 ± 0.08^{a+}	02.66 ± 0.03^{a+}	02.82 ± 0.10	2.37 ± 0.04
IV	04.70 ± 0.15^{b+}	01.16 ± 0.05^{b+}	02.76 ± 0.04^{b+}	02.42 ± 0.17	2.46 ± 0.10
V	06.39 ± 0.31^{c+}	01.42 ± 0.05^{c+}	03.25 ± 0.15^{c+}	02.32 ± 0.06^{d}	2.41 ± 0.13
VI	02.59 ± 0.03	00.64 ± 0.02	02.29 ± 0.03	02.90 ± 0.30	2.28 ± 0.04

$^{a+,b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to Group I values, while ^{d} represents a significant decrease at $p<0.05$ when compared to Group I values.
I = 10 ml/kg/day of distilled water.
II = 0.3 mg/kg/day of clomiphene dissolved in distilled water.
III = 100 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
IV = 200 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
V = 400 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
VI = 20 mg/kg/day of Vitamin C dissolved in distilled water.

HU caused significant ($p<0.05$ and $p<0.001$) dose related increases in the testicular tissue levels of SOD, CAT, and TBARS when compared to both untreated control (Group I) and clomiphene-treated (Group II) values (Table 5). Similarly, oral treatments with 20 mg/kg/day of Vitamin C had similar effects on the testicular tissue SOD, CAT, and TBARS recorded for rats treated with 400 mg/kg/day of *HU* (Table 5).

3.7. Effect of Oral Treatment with 100-400 mg/kg/day of Hunteria umbellata Water Seed Extract on Rat Testicular Tissue GSH, GSH-Px, and GSR. Treatments with 100-400 mg/kg/day of *HU* caused significant ($p<0.05$, $p<0.01$, and $p<0.001$) dose related testicular tissue levels of GSH, GSH-Px, and GSR compared to untreated control (Group I) and clomiphene-treated (Group II) values (Table 6). However, the values obtained for 400 mg/kg/day of *HU* were comparable with those recorded for Vitamin C-treated rats (Table 6). It is also worthy to note that treatments with 0.3 mg/kg/day of clomiphene had no profound alterations ($p>0.05$) in the testicular tissue levels of GSH, GSH-Px, and GSR (Table 6).

4. Discussion

Infertility is the inability of a couple of opposite sex to achieve a clinical pregnancy after 52 weeks or more of regular unprotected sexual intercourse. In other words, it is a complete failure of a sexually competent and active, noncontracepting couple to achieve pregnancy in one or more years despite regular sexual exposures [25]. Recent epidemiological data has shown that approximately 15% of married/unmarried couples are affected by infertility for which only 40-50% cases are attributable to male infertility [26] which itself could be due to either azoospermia or erectile dysfunction [27]. Identifiable factors (reversible/irreversible) influencing male fertility as reported by previous studies include drugs (such as anabolic steroid, replacement testosterone, and opiates), testicular varicocele, undescended testes, urinary tract infections, testicular tumor, hormonal imbalances, premature or retrograde ejaculation, prolonged heat exposure, obesity, older age, cigarette smoking, alcohol, heavy metals, pesticides, oxidative stress, genetic factors, and different environmental and nutritional factors [28–34]. Thus, in managing male infertility, treatment plans are designed alongside the

Table 5: Effect of repeated daily oral treatments with 100-400 mg/kg/day of *Hunteria umbellata* aqueous seed extract on testicular SOD, CAT, and TBARS of treated rats.

Groups	SOD (U/mg protein)	CAT (U/mg protein)	TBARS (nM/min/mg protein)
I	05.48 ± 0.11	20.77 ± 0.20	13.46 ± 0.06
II	05.37 ± 0.11	20.42 ± 0.19	13.45 ± 0.07
III	05.59 ± 0.08	20.79 ± 0.15	13.36 ± 0.08
IV	06.00 ± 0.08^{c+}	21.37 ± 0.21^{a+}	13.97 ± 0.06^{c+}
V	06.25 ± 0.02^{c+}	22.74 ± 0.03^{c+}	14.49 ± 0.12^{c+}
VI	06.92 ± 0.04^{c+}	23.51 ± 0.16^{c+}	16.89 ± 0.06^{c+}

$^{a+}$ and $^{c+}$ represent significant increases at $p<0.05$ and $p<0.001$, respectively, when compared to Group I values.
I = 10 ml/kg/day of distilled water.
II = 0.3 mg/kg/day of clomiphene dissolved in distilled water.
III = 100 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
IV = 200 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
V = 400 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
VI = 20 mg/kg/day of Vitamin C dissolved in distilled water.

Table 6: Effect of repeated daily oral treatments with 100-400 mg/kg/day of *Hunteria umbellata* aqueous seed extract on testicular GSH, GSH-Px and GSR of treated rats.

Groups	GSH (μM/g tissue)	GSH-Px (nM/mg protein)	GSR (nM/min/mg protein)
I	0.36 ± 0.02	140.00 ± 1.09	207.00 ± 2.05
II	0.35 ± 0.02	137.60 ± 1.61	203.20 ± 1.28
III	0.40 ± 0.02	139.10 ± 1.08	204.40 ± 1.80
IV	0.59 ± 0.01^{c+}	143.30 ± 0.53^{a+}	215.00 ± 2.22^{b+}
V	0.69 ± 0.05^{c+}	144.10 ± 0.17^{a+}	217.80 ± 0.67^{c+}
VI	0.76 ± 0.03^{c+}	148.10 ± 0.66^{a+}	230.60 ± 1.71^{c+}

$^{a+,b+}$ and $^{c+}$ represent significant increases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively, when compared to Group I values.
I = 10 ml/kg/day of distilled water.
II = 0.3 mg/kg/day of clomiphene dissolved in distilled water.
III = 100 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
IV = 200 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
V = 400 mg/kg/day of *Hunteria umbellata* aqueous seed extract dissolved in distilled water.
VI = 20 mg/kg/day of Vitamin C dissolved in distilled water.

identifiable possible cause and these interventions could be either medical or surgical [27, 35].

Apart from orthodox treatment of infertility, infertile couples are known to concomitantly use traditional/complementary medicine as therapeutic alternatives for their infertility since 25% of modern drugs are either plant-based or of plant origin [36]. More so, up to 80% of the world's population is either partially or wholly dependent on herbal products for the treatment of their medical conditions [37]. However, in evaluating pharmacological activities of male fertility-enhancing medicinal plants, scientific investigations are done *in vitro* (using culture cell lines) and *in vivo* (using experimental animals and humans) models. For examples, extracts obtained from *Vanda tessellata* [38], *Turnera diffusa* and *Pfaffia paniculata* [39], *Eurycoma longifolia* [40], *Terminalia catappa* [41], *Butea frondosa* [42], *Curculigo orchioides* [43], *Panax quinquefolius* (ginseng) [44], and *Lepidium meyenii* have been investigated and reported to enhance aphrodisiacs while extracts obtained from *Astragalus membranaceus, Asparagus racemosus, Withania somnifera, Andrographis paniculata*, and *Acanthopanax senticosus* were also reported to improve sperm parameters [45, 46].

In this study we evaluated the possible fertility-enhancing and fertility mechanism of action of *HU* in male Wistar rats for 60 days as an alternative/complimentary to existing fertility-boosting drugs bearing in mind their high cost and untoward side effects associated with their clinical use. In doing this, male Wistar rats were gavaged with graded doses (100, 200, and 400 mg/kg/day) of HU in male Wistar rats for 60 consecutive days using endpoints such as the somatogonadal index, semen analysis parameters, and gonadal hormone profile as well as the testicular tissue antioxidant profile.

Hormonal analysis of *HU*-treated rats clearly showed the extract to significantly improve circulating sera TS, FSH, and LH while it decreased circulating prolactin. Literature has shown that serum elevation in the testosterone, FSH, and LH improves sperm quality in relation to sperm count, volume, motility, and morphology [43]. FSH and LH are known to influence the fate of germ cells and this influence is mediated via their actions on specific transmembrane receptors, Follicle Stimulating Hormone receptor (FSH-R), and Luteinizing Hormone receptor (LH-R) that are overtly expressed in the Sertoli cells and interstitial Leydig cells,

respectively [47–50]. Scientific evidences abound for the critical role of the LH-testosterone signaling pathway in priming and sustaining the process of spermatogenesis in the extratubular Leydig cells while FSH regulates spermatocytogenesis and spermatogenesis by influencing both the germinal epithelium and the Sertoli cells [51, 52]. Thus, interplay of FSH, LH, and testosterone strongly influences quantitative and qualitative spermatogenesis. In this study, repeated oral treatment with 100-400 mg/kg/day of *HU* stimulated significant spermatogenesis which was characterized by increased sperm volume, count, progressive motility, and improved sperm morphology. This improvement in sperm quality could have been due to improved steroidogenesis characterized by increased circulating TS, FSH, and LH which have been widely reported to enhance spermatogenesis [53]. Clomiphene like tamoxifen is a known anti-estrogenic analogue which blocks estrogen from interacting with the anterior pituitary gland to induce increased LH, FSH and TS syntheses [54]. Thus, the mechanism of fertility enhancement by HU appears to be similar to that of clomiphene.

Another notable finding of this study is the nonsignificant alteration in circulating prolactin levels for treated groups. Physiologically, prolactin suppresses LH and FSH secretion which invariably lowers circulating TS levels and decreases sexual drive. This assertion is in complete agreement with elevated FSH, LH, and TS levels in the *HU*-treated groups. Previous studies have reported that any plant with aphrodisiac/male fertility enhancing potential often profoundly lowers serum PRL and enhances circulating LH and FSH levels and by extension enhances circulating TS levels [55, 56] with which our results are in complete agreement.

Medicinal plants rich in certain secondary metabolites such as phenolic acids, flavonoids, flavones, alkaloids, and antioxidant vitamins (e.g., Vitamins A, C, D, and E) have been reported to stimulate and enhance spermatogenesis [57–61]. However, our previous studies have reported *HU* to be abundantly rich in alkaloids, flavonoids, tannins, and glycosides [12]. Thus, the relative abundance of these phytochemicals could have been responsible for the increased spermatogenesis observed in this study.

The cause of infertility in about 50% of all infertile couples is attributable to male factors either solely or partly for which oxidative stress is one of the factors [62]. Excessive production of free radicals or reactive oxygen species (ROS) has implicated sperm damage and as such ROS have been extensively studied as in the etiology of male infertility. Superoxide anion, hydroxyl radical, and hydrogen peroxide are some of the major ROS present in seminal plasma and these have been implicated in male infertility [63–66]. Under normal physiological conditions, small and negligible amount of ROS is generated by spermatozoa and this is essential for spermatozoa capacitation and acrosomal reaction. However, when ROS generation is overwhelming, it could irreversibly bind to the spermatozoa plasma membrane- and cytoplasmic-rich polyunsaturated fatty acids resulting in lipid peroxidation and cell death of spermatozoa [67].

Antioxidants including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) often free scavenge ROS to protect spermatozoa from the deleterious effect of the generated ROS [68, 69]. Semen is also contains a variety of endogenous nonenzymatic antioxidant molecules such as vitamins A, C, D, and E, pyruvate, glutathione, and carnitine [70]. These antioxidants compensate for the loss of sperm cytoplasmic enzymes during spermatogenesis, which in turn diminishes endogenous repair mechanisms and enzymatic defenses [71–73]. In view of the established direct relationship between male fertility and testicular antioxidant status, *HU* significantly improved the antioxidant status of the treated rat testicles as indicated by increased levels of SOD, CAT, GSH, GSH-Px, GSR, and TBARS, which are in consonance with our previous *in vitro* [18] and *in vivo* [74] findings and that recently are reported by Oboh *et al.* [10]. Thus, these results clearly demonstrate the potential of *HU* in significantly improving the spermatogenic parameters via its antioxidant mechanism. Also, literature has shown antioxidants to improve various oxidative processes including spermatogenesis and steroidogenesis [68, 75], thus lending credence to the observed spermatogenic and steroidogenic effects of *HU* in the treated rats to be attributed to its high antioxidant profile.

5. Conclusion

Overall, prolonged oral treatments with 100-400 mg/kg/day of *HU* for 60 days significantly improved sperm function (as measured by sperm motility, count, viability, and morphology), mediated via increased spermatogenesis, steroidogenesis, and antioxidant mechanisms.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflicts of interest to declare. Adejuwon Adewale Adeneye is a Professor in the Department of Pharmacology, Therapeutics, and Toxicology, Faculty of Basic Clinical Sciences, Lagos State University College of Medicine, and the Principal Investigator of the present study. Joseph Abayomi Olagunju is a Professor in the Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, and Coinvestigator of this study. Babatunde Adekunle Murtala is Chief Technologist, Department of Physiology, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, and a Coinvestigator of the present study.

Authors' Contributions

Adejuwon Adewale Adeneye conceived, designed, and conducted the study. He also analyzed and interpreted the data and was responsible for the manuscript writing. Babatunde Adekunle Murtala conducted the semen analysis while Joseph Abayomi Olagunju also partook in the study design and data interpretation.

Acknowledgments

The authors thankfully acknowledge the full financial support given for this research work through the TETFUND Institution Based Research Fund, Second (2^{nd}) Batch Research Project disbursement for the year 2016 cycle. Our research team also acknowledges the contributions of the Vice Chancellor and Management of Lagos State University (LASU) headed by Prof. Olanrewaju A. Fagbohun as well as the LASU TETFUND Desk Officer, Dr. Suraj Mudasiru, for facilitating and ensuring timely disbursement of fund for this research work. While the research team also duly acknowledges the technical support staff of the Animal House, LASUCOM, for the care of the Experimental Animal used during the period of the study, the technical support of Mr. Sunday O. Adenekan of BIOLIFE CONSULTS in the area of Oxidative Stress analysis is much appreciated. This research work was fully sponsored by the Federal Government of Nigeria through Tertiary Education Fund (TETFund) Institutional Based Research Grant.

References

[1] A. M. Isidori, C. Pozza, D. Gianfrilli, and A. Isidori, "Medical treatment to improve sperm quality," *Reproductive BioMedicine Online*, vol. 12, no. 6, pp. 704–714, 2006.

[2] M. E. Thoma, A. C. McLain, J. F. Louis et al., "The prevalence of infertility in the United States as estimated by the current duration approach and a traditional constructed approach," *Fertililty & Sterility*, vol. 99, no. 5, pp. 1324–1331, 2013.

[3] M. Jedlinska, G. Bomba, K. Jakubowski, T. Rotkiewicz, B. Jana, and A. Penkowski, "Impact of oxidative stress and supplementation with vitamins E and C on testes morphology in rats," *The Journal of Reproduction and Development*, vol. 52, no. 2, pp. 203–209, 2006.

[4] R. M. Sargis and A. M. Davis, "Evaluation and treatment of male hypogonadism," *Journal of the American Medical Association*, vol. 319, no. 13, pp. 1375-1376, 2018.

[5] S. Arver, A. Dobs, A. Meikle, R. Allen, S. Sanders, and N. Mazer, "Improvement of sexual function in testosterone deficient men treated for 1 year with a permeation enhanced testosterone transdermal system," *The Journal of Urology*, vol. 155, no. 5, pp. 1604–1608, 1996.

[6] S. N. Seidman, "Hormonal aspects of sexual dysfunction: The therapeutic use of exogenous androgens in men and women," *Current Psychiatry Reports*, vol. 2, no. 3, pp. 215–222, 2000.

[7] K. W. Leung and A. S. Wong, "Ginseng and male reproductive function," *Spermatogenesis*, vol. 3, no. 3, Article ID e26391, 2014.

[8] G. F. Gonzales, A. Ruiz, C. Gonzales, L. Villegas, and A. Cordova, "Effect of *Lepidium meyenii* (maca) roots on spermatogenesis of male rats," *Asian Journal of Andrology*, vol. 3, no. 3, pp. 231–233, 2001.

[9] G. F. Gonzales, A. Cordova, C. Gonzales, A. Chung, K. Vega, and A. Villena, "*Lepidium meyenii* (Maca) improved semen parameters in adult men," *Asian Journal of Andrology*, vol. 3, no. 4, pp. 301–304, 2001.

[10] G. Oboh, A. A. Adebayo, A. O. Ademosun, and O. A. Abegunde, "Aphrodisiac effect of Hunteria umbellata seed extract: modulation of nitric oxide level and arginase activity in vivo," *Pathophysiology*, vol. 26, no. 1, pp. 39–47, 2019.

[11] A. Falodun, Z. A. Nworgu, and M. O. Ikponmwonsa, "Phytochemical components of Hunteria umbellata (K. Schum) and its effect on isolated non-pregnant rat uterus in oestrus.," *Pakistan Journal of Pharmaceutical Sciences*, vol. 19, no. 3, pp. 256–258, 2006.

[12] A. Adeneye and O. Adeyemi, "Hypoglycemic effects of the aqueous seed extract of Hunteria umbellata in normoglycemic and glucose and nicotine induced hyperglycemic rats," *International Journal of Natural Products Research*, vol. 2, no. 1, pp. 9–18, 2009.

[13] I. N. Ibeh, M. Idu, and I. M. Ejimadu, "Toxicological assessment of Abeere seed Hunteria umbellata K. Schum (Apocynaceae)," *Biociência*, vol. 15, no. 1, pp. 4–7, 2007.

[14] I. Igbe, E. K. I. Omogbai, and R. I. Ozolua, "Hypoglycemic activity of aqueous seed extract of Hunteria umbellata in normal and streptozotocin-induced diabetic rats," *Pharmaceutical Biology*, vol. 47, no. 10, pp. 1011–1016, 2009.

[15] A. A. Adeneye, O. O. Adeyemi, E. O. Agbaje, and M. O. Sofidiya, "The novel antihyperglycaemic action of Hunteria umbellata seed fractions mediated via intestinal glucose uptake inhibition," *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 9, no. 1, pp. 17–24, 2012.

[16] A. A. Adeneye, O. O. Adeyemi, and E. O. Agbaje, "Anti-obesity and antihyperlipidaemic effect of *Hunteria umbellata* seed extract in experimental hyperlipidaemia," *Journal of Ethnopharmacology*, vol. 130, no. 2, pp. 307–314, 2010.

[17] A. A. Adeneye, O. O. Adeyemi, E. O. Agbaje, and A. A. F. Banjo, "Evaluation of the toxicity and reversibility profile of the aqueous seed extract of hunteria umbellata (k. schum.) hallier f. in rodents," *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 7, no. 4, pp. 350–369, 2010.

[18] A. A. Adejuwon, S. M. Oluwatoyin, and A. O. Sunday, "Anti-inflammatory and antioxidant activities of hunteria umbellata seed fractions," *Pharmacologia*, vol. 2, no. 6, pp. 165–171, 2011.

[19] United States National Institutes for Health publication, no. 85-23, 1985.

[20] A. Flores, R. Wiff, and E. Díaz, "Using the gonadosomatic index to estimate the maturity ogive: application to Chilean hake (Merluccius gayi gayi)," *ICES Journal of Marine Science*, vol. 72, no. 2, pp. 508–514, 2014.

[21] R. P. Amann, "Use of animal models for detecting specific alterations in reproduction," *Fundamental and Applied Toxicology*, vol. 2, no. 1, pp. 13–26, 1982.

[22] I. H. Shaik and R. Mehvar, "Rapid determination of reduced and oxidized glutathione levels using a new thiol-masking reagent and the enzymatic recycling method: application to the rat liver and bile samples," *Analytical and Bioanalytical Chemistry*, vol. 385, no. 1, pp. 105–113, 2006.

[23] J. Zhang, R. Chen, Z. Yu, and L. Xue, "Superoxide dismutase (SOD) and catalase (CAT) activity assay protocols for caenorhabditis elegans," *Bio-Protocol*, vol. 7, no. 16, Article ID e2505, 2017.

[24] T. Iwase, A. Tajima, S. Sugimoto et al., "A simple assay for measuring catalase activity: a visual approach," *Scientific Reports*, vol. 3, no. 1, article no. 3081, 2013.

[25] F. Zegers-Hochschild, G. D. Adamson, J. deMouzon et al., "The international committee for monitoring assisted reproductive technology (ICMART) and World Health Organization (WHO) revised glossary on ART terminology, 2009," *Fertility and Sterility*, vol. 92, no. 5, pp. 2684–2687, 2009.

[26] F. Lotti and M. Maggi, "Sexual dysfunction and male infertility," *Nature Reviews Urology*, vol. 15, no. 5, pp. 287–307, 2018.

[27] M. Naz and M. Kamal, "Classification, causes, diagnosis and treatment of male infertility: a review," *Oriental Pharmacy and Experimental Medicine*, vol. 17, no. 2, pp. 89–109, 2017.

[28] M. Fraczek and M. Kurpisz, "The redox system in human semen and peroxidative damage of spermatozoa," *Postepy Hig Med Dosw*, vol. 59, pp. 523–534, 2005.

[29] G. Collodel, E. Moretti, V. Fontani et al., "Effect of emotional stress on sperm quality," *Indian Journal of Medical Research*, vol. 128, no. 3, pp. 254–261, 2008.

[30] P. Gharagozloo and R. J. Aitken, "The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy," *Human Reproduction*, vol. 26, no. 7, pp. 1628–1640, 2011.

[31] F. M. Onyije, "Drug: a major cause of infertility in male," *Asian Journal of Medical Pharmaceutical Research*, vol. 2, no. 2, pp. 30–37, 2012.

[32] W. E. J. Olooto, "Infertility in male; risk factors, causes and management- a review," *Journal Microbiology and Biotechnological Research*, vol. 2, no. 4, pp. 641–664, 2012.

[33] F. Gimenes, R. P. Souza, J. C. Bento et al., "Male infertility: a public health issue caused by sexually transmitted pathogens," *Nature Reviews Urology*, vol. 11, no. 12, pp. 672–687, 2014.

[34] L. I. Kolesnikova, S. I. Kolesnikov, N. A. Kurashova, and T. A. Bairova, "Causes and factors of male infertility," *Annals of The Russian Academy of Medical Sciences*, vol. 70, no. 5, p. 579, 2015.

[35] R. P. Kaur, V. Gupta, A. S. Christopher, and P. Bansal, "Potential pathways of pesticide action on erectile function—a contributory factor in male infertility," *Asian Pacific Journal of Reproduction*, vol. 4, no. 4, pp. 322–330, 2015.

[36] R. Reid, A. Steel, J. Wardle, A. Trubody, and J. Adams, "Complementary medicine use by the Australian population: a critical mixed studies systematic review of utilisation, perceptions and factors associated with use," *BMC Complementary and Alternative Medicine*, vol. 16, no. 1, pp. 176–198, 2016.

[37] S. M. K. Rates, "Plants as source of drugs," *Toxicon*, vol. 39, no. 5, pp. 603–613, 2001.

[38] P. K. Suresh Kumar, A. Subramoniam, and P. Pushpangadan, "Aphrodisiac activity of Vanda tessellata (Roxb.) Hook. ex Don extract in male mice," *Indian Journal of Pharmacology*, vol. 32, no. 5, pp. 300–304, 2000.

[39] R. Arletti, A. Benelli, E. Cavazzuti, G. Scarpetta, and A. Bertolini, "Stimulating property of *Turnera diffusa* and *Pfaffia paniculata* extracts on the sexual behavior of male rats," *Psychopharmacology*, vol. 143, no. 1, pp. 15–19, 1999.

[40] H. H. Ang, S. Ikeda, and E. K. Gan, "Evaluation of the potency activity of aphrodisiac in *Eurycoma longifolia* Jack," *Phytotherapy Research*, vol. 15, no. 5, pp. 435-436, 2001.

[41] W. D. Ratnasooriya and M. G. Dharmasiri, "Effects of Terminalia catappa seeds on sexual behaviour and fertility of male rats," *Asian Journal of Andrology*, vol. 2, no. 3, pp. 213–219, 2000.

[42] S. Ramachandran, Y. Sridhar, S. K. G. Sam et al., "Aphroidisiac activity of *Butea frondosa* Koen. ex Roxb. extract in male rats," *Phytomedicine*, vol. 11, no. 2-3, pp. 165–168, 2004.

[43] N. S. Chauhan and V. K. Dixit, "Spermatogenic activity of rhizomes of *Curculigo orchioides* gaertn in male rats," *International Journal of Applied Research in Natural Products*, vol. 1, no. 2, pp. 26–31, 2008.

[44] M. A. Abdel-Wahhab, A. A. El-Nekeety, S. E. Aly, W. J. Yoon, Y. T. Kim, and M. H. Park, "Improvement of sexual behavior in male rats via dietary supplementation with Panax ginseng extract standardized with ginsenoside Rg3," *Journal of Medical Sciences (Faisalabad)*, vol. 13, no. 5, pp. 337–345, 2013.

[45] E. A. Nantia, P. F. Moundipa, T. K. Monsees, and S. Carreau, "Medicinal plants as potential male anti-infertility agents: a review," *Basic and Clinical Andrology*, vol. 19, no. 3, pp. 148–158, 2009.

[46] M. E. Light, S. G. Sparg, G. I. Stafford, and J. Van Staden, "Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 127–130, 2005.

[47] W. H. Walker, "Testosterone signaling and the regulation of spermatogenesis," *Spermatogenesis*, vol. 1, no. 2, pp. 116–120, 2011.

[48] N. Sofikitis, N. Giotitsas, P. Tsounapi, D. Baltogiannis, D. Giannakis, and N. Pardalidis, "Hormonal regulation of spermatogenesis and spermiogenesis," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 109, no. 3-5, pp. 323–330, 2008.

[49] G. Escott, L. da Rosa, and E. Loss, "Mechanisms of hormonal regulation of sertoli cell development and proliferation: a key process for spermatogenesis," *Current Molecular Pharmacology*, vol. 7, no. 2, pp. 96–108, 2015.

[50] S. Ramaswamy and G. F. Weinbauer, "Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone," *Spermatogenesis*, vol. 4, no. 2, p. e996025, 2015.

[51] L. O'Donnell, S. J. Meachem, P. G. Stanton, and R. I. McLachlan, "Endocrine regulation of spermatogenesis," in *Knobil and Neill's Physiology of Reproduction*, J. D. Neill, T. M. Plant, D. W. Pfaff et al., Eds., pp. 1017–1070, Elsevier, Sydney, Australia, 2006.

[52] Z. K. Ismael, L. A. Al-Anbari, and H. A. L. Mossa, "Relationship of FSH, LH, DHEA and testosterone levels in serum with sperm function parameters in infertile men," *Journal of Pharmaceutical Sciences and Research*, vol. 9, no. 11, pp. 2056–2061, 2017.

[53] M. S. Khan, I. Ali, F. Tahir, and G. M. Khan, "Simultaneous analysis of the three hormones involved in spermatogenesis and their interrelation ratios," *Pakistan Journal of Pharmaceutical Sciences*, vol. 21, no. 4, pp. 344–349, 2008.

[54] A. E. Willets, J. M. Corbo, and J. N. Brown, "Clomiphene for the Treatment of Male Infertility," *Reproductive Sciences*, vol. 20, no. 7, pp. 739–744, 2013.

[55] R. Kenjale, R. Shah, and S. Sathaye, "Effects of *Chlorophytum borivilianum* on sexual behaviour and sperm count in male rats," *Phytotherapy Research*, vol. 22, no. 6, pp. 796–801, 2008.

[56] N. J. Gakungo, K. Mugisha, D. Owiny, and P. Waako, "Effects of crude aqueous leaf extracts of Citropsis articulata and Mystroxylon aethiopicum on sex hormone levels in male albino rats," *International Journal of Pharmaceutical Sciences Invention*, vol. 3, no. 1, pp. 5–17, 2014.

[57] A. Khaki, F. Fathiazad, M. Nouri et al., "The effects of Ginger on spermatogenesis and sperm parameters of rat," *Iranian Journal of Reproductive Medicine*, vol. 7, no. 1, pp. 7–12, 2009.

[58] A. Ghasemzadeh, H. Z. E. Jaafar, and A. Rahmat, "Antioxidant activities, total phenolics and flavonoids content in two varieties of malaysia young ginger (*Zingiber officinale* Roscoe)," *Molecules*, vol. 15, no. 6, pp. 4324–4333, 2010.

[59] F. Mohammadi, H. Nikzad, A. Taherian, J. A. Mahabadi, and M. Salehi, "Effects of herbal medicine on male infertility," *Anatomical Sciences*, vol. 10, no. 4, pp. 3–16, 2013.

[60] D. Jiang, A. Coscione, L. Li, and B.-Y. Zeng, "Effect of chinese herbal medicine on male infertility," *International Review of Neurobiology*, vol. 135, pp. 297–311, 2017.

[61] A. Mehrotra, D. K. Katiyar, A. Agarwal, V. Das, and K. K. Pant, "Role of total antioxidant capacity and lipid peroxidation in fertile and infertile men," *Biomedical Research (India)*, vol. 24, no. 3, pp. 347–352, 2013.

[62] M. R. Moein, S. Vahidi, J. Ghasemzadeh, and N. Tabibnejad, "Comparison of reactive oxygen species in neat and washed semen of infertile men," *Iranian Journal of Reproductive Medicine*, vol. 12, no. 5, pp. 301–306, 2014.

[63] A. Agarwal, R. A. Saleh, and M. A. Bedaiwy, "Role of reactive oxygen species in the pathophysiology of human reproduction," *Fertility and Sterility*, vol. 79, no. 4, pp. 829–843, 2003.

[64] H. Wagner, J. W. Cheng, and E. Y. Ko, "Role of reactive oxygen species in male infertility: An updated review of literature," *Arab Journal of Urology*, vol. 16, no. 1, pp. 35–43, 2019.

[65] B. J. Kerr, "Normal spermatogenesis," in *Male reproductive dysfunction pathophysiology and treatment*, R. F. Kandeel, S. R. Swerdloff, and L. J. Pryor, Eds., pp. 57–70, Informa Health Care, New York, NY, USA, 2007.

[66] E. de Lamirande and C. O'Flaherty, "Sperm activation: role of reactive oxygen species and kinases," *Biochimica et Biophysica Acta—Proteins and Proteomics*, vol. 1784, no. 1, pp. 106–115, 2008.

[67] N. Kemal Duru, M. Morshedi, and S. Oehninger, "Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa," *Fertility and Sterility*, vol. 74, no. 6, pp. 1200–1207, 2000.

[68] S. A. Sheweita, A. M. Tilmisany, and H. Al-Sawaf, "Mechanisms of male infertility: role of antioxidants," *Current Drug Metabolism*, vol. 6, no. 5, pp. 495–501, 2005.

[69] R. J. Aitken, T. B. Smith, M. S. Jobling, M. A. Baker, and G. N. De Iuliis, "Oxidative stress and male reproductive health," *Asian Journal of Andrology*, vol. 16, no. 1, pp. 31–38, 2014.

[70] A. Agarwal, K. P. Nallella, S. S. R. Allamaneni, and T. M. Said, "Role of antioxidants in treatment of male infertility: an overview of the literature," *Reproductive BioMedicine Online*, vol. 8, no. 6, pp. 616–627, 2004.

[71] R. J. Aitken, K. T. Jones, and S. A. Robertson, "Reactive oxygen species and sperm function—in sickness and in health," *Journal of Andrology*, vol. 33, no. 6, pp. 1096–1106, 2012.

[72] M. T. P. Gilbert, T. Haselkorn, M. Bunce et al., "The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when?" *PLoS ONE*, vol. 2, no. 6, article no. e537, 2007.

[73] T. T. Turner and J. J. Lysiak, "Oxidative stress: a common factor in testicular dysfunction," *Journal of Andrology*, vol. 29, no. 5, pp. 488–498, 2008.

[74] A. A. Adeneye, S. O. Adenekan, O. O. Adeyemi, and E. O. Agbaje, "Glucose utilization and anti-oxidative mechanisms of the aqueous Hunteria umbellata seed extract in alloxan induced diabetic rats," *Nigerian Journal of Physiological Sciences*, vol. 29, no. 1, pp. 071–079, 2014.

[75] P. Murugesan, T. Muthusamy, K. Balasubramanian, and J. Arunakaran, "Effects of vitamins C and E on steroidogenic enzymes mRNA expression in polychlorinated biphenyl (Aroclor 1254) exposed adult rat Leydig cells," *Toxicology*, vol. 232, no. 3, pp. 170–182, 2007.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 5376439, 13 pages
https://doi.org/10.1155/2019/5376439

Research Article

Fushiming Capsule Attenuates Diabetic Rat Retina Damage via Antioxidation and Anti-Inflammation

Mengshan He,[1] Pan Long,[2] Lunfeng Guo,[3] Mingke Zhang,[4] Siwang Wang,[1] and Hongling He[5]

[1]*Department of Chinese Material Medical and Natural Medicines, Fourth Military Medical University, Xi'an, Shaanxi 710032, China*
[2]*Center of Clinical Aerospace Medicine, Fourth Military Medical University, Xi'an, Shaanxi 710032, China*
[3]*Department of Pharmacy, Central Hospital of Ankang City, Ankang 725000, Shaanxi, China*
[4]*Xi'an Lejian Biological Technology Co., Ltd., Xi'an 710032, Shaanxi, China*
[5]*Academic Journals Publishing Center of Education Department, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, China*

Correspondence should be addressed to Siwang Wang; wangsiw@fmmu.edu.cn and Hongling He; hhl.09@163.com

Received 26 January 2019; Accepted 21 March 2019; Published 18 July 2019

Guest Editor: José C. T. Carvalho

Aims. Diabetic retinopathy (DR) remains one of the leading causes of acquired blindness. Fushiming capsule (FSM), a compound traditional Chinese medicine, is clinically used for DR treatment in China. The present study was to investigate the effect of FSM on retinal alterations, inflammatory response, and oxidative stress triggered by diabetes. *Main Methods*. Diabetic rat model was induced by 6-week high-fat and high-sugar diet combined with 35 mg/kg streptozotocin (STZ). 30 days after successful establishment of diabetic rat model, full field electroretinography (ffERG) and optical coherence tomography (OCT) were performed to detect retinal pathological alterations. Then, FSM was administered to diabetic rats at different dosages for 42-day treatment and diabetic rats treated with Calcium dobesilate (CaD) capsule served as the positive group. Retinal function and structure were observed, and retinal vascular endothelial growth factor-α (VEGF-α), glial fibrillary acidic (GFAP), and vascular cell adhesion protein-1 (VCAM-1) expressions were measured both on mRNA and protein levels, and a series of blood metabolic indicators were also assessed. *Key Findings*. In DR rats, FSM (1.0g/kg and 0.5g/kg) treatment significantly restored retinal function (a higher amplitude of b-wave in dark-adaptation 3.0 and OPs2 wave) and prevented the decrease of retinal thickness including inner nuclear layer (INL), outer nuclear layer (ONL), and entire retina. Additionally, FSM dramatically decreased VEGF-α, GFAP, and VCAM-1 expressions in retinal tissues. Moreover, FSM notably improved serum antioxidative enzymes glutathione peroxidase, superoxide dismutase, and catalase activities, whereas it reduced serum advanced glycation end products, methane dicarboxylic aldehyde, nitric oxide, and total cholesterol and triglycerides levels. *Significance*. FSM could ameliorate diabetic rat retina damage possibly via inhibiting inflammation and improving antioxidation.

1. Introduction

Diabetic retinopathy (DR) is a kind of neurovascular complication of diabetes [1]. Despite the availability of effective treatment, DR remains one of the most common causes of blindness among working age adults [2]. It is expected that the global number of diabetes patients will double by 2030 comparing with about 400 million in 2017 [3, 4]. Furthermore, more than a third of people with diabetes have symptoms of DR [5]. Currently, the main effective methods for DR treatment include laser photocoagulation, vitrectomy, and intravitreal injections of corticosteroids or anti-VEGF agents [2, 6]. These mentioned methods exhibit modest clinical effects, but none is yet to fully deaden clinical progression or reverse retinal damage [7]. Additionally, the frequent intraocular injections probably cause side effects involving corneal scarring and intraocular infection, not to mention the expenses related to frequent ophthalmology clinician visits. The alternative solution is limited for patients, which makes it an urgency to find new medications.

Fushiming capsule (FSM), a compound traditional Chinese medicine, is composed of bioactivity components, such

as puerarin, ginsenosides Rb1, ligustilide, aurantio obtusin, and chrysophanol [8]. Puerarin, being a vasodilator [9, 10], can reduce the oxidative stress damage of the retina [11, 12] and protect the retina through inhibiting inflammation and neuronal damage induced by proinflammatory factors [13, 14]. Ginsenosides Rb1 exhibits neuroprotective potential [15] and cytoprotective effects against oxidative stress-mediated apoptosis and other neuronal disturbances [16]. It is reported that ligustilide, which is responsible for the major bioactivities of Angelica sinensis, presents anti-inflammatory [17] and antioxidative [18] potential and prevents accumulation of platelets in blood vessels [19]. Aurantio obtusin, the major bioactivity component of cassia seed [20, 21], has various of biological properties, such as antioxidation, anticoagulating, and antihypertension [22, 23]. According to previous researches, Chrysophanol had antioxidative, anti-inflammatory, antiaging, and neuroprotection effects in Alzheimer disease (AD), cardiac injury, and diabetes [24–28]. Additionally, lycium barbarum and whitmania pigra are also constituents of FSM [8]. Thereinto, lycium barbarum could attenuate hypoxia and mitochondrial stress [29] and protect the function and viability of the retinal cells [30] in DR. Besides, whitmania pigra is applied clinically as a traditional Chinese medicine because of its anticoagulant and antithrombosis effects. Hence, this compound traditional Chinese medicine has potentially specific benefits for regulating the vascular microenvironment and resisting oxidation and inflammation.

It is well known that Calcium dobesilate (calcium 2,5-dihydroxybenzenesulfonic acid, CaD) has been prescribed for decades to some diabetic patients in the early stage of DR to stave off its progression [31]. According to the previous study, this synthetic compound exhibited systemic anti-inflammatory and antioxidative properties in the treatment of microcirculatory disorders [32]. Therefore, in this work, CaD was administered in the positive group, for 42 days, to diabetic animals, after 30 days of diabetes.

Among the factors implicated in the pathogenesis of DR, hyperglycemia-mediated inflammation and oxidative stress are considered as major relevant instigators of DR [33]. Inflammatory process, such as cytokine network and leukocyte adhesion (e.g., vascular cell adhesion protein-1 (VCAM-1)), induces vascular hyperpermeability and blood-retinal barrier breakdown [34]. Oxidative stress can directly or indirectly stimulate the release of proinflammatory cytokines, vascular endothelial growth factor-α (VEGF-α), and nitric oxide (NO), which results in retinal cell damage, pathological neovascularization, and the succeeding development of DR pathogenesis. Additionally, inflammation responses and low activities of antioxidative enzymes such as glutathione peroxidase (GSH-Px), Catalase (CAT), and superoxide dismutase (SOD) contribute to further increasing accumulation of reactive oxygen species (ROS) [35, 36]. This results in enhanced oxidative stress and aggravated retinal neuron damage and cell apoptosis. Moreover, hyperglycemia also causes Müller cells to produce proinflammatory cytokines to restore the retinal homeostasis [37, 38]. This is characterized by upregulation of glial fibrillary acidic (GFAP) and VEGF, which leads to a glial reaction and blood barrier hyperpermeability [39].

Based on the above observations, the present study was designed to test whether Fushiming capsule, a specific compound traditional Chinese medicine, could alleviate diabetic retina damage induced by high-fat and high-sugar diet combined with a low dose STZ injection and to determine how Fushiming capsule modulates oxidative stress and inflammatory responses.

2. Materials and Methods

2.1. Animals. Male Sprague Dawley (SD) rats (6-8 weeks old, 180-220 g) were obtained from the Laboratory Animal Center of Fourth Military Medical University in Xi'an, Shaanxi Province, China (license No.2014270138S). Rats were maintained under standard laboratory conditions, with 23°C ± 3°C room temperature, 40-65% humidity, and 12 h light/dark cycles, and were provided with ad libitum. All experiments were performed in accordance with the ARVO Statements for the use of Animals in Ophthalmic and Vision Research. All protocols were approved by the research ethics committee for care of laboratory animals at Fourth Military Medical University and experiments were conducted in compliance with its guidelines for experimental animals.

2.2. Diabetic Retinopathy Rat Model and Drug Administration. SD rats were fed with high-fat and high-sugar diet (breeding rodent material 54.6%, lard 16.9%, sucrose 14%, casein 10.2%, premix 2.1%, and maltodextrin 2.2%) (Slac Laboratory Animal, Shanghai, China) for 6 weeks after the rats were allowed to acclimate for 3 days. Then, the diabetic rat model was induced by a single intraperitoneal injection of streptozotocin (35 mg/kg body weight [40]) (Sigma, USA) after 6-week high-fat and high-sugar diet. Seventy-two hours after STZ injection, blood glucose level was measured by ACCU-CHEK Performa (Roche, Germany) and rats showing a blood glucose level above 16.7 mmol/L were considered as diabetes mellitus and selected for the study feeding with high-fat and high-sugar diet. After 30 days, ERG and OCT were performed for observing pathological changes in diabetic retinas. Then, according to the amplitude of the electroretinogram OPs2 wave, 40 successfully constructed diabetic retinopathy (DR) model rats were selected and randomly divided into 5 groups (n=8): untreated diabetic model group (normal saline, equal volume), positive group (calcium dobesilate (CaD) capsule, 0.2 g/kg, Guizhou Tian'an Pharmaceutical Co., Ltd., China) (CaD group), FSM high-dose group (FSM, 1.0 g/kg, Xi'an Lejian Biological Technology Co., Ltd., China), FSM medium-dose group (FSM, 0.5 g/kg), and FSM low-dose group (FSM, 0.25 g/kg), and different treatments were given for 42 consecutive days. In addition, 8 normal rats with matched ages were selected as the normal group (normal saline, equal volume) and were fed with ordinary diet. The method of administration is gavage, once a day. The rats were monitored throughout the study for body weight and blood glucose and were maintained on their respective diets throughout the treatment period.

2.3. Full Field Electroretinography (ffERG). Full field electroretinography (ffERG) measurements were obtained at 30

d after diabetic rat model established and 42 d posttreatment (72 d of total duration of diabetes). ERG operation procedures were performed like the method described previously [41, 42] which was in compliance with the ISCEV guidelines. The ffERG recording items covered dark-adapted 0.01 response, dark-adapted 3.0 response, dark-adapted oscillatory potential response, light-adapted 3.0 response, and light-adapted flicker response. Specifically, animals were placed in a dark environment for dark adaption overnight (>12 h) and prepared for recording under a dim red-light condition. Anesthesia was applied with intraperitoneal (IP) injection of 3 mL/kg 1% sodium pentobarbital (Sigma, USA) and 50 μL sumianxin II (Jilin Shengda Animal Pharmaceutical Co., Ltd., China). The pupils were dilated with 0.5% tropicamide solution (Shenyang Xingji Corporation, China). FfERG was recorded using the full-field (Ganzfeld) stimulation and a computer system (RETI port, Roland Consult GmbH, Germany) with custom-made silver chloride electrodes. The active electrode was a ring electrode placed at the center of the cornea. Stainless steel needle electrodes were placed in the cheek and tail to serve as the reference and ground leads, respectively. Levofloxacin eye drops (Suzhou, China) were used three times a day after ERG testing to avoid infection.

2.4. Optical Coherence Tomography (OCT). Optical coherence tomography (OCT) images were taken to observe structural changes of the retina at 30 d after diabetic rat model established and 42 d posttreatment (72 d of total duration of diabetes). OCT scans were performed with a Micron IV fundus camera and an OCT Scan Head equipped with a mouse objective lens. Linear OCT scans consisted of a series of 1024 single point A-Scans. Rat eyes had previously been dilated with 0.5% tropicamide. And hydroxyl ethyl cellulose l (Bausch & Lomb Freda, China) was used as a coupling gel. Fundus and OCT images were captured from 20 positions for each eye using a Retinal Imaging System (OPTO-RIS, OptoProbe, Canada) and 4D-ISOCT Microscope Imaging System (ISOCT, OptoProbe, Canada).

2.5. Determination of the Retinal VEGF-α, GFAP, and VCAM-1 mRNA Expression. After blood samples were taken, the left eyes of rats were removed and then the retina tissues were collected at 42 d posttreatment (72 d of total duration of diabetes). Total RNA was extracted from the retinal tissues using Trizol Reagent (Servicebio, G3013, Wuhan, China). RNA concentration was detected by Nanodrop 2000 (Thermo, New York, USA). cDNA was synthesized according to the manufacturer's instructions of RevertAid M-MuLV cDNA synthesis kit (Thermo, #k1622, USA). The fluorescent quantitative polymerase chain reaction (FQ-PCR) was performed using ABI Stepone plus real-time PCR device (New York, USA) according to the protocol of FastStart Universal SYBR-Green Master kit (Roche, Mannheim, Germany). Cycling conditions were as follows: 95°C, 10 min, and 40 cycles of 95°C, 15 s, 60°C, 60 s. The primers of VEGF-α were GAGCAGAAAGCCCATGAAGTG (forward) and ACTCCAGGGCTTCATCATTGC (reverse). The primers of GFAP were AGTCGGCGAGTTACCAGGAG (forward) and TTAATGACCTCGCCATCCCG (reverse). The primers of VCAM-1 were TGAACCCAAACAAAGGCAGAGTA (forward) and TTGGGAGTTGGAAAACCATCAC (reverse). The primers of β-actin were TGCTATGTTGCCCTAGACTTCG (forward) and GTTGGCATAGAGGTCTTTACGG (reverse). Quantification of VEGF-α, GFAP, and VCAM-1 mRNA was normalized to β-actin mRNA, and analysis was performed using $2-\Delta\Delta$Ct method. The final results were expressed as percentage relative to the normal group.

2.6. Determination of the Retinal VEGF-α, GFAP, and VCAM-1 Protein Expressions. The retinas tissues were separated and homogenized on ice in RIPA buffer (Beyotime, Nantong, Jiangsu Province, China) supplemented with 1:100 of proteinases/phosphatase inhibitor at 42 d posttreatment (72 d of total duration of diabetes) (n=4). Then, the lysates were centrifuged at 12,000 rpm at 4°C for 15 min to obtain the supernatant. A bicinchoninic acid (BCA) protein quantitation kit (Servicebio, Wuhan, China) was applied to determine the concentration of the protein. Equal amount of protein was denatured by boiling in loading sample buffer and then 30 μg protein from each sample was loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using gel (5%, 12%). Next, the proteins were transferred onto PVDF membrane (Millipore, USA) at 120 V for 100 min. The membranes were incubated with 5% non-fat milk solution (Servicebio, Wuhan, China) for 2 h at room temperature and then reacted with VEGF-α (1:1000; GB11034, Servicebio, Wuhan, China), GFAP (1:2000; GB11096, Servicebio, Wuhan, China), VCAM-1 (1:1000; 11444-1-AP, Proteintech Group, Wuhan, China), and β-actin (1:1000; GB12001, Servicebio, Wuhan, China) at 4°C overnight. The membranes were then incubated with HRP-conjugated secondary antibody (1:30000; GB23303, GB23301; Servicebio, Wuhan, China) at room temperature for 1 h and then enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, US) was used for protein visualization. The intensity of immunoreactivity was quantified by densitometry using Image J software (NIH).

2.7. Measurement of Blood Metabolic Indicators. After drug had been administered for 42 days (72 d of total duration of diabetes), fasted (12 h) rats were weighed and anesthetized (1% sodium pentobarbital (Sigma, USA), 3 mL/kg, i.p.). Blood specimens were collected from the vena cava. Serum samples were isolated after centrifugation of whole blood at 3500 r/min at 4°C for 15 min and were stored at −80°C. Enzyme activities of serum antioxidant parameters glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), and levels of serum oxidative stress parameter methane dicarboxylic aldehyde (MDA) and proinflammatory indicator nitric oxide (NO) were assessed using commercially available ultraviolet spectrometry method kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), according to the manufacturer's instructions. The levels of serum glucose (GLU), triglycerides (TG), and total cholesterol (TC) were estimated using commercially available kits from Huili Biotech (Changchun, China), according

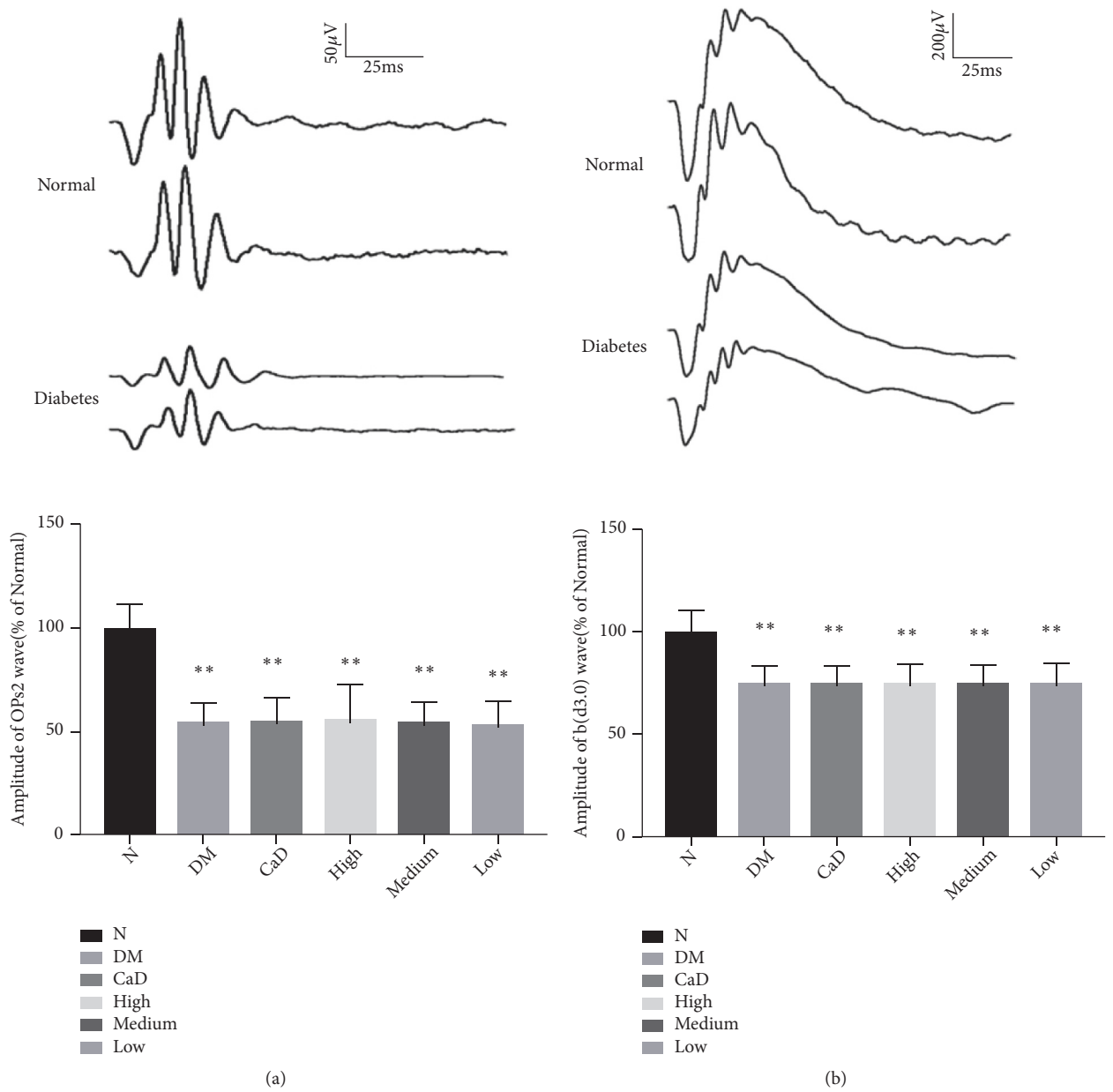


Figure 1: Diabetic rats present decreasing ERG responses after DR rat model established. (a) Dark-adapted OPs2 wave; (b) b-wave in dark-adaptation 3.0 response. Values are presented as mean ± SD, n=10, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group.

to the manufacturer's instructions. Additionally, advanced glycation end-product formations (AGEs) level was detected by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp., Wuhan, China), according to the manufacturer's instruction.

2.8. Statistical Analysis. All data were expressed as mean ± standard deviation. All statistical analyses were conducted using the SPSS version 19.0 software package (IBM, Chicago, IL, USA). One-way analysis of variance (ANOVA) test was performed, and post hoc multiple comparisons were conducted with LSD. $P < 0.05$ was regarded to be significant.

3. Results

3.1. Full Field Electroretinography (ffERG)

3.1.1. Full Field Electroretinography (ffERG) before the Treatment. Full field electroretinography (ffERG) was performed to evaluate the integral retinal function formed by various cell types in the retina. The b wave (dark-adaptation 3.0 response) reflects the electrical activity of cone cell and rod cell. OPs wave indirectly reflects the function of retinal blood vessels. As a typical representative of OPs wave, OPs2 wave can reflect the overall level of OPs wave. At 30 d after diabetic rat model established (before the treatment), as shown in Figure 1, it

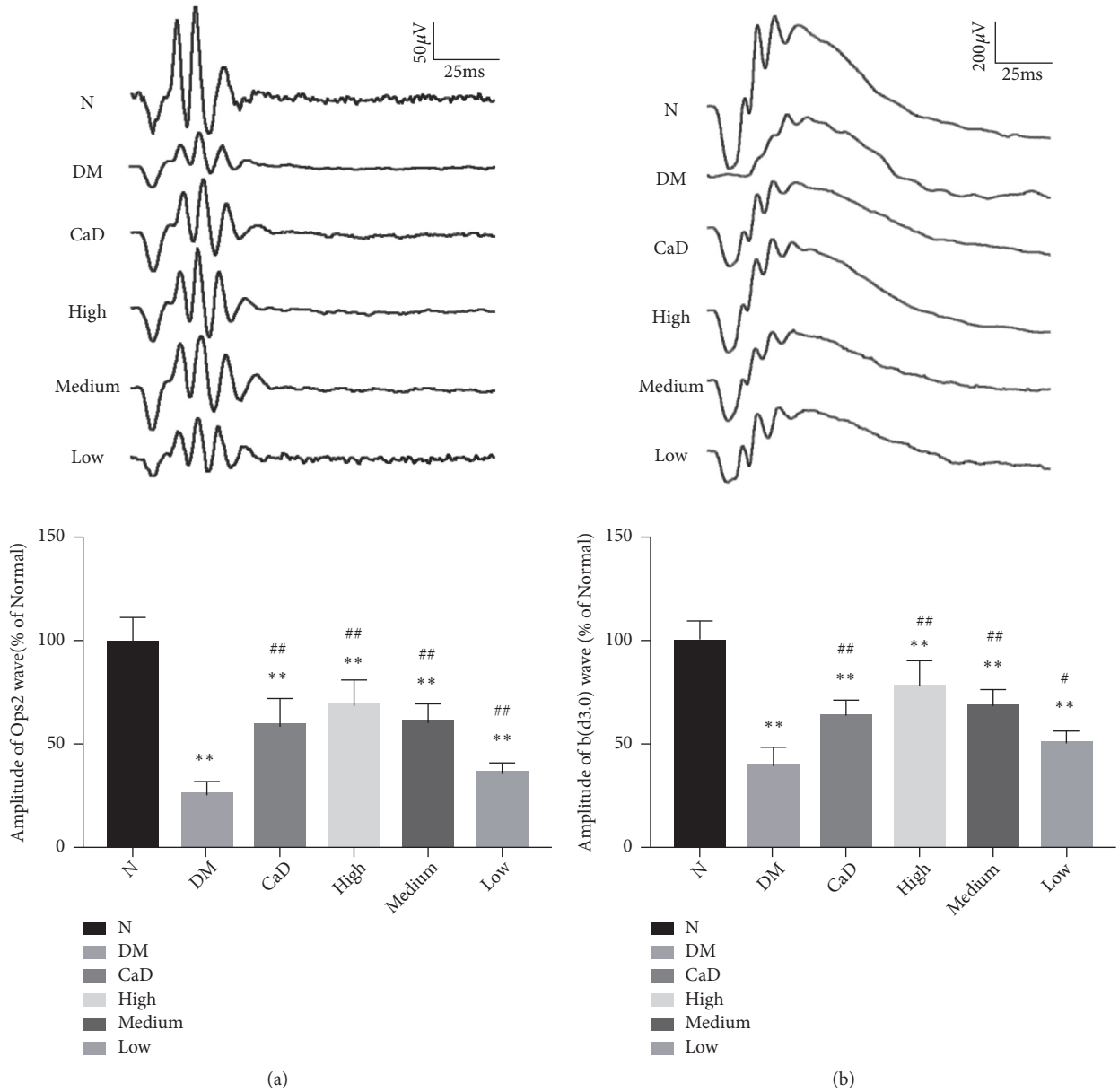


Figure 2: FSM protects diabetic rat ERG after 42-d treatment (72 d of total duration of diabetes). (a) OPs2 wave in different groups; (b) b-wave in dark-adaptation 3.0 response in different groups. Values are presented as mean ± SD, n=8. $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

was found that OPs2 wave decreased to 50-60% of normal eyes (all $p < 0.01$) and b (d3.0) wave decreased to 70-80% of normal eyes (all $p < 0.01$). According to the amplitude of OPs2 wave, diabetic rats were randomly divided into 5 groups as previously described, and the age matching normal SD rats were selected as normal group. The final results were expressed as percentage relative to the normal group.

3.1.2. Full Field Electroretinograms (ffERG) after the Treatment. After 42-d treatment (72 d of total duration of diabetes), rats in each group as previously described underwent ERG tests. As shown in Figure 2, compared with normal group, ERG showed that all diabetic group exhibited a significant decreasing amplitude of OPs2 wave (all $p < 0.01$) and b (d3.0) wave (all $p < 0.01$). Notably, in untreated diabetic model group, OPs2 wave decreased to 26% of normal group, and b (d3.0) wave decreased to 40% of normal group. However, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group presented an obviously higher amplitude of OPs2 and b (d3.0) wave (OPs2: CaD, $p < 0.01$; FSM high-dose, $p < 0.01$; FSM medium-dose, $p < 0.01$; FSM low-dose, $p < 0.01$; d3.0 response: CaD, $p < 0.01$; FSM high-dose, $p < 0.01$; FSM medium-dose, $p < 0.01$; FSM low-dose, $p < 0.05$) compared with untreated diabetic model group. The final results were expressed as percentage relative to the normal group.

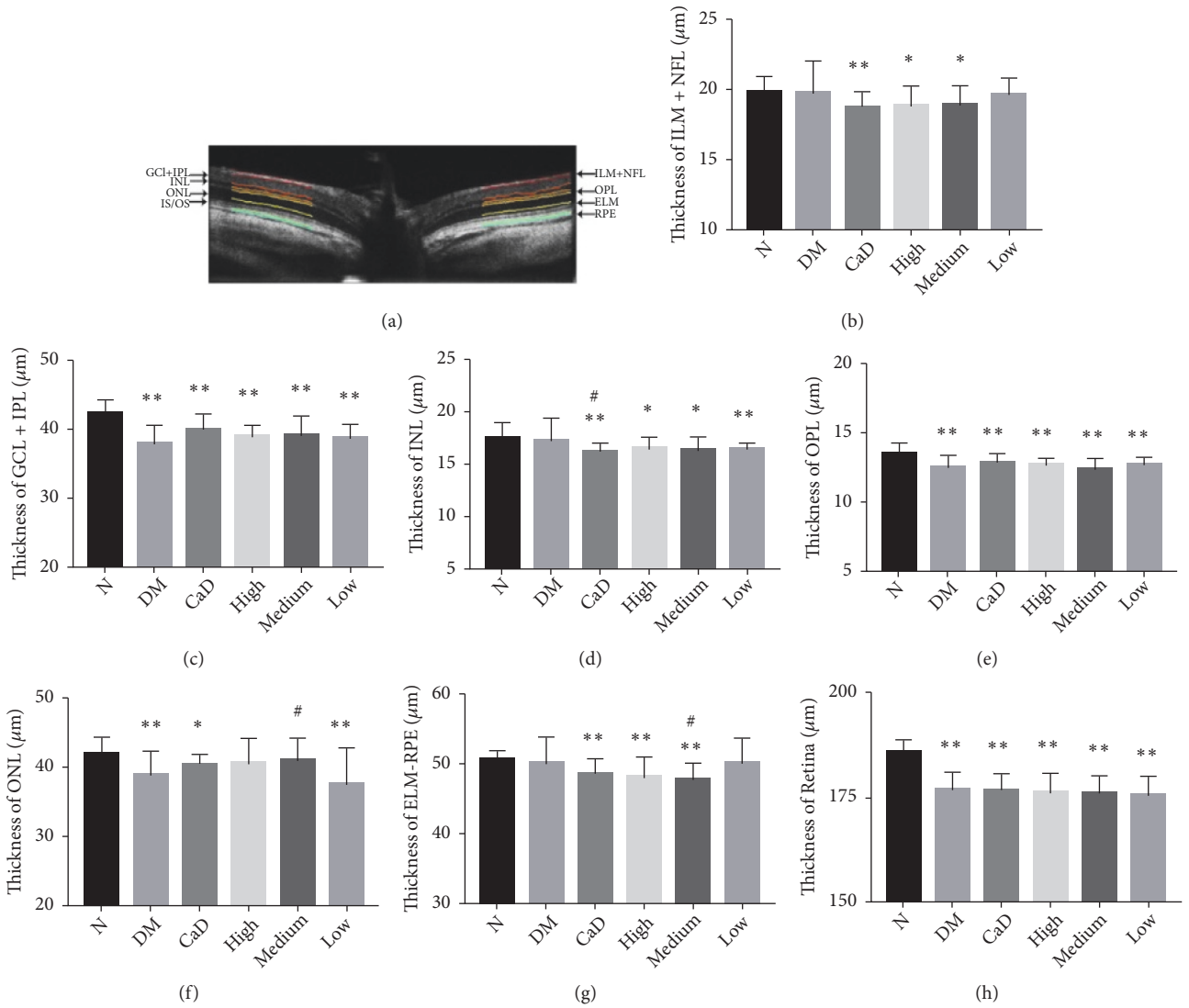


Figure 3: Diabetic rat retinal thickness is decreasing at 30 d after diabetic rat model established (before the treatment). (a) Representative OCT cross-sectional image of retina. (b) The thickness of ILM + NFL; (c) the thickness of GCL + IPL; (d) the thickness of INL; (e) the thickness of OPL; (f) the thickness of ONL; (g) the thickness of EML to RPE; (h) the thickness of entire retina. ILM+NFL: internal limiting membrane and nerve fiber layer; GCL + IPL: ganglion cell layer and inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; EML to RPE: external limiting membrane to retinal pigment epithelium. Values are presented as mean ± SD, n=10, $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group;$^{\#}p < 0.05$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

3.2. OCT Analysis

3.2.1. OCT Analysis before the Treatment. OCT was applied to evaluate retinal morphometric structure. As shown in Figure 3, the total retinal thickness decreased significantly at 30 d after diabetic rat model established. Particularly, ganglion cell layer (GCL) and inner plexiform layer (IPL), outer plexiform layer (OPL), and entire retina thickness became thinner (all $p < 0.01$). Thus, diabetic rats exhibited a thinner retinal thickness, which indicated to some extent that the retinal structure alterations occurred.

3.2.2. OCT Analysis after the Treatment. OCT was examined to observe retinal structural alterations after 42-d treatment (72 d of total duration of diabetes). As shown in Figure 4, the retina thickness of diabetic rats, especially, ganglion cell layer and inner plexiform layer (GCL and IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and entire retinal thickness were declining compared with normal group (all $p < 0.05$). Additionally, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group showed a lower decrease in INL, ONL, and total retinal thickness (all $p < 0.01$) compared with untreated diabetic rats. Moreover, CaD group and FSM high-dose group had thicker GCL and IPL thickness than untreated diabetic rats (all $p < 0.01$). Besides, CaD group, FSM high-dose, and FSM medium-dose group showed thicker external limiting membrane (ELM) and photoreceptor inner/outer segment (IS/OS) thickness

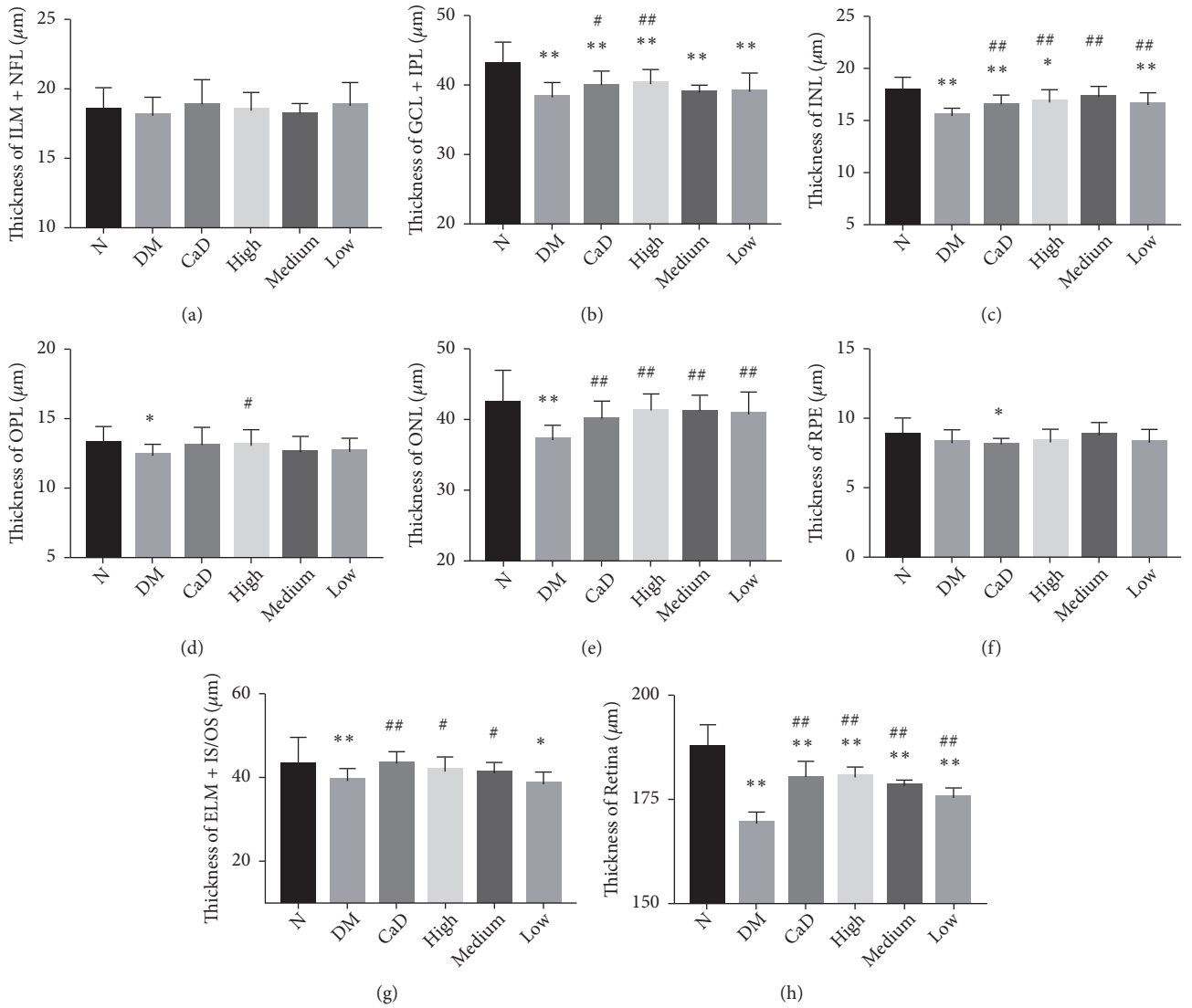


Figure 4: FSM protects diabetic rat retinal morphometric structure after 42-d treatment (72 d of total duration of diabetes). (a) The thickness of ILM & NFL; (b) the thickness of GCL + IPL; (c) the thickness of INL; (d) the thickness of OPL; (e) the thickness of ONL; (f) the thickness of RPE; (g) the thickness of EML + IS/OS; (h) the thickness of entire retina. ILM+NFL: internal limiting membrane and nerve fiber layer; GCL + IPL: ganglion cell layer and inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium; IS/OS + ELM: photoreceptor inner segment/outer segment and external limiting membrane. Values are presented as mean ± SD, n=8. $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

than untreated diabetic rats (all $p < 0.05$). FSM and CaD both showed protective effect on retinal structure integrity. As for the entire thickness of internal limiting membrane (ILM) and nerve fiber layer (NFL), there existed no significant difference among different groups (all $p > 0.05$).

3.3. VEGF-α, GFAP, and VCAM-1 mRNA Expressions. In order to evaluate the potential anti-inflammatory properties and effect on glial cell reactivity, VEGF-α, GFAP, and VCAM-1 mRNA levels were analyzed by FQ-PCR after 42-d treatment (72 d of total duration of diabetes). As is shown in Figure 5, the expressions of VEGF-α, GFAP, and VCAM-1 in all diabetic groups, excepting the expression of GFAP in FSM high-dose group, were incredibly more than those in normal group (all $p < 0.05$). Nevertheless, the expression of GFAP in CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group was decreasing compared with that in untreated diabetic model group (all $p < 0.05$). Moreover, the expressions of VEGF-α and VCAM-1 in CaD group, FSM high-dose, and FSM medium-dose group were less than those in untreated diabetic model group (all $p < 0.05$).

3.4. VEGF-α, GFAP, and VCAM-1 Protein Expressions. After 42-d treatment (72 d of total duration of diabetes), proinflammatory molecular VEGF-α, GFAP, and Vcam-1 protein expressions were detected by western blot method. As is

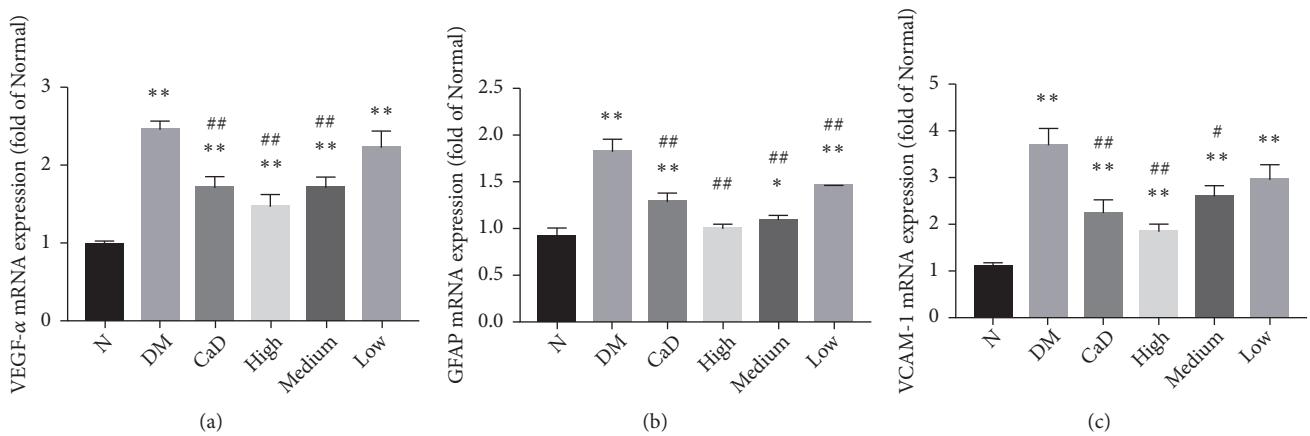


Figure 5: FSM decreases diabetic rat retinal VEGF-α, GFAP, and VCAM-1 mRNA levels after 42-d treatment (72 d of total duration of diabetes). The mRNA levels were assessed by FQ-PCR. (a) Values of VEGF-α. (b) Values of GFAP. (c) Values of VCAM-1. Data are presented as percentage of control and values are presented as mean ± SD, n=3-4. $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

shown in Figure 6, the expressions of VEGF-α, GFAP, and VCAM-1 in all diabetic groups, excepting the expression of GFAP in FSM high-dose group, were notably more than those in normal group, in agreement with the results obtained by FQ-PCR. However, the expressions of VEGF-α and GFAP in CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group were less than those in untreated diabetic model group (all $p < 0.05$). Additionally, the expression of VCAM-1 in CaD group and FSM high-dose and FSM medium-dose groups was less than that in untreated diabetic model group (all $p < 0.05$), in consistence with the results obtained by FQ-PCR.

3.5. Blood Metabolic Indicators

3.5.1. The Level of GLU, TC, and TG. As compared with normal rats, serum levels of GLU, TC, and TG in diabetic rats were markedly increased (all $p < 0.05$) (Figure 7). There is no significant difference in GLU among diabetic groups (all $p > 0.05$). After 42-d treatment (72 d of total duration of diabetes), CaD group, FSM high-dose group, and FSM medium-dose group notably decreased TC level (all $p < 0.01$ versus the untreated diabetic model group). Additionally, CaD group and FSM medium-dose group significantly decreased TG level (all $p < 0.05$ versus the untreated diabetic model group).

3.5.2. The Level of Detrimental Accumulation of AGEs, MDA, and NO. Serum AGEs, MDA, and NO levels were measured to assess the degree of oxidative stress and inflammatory condition/nitrosative stress in rats after 42-d treatment (72 d of total duration of diabetes). As compared with normal rats, serum levels of AGEs, MDA, and NO in diabetic rats, excepting the levels of MDA in FSM high-dose and medium-dose group, were markedly increased (all $p < 0.05$) (Figure 8). As compared with untreated diabetic model group, CaD group, FSM high-dose group, and FSM medium-dose group notably decreased AGEs and NO levels (all $p < 0.05$). Additionally, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group notably decreased MDA level (all $p < 0.01$ versus the untreated diabetic model group).

3.5.3. The Activity of Antioxidant Enzymes GSH-Px, SOD, and CAT. Considering the antioxidant stress property of FSM, the effects of this compound were evaluated on serum GSH-Px, SOD, and CAT activities after 42-d treatment (72 d of total duration of diabetes). As compared with normal rats, serum levels of GSH-Px, SOD, and CAT in diabetic rats, excepting the levels of SOD and CAT in FSM high-dose group, were markedly decreased (all $p < 0.05$) (Figure 9). However, CaD group, FSM high-dose, and FSM medium-dose group notably increased GSH-Px level (all $p < 0.05$ versus the untreated diabetic model group). FSM high-dose and FSM medium-dose group significantly increased SOD level (all $p < 0.05$ versus the untreated diabetic model group). Additionally, CaD group and FSM high-dose group significantly decreased CAT level (all $p < 0.05$ versus the untreated diabetic model group).

4. Discussion

Diabetic retinopathy (DR) is the leading cause of visual impairment and preventable blindness [1], which represents a significant socioeconomic burden for healthcare systems worldwide [7]. In the study, we evaluated the effect of compound traditional Chinese medicine FSM on DR treatment. A better retinal function and structure were found in FSM treatment groups because of an enhanced ability of antioxidation and anti-inflammation.

In the present study, diabetes was established by 6-week high-fat and high-sugar diet combined with 35 mg/kg streptozotocin (STZ) injection. We found that, at 30 days post diabetic rat model established, retinal function in the diabetic rats was decreased, both b (d3.0) wave and OPs2 wave compared with normal rats. Furthermore, diabetic rats

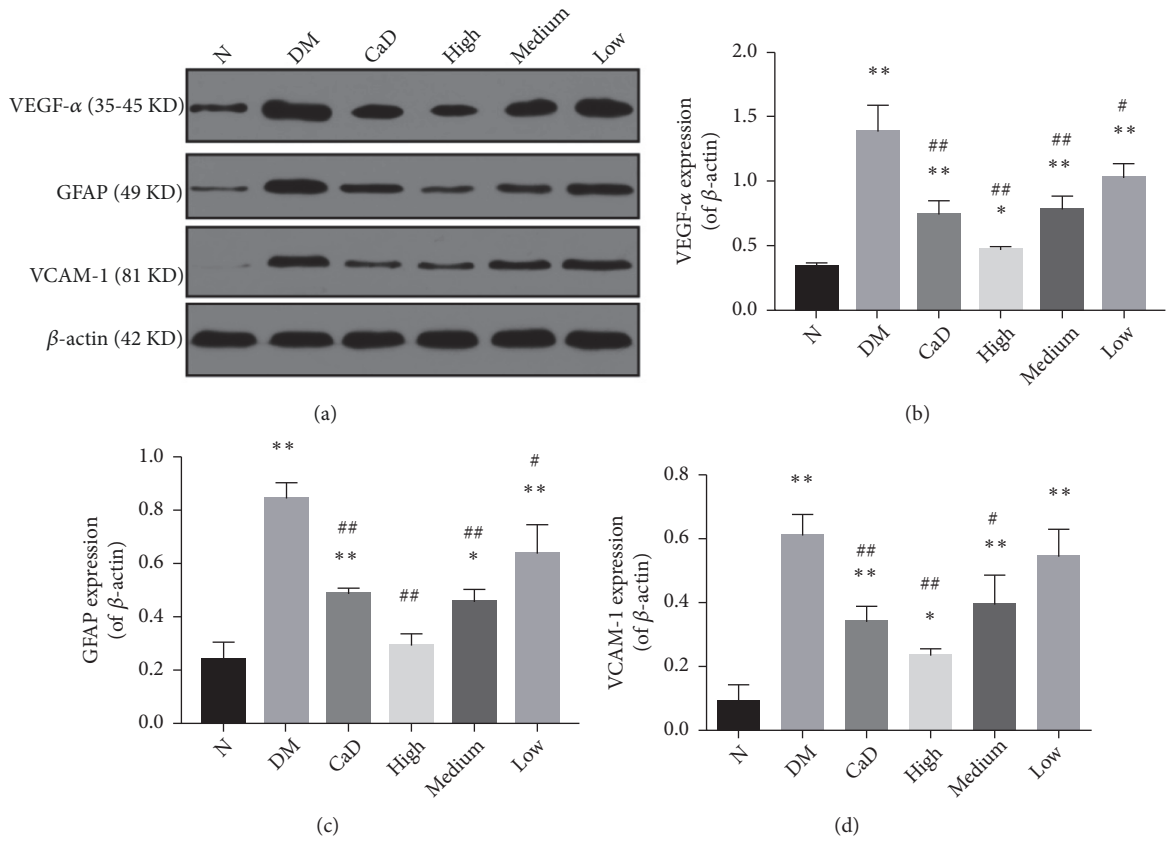


Figure 6: FSM decreases diabetic rat retinal VEGF-α, GFAP, and VCAM-1 protein levels after 42-d treatment (72 d of total duration of diabetes). (a) VEGF-α, GFAP, and VCAM-1 representative western blots, with the respective loading control (β-actin); (b) relative density of immunoblot of VEGF-α; (c) relative density of immunoblot of GFAP; (d) relative density of immunoblot of Vcam-1. Values are presented as mean ± SD, n=3-4. $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

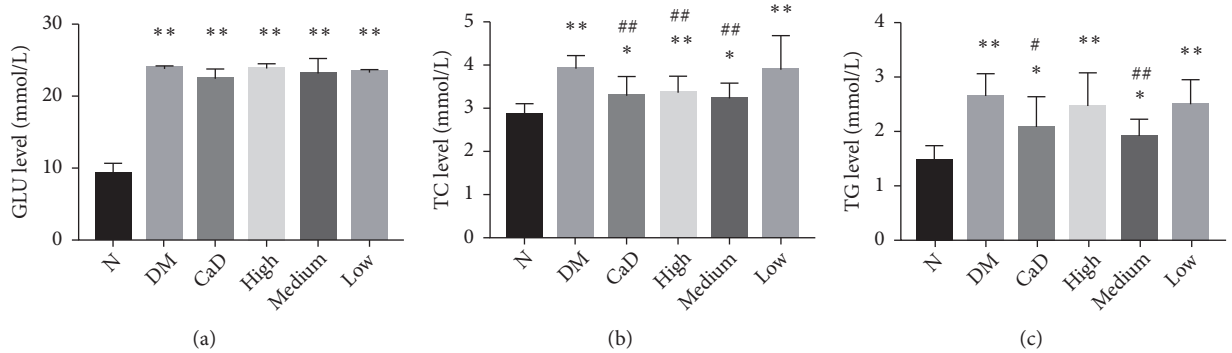


Figure 7: Effects of FSM on serum level of GLU, TC, and TG after 42-d treatment (72 d of total duration of diabetes). (a) The serum level of GLU level; (b) the serum level of TC; (c) the serum level of TG. Values are presented as mean ± SD, n=8. $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

were randomly grouped by OPs2 indicator. It is reported that oscillatory potentials (OPs) are considered the most relevant ERG test for DR diagnosis and progression [43]. Clinical research found that a frequent reduction or absence of OPs was shown in diabetic patients even in a preclinical stage of retinopathy, and the lower the amplitude of OPs2 is, the severer the DR is [44]. Otherwise, the reduction of retina thickness showed in OCT affirmed the retina morphological alterations. After 42-day treatment, retinal function in the FSM treated groups was restored in both b wave and OPs

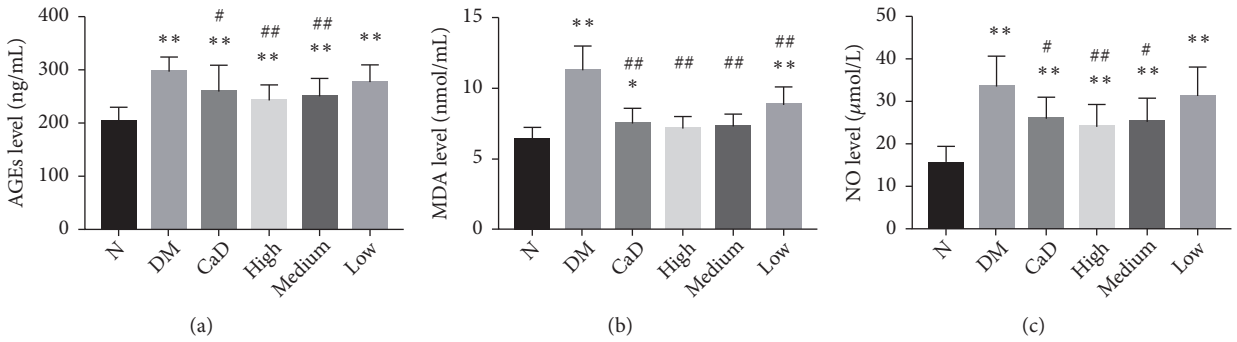


Figure 8: FSM downregulates the detrimental accumulation of AGEs, MDA, and NO after 42-d treatment (72 d of total duration of diabetes). (a) The serum level of AGEs; (b) the serum level of MDA; (c) the serum level of NO. Values are presented as mean ± SD, n=8. $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

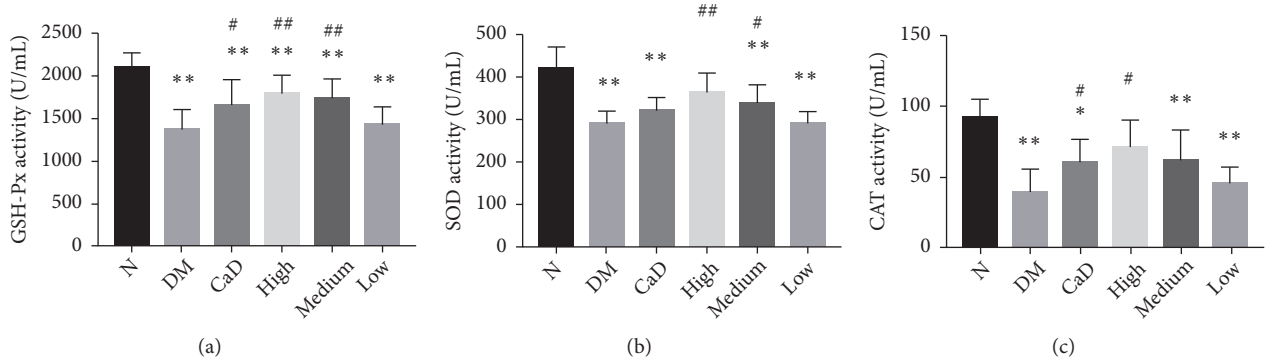


Figure 9: FSM upregulates the activity of serum antioxidant enzyme GSH-Px, SOD, and CAT after 42-d treatment (72 d of total duration of diabetes). (a) The serum activity of GSH-Px; (b) the serum activity of SOD; (c) the serum activity of CAT. Values are presented as mean ± SD, n=8. $*p < 0.05$, $**p < 0.01$: untreated diabetic model group, CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* normal group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$: CaD group, FSM high-dose, FSM medium-dose, and FSM low-dose group *vs* untreated diabetic model group.

which reflected functions of on & off bipolar cells and the vascular [45], compared with those of the untreated diabetic model group. Thus, FSM could rescue the retinal function and protect the function of retinal blood vessels. Additionally, FSM could significantly attenuate retinal structures damage induced by diabetes. Specifically, the thicknesses of INL, ONL, and entire retina in FSM groups were less decreasing, which revealed that the protective effect of FSM on the retinal structure contributed to resisting the retinal cells electrical activity decline.

Our results suggested a vascular endothelial dysfunction in diabetic rat retina, as estimated by assays of VCAM-1 protein and VCAM-1 mRNA expression. It was found that the upregulation of VCAM-1, a cell adhesion molecule, associated to increased inflammation-related genes transcription such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and nuclear factor-kappa B (NF-κB) [46]. Thus, we speculated that the enhanced endothelial activation reflected the inflammatory nature in this hyperglycemic state [47]. Interestingly, hyperglycemia also caused the stress condition of astrocytes and Müller cells, which was suggested by the increased expression of retinal GFAP in DR rats and these findings were in agreement with previous studies [38, 48]. The induction of GFAP expression usually occurred after retinal damage and indicated that the retina was undergoing degeneration [49]. It was worth mentioning that the upregulation of GFAP also initiated proinflammatory cytokines release [50]. Our findings suggested that FSM and CaD could relieve the inflammation by inhibiting the increase of VCAM-1 and GFAP expression. Furthermore, FSM and CaD reduced the upregulated expression of retinal VEGF-α, a major angiogenic factor, in DR rats. VEGF-α was considered to associate with oxidative stress and inflammatory response and induce retinal neovascularization, vascular leakage, and macular edema [51–53]. Additionally, the effect of CaD on VEGF-α was confirmed by other researches [54, 55], but FSM probably revealed better effects, at least according to the PCR and western blot results in our research. Our results presented that the compound traditional Chinese medicine FSM could downregulate the expression of VEGF-α to depress angiogenesis of DR, which may provide a new option or supplement for patients.

It was noteworthy that diabetic rats showed not only upregulation of inflammatory mediators and endothelial

activation in the retina, but also a systemic inflammatory response and oxidative stress in this model. It was reported that high serum NO production would increase systemic oxidative damage and nitrosative stress, which was related to increased severity of DR [18, 56, 57]. Moreover, upregulation of ROS could lead to enhanced activation of NF-kB, which, in turn, increased the release of proinflammatory cytokines and NO. Therefore, oxidative stress might indirectly contribute to DR through stimulating inflammation or directly contribute to the DR via oxidative damage to cells. Additionally, MDA, one of the breakdown products of peroxidized polyunsaturated fatty acids, was considered to be sensitive and reliable for the assessment of oxidative stress [58, 59]. AGEs accumulated under hyperglycemic conditions were reported to increase proinflammatory mediators, stimulate ROS production, cause retinal microvascular endothelial cells dysfunction, and enhance NO and VEGF secretion. They all occurred against a background of the various metabolic derangements that were inherent to diabetes [60]. Our results revealed that, compared with untreated diabetes, serum NO, MDA, and AGEs concentrations were downregulated and antioxidative SOD, GSH-Px, and CAT activities were upregulated by FSM, which indicated that FSM could protect the retinal structure and function via regulating nitrogen species homeostasis, reducing oxidative stress, and ameliorating inflammatory response.

Moreover, dyslipidemia was found in DR rats. Long-term effects of dyslipidemia might be of importance for progression of DR and it was associated with retinal hard exudate and visual loss [61]. Reducing the levels of serum triglycerides showed an effect on the progression of DR, as reported in several observation studies [1]. In consistence with those reports, our data supported the fact that TC and TG levels were increased in DR rats. Furthermore, FSM depressed serum TC and TG level, and this might be related to the hypolipidemic activity of puerarin, the main compound constituting FSM [8], which could rapidly be absorbed in the small intestines [62] and increase the fecal excretion of TG, promote the oxidation of fatty acids, and suppress the synthesis of fats [63].

In this study, we speculated that the active ingredients puerarin, ginsenosides Rb1, ligustilide, aurantio obtusin, and chrysophanol of FSM could protect retinal structure and promote retinal function via sustaining a better retinal microenvironment due to their anti-inflammatory, antioxidative, and anticoagulating abilities, which was proved by numerous researches [9–28]. Interestingly, puerarin was also reported to inhibit IL-1β-induced leukostasis by regulating the expression of VCAM-1 [14], and this at least partially supported our findings.

Our findings not only revealed the effects of FSM on retinal damage but also determined the pharmacological effect of CaD that was confirmed by other studies, and our results were consistent with those previous reports about CaD. In this regard, FSM showed satisfying benefits on protecting retinal function and structure and blood homeostasis, which resembled or rivaled CaD. However, lack of research on further mechanism of the major compounds of FSM may be the limitation in our study. In the next step, we would like to thoroughly evaluate the accurate mechanisms of the components of FSM on DR.

5. Conclusion

In summary, FSM exerted a multifaceted action on protecting the diabetic rat retinal function and morphological structure integrity. The potential mechanism of FSM protective role would be associated with antioxidant effects directly by scavenging free radicals or indirectly by increasing the antioxidant enzymes, regulating nitrogen species homeostasis, and ameliorating inflammatory response. Therefore, FSM could be contemplated as an alternative candidate for treatment of DR.

Data Availability

The original data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Mengshan He and Pan Long are the co-first authors and contributed equally to the work.

Acknowledgments

This work was supported by Shaanxi Science and Technology Innovation Project (grant no. 2015SF2-08-01, 2017ZDCXL-SF-01-01, and S2018-ZC-GCZXXY-SF-0005).

References

[1] S. D. Solomon, E. Chew, E. J. Duh et al., "Diabetic retinopathy: A position statement by the American Diabetes Association," *Diabetes Care*, vol. 40, no. 3, pp. 412–418, 2017.

[2] R. Muc, A. Saracen, and I. Grabska-Liberek, "Associations of diabetic retinopathy with retinal neurodegeneration on the background of diabetes mellitus. Overview of recent medical studies with an assessment of the impact on healthcare systems.," *Open Medicine (Poland)*, vol. 13, no. 1, pp. 130–136, 2018.

[3] W. R. Rowley, C. Bezold, Y. Arikan, E. Byrne, and S. Krohe, "Diabetes 2030: insights from yesterday, today, and future trends," *Population Health Management*, vol. 20, no. 1, pp. 6–12, 2017.

[4] M. E. Hartnett, W. Baehr, and Y. Z. Le, "Diabetic retinopathy, an overview," *Vision Research*, vol. 139, pp. 1–6, 2017.

[5] R. Lee, T. Y. Wong, and C. Sabanayagam, "Epidemiology of diabetic retinopathy, diabetic macular edema and related vision loss," *Eye and Vision*, vol. 2, article 17, 2015.

[6] M. Whitehead, S. Wickremasinghe, A. Osborne, P. Van Wijngaarden, and K. R. Martin, "Diabetic retinopathy: a complex pathophysiology requiring novel therapeutic strategies," *Expert Opinion on Biological Therapy*, vol. 18, pp. 1–14, 2018.

[7] C. Hernández, A. Simó-Servat, P. Bogdanov, and R. Simó, "Diabetic retinopathy: new therapeutic perspectives based on pathogenic mechanisms," *Journal of Endocrinological Investigation*, vol. 40, no. 9, pp. 925–935, 2017.

[8] X. Huimin, Z. Qiao, H. Yue, L. Yang, Z. Mingke, and W. Siwang, "Study on quality standard for fushiming capsule," *Journal of China Pharmacy*, vol. 29, pp. 749–753, 2018.

[9] K. H. Wong, G. Q. Li, K. M. Li, V. Razmovski-Naumovski, and K. Chan, "Kudzu root: traditional uses and potential medicinal benefits in diabetes and cardiovascular diseases," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 584–607, 2011.

[10] J. K. Prasain, N. Peng, R. Rajbhandari, and J. M. Wyss, "The Chinese Pueraria root extract (*Pueraria lobata*) ameliorates impaired glucose and lipid metabolism in obese mice," *Phytomedicine*, vol. 20, no. 1, pp. 17–23, 2012.

[11] Y. Cai, X. Zhang, X. Xu, and Y. Yu, "Effects of puerarin on the retina and STAT3 expression in diabetic rats," *Experimental and Therapeutic Medicine*, vol. 14, pp. 5480–5484, 2017.

[12] J. Kim, K. M. Kim, C.-S. Kim et al., "Puerarin inhibits the retinal pericyte apoptosis induced by advanced glycation end products in vitro and in vivo by inhibiting NADPH oxidase-related oxidative stress," *Free Radical Biology & Medicine*, vol. 53, no. 2, pp. 357–365, 2012.

[13] Y. Teng, H. Cui, M. Yang et al., "Protective effect of puerarin on diabetic retinopathy in rats," *Molecular Biology Reports*, vol. 36, no. 5, pp. 1129–1133, 2009.

[14] X. X. M. W. Zhu, "The effect of puerarin against IL-1 β-mediated leukostasis and apoptosis in retinal capillary endothelial cells (TR-iBRB2)," *Molecular Vision*, vol. 20, pp. 1815–1823, 2014.

[15] C. Fernández-Moriano, E. González-Burgos, I. Iglesias, R. Lozano, and M. P. Gómez-Serranillos, "Evaluation of the adaptogenic potential exerted by ginsenosides Rb1 and Rg1 against oxidative stress-mediated neurotoxicity in an in vitro neuronal model," *PLoS ONE*, vol. 12, no. 8, Article ID e182933, 2017.

[16] J. Wee, K. Park, and A. Chung, *Biological Activities of Ginseng and Its Application to Human Health*, 2011.

[17] W. Chao, Y. Hong, M. Chen, and B. Lin, "Inhibitory effects of Angelica sinensis ethyl acetate extract and major compounds on NF-kappaB trans-activation activity and LPS-induced inflammation," *Journal of Ethnopharmacology*, vol. 129, no. 2, pp. 244–249, 2010.

[18] S. Tasneem, B. Liu, B. Li, M. I. Choudhary, and W. Wang, "Molecular pharmacology of inflammation: Medicinal plants as anti-inflammatory agents," *Pharmacological Research*, vol. 139, pp. 126–140, 2019.

[19] W.-L. Wei, R. Zeng, C.-M. Gu, Y. Qu, and L.-F. Huang, "Angelica sinensis in China-A review of botanical profile, ethnopharmacology, phytochemistry and chemical analysis," *Journal of Ethnopharmacology*, vol. 190, pp. 116–141, 2016.

[20] Z. Bu, L. Lv, X. Li, C. Chu, and S. Tong, "pH-zone-refining elution–extrusion countercurrent chromatography: separation of hydroxyanthraquinones from Cassiae semen," *Journal of Separation Science*, vol. 40, no. 21, pp. 4281–4288, 2017.

[21] Q. Chen, Z. Xu, P. Yu, and P. Cen, "Advances in biochemical study of active anthraquinone constituents in Chinese traditional herb semen Cassiae," *Zhong Yao Cai*, vol. 25, no. 6, pp. 442–445, 2002.

[22] S. Li, Q. Li, X. Lv et al., "Aurantio-obtusin relaxes systemic arteries through endothelial PI3K/AKT/eNOS-dependent signaling pathway in rats," *Journal of Pharmacological Sciences*, vol. 128, no. 3, pp. 108–115, 2015.

[23] K. S. Kwon, J. H. Lee, K. S. So et al., "Aurantio-obtusin, an anthraquinone from cassiae semen, ameliorates lung inflammatory responses," *Phytotherapy Research*, vol. 32, no. 8, pp. 1537–1545, 2018.

[24] F. Lin, C. Zhang, X. Chen et al., "Chrysophanol affords neuroprotection against microglial activation and free radical-mediated oxidative damage in BV2 murine microglia," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 3, pp. 3447–3455, 2015.

[25] J. Zhang, C. L. Yan, S. Wang, Y. Hou, G. P. Xue, and L. Zhang, "Chrysophanol attenuates lead exposure-induced injury to hippocampal neurons in neonatal mice," *Neural Regeneration Research*, vol. 9, no. 9, pp. 924–930, 2014.

[26] C. Shen, J. Jiang, L. Yang, D. Wang, and W. Zhu, "Anti-ageing active ingredients from herbs and nutraceuticals used in traditional Chinese medicine: pharmacological mechanisms and implications for drug discovery," *British Journal of Pharmacology*, vol. 174, pp. 1395–1425, 2016.

[27] X. Chu, S. Zhou, R. Sun et al., "Chrysophanol relieves cognition deficits and neuronal loss through inhibition of inflammation in diabetic mice," *Neurochemical Research*, vol. 43, no. 4, pp. 972–983, 2018.

[28] Y. Lian, X. Xia, H. Zhao, and Y. Zhu, "The potential of chrysophanol in protecting against high fat-induced cardiac injury through Nrf2-regulated anti-inflammation, anti-oxidant and anti-fibrosis in Nrf2 knockout mice," *Biomedicine & Pharmacotherapy*, vol. 93, pp. 1175–1189, 2017.

[29] L. Tang, Y. Zhang, Y. Jiang et al., "Dietary wolfberry ameliorates retinal structure abnormalities in db/db mice at the early stage of diabetes," *Experimental Biology and Medicine*, vol. 236, no. 9, pp. 1051–1063, 2011.

[30] C.-K. Hu, Y.-J. Lee, C. M. Colitz, C.-J. Chang, and C.-T. Lin, "The protective effects of Lycium barbarum and Chrysanthemum morifolum on diabetic retinopathies in rats," *Veterinary Ophthalmology*, vol. 15, no. 2, pp. 65–71, 2012.

[31] P. Voabil, J. Liberal, E. C. Leal et al., "Calcium dobesilate is protective against inflammation and oxidative/nitrosative stress in the retina of a type 1 diabetic rat model," *Ophthalmic Research*, vol. 58, no. 3, pp. 150–161, 2017.

[32] R. Sevin and J. F. Cuendet, "Five years' experimentation with calcium dobesilate in the treatment of retinopathies," *Ophthalmologica*, vol. 170, no. 2-3, pp. 232–238, 1975.

[33] S. Roy, T. S. Kern, B. Song, and C. Stuebe, "Mechanistic insights into pathological changes in the diabetic retina: implications for targeting diabetic retinopathy," *The American Journal of Pathology*, vol. 187, no. 1, pp. 9–19, 2017.

[34] H. Karbasforooshan and G. Karimi, "The role of SIRT1 in diabetic retinopathy," *Biomedicine & Pharmacotherapy*, vol. 97, pp. 190–194, 2018.

[35] M. Brownlee, "The pathobiology of diabetic complications: a unifying mechanism," *Diabetes*, vol. 54, no. 6, pp. 1615–1625, 2005.

[36] S. Kubota, Y. Ozawa, T. Kurihara et al., "Roles of AMP-activated protein kinase in diabetes-induced retinal inflammation," *Investigative Ophthalmology & Visual Science*, vol. 52, no. 12, pp. 9142–9148, 2011.

[37] M. Mizutani, C. Gerhardinger, and M. Lorenzi, "Muller cell changes in human diabetic retinopathy," *Diabetes*, vol. 47, no. 3, pp. 445–449, 1998.

[38] E. Lieth, A. J. Barber, B. Xu et al., "Glial reactivity and impaired glutamate metabolism in short-term experimental diabetic retinopathy," *Diabetes*, vol. 47, no. 5, pp. 815–820, 1998.

[39] H. F. Dvorak, L. F. Brown, M. Detmar, and A. M. Dvorak, "Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis," *The American Journal of Pathology*, vol. 146, no. 5, pp. 1029–1039, 1995.

[40] X. Chen, L. Wang, Y. Wu et al., "Effect of puerarin in promoting fatty acid oxidation by increasing mitochondrial oxidative capacity and biogenesis in skeletal muscle in diabetic rats," *Nutrition & Diabetes*, vol. 8, no. 1, 2018.

[41] W. Yan, P. Long, T. Chen et al., "A natural occurring mouse model with adgrv1 mutation of usher syndrome 2C and characterization of its recombinant inbred strains," *Cellular Physiology and Biochemistry*, vol. 47, no. 5, pp. 1883–1897, 2018.

[42] M. He, P. Long, W. Yan et al., "ALDH2 attenuates early-stage STZ-induced aged diabetic rats retinas damage via Sirt1/Nrf2 pathway," *Life Sciences*, vol. 215, pp. 227–235, 2018.

[43] N. Pescosolido, A. Barbato, A. Stefanucci, and G. Buomprisco, "Role of electrophysiology in the early diagnosis and follow-up of diabetic retinopathy," *Journal of Diabetes Research*, vol. 2015, Article ID 319692, 8 pages, 2015.

[44] C. D. Luu, J. A. Szental, S.-Y. Lee, R. Lavanya, and T. Y. Wong, "Correlation between retinal oscillatory potentials and retinal vascular caliber in type 2 diabetes," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 1, pp. 482–486, 2010.

[45] D. L. McCulloch, M. F. Marmor, M. G. Brigell et al., "ISCEV Standard for full-field clinical electroretinography (2015 update)," *Documenta Ophthalmologica*, vol. 130, pp. 1–12, 2015.

[46] C. Gustavsson, C.-D. Agardh, A. V. Zetterqvist, J. Nilsson, E. Agardh, and M. F. Gomez, "Vascular cellular adhesion molecule-1 (VCAM-1) expression in mice retinal vessels is affected by both hyperglycemia and hyperlipidemia," *PLoS ONE*, vol. 5, no. 9, Article ID e12699, pp. 1–12, 2010.

[47] T. Khalfaoui, G. Lizard, and A. Ouertani-Meddeb, "Adhesion molecules (ICAM-1 and VCAM-1) and diabetic retinopathy in type 2 diabetes," *Journal of Molecular Histology*, vol. 39, no. 2, pp. 243–249, 2008.

[48] V. Asnaghi, C. Gerhardinger, T. Hoehn, A. Adeboje, and M. Lorenzi, "A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat," *Diabetes*, vol. 52, no. 2, pp. 506–511, 2003.

[49] A. K. H. Cheung, M. K. L. Fung, A. C. Y. Lo et al., "Aldose reductase deficiency prevents diabetes-induced blood-retinal barrier breakdown, apoptosis, and glial reactivation in the retina of *db*/*db* mice," *Diabetes*, vol. 54, no. 11, pp. 3119–3125, 2005.

[50] A. Araszkiewicz and D. Zozulinska-Ziolkiewicz, "Retinal neurodegeneration in the course of diabetes-pathogenesis and clinical perspective," *Current Neuropharmacology*, vol. 14, no. 8, pp. 805–809, 2016.

[51] J. Wang, E. Xu, M. H. Elliott, M. Zhu, and Y.-Z. Le, "Müller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage," *Diabetes*, vol. 59, no. 9, pp. 2297–2305, 2010.

[52] A. M. Joussen, V. Poulaki, M. L. Le et al., "A central role for inflammation in the pathogenesis of diabetic retinopathy," *The FASEB Journal*, vol. 18, no. 12, pp. 1450–1452, 2004.

[53] J. Fehér, S. Taurone, M. Spoletini et al., "Ultrastructure of neurovascular changes in human diabetic retinopathy," *International Journal of Immunopathology and Pharmacology*, vol. 31, Article ID 1042259236, 2017.

[54] O. Simó-Servat, C. Solà-Adell, P. Bogdanov, C. Hernández, and R. Simó, "Mechanisms of retinal neuroprotection of calcium dobesilate: therapeutic implications," *Neural Regeneration Research*, vol. 12, no. 10, pp. 1620–1622, 2017.

[55] Y. Zhou, J. Yuan, C. Qi, X. Shao, S. Mou, and Z. Ni, "Calcium dobesilate may alleviate diabetes-induced endothelial dysfunction and inflammation," *Molecular Medicine Reports*, vol. 16, no. 6, pp. 8635–8642, 2017.

[56] U. Förstermann and W. C. Sessa, "Nitric oxide synthases: regulation and function," *European Heart Journal*, vol. 33, no. 7, pp. 829–837, 2012.

[57] S. Sharma, S. Saxena, K. Srivastav et al., "Nitric oxide and oxidative stress is associated with severity of diabetic retinopathy and retinal structural alterations," *Clinical & Experimental Ophthalmology*, vol. 43, no. 5, pp. 429–436, 2015.

[58] S. Kulaksizoglu and A. Karalezli, "Aqueous humour and serum levels of nitric oxide, malondialdehyde and total antioxidant status in patients with type 2 diabetes with proliferative diabetic retinopathy and nondiabetic senile cataracts," *Canadian Journal of Diabetes*, vol. 40, no. 2, pp. 115–119, 2016.

[59] S. Mehrzadi, M. Motevalian, M. Rezaei Kanavi, I. Fatemi, H. Ghaznavi, and M. Shahriari, "Protective effect of melatonin in the diabetic rat retina," *Fundamental & Clinical Pharmacology*, vol. 32, no. 4, pp. 414–421, 2018.

[60] J. Xu, L.-J. Chen, J. Yu et al., "Involvement of advanced glycation end products in the pathogenesis of diabetic retinopathy," *Cellular Physiology and Biochemistry*, vol. 48, no. 2, pp. 705–717, 2018.

[61] G. R. Barile, S. I. Pachydaki, S. R. Tari et al., "The RAGE axis in early diabetic retinopathy," *Investigative Ophthalmology & Visual Science*, vol. 46, no. 8, pp. 2916–2924, 2005.

[62] J. K. Prasain, K. Jones, N. Brissie, R. Moore, J. M. Wyss, and S. Barnes, "Identification of puerarin and its metabolites in rats by liquid chromatography-tandem mass spectrometry," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 12, pp. 3708–3712, 2004.

[63] G. Zheng, L. Lin, S. Zhong, Q. Zhang, and D. Li, "Effects of puerarin on lipid accumulation and metabolism in high-fat diet-fed mice," *PLoS ONE*, vol. 10, no. 3, Article ID e0122925, 2015.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 9698727, 8 pages
https://doi.org/10.1155/2019/9698727

Research Article

Analgesic Effects of *Cnidium officinale* Extracts on Postoperative, Neuropathic, and Menopausal Pain in Rat Models

Eun Yeong Lim,[1,2] Jae Goo Kim,[1] Jaekwang Lee,[1] Changho Lee,[1] Jaewon Shim,[1] and Yun Tai Kim[1,2]

[1]*Division of Functional Food Research, Korea Food Research Institute, 245, Nongsaengmyeong-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do 55365, Republic of Korea*
[2]*Department of Food Biotechnology, Korea University of Science & Technology, 217 Gajeong-ro, Yuseong-gu, Daejeon 34113, Republic of Korea*

Correspondence should be addressed to Jaewon Shim; jwshim@kfri.re.kr and Yun Tai Kim; ytkim@kfri.re.kr

Received 22 February 2019; Accepted 2 June 2019; Published 16 June 2019

Guest Editor: José C. T. Carvalho

Cnidium officinale, widely cultivated in East Asia, has been reported to exhibit pharmacological efficacy in various disorders. However, little has been reported on its role as a pain killer. In this study, we reveal that the *C. officinale* extract (COE) has great efficacy as a novel analgesic in various *in vivo* pain models. Administration of COE attenuated hypersensitivity in all postoperative, neuropathic, and menopausal pain models. Decreased hyperalgesia was confirmed by a mechanical withdrawal threshold assay and ultrasonic vocalization call analysis. In addition, application of COE inhibited the induction of the proinflammatory cytokines and calpain-3 on dorsal root ganglion neurons in a spared nerve injury rat model. Treatment with ferulic acid, which was identified as one of the components of COE by HPLC analysis, alleviated nociceptive behaviors. Our findings suggest that ferulic acid is an active compound from COE, and COE is a potential phytomedical source for pain relief by inhibiting the process of inflammation.

1. Introduction

Pain is a basic sense essential to the survival of all living things [1]. Pain can signal the need to escape from dangerous stimuli and serve to minimize damage. Despite these benefits, unmanageable and long-lasting pain is one of the principal causes of poor quality of life, which is why many researchers are studying the mechanisms and causes of pain and are looking for novel materials to lessen pain. Although analgesic drugs are currently available and efficient for pain reduction, their repetitive application can result in several side effects, such as physical dependence and tolerance [2, 3]. Several studies also reported that nonsteroidal anti-inflammatory drugs (NSAIDs) might cause gastrointestinal lesions or renal and liver failure [4, 5]. Therefore, there is an emerging need for the discovery of novel materials for producing effective and safe analgesics [6, 7]. Many recent studies have evaluated innovative medical materials based on natural products [8–10].

Cnidium officinale, called "Chunkung," is a flowering plant widely cultivated in Korea, China, and Japan. *C. officinale* is traditionally used in Korea to attenuate pain and increase stamina [11]. Several studies have revealed its beneficial effects, including its antioxidant, anti-inflammatory, anticancer, antiangiogenic, and neuronal cell survival effects, in diverse health problems [12–16]. In one study, *C. officinale* extracts (COE) inhibited an LPS-induced increase in TNF-alpha and IL-12 in BV2 cells [12]. However, little is known of COE's ability to alleviate pain.

Therefore, in this study, we investigated the pain-relieving efficacy of COE in various *in vivo* pain models: a postoperative pain model [17], a neuropathic pain model [18], and a menopausal pain model [19]. In addition, we attempted to determine whether application of COE could be related to the expression of proinflammatory cytokines in a neuropathic pain model. We also showed that ferulic acid, an active compound of COE, could attenuate hyperalgesia.

2. Materials and Methods

2.1. Experimental Animals. All animal experiments complied with the guidelines of the Korea Food Research Institutional Animal Care and Use Committee (KFRI-M-13003-1). Male Sprague-Dawley (SD) rats were housed, two rats per cage, under a controlled temperature (23°C) and a 12-hour light/dark cycle (Samtako Bio Korea, Gyeonggi-do, Korea). The rats were housed for 1 week for acclimation before the experiments and were anesthetized with 2% isoflurane for the surgery.

2.2. Plantar Incision (PI) for Postoperative Pain Rat Model. PI surgery was performed as previously described [17]. Briefly, after rats were anesthetized with 2% isoflurane, a longitudinal incision of approximately 1 cm was performed with a scalpel, through the skin and fascia of the plantar aspect of the paw, starting 0.5 cm from the proximal edge of the heel and extending toward the toes. The plantar muscle was elevated and incised longitudinally. After hemostasis with gentle pressure, the skin was opposed with two single interrupted sutures using polyamide monofilaments. The animals were allowed to recover in a recovery chamber and their home cages. Rats were divided into five treatment groups after PI: (1) PI + vehicle, (2) PI + naproxen (30 mg/kg), (3) PI + COE (30 mg/kg), (4) PI + COE (100 mg/kg), and (5) PI + COE (300 mg/kg). COE was dissolved in distilled water. COE was given per os (p.o.) immediately after PI.

2.3. Spared Nerve Injury (SNI) Rat Model of Neuropathic Pain. SNI was performed as previously described [20]. The procedure consisted of an axotomy and ligation of the tibial and common peroneal nerves, leaving the sural nerve intact. The common peroneal and tibial nerves were ligated tightly with 5.0 silk and sectioned distal to the ligation, removing 2~4 mm of the distal nerve stump. Great care was taken to avoid any contact with or stretching of the intact sural nerve. The skin was opposed with two single interrupted sutures using polyamide monofilaments. The sciatic nerve and its branches were identically exposed in the sham control rat group, but they were neither transected nor ligated. Rats were divided into five treatment groups after the SNI surgery: (1) SNI + vehicle, (2) SNI + naproxen (30 mg/kg), (3) SNI + COE (30 mg/kg), (4) SNI + COE (100 mg/kg), and (5) SNI + COE (300 mg/kg). COE was also administered once daily p.o. immediately following surgery, and treatment was continued for 15 consecutive days.

2.4. Ovariectomy (OVX) Rat Model of Menopausal Pain. Eight-week-old female SD rats were purchased from Samtako Bio Korea (Gyeonggi-do, Korea). After acclimation for 1 week, rats were anesthetized using 2% isoflurane. Ovaries were removed bilaterally. The ovaries of rats in the sham group were merely touched using forceps. After recovery for 1 week, rats were divided into the following treatment groups: (1) sham + vehicle, (2) OVX + vehicle, (3) OVX + 17β-estradiol (E2, 10 μg/kg once daily, i.p), (4) OVX + COE 30 mg/kg, (5) OVX + COE 100 mg/kg, and (6) OVX + COE 300 mg/kg. COE was dissolved in distilled water for oral administration at the desired doses in a volume of 2 mL/kg once daily. E2 was dissolved in distilled water with 1% DMSO and 0.1% Tween 20. All groups were treated for 8 weeks.

2.5. Ultrasonic Vocalization (USV) Analysis. Pain-induced USV analysis was performed as previously described [21]. Adult rats were monitored for 22–27 kHz USV emissions after PI, and USVs were scored for 10 min using Sonotrack ultrasonic microphones (Metris B.V., KA, Hoofddorp, Netherlands) placed 25–30 cm from the heads of the animals. The emitted 'calls' were counted using Sonotrack 2.2.1 software.

2.6. Mechanical Withdrawal Threshold (MWT) Analysis. Animals were placed on an elevated wire grid, and the plantar surface of the paw was stimulated using a series of von Frey monofilaments of ascending force (Stoelting, Wood Dale, IL, USA). The MWT was the lowest force that evoked a brisk withdrawal response to one of three repetitive stimuli. A baseline measurement was obtained prior to surgery and at 6 and 24 h after surgery for PI and 3, 6, 9, 12, and 15 days after surgery for SNI to determine the time course of mechanical hyperalgesia.

2.7. Cytokine Analysis. All cytokine (interferon (IFN)-γ, interleukin (IL)-6, IL-12, and IL-2) levels in isolated L4, L5, and L6 dorsal root ganglion (DRG) of SNI rats were measured using a multiplex ELISA cytokine assay according to the manufacturer's instructions (Quansys Biosciences, Logan, UT, USA).

2.8. Immunohistochemistry. DRG were extracted 3 days after SNI and postfixed overnight in 4% paraformaldehyde. Immunofluorescent staining was performed on cryosections (20 μm). Sections were incubated in a solution containing 0.3% Triton X-100 and 3% goat serum for 1 hour at 25°C. Sections were incubated with a calpain-3 antibody (1:200, Sigma, St. Louis, MO, USA) overnight at 4°C. Sections were rinsed five times and incubated with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, 1:500, Invitrogen, Carlsbad, CA, USA) for 1 h at 25°C. Sections were mounted in antifade medium and stored at 4°C.

2.9. HPLC Conditions. Analytical HPLC analysis was performed using a Jasco HPLC system (Jasco, Hachioji, Tokyo, Japan), which comprised a PU-980 pump and an AS-950-10 autosampler equipped with an MD-2010 Plus multiwavelength detector. Chromatographic separation was performed using a Waters XTerra® RP18 (4.6 mm × 150 mm, particle size 3.5 μm) column. Ultraviolet (UV) detection was measured at 280 nm. A reverse-phase HPLC assay was performed using a mixture of 0.1% acetic acid (A) and 95% methanol in 0.1% acetic acid (B) as the mobile phase. Samples were eluted using the following gradient: 80% A and 20% B as initial conditions, 45% A and 55% B for 6 min, 15% A and 85% B for 5 min, and 80% A and 20% B for 5 min. The flow rate was 1 mL/min, and the column was maintained at 35°C. UV detection was measured at 280 nm. The injection volume was 10 μl of

Table 1: Chemical compounds content and retention time of COE.

Compound	RT (min)	Content (%)
Chlorogenic acid	6.943	1.8176
Ferulic acid	7.945	0.8805
Senkyunolide A	13.152	1.1817
(Z)-Ligustilide	14.445	1.1775

solution. The standard calibration curves of chlorogenic and ferulic acids exhibited linearity ($r^2 > 0.999**$) in the range of 3.125~50 μg/ml. The concentrations of chlorogenic acid and ferulic acid were 18.176 and 8.805 mg/g, respectively.

2.10. Statistical Analysis. Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, using Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) for multigroup comparisons. All data are presented as the mean standard error mean (SEM). $*$, $**$, and $***$ indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3. Results and Discussion

3.1. Mechanical Hypersensitivity Was Reduced by Oral Application of COE in Postoperative Pain Model. To assess the efficacy of COE in pain relief, we performed pain-evaluating behavioral tests in postoperative pain rat models. Postoperative pain was induced by a PI to observe the effective reduction of pain. After a longitudinal incision, approximately 1 cm in length, had been made with a scalpel, von Frey experiments were performed independently nine times. Naproxen treatment was used as a pain-reducing control drug through intraperitoneal injection. MWT were measured at 0, 5, and 24 hours after the PI. In the control group (rats without pre- and postoperative treatment of COE), the threshold declined rapidly from 51.500 ± 4.439 (g) to 0.560 ± 0.132 (g) and 0.457 ± 0.120 (g) at 5 and 24 hours after the incision, respectively, indicating that postoperative pain resulting from the PI sufficiently confirmed the pain response. In the group of rats to which 100 mg/kg COE was administered, the threshold reached 4.000 ± 0.777 (g) and 3.200 ± 0.730 (g), at 5 and 24 hours after the incision, respectively. These were significantly higher in the COE-treated group than in the control group at both 5 and 24 hours after incision. A higher threshold value indicates a reduction in hyperalgesia, and the above result suggests that COE relieves postoperative pain. To obtain a more convincing result about the pain-relieving effect of COE, we performed the MWT assay in the 300 mg/kg COE-treated group. As expected, pain relief was found to be COE dose dependent; thresholds reached 4.800 ± 0.800 (g) and 7.600 ± 0.748 (g) at 5 and 24 hours after the incision, respectively ($p < 0.05$, Figure 1(a), Table 1). However, treatment with 30 mg/kg COE did not seem to be enough to reduce hyperalgesia (0.752 ± 0.322 (g) and 0.864 ± 0.340 (g) at 5 and 24 hours after the incision, respectively).

Measuring the USV call is valuable for evaluating emotional status in rodents, as the USV call at 22 to 27 kHz usually occurs when the animals feel pain. We assessed the USV calls of rats after PI to confirm the ameliorative effect of COE. Oral administration of COE after PI reduced USV calls. The number of USV calls in the control group was 13.143 ± 1.792 at 6 hours and 13.429 ± 2.635 at 24 hours after incision, while those in the 100 mg/kg COE-treated group remained at 5.800 ± 1.533 at 6 hours and 5.500 ± 2.634 at 24 hours after incision (Figure 1(b)). An obvious pattern of reduction in USV calls was also detected in the 300 mg/kg treated group (1.200 ± 0.200 at 6 hours, 2.800 ± 0.583 at 24 hours after incision). As in the MWT assay, statistical significance was not observed between the 30 mg/kg COE-treated and the control groups.

3.2. Oral Application of COE Attenuated Neuropathic Pain by Inhibiting the Expression of Proinflammatory Cytokines and Calpain-3 in DRG Neurons. We assessed whether COE could ameliorate neuropathic pain in addition to postoperative pain. To induce neuropathic pain, we performed a SNI. SNI is a canonical method for causing neuropathic pain because it exhibits high reproducibility. The SNI procedure consists of ligation of the tibial and peroneal nerves after axotomy. We then evaluated MWT through von Frey assays every 3 days after the procedure. In the sham group, in which the sciatic nerves were exposed but neither transected nor ligated, MWT remained almost unchanged (Figure 2(a)). However, the control rats showed severe susceptibility 3 days after surgery. As in the postoperative pain experiment, naproxen was used as the positive control drug for pain reduction. The threshold scores were significantly higher in the groups treated with naproxen or COE than in the groups without any treatment (control), indicating that rats treated with COE were less susceptible to neuropathic pain than control rats (Figure 2(a)). These results show that COE is effective in a neuropathic pain model as well as in a postoperative pain model.

Inflammation is believed to be involved in the process of pain [10, 22]. Pain is associated with tissue injury and inflammation [22, 23] and is a characteristic symptom of arthritis [24]. Inflammatory mediators contain cytokines released from injured tissue to activate and sensitize nerve terminals. Recent studies have reported that not only immune cells but also DRG neurons release proinflammatory cytokines [25]. It has been reported that proinflammatory IL-1β expression is enhanced by crush injuries to peripheral nerves and causes increased levels of substance P and prostaglandin E2 in neuronal cells [26]. To elucidate the efficacy of COE in inflammation, we measured the expression levels of representative proinflammatory cytokines, IL-6, IFN-γ, IL-12, and IL-2 from DRG neurons after SNI. At 15 days after SNI, the expression levels of cytokines (IL-6: 80.195 ± 3.570, IFN-γ: 86.390 ± 4.376, IL-12: 40.391 ± 2.224, and IL-2: 12.554 ± 0.716) increased significantly compared with those after the sham operation (IL-6: 48.214 ± 0.999, IFN-γ: 47.298 ± 1.853, IL-12: 21.406 ± 1.017, and IL-2: 7.156 ± 0.337). Administration of COE (300 mg/kg) before and after the procedure resulted in a decrease of proinflammatory cytokine expression levels in DRG neurons, despite SNI (IL-6: 56.917 ± 0.661, IFN-γ: 55.189 ± 1.927, IL-12: 23.157 ± 1.166, and IL-2: 7.623 ± 0.248) (Figure 2(b)). Therefore, it is thought that application of COE shows an analgesic effect by reducing the expression

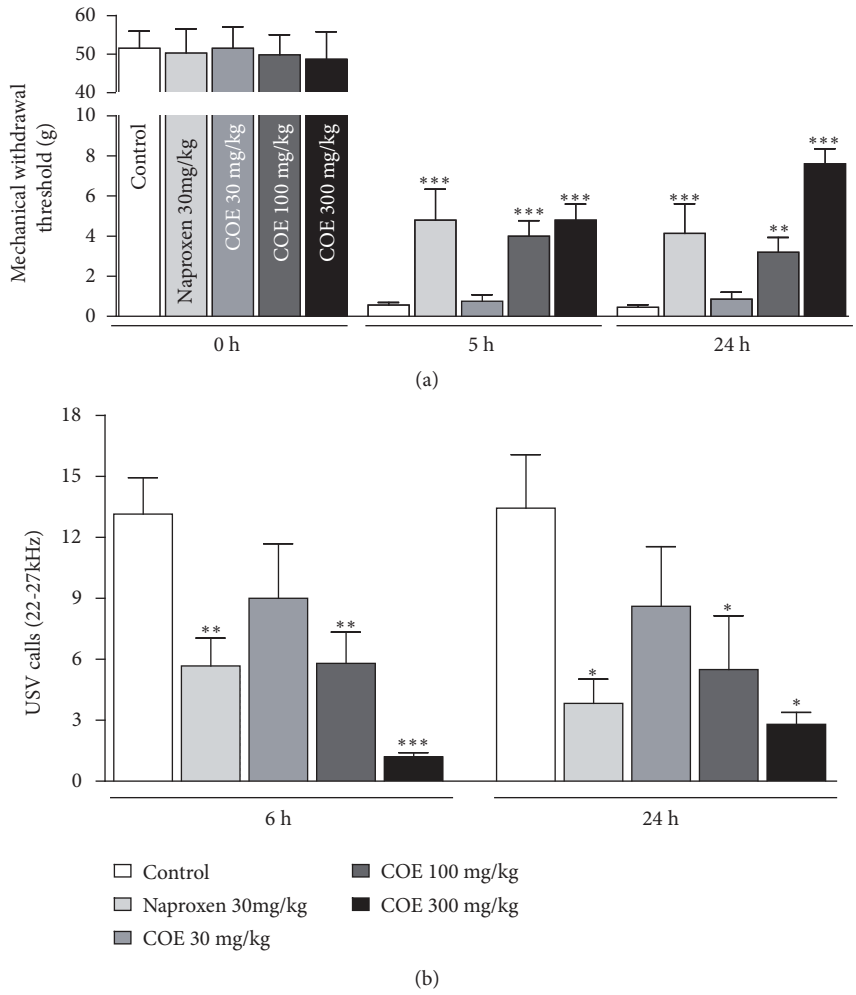


Figure 1: Reduced mechanical sensitivity by application of *C. officinale* extracts (COE) in a postoperative pain model. (a) Compared with the nontreated group, the groups treated with COE (100, 300 mg/kg) showed significantly higher mechanical withdrawal threshold at both 5 and 24 hours after surgery. Naproxen (30 mg/kg) was used as the positive control analgesic. (b) The quantification of 22–27 kHz USV calls revealed a significant difference between the COE-treated and the control groups. All data are the means ± SEM (n = 9 per group). The asterisks indicate significant difference from the control group, $* \ p < 0.05$, $** \ p < 0.01$, and $*** \ p < 0.001$.

levels of cytokines and inhibiting the inflammatory cascade. Despite this finding, further studies are needed to define the active and effectual components in COE and the exact mechanism on how it inhibits the expression of proinflammatory cytokines.

To find genes associated with neuropathic pain on DRG neurons, we performed a microarray analysis comparing the DRG of SNI in sham groups. Calpain-3 is one of the genes upregulated by neuropathic pain (data not shown). It is a member of the calpain family of calcium-dependent intracellular proteases. Its mRNA levels are high in the muscle and it is known to be involved in muscle regeneration [27, 28]. The relationship of calpain-3 and pain was not investigated previously, and the present study is the first to suggest an association of calpain-3 with neuropathic pain. Well-known calpain family proteins are calpain-1 and -2, which are associated with neuropathic pain [29, 30]. We confirmed the increase in calpain-3 in the SNI model by immunostaining with calpain-3 antibody in L5 DRG sections. Oral administration of COE significantly decreased calpain-3 upregulation. The relative intensity of fluorescence of calpain-3 increased in the control group (233.633 ± 5.229) compared to that in the sham group (100.000 ± 9.662), which was consistent with the microarray data. This increase disappeared in the group treated with 300 mg/kg COE (114.067 ± 7.358) (Figures 2(c) and 2(d)). This result indicated that COE attenuated neuropathic pain by inhibiting the expression of calpain-3.

3.3. Mechanical Hypersensitivity Was Reduced by Oral Application of COE in Menopausal Pain Model. The above-mentioned experiments confirmed the efficacy of COE in postoperative and neuropathic pain. An OVX model was used to examine its effect in menopausal pain [19]. We evaluated whether COE attenuated OVX-induced pain sensitivity. Oral application of COE reduced menopausal pain sensitivity in a concentration-dependent manner (24.04 ±

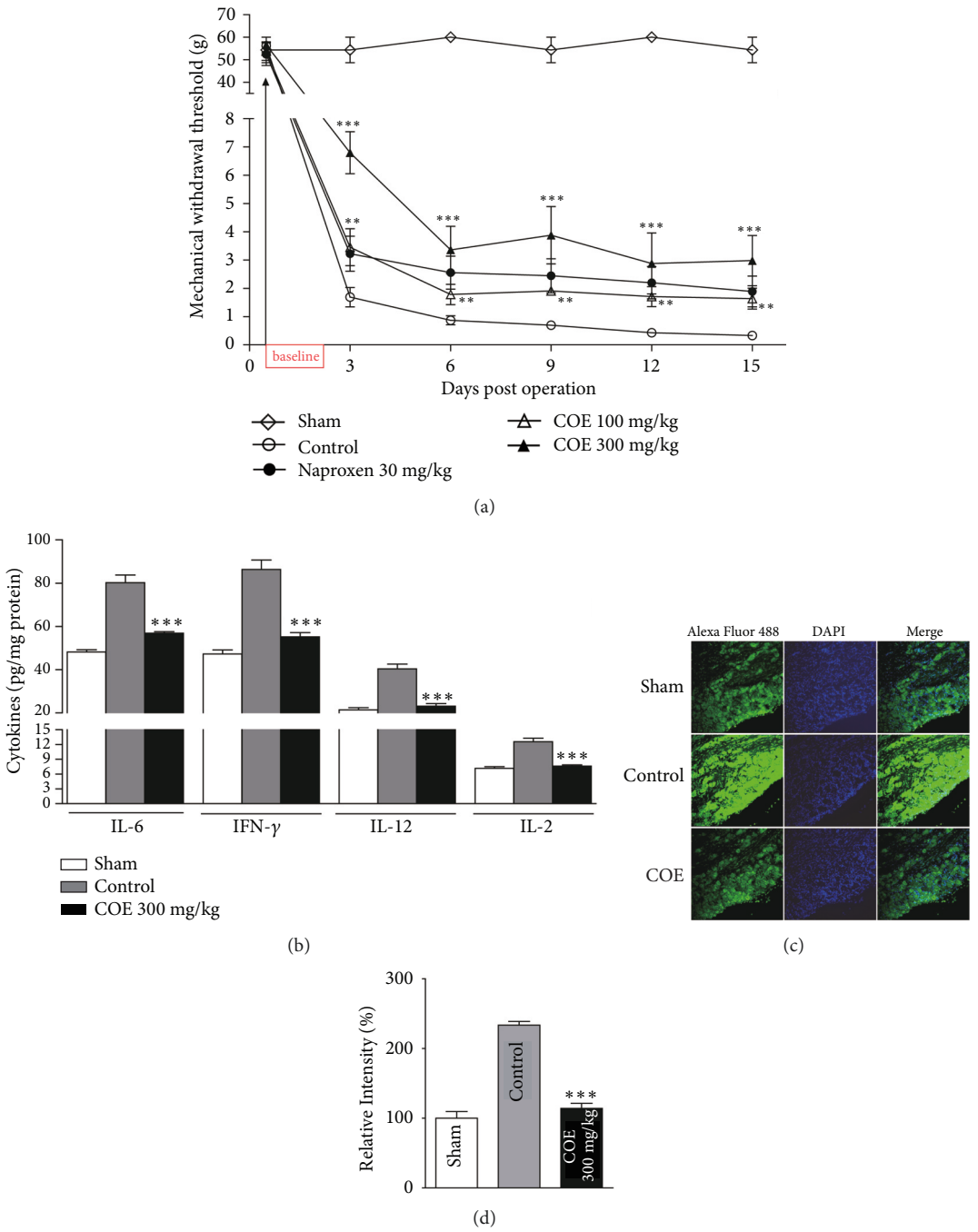


FIGURE 2: The effect of COE in the SNI neuropathic pain model. (a) Oral administration of COE significantly attenuated hypersensitivity in response to von Frey stimulation from 3 to 15 days in an SNI neuropathic model. The MWTs of the group treated with COE or naproxen remained higher than those of the untreated control group. (b) Administration of COE significantly inhibited the induction of IL-6, IFN-γ, IL-12, and IL-2 expression in DRG neurons of a neuropathic rat model by ELISA. Representative images (c) and relative fluorescence intensity (d) against calpain-3 (Alexa Fluor 488) revealed that COE attenuated the expression of calpain-3. Data are the means ± SEM (n = 9 per group). The asterisks indicate significant difference from the control group, ** $p < 0.01$ and *** $p < 0.001$.

7.303 g at 30 mg/kg, 25.63 ± 8.187 g at 100 mg/kg, and 29.00 ± 6.974 g at 300 mg/kg) (Figure 3). Menopause occurs when the amount of estrogen released from the ovaries decreases as a woman ages, and it may cause pain sensitivity [31]. Pain sensitivity increases in the abdomen, hind limbs, and proximal tail of OVX rats, and these symptoms are alleviated following hormone administration [32]. Estradiol administration reversed OVX-induced hypersensitivity.

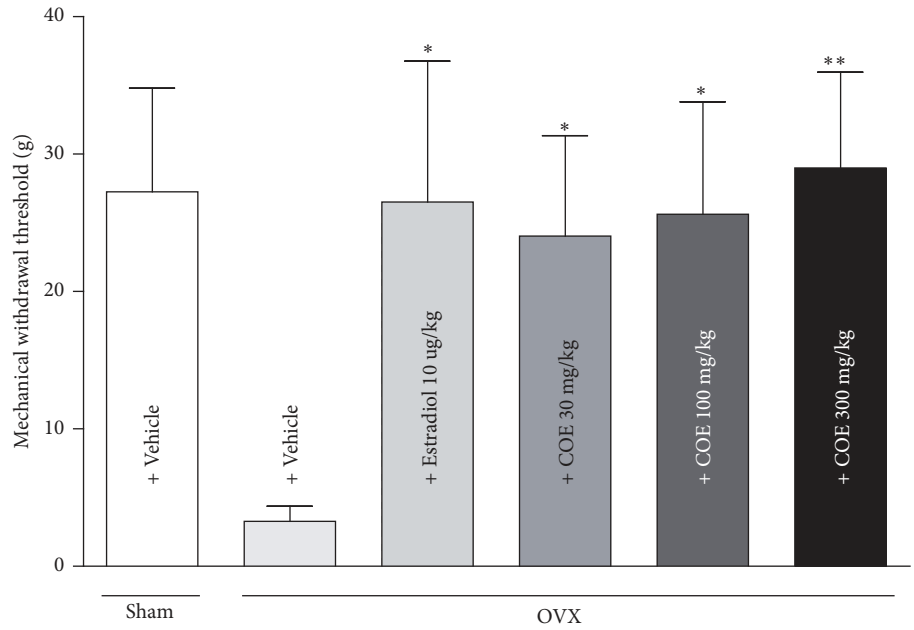


Figure 3: Reduced mechanical sensitivity by application of COE in OVX rats. COE alleviated pain sensitivity in menopausal pain model. Data are the means ± SEM (n = 9 per group). The asterisks indicate significant difference from the control group, $*$ $p < 0.05$ and $**$ $p < 0.01$.

3.4. The Active Compound of COE, Ferulic Acid, Attenuated Postoperative Pain. To identify the components of COE that relieved pain, HPLC was performed and ferulic acid was isolated. It was orally administered after induction of postoperative pain. The significant development of mechanical allodynia was confirmed by a decrease in the withdrawal threshold values (56.60 ± 3.400 at 0 h to 0.600 ± 0.073 at 24 h after PI). The MWTs of the ipsilateral hind paw 24 h after surgery demonstrated that a single ferulic acid treatment (40 to 100 mg/kg) attenuated mechanical allodynia (2.033 ± 0.366 at 40 mg/kg and 1.350 ± 0.155 at 100 mg/kg). Similar effects were observed for naproxen (30 mg/kg) administration, which was used as a positive control (4.133 ± 1.481 g) (Figure 4(a)). USV calls (22-27 kHz) in the ferulic acid-treated group (8.250 ± 1.652 calls at 40 mg/kg and 1.625 ± 0.460 calls at 100 mg/kg) also decreased compared to those in the control group (17.000 ± 3.464 calls) 24 hours after PI (Figure 4(b)). The major components of COE include ferulic acid, senkyunolide I, senkyunolide H, senkyunolide A, Z-ligustilide, and levistolide A [33]. Based on this, we could detect chlorogenic acid, ferulic acid, senkyunolide A, and Z-ligustilide from COE using HPLC (Table 1). Ferulic acid is one of the most abundant phenolic acids in plants and a major component of COE. It reduces collagen-induced inflammatory cytokine production [34]. Ferulic acid exhibited antinociceptive effects in a chronic neuropathic pain model induced by constriction injury and also alleviated pain in other models of neuropathic pain, such as the chemotherapy-induced neuropathy model [35]. Although ferulic acid has been reported to aid in alleviating chronic pain, this is the first study showing that ferulic acid could help alleviate postoperative pain.

4. Conclusions

In this study, we demonstrated that COE has great analgesic efficacy. Oral administration of COE attenuated hypersensitivity in *in vivo* models of postoperative, neuropathic, and menopausal pain. In addition, application of COE inhibited the induction of the proinflammatory cytokines and calpain-3, which was induced by neuropathic pain on DRG neurons. Ferulic acid was expected to be a potent active compound of COE for pain relief from HPLC analysis. Ferulic acid effectually alleviated mechanical hypersensitivity in a postoperative pain model. Although further studies about the exact working mechanism of COE in pain relief are required, COE might be a novel natural product for reducing hypersensitivity in postoperative, neuropathic, and menopausal pain.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Eun Yeong Lim and Jae Goo Kim contributed equally to this work.

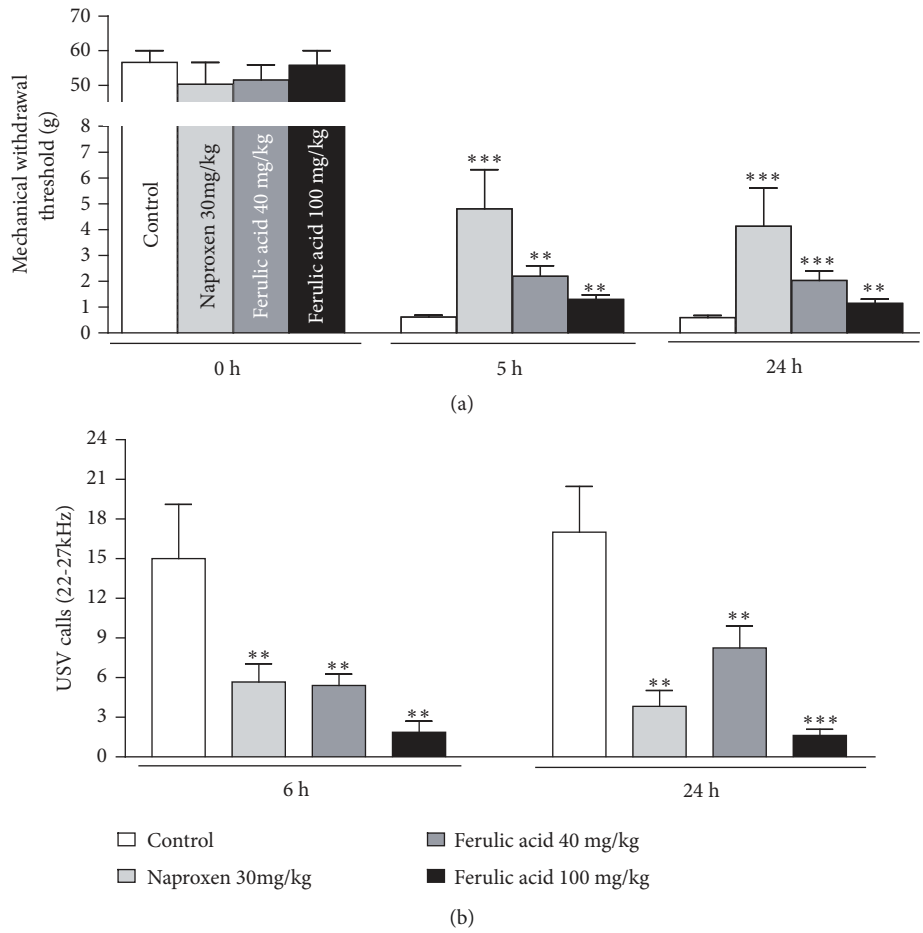


Figure 4: Reduced pain sensitivity by application of ferulic acid in a postoperative pain model. (a) Compared with the nontreated group, the group treated with ferulic acid showed a significantly higher mechanical withdrawal threshold (a) and less USV calls (b) at both 5 and 24 hours after surgery in a dose-dependent manner. Naproxen (30 mg/kg, n = 9) was used as the positive control analgesic. Data are the means ± SEM (n = 9 per group). The asterisks indicate significant difference from the control group, ∗∗ $p < 0.01$ and ∗ ∗ ∗ $p < 0.001$.

Acknowledgments

This research was supported by Main Research Program [E0164500-04] of the Korea Food Research Institute (KFRI) and by Basic Science Research Program [NRF-2017R1C1B2006455] through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning.

References

[1] B. Gerber, A. Yarali, S. Diegelmann, C. T. Wotjak, P. Pauli, and M. Fendt, "Pain-relief learning in flies, rats, and man: Basic research and applied perspectives," *Learning & Memory*, vol. 21, no. 4, pp. 232–252, 2014.

[2] I. Gilron and T. J. Coderre, "Emerging drugs in neuropathic pain," *Expert Opinion on Emerging Drugs*, vol. 12, no. 1, pp. 113–126, 2007.

[3] L. Manchikanti, M. V. Boswell, and J. A. Hirsch, "Lessons learned in the abuse of pain-relief medication: A focus on healthcare costs," *Expert Review of Neurotherapeutics*, vol. 13, no. 5, pp. 527–544, 2013.

[4] P. N. Praveen Rao and E. E. Knaus, "Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond," *Journal of Pharmacy & Pharmaceutical Sciences*, vol. 11, no. 2, pp. 81–110, 2008.

[5] A. Gupta and M. Bah, "NSAIDs in the treatment of postoperative pain," *Current Pain and Headache Reports*, vol. 20, no. 11, article no. 62, 2016.

[6] V. Brower, "New paths to pain relief," *Nature Biotechnology*, vol. 18, no. 4, pp. 387–391, 2000.

[7] E. Y. Lim and Y. T. Kim, "Food-derived natural compounds for pain relief in neuropathic pain," *BioMed Research International*, vol. 2016, Article ID 7917528, 12 pages, 2016.

[8] M. Liu, L. Zhou, Z. Chen, and C. Hu, "Analgesic effect of iridoid glycosides from Paederia scandens (LOUR.) MERRILL (Rubiaceae) on spared nerve injury rat model of neuropathic pain," *Pharmacology Biochemistry & Behavior*, vol. 102, no. 3, pp. 465–470, 2012.

[9] K. S. Kuehl, "Cherry juice targets antioxidant potential and pain relief," *Medicine and Sport Science*, vol. 59, pp. 86–93, 2012.

[10] J. C. Maroon, J. W. Bost, M. K. Borden, K. M. Lorenz, and N. A. Ross, "Natural antiinflammatory agents for pain relief in athletes," *Neurosurgical Focus*, vol. 21, no. 4, article no. E11, 2006.

[11] H. Kim and M.-J. Song, "Oral traditional plant-based therapeutic applications for pain relief recorded in North Jeolla province, Korea," *Indian Journal of Traditional Knowledge*, vol. 12, no. 4, pp. 573–584, 2013.

[12] S. H. Lee, J. H. Lee, E. Y. Oh et al., "Ethanol extract of Cnidium officinale exhibits anti-inflammatory effects in BV2 microglial cells by suppressing NF-κB nuclear translocation and the activation of the PI3K/Akt signaling pathway," *International Journal of Molecular Medicine*, vol. 32, no. 4, pp. 876–882, 2013.

[13] M. Ramalingam and P. Yong-Ki, "Free radical scavenging activities of cnidium officinale makino and ligusticum chuanxiong hort. methanolic extracts," *Pharmacognosy Magazine*, vol. 6, no. 24, pp. 323–330, 2010.

[14] H. Hong, J. C. An, J. F. De La Cruz, and S.-G. Hwang, "Cnidium officinale makino extract induces apoptosis through activation of caspase-3 and p53 in human liver cancer HepG2 cells," *Experimental and Therapeutic Medicine*, vol. 14, no. 4, pp. 3191–3197, 2017.

[15] Y. M. Lee, Y.-R. Lee, C.-S. Kim et al., "Cnidium officinale extract and butylidenephthalide inhibits retinal neovascularization in vitro and in vivo," *BMC Complementary and Alternative Medicine*, vol. 16, no. 1, article no. 231, 2016.

[16] J. M. Kim, D. Son, P. Lee, K. J. Lee, H. Kim, and S. Y. Kim, "Ethyl acetate soluble fraction of Cnidium officinale MAKINO inhibits neuronal cell death by reduction of excessive nitric oxide production in lipopolyccharide-treated rat hippocampal slice cultures and microglia cells," *Journal of Pharmaceutical Sciences*, vol. 92, no. 1, pp. 74–78, 2003.

[17] T. J. Brennan, E. P. Vandermeulen, and G. F. Gebhart, "Characterization of a rat model of incisional pain," *PAIN*, vol. 64, no. 3, pp. 493–501, 1996.

[18] A.-F. Bourquin, M. Süveges, M. Pertin et al., "Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse," *Pain*, vol. 122, no. 1-2, pp. 14–16, 2006.

[19] L.-H. Li, Z.-C. Wang, J. Yu, and Y.-Q. Zhang, "Ovariectomy results in variable changes in nociception, mood and depression in adult female rats," *PLoS ONE*, vol. 9, no. 4, Article ID e94312, 2014.

[20] I. Decosterd and C. J. Woolf, "Spared nerve injury: an animal model of persistent peripheral neuropathic pain," *PAIN*, vol. 87, no. 2, pp. 149–158, 2000.

[21] D. W. Lim, J. G. Kim, D. Han, and Y. T. Kim, "Analgesic effect of Harpagophytum procumbens on postoperative and neuropathic pain in rats," *Molecules*, vol. 19, no. 1, pp. 1060–1068, 2014.

[22] W.-H. Su, M.-H. Cheng, W.-L. Lee et al., "Nonsteroidal anti-inflammatory drugs for wounds: pain relief or excessive scar formation?" *Mediators of Inflammation*, vol. 2010, Article ID 413238, 8 pages, 2010.

[23] R. D. Gosselin, M. A. Dansereau, M. Pohl et al., "Chemokine network in the nervous system: a new target for pain relief," *Current Medicinal Chemistry*, vol. 15, no. 27, pp. 2866–2875, 2008.

[24] K. N. Wai Ying and A. While, "Pain relief in osteoarthritis and rheumatoid arthritis: TENS," *British Journal of Community Nursing*, vol. 12, no. 8, pp. 364–371, 2007.

[25] L. Leung and C. M. Cahill, "TNF-alpha and neuropathic pain - a review," *Journal of Neuroinflammation*, vol. 7, 2010.

[26] H. U. Zeilhofer, "The glycinergic control of spinal pain processing," *Cellular and Molecular Life Sciences*, vol. 62, no. 18, pp. 2027–2035, 2005.

[27] S. Hauerslev, M.-L. Sveen, M. Duno, C. Angelini, J. Vissing, and T. O. Krag, "Calpain 3 is important for muscle regeneration: evidence from patients with limb girdle muscular dystrophies," *BMC Musculoskeletal Disorders*, vol. 13, no. 1, article no. 43, 2012.

[28] J. S. Beckmann and M. Spencer, "Calpain 3, the 'gatekeeper' of proper sarcomere assembly, turnover and maintenance," *Neuromuscular Disorders*, vol. 18, no. 12, pp. 913–921, 2008.

[29] J. O. Nwankwo, J. Lei, J. Xu, A. Rivera, K. Gupta, and A. H. Chishti, "Genetic inactivation of calpain-1 attenuates pain sensitivity in a humanized mouse model of sickle cell disease," *Haematologica*, vol. 101, no. 10, pp. e397–e400, 2016.

[30] Y. Zang, S.-X. Chen, G.-J. Liao et al., "Calpain-2 contributes to neuropathic pain following motor nerve injury via up-regulating interleukin-6 in DRG neurons," *Brain, Behavior, and Immunity*, vol. 44, pp. 37–47, 2015.

[31] M. Martínez-Jauand, C. Sitges, J. Femenia et al., "Age-of-onset of menopause is associated with enhanced painful and non-painful sensitivity in fibromyalgia," *Clinical Rheumatology*, vol. 32, no. 7, pp. 975–981, 2013.

[32] R. Sanoja and F. Cervero, "Estrogen-dependent abdominal hyperalgesia induced by ovariectomy in adult mice: a model of functional abdominal pain," *PAIN*, vol. 118, no. 1-2, pp. 243–253, 2005.

[33] T. Yi, K. S. Leung, G. H. Lu, and H. Zhang, "Comparative analysis of *Ligusticum chuanxiong* and related umbelliferous medicinal plants by high performance liquid chromatography-electrospray ionization mass spectrometry," *Planta Medica*, vol. 73, no. 4, pp. 392–398, 2007.

[34] H. Zhu, Q.-H. Liang, X.-G. Xiong et al., "Anti-inflammatory effects of the bioactive compound ferulic acid contained in oldenlandia diffusa on collagen-induced arthritis in rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 573801, 10 pages, 2014.

[35] B. Vashistha, A. Sharma, and V. Jain, "Ameliorative potential of ferulic acid in vincristine-induced painful neuropathy in rats: an evidence of behavioral and biochemical examination," *Nutritional Neuroscience*, vol. 20, no. 1, pp. 60–70, 2017.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 9585727, 11 pages
https://doi.org/10.1155/2019/9585727

Research Article

The Anti-Inflammatory Effect of Feiyangchangweiyan Capsule and Its Main Components on Pelvic Inflammatory Disease in Rats via the Regulation of the NF-κB and BAX/BCL-2 Pathway

Yao Li,[1] Qian Yang,[2] Zhi-hui Shi,[3] Min Zhou,[4] Li Yan,[2] Hua Li,[2] Yan-hua Xie,[1] and Si-wang Wang[1,2]

[1]*The College of Life Sciences, Northwest University, Xi'an, 710069, China*
[2]*Department of Natural Medicine, School of Pharmacy, The Fourth Military Medical University, Xi'an, 710032, China*
[3]*Shaanxi Junbisha Pharmaceutical Limited Company, Xianyang, 712000, China*
[4]*School of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang 712000, China*

Correspondence should be addressed to Yan-hua Xie; xieyanh@fmmu.edu.cn and Si-wang Wang; wangsiw@fmmu.edu.cn

Received 9 December 2018; Accepted 23 April 2019; Published 13 June 2019

Guest Editor: Caio P. Fernandes

Although gastroenteritis and pelvic inflammatory disease (PID) occur in the gastrointestinal tract and pelvis, respectively, they display similar pathogeneses. The incidence of inflammation in these conditions is usually associated with dysbacteriosis, and, at times, they are caused by the same pathogenic bacteria, *Escherichia coli* and *Streptococcus aureus*. Feiyangchangweiyan capsule (FYC) is a traditional Chinese patent medicine that is widely used to treat bacterial dysentery and acute and chronic gastroenteritis. However, whether it has an effect on PID is unclear. The aim of this study was to investigate the anti-inflammatory effect of FYC and its main components, gallic acid (GA), ellagic acid (EA), and syringin (SY), on a pathogen-induced PID model and illustrate their potential mechanism of action. Female specific pathogen-free SD rats (n = 1110) were randomly divided into control, PID, FYC, GA, EA, SY, GA + EA, GA + SY, EA + SY, GA + EA + SY, and Fuke Qianjin capsule (FKC) positive groups. Histological examination and enzyme-linked immunosorbent assay (ELISA) were carried out as well as western blot analysis to detect the expression of NF-κB, BAX, BCL-2, and JNK. In this study, FYC and its main components dramatically suppressed the infiltration of inflammatory cells, reduced the production of IL-1β, TNF-α, and MCP-1, and elevated the IL-10 level to varying degrees. We also found that FYC and its main components inhibited the expression of BAX induced by infection and increased the expression of Bcl-2. FYC, GA, EA, and SY could also block the activation of the NF-κB pathway. Finally, we found that the phosphorylation of JNK could be decreased by FYC, GA, and SY. FYC and its main components exhibit anti-inflammatory effect on a pathogen-induced PID model by regulating the NF-κB and apoptosis signaling pathways.

1. Introduction

Pelvic inflammatory disease (PID) is the most frequently observed disorder of the female upper genital tract due to pathogens [1]. Many pathogenic microorganisms have been implicated in this disorder including the sexually transmitted bacteria, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, and the increase in vaginal flora such as *Gardnerella vaginalis*, *Streptococcus aureus,* and *Escherichia coli* [2, 3]. There are no evident initial PID symptoms in patients; however, as time progresses, the disorder becomes recurrent or chronic, and its sequelae of infertility, ectopic pregnancy, and chronic pelvic pain serve as important public health issues [4, 5]. The consequences of PID can be severe and treatment delay may contribute to the chance of infertility [6, 7]. Antibiotic therapies are the first choice of treatment for PID, but when used on a regular basis, they increase the likelihood of antibiotic resistance. Therefore, it is necessary to develop new complementary and alternative medicine to treat PID.

Feiyangchangweiyan capsule (FYC), a traditional Chinese medicine (TCM), is composed of *Euphorbia hirta* L., *Polygonum chinense* L., and *Ilex rotunda* Thunb. This traditional

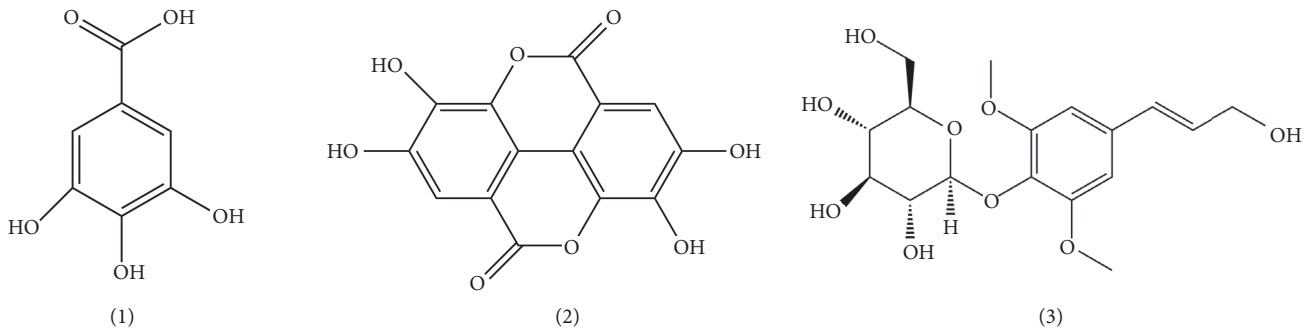


Figure 1: Chemical structures of gallic acid (1), ellagic acid (2), and syringin (3).

Chinese patent medicine was approved by China's Food and Drug Administration (CFDA) and is manufactured by Shaanxi Junbisha Pharmaceutical Limited Company. FYC is widely used to treat bacterial dysentery, acute gastroenteritis, and chronic gastroenteritis [8]. However, whether it has an effect on PID is unclear, and its active ingredients and the mechanisms it employs to treat gastrointestinal inflammation remain unknown. Monarch, minister, assistant, and guide play an important role in TCM prescription but the anti-inflammatory effect of FYC is prioritized when considering its therapeutic properties and functions.

E. hirta belongs to the plant family Euphorbiaceae and genus *Euphorbia* [9]. Its ethanolic and aqueous extracts have been reported to inhibit the growth of organisms, such as *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, to varying degrees [10–13]. The extract of aerial parts of *E. hirta* displayed dose-dependent anti-inflammatory effects in the phorbol acetate-induced ear inflammation mice model [14]. *P. chinense*, a perennial herb belonging to the family Polygonaceae, is widely used as a folk medicine for different purposes worldwide. In China, *P. chinense* is commonly consumed as a treatment for diarrhea and enteritis [15]. Furthermore, it is used to treat inflammation of the female genital tract in TCM clinical practice [16]. *I. rotunda* belongs to the family Aquifoliaceae and has been traditionally used to treat common cold, urinary tract infection, and cardiovascular disease. Its extract has been demonstrated to display anti-inflammatory and antioxidative effects [17, 18].

FYC contains a variety of components, but its anti-inflammatory and antibacterial activity can be attributed to the following elements: flavonoids, phenolic acids, ascorbic acid, etc. [19]. Our previous studies demonstrated that the main effective components of FYC are gallic acid (GA **1**, Figure 1), ellagic acid (EA **2**, Figure 1), and syringin (SY **3**, Figure 1) [19]. Microbial components have been shown to be involved in the pathogenesis of enterogastritis as well as PID. To add, the interaction between gastrointestinal microbiota and vaginal flora has been widely confirmed to impact this process. Therefore, based on the above theories and our preresearch data of the antibacterial effect of FYC, we endeavored to investigate the pharmacological effect and mechanism of FYC and its principal components on PID in rats.

2. Materials and Methods

2.1. Materials. *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) were purchased from American Type Culture Collection (Manassas, VA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), monocyte chemotactic protein-1 (MCP-1), and caspase-3 were obtained from MultiSciences (Hangzhou, China). Nuclear factor κB (NF-κB), IκB, BCL-2, BAX, JNK, and β-actin antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). FYC was provided by Shaanxi Junbisha Pharmaceutical Co. Ltd. (Xianyang, China). Fuke Qianjin capsule (FKC) was purchased from Zhuzhou Qianjin Pharmaceutical Co. Ltd. (Hunan, China). GA ($C_7H_6O_5$, FW = 170.12, purity ≥99.9%; the main active constituent of *E. hirta*), EA ($C_{14}H_6O_8$, FW = 302.19, purity ≥98.8%; the main active constituent of *P. chinense*), and SY ($C_{17}H_{24}O_9$, FW = 372.37, purity ≥98.5%; the main active constituent of *I. rotunda*) were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China).

2.2. Rat PID Model Construction and Sample Collection. Female specific pathogen-free SD rats (weight, 180–220 g; age, 8-week-old) were obtained from the Experimental Animal Center of the Fourth Military Medical University. The experimental protocols were approved by the Laboratory Animal Center of the Academy of the Fourth Military Medical University. The animals were housed under a 12-h dark-light cycle (light on, from 7:00 to 19:00) under a temperature of 22–24°C and relative humidity of 60–65%. All rats were randomly divided into the control, PID, FYC (1.2 g/kg), GA (210 mg/kg), EA (30 mg/kg), SY (35 mg/kg), GA (105 mg/kg) + EA (15 mg/kg), GA (105 mg/kg) + SY (18 mg/kg), EA (15 mg/kg) + SY (18 mg/kg), GA (70 mg/kg) + EA (10 mg/kg) + SY (12 mg/kg), and FKC (2.4 g/kg) groups, each with 10 rodents. After acclimation for 7 days, the PID model was established using improved methods reported previously [20–22]. Rats were anesthetized intraperitoneally with 30 mg/kg of pentobarbital. The lower abdomen was shaved and swabbed with Betadine. A 1-cm incision was made ventrally in the skin, and the peritoneum was directly and bluntly dissected over the cervix. Microbe-mixing solution with *E. coli* (1×10^8 CFU/mL) and *S. aureus* (1×10^8 CFU/mL)

was prepared, uterine horns were exposed, and 50 μL of microbe-mixing solution was immediately injected into the uteri proximal to the distal of the branching point in the direction of the endocervix by use of an insulin syringe fitted with a 30G needle. The skin was then closed with a standard surgical staple. Control group rats were inoculated intracervically with sterile saline. After 24 h of infection, each group of rats was orally administered the corresponding drugs. After 14 days, rats were sacrificed by cervical dislocation, and blood samples and uterus were collected and stored at −80°C for further evaluations. Meanwhile, a fraction of the uterus was immersed in neutral-buffered formalin (10%) for hematoxylin-eosin (H&E) staining.

2.3. Evaluation of Uterus Appearance. A researcher, blinded to the grouping, scored the degree of inflammation in rat uterus based on the apparent expression. The appearance of the uterus included edema, hyperemia, and white pus within the uterine cavity. Based on different intensities, each feature was counted on a three-point scale: 0 (not present), 1 (moderately visible), and 2 (severe). The weight of the uterus was then taken and used to calculate the uterine index (weight of horn/body weight of rat × 100).

2.4. Histological Evaluation. For histological evaluation, the uterus was cut into 2-μm sections and stained with hematoxylin and eosin. Three parts of each slide (tissue) were evaluated under low-power microscopy field (×200). The extent of inflammatory cell infiltration was evaluated by the semiconducted method (grade, 0 to 3).

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). The amounts of IL-1β, TNF-α, IL-10, and MCP-1 in serum were determined with ELISA kits (MultiSciences). The concentrations of cytokines and chemokines were expressed as pg/mL protein of homogenate.

2.6. Western Blot Analysis. Tissue samples of the upper genital tract were homogenized and lysed in ice-cold RIPA lysis buffer, then mixed with 2× SDS-PAGE sample buffer, boiled for 10 min, and then resolved by 10% SDS-PAGE. The proteins were then transferred onto polyvinylidene difluoride (Millipore, Billerica, MA, USA) membranes, blocked at 37°C for 60 min with 5% nonfat dry milk, and then reacted with properly diluted monoclonal antibodies (1:1000) including NF-κB p65, p-NF-κB p65, p-IκBα, Bα, BAX, BCL-2, JNK, and p-JNK. After washing, the membranes were incubated with peroxidase-linked goat anti-rabbit IgG secondary antibody (1:1000; Santa Cruz Biotechnology) at 37°C for 1 h. Protein bands were detected using horseradish peroxidase-conjugated goat anti-mouse IgG antibodies followed by an enhanced chemiluminescence reaction (Pierce Biotechnology, USA).

2.7. Statistical Analyses. All data are presented as mean ± standard deviation (SD). The differences between two datasets were evaluated using Student's t-test with SPSS 18.0 statistical software (SPSS, USA). One-way analysis of variance (ANOVA) was used to compare the difference between more than two datasets. Difference was considered statistically significant when the p value was <0.05.

3. Results

3.1. Uterine Appearance and Histopathological Changes. To assess the anti-inflammatory activity of the main components of FYC, uterine appearance was scored as shown in Figure 2. We found no obvious changes in the uterine horns in the control group; however, in the PID group, the uteri showed different degrees of lesion with uterine enlargement, hyperemia, white pus within the uterine cavity, and uterine wall thickening. The uterine index (weight of horn/body weight of rat × 100) of the PID group was much higher than that of the control group. However, these lesions were significantly attenuated by the administration of FYC and its main components, and the scores for the inflammation of uterine appearance showed dramatic differences between the PID and treatment groups.

The results of the histological assessment performed with a light microscope are presented in Figure 3. In the control group, the luminal epithelium was integrated into the endometrium, the glands were enlarged, and the smooth muscles were arranged regularly with no degeneration. As shown in Figure 3, the uteri from the PID group exhibited severe pathological changes, (e.g., hyperemia, hemorrhage, and the shedding of epithelial cells). Moreover, the uterine tissues were infiltrated by masses of inflammatory cells, most of which were polymorphonuclear (PMN) cells. These results demonstrate the occurrence of inflammation in the upper genital tract. Nevertheless, compared to the PID group, histopathological changes and the infiltration of inflammatory cells in the drug intervention groups were significantly decreased in the upper genital tract. Semiquantitative results also showed notable differences in the histopathological changes between the PID group and medicated groups.

3.2. Effect of FYC and Its Main Components on the Excessive Production of Cytokines and Chemokines. Inflammatory response and inflammatory cell infiltration are related to the excessive production of cytokines and chemokines. In this research, we used ELISA kits to measure IL-1β, TNF-α, IL-10, and MCP-1 in the serum of rats. As shown in Figure 4, IL-1β, TNF-α, and MCP-1 levels were dramatically increased in the PID group compared to the control group. Meanwhile, IL-10 was sharply decreased in the PID group compared to the control group. After oral administration of FYC and its main components, the pathogen-induced production of IL-1β, TNF-α, and MCP-1 was reduced by varying degrees. In addition, IL-10 in the serum from drug intervention groups was increased by varying degrees.

3.3. Effect of FYC and Its Main Components on Apoptosis in Response to PID. Considering that the proapoptotic factors (BAX) and antiapoptotic genes (BCL-2) are largely associated with the progression of apoptosis, the expression levels of these apoptosis-related proteins were analyzed to investigate the mechanism of FYC and its main components in the upper genital tract. As shown in Figure 5, the level of BAX was

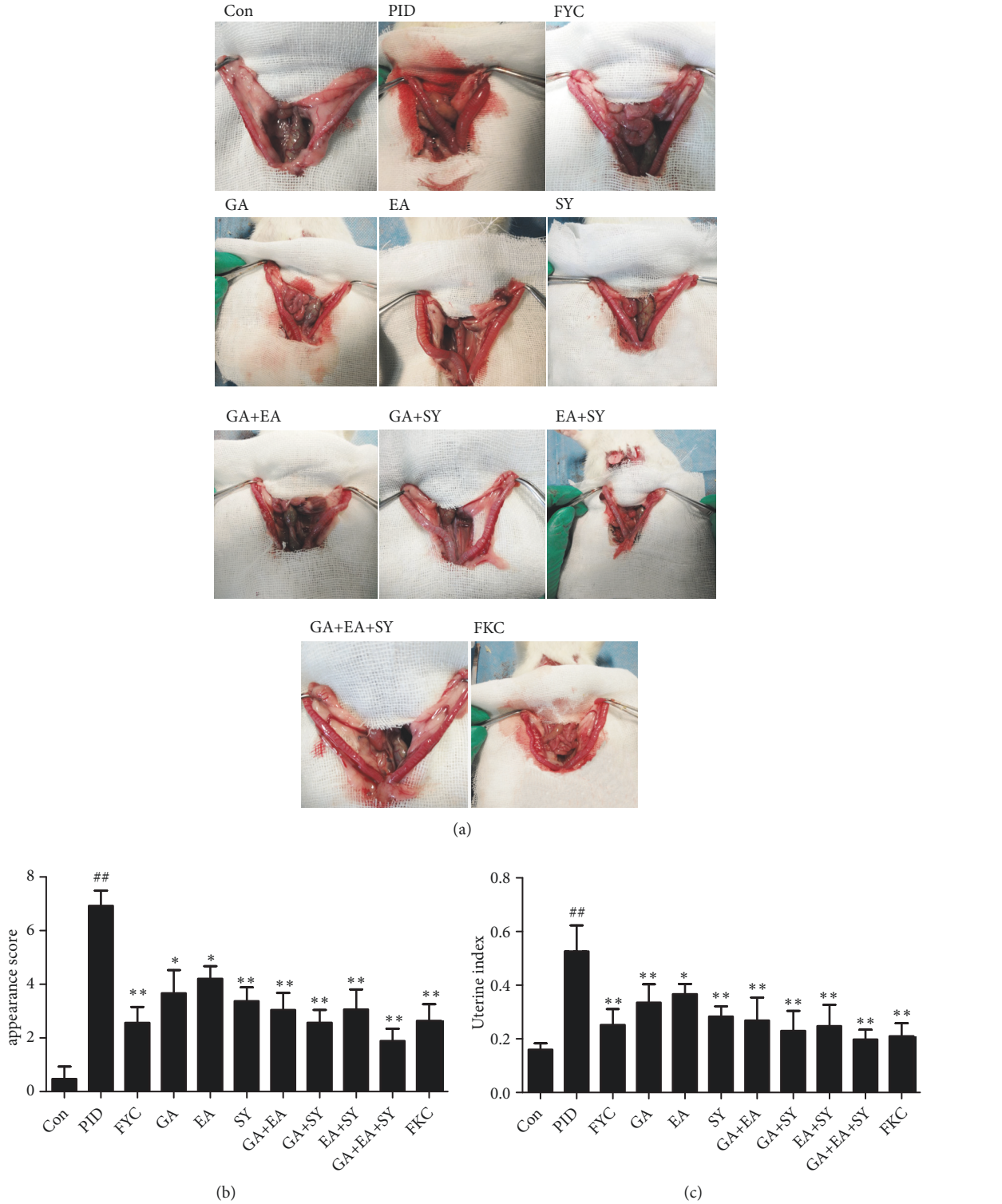
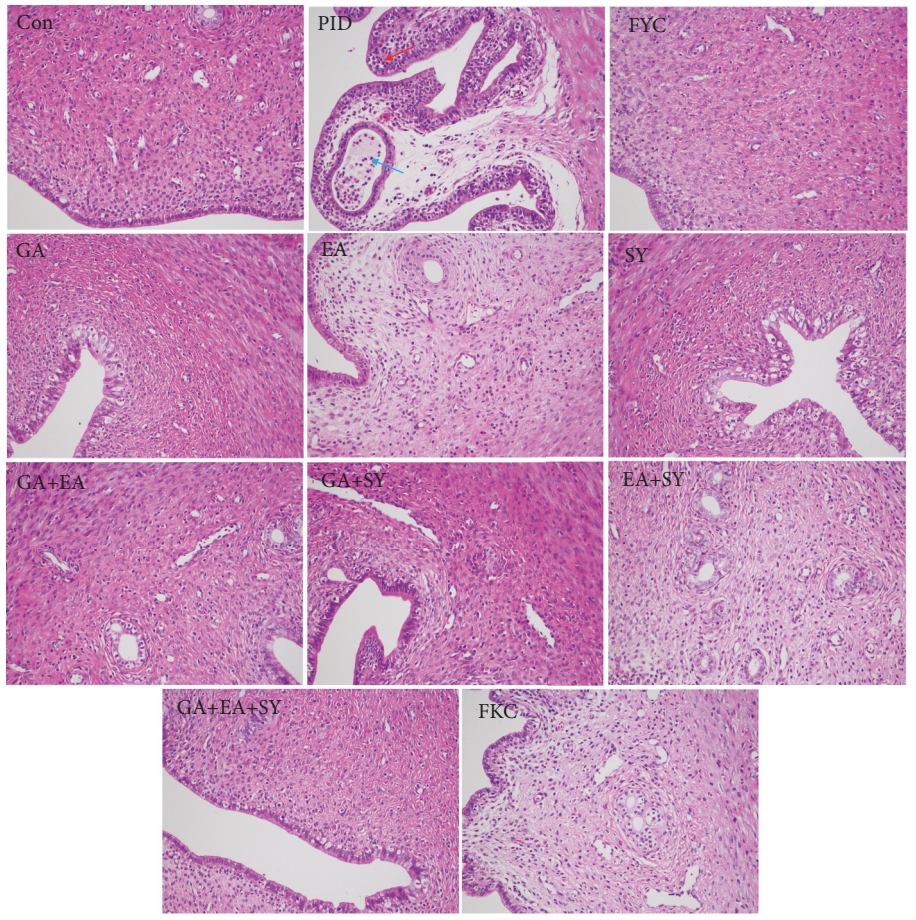
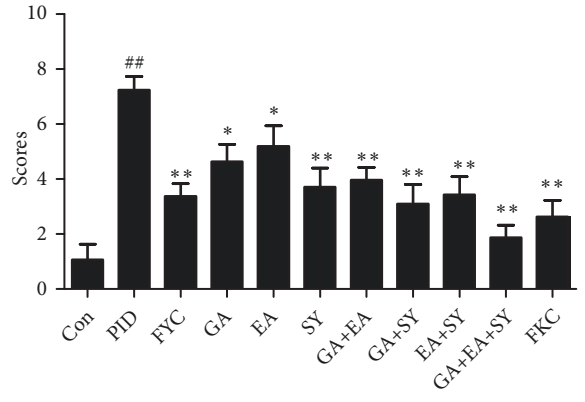


Figure 2: Effect of FYC and its main components on pathogen-induced inflammation of the uterus. (a) Appearance of the uterus in the different groups. White pus within the uterine cavity is indicated as ↑. (b) Semiquantitative scores of the inflammation for the appearance of uterus. (c) Index of the uterus for each group. Each bar represents the mean ± SD (##$P < 0.01$ vs. control group, *$P < 0.05$, **$P < 0.01$ vs. PID group).



(a)



(b)

Figure 3: Effect of FYC and its main components on the pathogen-induced infiltrations of inflammatory cells in the upper genital tract. (b) Typical micrographs (×200) of the uterus stained with H&E. Representative inflammatory cells are indicated as ↑. (b) Semiquantitative scores of the infiltration of inflammatory cells. Each bar represents the mean ± SD (##$P < 0.01$ vs. control group, *$P < 0.05$, **$P < 0.01$ vs. PID group).

significantly increased in the PID group whereas that of BCL-2 was decreased. After oral administration of FYC and its main components, the expression level of BAX was largely reduced and BCL-2 was increased. These results illustrate that apoptosis can be induced by bacterial infection, and FYC and its main components have an effect on apoptosis.

3.4. Effect of FYC and Its Main Components on NF-κB Activation and JNK Expression. In the uterus, the NF-κB pathway plays a pivotal role in inflammatory response. Hence, to investigate the underlying mechanism of the anti-inflammatory effects of FYC and its main components, NF-κB p65, p-NF-κB p65, and p-IκBα and the protein level

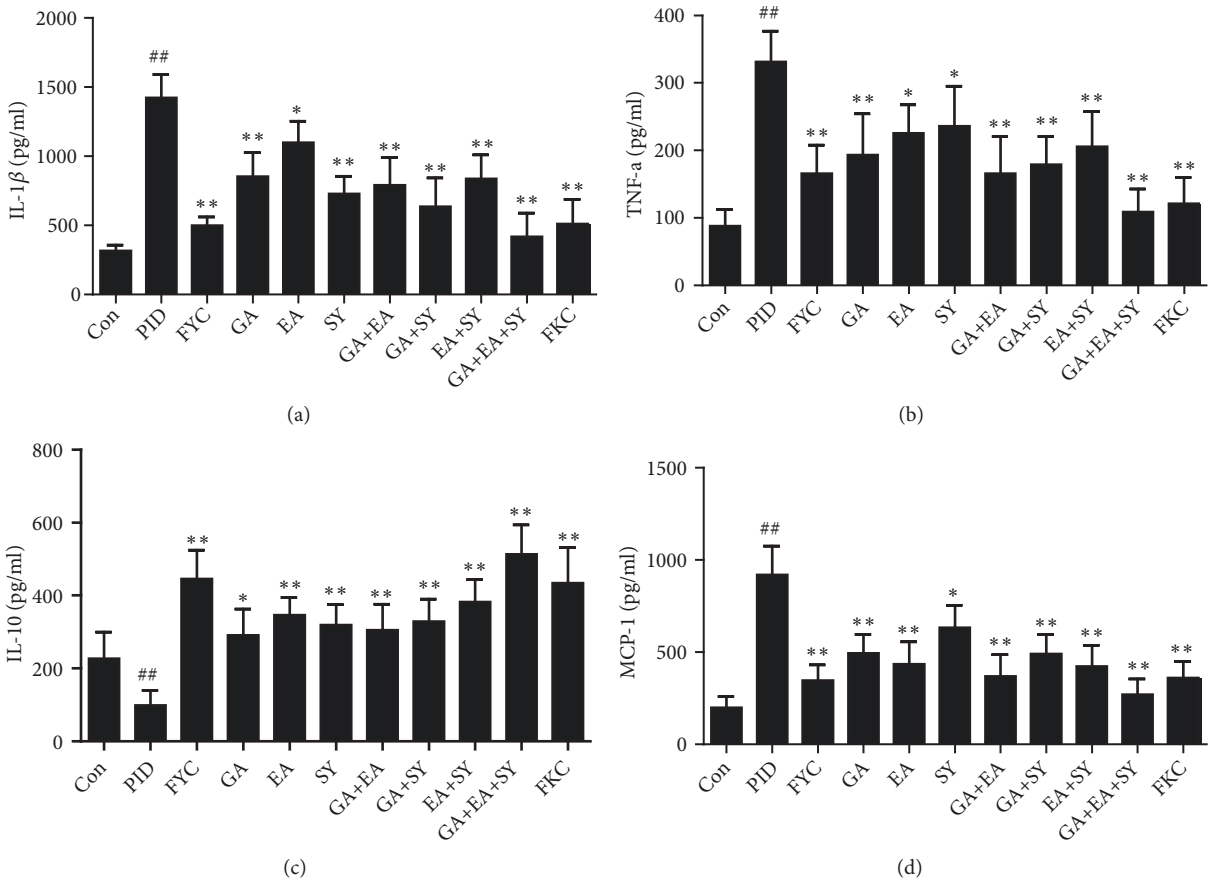


Figure 4: Effect of FYC and its main components on the pathogen-induced over-production of IL-1β, TNF-α, and MCP-1 and the reduction of IL-10 in the upper genital tract. Each bar represents mean ± SD (##$P < 0.01$ vs. control group, $*P < 0.05$, $**P < 0.01$ vs. PID group).

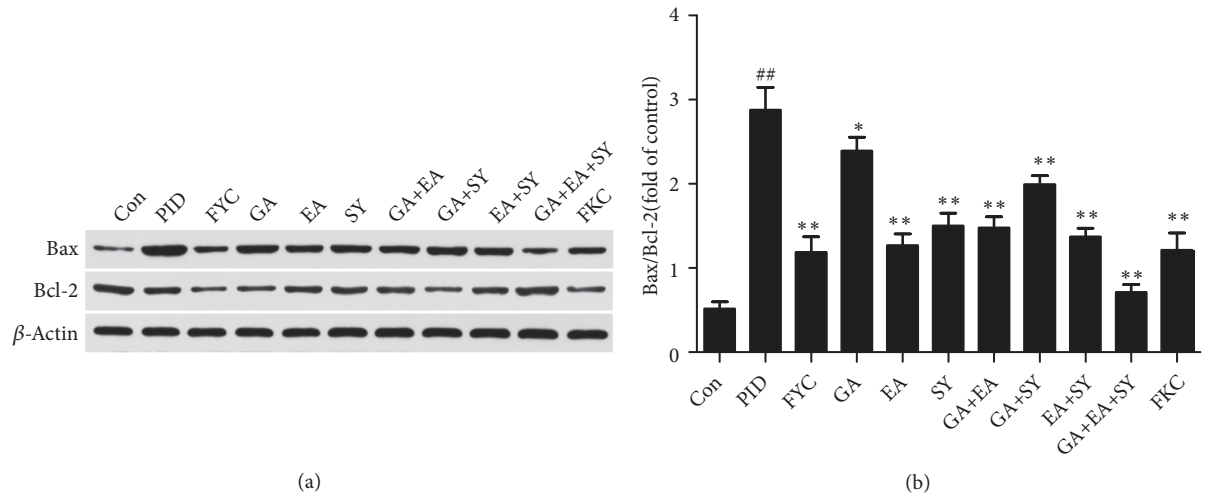


Figure 5: Effect of FYC and its main components on the pathogen-induced over-production of BAX/BCL-2 in the upper genital tract. Each bar represents the mean ± SD (##$P < 0.01$ vs. control group, $*P < 0.05$, $**P < 0.01$ vs. PID group).

of upstream effectors of the NF-kB signaling pathway (p-JNK/JNK) were detected by western blot analysis. As shown in Figure 6, compared to the control group, the expression of p-NF-κB p65, p-IκBα, and p-JNK was significantly increased in the PID group but was alleviated to varying degrees by treatment with FYC, GA, and SY.

4. Discussion

PID is a common disease in women and is referred to as inflammation of the upper genital tract including the ovaries, fallopian tubes, and their surrounding structures [23]. It is widely believed that *C. trachomatis* and *N. gonorrhoeae* are

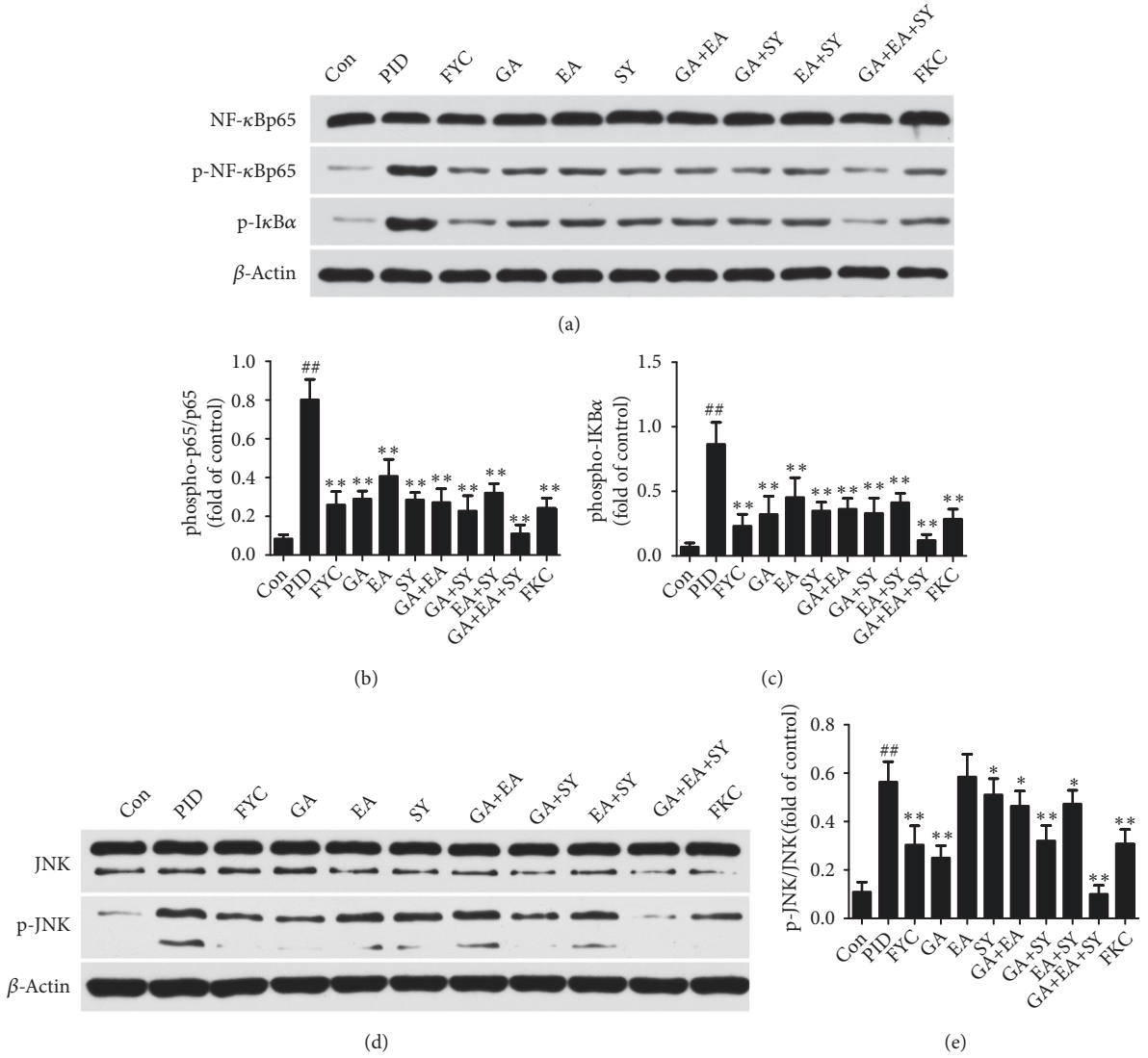


Figure 6: Effect of FYC and its main components on the pathogen-induced over-production of p-NF-κB and p-JNK in the upper genital tract. Each bar represents the mean ± SD (##$P < 0.01$ vs. control group, *$P < 0.05$, **$P < 0.01$ vs. PID group).

the main pathogens associated with PID, but *C. trachomatis* or *N. gonorrhoeae* infection may also permit the entry of opportunistic pathogen (such as *Mycoplasma hominis* and other anaerobes) into the upper genital tract [24, 25]. PID is a common cause of morbidity in young women. In the UK and the US, approximately 4% to 12% of young women are infected with chlamydia [26], 5% to 30% of whom are at risk of developing PID, if left untreated [27]. Of these PID patients, 10% to 20% will become infertile, 40% will develop chronic pelvic pain, and 10% who conceive will have an ectopic pregnancy [28]. In China, pathogenic bacteria causing PID also include *E. coli* and *S. aureus*. This phenomenon of pathogenic bacterial difference may akin to the living habits and environment in each country. Therefore, we attempted to use *E. coli* and *S. aureus* to provoke an augmented inflammation in the upper genital tract to construct the model used.

According to microecology, an organism is a large microecosystem. Vaginal flora and intestinal flora are the two main components of the human body's microecosystem and the balance of the two is interdependent as stated in the TCM theory [29]. Based on the literature search, every traditional Chinese medicine has antibacterial and anti-inflammatory effects; therefore, we assumed that FYC may also have a therapeutic effect on PID and this hypothesis was verified in our previous experiments. To investigate the potential mechanism of the main component of FYC and illustrate the multitarget effect of TCM, we selected GA, EA, and SY, which are regarded as the main components of FYC in our previous pharmacokinetic study, to treat rats with PID.

Neutrophils often appear at the sites of inflammation in different pathogen-infected tissues. When a mass of neutrophils reach the infection site of a tissue, they will release many inflammatory factors, oxygen free radicals, and

proteolytic enzymes to kill the pathogens. However, excessive products will also induce tissue damage and lead to structural disease in the upper genital tract. Patton et al. [30] reported that tissue damage including epithelial cell degeneration occurs in close approximation to lymphocytes and, in our study, many neutrophils and lymphocytes infiltrated into the epithelium of the upper genital tract of rats with PID, and each therapeutic group showed a better effect on attenuating the infiltration of inflammatory cells and alleviating this tissue damage.

Toll-like receptors (TLRs) are typically expressed in epithelial cells and mediate the innate immune system [31]. After pathogen infection and recognition of immunogens by local TLRs, the innate and adaptive immune systems are stimulated to fight against infection. Compared to the adaptive immune system, the innate immune system initiates more quickly and efficiently responds to the infection via surface defenses, cytokine elaboration, complement activation, and phagocytic responses [32, 33]. The pro- or anti-inflammatory cytokines produced by the host's immune system play essential roles in the inflammatory response. For instance, IL-1β, IL-10, MCP-1, neutrophils, or macrophages serve protective or destructive roles in PID [34, 35] and, in our study, cytokines and chemokines involving IL-1β, TNF-α, and MCP-1 were increased, whereas IL-10 was degraded in the uterus of rats with PID. After an oral administration of FYC, GA, EA, SY, and any two or three random combinations of GA, EA, and SY, respectively, IL-1β, TNF-α, and MCP-1 were significantly decreased whereas IL-10 was increased in a different manner. Furthermore, the effects of the GA + EA + SY group were better than the GA + EA, GA + SY, and EA + SY groups and were superior to the GA, EA, and SY groups.

Apoptosis is perceived as an important part of various processes involving normal cell turnover, hormone-dependent atrophy, and chemical-induced cell death [20]. However, inappropriate apoptosis may aggravate damage caused by some diseases, such as inflammation, tumor, or other human neurodegenerative diseases. Necrosis is commonly induced by aggressive inflammatory response, inappropriate apoptosis, and highly regulated genetic and biochemical processes [36] whereas apoptosis plays a critical role in the outcome of pathogenic infections [37]. The BCL-2 family is most notable for its regulation of apoptosis and the consistent proteins from this family can promote or inhibit apoptosis, such as the proapoptotic BAX and the antiapoptotic BCL-2 [38, 39]. Compared to the control group, the expression of BAX on the pelvic of rats with inflammation was found to increase and BCL-2 expression was dramatically suppressed. Administering FYC and its main components reversed these changes, especially EA and SY treatment, and the function of the combination group was significantly superior to that of the monomer groups.

The classic NF-κB pathway can be activated by the IRAK complex during inflammation or an immune response in an organism. NF-κB is a transcription factor retained in the cytoplasm when bound to IκB family members without any activity [40, 41]. Based on a report, an infection of the uterus caused by pathogens results in the phosphorylation of the IκB family and leads to ubiquitination and subsequent degradation. IκB degradation results in the translocation of NF-κB dimers to the nucleus and promotes the expression of inflammatory mediators, such as IL-1β and IL-6 [42–44]. These inflammatory mediators act as a positive feedback for further activation of NF-κB, and they result in a subsequent production of more proinflammatory mediators [45]. Several pathogens such as *E. coli* and *M. pneumonia* could induce the activation of the NF-κB pathway [46–49] and, according to some reports, MAPK, especially c-Jun NH (2)-terminal kinase (JNK) as the upstream kinase in the NF-κB signaling pathway, can induce the activation of NF-κB under some conditions [50]. JNK and p38 MAPK were induced and activated by a variety of cellular stresses, such as inflammatory cytokines [51], and their activation contributes to cell apoptosis and death [52]. Some researchers found that viral infections can induce the activation of JNK and p38 MAPK and cause injury to patients [53]. As shown in Figure 7 and as demonstrated in our previous research, JNK phosphorylation was decreased by FYC. Our results also showed that a mixture of bacterial infections activated the NF-κB pathway in the uterus of rats with PID; however, following an oral administration of FYC, GA, EA, and SY, the expression of p-NF-κB and p-IκB was suppressed. p-JNK was also found to be significantly reduced by FYC and GA. Generally, FYC and its main components may exert their anti-inflammatory effects by blocking the activation of the NF-κB pathway and regulating the apoptosis signaling pathway. To add, the anti-inflammatory effect of the compatibility group (GA + EA + SY) was superior to the FYC group; however, this difference may be due to the restriction of the FYC dose administered. The phytochemical characteristics of traditional Chinese medicines and their compatibility application are clearly worthy of further investigation.

5. Conclusions

FYC and its main components (GA, EA, and SY) showed dramatic anti-inflammatory activity in rats with pathogen-induced PID, especially in the group that contained the three primary components of FYC (GA + EA + SY). This led to the inhibition of neutrophil and lymphocyte infiltration and a reduction in the excessive production of cytokines or chemokines. The active ingredients of FYC (GA, EA, and SY) were demonstrated to be bioactive components, providing the theory basis for its compatibility as a TCM. Finally, the potential mechanism of this effect was revealed to be related to the regulation of the NF-κB and apoptosis pathways.

Data Availability

All data about this research are included in the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

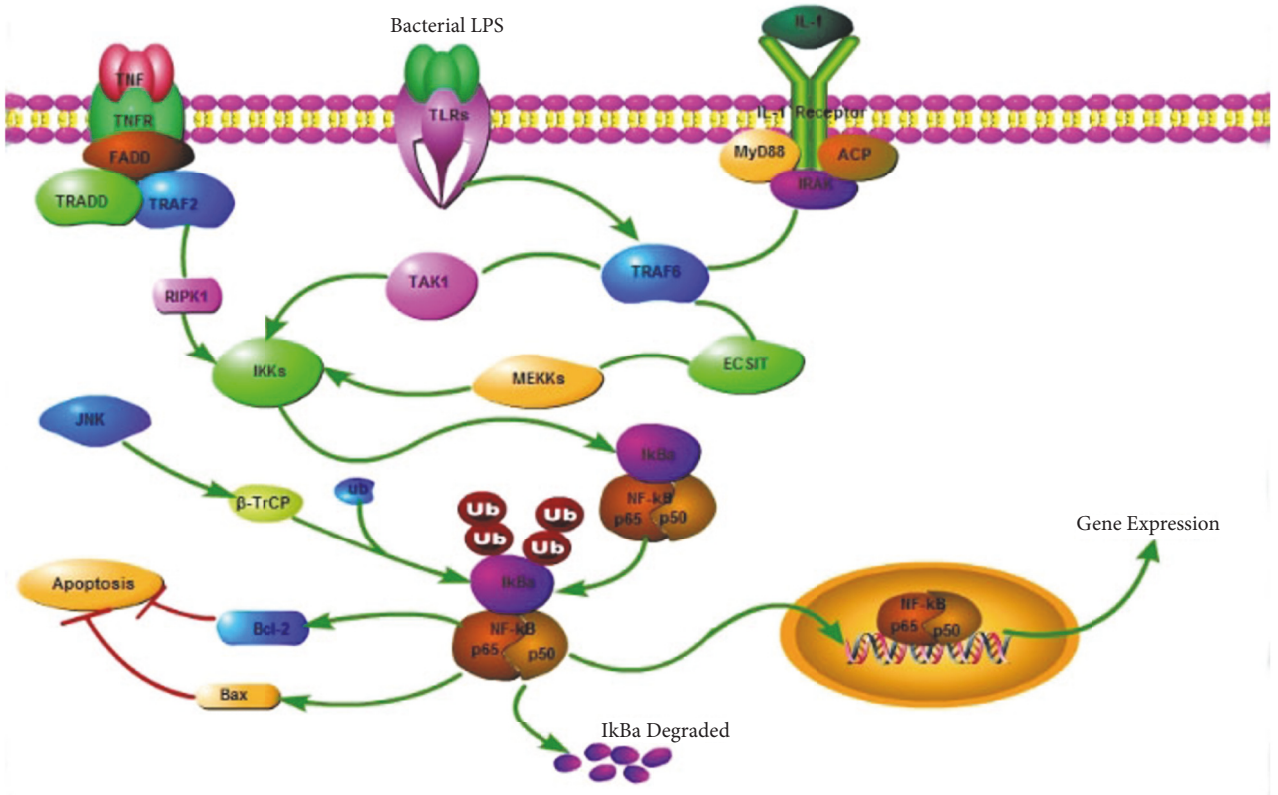


Figure 7: Signaling pathway.

Authors' Contributions

The work presented here was carried out in collaboration between all authors. Si-wang Wang and Yan-hua Xie defined the research theme and revised the manuscript critically. Yao Li and Qian Yang designed methods and experiments, carried out the laboratory experiments, and wrote the paper. Zhi-hui Shi, Min Zhou, Li Yan, and Hua Li collected and analyzed the data and interpreted the results. All authors have read and approved the final manuscript. Yao Li and Qian Yang contribute equally to the work.

Acknowledgments

This work was supported by a grant from the Science and Technology Innovation Project of Shaanxi province in China (No. 2015SF2-08-01), National Natural Science Foundation of China No. 81500192 and Shaanxi Province Science and Technology Research and Development Program Grant (No. 2017ADXM-SF-019), and Key Research Laboratory of Chinese Medicine and Natural Medicine in Shaanxi Province (No. 2015-164).

References

[1] S. S. Witkin, E. Minis, A. Athanasiou, J. Leizer, I. M. Linhares, and C. J. Papasian, "Chlamydia trachomatis: the persistent pathogen," *Clinical and Vaccine Immunology*, vol. 28, pp. 684–689, 2017.

[2] C. L. Haggerty, "Evidence for a role of Mycoplasma genitalium in pelvic inflammatory disease," *Current Opinion in Infectious Diseases*, vol. 21, no. 1, pp. 65–69, 2008.

[3] R. B. Ness, K. E. Kip, S. L. Hillier et al., "A cluster analysis of bacterial vaginosis-associated microflora and pelvic inflammatory disease," *American Journal of Epidemiology*, vol. 162, no. 6, pp. 585–590, 2005.

[4] D. Rui, D. Fuhrich, and J. D. C. Ross, "A review of antibiotic therapy for pelvic inflammatory disease," *International Journal of Antimicrobial Agents*, vol. 46, no. 3, pp. 272–277, 2015.

[5] M. Sinreih, M. Anko, N. H. Kene, V. Kocbek, and T. L. Rižner, "Expression of AKR1B1, AKR1C3 and other genes of prostaglandin F2α biosynthesis and action in ovarian endometriosis tissue and in model cell lines," *Chemico-Biological Interactions*, vol. 234, pp. 320–331, 2015.

[6] S. D. Hillis, R. Joesoef, P. A. Marchbanks, J. N. Wasserheit, W. Cates, and L. Westrom, "Delayed care of pelvic inflammatory disease as a risk factor for impaired fertility," *American Journal of Obstetrics & Gynecology*, vol. 168, no. 5, pp. 1503–1509, 1993.

[7] A. Bitew, Y. Abebaw, D. Bekele, and A. Mihret, "Prevalence of bacterial vaginosis and associated risk factors among women complaining of genital tract infection," *International Journal of Microbiology*, vol. 2017, Article ID 4919404, 8 pages, 2017.

[8] B. Li, "The curative effect of feiyang gastroenteritis tablets combined with levofloxacin in the treatment of acute or chronic gastroenteritis," *CHSM*, vol. 6, pp. 131-132, 2015.

[9] S. Kumar, R. Malhotra, and D. Kumar, "Euphorbia hirta: Its chemistry, traditional and medicinal uses, and pharmacological activities," *Pharmacognosy Reviews*, vol. 4, no. 7, pp. 58–61, 2010.

[10] B. Rao, K. Loganathan, K. Elumalai et al., "Antibacterial and antifungal activity of Euphorbia hirta l. Leaves: A comparative study," *Journal of Pharmacy Research*, vol. 3, pp. 548-549, 2010.

[11] P. N. B. Patel and K. C. Patel, "Antibacterial activity of E. hirta ethanomedicinal plant against gram negative UTI pathogens," *International Journal of Pharmaceutical Research and Allied Sciences*, vol. 3, pp. 24–29, 2014.

[12] Q. Shi, Y. Sun, and D. Meng, "Phytochemical and cytotoxic studies on the roots of Euphorbia fischeriana," *Bioorganic & Medicinal Chemistry Letters*, vol. 27, no. 2, pp. 266–270, 2017.

[13] S. Perumal and R. Mahmud, "Chemical analysis, inhibition of biofilm formation and biofilm eradication potential of Euphorbia hirta L. against clinical isolates and standard strains," *BMC Complementary and Alternative Medicine*, vol. 13, p. 346, 2013.

[14] M.-C. Lanhers, J. Fleurentin, P. Dorfman, F. Mortier, and J.-M. Pelt, "Analgesic, antipyretic and anti-inflammatory properties of Euphorbia hirta," *Planta Medica*, vol. 57, no. 3, pp. 225–231, 1991.

[15] H.-T. Xiao, S.-W. Tsang, H.-Y. Qin et al., "A bioactivity-guided study on the anti-diarrheal activity of Polygonum chinense Linn," *Journal of Ethnopharmacology*, vol. 149, no. 2, pp. 499–505, 2013.

[16] M. Kassim, M. Achoui, M. R. Mustafa, M. A. Mohd, and K. M. Yusoff, "Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity," *Nutrition Research*, vol. 30, no. 9, pp. 650–659, 2010.

[17] M. H. Kim, K. H. Park, M. H. Oh et al., "Two new hemiterpene glycosides from the leaves of Ilex rotunda. thunb," *Archives of Pharmacal Research*, vol. 35, no. 10, pp. 1779–1784, 2012.

[18] Y. J. Kim, E. B. Jung, M. S. Lee et al., "Rotundarpene inhibits toll-like receptor 2 activation-induced production of inflammatory mediators in keratinocytes by suppressing the Akt and NF-kappaB pathways," *International Immunopharmacology*, vol. 18, no. 2, pp. 325–332, 2014.

[19] D. Zhou, Q. Yang, Y. Zhe et al., "Fingerprint analysis and quantitative Determination of fourteen active components in the traditional Chinese medicinal preparation Feiyangchangweiyan Capsule by HPLC-DAD-ESI-MS/MS," *Iranian Journal of Pharmaceutical research*, vol. 18, no. 2, pp. 948–960, 2019.

[20] L.-J. Zhang, J.-Y. Zhu, M.-Y. Sun et al., "Anti-inflammatory effect of Man-Pen-Fang, a Chinese herbal compound, on chronic pelvic inflammation in rats," *Journal of Ethnopharmacology*, vol. 208, pp. 57–65, 2017.

[21] D. Xu, H. Xiong, Z. Xiao et al., "Uterine cytokine profile in a rat model of endometritis," *American Journal of Reproductive Immunology*, vol. 73, no. 3, pp. 214–220, 2015.

[22] R. G. Rank, H. M. Lacy, A. Goodwin et al., "Host chemokine and cytokine response in the endocervix within the first developmental cycle of Chlamydia muridarum," *Infection and Immunity*, vol. 78, no. 1, pp. 536–544, 2010.

[23] J. L. Arredondo, V. Diaz, H. Gaitan et al., "Oral clindamycin and ciprofloxacin versus intramuscular ceftriaxone and oral doxycycline in the treatment of mild-to-moderate pelvic inflammatory disease in outpatients," *Clinical Infectious Diseases*, vol. 24, no. 2, pp. 170–178, 1997.

[24] S. S. Patnaik, A. S. Laganà, S. G. Vitale et al., "Etiology, pathophysiology and biomarkers of interstitial cystitis/painful bladder syndrome," *Archives of Gynecology and Obstetrics*, vol. 295, no. 6, pp. 1341–1359, 2017.

[25] T. Spencer, P. Umeh, E. Irokanulo et al., "Bacterial isolates associated with pelvic inflammatory disease among female patients attending some hospitals in Abuja, Nigeria," *African Journal of Infectious Diseases*, vol. 8, no. 1, 2013.

[26] A. M. Burnett, C. P. Anderson, and M. D. Zwank, "Laboratory-confirmed gonorrhea and/or chlamydia rates in clinically diagnosed pelvic inflammatory disease and cervicitis," *The American Journal of Emergency Medicine*, vol. 30, no. 7, pp. 1114–1117, 2012.

[27] S. D. Datta, E. Torrone, D. Kruszon-Moran et al., "Chlamydia trachomatis trends in the United States among persons 14 to 39 years of age, 1999-2008," *Sexually Transmitted Diseases*, vol. 39, no. 2, pp. 92–96, 2012.

[28] W. L. Risser, J. M. Risser, and A. L. Risser, "Current perspectives in the USA on the diagnosis and treatment of pelvic inflammatory disease in adolescents," *Adolescent Health, Medicine and Therapeutics*, vol. Volume 8, pp. 87–94, 2017.

[29] Z. Tao-Hu, S. Yun-Chen, and X. Feng-Ruan, "Discussion on the relationship between intestinal and vaginal microecological balance in spleen and kidney related theory," *Lishizhen Medicine and Materia Medica Research*, vol. 27, pp. 419-420, 2016.

[30] D. L. Patton, M. Askienazy-Elbhar, J. Henry-Suchet et al., "Detection of Chlamydia trachomatis in fallopian tube tissue in women with postinfectious tubal infertility," *American Journal of Obstetrics & Gynecology*, vol. 171, no. 1, pp. 95–101, 1994.

[31] M. T. Mascellino, P. Boccia, and A. Oliva, "Immunopathogenesis in chlamydia trachomatis infected women," *ISRN Obstetrics and Gynecology*, vol. 2011, Article ID 436936, 9 pages, 2011.

[32] H. Sellami, N. Said-Sadier, A. Znazen, R. Gdoura, D. M. Ojcius, and A. Hammami, "Chlamydia trachomatis infection increases the expression of inflammatory tumorigenic cytokines and chemokines as well as components of the Toll-like receptor and NF-κB pathways in human prostate epithelial cells," *Molecular and Cellular Probes*, vol. 28, no. 4, pp. 147–154, 2014.

[33] A. E. King, N. Wheelhouse, S. Cameron et al., "Expression of secretory leukocyte protease inhibitor and elafin in human fallopian tube and in an in-vitro model of Chlamydia trachomatis infection," *Human Reproduction*, vol. 24, no. 3, pp. 679–686, 2009.

[34] X. Xie, M. Yang, Y. Ding, and J. Chen, "Microbial infection, inflammation and epithelial ovarian cancer (Review)," *Oncology Letters*, vol. 14, no. 2, pp. 1911–1919, 2017.

[35] E. P. Hanson, L. Monaco-Shawver, L. A. Solt et al., "Hypomorphic nuclear factor-κB essential modulator mutation database and reconstitution system identifies phenotypic and immunologic diversity," *The Journal of Allergy and Clinical Immunology*, vol. 122, no. 6, pp. 1169–1177.e16, 2008.

[36] C. S. Boyd and E. Cadenas, "Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis," *Biological Chemistry*, vol. 383, no. 3-4, pp. 411–423, 2002.

[37] P. Zhou, S. Lu, Y. Luo et al., "Attenuation of TNF-α-induced inflammatory injury in endothelial cells by ginsenoside Rb1 via inhibiting NF-κB, JNK and p38 signaling pathways," *Frontiers in Pharmacology*, vol. 8, no. 464, 2017.

[38] K. M. Murphy, V. Ranganathan, M. L. Farnsworth, M. Kavallaris, and R. B. Lock, "Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells," *Cell Death & Differentiation*, vol. 7, no. 1, pp. 102–111, 2000.

[39] R. A. Kirkland, G. M. Saavedra, B. S. Cummings, and J. L. Franklin, "Bax regulates production of superoxide in both apoptotic and nonapoptotic neurons: role of caspases," *The Journal of Neuroscience*, vol. 30, no. 48, pp. 16114–16127, 2010.

[40] S. Ghosh, M. J. May, and E. B. Kopp, "NF-κB and rel proteins: evolutionarily conserved mediators of immune responses," *Annual Review of Immunology*, vol. 16, pp. 225–260, 1998.

[41] R. Janssen, A. van Wengen, M. A. Hoeve et al., "The same IkappaBalpha mutation in two related individuals leads to completely different clinical syndromes," *The Journal of Experimental Medicine*, vol. 5, pp. 559–568, 2004.

[42] W. Zou, H. Zhou, J. Hu et al., "Rhizoma Smilacis Glabrae inhibits pathogen-induced upper genital tract inflammation in rats through suppression of NF-κB pathway," *Journal of Ethnopharmacology*, vol. 202, pp. 103–113, 2017.

[43] S. M. Borghi, V. Fattori, K. W. Ruiz-Miyazawa et al., "Leishmania (L). amazonensis induces hyperalgesia in balb/c mice: Contribution of endogenous spinal cord TNFα and NFκB activation," *Chemico-Biological Interactions*, vol. 268, pp. 1–12, 2017.

[44] J. He, X. You, Y. Zeng, M. Yu, L. Zuo, and Y. Wu, "Mycoplasma genitalium-derived lipid-associated membrane proteins activate NF-kappaB through toll-like receptors 1, 2, and 6 and CD14 in a MyD88-dependent pathway," *Clinical and Vaccine Immunology*, vol. 16, no. 12, pp. 1750–1757, 2009.

[45] V. Bours, G. Bonizzi, M. Bentires-Alj et al., "NF-κB activation in response to toxical and therapeutical agents: Role in inflammation and cancer treatment," *Toxicology*, vol. 153, no. 1-3, pp. 27–38, 2000.

[46] T. Into, K. Kiura, M. Yasuda et al., "Stimulation of human Toll-like receptor (TLR) 2 and TLR6 with membrane lipoproteins of Mycoplasma fermentans induces apoptotic cell death after NF-κB activation," *Cellular Microbiology*, vol. 6, no. 2, pp. 187–199, 2004.

[47] C. L. McGowin, M. Liang, D. H. Martin, and R. B. Pyles, "Mycoplasma genitalium-encoded MG309 activates NF-κB via toll-like receptors 2 and 6 to elicit proinflammatory cytokine secretion from human genital epithelial cells," *Infection and Immunity*, vol. 77, no. 3, pp. 1175–1181, 2009.

[48] T. Shimizu, Y. Kida, and K. Kuwano, "A dipalmitoylated lipoprotein from Mycoplasma pneumoniae activates NF-κB through TLR1, TLR2, and TLR6," *The Journal of Immunology*, vol. 175, no. 7, pp. 4641–4646, 2005.

[49] C. Picard, J.-L. Casanova, and A. Puel, "Infectious diseases in patients with IRAK-4, MyD88, NEMO, or IκBα deficiency," *Clinical Microbiology Reviews*, vol. 24, no. 3, pp. 490–497, 2011.

[50] Y. Pan, X. Zhang, and Y. Wang, "Targeting JNK by a new curcumin analog to inhibit NF-kB-mediated expression of cell adhesion molecules attenuates renal macrophage infiltration and injury in diabetic mice," *PLoS ONE*, vol. 8, no. 11, Article ID e79084, 2013.

[51] W. L. Thompson and L. J. Van Eldik, "Inflammatory cytokines stimulate the chemokines CCL2/MCP-1 and CCL7/MCP-7 through NFκB and MAPK dependent pathways in rat astrocytes," *Brain Research*, vol. 1287, pp. 47–57, 2009.

[52] J. H. Kim, S. H. Jung, Y. I. Yang et al., "Artemisia leaf extract induces apoptosis in human endometriotic cells through regulation of the p38 and NFkappaB pathways," *Journal of Ethnopharmacology*, vol. 145, no. 3, pp. 767–775, 2013.

[53] F. Xia, C. Wang, Y. Jin et al., "Luteolin protects HUVECs from TNF-α-induced oxidative stress and inflammation via its effects on the Nox4/ROS-NF-κB and MAPK pathways," *Journal of Atherosclerosis and Thrombosis*, vol. 21, no. 8, pp. 768–783, 2014.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7318616, 7 pages
https://doi.org/10.1155/2019/7318616

Research Article

Effect of Chaihu Shugan Powder-Contained Serum on Glutamate-Induced Autophagy of Interstitial Cells of Cajal in the Rat Gastric Antrum

Ren-Qian Tan,[1] **Zhi Zhang**,[1] **Jing Ju**,[1] **and Jiang-Hong Ling**[2]

[1]*The First Affiliated Hospital, Guangxi Medical University, Nanning 530021, China*
[2]*Shuguang Affiliated Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200021, China*

Correspondence should be addressed to Jiang-Hong Ling; 459183870@qq.com

Received 26 December 2018; Revised 19 April 2019; Accepted 8 May 2019; Published 29 May 2019

Academic Editor: Lucindo Q. Júnior

Gastrointestinal (GI) motility disorder is caused by excessive autophagy of the interstitial cells of Cajal (ICC). Chaihu Shugan Powder (CSP) is a traditional Chinese medicine with therapeutic benefits in GI motility disorders; however, the underlying mechanism of its therapeutic effect in GI disorders, especially autophagy of ICC, remains unclear. Thus, this study investigated the effects of CSP-contained serum on glutamate-induced autophagy in rat gastric ICC, exploring its underlying mechanism. In vitro cultured rat stomach ICC were identified by fluorescence microscopy and then stimulated with glutamate (5 mmol/L) for 3 h to establish the autophagy model. These cells were then treated with 10% CSP-containing serum or the autophagy inhibitor 3-methyladenine (3-MA; 5 mmol/L) for 24 h. The control group was cultured with only 10% serum containing physiological saline. The viability of ICC was measured by the CCK-8 assay. The ultrastructure and autophagosomes of ICC were observed using transmission electron microscopy. LC3 expression was detected by immunofluorescence, and LC3, Beclin1, Bcl2, and PI3KC3 expression was detected by western blot analysis. Transmission electron microscopy showed abundant endoplasmic reticulum, mitochondria, and other organelles in the control group, whereas the cells in the autophagy model control group had clear autophagic vacuoles, which were not apparent in both CSP and 3-MA groups. ICC viability was significantly increased by CSP and 3-MA interventions ($P < 0.01$), accompanied by a decrease in LC3 fluorescence ($P < 0.01$). Moreover, the expression levels of LC3II/I, Beclin1, and PI3KC3 were significantly decreased (all $P < 0.01$) with CSP and 3-MA treatment, while Bcl2 expression level was higher than that of the model group ($P < 0.01$). Thus, CSP can reduce autophagic damage by enhancing Bcl2 expression and downregulating the expression of LC3, Beclin1, and PI3KC3 to protect ICC. These results highlight the potential of CSP in the treatment of GI motility disorders.

1. Introduction

Interstitial cells of Cajal (ICC) have been widely recognized as pacemakers and regulators of gastrointestinal (GI) motility and have therefore become the focus of research into the mechanism of GI motility disorders. Changes in the structure and number of ICC can often lead to GI function disorders, including idiopathic achalasia, diabetic gastropathy, chronic idiopathic intestinal pseudoobstruction, constipation, and functional dyspepsia [1–6]. Although the precise mechanism linking ICC changes with GI motility disorders has not yet been elucidated, our previous study [7] showed that excessive autophagy can lead to changes in the number and morphology of ICC. Autophagy is a highly conserved cellular protection process that is widely present in eukaryotic cells, involving the formation of autophagic vacuoles by the lysosomal pathway, thereby eradicating the cell's own senescent and damaged organelles or misfolded proteins to maintain homeostasis of the cellular environment [8]. However, uncontrolled autophagy of ICC can lead to slow-wave potentials, contraction rhythms of the smooth muscle, and loss of regulation in the transfer of neurotransmitters, contributing to GI motility disorders. Therefore, we hypothesized that GI motility disorder could be prevented by regulating the autophagy level of ICC [9].

Chaihu Shugan Powder (CSP) is a classical and effective prescription of traditional Chinese medicine (TCM), with strong and abundant evidence for its good therapeutic effects on GI motility disorders [10–14]. CSP was first described in the book "*Jing Yue Quan Shu*" written by Zhang Jiebin during the time of the Ming Dynasty. It consists of the following seven Chinese herbs: *Radix Bupleuri, Aurantii nobilis pericarpium, Paeonia, Szechwan Lovage rhizome, Fructus Aurantii, Nutgrass Galingale rhizome, and Glycyrrhiza uralensis*, traditionally administered with a dose ratio of 4:4:3:3:3:3:1. According to TCM theory, CSP effects include soothing stagnated Gan-qi, relieving the liver, regulating Qi, and relieving pain. CSP has been used to relieve bloating, abdominal pain, belching, anorexia, nausea, bitterness, acid reflux, constipation, and sloppy stool. The ingredients of the CSP decoction are mainly *albiflorin, ferulic acid, paeoniflorin, liquiritin, isoliquiritin, isoliquiritigenin, narirutin, naringin, naringenin, hesperidin, hesperetin,neohesperidinn, glycyrrhizic acid, alpha-cyperone, and 18-beta-glycyrrhizic acid* [15]. Zhang et al. [16] reported that CSP and its absorbed compound ferulic acid (FA) induced prokinetics via inhibiting serotonin, regulating the hypothalamic-pituitary axis, while increasing ghrelin and stimulating jejunal contraction simultaneously. Our previous study [17] showed that CSP can inhibit excessive autophagy of gastric antrum ICC in functional dyspepsia rats, thus promoting gastrointestinal motility. However, the efficacy of CSP in the treatment of GI motility disorders and the underlying mechanism are unknown. The application and popularization of drug-contained serum technology have broadened the research field of the effects of Chinese medicine compounds at the cellular level [18–20]. Bochu et al. [21] suggested the use of drug-contained serum in an isolated reaction system is suitable for investigations of pharmacological mechanisms, since this serum can not only prevent the physical and chemical properties of crude drug preparations from interfering with in vitro experiments but also can reflect the digestion and absorption of the Chinese medicine in the GI tract, and its subsequent biotransformation, thereby better reflecting the process that produces pharmacological effects. Therefore, the aim of the present study was to examine the effects of a CSP-contained serum on autophagy in the ICC of rats and to explore the underlying pharmacological mechanism in relation to autophagy.

2. Materials and Methods

2.1. Animals. The research was conducted according to protocols approved by the Experimental Animal Ethics Committee of Guangxi Medical University (No. 201611017) and was conducted in compliance with the Care and Use of Laboratory Animals guideline published by the US National Institutes of Health (NIH; publication No. 85-23; 1996). All efforts were made to minimize animal suffering.

Sprague-Dawley rats were obtained from Experimental Animal Center of Guangxi Medical University (Guang'xi, China; License no. SCXK (Gui) 2014-0002; Guang'xi, China) and randomly divided into two groups: group A (n = 20, 10 males and 10 females, 250 ± 20 g), which were used to prepare the drug-contained serum, and group B (n = 20, 10 males and 10 females, 150 ± 20 g), which were used for the isolation of ICC. All rats were acclimated in cages for three days prior to the experiments and maintained under a 12 h/12 h light cycle at 21 ± 2°C and 50 ± 5% relative humidity, with unlimited access to standard food and water.

2.2. Preparation of Drug-Contained Serum. CSP was prepared according to The People's Republic of China Pharmacopeia 1st Volume conventional dosage as follows: 6 g *Radix Bupleuri*, 4.5 g *Paeonia*, 4.5 g *Szechwan Lovage rhizome*, 4.5 g *Fructus Aurantii*, 6 g *Aurantii nobilis pericarpium*, 4.5 g *Nutgrass Galingale rhizome*, and 1.5 g *Glycyrrhiza uralensis*; all component herbs were purchased from the pharmacy of The First Affiliated Hospital of Guangxi Medical University. For the experiments, CSP was used at 10 times the normal recommended dose for a 60-kg adult (according to the Chinese Medicine book *Jing Yue Quan Shu*, based on the surface area conversion algorithm for the standard rat body [22] and was concentrated to 105% (1.05 g/mL).

Rats of group A were randomly divided into two groups and were intragastrically administered with physiological saline and the CSP decoction, respectively, at a concentration of 1.5 mL/100 g twice daily at 12 h intervals for three consecutive days. Under anesthesia, blood was extracted from the abdominal aorta 1h after the last administration, and the serum was collected after standing and centrifugation. The serum was then inactivated in water (56°C, 0.5 h), filtered, sterilized by a 0.22 μm microporous membrane filter, and stored at -80°C until use. At the end of the experiment, all rats were sacrificed by cervical dislocation.

2.3. ICC Isolation and Culture. The 20 rats in group B were euthanized by cervical dislocation. The stomachs were isolated and the antrum was dissected out for subsequent use. After longitudinal laparotomy, the antrum was immediately dissected, opened along the lesser curvature of the stomach, and rinsed free of content with ice-cold D-Hanks solution. The gastric mucosa was carefully peeled away from the smooth muscle layers with sharp forceps, and then cut into small pieces of approximately 1 × 1 mm. The pieces were then incubated in a Ca^{2+}-free dispersal solution containing collagenase type II (1.3 mg/mL; Sigma, USA) for approximately 50 min at 37°C. Single cells were obtained by gentle agitation for 5 min with a wide-bore pipette, and the dispersed cells were filtered through 200 μm mesh filters. The dispersed cells were plated onto a 25 mm diameter culture flask and cultured at 37°C in a 95% O_2-5% CO_2 incubator in ICC growth medium (M199; Hyclone, USA) supplemented with 2% antibiotics and 25 ng/mL murine stem cell factor (Sigma, USA). ICC were identified immunologically by incubation with rabbit anti-rat c-Kit monoclonal antibody (Pierce, USA) at a dilution of 1:200 for 60 min, which allowed for differentiation of ICC from other cell types in the culture based on morphological differences detected by light and fluorescence microscopy.

2.4. Autophagy Model Establishment and Treatments. The autophagy model was established according to recent methods reported by Tan et al. [23] using incubation with 5 mol/L glutamate (L-glutamic acid; Sigma, USA) for 3 h. For

comparison, the control group was cultured with 10% serum containing physiological saline. The cells in the autophagy group were then treated with or without 10% serum containing CSP or 5 mol/L 3-methyladenine (3-MA), an autophagy inhibitor.

2.5. Cell Viability. The viability of ICC in the different groups was assessed with Cell Counting Kit-8 (CCK-8; Dojindo, Japan). In brief, ICC were seeded at a density of 1×10^4 cells/well in 96-well flat-bottomed microplates. Once the cells attached to the bottom of the culture vessel, the culture medium was removed from each well. Different concentrations of the respective culture medium for each group were added to the wells, and the cells were cultured for 24 h. CCK-8 reagent (10 μL) was added to each well of a 96-well flat-bottomed microplate that contained 100 μL of culture medium to reach a final concentration of 10 μL/100 μL and incubated for an additional 4 h at 37°C. The absorbance rate was measured at 450 nm on an auto microplate reader (Multiskan FC, Thermo, USA). All experiments were conducted in triplicate on six separate occasions.

2.6. Transmission Electron Microscopy. After centrifugation, the cells were fixed for 2 h with 3% glutaraldehyde in phosphate buffered saline (PBS, pH 7.2). After rinsing with PBS, the samples were post-fixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated in a graded series of acetone, and embedded in Eponate 812 resin. Ultrathin sections were cut into 50–70 nm thick slices, double-stained with uranyl acetate and lead citrate, and observed on a Hitachi (Japan) transmission electron microscope.

2.7. Immunofluorescence. ICC in the logarithmic growth phase was plated onto sterile glass coverslips in a 24-well flat-bottomed microplate at a density of 5×10^3 cells/well. Once the cells attached to the bottom of the culture vessel, ICC was cultured according to the group requirements mentioned above. The culture medium was removed from the coverslips, all of the cultured cells were fixed with ice-cold paraformaldehyde for 10 min and then washed in PBS three times for 5 min each. The samples were incubated in 5% nonfat milk for 10 min at room temperature to reduce nonspecific staining. After incubation with an anti-LC3 antibody (1:100; Novus, USA) at 4°C overnight; the samples were washed in PBS three times for 10 min each. Immunoreactivity was detected with a fluorescein isothiocyanate-conjugated secondary antibody (anti-rabbit IgG, 1:200) in the dark for 30 min at 37°C. The nuclei were labeled with 4′,6-diamidino-2-phenylindole (Beyotime, China). The sections were examined by fluorescence microscopy (Upright fluorescence microscope; Nikon, Japan), respectively.

2.8. Western Blot. The cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer containing a protease inhibitor cocktail. The protein samples were harvested, resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk for 2 h, the membranes were incubated overnight at 4°C with the following primary antibodies: LC3 (1:1000; Novus, USA), Beclin1 (1:1000; Novus, USA), Bcl2 (1:1000; CST, USA), PI3KC3 (1:1000; CST, USA), and GAPDH (1:1000; SAB, USA). After washing in PBS (supplemented with 0.1% Tween 20) three times for 5 min each, the samples were incubated with the appropriate secondary antibody and developed with enhanced chemiluminescence.

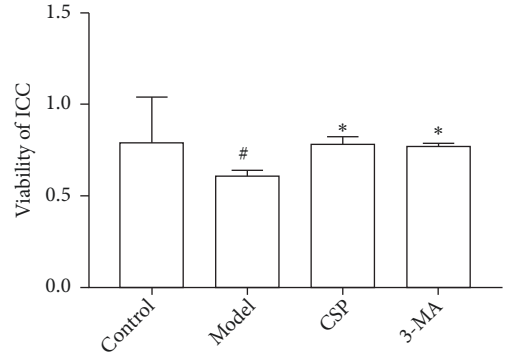


Figure 1: The viability of interstitial cells of Cajal (ICC) under glutamate-induced autophagy with and without treatment of the known autophagy inhibitor, 3-MA, or Chaihu Shugan powder (CSP). $^{\#}P < 0.01$ versus control group; $^{*}P < 0.05$ versus model group (N = 6 per group).

2.9. Statistical Analyses. Data are presented as mean ± standard deviation. Differences between groups were assessed using one-way analysis of variance followed by Tukey's test with SPSS 23.0 software. All statistical tests were two-tailed, and a P value < 0.05 was considered statistically significant.

3. Results

3.1. ICC Viability. The CCK-8 assay demonstrated that the OD value of the model group significantly decreased compared to that of the control group ($P< 0.001$), whereas the OD values of the CSP and 3-MA groups were significantly increased compared to that of the model group (both $P< 0.001$) (Figure 1).

3.2. Ultrastructure of ICC. Transmission electron microscopy revealed that ICC in the control group contained abundant organelles, such as endoplasmic reticulum and mitochondria, without obvious autophagosomes and autophagic vacuoles. There was an apparent number of autophagic vacuoles and vacuoles in the model group than in the normal control group, suggesting autophagy in ICC. Interestingly, CSP and 3-MA treatment significantly inhibited autophagy (Figure 2).

3.3. Protein Expression of Autophagy Markers. Compared with the control group, the fluorescent intensity of LC3 in the ICC of the model group was significantly increased ($P< 0.01$), whereas the LC3 fluorescence of the CSP and 3-MA groups was significantly lower than that of the model group

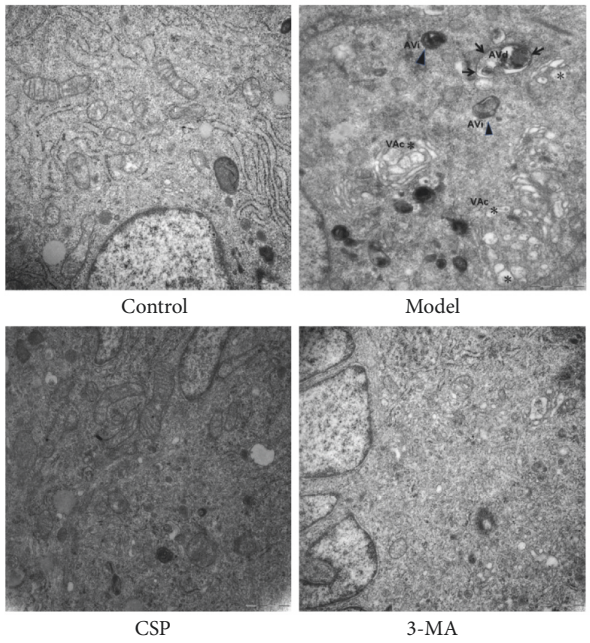


Figure 2: Evidence of autophagy and the ultrastructure of interstitial cells of Cajal (ICC) observed under transmission electron microscopy (×30, 000). ▲: initial autophagic vacuoles, AVi; ⟶: degrading autophagic vacuoles, and AVd; ∗: vacuole, VAc.

($P< 0.05$) (Figure 3). This finding was further confirmed by western blot analysis (Figure 4), which showed that there was a significant increase in the levels of LC3, PI3KC3, and Beclin1, but decrease in Bcl2 protein expression in the model group ($P< 0.05$). However, the CSP and 3-MA treatment markedly reversed the abnormal changes of these proteins ($P< 0.05$), suggesting that CSP can reduce autophagy in ICC.

3.4. Discussion. Recently, ICC have emerged as the basic determinant of GI motility, and thus serve as an ideal tool for investigating the mechanisms of GI motility and determining treatment strategies for related disorders [24, 25]. According to the theory of TCM, the liver controls conveyance and dispersion, loving rising, and smooth, hating depression. Presence of external pathogens, emotional disorders, dietary irregularities, and spleen-stomach weakness lead to liver dysfunction. Additionally, the pattern of liver qi invading the stomach, leading to spleen and stomach dysfunction is considered to be the main cause of GI motility disorders. CSP is a classical and effective prescription representative of the Shugan Qi method. It can soothe the liver and regulate qi, move qi to relieve pain, promote digestion, and remove food stagnation that can further relieve clinical symptoms of GI motility disorder and eventually restore normal digestive function. Although CSP has been used as an effective prokinetic for GI motility disorders in TCM [16, 26], the underlying mechanism of its effects remains unclear so far. Here, we demonstrate a clear attenuating effect of CSP on excessive autophagy of ICC that could contribute to dysregulated GI motility.

Autophagy is a biological process through which cells maintain their homeostasis and physiology [27]. Under stress, autophagy is responsible for the removal of damaged organelles, production of energy for cells, and maintenance of internal environment stability. However, excessive autophagy can induce cell death. LC3 is a homolog of yeast autophagy-associated protein 8 (Atg8) and is essential for autophagy. It is involved in the formation of autophagic vesicles and therefore is an important indicator of autophagic activity [28]. LC3II is a widely used autophagy marker; when autophagy occurs in cells, LC3I, which mainly exists in the cytoplasm, is ubiquitin-modified and converts to the LC3II form with phosphatidyl ethanolamine on the surface of the autophagic foam membrane. Its content is proportional to the number of autophagic vacuoles. By examining LC3II/LC3I, we can determine the number of autophagosomes and infer the strength of autophagy [29].

Autophagic activity is regulated by many signaling pathways, and the combination of PI3KC3 and Beclin1 to form autophagosomes is the main positive regulatory mechanism. PI3KC3 is a homolog of Vps34, which phosphorylates the third site protein of phosphatidylinositol in eukaryotes to form a complex with Beclin1. siRNA silences the expression of PI3KC3 and attenuates the expression of autophagy [30]. Beclin1 is a homologous gene of yeast ATG6 and is also the most important regulator of the positive regulatory mechanism [31]. Absence of Beclin1 weakens autophagic activity. The reintroduction of Beclin1 can effectively regulate autophagy pathway and enhance cell death metabolism [32]. Beclin1 interacts with many proteins to form multiple functionally distinct complexes [33]. Through the formation of complexes with PI3KC3, Beclin1 regulates the localization of autophagy precursors and in turn regulates autophagic activity [34]. Contrastingly, Bcl2 acts as an inhibitor of autophagy and apoptosis and protects cells [35, 36]. Although upregulated Beclin1 expression in mammalian cells can induce autophagy [37], Bcl2 can inhibit the Beclin1-dependent autophagy by forming a complex with Beclin1. Thus, Beclin1 is a key factor in regulating and maintaining the balance between autophagy and apoptosis in cells; when Beclin1 forms a complex with PI3KC3, it activates the process of autophagy, and when Beclin1 forms a complex with Bcl2, it activates apoptosis.

Glutamate is an important amino acid that maintains normal physiological functions of the body and is involved in the synthesis of various proteins. Oudenhove et al. [38] suggested that an excess amount of glutamate could induce neuronal cell autophagy due to its excitotoxic and oxidative stress impairment. Chen et al. [39] suggested that glutamate could induce the formation and recruitment of PI3KC3 and Beclin1, form complexes, and participate in the formation of autophagic membranes. However, the glutamate-induced activation of autophagy in ICC is not clear. In the present study, glutamate treatment markedly reduced the viability of rat ICC and increased the number of autophagic vacuoles. Additionally, increased expression of autophagy markers such as LC3, Beclin1, and PI3KC3 and reduced expression of the apoptosis regulator protein Bcl2 was observed. This indicated that the glutamate-mediated induction of autophagy

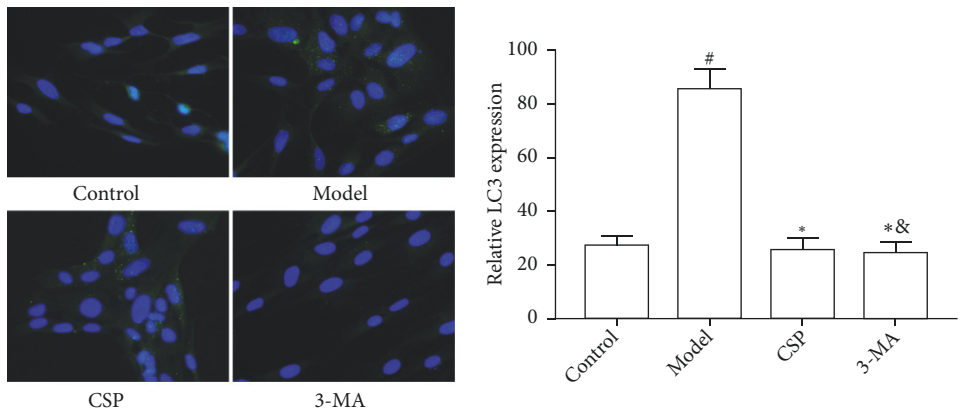


FIGURE 3: Observation of LC3 in the interstitial cells of Cajal (ICC) of each group under fluorescence microscopy (×200). $^{\&}P < 0.05$ versus control; $^{\#}P < 0.01$ versus control; $*P < 0.01$ versus model. (N = 15 per group).

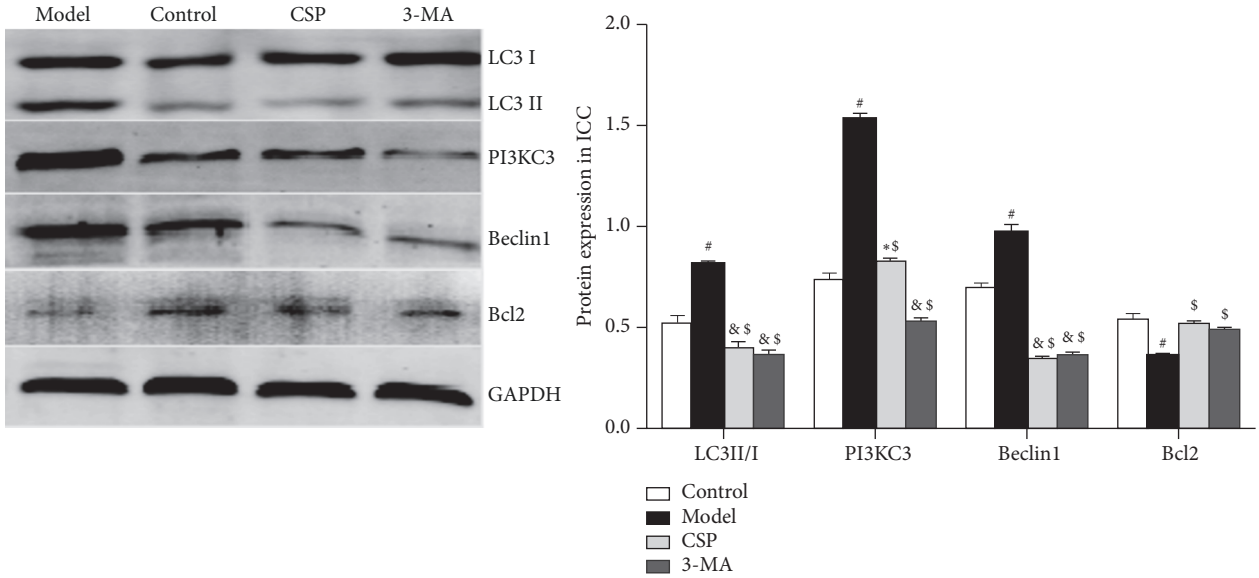


FIGURE 4: LC3, PI3KC3, Beclin1, and Bcl2 protein expression in interstitial cells of Cajal (ICC). The expression levels were normalized to that of the housekeeping protein GAPDH. $^{\&}P < 0.05$ versus control; $^{\#}P < 0.01$ versus control; $^{\$}P < 0.01$ versus model; $^{*}P < 0.05$ versus 3-MA (N = 3 per group).

was successful. Furthermore, treatment with the known autophagy inhibitor 3-MA inhibited autophagy and the same effect was also detected for CSP. Thus, our study suggests that this mechanism might involve increasing the levels of autophagy-related proteins while simultaneously inhibiting the proteins that negatively regulate autophagy.

Importantly, both CSP and 3-MA could improve the autophagic damage induced by glutamate. Klionsky et al. [40] suggested that the mechanism by which 3-MA inhibits autophagy may involve suppressing the expression of PI3KC3 and thereby preventing the formation of autophagosomes. Chen et al. [41] also found that 3-MA could inhibit autophagy by reducing the expression of Beclin1. Therefore, our findings suggest that CSP may inhibit autophagy in the same manner as 3-MA.

Collectively, these results highlight the protective effect of CSP on excessive autophagy of ICC by reducing the high expression of LC3, Beclin1, and PI3KC3 proteins and enhancing the low expression of Bcl2.

3.5. Conclusions. Our data suggest that CSP shows significant gastroprokinetic effects by inhibiting excessive autophagy via prevention of Bcl2-Beclin1 complex dissociation.

These effects highlight CSP as a strong candidate for the treatment of GI motility disorders. However, the effects induced by TCM are currently difficult to reproduce because of limited information available regarding the absorbed bioactive compounds. To resolve this issue and develop a novel prokinetic strategy based on CSP, further research is required to identify the specific compounds responsible

for the inhibitory effects of CSP and to determine their underlying mechanisms of action.

List of Abbreviations

3-MA: 3-Methyladenine
CSP: Chaihu Shugan powder
TCM: Traditional Chinese medicine
CCK-8: Cell Counting Kit-8
ICC: Interstitial cells of Cajal;
GI: Gastrointestinal
PBS: Phosphate-buffered saline.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

The research was conducted according to protocols approved by Guangxi Medical University Institutional Ethical Committee (no. 201611016; no. 201611017) and was conducted in compliance with the guidelines of Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH; publication nos. 85-23; 1996). All efforts were made to minimize animal suffering.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jiang-Hong Ling conceived and designed the study. Zhi Zhang and Ren-Qian Tan completed the experiments. Ren-Qian Tan and Jing Ju performed data analyses and drafted the initial manuscript. All authors read and approved the final manuscript. Ren-Qian Tan and Zhi Zhang contributed equally to this work.

Funding

This work was supported by grants from the National Natural Science Foundation of China (No. 81560763).

Acknowledgments

We thank Li-Min Zhang and Xing Lin for suggestions on the revision of our manuscript.

References

[1] D. S. Pardi, S. M. Miller, D. L. Miller et al., "Paraneoplastic dysmotility: loss of interstitial cells of Cajal," *American Journal of Gastroenterology*, vol. 97, no. 7, pp. 1828–1833, 2002.

[2] M. Nakahara, K. Isozaki, S. Hirota et al., "Deficiency of KIT-positive cells in the colon of patients with diabetes mellitus," *Journal of Gastroenterology and Hepatology*, vol. 17, no. 6, pp. 666–670, 2002.

[3] C. J. Streutker, J. D. Huizinga, F. Campbell, J. Ho, and R. H. Riddell, "Loss of CD117 (c-kit)- and CD34-positive ICC and associated CD34-positive fibroblasts defines a subpopulation of chronic intestinal pseudo-obstruction," *The American Journal of Surgical Pathology*, vol. 27, no. 2, pp. 228–235, 2003.

[4] V. Villanacci, V. Annese, A. Cuttitta et al., "An immunohistochemical study of the myenteric plexus in idiopathic achalasia," *Journal of Clinical Gastroenterology*, vol. 44, no. 6, pp. 407–410, 2010.

[5] J. Yin and J. D. Z. Chen, "Roles of interstitial cells of Cajal in regulating gastrointestinal motility: In vitro versus in vivo studies," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 4, pp. 1118–1129, 2008.

[6] K. M. Sanders, T. Ördög, and S. M. Ward, "Physiology and pathophysiology of the interstitial cells of Cajal: From bench to bedside. IV. Genetic and animal models of GI motility disorders caused by loss of interstitial cells of Cajal," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 282, no. 5, pp. G747–G756, 2002.

[7] L. Zhang, L. Zeng, J. Deng et al., "Investigation of autophagy and differentiation of myenteric interstitial cells of Cajal in the pathogenesis of gastric motility disorders in rats with functional dyspepsia," *Biotechnology and Applied Biochemistry*, vol. 65, no. 4, pp. 533–539, 2018.

[8] T. Watanabe, A. Kuma, and N. Mizushima, "Physiological role of autophagy in metabolism and its regulation mechanism," *Nihon Rinsho. Japanese Journal of Clinical Medicine*, vol. 69, pp. 775–781, 2011.

[9] Y. X. Ji, J. H. Ling, L. M. Pang, G. H. Huang, L. Jiang, and C. W. Zheng, "The interstitial cells of Cajal, autophagy and gastrointestinal motility disorders," *Journal of Guangxi Medical University*, vol. 30, pp. 485–487, 2013.

[10] X. Y. Su and Y. Y. Chen, "Treatment of functional dyspepsia with Chaihu Shugan powder: a meta-analysis," *Chinese Journal of Integrated Traditional and Western Medicine*, vol. 26, pp. 384–386, 2016.

[11] W. Q. Chen and H. G. Yang, "Efficacy of modified Chaihu Shugan powder on 61 cases with irritable bowel syndrome," *New Journal of Traditional Chinese Medicine*, vol. 35, article no 37, 2003.

[12] C. A. Qiao, "Clinical study of modified Chaihu Shugan powder on functional dyspepsia," *Journal of Sichuan Traditional Chinese Medicine*, vol. 28, pp. 85-86, 2010.

[13] Q. Z. Cui and X. Y. Wang, "Treatment of 54 cases of constipation with Chaihu Shugan powder," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 14, pp. 25–38, 2008.

[14] N. Yang, X. Jiang, X. Qiu, Z. Hu, L. Wang, and M. Song, "Modified Chaihu Shugan powder for functional dyspepsia: meta-analysis for randomized controlled trial," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 791724, 10 pages, 2013.

[15] R. Fan, X. Huang, Y. Wang et al., "Ethnopharmacokinetic-and activity-guided isolation of a new antidepressive compound from Fructus Aurantii found in the traditional chinese medicine Chaihu-Shugan-San: a new approach and its application," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 607584, 2012.

[16] Y.-J. Zhang, X. Huang, Y. Wang et al., "Ferulic acid-induced anti-depression and prokinetics similar to Chaihu-Shugan-San via polypharmacology," *Brain Research Bulletin*, vol. 86, no. 3-4, pp. 222–228, 2011.

[17] L. J. Zeng, J. H. Ling, J. Deng, Y. J. Wang, Z. Zhang, and Z. J. Lei, "Effect of Chaihu Shugan powder on autophagy of interstitial cells of interstitial Cajal in gastric antrum of rats with functional dyspepsia," *Lishizhen Medicine and Materia Medica Research*, vol. 28, pp. 1041–1044, 2017.

[18] Z. Q. Liu, D. k. Yin, L. Han et al., "Protective effect of medicated serum prepared with Taohong Siwu Tang on hydrogen peroxide-induced human umbilical vein endothelial cells," *Zhongguo Zhong Yao Za Zhi*, vol. 38, pp. 402–406, 2013.

[19] X. Guo, M. L. Li, X. L. Liu, B. Jia, H. X. Shi, and T. Shen, "Effect of aconite containing serum on L-type calcium channel in rat ventricular myocytes," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 11, pp. 111–115, 2015.

[20] S. Xu, Y. Dong, K. Yang, XY. Gong, and N. Li, "Effect of Sanzi Yangqin Tang on CysLTs mediated inflammatory pathways in bronchial epithelial cells," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 11, pp. 137–141, 2016.

[21] W. Bochu, Z. Liancai, and C. Qi, "Primary study on the application of Serum Pharmacology in Chinese traditional medicine," *Colloids and Surfaces B: Biointerfaces*, vol. 43, no. 3-4, pp. 194–197, 2005.

[22] S. Y. Xu, R. L. Bian, and X. Chen, *Experimental Methodology of Pharmacology*, People's Medical Publishing House, China, 3rd edition, 2002.

[23] R. Q. Tan, Z. Zhang, H. E. Ning, L. M. Zhang, Y. Wang, and J. H. Ling, "Establishment of autophagy model of rat gastric interstitial cells of Cajal induced by glutamic acid," *Chinese Journal of Pathophysiology*, vol. 34, pp. 1532–1536, 2018.

[24] S. M. Ward, A. J. Burns, S. Torihashi, and K. M. Sanders, "Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine," *The Journal of Physiology*, vol. 480, no. 1, pp. 91–97, 1994.

[25] K. M. Sanders, "A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract," *Gastroenterology*, vol. 111, no. 2, pp. 492–515, 1996.

[26] Y.-H. Li, C.-H. Zhang, S.-E. Wang, J. Qiu, S.-Y. Hu, and G.-L. Xiao, "Effects of Chaihu Shugan San on behavior and plasma levels of corticotropin releasing hormone and adrenocorticotropic hormone of rats with chronic mild unpredicted stress depression," *Journal of Chinese Integrative Medicine*, vol. 7, no. 11, pp. 1073–1077, 2009.

[27] V. Tuloup-Minguez, A. Hamaï, A. Greffard, V. Nicolas, P. Codogno, and J. Botti, "Autophagy modulates cell migration and β1 integrin membrane recycling," *Cell Cycle*, vol. 12, no. 20, pp. 3317–3328, 2013.

[28] Y. Kabeya, N. Mizushima, T. Ueno et al., "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing," *EMBO Journal*, vol. 19, no. 21, pp. 5720–5728, 2000.

[29] S. J. Cherra III, S. M. Kulich, G. Uechi et al., "Regulation of the autophagy protein LC3 by phosphorylation," *The Journal of Cell Biology*, vol. 190, no. 4, pp. 533–539, 2010.

[30] G. Juhász, J. H. Hill, Y. Yan et al., "The class III PI (3) K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila," *The Journal of Cell Biology*, vol. 181, no. 4, pp. 655–666, 2008.

[31] C. He and B. Levine, "The Beclin 1 interactome," *Current Opinion in Cell Biology*, vol. 22, no. 2, pp. 140–149, 2010.

[32] J. Y. Shin, S.-H. Hong, B. Kang, A. Minai-Tehrani, and M.-H. Cho, "Overexpression of beclin1 induced autophagy and apoptosis in lungs of K-rasLA1 mice," *Lung Cancer*, vol. 81, no. 3, pp. 362–370, 2013.

[33] M. C. Maiuri, A. Criollo, and G. Kroemer, "Crosstalk between apoptosis and autophagy within the Beclin 1 interactome," *EMBO Journal*, vol. 29, no. 3, pp. 515-516, 2010.

[34] F. Scarlatti, R. Maffei, I. Beau, P. Codogno, and R. Ghidoni, "Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells," *Cell Death & Differentiation*, vol. 15, no. 8, pp. 1318–1329, 2008.

[35] F. Strappazzon, M. Vietri-Rudan, S. Campello et al., "Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy," *EMBO Journal*, vol. 30, no. 7, pp. 1195–1208, 2011.

[36] Z.-J. Sun, L. Zhang, B. Hall, Y. Bian, J. S. Gutkind, and A. B. Kulkarni, "Chemopreventive and chemotherapeutic actions of mTOR inhibitor in genetically defined head and neck squamous cell carcinoma mouse model," *Clinical Cancer Research*, vol. 18, no. 19, pp. 5304–5311, 2012.

[37] X. H. Liang, S. Jackson, M. Seaman et al., "Induction of autophagy and inhibition of tumorigenesis by beclin 1," *Nature*, vol. 402, no. 6762, pp. 672–676, 1999.

[38] L. Van Oudenhove and Q. Aziz, "The role of psychosocial factors and psychiatric disorders in functional dyspepsia," *Nature Reviews Gastroenterology & Hepatology*, vol. 10, no. 3, pp. 158–167, 2013.

[39] Z. Y. Chen, M. G. Chen, T. T. Lu, G. Z. Ni, X. F. Liu, and G. L. Xu, "Autophagy is activated during glutamicacid-induced cortical neuron injury," *Chinese Journal of Pathophysiology*, vol. 27, pp. 62–66, 2011.

[40] D. J. Klionsky, P. Codogno, A. M. Cuervo et al., "A comprehensive glossary of autophagy-related molecules and processes," *Autophagy*, vol. 6, no. 4, pp. 438–448, 2010.

[41] Z. Chen, T. Lu, X. Yue et al., "Neuroprotective effect of ginsenoside Rb1 on glutamate-induced neurotoxicity: With emphasis on autophagy," *Neuroscience Letters*, vol. 482, no. 3, pp. 264–268, 2010.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 3940397, 15 pages
https://doi.org/10.1155/2019/3940397

Research Article

Ethnobotanical Study of Cultivated Plants in Kaišiadorys District, Lithuania: Possible Trends for New Herbal Based Medicines

Zivile Pranskuniene[1,2] **Kristina Ratkeviciute**,[1] **Zenona Simaitiene**,[3] **Andrius Pranskunas**[4] **and Jurga Bernatoniene**[1,2]

[1]*Department of Drug Technology and Social Pharmacy, Lithuanian University of Health Sciences, Kaunas, Lithuania*
[2]*Institute of Pharmaceutical Technologies, Lithuanian University of Health Sciences, Kaunas, Lithuania*
[3]*The Museum of History of Lithuanian Medicine and Pharmacy, Lithuania*
[4]*Department of Intensive Care Medicine, Lithuanian University of Health Sciences, Kaunas, Lithuania*

Correspondence should be addressed to Zivile Pranskuniene; zivile.pranskuniene@lsmuni.lt

Received 6 February 2019; Revised 18 March 2019; Accepted 23 April 2019; Published 26 May 2019

Guest Editor: Caio P. Fernandes

Background. Despite the growing body of ethnobotanical studies in Europe, publications are scarce in Lithuania. Ethnobotanical study in Kaišiadorys district is one of the few from this field done in Lithuania. Lithuania is divided into five ethnographic regions, and Kaišiadorys district is an area which borders with the three of them (Aukštaitija, Dzūkija, and Suvalkija), which determines the ethnographic distinctiveness of the area. The aim of this study was to determine the occurrence of cultivated medicinal plants, their families and pharmaceutical forms used in Kaišiadorys district, Lithuania, and to assess the conformity of medicinal plant materials used by respondents with the recommendations for medicinal plant materials in the World Health Organization monographs. *Methods*. The field work was conducted in periods of time from July 2016 to October 2017. During this ethnobotanical research, 30 people were interviewed, 25 of whom agreed to communicate. The average age of respondents was 65 years. Information was collected using semistructured and structured interviews. The obtained information was recorded indicating ethnic names of plants, their preparation techniques, parts used, modes of administration, and application for therapeutic purposes. *Results*. Respondents mentioned 71 species of cultivated medicinal plants from 38 families, used for therapeutic purposes and indicated which parts of the plant they use, how they prepare them, indications for use, and ways of administration. The most commonly cited families were Asteraceae (20.5%), Lamiaceae (13.9%), and Apiaceae (12.8%); most popular plants, cited more than 20 times, were *Tilia cordata* L., *Matricaria recutita* L., *Calendula officinalis* L., *Carum carvi* L., and *Artemisia absinthium* L. The frequently used plant parts were flowers (mentioned 35.8%), leaves (16.3%), roots and tubers (16.1%), herb (14.8%), and seeds (7.0%). Diseases most frequently treated were digestive (21.5% of citations), respiratory (21.3 % of citations), mental and behavioral (11.0%) disorders, certain infections and parasitic diseases (10.1%), and diseases of genitourinary system (9.1%). *Conclusions*. Only 19 of the cultivated medicinal plant species mentioned by interviewed persons are described in the World Health Organization monographs. This means that the remaining 52 species are used without the World Health Organization approved medical indications, based solely on the folk medicine knowledge and experience. This study showed that the folk use of plants is strongly rooted in daily practice in the studied area.

1. Introduction

Next to the modern medicine with new medicines being developed and improved, there remains an interest in traditional ways of folk medicine. People are a part of nature, so naturally, from ancient times, natural resources have been used to keep them healthy or to treat illness. Lithuanian folk medicine, which encompasses various medical knowledge, beliefs, and treatments, existing in the traditional rural community, has deep traditions that are preserved as the nation's cultural heritage [1]. Scientists emphasize the need to integrate folk medicine, or traditional medicine,

into the public health system [2]. Research of the knowledge on local medicinal plants and raw materials creates new hypotheses about their impact on human body [3]. The combination of analysis of traditionally used medicinal substances and modern clinical trials may play an important role in the development of new herbal medicinal product formulations based on ethnic heritage [4]. If in the eighties and nineties pharmaceutical companies focused on the synthesis of new medicines, nowadays, the natural medicinal products are being more and more sought for, while using advanced technologies. A new concept for pharmaceutical development was defined in the 1960s: the development of new drugs from natural raw materials is a future perspective; vegetable and animal raw materials could serve as a cheaper "intermediate" product for semisynthetic drugs. Production using plentiful historically approved raw materials would be much more cost-effective for new pharmaceuticals than the full-scale drug synthesis [5].

Medicinal plants are important in social, economic, and cultural terms; however, the traditional knowledge of plant-based materials, especially of less known plants, is constantly diminishing [6]. How often herbal preparations are used depends on their availability and traditions. Scientists are considering how to integrate traditional medical knowledge into the public health system as a complementary treatment tool, effects of which are based on clinical trials [2]. Although modern studies of herbal medicinal preparations are often launched from herbal preparations used in traditional medicine, the new unknown indications for them are still getting discovered [7]. According to the WHO, about 80% of the population in developed countries use products for health care derived from medicinal plants and 30% of all medicines sold in the world contain plant components [8]. In Europe, over 1,300 local plants are recognized in the medicine [9].

In Lithuania, the history of using plant-based materials is rich and can be analyzed geographically, culturally, and linguistically. Medicinal plants have been collected or grown for a long time. Also various spices, imported vegetable raw materials, and knowledge of their use have become available through trade routes [10]. Scientists believe that traditional Lithuanian medicine uses 462 spontaneous, adventive, or introduced higher plant species, five fungi, two lichens, one moss, and one alga species [11]. Since old times in Lithuania, plants grown in flower beds had not only decorative purposes. Most types of ornamental plants traditionally grown in Lithuania were used for treatment. Farmstead landscaping and the appearing of flower beds have been influenced by the Valakas land reform performed in the Grand Duchy of Lithuania and by the establishment of monasteries. Fruit and vegetable gardens and flower beds were built at the monasteries, flowers, vegetables, and shrubs and fruit trees were grown there. For example, the pharmacy of Pažaislis Monastery (founded in 1662) was like a laboratory where monks worked with medicinal plants, studied medical literature, manuals and pharmacopoeia, and created recipes. The monasteries' recipes abounded in both local and imported medicinal raw materials [12]. Until the beginning of the 20th century, imported raw materials used to reach Europe through London, Amsterdam, or Hamburg from Java, e.g., *Cort. Chinae*, South America, e.g., *Lign. Guajaci, Rad. Rhei, Flor. Chamomillae, Aloe,* and *Sanguis Draconis* [10]. Jurgis Ambraziejus Pabrėža the vicar of the Kretinga Monastery, the first botanist of the 19th century Western Lithuania, and the author of the first Lithuanian botanical terminology work (J. Pabrėža manuscript "Taislius augyminis" (Remedies of Plant Origin)) was included in the UNESCO World Memory Program in 2008. In the Kretinga monastery he cultivated about 400 plant species in plots. J. A. Pabrėža classified plants by their purpose: (1) those used for decorations, (2) those used in medicines, (3) those used by farmers and craftsmen, and (4) dye plants [13]. Modern ethnopharmaceutical research in Europe is growing rapidly, especially in Southern European countries, Spain, and Italy. Now it focuses on "unnoticed" Eastern European countries, Poland, Romania, Bulgaria, and Lithuania [14]. Despite the increasing number of ethnopharmaceutical studies in Europe, the number of publications from Lithuania in scientific journals is minimal [15–17]. Several previous studies have shown that it is important to collect and systematize this information as quickly as possible, to preserve it as a part of traditional Lithuanian heritage and to successfully use it in further research.

Ethnobotanical study in Kaišiadorys region is one of the few from this field done in Lithuania. Lithuania is divided into five ethnographic regions, among them Kaišiadorys district borders with Aukštaitija, Dzūkija, and Suvalkija. This fact determines the ethnographic distinctiveness of the region. Scientists in Lithuania seek to create unique herbal medicinal products from traditionally used plants, the use of which has been approved historically, taking into account the needs of today's consumer.

Tasks of the work are to determine the distribution of cultivated medicinal plants in Kaišiadorys district, Lithuania, their families and pharmaceutical forms, to perform the analysis of indications for the use of medicinal plants and to identify the types of cultivated medicinal plants that had been used / still are used for treatment in Lithuanian traditional medicine and are possibly suitable for the formulation of herbal medicinal preparations based on ethnic heritage, and to assess the conformity of plant materials used by the respondents with recommendations in the World Health Organization monographs.

2. Material and Methods

2.1. Study Area. The survey was carried out in Kaišiadorys district, located in the middle part of Lithuania, Kaunas region, in Kaunas Lagoon, the rivers Nemunas and Neris. This area is interesting because it has intertwined dialects, cultural, architectural, and art traditions, and customs of three Lithuanian ethnographic regions. Theoretically, Kaišiadorys district belongs to Aukštaitija ethnographic region, but it also borders with Dzūkija and Suvalkija [18]. The different traits of the ethnographic regions and their traditions determine the peculiarities of traditional folk medicine in these different areas.

Figure 1: Study area.

Kaišiadorys is a town located 67 km west of Vilnius and 37 km east of Kaunas [19]. The name of the town was first mentioned in 1590 under name of Košeidarova being mentioned. In 1902, Kaišiadorys settlement became a town. The growth of the town was determined by the large flows of people traveling by railway, passed through Kaišiadorys and transferred to another railway line, which led to the establishment of the main businesses: shops, hotels, and inns, the majority of the population being Jews [19]. In earlier times the majority of the residents of Kaišiadorys and the surrounding towns were Jews, and although their traditional folk medicine would also be a valuable subject for research, Jewish respondents were not recorded in this study.

Kaišiadorys became the district center in 1950 [19]. The area of the district is 1087 kmÂš, which makes 1.7% of the total area of Lithuania. According to the data of 2015, 31 624 inhabitants lived in the district.

There are two towns in the district, Kaišiadorys and Žiežmariai, three settlements, Kruonis, Rumšiškės, and Žasliai, and 384 villages [20].

Respondents living in Kaišiadorys and Žiežmariai and in the 12 villages of the district were interviewed during the research. Figure 1 shows the territory of Kaišiadorys district with the marked areas of survey.

63% of the Kaišiadorys district population lives in villages [20]. The majority of the survey respondents also were rural residents: 28% of the respondents lived in the urban area but came from rural areas, while the remaining 72% lived in villages.

The diversity and occurrence of plants in both the country and the region are determined by geographical location and climatic conditions. The territory of Lithuania is in the mid-latitude climate zone; only the Baltic coastal climate area is closer to the Western European climate [21]. The standard climate normal in Lithuania (1981-2010) shows 18°C being the highest and -3°C being the lowest monthly average temperature [22]. The maximum average monthly precipitation based on the standard climate normal (1981-2010) is about 75-80 mm and the lowest is about 35-40 mm [23].

In the north-western part of Kaišiadorys district territory there is a lowland of the lower reaches of Neris, in the south-east of the Dzūkai heights, characterized by ash forests with hornbeams, and the Aukštadvaris forest. The average temperature of January is −5.1°C and July + 17.5°C. In Kaišiadorys district, 631 mm of precipitation falls annually. There are 39 lakes and eight ponds in the district. The forest area of Kaišiadorys district is 31.1%. 63% of all trees are coniferous: 38% pine and 25% spruce. In mixed forests, usually grow broad-leaved trees and spruce trees. The largest swamp is Palaraistis. The territory of Kaišiadorys district includes parts of Kaunas Lagoon and Aukštadvaris regional parks and a few reserves: Lapainis botanical, Būda and Kaukinė botanical-zoological, Lomena, Budeliai, and Strošiūnai landscape, and Gabriliava pedological reserve [20].

2.2. Methods. The purpose of the study was explained to each interviewed person, prior informed consent was obtained from all interviewees, and all conversations were recorded and encoded following the Code of Ethics of the International Society of Ethnobiology [24]. The research was approved by the Bioethics Center of Lithuanian University of Health Sciences (No. BEC-FF-18). Approval to carry out this type of research was obtained from the authorities of local community. Field work was conducted in periods from July 2016 to October 2017. During this ethnobotanical research, 30 people were interviewed, 25 of whom agreed to communicate, and 24 of them were females and 1 male. As we have mentioned in another study done in Lithuania, the main reason for higher number of women was the fact that traditional knowledge of herbal medicine in Lithuania was passed down through the female line [17]. The majority of respondents in the study (64%) were of older generation, 24% of whom were aging adults (60-74 years) and 40% were elderly (75-90 years). Middle-aged (30-59 years) people made up 36% of the respondents. Many of our respondents lived in lonely farmsteads with the nearest farmstead or settlement a few kilometers away. Information was collected using semistructured and structured interviews. Respondent search used guides and random selection and snow ball techniques. In order to reduce the number of random respondents and

to form a target group more efficiently, in the search of respondents, guides who knew the respondents and their possible experience in traditional folk medicine and in herbal medicine were used. The guides of our study were two women: a resident of Paparčiai village, the chairman of the community and the employee of the culture center, and a long-time head of Ringailiai folklore team, who also worked in the culture center. Their work specifics provided them with an opportunity to get to know the people of the country and to make contacts with them. A field study conducted with a guide is rewarded by the favor and openness of the respondents and is likely to capture more ethnopharmaceutical information during a structured interview. At the end of each interview, the respondents were asked whether they knew more people who were engaged in herbal medicine or were interested in traditional folk medicine; thus the target group was increased applying the snowball method, where one respondent mentions another potential respondent.

The first meeting with the interviewed persons occurred at their homes or gardens. During interviews, the investigator used a complete questionnaire and wrote down himself the answers to the questions. The obtained information was recorded indicating ethnic names of plants, their preparation techniques, parts used, modes of administration, and application for therapeutic purposes. Much attention was paid to the sources of ethnobotanical knowledge obtained by the respondents.

In order to get more information which the respondents naturally missed during the first meeting, some respondents were met more than once. Parts of plants were identified using writings on traditional Lithuanian flora [25–27]. Also, when possible, the respondents were asked to supply a fresh sample of each plant mentioned for the taxonomic determination and name it in a local dialect. Taxonomic identification, botanical nomenclature, and family assignments followed the Plant List database [28] and the Angiosperm Phylogeny Group IV [29]. The frequency of reports indicates the number of respondents who mentioned the use of each species (Table 1).

3. Results and Discussion

3.1. Demographic Data of the Respondents and Sources of Knowledge. In the ethnobotanical survey in Kaišiadorys district, 25 respondents, 90% of whom lived in villages, were interviewed in the structured interviews. The authenticity of ethnopharmaceutical knowledge of Kaišiadorys district could have been influenced by the previous place of residence of the respondents, as it might be based on the knowledge from there, but majority of the respondents lived in Kaišiadorys district, and only 16% of them lived previously in another area. Taking into account the fact that the older rural inhabitants tend to preserve old traditions of their country and community, it can be expected to have obtained pure ethnobotanical knowledge of Kaišiadorys district. The average age of respondents was 65 years.

The higher number of older generation respondents allowed expecting obtaining genuine local ethnobotanical knowledge passed down the generations, rather than being based on the information presented by modern media. The education, profession, and sources of folk medicinal knowledge may also influence the authenticity of ethnopharmaceutical knowledge. All older respondents (40%) had completed grades 3, 4, or 7, which is an incomplete secondary education, while the remaining elderly and middle-aged respondents had completed secondary education (28%), upper secondary education (16%), or higher education (4%).

Respondents, who had not completed their secondary education, when talking about the profession, mentioned working in collective farm, singing in church, community-specific rituals, and making crafts; those with higher education mentioned professions such as folklore team leader, ceramicist, ethnocultural teacher, nurse assistant, and nurse.

Most of the respondents did not have higher education and had not even acquired secondary education, and the mentioned professions, such as collective farm work, head of folklore team, making crafts, or ethnocultura teacher, suggested that respondents were closely related to folk traditions; therefore, the ethnobotanical knowledge obtained during the research is based on the knowledge of traditional folk medicine of Kaišiadorys district, passed and saved from generation to generation. Respondents provided sources of ethnobotanical information, from which they gathered knowledge about the use of medicinal plants for the treatment of various disorders. The sources of ethnopharmaceutical knowledge of the respondents are presented in Figure 2.

Most respondents learned to treat with herbs from their parents, grandparents (92.0% of citations), and from their neighbors or acquaintances (68.0% of citations), which indicates the existence of an oral tradition when information is passed by word of mouth. However, not any less important source of ethnobotanical knowledge were books and newspapers (76.0% of citations), readily available nowadays and useful for both remembering forgotten information and supplementing existing knowledge. A smaller part of the material collected in the study was based on the knowledge from the mass media (40.0% of citations), which was less popular among the older generation. Respondents also relied on information provided by a family doctor or pharmacist (28.0% of citations) and other sources (4.0% of citations) identified as knowledge derived from sorcerers.

In order to preserve the knowledge of traditional folk medicine, it is important to write it down or pass it on to others. 64% of respondents said that they passed on their knowledge to other people, who were usually family members or close acquaintances, neighbours, or friends. Most often older respondents mentioned that they told their grandchildren about medicinal plants or shared the knowledge with their peers and neighbours.

3.2. Medicinal Plants Cultivated for Medicinal and Other Purposes. The primary function of home gardens generally is food production but it also serves other purposes, such as medicinal and ornamental ones. Plant cultivation at home has many advantages: for better harvests plant species are selected, land prepared and proper conditions for them are

Table 1: Cultivated herbal materials in Kaišiadorys district.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
Acoraceae	*Acorus calamus* L.	Gastric disorders (1)	Roots	Raw material	O. Ad.	D
		Pain of gum (1)	Roots	Compress	Ext.	
			Roots	Decoction	Ext., rinsing	
Alliaceae	*Allium cepa* L.	Weak organism (8), helminth (2)	Corm	Raw material	O. Ad.	C F
		Wounds (1)	Corm	Compress	Ext.	
Alliaceae	*Allium sativum* L.	Hypertension (3)	Corm	Raw material	O. Ad.	C F
		Helminth (9)	Corm	Raw material	O. Ad.	
			Roots	Decoction with milk	O. Ad.	
		Weak organism (7)	Corm	Raw material	O. Ad.	
		Inflammation of the throat (2)	Corm	Raw material	O. Ad.	
		Inflammation of the mucous membrane of the nose (1)	Corm	Raw material	O. Ad.	
		Oncology diseases (1)	Corm	Raw material	O. Ad.	
		Toothache (1)	Corm	Chewing or compress	Ext.	
Aloaceae	*Aloe arborescens* Mill.	Gastric wounds (2)	Leaves	Chopped leaves are mixed with ethanol and honey	O. Ad.	D
		Lung diseases (2), cough (2)	Leaves	Chopped leaves are mixed with honey	O. Ad.	
		Weak organism (1)	Leaves	Chopped leaves are mixed with honey and lemon juice	O. Ad.	
		Sore throat (1)	Leaves	3 chopped leaves are mixed with 3 chopped lemons, 12 quail eggs, and honey	O. Ad.	
		Skin wounds, burns (2)	Leaves	Juice	Ext.	
		Herpes (1)	Leaves	Juice	Ext.	
Apiaceae	*Anethum graveolens* L.	Hypertension (16)	Seeds, flowers, leaves, and aerial parts	Tea	O. Ad.	C F
Apiaceae	*Carum carvi* L.	Abdominal bloating (15)	Seeds	Tea	O. Ad.	C F
		Indigestion (8)	Seeds	Tea	O. Ad.	
		Diarrhea (3)	Seeds	Tea	O. Ad.	

Table 1: Continued.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
		Headache caused by fatigue and stress (1)	Seeds, flowers	Tea	O. Ad.	
		Lactation disorder (1)	Seeds	Tea	O. Ad.	
Apiaceae	*Daucus carota* L.	Gastric hyperacidity (1)	Roots	Raw material	O. Ad.	F
		Diarrhea (1)	Roots	Porridge	O. Ad., eating of porridge all day	
		Helminth (2)	Roots	Raw material	O. Ad.	
		Weak vision (1)	Roots	Raw material	O. Ad.	
		Tonisation (1)	Roots	Tea	O. Ad.	
Apiaceae	*Petroselinum crispum* Mill.	Inflammation of the bladder (5)	Aerial parts	Tea	O. Ad.	C F
Asteraceae	*Artemisia absinthium* L.	Indigestion (9), appetite (8), biliary disorders (2), diarrhea (6), and abdominal, gastric pain (6)	Flowers	Tea	O. Ad.	D
		Helminth (2)	Flowers	Tea	O. Ad.	
		Detoxification of the organism (1)	Flowers	Tea	O. Ad.	
		Insomnia (1)	Flowers	Tea	O. Ad.	
Asteraceae	*Calendula officinalis* L.	Inflammation (of the mouth, throat, stomach, intestine, and urinary tract) (19), painful menstruations (8)	Flowers	Tea	O. Ad.	D
		Inflammation of the mouth, throat (4)		Decoction	Ext., rinsing	
		Menstruations (10), inflammation of the urinary tract, vagina (6)		Decoction	Ext., wash of genital organs, bath	
		Wounds (1)	Flowers	Decoction	Ext., wash of wounds	
		Skin scars (1)	Flowers	Ointment	Ext.	
Asteraceae	*Echinacea purpurea* L. Moench	Weak organism (2)	Flowers	Tea	O. Ad.	D
Asteraceae	*Helianthus annuus* L.	Outgrowths (1)	Roots	Decoction	O. Ad.	D F
Asteraceae	*Inula helenium* L.	Eczema (1), bone cracks (1)	Roots	Ointment: chopped roots are heated with fats	Ext.	D
Asteraceae	*Matricaria recutita* L.	Gastric and intestinal disorders (12)	Flowers	Tea	O. Ad.	D C
		Abdominal bloating of infant (1)	Flowers	Tea	O. Ad.	
		Cold (2), cough (3), and runny nose (3)	Flowers	Tea	O. Ad.	
		Inflammation of the throat (3), mouth (3)	Flowers	Decoction	Ext., rinsing	
		Inflammation of the eyes (4), wounds (6)	Flowers, aerial parts	Decoction	Ext., wash	

Table 1: Continued.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
		Inflammation of the skin (1)	Flowers, aerial parts	Decoction	Ext., compress	
		Calming effect on children (1)	Flowers, aerial parts	Decoction	Ext., bath	
Asteraceae	*Silybum marianum* L. Gaertn.	Hepatic disorder (1)	Flowers	Tea	O. Ad.	D
		Detoxification of the organism (1)	Leaves	Tea	O. Ad.	
Asteraceae	*Tanacetum balsamita* L.	Being used for oral hygiene (1)	Aerial parts	Chewing	Ext.	D
Berberidaceae	*Berberis vulgaris* L.	Hepatic inflammation (1)	Roots	Decoction	O. Ad.	D
Betulaceae	*Betula pendula* Roth.	Blood thrombus (5)	Buds	Tea	O. Ad.	D
		Cough (1)	Buds	Tea	O. Ad.	
		Weak organism (1)	Buds	Tea	O. Ad.	
Boraginaceae	*Symphytum officinale* L.	Bone cracks (4), contusion (3), joint (2), muscle (1) pain, and sprain (1)	Roots	Ethanol infusion, chopped roots are mixed with butter or unsalted fats, compress	Ext., compress, rubbing	D
		Gastric ulcer (1)	Roots	Ethanol infusion	O. Ad.	
Brassicaceae	*Armoracia rusticana* G. Gaertn., B. Mey. & Scherb.	Headache (1)	Leaves	Compress	Ext.	F C
Brassicaceae	*Brassica oleracea* L. var. *capitata*	Colon cancer (1)	Leaves	Raw material	O. Ad.	F
		Blood clotting disorders (1)	Leaves	Raw material	O. Ad.	
		Constipation (1)	Leaves	Pickled	O. Ad.	
		Swelling (4), inflammation (4), and headache (4)	Leaves	Compress	Ext.	
Cannabaceae	*Humulus lupulus* L.	Anxiety (4)	Buds	Tea	O. Ad.	D
			Buds	Pillow	Ext.	
Chenopodiaceae	*Beta vulgaris* L. subsp. *vulgaris*	Anemia (3), lack of iron (3), increased blood clotting (3)	Roots	Raw material, juices	O. Ad.	F
		Weak organism (1)	Roots	Raw material, juices	O. Ad.	
Commelinaceae	*Callisia fragrans* (Lindl.) Woodson	Joint pain (1)	Leaves: it should be 9 parts of stem; then leaves are appropriate for use	40-degree ethanol infusion for compress	Ext.	
		Weak organism (1)	Leaves	Ethanol extract	O. Ad.	
Convallariaceae	*Convallaria majalis* L.	Contusion, swelling (1)	Aerial parts	Compress of water extract	Ext.	D

Table 1: Continued.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
Cupressaceae	*Juniperus communis* L.	Outgrowths (1)	Roots	Ethanol infusion	O. Ad.	D C F
		Blood detoxification (2)	Roots	Water infusion	O. Ad.	
Elaeagnaceae	*Hippophae rhamnoides* L.	Weak organism (1)	Fruits	Juice	O. Ad.	F D
		Gastric ulcer (1)	Fruits	Oil	O. Ad.	
Fabaceae	*Phaseolus vulgaris* L.	Hypertension (1)	Seeds	Eating of boiled or roasted seeds	O. Ad.	F D
Fabaceae	*Trifolium pratense* L.	Male potency disorders (2)	Flowers	Tea	O. Ad.	D
		Headache during menstruation, menopause (3)	Flowers	Tea	O. Ad.	
Fabaceae	*Trifolium repens* L.	Cold (2)	Flowers	Tea	O. Ad.	D
Fagaceae	*Quercus robur* L.	Diarrhea (4)	Bark	Decoction	O. Ad.	D F
Geraniaceae	*Pelargonium odoratissimum* (L.) L'Her.	Cold (2), inflammation (2)	Flowers, leaves	Tea	O. Ad.	D
		Being used for air disinfection (1)	Aerial parts	Fumigation	Ext.	
Grossulariaceae	*Ribes nigrum* L.	Cold (7), fever (3), and cough (1)	Leaves, fruits, and stem	Tea	O. Ad.	F D
		Weak organism (3)	Leaves, fruits, and stem	Tea, eating of fruits	O. Ad.	
		Blood clotting disorders (1)	Leaves, fruits, and stem	Tea	O. Ad.	
		Insomnia, anxiety (2)	Leaves	Tea	O. Ad.	
		Being used for regulation of glucose for diabetics (1)	Leaves, fruits, and stem	Tea	O. Ad.	
Grossulariaceae	*Ribes uva-crispa* L.	Renal disorders (1)	Flowers	Tea	O. Ad.	D F
Hippocastanaceae	*Aesculus hippocastanum* L.	Vein disease (1)	Flowers	Ethanol infusion	Ext., rubbing	D
Lamiaceae	*Agastache foeniculum* (Pursh) Kuntze	Depression (4)	Leaves, flowers	Tea	O. Ad.	D F C
			Leaves, flowers	Pillow	Ext.	
		Cold (1)	Leaves, flowers	Tea	O. Ad.	
		Hypertension (1)	Leaves, flowers	Tea	O. Ad.	
Lamiaceae	*Elsholtzia ciliata* (Thunb.) Hyl.	Appetite disorders (1)	Flowers	Tea	O. Ad.	D C
		Diuresis disorders (1)	Flowers	Tea	O. Ad.	

Table 1: Continued.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
Lamiaceae	*Melissa officinalis* L.	Anxiety, insomnia (14)	Aerial parts, leaves	Tea	O. Ad.	D C
		Diuresis and release of intestinal gas disorders (1)	Aerial parts	Tea	O. Ad.	
		Appetite disorders (1)	Aerial parts	Tea	O. Ad.	
Lamiaceae	*Mentha x piperita* L.	Anxiety (14), insomnia (14)	Leaves, aerial parts	Tea	O. Ad.	D C F
		Indigestion (6), diarrhea (1), and meteorism (1)	Leaves, aerial parts	Tea	O. Ad.	
		Acne (1)	Aerial parts	Compress of strong tea	Ext.	
Lamiaceae	*Nepeta cataria* L.	Headache (1)	Aerial parts	Tea	O. Ad.	D
Lamiaceae	*Origanum vulgare* L.	Appetite disorders (1), indigestion (3)	Aerial parts	Tea	O. Ad.	D C F
Lamiaceae	*Salvia officinalis* L.	Sore throat (4)	Leaves	Decoction	Ext., rinsing of the throat	D
			Leaves	Tea	O. Ad.	
		Being used for strengthening of voice strings (1)	Leaves	Tea	O. Ad.	
		Mental illness (1), tremor of hands (1), fright (1), and stress (1)	Leaves	Tea	O. Ad.	
Linaceae	*Linum usitatissimum* L.	Gastric, esophageal inflammation (1)	Seeds	Decoction	O. Ad.	F D
Malvaceae	*Althaea officinalis* L.	Cough (1)	Roots	Tea	O. Ad.	D
Malvaceae	*Malva neglecta* Wallr.	Gastric hyperacidity (1)	Flowers	Raw material	O. Ad.	D
Oleaceae	*Syringa vulgaris* L.	Wounds (1)	Leaves	Compress	Ext.	D
		Nervousness (1)	White flowers	Tea	O. Ad.	
Onagraceae	*Chamaenerion angustifolium* L.	Gastric wounds (1), intestinal diseases (1)	Leaves	Infusion	O. Ad.	D
Paeoniaceae	*Paeonia sp.* L.	Fright (4)	Flower: pink flowers for girls and white flowers for boys (2)	Tea	O. Ad.	D
Pinaceae	*Pinus sylvestris* L.	Tuberculosis, (1), lung inflammation	Bud	Buds boiled with milk	O. Ad.	D
		Respiratory tract diseases (5), bronchitis, (5), and cough (5)	Cone	Ethanol infusion	O. Ad.	

Table 1: Continued.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
			Bud	Ethanol extract, ethanol infusion, tea, mixed with honey, mixed with sugar	O. Ad.	
			Bud	Bath, inhalation	Ext.	
		Gastric wounds (1)	Bud	Tea	O. Ad.	
Poaceae	*Avena sativa* L.	Lungs inflammation (1)	Seeds	Compress	Ext.	F
Poaceae	*Secale cereale* L.	Erysipelas (1)	Seeds	Powders	Ext.	
Primulaceae	*Primula veris* L.	Inflammation of the bladder (1)	Aerial parts	Tea	O. Ad.	D
		Cold (2)	Aerial parts	Tea	O. Ad.	
Rosaceae	*Agrimonia eupatoria* L.	Sore throat (1)	Aerial parts	Infusion	Ext., rinsing of the throat	
Rosaceae	*Aronia melanocarpa* (Michx.) Elliott.	Blood clotting disorders (1), lack of iron (1)	Fruits	Raw material	O. Ad.	D F
			Fruits	Tea	O. Ad.	
Rosaceae	*Crataegus monogyna* Jacq.	Cardiac disorders (4)	Fruits	Raw material	O. Ad.	D
			Flowers, bark	Ethanol infusion	O. Ad., used at the morning before eating	
Rosaceae	*Fragaria* sp. L.	Decreased hemoglobin in the blood (1)	Fruits	Raw material	O. Ad.	D F
Rosaceae	*Padus avium* Mill.	Erysipelas (2)	Flowers	Tea	O. Ad.	D
			Flowers	Bath, wash	Ext.	
Rosaceae	*Prunus cerasus* L.	Fever (1)	Stem	Tea	O. Ad.	D F
Rosaceae	*Rosa canina* L.	Weak organism (1)	Fruits	Tea	O. Ad.	D F
Rosaceae	*Rubus rosifolius* Sm.	Being used as vitamin source for diabetics (1)	Fruits	Raw material	O. Ad.	D F
Rosaceae	*Sorbus aucuparia* L.	Wounds (1)	Bark	Decoction	Ext., wash	D
		Being used as vitamin source (5)	Fruits	Raw material	O. Ad.	F
		Constipation (3)	Fruits, leaves	Tea, eating of fruits	O. Ad.	
		Cough (2)	Fruits	Tea, eating with honey	O. Ad.	
Rutaceae	*Ruta graveolens* L.	Being used to induce abortion (1)	Leaves, flowers	Tea	O. Ad.	D
		Being used for strengthening of voice strings (1)	Leaves	Chewing	O. Ad.	

Table 1: Continued.

Family	*Botanical name*	*Disorder* (frequency of citations)	*Part used*	Preparation	*Administration and usage* (O. Ad.: oral administration, Ext.: external use)	*Other purposes* (spice: C, decorative: D, and food: F)
Salicaceae	*Populus* sp. L.	Pain (1)	Bud	Ethanol decoction	Ext., compress, rubbing	D
		Disinfection of wounds (1)	Bud	Ethanol decoction	Ext., wash	
Scrophulariaceae	*Verbascum densiflorum* Bertol.	Cough (1), diseases of respiratory tract (1)	Aerial parts	Tea	O. Ad.	D
Scrophulariaceae	*Veronica officinalis* L.	Complicated wounds (1)	Leaves	Compress, compress with honey	Ext.	
		Toothache (1)	Aerial parts	Decoction	Ext., rinsing	
Solanaceae	*Solanum tuberosum* L.	Sinusitis (1), inflammation of the throat (1)	Roots	Compress of boiled roots	Ext.	F
		Cough (1)	Roots	Inhalation	O. Ad.	
		Gastric hyperacidity (4)	Roots	Raw material	O. Ad.	
Tiliaceae	*Tilia cordata* Mill.	Cold, cough, and fever (23)	Flowers	Tea	O. Ad.	D
		Gastric hyperacidity (1)	Bud	Chewing	O. Ad.	
		Diseases of the respiratory system (1)	Flowers	Inhalation	O. Ad.	
			Flowers	Bath	Ext.	
Tropaeolaceae	*Tropaeolum majus* L.	Inflammation of the bladder (2)	Flowers	Tea	O. Ad.	D
		Lungs inflammation (3), cold (2), and fever (2)	Flowers	Tea	O. Ad.	
		Gastric hyperacidity (1)	Flowers	Tea	O. Ad.	
Vibrionaceae	*Viburnum opulus* L.	Bronchitis (2), asthma (3), and cough (1)	Fruits	Eating with sugar, juice with honey	O. Ad.	
		Weak organism (1)	Fruits	Eating with honey	O. Ad.	
		Hypertension (1)	Fruits	Juice with honey	O. Ad.	

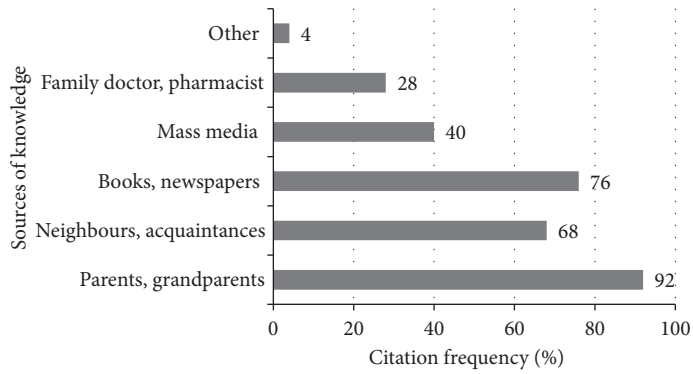


Figure 2: Respondents' sources of ethnobotanical knowledge.

chosen, and plants are always "at hand" [14]. The studies have distinguished the same plants used for various purposes: food (*Chamaenerion angustifolium* L., *Heracleum lanatum* Michx., etc.), medicines (*Heracleum lanatum*) or for household use (*Juniperus sibirica* Burgsd.) and for incense (*Leymus mollis* (Trin.) Hara), and *Urtica* sp. L. for fiber production, as well as data on local peoples' perception of the peculiarities and differences of plant cultivation [30]. The use of vegetable raw materials in households is also linked to their specific purposes [31].

Knowledge about cultivated plants and their use can be found in the material of the first Lithuanian ethnographic expeditions [32]. Ethnopharmaceutical research on ornamental plants in Lithuania has just started. There have been described 153 species of ornamental plants, including 17 trees, 13 shrubs, 123 herbaceous plants, and 13 species at home as indoor plants. 125 of the total number of studied plants were used for medical purposes (81.7%). The most abundant species belong to Asteraceae, Lamiaceae, Rosaceae, Solanaceae, and Fabaceae families [33]. Comparing therapeutic indications in Lithuanian folk medicine with the 19th century's and the modern medicine, most coincidence was found in the use of chamomile (*Matricaria recutita* L.), calendula (*Calendula officinalis* L.), absinth wormwood (*Artemisia absinthium* L.), horseheal (*Inula helenium* L.), and juniper. The most frequently used parts were aerial parts, leaves, and flowers, from which aqueous and ethanol extracts were commonly prepared [33].

Historically, in families women had more to do with plant species. In most societies cooking, healing with traditional medicines, dyeing cloth and yarns, gardening, and seed selection belong to female activities [34]. In Lithuania, the trend was the same [35]. It can be assumed that there were medicinal raw materials possible to find near the house in illness.

We registered 71 species of medicinal plants from 38 families used for therapeutic purposes. The results of the research are presented in Table 1. The most commonly cited families were Asteraceae (20.5%), Lamiaceae (13.9 %), and Apiaceae (12.8%). The most popular plants cited more than 20 times were *Tilia cordata* Mill., *Matricaria recutita, Calendula officinalis, Carum carvi* L., and *Artemisia absinthium*. The most commonly used plant for prophylaxis was *Tilia cordata* mentioned 25 times. Respondents pointed out that they usually use flowers to make tea for colds, fever, or cough, symptoms of flu, in order to promote sweating. One respondent also indicated that the flowers are used to prepare inhalations for respiratory diseases and buds to chew for increased gastric acidity. The second place is taken by chamomile (*Matricaria recutita*, cited 24 times). The herbal tea is prepared from its flowers for gastrointestinal disorders such as inflammation, pain, bloating, and diarrhea, less commonly for colds or coughs. One respondent emphasized that chamomile tea is given to breastfed babies to avoid abdominal colic. Respondents also pointed out that decoctions of both flowers and aerial parts can be used to wash eyes and infected wounds, applied as poultices in skin inflammation, to bath children, so that they are calm, and to rinse inflamed oral cavity or throat. Raspberry (*Rubus idaeus* L.) and calendula (*Calendula officinalis*) were mentioned 22 times. Raspberry stems and leaves are used to prepare tea for colds, fever, or cough. Respondents mentioned that they use marigold flowers for tea, decoction, or ointment. Tea is taken for mouth, throat, stomach, intestine, urinary tract, and uterus inflammation and in menstruation. The decoction is used for mouth or throat rinses, washes, or baths in menstruation and for treatment of vaginal and urinary tract inflammation. Marigold flowers ointment is used to treat skin scars. Ethnobotanical studies carried out in various European countries have found that terrestrial parts of medicinal plants are commonly used for treatment or prophylaxis [36–38]. The respondents mentioned that they do not use all parts of the plant together, but a different part in each case, knowing that those particular parts of the plant contain needed active ingredients and seeking to have the best effect in treating the respective ailments or diseases. Parts of plants the most used for treatment were flowers (mentioned 35.8%), leaves (16.3%), roots and tubers (16.1%), aerial parts (14.8%), and seeds (7.0%). The most popular modes of preparation were tea (47.5% of citations), raw material (23.4%), poultice (7.4%), and decoction (6.7%). According to the respondents' experience, tea is easily and quickly prepared by pouring hot water over raw materials and keeping for 5-10 minutes. The second way of use mentioned by respondents is a raw material or functional food. The popularity of this method of preparation may be due to the fact that it does not require much time or effort and is simple. It includes the use of fresh raw materials, for example, eating strawberries, wild strawberries, beetroot, or cabbage leaves, as well as raw material prepared as food. Throughout the world and Europe, the research has found that medicinal herbal raw materials were used for therapeutic purposes as well in the form of ready-to-eat vegetables, jams, or spices [39–41]. Talking about poultices, there were mentioned ways of their preparation such as putting fresh raw material directly on the affected parts of the body or using gauze soaked with infusions, extracts, or decoctions. Preparation of decoctions takes longer time, which is likely to have led to its less frequent use in the preparation of herbal raw materials. Other ways of preparing and using medicinal herbal raw materials include ointments, sprinkles, cushions, bath brooms, oil infusions, oil extraction, and growing plants indoors.

3.3. Indications for the Use and Comparison of Indications with the World Health Organization Monographs. Once the obtained information is structured in accordance with the system of International Classification of Diseases [42], it can be observed which diseases, disorders, or ailments are most commonly treated by respondents with collected herbal material. Data on the indications of use by respondents of medicinal herbal raw materials are presented in Figure 3. Medicinal plants were most frequently used for the treatment of digestive (21.5%) and respiratory (21.3%) diseases, mental and behavioral disorders (11.0%), certain infections and parasitic diseases (10.1%), and diseases of genitourinary system (9.1%).

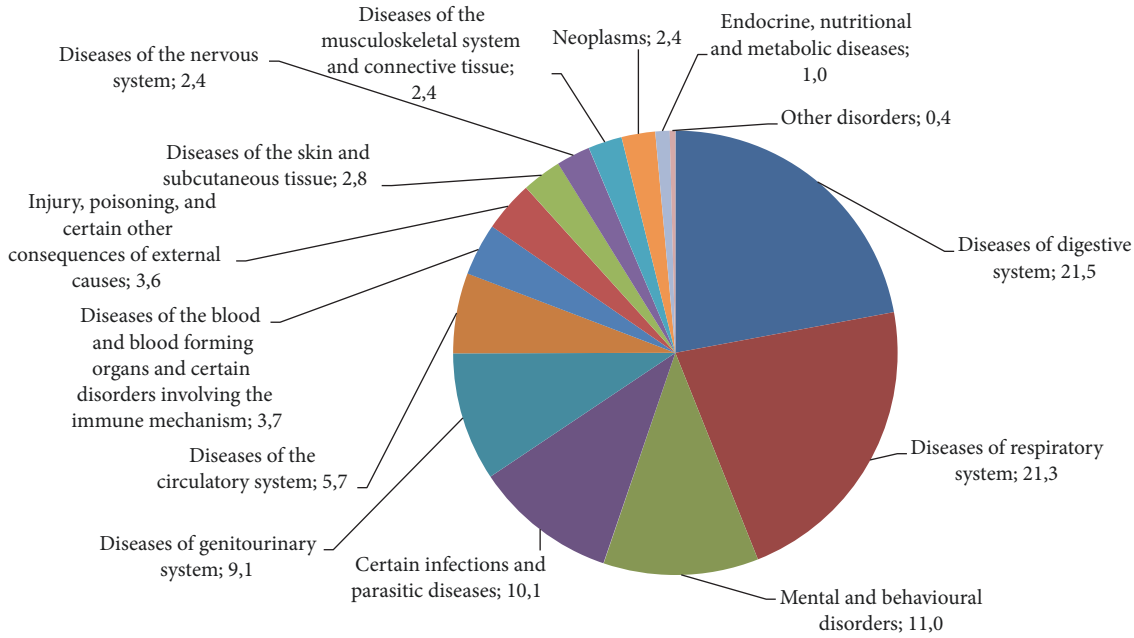


Figure 3: Indications of use by respondents of medicinal herbal raw materials (data are presented in %).

Gastrointestinal disorders most often mentioned by respondents were diarrhea, intestinal gas accumulation, gastric mucosal lesions, ulcers, digestive, and liver or gall bladder disorders. Respiratory ailments commonly referred to were pneumonia, flu, colds, bronchitis, asthma, or symptoms such as cough, sore throat, inflammation, and swelling of the nasal mucosa during a cold. As urinary system disorders, there were mentioned kidney disorders, kidney stones, urinary tract infections, and diuresis. Gender-related ailments include menstrual disorders such as pain, excessive bleeding, or total absence of secretions. Mental and behavioral disorders commonly referred to were anxiety, insomnia, and depression or fright.

According to the World Health Organization, the use of herbal raw materials for therapeutic purposes in developing countries has increased in recent decades [43, 44]. Although only a small proportion of medicinal plants have scientifically based indications for use, information on the safety and efficacy of medicinal herbs is important for both the average consumer and the healthcare professional. Therefore, one of the objectives of the World Health Organization's ongoing activities is to disseminate scientific information on folk medicine to ensure the safety and quality of medicinal products used in folk medicine [45]. According to the World Health Organization (WHO), there are currently few plant species that are scientifically tested and evaluated based on their medical application. There are even less plants that have been approved for safe and effective use for medical purposes. A guarantee of the safety, efficacy, and quality of medicinal plants in developed countries is challenging as people increasingly return to herbal remedies refusing to use chemical drugs [43]. According to the WHO recommendations on safety and efficacy, the use of medicinal herbal raw materials in Kaišiadorys district was compared with the recommended use of medicinal herbs in the WHO monographs.

Respondents in Kaišiadorys region mentioned 71 species of cultivated medicinal plants from 38 families used for therapeutic purposes. Only 19 of them are described in the WHO monographs.

While most of the indications for the use of medicinal herbal raw materials recorded in the study match the recommendations in the WHO monographs, some discrepancies have been observed; for example, the respondents used flowers of *Tilia cordata* for cold and cough as stated in the WHO monographs, but they used its buds as well, which were chewed in the presence of increased gastric acidity.

The WHO monographs have confirmed the external use of *Calendula officinalis*; however, respondents as well used the flower tea internally to treat inflammations of the throat, stomach, intestine, uterus, and urinary tract.

Indications for the use of common dill (*Anethum graveolens* L.) totally differed: the WHO monographs recommend the intake of fruit in the treatment of dyspepsia, gastritis, gas accumulation, and stomach pain, while the respondents used seeds, flowers, leaves, and herb internally for high blood pressure. Respondents not only used peppermint (*Mentha x piperita* L.) leaves internally, as indicated by the WHO, but also used the whole herb externally in acne.

The discrepancy between indications of use of medicinal raw herbal materials recorded in Kaišiadorys district and the WHO monographs shows that the information provided by respondents is not based on scientific literature or research, but on the long-standing experience of traditional folk medicine, which is passed down from generation to generation.

4. Conclusions

Only 19 of cultivated medicinal plant species mentioned by interviewed persons are described in the World Health Organization monographs. This means that the remaining 52 species were used without World Health Organization approved medical indications and were based solely on the folk medicine knowledge and experience. The study showed that the folk use of plants in the studied area was strongly rooted in daily practice. In Lithuania, researchers use traditional plants according to their historical background and try to create unique products suitable for today's user needs.

The most suitable for Lithuanian climatic conditions cultivated plants with historically justified medical applications can be a source of ideas for further research.

Data Availability

Collected material will be left in Lithuanian Museum of the History of Medicine and Pharmacy.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] V. Savoniakaite, *Lietuvos Etnologijos Ir Antropologijos Enciklopedija*, Lietuvos istorijos institutas, Vilnius, Lithuania, 2011.

[2] R. R. N. Alves and I. M. L. Rosa, "Biodiversity, traditional medicine and public health: where do they meet?" *Journal of Ethnobiology and Ethnomedicine*, vol. 3, article 14, 2007.

[3] T. B. Henfrey, *Ethnoecology, resource use, conservation and development in a Wapishana Community in the South Rupununi Guyana*, vol. 12, University of Kent, Canterbury, UK, 2002.

[4] M. Heinrich, J. Barnes, J. M. Prieto Garsia, S. Gibbons, and E. M. Williamson, *Fundamentals of Pharmacognosy and Phytotherapy*, Churchill Livingstone, London, 2004.

[5] R. A. Deno, T. D. Rowe, and D. C. Brodie, *The Profession of Pharmacy*, Philadelphia, Pa, USA, 2nd edition, 1966.

[6] C. P. Kala, "Current status of medicinal plants used by traditional Vaidyas in Uttaranchal, India," *Ethnobotany Research and Applications*, vol. 3, pp. 267–278, 2005.

[7] E. Aguirre-Hernández, A. L. Martínez, M. E. González-Trujano, J. Moreno, H. Vibrans, and M. Soto-Hernández, "Pharmacological evaluation of the anxiolytic and sedative effects of Tilia americana L. var. mexicana in mice," *Journal of Ethnopharmacology*, vol. 109, pp. 140–145, 2007.

[8] R. Verpoorte, "Medicinal plants: a recource for novel leads and drugs in K.G.Ramawat," in *Herbal Drugs: Ethnomedicine to Modern Medicine*, Udaipur, India, 2008.

[9] D. J. Leaman, "Sustainable wild collection of plants - make way for a new Standard," in *Convention on Biological Diversity*, 2008.

[10] A. Tschirch, "Handbuch der Pharmakognosie," Zweite, erweiterte Abteilung, Leipzig, pp. 395 -466, 1930.

[11] J. Radušienė and V. Janulis, "Vaistinių ir aromatinių augalų įvairovės tyrimo, jų panaudojimo bei išsaugojimo tendencijos," Kaunas, Medicina, 2004.

[12] Z. Kiaupa, "The History of Lithuania," p. 359, 2005.

[13] J. A. Pabrėža, "Taislius augyminis, T. I," pp. 1-223, Vilnius, 2009.

[14] C. H. Wildhaber, "A comparitive study of rural and urban allotments in Gravesham, Kent, U.K," in *Ethnobotany in the New Europe: People,Health and Wild Plant Recourses*, M. Pardo-de-Santayana, A. Pieroni, and R. K. Puri, Eds., Chapter 16, pp. 330–359, Berghahn press, Oxford, UK, 2013.

[15] Z. Petkeviciute, N. Savickiene, A. Savickas et al., "Urban ethnobotany study in Samogitia region, Lithuania," *Journal of Medicinal Plants Research*, vol. 4, no. 1, pp. 064–071, 2010.

[16] Z. Pranskuniene, J. Bernatoniene, Z. Simaitiene, A. Pranskunas, and T. Mekas, "Ethnomedicinal uses of honeybee products in lithuania: the first analysis of archival sources," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 9272635, 7 pages, 2016.

[17] Z. Pranskuniene, R. Dauliute, A. Pranskunas, and J. Bernatoniene, "Ethnopharmaceutical knowledge in Samogitia region of Lithuania: where old traditions overlap with modern medicine," *Journal of Ethnobiology and Ethnomedicine*, vol. 14, p. 70, 2018.

[18] Lietuvos etnografiniai regionai, Etninės kultūros globos taryba. Available at: http://www.ekgt.lt, 2018.

[19] O. Lukoševičius, Kaišiadorių bažnytinis paveldas, Kaišiadorių muziejus, 2018.

[20] R. Kaiiadoriu, Kaišiadorių muziejaus enciklopedija. Available at: http://www.kaisiadoriumuziejus, 2018.

[21] K. Rajonavimas, Lietuvos hidrometeorologijos tarnyba prie Aplinkos ministerijos. Available at: http://www.meteo.lt/lt/klimato-rajonavimas, 2018.

[22] V. Baltrunas, A. Bukantis, A. Cesnulevicius, Z. Gudzinskas, E. Kutorga, and G. Motuza, Gamta (Nature), Kaunas, Šviesa, 2006.

[23] Krituliai, Lietuvos hidrometeorologijos tarnyba prie Aplinkos ministerijos. Available at: http://www.meteo.lt/krituliai, 2018.

[24] International Society of Ethnobiology (2006), International Society of Ethnobiology Code of Ethics (with 2008 additions), http://ethnobiology.net/code-of-ethics.

[25] R. Jankevičienė, Botanikos vardų žodynas, Vilnius, Botanikos instituto leidykla, 1998.

[26] O. Ragažinskienė, S. Rimkienė, and V. Sasnauskas, aistinių augalų enciklopedija, Kaunas, 2005.

[27] K. Vilkonis, Lietuvos žaliasis rūbas, Kaunas, 2008.

[28] The Plant List.Version 1.1. Published on the Internet, 2013. Available at: http://www.theplantlist.org.

[29] P. F. Stevens, Angiosperm Phylogeny Website, version 13, 2012, Available at: http://www.mobot.org/MOBOT/research/APweb.

[30] L. Stecker, "They know all abouit their plants. Georg W.Steller (1709 -1746) and the first Ethnobotanical Study in Kamchatka," in *Pioneers in European Ethnobiology*, I. Svanberg and L. Luczaj, Eds., pp. 51–68, Upsala, Sweden, 2014.

[31] A. Ipolitova and I. Svanberg, "Field work in the Minusinsk Areas. Petr Ostrovskich (1870 -1940) Contributions to Khakas ethnobotany," in *Pioneers in European Ethnobiology*, I. Svanberg and L. Luczaj, Eds., pp. 131–140, Upsala, Sweden, 2014.

[32] R. Trimakas, Lietuvių liaudies medicina: etnografiniai ir folkloristiniai aspektai XIX a. pab. – XX a, Vilnius, 2008.

[33] U. Gudelytė, Tradiciškai Lietuvoje augintų dekoratyvinių augalų etnofarmacinis tyrimas, Kaunas, 2010.

[34] P. L. Howard, "Women and the Plant World," *An Exploration in Economic Botany*, vol. 58, no. 3, 2003.

[35] E. Šimkūnaitė, Lietuvių liaudies medicinos vaistingieji augalai, Mašinraštis, 1948.

[36] S. Vitalini, M. Iriti, C. Puricelli, D. Ciuchi, A. Segale, and G. Fico, "Traditional knowledge on medicinal and food plants used in Val San Giacomo (Sondrio, Italy) - An alpine ethnobotanical study," *Journal of Ethnopharmacology*, vol. 145, no. 2, pp. 517–529, 2013.

[37] N. Menković, K. Šavikin, S. Tasić et al., "Ethnobotanical study on traditional uses of wild medicinal plants in Prokletije Mountains (Montenegro)," *Journal of Ethnopharmacology*, vol. 133, no. 1, pp. 97–107, 2011.

[38] M. Kujawska and A. Pieroni, "Plants Used as Food and Medicine by Polish Migrants in Misiones, Argentina," *Ecology of Food and Nutrition*, vol. 54, no. 3, pp. 255–279, 2015.

[39] A. Dénes, N. Papp, D. Babai, B. Czúcz, and Z. Molnár, "Wild plants used for food by Hungarian ethnic groups living in the Carpathian Basin," *Acta Societatis Botanicorum Poloniae*, vol. 81, no. 4, pp. 381–396, 2012.

[40] I. Svanberg, "The use of wild plants as food in pre-industrial Sweden," *Acta Societatis Botanicorum Poloniae*, vol. 81, no. 4, pp. 317–327, 2012.

[41] K. Šavikin, G. Zdunić, N. Menković et al., "Ethnobotanical study on traditional use of medicinal plants in South-Western Serbia, Zlatibor district," *Journal of Ethnopharmacology*, vol. 146, no. 3, pp. 803–810, 2013.

[42] International Statistical Classification of Diseases and Related Health Problems (ICD) Available at: http://apps.who.int/classifications/icd10/browse/2016/en.

[43] WHO monographs on selected medicinal plants. Vol. 1. Geneva: World Health Organization; 1999. Available at: http://apps.who.int/medicinedocs/en/d/Js2200e/, 2018.

[44] WHO monographs on selected medicinal plants. Vol. 4. Spain: World Health Organization; 2009. http://apps.who.int/medicinedocs/en/m/abstract/Js16713e/, 2018.

[45] S. Evans, "Changing the knowledge base in Western herbal medicine," *Social Science & Medicine*, vol. 67, no. 12, pp. 2098–2106, 2008.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 2965821, 16 pages
https://doi.org/10.1155/2019/2965821

Research Article

Phyllanthus muellerianus (Euphorbiaceae) Restores Ovarian Functions in Letrozole-Induced Polycystic Ovarian Syndrome in Rats

Eveline Christiane Ndeingang, Patrick Brice Defo Deeh, Pierre Watcho, and Albert Kamanyi

Animal Physiology and Phytopharmacology Laboratory, University of Dschang, P.O. Box 67, Dschang, Cameroon

Correspondence should be addressed to Pierre Watcho; pwatcho@yahoo.fr

Received 4 February 2019; Accepted 23 April 2019; Published 14 May 2019

Guest Editor: Caio P. Fernandes

Polycystic ovarian syndrome (PCOS) is one of the common causes or female infertility. *Phyllanthus muellerianus* (Euphorbiaceae) is a plant used to treat various ailments including frequent menstruation and anovulation. We investigated the effects of *P. muellerianus* extracts on estrus cyclicity, lipid profile, oxidative stress-related markers, sex hormones, and ovarian architecture in letrozole-induced PCOS in rats. After induction of PCOS using letrozole (1 mg/kg/day), normal (n=6), and PCOS (n=108; distributed into 18 groups of 6 animals/group) rats were treated orally for 7 or 14 days with distilled water (10 ml/kg/day), clomiphene citrate (2 mg/kg/day), metformin (500 mg/kg/day), and aqueous or methanolic extract of *P. muellerianus* (30, 60, and 120 mg/kg). Estrus cyclicity, body, and sexual organ (ovaries and uterus) weights, biochemical and histological parameters were measured. There were letrozole-induced PCOS characterized by irregular estrus cyclicity, elevated ($p<0.05$-0.01) glycaemia, ovarian weight, triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol, malondialdehyde, luteinizing hormone (LH), and testosterone concentrations, but there were low ($p<0.05$-0.001) HDL cholesterol, estradiol, progesterone, catalase, peroxidase, and superoxide dismutase levels, compared with control. PCOS rats had multiple cysts compared with control. These reproductive, biochemical, and structural alterations were alleviated by *P. muellerianus* extracts. For instance, *P. muellerianus* restored the estrus cyclicity with a remarkable effect after 14 days of treatment. Moreover, *P. muellerianus* significantly decreased ($p<0.001$) LH and testosterone (both extracts; 30, 60, and 120 mg/kg) levels, but increased ($p<0.01$) estradiol (aqueous extract; 60 mg/kg) concentration. Cystic follicles were also decreased after plant application. *P. muellerianus* alleviated reproductive, hormonal, and structural alterations in PCOS rats. This plant could be useful in the management/treatment of reproductive and metabolic disorders related to PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS), the most prevalent hormonal disorders among women of reproductive age is a heterogeneous endocrine and metabolic disorder, causing irregular menstrual cycles, dyslipidemia, excessive body weight, oxidative stress, hyperandrogenism, and infertility [1, 2]. PCOS affects 5 to 10% of reproductive-aged women and 40% of affected women experience infertility, making this condition the leading cause of anovulatory infertility [3]. In women with PCOS, the normal ovarian function is disturbed mainly by hyperandrogenism and elevated level of luteinizing hormone (LH) [4], thus resulting in multiple cysts [5]. PCOS increased gonadotropin-releasing hormone (GnRH) pulse frequency, which favors LH production over follicle stimulating hormone (FSH) [6]. This increase in LH concentration subsequently promotes androgens production in the theca cells, while the relative FSH deficiency reduces the ability of granulosa cells to convert androgen into estrogen and impairs follicle maturation and ovulation [7].

Oxidative stress (OS) is also linked to a higher risk of infertility in patients with PCOS. Many studies have shown that OS-related biochemical parameters such as malondialdehyde (MDA), glutathione peroxidase (GPx), catalase, and

superoxide dismutase (SOD) are abnormal in patients with PCOS [8]. Moreover, OS is associated with overproduction of reactive oxygen species (ROS), which could cause DNA damage and mutations in tumor suppressor genes, leading to uncontrolled ovarian cells proliferation, development of multiple cysts, and infertility [9].

Letrozole is an aromatase inhibitor commonly used for the treatment of breast cancer, but it is associated with metabolic and reproductive disorders. Indeed, the inhibition of aromatase by letrozole decreases the conversion of androgens to estrogens, leading to an accumulation of androgens in the ovary [10]. Previous reports have shown that letrozole is efficient in establishing PCOS in rats [11, 12]. These animals developed many characteristics of human PCOS, including abnormal follicles [11], hyperglycemia [13], oxidative stress [9], and altered sex hormones (testosterone, estrogens, LH and FSH) levels [11].

The therapeutic treatment of PCOS involved the use of several drugs such as metformin and clomiphene citrate, but they are commonly associated with serious side effects. A new therapeutic approach with fewer side effects, easy availability, and broad spectrum is required. Previous studies reported the efficacy of some plants such as *Trigonella foenum-graecum* [2], *Ecklonia cava* [14] and *Allium fistulosum* [12] in the restoration of ovarian function in PCOS rats. *Phyllanthus muellerianus* (Kuntze) Exell (Euphorbiaceae) commonly called "Mbolongo" in eastern Cameroon, is a tropical plant extensively used for the treatment of digestive disorders, frequent menstruation and anovulation [15, 16]. Phytochemical screening of the barks and leaves of this plant revealed the presence of various components such as gallic acid, isoquercitrin, caffeic acid, geraniin, furosin, corilagin, astragalin, rutin, phaselic acid, methyl gallate, chlorogenic acid, and 3,5-o-dicaffeoylquinic [17]. Moreover, *P. muellerianus* possesses antihyperglycemic [18], antihyperlipidemic [19], antioxidant [20], and aphrodisiac [21] properties in normal rats, but its action in PCOS rats is unknown. Considering the above-mentioned pharmacological properties, *P. muellerianus* may be a good candidate for the treatment of PCOS-related metabolic and reproductive disorders. Therefore, the present study was undertaken to investigate the effects of aqueous and methanolic extracts of *P. muellerianus* on estrus cyclicity, blood glucose level, lipid profile, oxidative stress-related biochemical parameters, sex hormones, and ovarian histology in letrozole-induced PCOS in rats.

2. Materials and Methods

2.1. Plant Material and Preparation of Aqueous and Methanolic Extracts. The fresh roots of *P. muellerianus* were collected in April 2016 in Tonga, West Region of Cameroon. Botanical identification was done at the Cameroon National Herbarium in Yaoundé-Cameroon (voucher specimen N° BWPV03). Roots were cut, shade-dried for 7 days and powdered with an electric mixer.

The powder of *P. muellerianus* (250 g) was infused in boiling water (1500 ml) for 15 minutes and filtered. The filtrate obtained was dried in an oven (55°C) for 48 hours. At the end, 21.15 g of aqueous extract of *P. muellerianus* were obtained (extraction yield: 11.82%).

The methanolic extract of *P. muellerianus* was obtained by macerating 2500 g of powder in methanol (5000 ml) for 72 hours. The mixture was filtered and evaporated using a rotary evaporator (75°C) under reduced pressure. After evaporation, 26.68 g of methanolic extract was obtained (extraction yield: 9.37%).

2.2. Animals. Adult female Wistar rats (180-200 g) were obtained from the animal house of the Animal Biology Department, Faculty of Science of the University of Dschang, Cameroon. The animals were maintained at room temperature (22-23°C) with a reverse natural light-dark cycle (about 12 h of light and 12 h of dark cycle) and free access to a standard diet and water. Only rats with at least three consecutive regular appearance of estrus stages in order (estrus cyclicity) were used in the study. Throughout the treatment period, animals were weighed (twice a week) and vaginal smear was observed under microscope in order to identify the estrus stage. The project was presented and validated by the scientific committee of the Department of Animal Biology, University of Dschang (Date, 05.06.2015), which follows the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Economic Community guidelines; Directive 86/609/EEC, of the 24th November 1986 [22].

2.3. Animal Grouping and Induction of PCOS. Females with regular estrus cyclicity as described above were selected and distributed into 2 sets. The first set (control group, n=6) was administered orally with vehicle (0.9% NaCl solution) once daily. The second set (n=152) was orally treated for 21 days with letrozole (1 mg/kg/day) dissolved in 0.9% NaCl to induce PCOS. Vaginal smears were collected daily and examined microscopically for the identification of estrus stage. At the end of the induction period, rats of the first set and 6 rats of the second set were randomly selected, sacrificed by cervical dislocation under anesthesia (diazepam: 10 mg/kg and ketamine: 50 mg/kg). Biochemical and histological examinations were performed to confirm PCOS in rats. PCOS rats exhibited the main features of PCOS such as hyperglycemia, hyperandrogenism, and multiple cysts as reported previously [23]. Additionally, irregular estrus cyclicity (rats having a disturbed appearance of the four estrus stages) was the main criteria to select PCOS rats [23]. Subsequently, 108 PCOS rats were distributed into 18 groups (6 rats per group) and treated orally for 7 or 14 days with distilled water (10 ml/kg/day), clomiphene citrate (2 mg/kg/day), metformin (500 mg/kg/day), and aqueous or methanolic extract of *P. muellerianus* (30, 60, and 120 mg/kg/day). A healthy control group (n=6) was administered with distilled water (10 ml/kg/day) for 14 days. The effective dose of letrozole (1 mg/kg) and treatment period (21 days) were chosen from reference studies [11, 24]. Doses of *P. muellerianus* (30, 60 and 120 mg/kg/day) were selected from our pilot study (unpublished data). During the treatment period, vaginal smears were collected daily and examined microscopically for the identification of estrus stage. After 7 and 14 days of treatment,

animals were sacrificed and the body and organ (ovaries and uterus) weights, blood glucose level, lipid profile, oxidative stress-related biochemical parameters, sex hormones, and ovarian histology were evaluated.

2.4. Estrus Cycle Motoring. Vaginal smears were collected daily (8-10 a.m.) to determine the reproductive cycle of each animal. As described in our previous study [25], the predominance of nucleated epithelial cells was classified as proestrus (the first stage). The estrus (the second stage) was characterized by the presence of cornified squamous epithelial cells which occur in clusters. Metestrus (the third stage) is a mix of cell types with a predominance of leukocytes and a few nucleated and/or cornified squamous epithelial cells. Diestrus (the fourth stage) consists predominantly of leukocytes [26].

2.5. Oral Glucose Tolerance Test. This test was done before and after induction of PCOS, and after treatments with the reference drugs and plant extracts. Rats were fasted for 6 hours and glycemia was determined in a tail blood sample using a handheld glucometer (Accucheck-active, Roche Diagnostics) before (time 0) a single oral administration of glucose (2.5 g/kg) and at 30, 60, 90 and 120 minutes after administration [27].

2.6. Sacrifice and Sample Collection. After 7 or 14 days of treatment, rats were sacrificed by cervical dislocation under diazepam (10 mg/kg) and ketamine (50 mg/kg) anesthesia, and abdominal artery blood was collected into heparinized tubes. Plasma was obtained by centrifugation (3000 g for 10 min) and stored at -20°C until assayed for lipid profile, oxidative stress parameters, and sex hormones. After blood collection, ovaries and uterus were removed and weighed and histological study was done.

2.7. Lipid Profile, Oxidative Stress Parameters and Sex Hormones. The lipid profile, including plasmatic total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VHDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), was evaluated using standard colorimetric kits (CORMAY, Łomianki, POLAND) as described previously [28, 29]. The LDL-C and VLDL-C levels were calculated based on Friedewald's equation: LDL = TC – TG/5 – HDL; VLDL = TG/5 [28].

Plasmatic estradiol, LH, FSH, progesterone, and testosterone levels were measured using commercial kits as described previously [30, 31].

Malondialdehyde (MDA) level was estimated as described by Olszewska-Słonina et al. [32]. Total peroxidase contents were measured as described by Giustarini et al. [33]. Superoxides dismutase (SOD) and catalase activities were determined as described by Serra et al. [34] and Hadwan [35], respectively.

2.8. Histopathological Evaluation. Ovaries were processed step by step through 10% neutral formalin fixation (24 h), paraffin embedding, and longitudinally and serially sectioned at 4 μm with a microtome. The samples were stained with hematoxylin and eosin and assessed microscopically according to the methods described by Kafali et al. [11]. Preantral, antral, atretic and cystic follicles, and corpus luteum were identified [36].

2.9. Statistical Analysis. All data were expressed as mean ± SEM (standard error of the mean). ANOVA for repeated measures followed by Tukey HSD posttest was used for multiple comparisons. Statistical analysis was done using STATISTICA software Version 8.0 (StatSoft, Inc., Tulsa, USA). The significance of the difference was fixed at $p<0.05$.

3. Results

3.1. Effects of Letrozole on Estrus Cyclicity, Blood Glucose Level, Body and Ovarian Weights, and Sex Hormones after 21 Days of Treatment. On day 0 (before letrozole treatment), all rats had a regular estrus cyclicity. After 5, 10, 15, and 21 days of oral treatment with letrozole, 57, 59, 72, and 46% of rats had an irregular cyclicity, respectively. In the control group, all rats had a regular estrus cycle throughout the study period (Table 1). PCOS rats showed elevated blood glucose level ($p<0.01$-0.001), compared with control (Figure 1(a)). No significant change in body weight was observed in both groups (Figure 1(b)). In PCOS rats, the relative ovary weight was significantly increased ($p<0.05$), but no significant change in uterus weight was observed compared to the control group (Figures 1(c) and 1(d)). Letrozole-induced PCOS rats showed an elevated ($p<0.001$) testosterone and LH concentrations, with reduced ($p<0.001$) estradiol level (Figures 1(e)–1(g)). Moreover, section of ovary from control rats showed growing follicles (at different stages) and a large number of corpus luteum (Figure 1(h)) while those from PCOS rats exhibited many cystic and atretic follicles and decreased corpus luteum (Figure 1(i)). Overall, these features confirmed PCOS in rats after 21 days of letrozole treatment.

3.2. Effects of Different Treatments on Body Weight and Relative Weights of the Ovaries and Uterus. There was no change in the body weight of rats administered for 7 and 14 days with reference products or plant extracts, compared with control (Figure 2(a)).

In general, the relative weights of the ovaries were reduced in all groups (except control group) after 14 days of treatment compared to those treated for 7 days. A significant decrease in ovarian weight was noticed in animals treated for 7 days with the aqueous extract of *P. muellerianus* (30 mg/kg: $p<0.05$; 60 mg/kg: $p<0.01$) compared to the SOPK group. After 14 days of treatment, clomiphene citrate, and aqueous or methanolic extract of *P. muellerianus* significantly ($p<0.05$-0.01) decreased the ovarian weight (Figure 2(b)). Moreover, in rats treated for 14 days with the methanolic extract of *P. muellerianus* at 120 mg/kg, a significant decrease ($p<0.05$) in ovarian weight was noted, compared with rats treated for 7 days with the same dose (Figure 2(b)).

The methanolic extract of *P. muellerianus* (120 mg/kg) significantly increased ($p<0.05$) the uterus weight after 7

Table 1: Effects of 21 days of treatment with letrozole on the percentage of irregular estrus cycle.

Groups	Duration of treatment				
	D0	D1-5	D6-10	D11-15	D16-21
	Percentage of irregular estrus cycle (%)				
PCOS	0	57	59	72	46
Control	0	0	0	0	0

Number of rats per group=6. PCOS: polycystic ovarian syndrome. D: day.

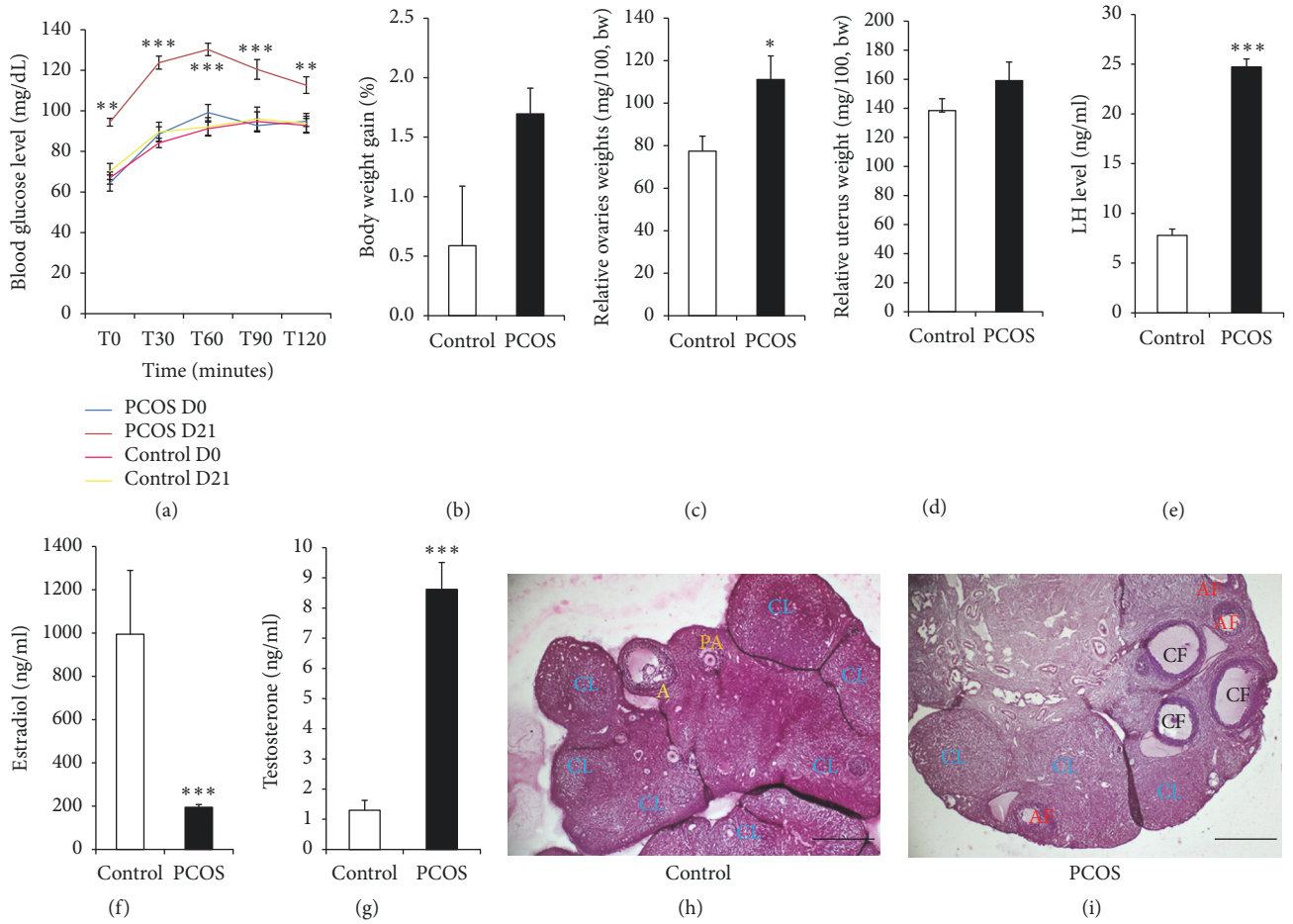


Figure 1: *Letrozole treatment of female rats resulted in reproductive and metabolic features of PCOS.* (a) Blood glucose tolerance test; (b) body weight gain; (c, d) relative ovary and uterus weights, respectively; (e-g) ovarian LH, estradiol, and testosterone levels, respectively. Values are means ± SEM, number of animals per group =6. ∗p<0.05; ∗∗p<0.01; ∗ ∗ ∗p<0.001: significantly different compared with control. (h) Section of ovary from a control rat showing growing follicles (at different stages) and a large number of corpus luteum. (i) Section of ovary from PCOS rats exhibiting many cystic and atretic follicles and decreased corpus luteum. CL: corpus luteum; CF: cystic follicle; AF: atretic follicle; PA: preantral follicle; A: antral follicle. Magnification x40. Calibration bar =100 μm.

days of treatment, compared with control. After 14 days of treatment, metformin, aqueous, and methanolic extracts of *P. muellerianus* (30 and 60 mg/kg) significantly (p<0.05-0.01) elevated the uterus weight, compared with SOPK group. The highest value was observed in rats administered for 14 days with the methanolic extract of *P. muellerianus* at 60 mg/kg (Figure 2(c)).

3.3. Effects of Different Treatments on Estrus Cyclicity. In the control group, all rats had a regular estrus cyclicity while all untreated PCOS rats had an irregular estrus cycle. Remarkably, the aqueous (120 mg/kg: 66.67%) and methanolic (30 mg/kg: 66.67%; 60 mg/kg: 55.56%) extracts of *P. muellerianus* increased the percentage of rats with regular estrus cyclicity compared with PCOS group (Table 2).

3.4. Effects of Different Treatments on Blood Glucose Tolerance Test. Blood glucose levels were significantly elevated (p<0.05-0.001) in untreated PCOS rats (0, 90 and 120 min), compared with control. In all PCOS groups (except rats treated with metformin for 14 days), blood glucose levels at 30, 60, 90, and 120 min were elevated, compared to

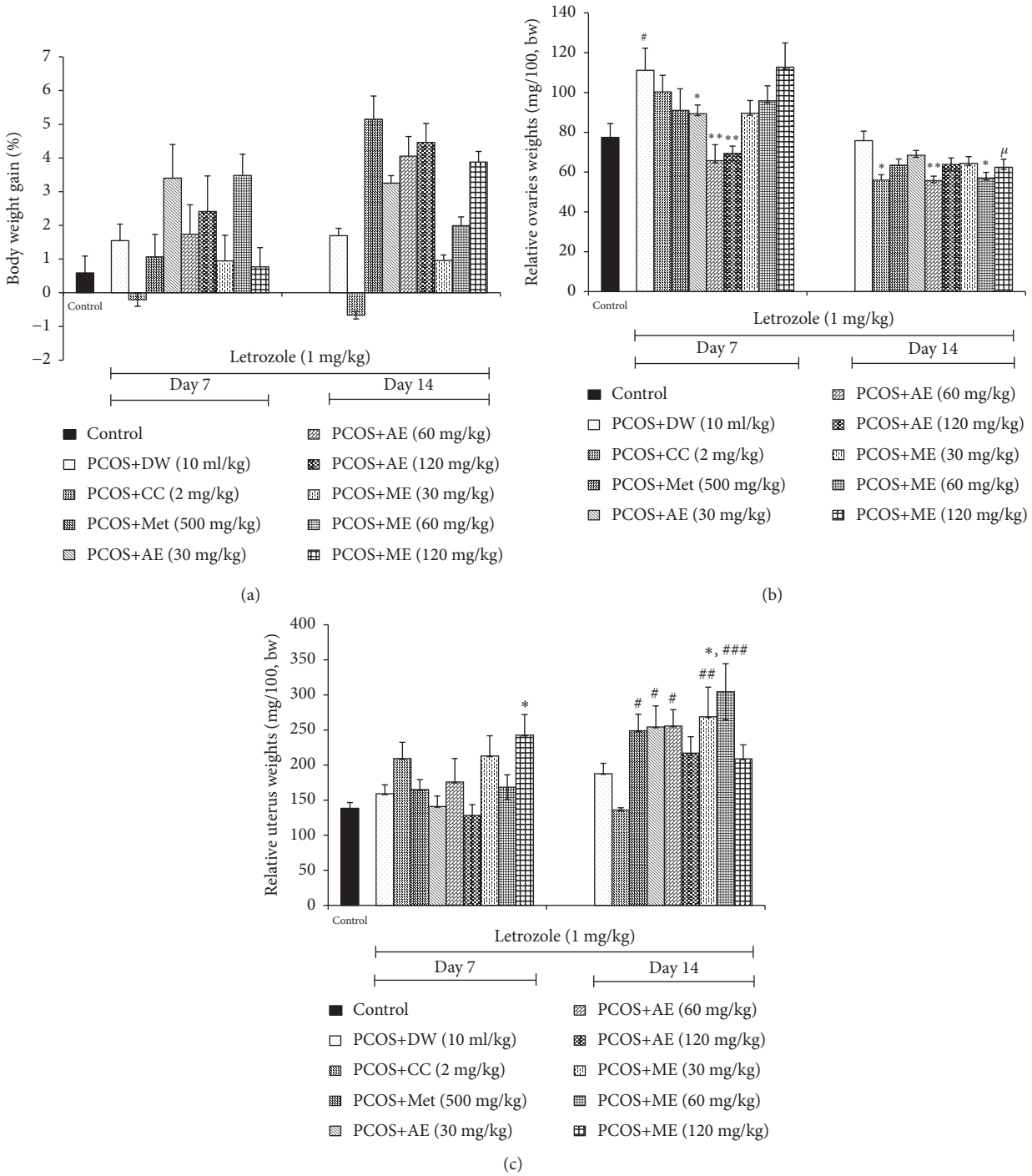


Figure 2: *Effects of clomiphene citrate, metformin, aqueous and methanolic extracts of P. mullerianus on body (a), ovaries (b) and uterus (c) weights after 7 and 14 days of treatment*. Values are means ± SEM, number of animals per group = 6. DW: distilled water; CC: clomiphene citrate; Met: metformin; AE: aqueous extract; ME: methanolic extract; $*p<0.05$; $**p<0.05$; $***p<0.05$: significantly different compared with control. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$: significantly different compared with PCOS group. $^{\mu}p<0.01$: significantly different compared with day 7 for the same group.

their respective basal values (before glucose administration). Interestingly, metformin (120 min), aqueous (60 mg/kg, 90 and 120 min), and methanolic (30 mg/kg, 120 min; 60 mg/kg, 30, 90 and 120 min) extracts of *P. mullerianus* significantly decreased ($p<0.05$-0.001) blood glucose level after 14 days of treatment, compared with PCOS group (Table 3).

3.5. Effects of Different Treatments on Lipid Profile

3.5.1. Effects on Triglycerides. TG level was significantly elevated ($p<0.01$) in untreated SOPK group, compared with control. In all SOPK-treated groups, no significant change in TG level was observed after 7 days of treatment. On the

Table 2: Percentage of PCOS rats with regular estrus cycle after treatments with clomiphene citrate, metformin, and aqueous and methanolic extracts of *P. mullerianus* for 14 days.

	Control	PCOS+DW (10 ml/kg)	PCOS+CC (2 mg/kg)	PCOS+MET (500 mg/kg)	PCOS+AE (30 mg/kg)	PCOS+AE (60 mg/kg)	PCOS+AE (120 mg/kg)	PCOS+ME (30 mg/kg)	PCOS+ME (60 mg/kg)	PCOS+ME (120 mg/kg)
D0-D4	100	0	0	0	0	22.22	0	22.22	0	0
D5-D9	100	0	33.33	22.22	22.22	0	44.44	11.11	44.44	33.33
D10-D14	100	0	44.44	33.33	22.22	33.33	66.67	66.67	55.56	44.44

Number of rats per group = 6. D: day; DW: distilled water; CC: clomiphene citrate; MET: metformin; AE: aqueous extract; ME: methanolic extract; PCOS: polycystic ovarian syndrome.

contrary, clomiphene citrate, metformin, and aqueous (30 and 60 mg/kg) or methanolic (60 and 120 mg/kg) extract of *P. muellerianus* significantly decreased ($p<0.05$-0.01) the TG level, compared to the untreated SOPK group (Figure 3(a)).

3.5.2. Effects on Total Cholesterol. Elevated ($p<0.01$) TC level was noticed after SOPK induction. However, a decrease ($p<0.05$) in TC level was observed after 7 days of *P. mullerianus* (aqueous extract at 120 mg/kg) application. Clomiphene citrate, aqueous (120 mg/kg), and methanolic (30, 60 and 120 mg/kg) extracts of *P. muellerianus* significantly lowered ($p<0.05$-0.001) the TC level after 14 days of treatment, compared with untreated SOPK group. The efficacy of the methanolic extract was more pronounced (Figure 3(b)).

3.5.3. Effects on HDL Cholesterol. The installation of SOPK was associated with a significant reduction ($p<0.01$) in ovarian HDL cholesterol level. However, it was significantly increased ($p<0.001$) 7 days after plant (aqueous extract, 30 and 60 mg/kg) administration. In all groups (except aqueous extract, 30 mg/kg) treated for 14 days with reference products or plant extracts, the HDL cholesterol level remained lower than that of control group. Interestingly, the aqueous extract of *P. muellerianus* (60 mg/kg) significantly increased ($p<0.05$) the HDL cholesterol level after 14 days of treatment, compared with SOPK group (Figure 3(c)).

3.5.4. Effects on LDL and VLDL Cholesterol. Letrozole treatment significantly increased ($p<0.01$) ovarian LDL and VLDL cholesterol levels, compared with control. LDL cholesterol concentration was significantly reduced ($p<0.01$) in rats administered for 7 days with aqueous and methanolic extracts of *P. muellerianus* (120 mg/kg), but no change ($p>0.05$) in VLDL cholesterol level was observed in all treated groups. After 14 days of treatment, the ovarian LDL cholesterol level was significantly lowered ($p<0.05$-0.001) in rats administered with aqueous (120 mg/kg) or methanolic (30, 60, and 120 mg/kg) extract of *P. muellerianus,* compared with SOPK group. VLDL cholesterol level was also reduced in rats administered for 14 days with clomiphene citrate ($p<0.05$), metformin ($p<0.001$), and aqueous (30, 60 mg/kg; $p<0.001$) or methanolic (60, 120 mg/kg; $p<0.05$) extract of *P. mullerianus* (Figures 3(d) and 3(e)).

3.6. Effects of Different Treatments on Oxidative Stress Parameters

3.6.1. Effects on MDA Level. Compared with the untreated SOPK group, we found that MDA level was significantly decreased ($p<0.05$-0.001) in rats administered with aqueous (30, 60 and 120 mg/kg) or methanolic (30 and 60 mg/kg) extract of *P. muellerianus* after 7 and 14 days of treatment (Figure 4(a)).

3.6.2. Effects on Catalase Activity. Catalase activity was significantly lowered ($p<0.01$) in rats after SOPK induction. However, females administered for 7 days with metformin and methanolic extract of *P. muellerianus* (60 mg/kg) exhibited high ($p<0.05$-0.01) catalase activity in the ovaries. After 14 days of treatment, catalase activity was significantly elevated in rats treated with clomiphene citrate ($p<0.01$) and aqueous extract of *P. muellerianus* (30 mg/kg; $p<0.05$), compared to the SOPK group (Figure 4(b)).

3.6.3. Effects on Total Peroxidases and SOD Activities. Ovarian total peroxidases ($p<0.01$) and SOD ($p<0.05$) activities were also decreased after SOPK induction. Remarkably, clomiphene citrate and aqueous extract of *P. muellerianus* (120 mg/kg) significantly elevated ($p<0.05$) total peroxidases and SOD activities, respectively, after 7 days of treatment. After 14 days of treatment, high total peroxidases activities ($p<0.05$) were noticed in rats administered with methanolic extract of *P. muellerianus* (120 mg/kg), compared to the SOPK group. Similarly, SOD activity was elevated ($p<0.05$) in rats treated for 14 days with aqueous (60 mg/kg) and methanolic (60 and 120 mg/kg) extracts of *P. muellerianus* (Figures 4(c) and 4(d)).

3.7. Effects of Different Treatments on Sex Hormones. After induction of PCOS, LH and testosterone levels were significantly increased while progesterone and estradiol concentrations were decreased compared with control.

Metformin and *P. muellerianus* (both extracts at all doses) significantly lowered ($p < 0.001$) LH level after 7 days of treatment. All rats (except those administered with aqueous extract, 30 mg/kg) treated for 14 days with plant extracts also had a low ($p < 0.001$) LH level, compared with PCOS group. In all groups, FSH concentrations were statistically unchanged after treatments. On the contrary, progesterone

Table 3: Effects of clomiphene citrate, metformin, and aqueous and methanolic extracts of *P. mullerianus* on glucose tolerance of rats after 7 and 14 days of treatment.

Groups	Doses	Blood glucose level (mg/dL) Time				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/kg	70.14 ± 3.99	89.57 ± 9.82	92.14 ± 4.55	96 ± 7.88	93.86 ± 4.86
7 days						
PCOS+Distilled water	10 ml/kg	96.13 ± 3.74*	128.63 ± 2.09$^{\mu\mu}$	121 ± 4.65$^{\mu\mu}$	137.13 ± 5.56*$^{,\mu\mu\mu}$	131.75 ± 5.09* *$^{,\mu\mu\mu}$
PCOS+Clomiphene citrate	2 mg/kg	77 ± 2.87	121.29 ± 10.17$^{\mu}$	125.57 ± 11.84$^{\mu}$	121.14 ± 9.79$^{\mu}$	118.43 ± 7.60$^{\mu}$
PCOS+Metformin	500 mg/kg	76.57 ± 3.21	127.29 ± 4.22$^{\mu\mu\mu}$	125.86 ± 3.28$^{\mu\mu}$	122.57 ± 3.56$^{\mu\mu}$	126.86 ± 5.47$^{\mu\mu}$
PCOS+Aqueous extract	30 mg/kg	75.13 ± 3.30	107.75 ± 7.43$^{\mu\mu}$	119.75 ± 4.31$^{\mu\mu}$	110.63 ± 4.13$^{\mu\mu}$	113.63 ± 3.63$^{\mu\mu}$
PCOS+Aqueous extract	60 mg/kg	72.38 ± 4.56	111.25 ± 3.36$^{\mu\mu}$	115.13 ± 6.89$^{\mu\mu\mu}$	111.63 ± 4.59$^{\mu\mu}$	105.38 ± 3.98$^{\mu}$
PCOS+Aqueous extract	120 mg/kg	77.38 ± 5.53	106.38 ± 6.09$^{\mu\mu}$	118.5 ± 5.15$^{\mu\mu}$	109.63 ± 4.83$^{\mu\mu}$	119.25 ± 3.95$^{\mu\mu}$
PCOS+Methanolic extract	30 mg/kg	88.71 ± 4.09	131.14 ± 5.88$^{\mu\mu\mu}$	138.71 ± 4.99$^{\mu\mu\mu}$	134.71 ± 6.14$^{\mu\mu\mu}$	126.71 ± 5.08$^{\mu\mu}$
PCOS+Methanolic extract	60 mg/kg	88.43 ± 2.58	143.43 ± 3.30$^{\mu\mu\mu}$	140.43 ± 6.44$^{\mu\mu\mu}$	145.43 ± 24.37$^{\mu\mu\mu}$	136 ± 5.27* *$^{,\mu\mu}$
PCOS+Methanolic extract	120 mg/kg	68.57 ± 2.09	129.57 ± 6.79$^{\mu\mu\mu}$	141.57 ± 3.60$^{\mu\mu\mu}$	144.29 ± 8.95$^{\mu\mu\mu}$	127.57 ± 3.33$^{\mu\mu}$
14 days						
PCOS+Distilled water	10 ml/kg	97.86 ± 4.97*	126.14 ± 2.26$^{\mu\mu}$	124.29 ± 2.85$^{\mu\mu}$	127.29 ± 3.09$^{\mu\mu}$	136.86 ± 2.38* * *$^{,\mu\mu\mu}$
PCOS+Clomiphene citrate	2 mg/kg	83.83 ± 2.94	112 ± 4.80$^{\mu\mu}$	118.83 ± 3.82$^{\mu\mu}$	118 ± 7.57$^{\mu\mu}$	115.67 ± 2.47$^{\mu\mu}$
PCOS+Metformin	500 mg/kg	91.75 ± 5.95	110.13 ± 7.69	114 ± 2.03	124.75 ± 2.33	108.5 ± 8.73$^{\#\#}$
PCOS+Aqueous extract	30 mg/kg	84.88 ± 3.28	110.63 ± 2.28$^{\mu}$	116.88 ± 5.62$^{\mu}$	122.38 ± 3.61$^{\mu}$	119.75 ± 3.96$^{\mu}$
PCOS+Aqueous extract	60 mg/kg	81.43 ± 2.35	112.71 ± 4.89$^{\mu}$	111 ± 5.01$^{\mu}$	103.14 ± 4.31$^{\#,\mu}$	96.57 ± 5.06$^{\#\#\#}$
PCOS+Aqueous extract	120 mg/kg	79.42 ± 3.74	121.57 ± 3.08$^{\mu}$	121.71 ± 3.98$^{\mu}$	123.14 ± 5.28$^{\mu}$	120.29 ± 3.87$^{\mu}$
PCOS+Methanolic extract	30 mg/kg	78.25 ± 4.81	108.5 ± 4.39$^{\mu}$	116.63 ± 7.29$^{\mu}$	107.75 ± 5.12$^{\mu}$	97.75 ± 4.22$^{\#\#\#}$
PCOS+Methanolic extract	60 mg/kg	78.67 ± 5.53	98.33 ± 3.48$^{\#}$	117 ± 4.67$^{\mu}$	103.67 ± 5.81$^{\#,\mu}$	102.5 ± 6.41$^{\#\#,\mu}$
PCOS+Methanolic extract	120 mg/kg	77.25 ± 2.92	126.38 ± 5.52$^{\mu\mu}$	132.13 ± 6.19$^{\mu\mu\mu}$	126.63 ± 3.77$^{\mu\mu}$	118 ± 3.85$^{\mu}$

The values are expressed as mean ± SEM; number of rats per group=6. PCOS: polycystic ovarian syndrome. *$p < 0.05$; **$p < 0.01$; * * *$p < 0.001$: significantly different compared with control. $^{\#}p<0.05$; $^{\#\#}p <0.01$; $^{\#\#\#}p <0.001$: significantly different compared with PCOS rats treated with distilled water.
$^{\mu}p<0.05$; $^{\mu\mu}p <0.01$; $^{\mu\mu\mu}p <0.001$: significantly different compared with the base line value (T0, before glucose administration).

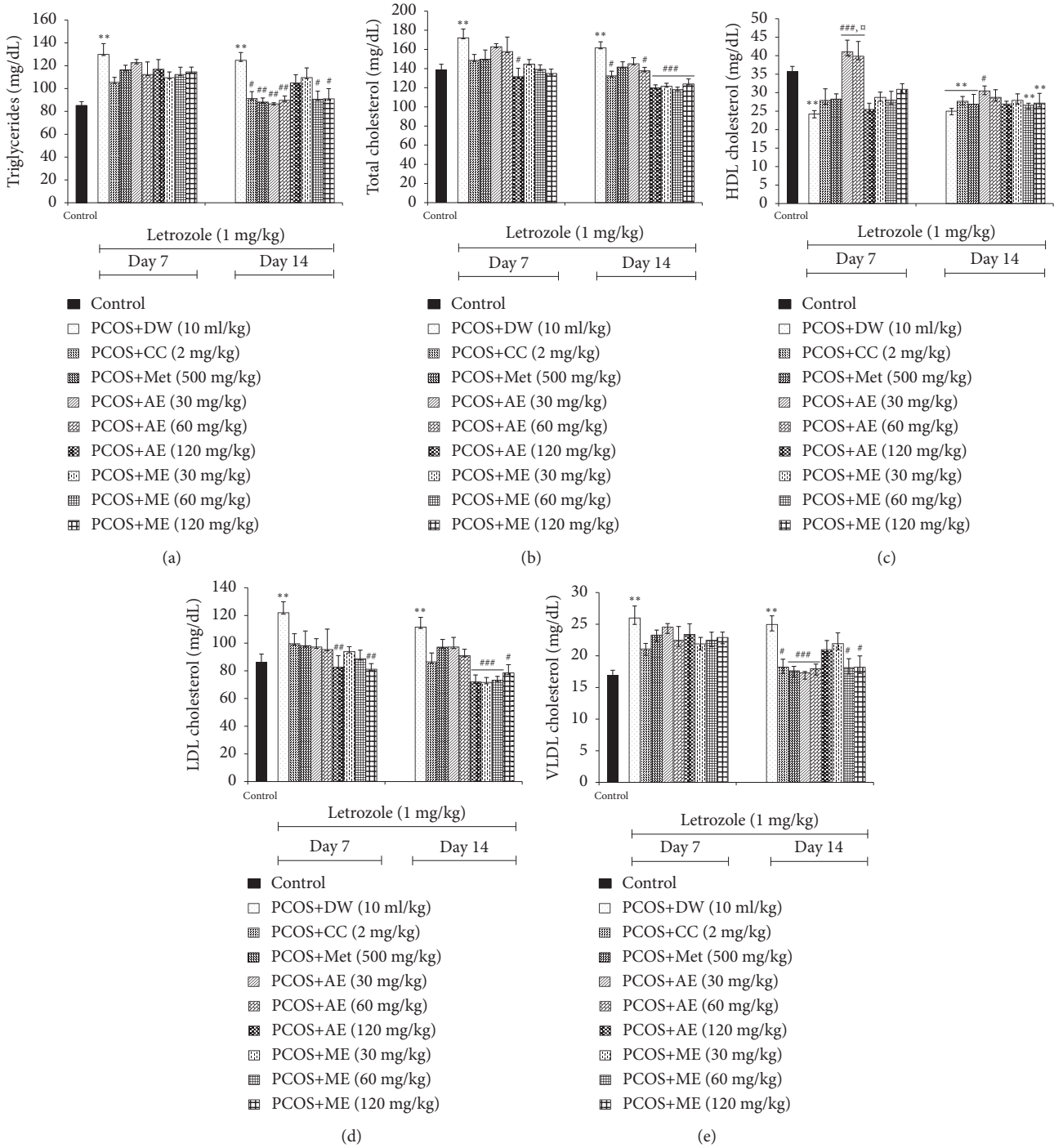


Figure 3: *Effects of clomiphene citrate, metformin, aqueous and methanolic extracts of P. mullerianus on triglycerides (a), total cholesterol (b), HDL cholesterol (c), LDL cholesterol (d), and VLDL cholesterol (e) after 7 and 14 days of treatment*. Values are means ± SEM, number of animals per group = 6. DW: distilled water; CC: clomiphene citrate; Met: metformin; AE: aqueous extract; ME: Methanolic extract; ∗p<0.05; ∗∗p<0.05; ∗ ∗ ∗p<0.05: significantly different compared with control. $^{\#}$p<0.05; $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001: significantly different compared with PCOS group.

levels remained low in rats administered for 7 days with aqueous (120 mg/kg) and methanolic (30 and 120 mg/kg) extracts of *P. muellerianus,* compared with control. Remarkably, a significant decrease ($p < 0.05$-0.001) in progesterone level was observed in rats treated with metformin (7 and 14 days), aqueous (7 days: 30 and 60 mg/kg; 14 days: 60 mg/kg), and methanolic (14 days: 60 mg/kg) extracts of *P. muellerianus,* compared with PCOS group. When compared with PCOS group, estradiol level was significantly increased ($p < 0.05$-0.001) in rats administered with clomiphene citrate (7 and

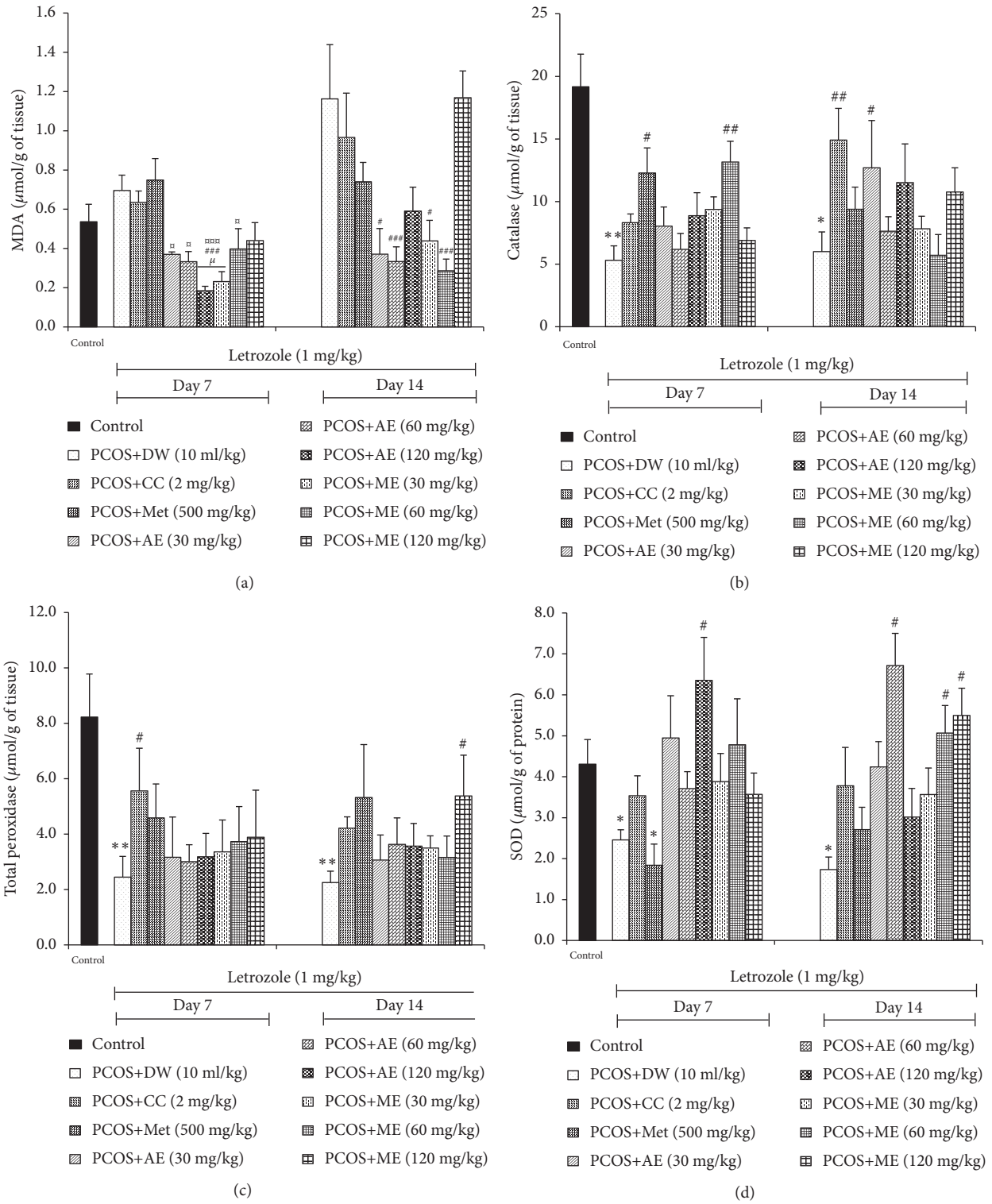


Figure 4: *Effects of clomiphene citrate, metformin, aqueous and methanolic extracts of P. mullerianus on malondialdehyde (a), catalase (b), total peroxidase (c) and superoxide dismutase (d) levels after 7 and 14 days of treatment*. Values are means ± SEM, number of animals per group = 6. DW: distilled water; CC: clomiphene citrate; Met: metformin; AE: aqueous extract; ME: methanolic extract; $*p<0.05$; $**p<0.05$: significantly different compared with control. $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$: significantly different compared with PCOS group. $^{¤}P<0.05$; $^{¤¤¤}P<0.001$: significantly different compared with metformin group.

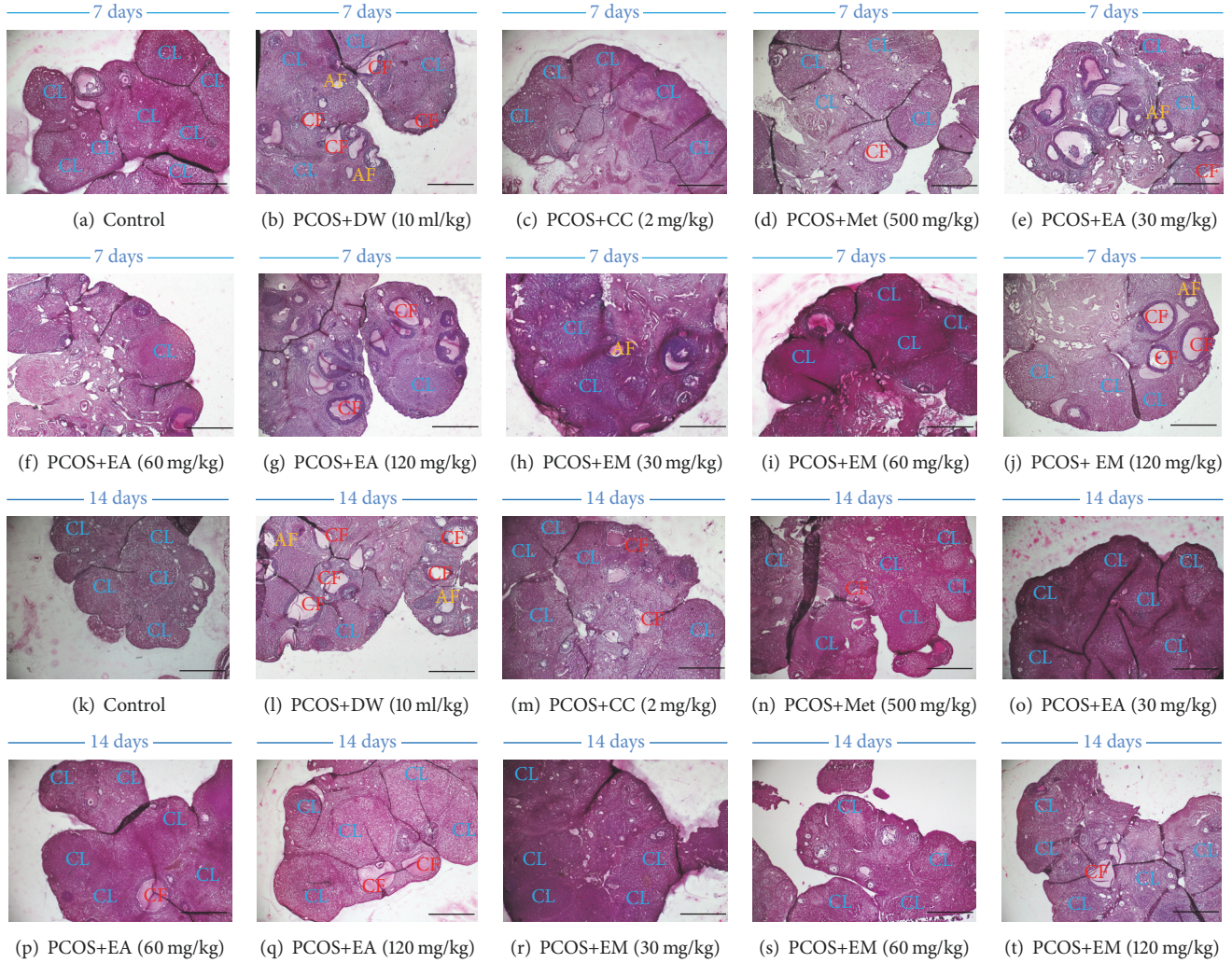


FIGURE 5: *Effects of clomiphene citrate, metformin, aqueous and methanolic extracts of P. mullerianus on ovarian histology after 7 and 14 days of treatment*. DW: distilled water; CC: clomiphene citrate; Met: metformin; AE: aqueous extract; ME: methanolic extract; CL: corpus luteum; CF: cystic follicle; AF: atretic follicle; PA: preantral follicle; A: antral follicle. Magnification x40. Calibration bar =100 μm.

14 days), metformin (7 days), and aqueous extract of *P. muellerianus* (7 and 14 days: 60 mg/kg). Metformin, aqueous and methanolic extracts of *P. muellerianus* (60 and 120 mg/kg) significantly reduced ($p < 0.05$-0.01) the testosterone level after 7 days of treatment. Testosterone concentration was also lowered in rats treated for 14 days with metformin and *P. muellerianus* (both extracts at all doses) (Table 4).

3.8. Effects of Different Treatments on Ovarian Histology. Letrozole treatment negatively affected the ovarian architecture by decreasing the number of corpus luteum and increasing cystic and atretic follicles (Figures 5(a), 5(b), 5(k), and 5(l)). Clomiphene citrate (Figures 5(c) and 5(m)), metformin (Figures 5(d) and 5(n)), and aqueous (Figures 5(e), 5(f), 5(g), 5(o), 5(p), and 5(q)) and methanolic (Figures 5(h), 5(i), 5(j), 5(r), 5(s), and 5(t)) extracts of *P. muellerianus* alleviated these detrimental effects of letrozole after 7 and 14 days of treatment. The efficacy of the plant extracts was more pronounced after 14 days of treatment.

4. Discussion

This study clearly showed that aqueous and methanolic extracts of *P. muellerianus* alleviated the reproductive and metabolic disorders in PCOS rats after 7 and 14 days of treatment. This therapeutic effect was characterized by the restoration of estrus cyclicity, the reduction of blood glucose level and oxidative stress as well as the improvement of lipid profile and sex hormones. The histological study of ovarian tissues also showed significant improvement in ovarian architecture (reduction of cystic follicles and elevated number of corpus luteum) after plant application, thus corroborating our findings that *P. muellerianus* is a potential agent for the treatment of reproductive and metabolic disorders related to PCOS.

The efficacy of letrozole (an aromatase inhibitor) in establishing PCOS in rats is well documented [11, 12]. It acts by inhibition of aromatase, leading to low conversion of androgens to estrogens, resulting in an excessive accumulation of androgens in the ovary [10]. The hormonal changes

Table 4: Effects of clomiphene citrate, metformin, aqueous and methanolic extracts of *P. mullerianus* on plasmatic LH, FSH, progesterone, estradiol, and testosterone levels after 7 and 14 days of treatment.

Groups	Doses	LH (ng/ml)	FSH (ng/ml)	Progesterone (ng/ml)	Estradiol (ng/ml)	Testosterone (ng/ml)
Control	10 ml/kg	7.79 ± 0.64	3.15 ± 0.03	56.15 ± 1.82	995 ± 195.17	1.30 ± 0.33
			7 days			
PCOS+Distilled water	10 ml/kg	24.72 ± 0.81*	3.28 ± 0.11	16.65 ± 1.14***	169 ± 20.98***	7.54 ± 1.31***
PCOS+Clomiphene citrate	2 mg/kg	17.98 ± 1.62	3.26 ± 0.03	16.71 ± 1.76***	858.75 ± 45.24$^{\#\#\#}$	4.95 ± 0.36
PCOS+Metformin	500 mg/kg	13.04 ± 0.83$^{\#\#\#}$	3.31 ± 0.16	32.86 ± 3.1$^{\#}$	645 ± 85.51$^{\#\#\#}$	1.85 ± 0.33$^{\#\#}$
PCOS+Aqueous extract	30 mg/kg	14.71 ± 1.21$^{\#\#\#}$	3.34 ± 0.10	37.39 ± 2.44$^{\#\#\#}$	332.5 ± 20.23	5.59 ± 1.10
PCOS+Aqueous extract	60 mg/kg	11.86 ± 0.94$^{\#\#\#}$	3.37 ± 0.05	44.56 ± 4.13$^{\#\#\#}$	648.5 ± 108.98$^{\#\#}$	2.61 ± 0.71$^{\#}$
PCOS+Aqueous extract	120 mg/kg	9.58 ± 0.13$^{\#\#\#}$	3.41 ± 0.02	17.56 ± 2.12***	306.5 ± 12.16	3.16 ± 0.67$^{\#}$
PCOS+Methanolic extract	30 mg/kg	9.25 ± 0.32$^{\#\#\#}$	3.23 ± 0.13	19.82 ± 2.24***	273.5 ± 8.42	6.45 ± 1.30
PCOS+Methanolic extract	60 mg/kg	9.77 ± 0.36$^{\#\#\#}$	2.97 ± 0.02	29.15 ± 1.09	415.75 ± 38.01	2.17 ± 0.32$^{\#\#}$
PCOS+Methanolic extract	120 mg/kg	11.84 ± 1.34$^{\#\#\#}$	3.29 ± 0.12	16.65 ± 1.78***	335 ± 21.20	3.15 ± 0.76$^{\#}$
			14 days			
PCOS+Distilled water	10 ml/kg	22.62 ± 2.12*	2.98 ± 0.05	15.74 ± 2.46***	294.75 ± 12.29***	8.62 ± 0.89***
PCOS+Clomiphene citrate	2 mg/kg	19.71 ± 2.86	3.22 ± 0.18	20.86 ± 1.83	782.5 ± 73.30$^{\#\#}$	4.14 ± 1.57
PCOS+Metformin	500 mg/kg	18.3 ± 2.34	2.93 ± 0.06	38.32 ± 3.59$^{\#\#}$	429 ± 27.99	1.49 ± 0.37$^{\#\#\#}$
PCOS+Aqueous extract	30 mg/kg	21.76 ± 2.52	3.03 ± 0.07	17.59 ± 1.89***	521 ± 84.79	4.05 ± 1.87$^{\#}$
PCOS+Aqueous extract	60 mg/kg	12.58 ± 1.30$^{\#\#\#}$	3.10 ± 0.05	43.61 ± 4.38$^{\#\#\#}$	608.5 ± 64.45$^{\#\#}$	1.92 ± 0.27$^{\#\#}$
PCOS+Aqueous extract	120 mg/kg	6.8 ± 1.39$^{\#\#\#}$	3.13 ± 0.10	30.60 ± 1.39	225.5 ± 35.25	1.53 ± 0.34$^{\#\#\#}$
PCOS+Methanolic extract	30 mg/kg	9.87 ± 0.29$^{\#\#\#}$	3.30 ± 0.07	23.87 ± 4.33	191.25 ± 4.87	1.70 ± 0.34$^{\#\#\#}$
PCOS+Methanolic extract	60 mg/kg	14.17 ± 1.63$^{\#\#\#}$	2.97 ± 0.11	36.32 ± 3.62$^{\#\#}$	363.75 ± 68.93	1.54 ± 0.36$^{\#\#\#}$
PCOS+Methanolic extract	120 mg/kg	9.51 ± 0.14$^{\#\#\#}$	3.05 ± 0.02	30.22 ± 3.96	275 ± 9.78	3.02 ± 0.11$^{\#}$

The values are expressed as mean ± SEM; number of rats per group=6. PCOS: polycystic ovarian syndrome. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$: significantly different compared with control. $^{\#}p<0.05$; $^{\#\#}p <0.01$; $^{\#\#\#}p <0.001$: significantly different compared with PCOS rats treated with distilled water.

negatively affect or stop the maturation of follicles, leading to anovulation [37]. Letrozole-induced PCOS model is a good method because animals developed many characteristics of human PCOS, including hyperandrogenism and abnormal follicles [11], hyperglycemia [13], and oxidative stress [9]. As expected, we observed in the current study that letrozole easily induced PCOS in rats after 21 days of continuous administration. These findings are similar to other reports [11, 14, 38].

The estrus cycle is negatively affected in rats with PCOS, mainly due to the alteration of steroid hormones, which regulate ovarian function [39]. In the present study, PCOS rats had irregular estrus cyclicity whereas control rats exhibited a regular one at the end of the induction period. Similarly, Rajan et al. [38] and Yang et al. [14] reported that letrozole-induced PCOS in rats is associated with prolonged estrus cycle. Interestingly, 44.44% of PCOS rats administered with clomiphene citrate had a regular estrus cycle after 14 days of treatment. This result is evident because clomiphene citrate is a first-line treatment for patients with PCOS. Treatment of PCOS rats with aqueous or methanolic extract of *P. muellerianus* restored the estrous cyclicity possibly by modulating the aromatization of androgens into estrogen, by lowering LH level, by improving circulating estradiol concentration and inducing ovulation.

Hyperglycemia is also considered as an important indicator of PCOS. The blood glucose level was significantly elevated in PCOS rats, compared with control. The insulin resistance and low glucose tolerance created by letrozole are mainly due to elevated androgen concentrations as reported by Desai et al. [40]. The effectiveness of metformin in the treatment of anovulatory infertility among patients with PCOS has been proven [41]. As reported in the literature [42], we observed in the current study that metformin-treated rats displayed a significantly decrease in blood glucose level, compared with untreated PCOS rats. Remarkably, *P. muellerianus* also decreased the glucose levels after treatment. We could therefore suggest that *P. muellerianus* reduces glucose resistance by controlling glucose homeostasis, improving insulin secretion and potentiating the insulin-mediated uptake of glucose.

PCOS has been linked to dyslipidemia. In untreated PCOS group, a significant reduction in HDL cholesterol, but elevated triglycerides, total cholesterol, LDL cholesterol and VLDL cholesterol were recorded, compared with control. These parameters were significantly improved after *P. muellerianus* application. These findings are similar to those of Romualdi et al. [43] and Rafraf et al. [44] on soy isoflavone and chamomile tea extract, respectively. The hyperlipidemia observed in untreated PCOS rats was also correlated with an upward trend in body weight and a significant increase in ovarian weight which could be due to the anabolic properties of letrozole, associated with fat accumulation and multiple cysts formation in the ovary, respectively [45]. The significant reduction in ovarian weight and elevated uterus weight in PCOS rats treated with plant extracts could indicate normal follicle formation and uterotonic effects of *P. muellerianus*. However, further studies are required to clarify these effects.

It has been reported that the antioxidant enzymes decline in patients with PCOS [46]. In the present study, notable changes in oxidative stress parameters were displayed in PCOS group. In this group, a significant increase in MDA level, but reduction in catalase, total peroxidases and SOD activities were noticed, compared with control. Jahan et al. [42] also demonstrated that letrozole-induced PCOS in rats is associated with oxidative stress in the ovaries. In the ovaries of PCOS rats, the oxidative stress-related biochemical parameters were significantly improved by *P. muellerianus*. Similarly, the efficacy of *Phoenix dactylifera*, *Clonorchis sinensis*, *Mentha piperita,* and *Thymus vulgaris* commonly used for the treatment of PCOS symptoms is linked to their antioxidant potentials and their ability to improve sex hormones and restore ovary function [46].

Measurement of sex hormone levels (especially testosterone, LH, and estradiol) is recommended for diagnosing PCOS [47]. Indeed, elevated serum testosterone and LH concentrations and low estradiol, progesterone, and FSH levels are the most consistent hormonal features to diagnose PCOS in woman [47]. In this study, letrozole-induced PCOS rats showed high LH and testosterone levels, but low estradiol and FSH concentrations, compared with control. These results matched those of some researchers [48, 49] and further confirm the PCOS condition. Of great interest, *P. muellerianus* treatment alleviated hyperandrogenism in rats as evidenced by significant decreased testosterone and LH levels after 7 and 14 days of oral administration, which could promote follicular development and induce ovulation. In parallel, *Ecklonia cava* extract improves sex hormones in PCOS rats by significantly upregulating FSH concentration and downregulating testosterone and LH levels [14]. Moreover, Bardei [50] demonstrated that rasp berry fruit extract induced a significant reduction in testosterone level and restored ovarian function in PCOS rats, due to its antioxidant potential. *Vitex agnus-castus* fruit extract also exhibited an antiandrogenic effect in PCOS rats by increasing aromatase process (increasing the conversion of testosterone into estradiol) leading to low testosterone level [51]. Because abnormal increased in testosterone level contributes to the pathogenesis of PCOS [11], the downregulation of this hormone after *P. muellerianus* treatment may have beneficial effects on reproductive disorders in PCOS. As observed in the current study, a decline in estrogen level in untreated PCOS rats is correlated with multiple cysts formation and low number of corpus luteum in the ovary [52]. However, *P. muellerianus* improved estradiol level after treatment. Since the abnormal increase in estradiol level is correlated with multiple cysts formation, abnormal estrus cyclicity and anovulation [53], the improvement in estradiol level after treatment with *P. muellerianus* may suggest a beneficial effect on ovarian function. The improvement in LH, estradiol, and testosterone levels by metformin corroborated previous studies which demonstrated that this drug improves ovarian-related markers and induces ovulation in mice with PCOS [54, 55].

PCOS has been reported to be associated with ovarian damage [42]. The ovary sections of PCOS rats revealed many cystic and atretic follicles and low number of corpus luteum

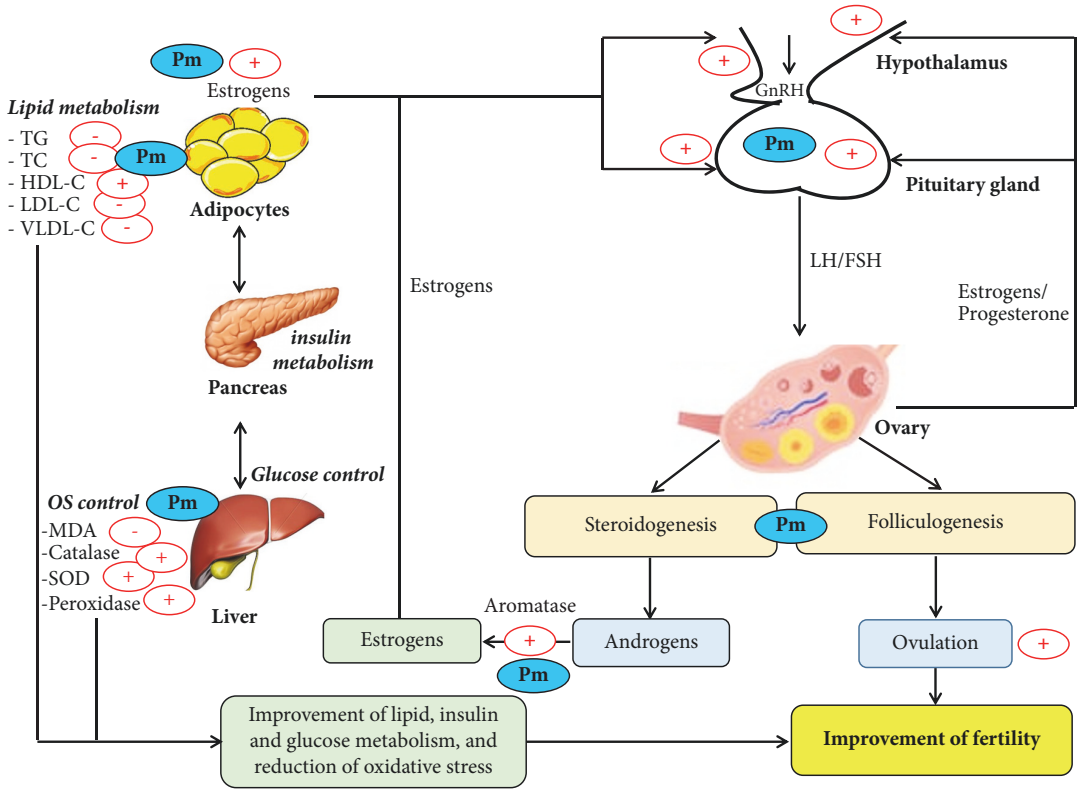


Figure 6: *Proposed mechanism of action of P. mullerianus.* Pm: *Phyllanthus muellerianus*; TG: triglycerides; TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; VLDL-C: very low density lipoprotein cholesterol; MDA: malondialdehyde; SOD: superoxide dismutase; GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle stimulating hormone. + = stimulation; - = inhibition.

[42, 56]. Moreover, the size of cystic follicles was larger than that of other follicles which can be correlated to the increase levels of intraovarian androgens [57]. Thickness of granulosa layer was lowered while that of theca layer was increased in different follicles of untreated PCOS rats. These detrimental effects of letrozole on ovary architecture were corrected by *P. muellerianus* extracts, probably due to its antioxidant and antiandrogenic properties.

To the best of our knowledge, the present study is the first to illustrate the beneficial effect of *P. muellerianus* in PCOS rats. Based on the results obtained, it could be proposed that *P. muellerianus* acts by (1) modulating the pulsatile release of GnRH, LH, and FSH, (2) amplifying the aromatization of androgens into estrogens, and (3) stimulating estrogens production by adipocytes, responsible for the restoration of estrus cyclicity and ovulation induction. Additionally, the beneficial effects of *P. muellerianus* could be mediated through its antihyperglycemic and antioxidant potentials (Figure 6). However, more mechanistic studies (transcription factor for instance) are needed to further support this mechanism.

5. Conclusion

The present study confirmed that letrozole-induced PCOS in rats is associated with reproductive and metabolic disorders. *P. muellerianus* restores estrus cyclicity, reduces blood glucose level and oxidative stress, improves lipid profile and sex hormones, and prevents ovarian damage in PCOS rats after 7 and 14 days of treatment. This plant might be considered as an alternative therapeutic remedy to treat reproductive and metabolic disorders in patients with PCOS.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

The present work was carried out with authors' personal funds.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Pierre Watcho, Eveline Christiane Ndeingang, and Patrick Brice Defo Deeh participated in the study design. Eveline

Christiane Ndeingang and Patrick Brice Defo Deeh collected the data and carried out the statistical analysis. Pierre Watcho, Eveline Christiane Ndeingang, Patrick Brice Defo Deeh, and Albert Kamanyi drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are grateful to the University of Dschang Cameroon, for the research facilities.

References

[1] H. Teede, A. Deeks, and L. Moran, "Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan," *BMC Medicine*, vol. 8, article 41, 2010.

[2] A. Swaroop, A. S. Jaipuriar, S. K. Gupta et al., "Efficacy of a novel fenugreek seed extract (Trigonella foenum-graecum, furocyst™) in polycystic ovary syndrome (PCOS)," *International Journal of Medical Sciences*, vol. 12, no. 10, pp. 825–831, 2015.

[3] B. M. Hassanzadeh, S. A. Emami, N. Mousavifar et al., "Effects seeds extract on insullin resistance in women with polycystic ovarian syndrome," *Iranian Journal of Pharmaceutical Research*, vol. 12, no. 2, pp. 475–481, 2013.

[4] B. Imani, M. J. C. Eijkemans, E. R. Te Velde, and B. C. J. M. Fauser, "A nomogram to predict the probability of live birth after clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility," *Fertility and Sterility*, vol. 77, no. 1, pp. 91–97, 2002.

[5] S. Franks, J. Stark, and K. Hardy, "Follicle dynamics and anovulation in polycystic ovary syndrome," *Human Reproduction Update*, vol. 14, no. 4, pp. 367–378, 2008.

[6] C. M. Burt Solorzano, J. P. Beller, M. Y. Abshire, J. S. Collins, C. R. McCartney, and J. C. Marshall, "Neuroendocrine dysfunction in polycystic ovary syndrome," *Steroids*, vol. 77, no. 4, pp. 332–337, 2012.

[7] C. R. McCartney, C. A. Eagleson, and J. C. Marshall, "Regulation of gonadotropin secretion: implications for polycystic ovary syndrome," *Seminars in Reproductive Medicine*, vol. 20, no. 4, pp. 317–326, 2002.

[8] M. Murri, M. Luque-Ramírez, M. Insenser, M. Ojeda-Ojeda, and H. F. Escobar-Morreale, "Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis," *Human Reproduction Update*, vol. 19, no. 3, pp. 268–288, 2013.

[9] T. Zuo, M. Zhu, and W. Xu, "Roles of oxidative stress in polycystic ovary syndrome and cancers," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 8589318, 14 pages, 2016.

[10] J. A. Garcia-Velasco, L. Moreno, A. Pacheco et al., "The aromatase inhibitor letrozole increases the concentration of intraovarian androgens and improves in vitro fertilization outcome in low responder patients: a pilot study," *Fertility and Sterility*, vol. 84, no. 1, pp. 82–87, 2005.

[11] H. Kafali, M. Iriadam, I. Ozardali, and N. Demir, "Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease," *Archives of Medical Research*, vol. 35, no. 2, pp. 103–108, 2004.

[12] Y. Lee, H. Yang, S. Lee, S. Kwon, E. Hong, and H. Lee, "Welsh onion root (*Allium fistulosum*) restores ovarian functions from letrozole induced-polycystic ovary syndrome," *Nutrients*, vol. 10, no. 10, p. 1430, 2018.

[13] J.-P. Zhu, Y.-C. Teng, J. Zhou, W. Lu, M.-F. Tao, and W.-P. Jia, "Increased mean glucose levels in patients with polycystic ovary syndrome and hyperandrogenemia as determined by continuous glucose monitoring," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 92, no. 2, pp. 165–171, 2013.

[14] H. Yang, S. Y. Lee, S. R. Lee et al., "Therapeutic effect of Ecklonia cava extract in letrozole-induced polycystic ovary syndrome rats," *Frontiers in Pharmacology*, vol. 9, p. 1325, 2018.

[15] H. M. Burkill, *The Useful Plants of West Tropical Africa*, vol. 2, Families E-1 Royal Botanic Gardens, Kew, Richmond, UK, 2nd edition, 1995.

[16] U. A. Katsayal and R. S. Lamal, "Preliminary phytochemical and antibacterial screening of the ethanolic stem bark extract of *Phyllanthus muellerianus*," *Nigerian Journal of Pharmaceutical Sciences*, vol. 8, no. 2, pp. 121–125, 2009.

[17] C. Agyare, M. Lechtenberg, A. Deters, F. Petereit, and A. Hensel, "Ellagitannins from *Phyllanthus muellerianus* (Kuntze) Exell.: Geraniin and furosin stimulate cellular activity, differentiation and collagen synthesis of human skin keratinocytes and dermal fibroblasts," *Phytomedicine*, vol. 18, no. 7, pp. 617–624, 2011.

[18] A. A. Adeneye, "The leaf and seed aqueous extract of *Phyllanthus amarus* improves insulin resistance diabetes in experimental animal studies," *Journal of Ethnopharmacology*, vol. 144, no. 3, pp. 705–711, 2012.

[19] X. Mao, L.-F. Wu, H.-L. Guo et al., "The genus *Phyllanthus*: an ethnopharmacological, phytochemical, and pharmacological review," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 7584952, 36 pages, 2016.

[20] Y. Boakye and C. Agyare, "Antimicrobial and antioxidant activities of geraniin and aqueous leaf extract of Phyllanthus muellerianus (Kuntze) Exell," *Planta Medica*, vol. 79, no. 13, 2013.

[21] K. D. Ben-Bala, "*Phyllanthus muellerianus* (Kuntze) Exell," in *PROTA (Plant Resources of Tropical Africa/Ressources Végétales de L'Afrique Tropicale)*, G. H. Schmelzer and A. Gurib-Fakim, Eds., Wageningen, Netherlands, 2008.

[22] EEC, "Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes," *Offical Journal of the European Communities*, vol. 358, pp. 1–29, 1986.

[23] H. Ghafurniyan, M. Azarnia, M. Nabiuni, and L. Karimzadeh, "The effect of green tea extract on reproductive improvement in estradiol valerate-induced polycystic ovary polycystic ovarian syndrome in rat," *Iranian Journal of Pharmaceutical Research*, vol. 14, no. 4, pp. 1215–1223, 2015.

[24] M. A. Rezvanfar, M. A. Rezvanfar, A. Ahmadi, H. A. Shojaei-Saadi, M. Baeeri, and M. Abdollahi, "Molecular mechanisms of a novel selenium-based complementary medicine which confers protection against hyperandrogenism-induced polycystic ovary," *Theriogenology*, vol. 78, no. 3, pp. 620–631, 2012.

[25] E. Ngadjui, P. A. Nkeng-Efouet, T. B. Nguelefack, A. Kamanyi, and P. Watcho, "High fat diet-induced estrus cycle disruption: effects of Ficus asperifolia," *Journal of Complementary and Integrative Medicine*, vol. 12, no. 3, pp. 205–215, 2015.

[26] S. K. Kulkarni, *Hand Book of Experimental Pharmacology*, New Delhi, India, 1999.

[27] C. K. Fofié, E. P. Nguelefack-Mbuyo, N. Tsabang, A. Kamanyi, and T. B. Nguelefack, "Hypoglycemic properties of the aqueous extract from the stem bark of ceiba pentandra in dexamethasone-induced insulin resistant rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 4234981, 11 pages, 2018.

[28] W. T. Friedewald, R. I. Levy, and D. S. Fredrickson, "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clinical Chemistry*, vol. 18, no. 6, pp. 499–502, 1972.

[29] T. J. Youmbissi, S. Djoumessi, C. Nouedoui et al., "Lipid profile of a group of hypertensive Cameroonians black African. [Profil lipidique d'un groupe d'hypertendus camerounais noirs africains]," *Médecine d'Afrique Noire*, vol. 48, pp. 305–314, 2001.

[30] M. G. Ropelato, M. C. G. Rudaz, M. E. Escobar et al., "Acute effects of testosterone infusion on the serum luteinizing hormone profile in eumenorrheic and polycystic ovary syndrome adolescents," *The Journal of Clinical Endocrinology & Metabolism*, vol. 94, no. 9, pp. 3602–3610, 2009.

[31] M. A. Rezvanfar, M. A. Rezvanfar, A. R. Shahverdi et al., "Protection of cisplatin-induced spermatotoxicity, DNA damage and chromatin abnormality by selenium nano-particles," *Toxicology and Applied Pharmacology*, vol. 266, no. 3, pp. 356–365, 2013.

[32] D. M. Olszewska-Słonina, D. Mątewski, R. Czajkowski et al., "The concentration of thiobarbituric acid reactive substances (TBARS) and paraoxonase activity in blood of patients with osteoarthrosis after endoprosthesis implantation," *Medical Science Monitor*, vol. 17, no. 9, pp. CR498–CR504, 2011.

[33] D. Giustarini, P. Fanti, E. Matteucci, and R. Rossi, "Micromethod for the determination of glutathione in human blood," *Journal of Chromatography B*, vol. 964, pp. 191–194, 2014.

[34] J. A. Serra, E. R. Marschoff, R. O. Domínguez et al., "Comparison of the determination of superoxide dismutase and antioxidant capacity in neurological patients using two different procedures," *Clinica Chimica Acta*, vol. 301, no. 1-2, pp. 87–102, 2000.

[35] M. H. Hadwan, "New method for assessment of serum catalase activity," *Indian Journal of Science and Technology*, vol. 9, no. 4, 2016.

[36] I. O. Gozukara, N. Pınar, O. Ozcan et al., "Effect of colchicine on polycystic ovary syndrome: an experimental study," *Archives of Gynecology and Obstetrics*, vol. 293, no. 3, pp. 675–680, 2016.

[37] M. Gopal, S. Duntley, M. Uhles, and H. Attarian, "The role of obesity in the increased of obstructive sleep apnea syndrome in patients with polycystic ovarian syndrome," *Sleep Medicine*, vol. 3, no. 5, pp. 401–404, 2002.

[38] R. K. Rajan, S. S. Kumar M., and B. Balaji, "Soy isoflavones exert beneficial effects on letrozole-induced rat polycystic ovary syndrome (PCOS) model through anti-androgenic mechanism," *Pharmaceutical Biology*, vol. 55, no. 1, pp. 242–251, 2017.

[39] J. Sun, C. Jin, H. Wu et al., "Effects of electro-acupuncture on ovarian P450arom, P450c17α and mRNA expression induced by letrozole in PCOS rats," *PLoS ONE*, vol. 8, no. 11, p. e79382, 2013.

[40] N. R. Desai, W. H. Shrank, M. A. Fischer et al., "Patterns of medication initiation in newly diagnosed diabetes mellitus: quality and cost implications," *American Journal of Medicine*, vol. 125, no. 3, pp. 1–7, 2012.

[41] N. P. Johnson, "Metformin use in women with polycystic ovary syndrome," *Annals of Translational Medicine*, vol. 2, no. 6, pp. 56–61, 2014.

[42] S. Jahan, F. Munir, S. Razak et al., "Ameliorative effects of rutin against metabolic, biochemical and hormonal disturbances in polycystic ovary syndrome in rats," *Journal of Ovarian Research*, vol. 9, no. 1, p. 86, 2016.

[43] D. Romualdi, B. Costantini, G. Campagna, A. Lanzone, and M. Guido, "Is there a role for soy isoflavones in the therapeutic approach to polycystic ovary syndrome? Results from a pilot study," *Fertility and Sterility*, vol. 90, no. 5, pp. 1826–1833, 2008.

[44] M. Rafraf, M. Zemestani, and M. Asghari-Jafarabadi, "Effectiveness of chamomile tea on glycemic control and serum lipid profile in patients with type 2 diabetes," *Journal of Endocrinological Investigation*, vol. 38, no. 2, pp. 163–170, 2015.

[45] N. Samy, M. Hashim, M. Sayed et al., "Clinical significance of inflammatory markers in polycystic ovary syndrome: their relationship to insulin resistance and body mass index," *Disease Markers*, vol. 26, no. 4, pp. 163–170, 2009.

[46] Z. Abasian, A. Rostamzadeh, M. Mohammadi, M. Hosseini, and M. Rafieian-kopaei, "A review on role of medicinal plants in polycystic ovarian syndrome: pathophysiology, neuroendocrine signaling, therapeutic status and future prospects," *Middle East Fertility Society Journal*, vol. 23, pp. 255–262, 2018.

[47] S. Bednarska and A. Siejka, "The pathogenesis and treatment of polycystic ovary syndrome: what's new?" *Advances in Clinical and Experimental Medicine*, vol. 26, no. 2, pp. 359–367, 2017.

[48] F. Orio, F. Giallauria, S. Palomba et al., "Metabolic and cardiopulmonary effects of detraining after a structured exercise training programme in young PCOS women," *Clinical Endocrinology*, vol. 68, no. 6, pp. 976–981, 2008.

[49] S. Palomba, F. Giallauria, A. Falbo et al., "Structured exercise training programme versus hypocaloric hyperproteic diet in obese polycystic ovary syndrome patients with anovulatory infertility: a 24-week pilot study," *Human Reproduction*, vol. 23, no. 3, pp. 642–650, 2008.

[50] K. Bardei, "The effects of hydro-alcoholic extract of raspberry fruit on ovarian follicles and serum parameters in poly cystic ovary syndrome-induced rat," *Armaghane Danesh*, vol. 19, pp. 955–968, 2015.

[51] G. Jelodar and K. Askari, "Effect of Vitex agnus-castus fruits hydroalcoholic extract on sex hormones in rat with induced polycystic ovary syndrome (PCOS)," *Physiology and Pharmacology*, vol. 16, no. 1, pp. 62–69, 2012.

[52] D. Dewailly, G. Robin, M. Peigne, C. Decanter, P. Pigny, and S. Catteau-Jonard, "Interactions between androgens, FSH, anti-Mullerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary," *Human Reproduction Update*, vol. 22, no. 6, pp. 709–724, 2016.

[53] E. Diamanti-Kandarakis, C. Christakou, and H. Kandarakis, "Polycystic ovarian syndrome: the commonest cause of hyperandrogenemia in women as a risk factor for metabolic syndrome," *Minerva Endocrinologica*, vol. 32, no. 1, pp. 35–47, 2007.

[54] V. Sander, C. G. Luchetti, M. E. Solano et al., "Role of the N, N′-dimethylbiguanide metformin in the treatment of female prepuberal BALB/c mice hyperandrogenized with dehydroepiandrosterone," *Reproduction*, vol. 131, no. 3, pp. 591–602, 2006.

[55] N. Johnson, "Metformin is a reasonable first-line treatment option for non-obese women with infertility related to anovulatory polycystic ovary syndrome - a meta-analysis of randomised trials," *Australian and New Zealand Journal of Obstetrics and Gynaecology*, vol. 51, no. 2, pp. 125–129, 2011.

[56] J. C. M. Riley and H. R. Behrman, "Oxygen radicals and reactive oxygen species in reproduction," *Experimental Biology and Medicine*, vol. 198, no. 3, pp. 781–791, 1991.

[57] V. Mahesh, T. Mills, C. Bagnell et al., "Animal models for study of polycystic ovaries and ovarian atresia," in *Regulation of Ovarian and Testicular Function*, V. B. Mahesh, D. S. Dhindsa, E. Anderson, and S. P. Kalra, Eds., vol. 219 of *Advances in Experimental Medicine and Biology*, Springer, Boston, MA, USA, 1987.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 3104741, 7 pages
https://doi.org/10.1155/2019/3104741

Research Article

Uncovering the Pharmacological Mechanism of *Chaibei Zhixian* Decoction on Epilepsy by Network Pharmacology Analysis

Jian Zhang,[1] Chenglong Zheng,[2] Siyuan Yuan,[1] Xiaoke Dong,[3] Le Wang,[3] Yong Wang,[4] Wei Wang,[1] Kuo Gao,[3] and Jinmin Liu[3]

[1]*School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing 100029, China*
[2]*Beijing Gulou Hospital of Traditional Chinese Medicine, Beijing, 10009, China*
[3]*Dongfang Hospital, Beijing University of Chinese Medicine, Beijing, 100078, China*
[4]*School of Life Science, Beijing University of Chinese Medicine, Beijing 100029, China*

Correspondence should be addressed to Wei Wang; wangwei@bucm.edu.cn, Kuo Gao; linfengtingchan@foxmail.com, and Jinmin Liu; jmvip@vip.163.com

Received 20 February 2019; Revised 29 March 2019; Accepted 21 April 2019; Published 12 May 2019

Guest Editor: José C. T. Carvalho

Objective. Epilepsy is a neuronal disorder that is characterized by epileptic seizures and linked with abnormal neural functioning in the brain. Traditional Chinese medicine (TCM) formula *Chaibei Zhixian* decoction (CZD) has been widely used for epilepsy in China while the pharmacological mechanisms are still unclear. In the present study, systematic and comprehensive network pharmacology was utilized for the first time to reveal the potential pharmacological mechanisms of CZD on epilepsy. *Methods*. Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and analysis platform was utilized for the development of an ingredients-targets database. After identifying epileptic targets of CZD, their interaction with other proteins was estimated based on protein-protein interaction network created from STITCH and gene ontology (GO) enrichment analysis utilizing Cytoscape-ClueGO plugin. *Results*. CZD formula was found to have 643 chemical ingredients, and the potential protein targets of these ingredients were 5230, as retrieved from TCMSP database. Twenty-six protein targets were found to be associated with epilepsy. Thirteen hub genes were regulated by CZD in epilepsy, including estradiol, ESR1, ESR2, SRC, CTNNB1, EP300, MAPK1, MAPK3, SP1, BRCA1, NCOA3, CHRM1, and GSK3B. The results of GO terms analysis showed that 8 GO terms were recovered in the form of 3 clusters, including negative regulation of protein kinase B signaling, positive regulation of interleukin-1 production, and microvillus assembly. *Conclusions*. Network pharmacology approach provides better understanding of the underlying pharmacological mechanisms of CZD on epilepsy. Estradiol, ESR1, ESR2, CTNNB1, EP300, MAPK1, MAPK3, BRCA1, and GSK3B are likely to be important molecules regulated by CZD in treatment of epilepsy. Negative regulation of protein kinase B signaling may play vital roles in the treatment of epilepsy by CZD.

1. Introduction

Epilepsy is a complex disorder involving neurological alterations that lead to the pathological development of recurrent seizures [1, 2]. Epilepsy affects millions of people worldwide and approximately one-third of patients suffer from cognitive impairment, particularly memory disruption [1, 3, 4]. First-line antiepileptic drugs have been given priority in the clinical treatment of epileptic seizures [1]. However, the risk of adverse effects from antiepileptic drugs is considerable and includes potential cognitive and behavioral effects [5]. Therefore, strategies that reduce the side effects of antiepileptic drugs or develop new drugs are urgently needed for epilepsy therapies.

Traditional Chinese medicine (TCM) has a long history in prevention and treatment of epilepsy in China [6, 7]. *Chaibei Zhixian* decoction (CZD), composed of Radix Bupleuri, Bulbus Fritillariae Thunbergii, Rhizoma Gastrodiae, Rhizoma Pinelliae, Rhizoma Acori Tatarinowii, Concha Ostreae, and Pheretima in a 4:3:5:3:3:10:2 ratio (Table 1), has

Table 1: Pharmaceutical ingredients of *Chaibei Zhixian* decoction.

Latin name	Species	Family	Part used
Radix Bupleuri	*Bupleurum Chinese* DC. *Bupleurum scorzonerifolium* Willd.	*Umbelliferae*	Roots
Bulbus Fritillariae Thunbergii	*Fritillaria thunbergii* Miq.	*Liliaceae*	Bulbs
Rhizoma Gastrodiae	*Gastrodia elata* Bl.	*Orchidaceae*	Rhizomes
Rhizoma Pinelliae	*Pinellia ternata* (Thunb.) Breit.	*Araceae*	Rhizomes
Rhizoma Acori Tatarinowii	*Acorus tatarinowii* Schott.	*Araceae*	Rhizomes
Concha Ostreae	*Ostrea gigas* Thunb. *Ostrea talienwhanensis* Crosse *Ostrea rivularis* Gould	*Ostreidae*	Concha
Pheretima	*Pheretima aspergillum* (E. Perrier)	*Megascolecidae*	Bodies

The ratio of these herbs was 4:3:5:3:3:10:2

been widely used in clinical treatment of epilepsy in China. Clinical study has shown that CZD is safe and effective for intractable epilepsy [8]. In addition, the combination of CZD with first-line antiepileptic drugs could reduce side effects and increase curative effects [9]. Some experimental studies have found CZD to have therapeutic effects on epilepsy by regulating multidrug resistance-associated protein 1, nuclear factor-kappa B, breast cancer resistance protein, and p-glycoprotein [10–13]. These studies all use traditional research method of single-drug, single-target, and single-pathway, but the TCM formula CZD has the characteristics of being multicomponent, multitarget, and multipathway. Thus, a new comprehensive and systematic evaluation of the pharmacological mechanism of CZD on epilepsy is critically needed.

Network pharmacology, including chemoinformatics, bioinformatics, network biology, and pharmacology, is a comprehensive method to uncover the bioactive components and potential mechanisms of TCM formulas from a systemic perspective [14]. In this study, the potential pharmacological mechanisms of CZD on epilepsy have been probed using network pharmacology, drug-target interaction databases, and a biological process analysis.

2. Methods

The first step of this study involved the retrieval of CZD constituents and their target proteins in *Homo sapiens*. Then the construction of CZD-target interaction network and its analysis was accomplished by using various GO terms. Finally, to assess the molecular mechanisms of CZD effects in epilepsy, Cytoscape along with its plugin ClueGO was utilized for GO enrichment analysis, followed by the analysis of biological processes.

2.1. Chemical Search and Their Target Retrieval. CZD contains Radix Bupleuri, Bulbus Fritillariae Thunbergii, Rhizoma Gastrodiae, Rhizoma Pinelliae, Rhizoma Acori Tatarinowii, Concha Ostreae, and Pheretima (Table 1). The chemical constituents present in these seven sources as well as the protein targets of these chemicals were searched via Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://5th.tcmspw.com/tcmsp.php) [15]. The duplications in these chemical constituents from various sources and their targets were removed. From the retrieved targets, epilepsy-related targets were screened via Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) and employed for further analysis.

2.2. Conversion of Target Proteins into Network and Its Analysis. STITCH 5.0 database (http://stitch.embl.de/) [16] was utilized for analysis of the interaction among the identified protein targets to systematically investigate the mode of action of CZD. STITCH database has been furnished with extensive information regarding protein interactions. The main sources of information about these interactions are genomic model evaluations and high-throughput experimental outcomes. The information about > 9 million proteins from more than 2000 organisms has been added to this database. The mechanism of action of CZD and its essential pharmacodynamic constituents was assessed by developing a protein interaction network. The optional setting for network construction was set as follows: number of interactors = not more than 10; minimum required interaction score = 0.700 [16].

2.3. GO Terms Analysis through ClueGO Plugin. After identifying typical biological features of the protein targets, the ingredients of protein interaction network obtained from STITCH were used in ClueGO-based analysis, and GO enrichment analysis was introduced for the segmentation of target genes in a hierarchically arranged manner. ClueGO was utilized as a plugin of Cytoscape 3.4.0 software to construct [17], visualize, and evaluate protein target network and to study the biological pathways [18]. ClueGO analysis was conducted at a level of significance of 0.05. This study assumed that the network was of medium type. In contradiction to the detailed and global networks, medium network establishes GO terms belonging to GO levels 4-8, having a medium number of associated genes, and a medium

Table 2: Node degree of the targets of CZD acquired via STITCH database.

Targets	Node Degree	Targets	Node Degree
ESR1	11	GSK3B	5
Estradiol	12	MAPK3	5
SRC	10	HTR2A	4
CTNNB1	8	MAPT	3
EP300	8	AXIN1	2
ESR2	8	CHRM5	2
MAPK1	8	HTR2C	2
SP1	8	NTRK2	2
BRCA1	7	SLC6A4	2
NCOA3	6	CYP1A2	1
CHRM1	5	NTF4	1

Table 3: Nature of action of functional targets of CZD acquired via STITCH.

Functional targets	Activation	Inhibition	Binding	Phenotype	Catalysis	Post-Trans. Mod.	Reaction	Expression	Score
CTNNB1	•	•	•		•	•	•		0.999
AXIN1	•	•	•		•	•	•		0.999
NCOA3			•						0.999
SRC	•	•	•		•	•	•	•	0.999
MAPT	•	•	•			•		•	0.999
SP1	•	•	•			•		•	0.999
BRCA1			•						0.999
NTF4	•		•						0.999
Estradiol	•	•	•		•			•	0.999

percentage of uploaded genes found. Furthermore, two-sided hypergeometric test along with Bonferroni correction was used in network analysis. Lastly, organic layout algorithm was employed to visualize the functional network.

3. Results

3.1. Chemical Search and Their Target Retrieval. TCMSP search resulted in the retrieval of 643 chemical ingredients in the five herbs, Radix Bupleuri, Bulbus Fritillariae Thunbergii, Rhizoma Gastrodiae, Rhizoma Pinelliae, and Rhizoma Acori Tatarinowii, and two animals, Concha Ostreae and Pheretima. The protein targets of these 643 chemical ingredients recovered from TCMSP database were 5230 in number (Supplemental Table 1). Some studies revealed that CZD could be used for treating epilepsy [8–13]. After removing the repeated protein targets, 941 protein targets still remain (Supplemental Table 2). Among them, 26 protein targets of CZD were found to be associated with epilepsy through scanning TCMSP database (Supplemental Table 3), followed by their standardization through UniProt database mapping (http://www.uniprot.org/).

3.2. Conversion of Target Proteins into Network and Its Analysis. The systematically selected protein targets with a probabilistic confidence score of 0.700 were plotted as an interaction network (Figure 1) by using STITCH database (accessed in Oct 2018). The number of nodes and edges of this network were 21 and 48, respectively. Out of 21, there were 11 physical and 10 functional interactions. STITCH is a database of known and predicted interactions between chemicals and proteins. The interactions include direct (physical) and indirect (functional) associations; they stem from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other (primary) databases. The nodes and the edges represent protein/gene targets and their interactions, respectively. In case of random selection of nodes, the expected number of edges of the acquired PPIN was 23. The statistics of PPIN enrichment had a very small p-value (3.56E-06), indicating arbitrary nature of nodes and a significant number of edges.

The average node degree and clustering coefficient are the other important features of PPIN. Degree is a topological parameter that refers to the number of connections between a node and other nodes and can be used to describe the characteristics (particularly centrality) of nodes in the network. Node degree is a quantitative feature of a node that represents the number of linkages of a node in a network. A higher degree means a stronger correlation. The average node degree refers to the mean number of associations of a protein in a PPIN at threshold score, whereas the connectivity degree of PPIN nodes is indicated by the clustering coefficient. An increase in the clustering coefficient results in the increase in network connectivity. The average node degree and clustering coefficient values were 4.57 and 0.57, respectively. A node

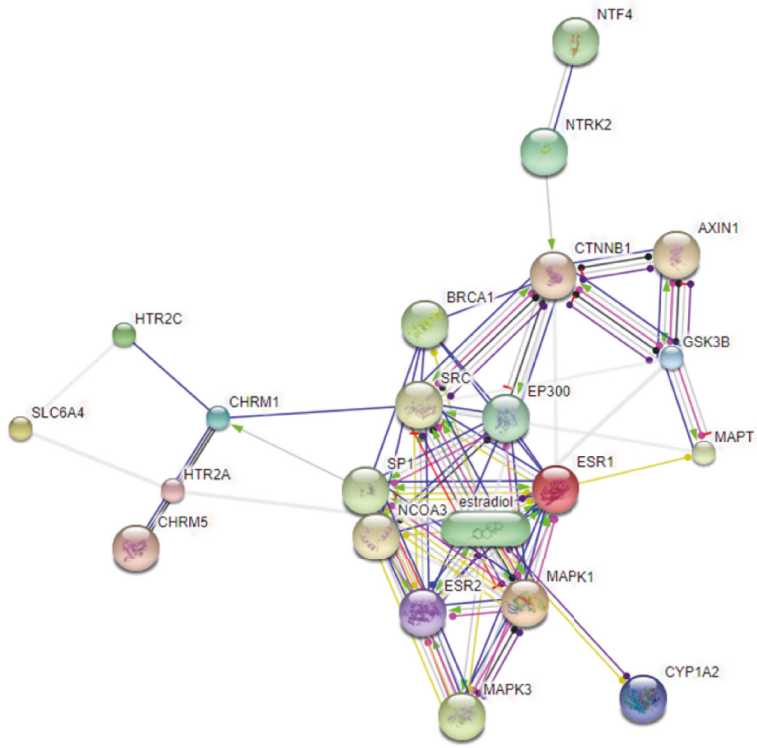


Figure 1: PPIN (action view) showing CZD targets. The colored edges indicate the nature of action, as interpreted here: activation ( ), inhibition ( ), binding ( ), catalysis ( ), phenotype ( ), posttranslational modification ( ), reaction ( ), and transcriptional regulation ( ). The effects of action are represented by the following symbols: positive ( ), negative ( ), and unspecified ( ). AXIN1: axin 1; NCOA3: nuclear receptor coactivator 3; BRCA1: breast cancer 1, early onset; CHRM1: cholinergic receptor, muscarinic 1; CHRM5: cholinergic receptor, muscarinic 5; ESR1: estrogen receptor 1; CTNNB1: catenin (cadherin-associated protein), beta 1; CYP1A2: cytochrome P450, family 1, subfamily A, polypeptide 2; ESR2: estrogen receptor 2; HTR2A: 5-hydroxytryptamine (serotonin) receptor 2A; GSK3B: glycogen synthase kinase 3 beta; MAPK1: mitogen-activated protein kinase 1; MAPK3: mitogen-activated protein kinase 3; HTR2C: 5-hydroxytryptamine (serotonin); MAPT: microtubule-associated protein tau; SP1: Sp1 transcription factor; receptor 2C; NTF4: neurotrophin 4; EP300: E1A binding protein p300; NTRK2: neurotrophic tyrosine kinase, receptor, type 2; SLC6A4: solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; SRC: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian).

is termed as hub if it has number of linkage higher than its average node degree. Thirteen hubs have higher node degree than the average node degree, including estrogen receptor 1 (ESR1), estradiol, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (SRC), catenin beta 1 (CTNNB1), E1A binding protein p300 (EP300), estrogen receptor 1 (ESR2), mitogen-activated protein kinase 1 (MAPK1), Sp1 transcription factor (SP1), breast cancer 1, early onset (BRCA1), nuclear receptor coactivator 3 (NCOA3), cholinergic receptor, muscarinic 1 (CHRM1), glycogen synthase kinase 3 beta (GSK3B), and mitogen-activated protein kinase 3 (MAPK3) (Table 2). In addition, the functional proteins except NCOA3 and BRCA1 could be activated by CZD, which, on the other hand, inhibit CTNNB1, AXIN1, SRC, MAPT, SP1, and estradiol (Table 3). All functional proteins, as listed in Table 3, can bind with CZD. Moreover, the catalysis, posttranslational modifications, reactions, and expression are also found to be affected by CZD (Table 3).

3.3. GO Terms through ClueGO Plugin. The annotation of biological functions was carried out by using GO terms and ClueGO plugin. This enrichment analysis of CZD targets resulted in the evolution of 8 GO terms which were ordered into 3 subgroups, including negative regulation of protein kinase B signaling, positive regulation of interleukin-1 production, and microvillus assembly (Table 4, Figure 2).

4. Discussion

Epilepsy affects millions of people worldwide and approximately one-third of patients suffer from cognitive deficits. Due to the side effects of first-line antiepileptic drugs, more effective treatments are still needed. The TCM formula CZD not only is safe and effective for intractable epilepsy but also reduces side effects and increase curative effects when in combination with first-line antiepileptic drugs. However, the underlying mechanism of CZD on epilepsy is still unclear and

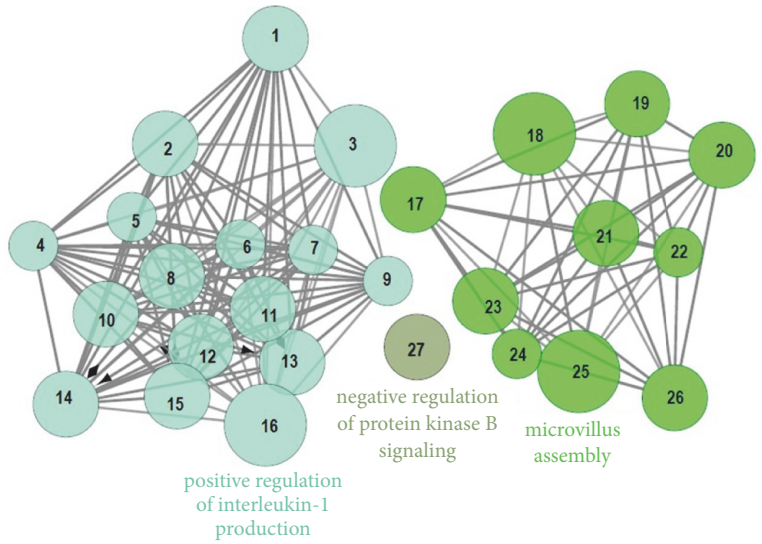


Figure 2: Targets involved in the biological effects. The most significant term in each stack is used to label the respective group. Node size is directly related to the term enrichment significance. The groups of GO terms having similar function are partially overlapped. [1-AIM2, 2-AZU1, 3-CALCA, 4-CARD8, 5-CASP1, 6-CASP5, 7-CCL19, 8-EGR1, 9-GSDMD, 10-HAVCR2, 11-HDAC2, 12-HMGB1, 13-HSPB1, 14-NOD1, 15-SMAD3, 16-TLR4, 17-ATP8B1, 18-EZR, 19-FSCN1, 20-FXYD5, 21-PLD1, 22-PRKCSH, 23-RAP1A, 24-RAPGEF2, 25-RAPGEF6, 26-SLC9A3R1, 27-PHLPP1].

Table 4: Recovery of GO terms and the associated genes.

GO ID	GO Term	Term p Value (¤)	Group p Value (¤)	Associated Genes Found
51898	Negative regulation of protein kinase B signaling	750.0E-6 (4.5E-3)	750.0E-6 (750.0E-6)	PHLPP1, SLC9A3R1
32732	Positive regulation of interleukin-1 production	6.7E-6 (140.0E-6)	64.0E-6 (190.0E-6)	AZU1, HMGB1, TLR4
30033	Microvillus assembly	1.7E-6 (39.0E-6)	420.0E-6 (840.0E-6)	RAP1A, RAPGEF2, SLC9A3R1

¤Corrected with Bonferroni step down.

remains unrevealed from a systemic point of view. Therefore, we adopted network pharmacology to further explore the mechanisms of CZD on epilepsy in this study. This systematic network pharmacology approach is a combination of various procedures, including retrieval of chemical ingredients of CZD, target search of these chemicals, development of network using these targets, and GO terms analysis. CZD formula was found to have 643 chemical ingredients, and the potential protein targets of these ingredients were 5230. Two aspects aroused our attention: first, 26 protein targets were found to be associated with epilepsy. Some of them are likely to be key molecules in the treatment of epilepsy with CZD. Second, GO terms analysis indicated that negative regulation of protein kinase B signaling, positive regulation of interleukin-1 production, and microvillus assembly have linkage with CZD treatment for epilepsy.

Network pharmacology analysis has shown that 13 hub genes were regulated by CZD in epilepsy, including estradiol, ESR1, ESR2, SRC, CTNNB1, EP300, MAPK1, MAPK3, SP1, BRCA1, NCOA3, CHRM1, and GSK3B. Among them, estradiol, ESR1, ESR2, CTNNB1, EP300, MAPK1, MAPK3, BRCA1, and GSK3B are closely related to epilepsy based on current studies. Thus, they are likely to be the main regulators of CZD in treatment of epilepsy. Estrogens affect neuronal excitability and have neuroprotective effects on seizure-induced hippocampal damage [19, 20]. Several studies have confirmed ESR was associated with epilepsy [21–24]. CTNNB1 has been implicated in epilepsy because of its altered postseizure expression [25, 26]. The dysfunction of CTNNB1-mediated signaling pathways leads to cortical malformation and increased seizure susceptibility [25]. EP300 may serve as potential targets for the treatment of epilepsy based on gene expression profile analysis of brain tissue of patients with epilepsy [27]. MAPK, as an important regulator of synaptic excitability, exerts an influence on epilepsy in animal models as well as human disease [27–30]. Interestingly, the component of CZD, gastrodin, has been reported to attenuate seizures by modulating the MAPK-associated inflammatory responses [31]. Variants in BRCA1-associated protein required for ATM activation-1

cause multifocal seizure syndrome [32, 33]. GSK3B activity protects neuronal networks from hyperactivation in response to epileptogenic stimuli [34]. Given that some main hubs of PPIN are closely related to epilepsy, they are likely to be important molecules regulated by CZD in treatment of epilepsy.

GO terms analysis revealed that negative regulation of protein kinase B signaling, positive regulation of interleukin-1 production, and microvillus assembly have linkage with CZD treatment for epilepsy. Protein kinase B, a serine/threonine-specific protein kinase, is involved in the regulation of binding phospholipids, phosphorylation, and ubiquitination but also modulates a wide array of cellular processes including cell apoptosis, metabolism, and proliferation [35]. Activation of the Akt signaling could alleviate neuronal apoptosis and oxidative stress [36]. The other two GO terms are positive regulation of interleukin-1 production and microvillus assembly. The former term represents a process in which the production of interleukin-1 is positively regulated, while the latter refers to the formation of microvillus. The positive regulation of interleukin-1 production has been found to be linked with three genes (i.e., AZU1, HMGB1, and TLR4). Microvillus assembly is regulated under the effect of three genes such as RAP1A, RAPGEF2, and SLC9A3R1. It has been reported that microvilli-like entities are linked with the inward movement of lethal capsulated neisseria meningitidis into vascular endothelial cells that may affect BBB resulting in seizures [37, 38].

In short, the present study has suggested various modes of CZD action against epilepsy, revealing that CZD profoundly enhances the performance of target genes involved in inhibiting epilepsy. The limitation of this study is that the bioactive components and targets found by network pharmacology analysis are the result of theoretical predictions and they should be verified by experiments. Further study will focus on using animal experiments and clinical trials to verify the hypothesis.

5. Conclusion

Network pharmacology analysis provides better understanding of the underlying pharmacological mechanisms of CZD on epilepsy. Our results revealed that estradiol, ESR1, ESR2, CTNNB1, EP300, MAPK1, MAPK3, BRCA1, and GSK3B are likely to be important molecules regulated by CZD in treatment of epilepsy. In addition, negative regulation of protein kinase B signaling may play vital roles in the treatment of epilepsy by CZD.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kuo Gao, Jinmin Liu, and Wei Wang conceived and designed the project. Jian Zhang, Chenglong Zheng, Siyuan Yuan, Le Wang, and Xiaoke Dong implemented the methods and conducted the analysis. Kuo Gao, Jian Zhang, and Yong Wang drafted the manuscript. Jinmin Liu and Wei Wang revised the manuscript. All authors read and approved the final manuscript. Jian Zhang and Chenglong Zheng contributed equally to this work.

Acknowledgments

This study was financially supported by National Natural Science Foundation of China (nos. 81373586 and 81774277), Young Teacher Project of Beijing University of Chinese Medicine (2019-JYB-JS-095), and China Postdoctoral Science Foundation (2018M641286).

Supplementary Materials

Supplementary 1. Supplemental Table 1: 5230 protein targets from the TCMSP database.

Supplementary 2. Supplemental Table 2: nonrepetitive 941 protein targets from the TCMSP database.

Supplementary 3. Supplemental Table 3: 26 protein targets of CZD associated with epilepsy through scanning TCMSP database.

References

[1] H. Yang, R. Zhang, C. Jia et al., "Neuronal protective effect of Songling Xuemaikang capsules alone and in combination with carbamazepine on epilepsy in kainic acid-kindled rats," *Pharmaceutical Biology*, vol. 57, no. 1, pp. 22–28, 2019.

[2] A. Bui, H. K. Kim, M. Maroso, and I. Soltesz, "Microcircuits in epilepsy: heterogeneity and hub cells in network synchronization," *Cold Spring Harbor Perspectives in Medicine*, vol. 5, no. 11, 2015.

[3] B. Bell, J. J. Lin, M. Seidenberg, and B. Hermann, "The neurobiology of cognitive disorders in temporal lobe epilepsy," *Nature Reviews Neurology*, vol. 7, no. 3, pp. 154–164, 2011.

[4] M. Maschio, L. Dinapoli, A. Fabi, D. Giannarelli, and T. Cantelmi, "Cognitive rehabilitation training in patients with brain tumor-related epilepsy and cognitive deficits: a pilot study," *Journal of Neuro-Oncology*, vol. 125, no. 2, pp. 419–426, 2015.

[5] G. Liu, N. Slater, and A. Perkins, "Epilepsy: treatment options," *American Family Physician*, vol. 96, no. 2, pp. 87–96, 2017.

[6] Z. Zhao, X. He, C. Ma et al., "Excavating anticonvulsant compounds from prescriptions of traditional chinese medicine in the treatment of epilepsy," *American Journal of Chinese Medicine*, vol. 46, no. 4, pp. 707–737, 2018.

[7] Y. Hijikata, A. Yasuhara, Y. Yoshida, and S. Sento, "Traditional Chinese medicine treatment of epilepsy," *The Journal of Alternative and Complementary Medicine*, vol. 12, no. 7, pp. 673–677, 2006.

[8] X. C. Zheng, S. F. Li, and J. M. Liu, "Clinical observation of single using Chaibei Zhixian decoction and combined medication

on intractable epilepsy," *Tianjin Journal of Traditional Chinese Medicine*, vol. 29, no. 03, pp. 224–227, 2012 (Chinese).

[9] C. H. Wu and X. L. Liao, "Randomized parallel controlled study on complicated partial seizure of refractory epilepsy treated with chaibei zhixian decoction combined with western medicine," *Journal of Practical Traditional Chinese & Internal Medicine*, vol. 32, no. 05, pp. 21–23, 2018 (Chinese).

[10] X. H. Wang, Z. R. Yan, Q. Zhang et al., "Effects of different doses of Kangxianling Decoction on type I collagen gene expression in rats with doxorubicin-induced nephropathy," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 30, no. 06, pp. 2062–2065, 2015 (Chinese).

[11] X. H. Wang, Z. R. Yan, Q. Zhang et al., "Effects of Chaibei Zhixian decoction on regulating the expression of P-gp/ Mdr1 in rat brain microvascular endothelial cells," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 31, no. 12, pp. 4961–4965, 2016 (Chinese).

[12] Z. R. Yan, Q. Zhang, X. H. Wang et al., "Role of Chaibei Zhixian decoction on regulating the expression of BCRP and NF-κB p65 in rat brain microvascular endothelial cells," *Global Traditional Chinese Medicine*, vol. 7, no. 04, pp. 241–246, 2014 (Chinese).

[13] X. C. Zheng and J. M. Liu, "Effects of Chaibei Zhixian decoction on the expressions of P-glycoprotein and MDR1 in intractable epileptic rats," *Journal of Traditional Chinese Medicine*, vol. 33, no. 11, pp. 4937–4940, 2018 (Chinese).

[14] W. Song, S. Ni, Y. Fu, and Y. Wang, "Uncovering the mechanism of Maxing Ganshi Decoction on asthma from a systematic perspective: a network pharmacology study," *Scientific Reports*, vol. 8, no. 1, Article ID 17362, 2018.

[15] J. Ru, P. Li, J. Wang et al., "TCMSP: a database of systems pharmacology for drug discovery from herbal medicines," *Journal of Cheminformatics*, vol. 6, no. 1, article 13, 2014.

[16] M. Kuhn, D. Szklarczyk, S. Pletscher-Frankild et al., "STITCH 4: integration of protein-chemical interactions with user data," *Nucleic Acids Research*, vol. 42, no. 1, pp. D401–D407, 2014.

[17] G. Bindea, B. Mlecnik, H. Hackl et al., "ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks," *Bioinformatics*, vol. 25, no. 8, pp. 1091–1093, 2009.

[18] X. Yao, H. Hao, Y. Li, and S. Li, "Modularity-based credible prediction of disease genes and detection of disease subtypes on the phenotype-gene heterogeneous network," *BMC Systems Biology*, vol. 5, no. 1, article 79, 2011.

[19] J. Velíšková, "Estrogens and epilepsy: why are we so excited?" *Neuroscientist*, vol. 13, no. 1, pp. 77–88, 2007.

[20] D. A. Iacobas, S. Iacobas, N. Nebieridze, L. Velíšek, and J. Velíšková, "Estrogen protects neurotransmission transcriptome during status epilepticus," *Frontiers in Neuroscience*, vol. 12, article 332, 2018.

[21] C. F. Zorumski, S. M. Paul, Y. Izumi, D. F. Covey, and S. Mennerick, "Neurosteroids, stress and depression: potential therapeutic opportunities," *Neuroscience & Biobehavioral Reviews*, vol. 37, no. 1, pp. 109–122, 2013.

[22] L. Velíšek, N. Nebieridze, T. Chachua, and J. Velíšková, "Anti-seizure medications and Estradiol for Neuroprotection in epilepsy: The 2013 update," *Recent Patents on CNS Drug Discovery*, vol. 8, no. 1, pp. 24–41, 2013.

[23] H. E. Scharfman and N. J. MacLusky, "Sex differences in the neurobiology of epilepsy: a preclinical perspective," *Neurobiology of Disease*, vol. 72, pp. 180–192, 2014.

[24] D. H. Pham, C. C. Tan, C. C. Homan et al., "Protocadherin 19 (PCDH19) interacts with paraspeckle protein NONO to co-regulate gene expression with estrogen receptor alpha (ERα)," *Human Molecular Genetics*, vol. 26, no. 11, pp. 2042–2052, 2017.

[25] V. E. Campos, M. Du, and Y. Li, "Increased seizure susceptibility and cortical malformation in β-catenin mutant mice," *Biochemical and Biophysical Research Communications*, vol. 320, no. 2, pp. 606–614, 2004.

[26] A. Rosiles, C. Rubio, C. Trejo, J. Gutierrez, L. Hernández, and C. Paz, "Participation of sox-1 expression and signaling of β-catenin in the pathophysiology of generalized seizures in cerebellum of rat," *CNS and Neurological Disorders - Drug Targets*, vol. 15, no. 1, pp. 3–6, 2016.

[27] D. Wang, X. Song, Y. Wang, X. Li, S. Jia, and Z. Wang, "Gene expression profile analysis in epilepsy by using the partial least squares method," *The Scientific World Journal*, vol. 2014, Article ID 731091, 5 pages, 2014.

[28] D.-M. Wu, Y.-T. Zhang, J. Lu, and Y.-L. Zheng, "Effects of microRNA-129 and its target gene c-Fos on proliferation and apoptosis of hippocampal neurons in rats with epilepsy via the MAPK signaling pathway," *Journal of Cellular Physiology*, vol. 233, no. 9, pp. 6632–6643, 2018.

[29] X. Zhu, Y. Chen, Y. Du, Q. Wan, Y. Xu, and J. Wu, "Astragaloside IV attenuates penicillin-induced epilepsy via inhibiting activation of the MAPK signaling pathway," *Molecular Medicine Reports*, vol. 17, no. 1, pp. 643–647, 2017.

[30] Z. Yang, J. Wang, C. Yu et al., "Inhibition of p38 MAPK signaling regulates the expression of EAAT2 in the brains of epileptic rats," *Frontiers in Neurology*, vol. 9, article 925, 2018.

[31] L. Chen, X. Liu, H. Wang, and M. Qu, "Gastrodin attenuates pentylenetetrazole-induced seizures by modulating the mitogen-activated protein kinase-associated inflammatory responses in mice," *Neuroscience Bulletin*, vol. 33, no. 3, pp. 264–272, 2017.

[32] S. Srivastava, H. E. Olson, J. S. Cohen et al., "BRAT1 mutations present with a spectrum of clinical severity," *American Journal of Medical Genetics Part A*, vol. 170, no. 9, pp. 2265–2273, 2016.

[33] S. A. Mundy, B. L. Krock, R. Mao, and J. J. Shen, "BRAT1-related disease-identification of a patient without early lethality," *American Journal of Medical Genetics Part A*, vol. 170, no. 3, pp. 699–702, 2016.

[34] M. Urbanska, P. Kazmierska-Grebowska, and T. Kowalczyk, "GSK3beta activity alleviates epileptogenesis and limits GluA1 phosphorylation," *EBioMedicine*, vol. 39, pp. 377–387, 2019.

[35] I. Hers, E. E. Vincent, and J. M. Tavaré, "Akt signalling in health and disease," *Cellular Signalling*, vol. 23, no. 10, pp. 1515–1527, 2011.

[36] A. Liu, M. Chu, and Y. Wang, "Up-regulation of trem 2 inhibits hippocampal neuronal apoptosis and alleviates oxidative stress in epilepsy via the PI3K/Akt pathway in mice," *Neuroscience Bulletin*, 2019.

[37] E. Eugène, I. Hoffmann, C. Pujol, P.-O. Couraud, S. Bourdoulous, and X. Nassif, "Microvilli-like structures are associated with the internalization of virulent capsulated Neisseria meningitidis into vascular endothelial cells," *Journal of Cell Science*, vol. 115, no. 6, pp. 1231–1241, 2002.

[38] M. R. Bower, M. Stead, R. S. Bower et al., "Evidence for consolidation of neuronal assemblies after seizures in humans," *The Journal of Neuroscience*, vol. 35, no. 3, pp. 999–1010, 2015.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 5801591, 11 pages
https://doi.org/10.1155/2019/5801591

Research Article

Identify the Key Active Ingredients and Pharmacological Mechanisms of Compound XiongShao Capsule in Treating Diabetic Peripheral Neuropathy by Network Pharmacology Approach

Meixiang Yu,[1] Xin Song,[2] Wanhua Yang ,[1] Ziwei Li,[1] Xiaoqin Ma,[1] and Chenxia Hao[1]

[1]*Department of Pharmacy, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China*
[2]*Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200120, China*

Correspondence should be addressed to Wanhua Yang; yangwanhuaxy@163.com

Received 29 January 2019; Revised 20 March 2019; Accepted 15 April 2019; Published 9 May 2019

Guest Editor: José C. T. Carvalho

Compound XiongShao Capsule (CXSC), a traditional herb mixture, has shown significant clinical efficacy against diabetic peripheral neuropathy (DPN). However, its multicomponent and multitarget features cause difficulty in deciphering its molecular mechanisms. Our study aimed to identify the key active ingredients and potential pharmacological mechanisms of CXSC in treating DPN by network pharmacology and provide scientific evidence of its clinical efficacy. CXSC active ingredients were identified from both the Traditional Chinese Medicine Systems Pharmacology database, with parameters of oral bioavailability ≥ 30% and drug-likeness ≥ 0.18, and the Herbal Ingredients' Targets (HIT) database. The targets of those active ingredients were identified using ChemMapper based on 3D-structure similarity and using HIT database. DPN-related genes were acquired from microarray dataset GSE95849 and five widely used databases (TTD, Drugbank, KEGG, DisGeNET, and OMIM). Next, we obtained candidate targets with therapeutic effects against DPN by mapping active ingredient targets and DPN-related genes and identifying the proteins interacting with those candidate targets using STITCH 5.0. We constructed an "active ingredients-candidate targets-proteins" network using Cytoscape 3.61 and identified key active ingredients and key targets in the network. We identified 172 active ingredients in CXSC, 898 targets of the active ingredients, 110 DPN-related genes, and 38 candidate targets with therapeutic effects against DPN. Three key active ingredients, namely, quercetin, kaempferol, and baicalein, and 25 key targets were identified. Next, we input all key targets into ClueGO plugin for KEGG enrichment and molecular function analyses. The AGE-RAGE signaling pathway in diabetic complications and MAP kinase activity were determined as the main KEGG pathway and molecular function involved, respectively. We determined quercetin, kaempferol, and baicalein as the key active ingredients of CXSC and the AGE-RAGE signaling pathway and MAP kinase activity as the main pharmacological mechanisms of CXSC against DPN, proving the clinical efficacy of CXSC against DPN.

1. Introduction

Diabetic peripheral neuropathy (DPN) is one of the most common complications of diabetes, affecting approximately 50% of people with diabetes [1–3]. Characterized by numbness, pain, paresthesia, and sensory loss, it becomes a major cause of disability and mortality [4–6]. Given the estimated global prevalence of diabetes of 693 million by 2045 [4], DPN is likely to affect as many as 346.5 million people worldwide, at a tremendous cost.

The pathogenesis of DPN is complex and multifactorial [7, 8]. It is generally accepted that chronic hyperglycemia induces the generation of advanced glycation end products (AGEs) in peripheral nervous tissue, and AGEs frequently result in neurological dysfunction mainly by modifying nervous structural proteins or overexpressing their receptors (RAGE) [9–11]. In addition, high glucose level activates mitogen-activated protein kinase (MAPK) in sensory neurons [8]. Moreover, elevated p38 and ERK activities may

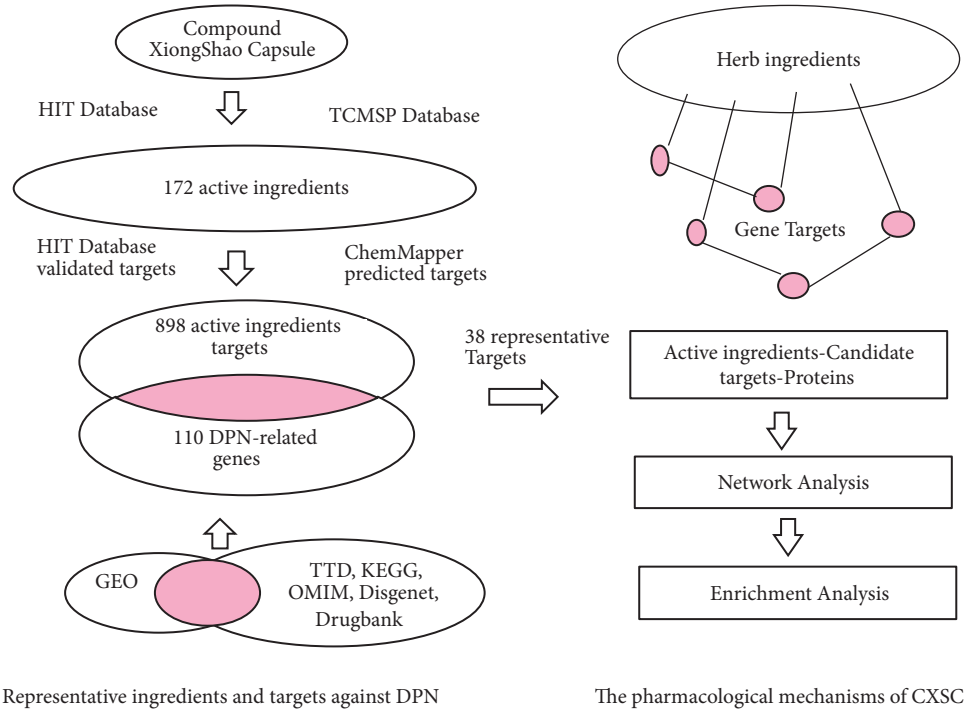


Figure 1: Flowcharts of the network pharmacology analysis. Left: summary of the identification of representative ingredients of CXSC and targets with therapeutic effects against DPN. Right: summary of the determination of the pharmacological mechanisms of CXSC.

cause neuron apoptosis, affecting neuronal functions and accelerating the progress of DPN [8, 12].

Despite advancements in the understanding of DPN pathogenesis, prevention and therapy of DPN mainly focus on glucose control and lifestyle modification [13]. There is still a lack of therapies against DPN pathogenesis other than aldose reductase inhibitors, i.e., epalrestat, and antioxidants, i.e., alpha-lipoic acid [14, 15]. In addition, these therapies show modest efficacy in clinical practice and exhibit certain adverse reactions, such as nausea, vomiting, and dizziness [14, 16]. Thus, safe and effective treatments of DPN that act on its mechanisms are urgently needed [13].

Recently, Traditional Chinese Medicine (TCM) has shown efficacy in treating DPN [17, 18]. Compound Xiong-Shao Capsule (CXSC) was produced from Buyang Huanwu Decoction which has been known for hundreds of years to possess significant neuroprotective properties [19–21] and certain clinical effect against DPN [22, 23]. CXSC has also been approved to use to treat DPN in Ruijin Hospital, Shanghai Jiao Tong University by the Shanghai Food and Drug Administration. CXSC is composed of 12 traditional Chinese herbs: Radix Paeoniae (RP), Radix Cyathulae (RC), Rhizoma Chuanxiong (RCX), Cortex Lyci (CL), Radix Saposhnikoviae (RS), Cassia Twig (CT), *Sargassum pallidum* (SP), *Polygonatum sibiricum* (PG), Astragali Radix (AgR), *Ramulus mori* (RM), *Silybum marianum* (SM), and *Orostachys fimbriata* (OF). CXSC has shown significant efficacy in clinical practice over several years, including improving whole-blood high-shear viscosity and improving incubation period and amplitude in the median or peroneal nerve of patients suffering from DPN [24]. Furthermore, some herbs in CXSC have been confirmed to exhibit positive pharmacological effects against DPN. For example, CL, AgR, and SM are involved in blood glucose control and show anti-inflammatory and inhibitory effects against oxidative stress [25–27].

However, the active ingredients of CXSC and their potential pharmacological mechanisms have not been fully studied. In a previous study, network pharmacology was used to discover the active ingredients and elucidate the mechanisms of herbal formulae [28]. Thus, our study aimed to use the network pharmacology approach to identify the key active ingredients of CXSC and their pharmacological mechanisms in treating DPN. A flowchart of the network pharmacology approach is presented in Figure 1.

2. Materials and Methods

2.1. Identification of Active Ingredients. Active ingredients of CXSC were collected both from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) Database [29] (http://lsp.nwu.edu.cn/, updated on May 31, 2014) according to the ADME parameters: oral bioavailability (OB) > 30% and drug-likeness (DL) > 0.18 [30] and from the Herbal Ingredients' Targets (HIT) Database [31] (http://lifecenter.sgst.cn/hit/, downloaded on July 31, 2018). By combining these two databases and removing any overlapping data, the active ingredients of CXSC were identified.

The TCMSP database is a unique TCM platform for identifying the relationships between drugs, targets, and diseases [29]. HIT is a database containing herbal ingredients and validated protein targets derived from more than 3250 literatures [31].

Table 1: The number of overlapped active ingredients between herbs.

Active ingredients	Total	Herbs
beta-sitosterol	7	*CT, CL, OF, PGS, RC, RP, RS*
Sitosterol	6	*CT, OF, PGS, RP, RS, RCX*
quercetin	5	*SM, AgR, OF, RC, SP*
Stigmasterol	3	*SM, CL, RP*
acetic acid	3	*AgR, CT, RP*
kaempferol	3	*AgR, OF, RM*
(-)-taxifolin	2	*SM, CT*
CLR	2	*SM, CL*
hederagenin	2	*AgR, CL*
FA	2	*AgR, RCX*
(+)-catechin	2	*CT, RP*
baicalein	2	*PGS, RP*
ecdysterone	2	*RC, RM*
Mandanol	2	*RS, RCX*

2.2. Targets of Active Ingredients. The validated targets of the active ingredients of CXSC were collected from the HIT database [32, 33]. In addition, the predicted targets of these active ingredients were obtained by using ChemMapper (http://www.lilab-ecust.cn/chemmapper/index.html), a web server for predicting potential drug targets based on 3D structure similarity [34], with 3D structure similarity of above 1.0 and prediction score of above 0 [32, 33]. Duplicates of the validated and predicted targets were eliminated, and the targets of the active ingredients of CXSC were screened.

2.3. Genes Related to DPN Obtained. Known genes of DPN were identified from five currently available databases using "diabetic peripheral neuropathy" as the keyword. In addition, the main differentially expressed genes (DEGs) between DPN patients and diabetic patients were extracted from microarray data GSE95849 [35] in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database [36], with a cut-off value of $P < 0.05$ and fold change |FC| of ≥ 1.5 [37]. DPN-related genes were identified after removal of duplicates.

The five databases were: the Therapeutic Target Database [38] (TTD, http://bidd.nus.edu.sg/group/cjttd/, last updated: 15th Sep. 2017); DrugBank [39] (http://www.drugbank.ca/, version:5.10); the Kyoto Encyclopedia of Genes and Genomes Pathway Database [40] (KEGG, https://www.kegg.jp/, downloaded:June. 2018); DisGeNET Database [41] (http://www.disgenet.org/web/DisGeNET/menu/home, version:5.0); Online Mendelian Inheritance in Man Database [42] (OMIM, http://www.omim.org/, last updated: 30th June. 2018).

2.4. Candidate Targets and Proteins Interacting with Candidate Targets. Candidate targets with therapeutic effects against DPN were identified by mapping the targets of the active ingredients of CXSC and the genes related to DPN [33]. The proteins that interacted with these candidate targets were screened by using STITCH 5.0 (http://stitch.embl.de/) [43], a database of known and predicted interactions between chemicals and proteins, with the species limited to "Homo Sapiens" and a confidence score of > 0.9.

2.5. Network Construction and Enrichment Analysis. A network of "active ingredients-candidate targets-proteins" was constructed using the Cytoscape 3.61 software [44]. The key active ingredients of CXSC and the key targets in this network were simultaneously identified by Network Analyzer plugin [45] using the following criterion: nodes with degree values exceeding twice the average value of all nodes in the network [46]. The degree value is the number of edges a node has in a network, which indicates how many active ingredients/targets/proteins one active ingredient/target/protein is related with. The larger the degree value, the more critical a role the node (active ingredient, target, or protein) is believed to play in the network [46]. A widely used visualization software, ClueGO [47], was used to explore the potential KEGG pathways and molecular functions of the key targets.

3. Results

3.1. Active Ingredients of CXSC. A total of 172 active ingredients (Supplementary Table 1) were identified in this study, of which 133 were collected from the TCMSP database and 39 were obtained from the HIT database. The numbers of active ingredients in RP, AgR, RCX, RS, CL, CT, PGS, SM, RM, RC, OF, and SP were 35, 22, 22, 21, 21, 13, 13, 13, 12, 7, 5, 5, and 4, respectively. The active ingredients that overlapped between herbs are shown in Table 1; for example, beta-sitosterol was identified in seven herbs, sitosterol was identified in six herbs, and quercetin was identified in five herbs.

3.2. Targets of the Active Ingredients of CXSC. A total of 898 targets of the active ingredients of CXSC (Supplementary Table 2) were identified in our study, including 229 validated targets and 669 predicted targets. Our analysis results showed that quercetin, baicalein, and kaempferol were the top three active ingredients targeting 308, 296, and 224 targets, respectively. Furthermore, there were 140 common targets of 12 herbs, 72 common targets of 11 herbs, 30 common targets of 10 herbs, 26 common targets of 9 herbs, 33 common targets

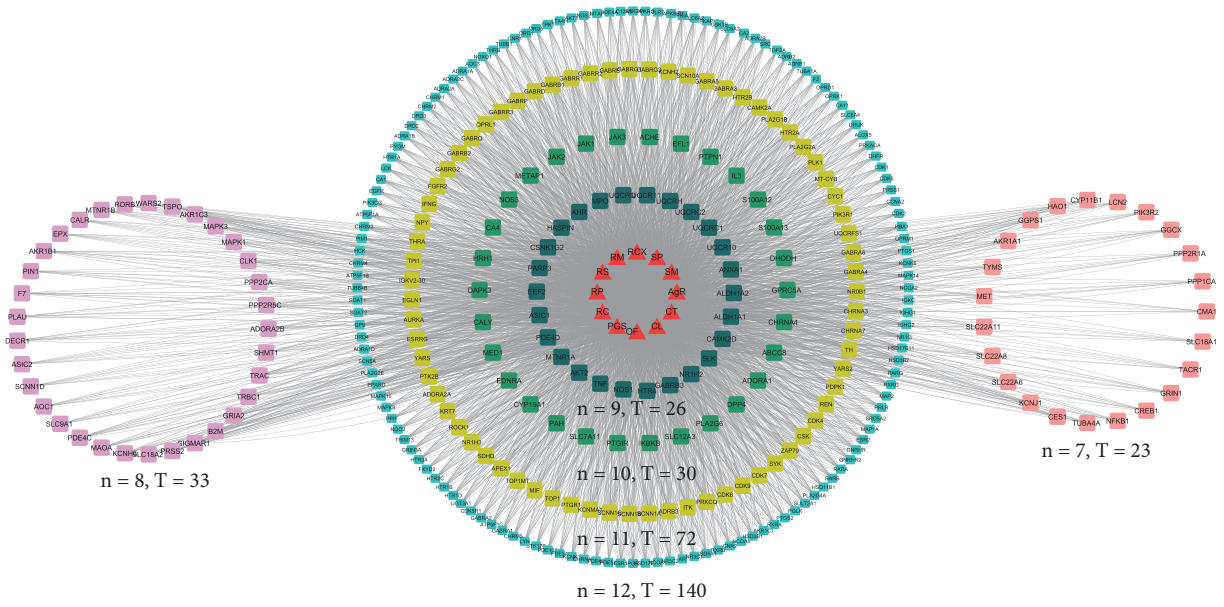


Figure 2: The targets that overlapped between at least 7 herbs. The triangle nodes represent the herbs, whereas the rectangle nodes represent the targets. The targets distributed in a circle are targeted by the same number of herbs. The number of herbs and number of targets are expressed as "n" and "T," respectively.

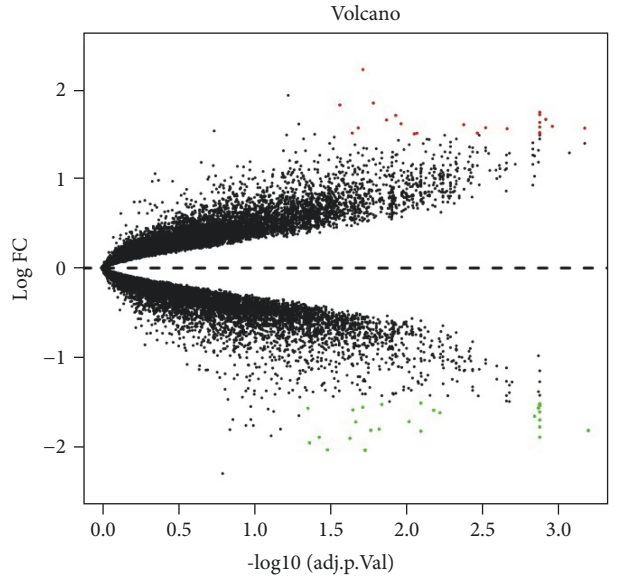


Figure 3: Volcano map of 51 DEGs. Red nodes represent upregulated genes and green nodes represent downregulated genes.

of 8 herbs, and 23 common targets of 7 herbs. The common targets of at least 7 herbs are shown in Figure 2.

3.3. Genes Related to DPN. A total of 51 DEGs were extracted from microarray data GSE95849, including 23 upregulated genes and 28 downregulated genes, as shown in Figure 3. After combination with the 59 known targets from the other five databases, a total of 110 DPN-related genes were identified (Supplementary Table 3).

3.4. Candidate Targets and Interacting Proteins. A total of 38 candidate targets were shown to have potential pharmacological effects against DPN, including 11 predicted targets (INS, MAPK14, MMP2, NOS3, SCN9A, SLC6A4, SLC6A3, OPRM1, TUBB1, ABCC8, and KCNJ11) and 27 validated targets (AKT1, BAX, BCL2, CASP3, JUN, MAPK1, MAPK8, TNF, VEGFA, IL1B, IL6, PRKCA, PRKCB, RELA, SELE, STAT1, PLAU, CCND1, COL1A1, CXCL8, EDN1, F3, ICAM1, ACE, SLC6A2, and CYP1A2). Specifically, TNF, IL1B, IL6, and CXCL8 were involved in inflammatory reactions; AKT1, BAX, BCL2, CASP3, and MAPK8 were related to apoptosis; and MAPK1, MAPK8, and MAPK14 were associated with MAPK activity. In addition, there were 86 proteins that interacted with those candidate targets.

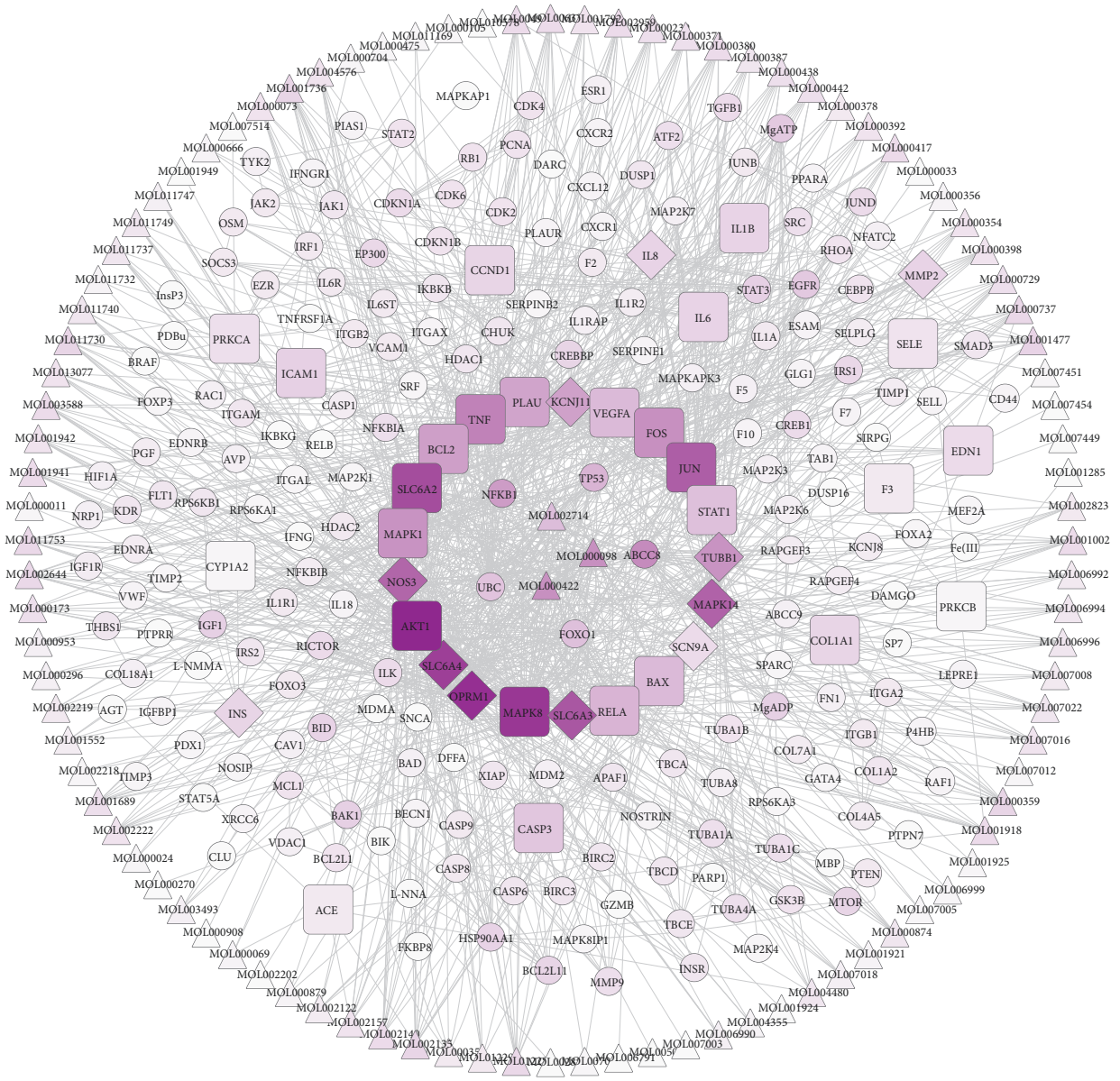
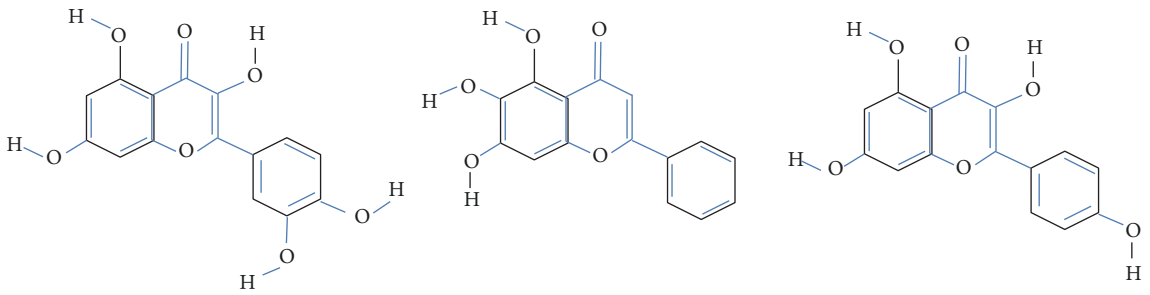


Figure 4: "Active ingredients-candidate targets-proteins" network. The triangle nodes represent active ingredients, the rectangle nodes represent validated targets, the diamond nodes represent predicted targets, and the circular nodes represent interacting proteins. The color of the nodes is shown in a gradient from purple to transparent according to descending order of the degree value. The three key active ingredients and 25 key targets are listed in the center circle.

3.5. Key Active Ingredients and Key Targets in the Network. An "active ingredients-candidate targets-proteins" network of 38 candidate targets and their interacting proteins was constructed using the Cytoscape 3.61 software (Figure 4). There were 328 nodes and 1396 edges in the network, with an average degree value of 8.767, as calculated by using the NetworkAnalyzer plugin. There were 28 nodes with degree values exceeding 18 (twice the average value of the degree), comprising of three active ingredients (quercetin, kaempferol, and baicalein) representing the key active ingredients (Figure 5) and 25 targets representing the key targets (Table 2).

3.6. KEGG Pathways and Molecular Functions Enriched. The enrichment analysis results showed enrichment in the KEGG signaling pathways and molecular functions. As shown in Figure 6, the following three KEGG pathways were enriched: the AGE-RAGE signaling pathway in diabetic complications, B cell receptor signaling pathway, and cocaine addiction pathway, with the AGE-RAGE signaling pathway accounting for 88% of all the pathways enriched. The enriched molecular functions were divided into three groups: MAP kinase activity, phosphatase binding, and neurotransmitter: sodium symporter activity (Figure 7).



Quercetin (MOL000098) Baicalein (MOL002714) Kaempferol (MOL000422)

Figure 5: The chemical structure of the 3 key active ingredients.

Table 2: The information about the 25 key targets.

Gene	UniProt	Description	Degree	Source
AKT1	P31749	RAC-alpha serine/threonine-protein kinase	83	validated
OPRM1	P35372	Mu-type opioid receptor	76	predicted
MAPK8	P45983	Mitogen-activated protein kinase 8	72	validated
SLC6A4	P31645	Sodium-dependent serotonin transporter	67	predicted
SLC6A2	P23975	Sodium-dependent noradrenaline transporter	60	validated
SLC6A3	Q01959	Sodium-dependent dopamine transporter	56	predicted
JUN	P05412	Transcription factor AP-1	53	validated
MAPK14	Q16539	Mitogen-activated protein kinase 14	51	predicted
NOS3	P29474	Nitric oxide synthase	50	predicted
TNF	P01375	Tumor necrosis factor	40	validated
ABCC8	Q09428	ATP-binding cassette sub-family C member 8	37	proteins
FOS	P01100	Proto-oncogene c-Fos	35	validated
TUBB1	Q9H4B7	Tubulin beta-1 chain	34	predicted
MAPK1	P28482	Mitogen-activated protein kinase 1	34	validated
NFKB1	P19838	Nuclear factor NF-kappa-B p105 subunit	31	proteins
BCL2	P10415	Apoptosis regulator Bcl-2	30	validated
KCNJ11	Q14654	ATP-sensitive inward rectifier potassium channel 11	29	predicted
PLAU	P00749	Urokinase-type plasminogen activator	28	validated
RELA	Q04206	Transcription factor p65	23	validated
TP53	P04637	Cellular tumor antigen p53	23	proteins
BAX	Q07812	Apoptosis regulator BAX	21	validated
VEGFA	P15692	Vascular endothelial growth factor A	21	validated
STAT1	P42224	Signal transducer and activator of transcription 1-alpha/beta	19	validated
FOXO1	Q12778	Forkhead box protein O1	19	proteins
UBC	P0CG48	Polyubiquitin-C	18	proteins

4. Discussion

TCM pharmacological mechanisms have always been associated with multiple components and multiple targets that are difficult to explain. However, the holistic ideas of TCM have been revealed by emerging network pharmacology approaches studying the relationships between drugs, targets, and diseases [28]. In our study, the active ingredients of CXSC, targets of these active ingredients, and genes related to DPN were identified comprehensively from the databases. Next, an "active ingredient-target-protein" network with 328 nodes and 1396 edges was constructed by the Cytoscape software. After analyzing the network, three key active ingredients and 25 key targets of CXSC were determined, and the pharmacological mechanisms of CXSC in treating DPN were elucidated by enrichment analysis of the 25 key targets.

4.1. Quercetin, Kaempferol, and Baicalein as the Key Active Ingredients of CXSC. The "active ingredients-targets-proteins" network showed quercetin, kaempferol, and baicalein as the key active ingredients of CXSC that exhibit therapeutic effects against DPN. Moreover, these key ingredients targeted 308 (34.3%), 296 (33.0%), and 244 (27.2%) targets, respectively. Furthermore, these three key ingredients targeted 22 of the 38 candidate targets with therapeutic effects against

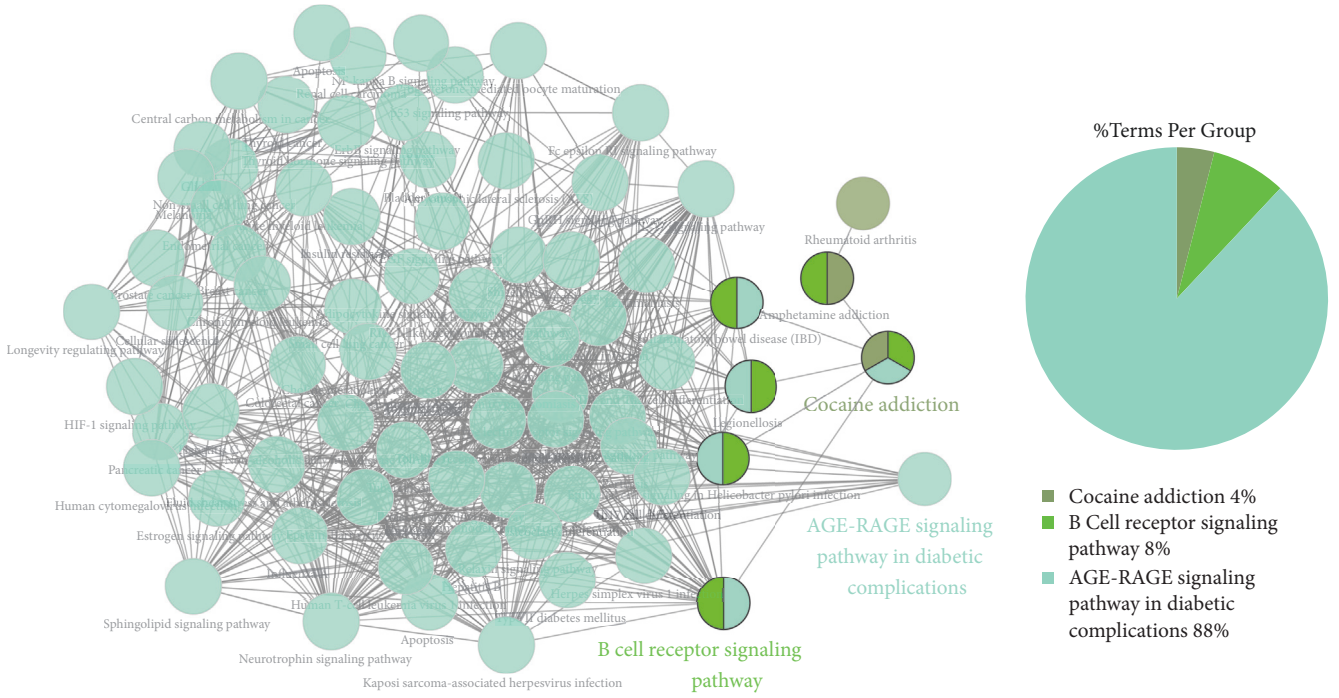


Figure 6: KEGG signaling pathways.

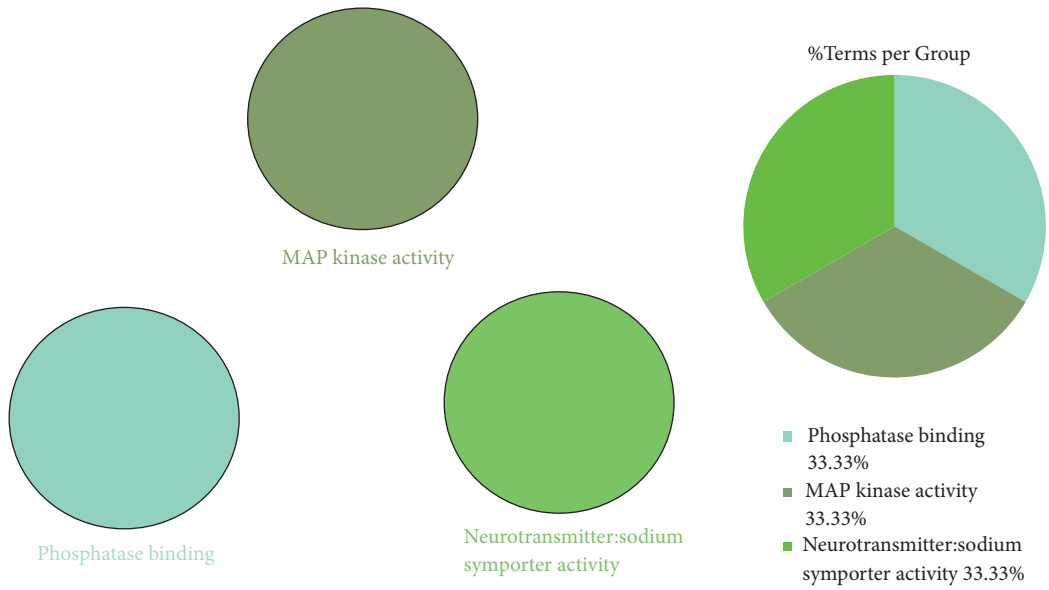


Figure 7: Molecular functions analysis.

DPN, as well as 21 of the 25 key targets of CXSC, as shown in Figure 8. Interestingly, several studies have previously shown that quercetin may be helpful against diabetic neuropathy by attenuating cold allodynia and hyperalgesia [48, 49], maintaining the density of the general neuronal population [50], and restoring sciatic nerves injuries [51] in streptozotocin-(STZ-) induced diabetic rats. Kaempferol [52] and baicalein [53] also showed neuroprotective effect against STZ-induced diabetic neuropathy. Thus, we speculated that CXSC exhibited significant therapeutic effect against DPN mainly through the synergistic actions of quercetin, kaempferol, and baicalein.

4.2. Regulations of the AGE-RAGE Pathway and MAPK Activity as the Pharmacological Mechanisms of CXSC Therapeutic Effect against DPN. Kaempferol and baicalein, the key active ingredients of CXSC, were previously confirmed to reduce the formation of AGEs, thereby reducing inflammatory responses in diabetic rat nerves [52, 54]; thus, these two compounds may exert therapeutic effect against DPN.

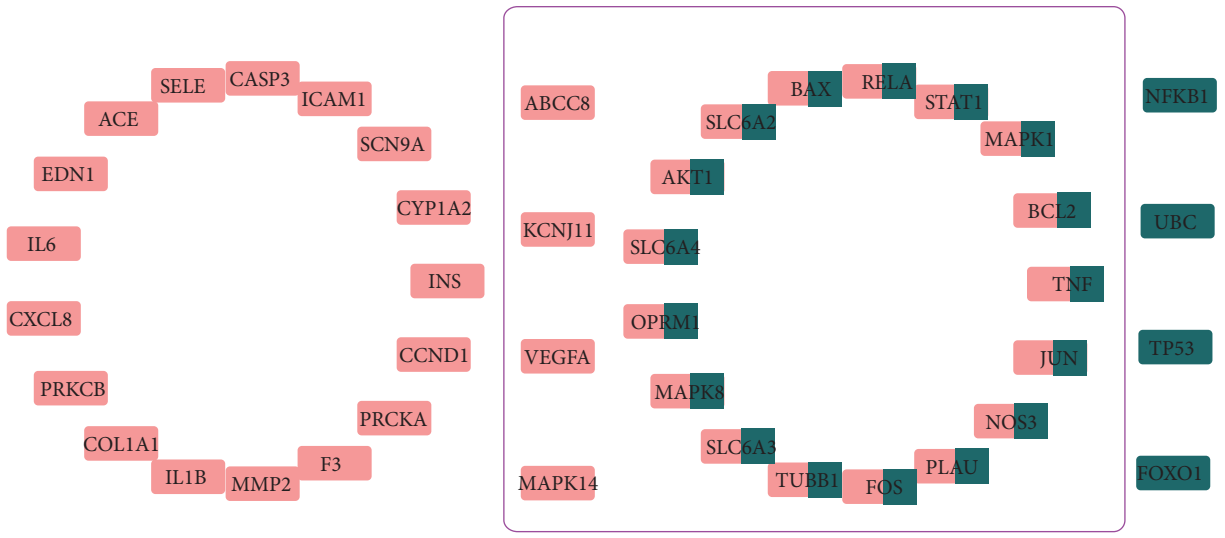


Figure 8: Data of the three key active ingredients (quercetin, kaempferol, and baicalein). Pink rectangles represent 38 candidate targets with therapeutic effects against DPN, whereas green rectangles represent the 25 key targets. Two-colored rectangles are targets that overlapped between the two categories. Targets in the purple box are targets of quercetin, kaempferol, and baicalein.

Quercetin, another key active ingredient of CXSC, protected rat dorsal root ganglion neurons against high glucose-induced injury *in vitro* through dose-dependent inhibition of the NF-κB signaling pathway; thus, this compound may be beneficial as a treatment of diabetic neuropathy [55]. Furthermore, activation and perpetuation of the AGE-RAGE signaling pathway have been reported in diabetic neuropathy [9]. AGEs act on RAGEs to allow sustained activation of NF-κB, which aggravates inflammatory reactions and leads to neuronal dysfunction [9, 11]. Lukic I.K [10] identified the AGE-RAGE signaling pathway as a new therapeutic target of neuronal dysfunction. In our study, the AGE-RAGE signaling pathway of diabetic complications accounted for 88% of all the enriched pathways, suggesting that the therapeutic effect of CXSC against DPN was mainly related to reduction in AGE formation and inhibition of NF-κB.

In addition, AGEs can activate p38 MAPK, leading to neuron apoptosis [56]. MAPK may play important roles in neuronal cell death or regeneration [57, 58]. For example, p38 MAPK was reported to aggravate oxidative stress and inflammation through induction of nitric oxide synthase, thereby resulting in NO production, as well as through regulation of the production of cytokines, such as TNF and IL-10, leading to neuronal cell death [59, 60]. Notably, increased phosphorylation of MAPK has been detected in diabetic animal models [61–63] and in the peripheral nerves of patients with DPN [12]. p38 MAPK, especially has been proved to be associated with DPN pathogenesis, such as increased mechanical hyperalgesia [64–66] and reduced nerve conduction velocity [67]. Thus, adjustment in the activity of MAPK may be the basis of preventive treatments of diabetic neuropathy [9]. Fortunately, tetramethylpyrazine, a main ingredient of RCX in CXSC, was found to block MAPK and suppress reactive oxygen species in N9 microglial cells, which may shed light on future treatments of neurodegenerative diseases [68]. Baicalein, one of the three key active ingredients identified in the current study, counteracts diabetes-associated p38 MAPK phosphorylation and oxidative-nitrosative stress, targeting several mechanisms implicated in DPN [53]. Therefore, the effects of CXSC on MAPK regulation against DPN may be attributed to tetramethylpyrazine and baicalein. Our molecular function analysis also indicated that the therapeutic effect of CXSC against DPN was related to MAPK activity. Our results were consistent with those of previous studies, which indicated that p38 MAPK activation mediates RAGE-induced, NF-κB-dependent secretion of proinflammatory cytokines, leading to accelerated inflammation. Activation of the AGE-RAGE pathway and MAPK appeared to be present not only in DPN but also in diabetic nephropathy [69] and diabetic keratopathy [56]. Thus, further studies are needed to investigate whether CXSC is effective against other diabetic complications.

5. Conclusions

In conclusion, the multicomponent and multitarget features of the therapeutic effects of CXSC against DPN were effectively elucidated through network pharmacology approach. Quercetin, kaempferol, and baicalein were determined as the key active ingredients of CXSC. In addition, the AGE-RAGE signaling pathway and regulation of MAPK activity were shown as the main pharmacological mechanisms of the therapeutic effects of CXSC against DPN, thereby providing scientific evidence of the clinical efficacy of CXSC against DPN.

Data Availability

The data of our research can be acquired from the Supplementary Materials uploaded with this article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Yu Meixiang and Yang Wanhua designed, wrote, and revised the manuscript. Yu Meixiang and Song Xin performed the experiments; Li Ziwei, Ma Xiaoqin, and Hao Chenxia revised the manuscript and provided technical or material support.

Acknowledgments

This project was supported by the key scientific research projects of the Science and Technology Commission of Shanghai (no.17401901100).

Supplementary Materials

Supplementary Table 1. 172 active ingredients in Compound XiongShao Capsule. 172 active ingredients in Compound XiongShao Capsule were downloaded from both the Traditional Chinese Medicine Systems Pharmacology (TCMSP) Database (http://lsp.nwu.edu.cn/, updated on May 31, 2014) according to an ADME principle: oral bioavailability (OB) > 30% and drug-likeness (DL) > 0.18 and the Herbal Ingredients' Targets (HIT) Database (downloaded on July 31, 2018). Supplementary Table 2. 898 targets of active ingredients in Compound XiongShao Capsule. The validated targets of the active ingredients in Compound XiongShao Capsule were extracted from HIT database. At the same time, the predicted targets of the active ingredients, which were saved as "MOL2" file format and then inputted into the ChemMapper (http://www.lilab-ecust.cn/chemmapper/index.html, downloaded in August 2018), were selected if the 3D similarity was above 1.0 and the prediction score was >0 as well. Eliminate duplicates, respectively, the targets of active ingredients were obtained. Supplementary Table 3. 110 diabetic peripheral neuropathy-related genes. The main differentially expressed genes (DEGs) of diabetic peripheral neuropathy (DPN) were extracted from Microarray data GSE95849 including six diabetic samples and six DPN samples in the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) with cut-off values of $P < 0.05$ and fold change $|FC| \geq 1.5$. Simultaneously, known genes related to DPN were obtained from five currently available databases using "diabetic peripheral neuropathy" as the keyword: (1) the Therapeutic Target Database (TTD) (http://bidd.nus.edu.sg/group/cjttd/, last updated:15th Sep. 2017). (2) DrugBank (http://www.drugbank.ca/, version: 5.10). (3) the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (https://www.kegg.jp/, downloaded:June. 2018) (4) DisGeNET Database (http://www.disgenet.org/web/DisGeNET/menu/home, version:5.0). (5) Online Mendelian Inheritance in Man (OMIM) Database (http://www.omim.org/, last updated:30th June. 2018). At last, DPN-related genes were collected after eliminating duplicates. *(Supplementary Materials)*

References

[1] M. Turns, "The diabetic foot: An overview for community nurses," *British Journal of Community Nursing*, vol. 17, no. 9, pp. 422–433, 2012.

[2] C. J. M. Alleman, K. Y. Westerhout, M. Hensen et al., "Humanistic and economic burden of painful diabetic peripheral neuropathy in Europe: a review of the literature," *Diabetes Research and Clinical Practice*, vol. 109, no. 2, pp. 215–225, 2015.

[3] J. C. Won, S. S. Kim, K. S. Ko, and B.-Y. Cha, "Current status of diabetic peripheral neuropathy in korea: Report of a hospital-based study of type 2 diabetic patients in korea by the diabetic neuropathy study group of the korean diabetes association," *Diabetes & Metabolism*, vol. 38, no. 1, pp. 25–31, 2014.

[4] N. Cho, J. Shaw, S. Karuranga et al., "IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045," *Diabetes Research and Clinical Practice*, vol. 138, pp. 271–281, 2018.

[5] S. Tesfaye, A. J. Boulton, and P. J. Dyck, "Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatment," *Diabetes Care*, vol. 33, no. 10, pp. 2285–2293, 2010.

[6] T. Didangelos, J. Doupis, and A. Veves, "Painful diabetic neuropathy: clinical aspects," *Handbook of Clinical Neurology*, vol. 126, pp. 53–61, 2014.

[7] J. W. Albers and R. Pop-Busui, "Diabetic neuropathy: mechanisms, emerging treatments, and subtypes," *Current Neurology and Neuroscience Reports*, vol. 14, p. 473, 2014.

[8] D. W. Zochodne, "Mechanisms of diabetic neuron damage: Molecular pathways," *Handbook of Clinical Neurology*, vol. 126, pp. 379–399, 2014.

[9] R. Wada and S. Yagihashi, "Role of advanced glycation end products and their receptors in development of diabetic neuropathy," *Annals of the New York Academy of Sciences*, vol. 1043, pp. 598–604, 2005.

[10] I. K. Lukic, P. M. Humpert, P. P. Nawroth, and A. Bierhaus, "The RAGE pathway: activation and perpetuation in the pathogenesis of diabetic neuropathy," *Annals of the New York Academy of Sciences*, vol. 1126, pp. 76–80, 2008.

[11] R. Ramasamy, S. F. Yan, and A. M. Schmidt, "Receptor for AGE (RAGE): signaling mechanisms in the pathogenesis of diabetes and its complications," *Annals of the New York Academy of Sciences*, vol. 1243, pp. 88–102, 2011.

[12] T. Purves, A. Middlemas, S. Agthong et al., "A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy," *The FASEB Journal*, vol. 15, no. 13, pp. 2508–2514, 2001.

[13] R. Pop-Busui, A. J. M. Boulton, E. L. Feldman et al., "Diabetic neuropathy: A position statement by the American diabetes association," *Diabetes Care*, vol. 40, no. 1, pp. 136–154, 2017.

[14] N. Hotta, R. Kawamori, M. Fukuda, and Y. Shigeta, "Long-term clinical effects of epalrestat, an aldose reductase inhibitor, on progression of diabetic neuropathy and other microvascular complications: Multivariate epidemiological analysis based on patient background factors and severity of diabetic neuropathy," *Diabetic Medicine*, vol. 29, no. 12, pp. 1529–1533, 2012.

[15] D. Ziegler, P. A. Low, W. J. Litchy et al., "Efficacy and safety of antioxidant treatment with α-lipoic acid over 4 years in diabetic polyneuropathy: the NATHAN 1 trial," *Diabetes Care*, vol. 34, no. 9, pp. 2054–2060, 2011.

[16] D. Ziegler, A. Ametov, A. Barinov et al., "Oral treatment with α-lipoic acid improves symptomatic diabetic polyneuropathy," *Diabetes Care*, vol. 29, no. 11, pp. 2365–2370, 2006.

[17] X. Yang, W. Yao, H. Liu, Y. Gao, R. Liu, and L. Xu, "Tangluoning, a traditional Chinese medicine, attenuates in vivo and in vitro diabetic peripheral neuropathy through modulation of PERK/Nrf2 pathway," *Scientific Reports*, vol. 7, no. 1, Article ID 1014, 2017.

[18] Y. Tong and H. Hou, "Effects of Huangqi Guizhi Wuwu Tang on diabetic peripheral neuropathy," *The Journal of Alternative and Complementary Medicine*, vol. 12, no. 6, pp. 506–509, 2006.

[19] A. Chen, H. Wang, J. Zhang et al., "BYHWD rescues axotomized neurons and promotes functional recovery after spinal cord injury in rats," *Journal of Ethnopharmacology*, vol. 117, no. 3, pp. 451–456, 2008.

[20] K.-J. Kim and U. Namgung, "Facilitating effects of Buyang Huanwu decoction on axonal regeneration after peripheral nerve transection," *Journal of Ethnopharmacology*, vol. 213, pp. 56–64, 2018.

[21] I. A. Chang, H. D. Lim, K. J. Kim, H. Shin, and U. Namgung, "Enhanced axonal regeneration of the injured sciatic nerve by administration of Buyang Huanwu decoction," *Journal of Ethnopharmacology*, vol. 194, pp. 626–634, 2016.

[22] T. Ji, "Clinical observation on treating diabetic peripheral neuropathy with Buyang Huanwu decoction," *Clinical Journal of Chinese Medicine*, vol. 6, pp. 74-75, 2014.

[23] J. Liu, "Efficacy observation on treating diabetic peripheral neuropathy with the Buyang Huanwu decoction," *Clinical Journal of Chinese Medicine*, vol. 7, pp. 173-174, 2015.

[24] H. J. Cui, W. R. Zhu, J. J. Zhou et al., "Clinical observation of compound xiongshao capsules in the treatment of diabetic peripheral neuropathy," *China Pharmacy*, vol. 29, pp. 223–228, 2018.

[25] D. Gao, Q. Li, Z. Liu et al., "Hypoglycemic effects and mechanisms of action of Cortex Lycii Radicis on alloxan-induced diabetic mice," *Yakugaku Zasshi*, vol. 127, no. 10, pp. 1715–1721, 2007.

[26] M. Ryu, E. H. Kim, M. Chun et al., "Astragali Radix elicits anti-inflammation via activation of MKP-1, concomitant with attenuation of p38 and Erk," *Journal of Ethnopharmacology*, vol. 115, no. 2, pp. 184–193, 2008.

[27] H. Z. Toklu, T. Tunali Akbay, A. Velioglu-Ogunc et al., "Silymarin, the Antioxidant Component of Silybum marianum, Prevents Sepsis-Induced Acute Lung and Brain Injury," *Journal of Surgical Research*, vol. 145, no. 2, pp. 214–222, 2008.

[28] S. Li and B. Zhang, "Traditional Chinese medicine network pharmacology: theory, methodology and application," *Chinese Journal of Natural Medicines*, vol. 11, pp. 110–120, 2013.

[29] J. Ru, P. Li, J. Wang et al., "TCMSP: a database of systems pharmacology for drug discovery from herbal medicines," *Journal of Cheminformatics*, vol. 6, no. 1, article 13, 2014.

[30] X. Deng, X. Xing, G. Sun et al., "Guanxin danshen formulation protects against myocardial ischemia reperfusion injury-induced left ventricular remodeling by upregulating estrogen receptor beta," *Frontiers in Pharmacology*, vol. 8, p. 777, 2017.

[31] H. Ye, L. Ye, H. Kang et al., "HIT: Linking herbal active ingredients to targets," *Nucleic Acids Research*, vol. 39, no. 1, pp. D1055–D1059, 2011.

[32] L. Gao, X. Wang, Y. Niu et al., "Molecular targets of Chinese herbs: a clinical study of hepatoma based on network pharmacology," *Scientific Reports*, vol. 6, no. 1, Article ID 24944, 2016.

[33] L. Gao, K. Wang, Y. Zhou, J. Fang, X. Qin, and G. Du, "Uncovering the anticancer mechanism of Compound Kushen Injection against HCC by integrating quantitative analysis, network analysis and experimental validation," *Scientific Reports*, vol. 8, no. 624, 2018.

[34] J. Gong, C. Cai, X. Liu et al., "ChemMapper: a versatile web server for exploring pharmacology and chemical structure association based on molecular 3D similarity method," *Bioinformatics*, vol. 29, no. 14, pp. 1827–1829, 2013.

[35] L. Luo, L.-D. Ji, J.-J. Cai et al., "Microarray analysis of long noncoding rnas in female diabetic peripheral neuropathy patients," *Cellular Physiology and Biochemistry*, vol. 46, no. 3, pp. 1209–1217, 2018.

[36] T. Barrett, S. E. Wilhite, P. Ledoux et al., "NCBI GEO: archive for functional genomics data sets—update," *Nucleic Acids Research*, vol. 41, no. 1, pp. D991–D995, 2013.

[37] Y. Tian, B. Stamova, G. C. Jickling et al., "Effects of gender on gene expression in the blood of ischemic stroke patients," *Journal of Cerebral Blood Flow & Metabolism*, vol. 32, no. 5, pp. 780–791, 2012.

[38] X. Chen, Z. L. Ji, and Y. Z. Chen, "TTD: therapeutic target database," *Nucleic Acids Research*, vol. 30, no. 1, pp. 412–415, 2002.

[39] D. S. Wishart, C. Knox, A. C. Guo et al., "DrugBank: a knowledgebase for drugs, drug actions and drug targets," *Nucleic Acids Research*, vol. 36, pp. D901–D906, 2008.

[40] M. Kanehisa, "The KEGG database," *Novartis Foundation Symposia*, vol. 247, pp. 91–103, 2002.

[41] J. Piñero, N. Queralt-Rosinach, À. Bravo et al., "DisGeNET: a discovery platform for the dynamical exploration of human diseases and their genes," *Database*, vol. 2015, Article ID bav028, 2015.

[42] A. Hamosh, A. F. Scott, J. S. Amberger, C. A. Bocchini, and V. A. McKusick, "Online Mendelian Inheritance in Man (OMIM), a knowledgebase of human genes and genetic disorders," *Nucleic Acids Research*, vol. 33, pp. D514–D517, 2005.

[43] M. Kuhn, C. von Mering, M. Campillos, L. J. Jensen, and P. Bork, "STITCH: interaction networks of chemicals and proteins," *Nucleic Acids Research*, vol. 36, no. 1, pp. D684–D688, 2008.

[44] M. E. Smoot, K. Ono, J. Ruscheinski, P. L. Wang, and T. Ideker, "Cytoscape 2.8: new features for data integration and network visualization," *Bioinformatics*, vol. 27, no. 3, pp. 431-432, 2011.

[45] H. de Jong, J. Geiselmann, C. Hernandez, and M. Page, "Genetic network analyzer: Qualitative simulation of genetic regulatory networks," *Bioinformatics*, vol. 19, no. 3, pp. 336–344, 2003.

[46] S. Li, Z. Q. Zhang, L. J. Wu, X. G. Zhang, Y. D. Li, and Y. Y. Wang, "Understanding ZHENG in traditional Chinese medicine in the context of neuro-endocrine-immune network," *IET Systems Biology*, vol. 1, no. 1, pp. 51–60, 2007.

[47] G. Bindea, B. Mlecnik, H. Hackl et al., "ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks," *Bioinformatics*, vol. 25, no. 8, pp. 1091–1093, 2009.

[48] N. P. Kumar, A. R. Annamalai, and R. S. Thakur, "Antinociceptive property of emblica officinalis gaertn (Amla) in high fat dietfed/low dose streptozotocin induced diabetic neuropathy in rats," *Indian Journal of Experimental Biology (IJEB)*, vol. 47, no. 9, pp. 737–742, 2009.

[49] M. Anjaneyulu and K. Chopra, "Quercetin attenuates thermal hyperalgesia and cold allodynia in STZ-induced diabetic rats," *Indian Journal of Experimental Biology (IJEB)*, vol. 42, no. 8, pp. 766–769, 2004.

[50] P. E. B. Ferreira, C. R. P. Lopes, A. M. P. Alves et al., "Diabetic neuropathy: an evaluation of the use of quercetin in the cecum of rats," *World Journal of Gastroenterology*, vol. 19, no. 38, pp. 6416–6426, 2013.

[51] I. C. Chis, A. Clichici, A. L. Nagy, A. Oros, C. Catoi, and S. Clichici, "Quercetin in association with moderate exercise training attenuates injuries induced by experimental diabetes in sciatic nerves," *Journal of Physiology and Pharmacology*, vol. 68, pp. 877–886, 2017.

[52] L. Kishore, N. Kaur, and R. Singh, "Effect of Kaempferol isolated from seeds of Eruca sativa on changes of pain sensitivity in Streptozotocin-induced diabetic neuropathy," *Inflammopharmacology*, vol. 26, no. 4, pp. 993–1003, 2018.

[53] R. Stavniichuk, V. R. Drel, H. Shevalye et al., "Baicalein alleviates diabetic peripheral neuropathy through inhibition of oxidative-nitrosative stress and p38 MAPK activation," *Experimental Neurology*, vol. 230, no. 1, pp. 106–113, 2011.

[54] X. Wang, L. I. Cai-Na, and Z. F. J. C. J. o. C. P. Shen, "Advances on the protective mechanism study of baicalein on diabetic peripheral neuropathy," 2018.

[55] Y. Shi, X.-C. Liang, H. Zhang, Q.-L. Wu, L. Qu, and Q. Sun, "Quercetin protects rat dorsal root ganglion neurons against high glucose-induced injury in vitro through Nrf-2/HO-1 activation and NF-κB inhibition," *Acta Pharmacologica Sinica*, vol. 34, no. 9, pp. 1140–1148, 2013.

[56] L. Shi, X. Yu, H. Yang, and X. Wu, "Advanced glycation end products induce human corneal epithelial cells apoptosis through generation of reactive oxygen species and activation of JNK and p38 MAPK pathways," *PLoS ONE*, vol. 8, no. 6, Article ID e66781, 2013.

[57] S. J. Harper and P. LoGrasso, "Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38," *Cell Signal*, vol. 13, pp. 299–310, 2001.

[58] K. Takeda and H. Ichiijo, "Neuronal p38 MAPK signalling: An emerging regulator of cell fate and function in the nervous system," *Genes to Cells*, vol. 7, no. 11, pp. 1099–1111, 2002.

[59] J. C. Lee, S. Kumar, D. E. Griswold, D. C. Underwood, B. J. Votta, and J. L. Adams, "Inhibition of p38 MAP kinase as a therapeutic strategy," *International Journal of immunopharmacology*, vol. 47, no. 2-3, pp. 185–201, 2000.

[60] G.-H. Jeohn, C. L. Cooper, B. Wilson et al., "p38 MAP kinase is involved in lipopolysaccharide-induced dopaminergic neuronal cell death in rat mesencephalic neuron-glia cultures," *Annals of the New York Academy of Sciences*, vol. 962, pp. 332–346, 2002.

[61] R. Stavniichuk, H. Shevalye, H. Hirooka, J. L. Nadler, and I. G. Obrosova, "Interplay of sorbitol pathway of glucose metabolism, 12/15-lipoxygenase, and mitogen-activated protein kinases in the pathogenesis of diabetic peripheral neuropathy," *Biochemical Pharmacology*, vol. 83, no. 7, pp. 932–940, 2012.

[62] H. Cheng, J. Dauch, J. Hayes, Y. Hong, and E. Feldman, "P38 mediates mechanical allodynia in a mouse model of type 2 diabetes," *Molecular Pain*, vol. 6, no. 28, 2010.

[63] V. R. Drel, P. Pacher, R. Stavniichuk et al., "Poly(ADP-ribose) polymerase inhibition counteracts renal hypertrophy and multiple manifestations of peripheral neuropathy in diabetic Akita mice," *International Journal of Molecular Medicine*, vol. 28, no. 4, pp. 629–635, 2011.

[64] L. Daulhac, C. Mallet, C. Courteix et al., "Diabetes-induced mechanical hyperalgesia involves spinal mitogen-activated protein kinase activation in neurons and microglia via N-methyl-D-aspartate- dependent mechanisms," *Molecular Pharmacology*, vol. 70, no. 4, pp. 1246–1254, 2006.

[65] L. Daulhac, V. Maffre, C. Mallet et al., "Phosphorylation of spinal N-methyl-d-aspartate receptor NR1 subunits by extracellular signal-regulated kinase in dorsal horn neurons and microglia contributes to diabetes-induced painful neuropathy," *European Journal of Pain*, vol. 15, no. 2, pp. 169–e12, 2011.

[66] S. A. Price, N. J. Gardiner, B. Duran-Jimenez, L. A. H. Zeef, I. G. Obrosova, and D. R. Tomlinson, "Thioredoxin interacting protein is increased in sensory neurons in experimental diabetes," *Brain Research*, vol. 1116, no. 1, pp. 206–214, 2006.

[67] S. A. Price, S. Agthong, A. B. Middlemas, and D. R. Tomlinson, "Mitogen-activated protein kinase p38 mediates reduced nerve conduction in experimental diabetic neuropathy: interactions with aldose reductase," *Diabetes*, vol. 53, no. 7, pp. 1851–1856, 2004.

[68] H.-T. Liu, Y.-G. Du, J.-L. He et al., "Tetramethylpyrazine inhibits production of nitric oxide and inducible nitric oxide synthase in lipopolysaccharide-induced N9 microglial cells through blockade of MAPK and PI3K/Akt signaling pathways, and suppression of intracellular reactive oxygen species," *Journal of Ethnopharmacology*, vol. 129, no. 3, pp. 335–343, 2010.

[69] Y. S. Kanwar, L. Sun, P. Xie, F. Y. Liu, and S. Chen, "A glimpse of various pathogenetic mechanisms of diabetic nephropathy," *Annual Review of Pathology*, vol. 6, pp. 395–423, 2011.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 3583067, 18 pages
https://doi.org/10.1155/2019/3583067

Review Article

Metabolic and Epigenetic Action Mechanisms of Antidiabetic Medicinal Plants

Siba Shanak,[1] Bashar Saad,[1,2] and Hilal Zaid [1,2]

[1]*Faculty of Sciences, Arab American University Palestine, P.O Box 240, Jenin, State of Palestine*
[2]*Qasemi Research Center- Al-Qasemi Academy, P.O Box 124, Baqa El-Gharbia 30100, Israel*

Correspondence should be addressed to Hilal Zaid; hilal.zaid@gmail.com

Received 27 January 2019; Accepted 17 April 2019; Published 5 May 2019

Guest Editor: José C. T. Carvalho

Diabetes is a predominant metabolic disease nowadays due to the off-beam lifestyle of diet and reduced physical activity. Complications of the illness include the gene-environment interactions and the downstream genetic and epigenetic consequences, e.g., cardiovascular diseases, tumor progression, retinopathy, nephropathy, neuropathy, polydipsia, polyphagia, polyuria, and weight loss. This review sheds the light on the mechanistic insights of antidiabetic medicinal plants in targeting key organs and tissues involved in regulating blood glucose homeostasis including the pancreas, liver, muscles, adipose tissues, and glucose absorption in the intestine. Diabetes is also involved in modulating major epigenetic pathways such as DNA methylation and histone modification. In this respect, we will discuss the phytochemicals as current and future epigenetic drugs in the treatment of diabetes. In addition, several proteins are common targets for the treatment of diabetes. Some phytochemicals are expected to directly interact with these targets. We lastly uncover modeling studies that predict such plausible interactions. In conclusion, this review article presents the mechanistic insight of phytochemicals in the treatment of diabetes by combining both the cellular systems biology and molecular modeling.

1. Background

Diabetes mellitus (DM) is a metabolic ailment resulting from insulin resistance or the reduced secretion of insulin. Insulin disorders result in distorted carbohydrate, fat and protein metabolism, and the increased levels of serum glucose. Uncontrolled hyperglycemia may end up damaging blood vessels and causing macrovascular (atherosclerotic) and microvascular (retinopathy and nephropathy) disorders [1]. Besides, HDL/LDL ratio in the serum decreases [2]. It is accepted that diabetes is a result of the imbalanced regulation at the genetic and epigenetic levels. Pancreatic β-cell differentiation is controlled by several genes such as GLP1 receptor, PAX4, and PDX. These genes are regulated at the epigenetic level. Additionally, some factors that are involved in insulin resistance, such as NF-kB, osteopontin, and Toll-like receptors, are epigenetically regulated.

Healthy lifestyle can be used to alleviate hyperglycemia. Nonetheless, this might fail to treat diabetes in a large number of cases. In this regard, medications should be introduced. Thus, understanding the different molecular and cellular mechanisms of action for the glycemic control helps in planning and introducing active chemicals for the treatment of diabetes. Several pharmaceutical drugs present in the market have limited actions and many side effects, e.g., biguanides and sulphonylurea. The scientific introduction of medicinal plants is a good alternative for the treatment of diabetes [3]. Indeed, active phytochemical should be screened and validated to test for their efficacy and toxicity. Herein, we summarize the mechanisms of action for the antidiabetic activity of drugs, with the emphasis on plants and their active phytochemicals. Moreover, the inhibition of epigenetic marks associated with diabetes is detailed. Herein, light is shed on medicinal plants and active ingredients that target diabetes via epigenetic mechanisms. These epigenetic drugs, or "epidrugs", target DNA methyltransferases, histone-modifying enzymes, e.g., histone deacetylases, histone acetyltransferases, protein arginine methyltransferases, histone methyltransferases, and histone demethylases.

Moreover, phytochemical "lead compounds" that target diabetes are currently screened via methods of *in silico*

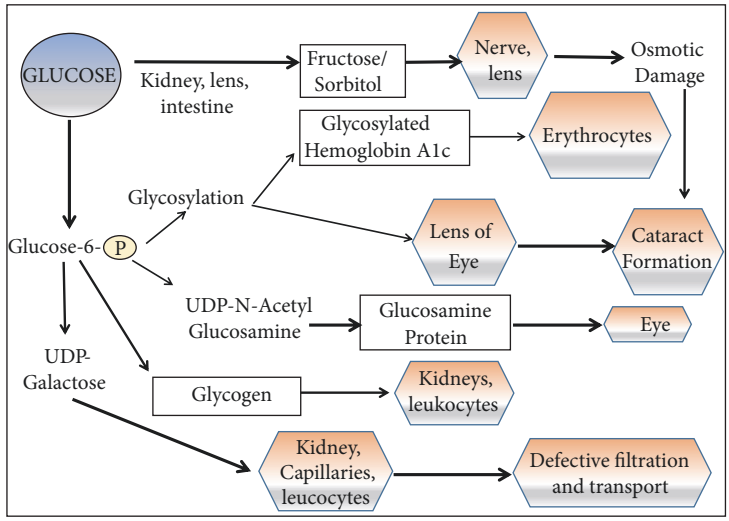


Figure 1: *Consequences of hyperglycemia.* As a result of osmotic imbalance and many others, hyperglycemia contributes to damage in several organs, e.g., eye, kidney, leukocytes, and capillaries. Figure 1 is reproduced from Saad et al. (2017) ([under the Creative Commons Attribution License/public domain)" [14].

drug design. In this review, we introduce protein targets for the antidiabetic drugs. Additionally, we show that several plants and the derived phytochemicals were put under the microscope by methods of computer-aided drug design. These plants include dried leaves of green tea, pomegranate, complex phenols in olive oil, linalool of *C. sativum*, *Papaver somniferum*-derived papaverine, and components of ginger, as well as *Euphorbia thymifolia* Linn. extracts. Hence, we concentrate on the cellular and molecular levels in drug design. It is thus worth mentioning that phytochemical screening for the treatment of diabetes is transferred into a new epoch.

2. Introduction

Diabetes mellitus (DM) is a metabolic disorder, where insulin resistance or the reduced levels of secreted insulin cause hyperglycemia [4]. Insulin is a key anabolic hormone, involved in signalling cascades that regulate complex carbohydrates, fats, and proteins synthesis. According to the World Health Organization (WHO), more than 422 million people worldwide were diabetic in 2014, and this number is expected to double in 2040. The prevalence of diabetes is the highest in the Middle East (13.7% in 2014), where the number of diabetic patients reached 43 million in 2014 [5].

Based on the etiology of DM, two main types of diabetes are known. Type I diabetes has very low prevalence. In most cases of this form, autoimmune mechanisms target the pancreatic β-cells to destruction, what drives the necessity for insulin replacement therapy [4, 6]. Type II diabetes is more predominant, and it results from insulin resistance in target tissues or the shortage in insulin secretion [4].

Since insulin signalling serves as a metabolic hub, carbohydrate, fat, and protein metabolism are drastically distorted after the increase in blood glucose levels. Fasting glycemia, postprandial glycemia, and haemoglobin A1C levels are elevated to ≥7mM, ≥11mM, and 6.5%, respectively [7]. Uncontrolled hyperglycemia for prolonged periods results in the destruction of blood vessels supplying the body organs, with the consequence of heart, eyes, kidneys, and nerves system damage. As a result, macrovascular (atherosclerotic) and microvascular (retinopathy and nephropathy) disorders follow. These complications are the leading causes of mortality in diabetic patients [1]. Moreover, levels of serum LDL increase, whereas the serum levels of HDL decrease [2]. Additional complications include blurred vision, polyuria, polydipsia, polyphagia, and weight loss [8]. Figure 1 introduces the causal relationship between hyperglycemia and the resulting damage in different organ systems. Extracellular hyperglycemia ends up in oxidative stress due to the oxidation and glycation reactions between reducing sugars and proteins [9]. Consequently, glycoxidation products, such as Nε-(carboxymethyl)-lysine and Nε-(carboxymethyl)-hydroxylysine, as well as free radicals, accumulate in tissue collagen of diabetic patients, causing metabolic stress, tissue damage, and cell death [10]. Among the consequences of tissue damage are changes in eye refraction [11], infiltration difficulties in the kidney [12], and others.

It is widely appreciated that genetic and epigenetic factors predispose to diabetes. Major genes that control β-cell differentiation, such as GLP1 receptor, PAX4, and PDX1, are epigenetically regulated. Epigenetics also induce insulin resistance through proinflammatory effects on some factors, as NF-kB, osteopontin, and Toll-like receptors [13].

To avoid hyperglycemia or alleviate the symptoms, preventive strategies through nonpharmacological approaches can be followed. Healthy diet, exercise, and weight loss can adjust glucose serum levels and improve normal glucose metabolism. When lifestyle change fails to treat diabetes, medications become a necessity. In type I DM, therapeutic insulin replacement is introduced. Additionally, pancreatic islets can be transplanted [16]. Drugs were on the other hand designed to target type II DM, with variant modes of actions. Some drugs induce an increased production and secretion of

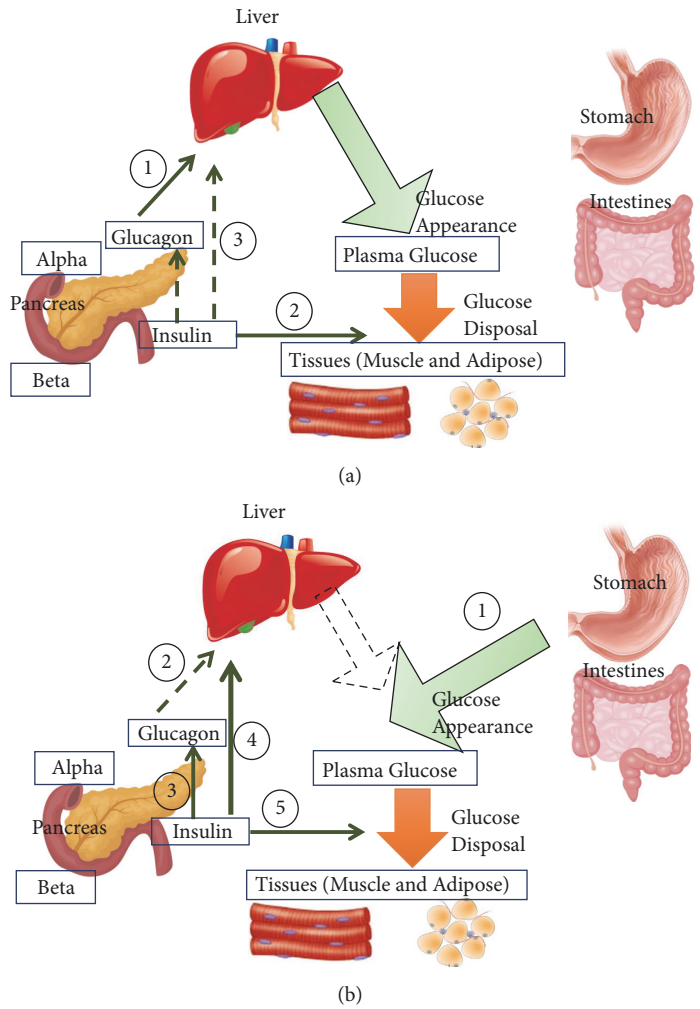


FIGURE 2: *Glucose homeostasis: role of insulin and glucagon.* In the fasting state (a), serum glucose is derived from glycogenolysis under the regulations of glucagon (1). Insulin controls glucose disposal at its basal levels (2). Since glucose levels are not high, low levels of insulin have minimal role in supressing glucose appearance in the serum (via glycogenolysis and gluconeogenesis) (3). In the fed state (b), glucose in the plasma is derived from nutrition "stomach and intestine" (1). Glucagon secretion and effect are supressed as a result of insulin secretion (2,3). Communication within the islet cells of the pancreas contributes to this inhibition. (4) As a result, gluconeogenesis and glycogenolysis are supressed in the liver. Glucose disposal is activated in peripheral organs (5) [15]. Figure 2 is reproduced from Saad et al. (2017) ([under the Creative Commons Attribution License/public domain)" [14].

insulin in the β-pancreatic cells. Other drugs promote insulin sensitivity in the target tissues. Liver tissue is responsible for buffering blood glucose and secreting glucose to the bloodstream to retain glucose homeostasis. Thus, hepatic enzymes involved in gluconeogenesis and glycogenolysis are inhibited via insulin signalling. On the other hand, skeletal muscles and adipose tissues are stimulated to increase glucose uptake [17]. Additionally, abnormal lipolysis induces hyperglycemia, reduced insulin secretion, and/or glucose uptake. Thus, some drugs that target diabetes are designed to inhibit lipolysis. Abnormal lipolysis also results in lipotoxicity, with the accumulation of toxic lipid metabolites (ceramide, diacylglycerol, and fatty acyl CoA) in adipocytes, muscles, liver, and the pancreas. Cardiovascular diseases are a major consequence of lipolysis [18]. Such complications cannot be treated via the aforementioned mechanisms and need agents that regulate the vascular homeostasis to relieve the injury and reduce the inflammation [19]. The most direct route for glycemic control is via the inhibition of glucose absorption in the intestinal walls. Thus enzymes that digest complex polysaccharides into simpler absorbable forms are inhibited [20] via, for example, alpha-glucosidase competitive inhibitors [21]. Major complications of diabetes include a plausible increase in the inflammatory responses. Thus, anti-inflammatory drugs are also designed to alleviate the side effects of diabetes [22].

The scope of glycemic control at the organ level is introduced in Figure 2. Different organ systems collaborate to maintain serum glucose in the fasting and postprandial states.

Many drugs are nowadays available in the market that are restricted by their pharmacokinetic properties, limited action, and side effects, e.g., biguanides, sulphonylurea.

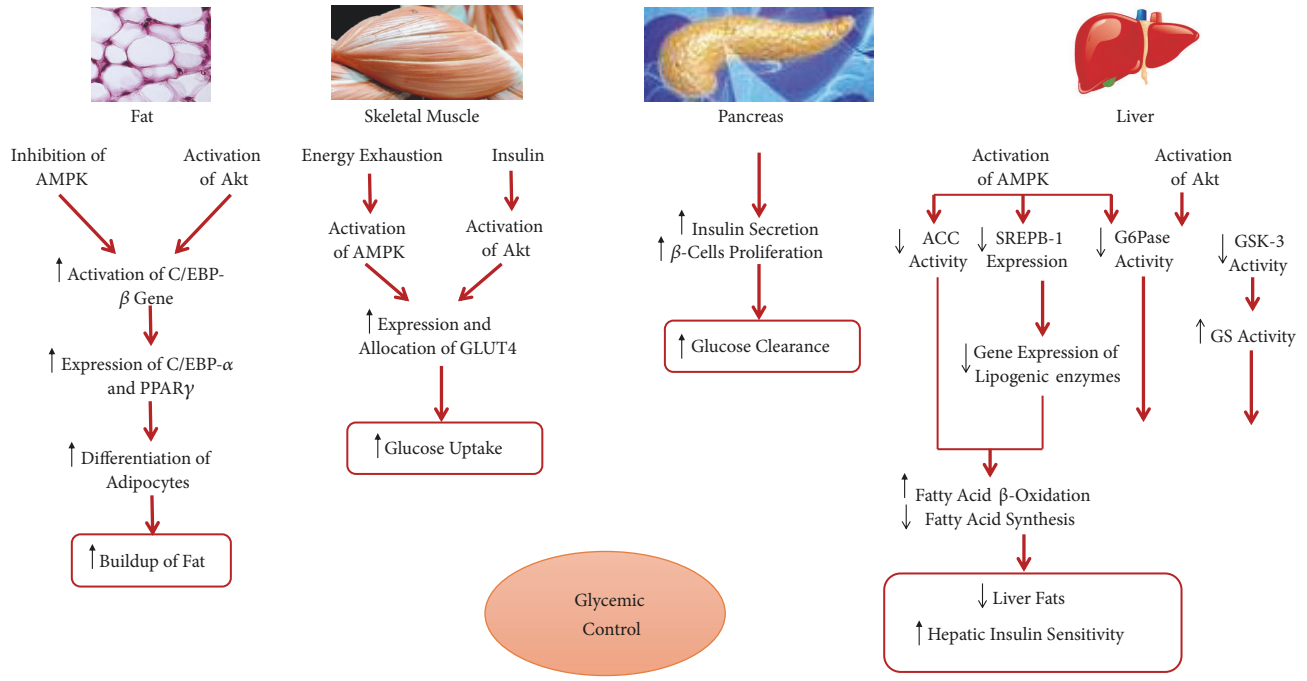


FIGURE 3: *Contribution of the major tissues to the glycemic control via insulin-dependent Akt and insulin-independent AMPK signaling cascades [14].* AMPK: AMP-activated protein kinase; Akt: protein kinase B; CEBP: Ccaat-enhancer binding protein; GLUT: glucose transporter; ACC: acetyl-CoA carboxylase; SREPB-1: sterol regulatory element binding protein 1; G6Pase: glucose-6-phosphatase; GSK: glycogen synthase kinase; GS: glycogen synthase. Figure 3 is reproduced from Saad et al. (2017) ([under the Creative Commons Attribution License/public domain)" [14].

Medicinal plants are used in all known traditional medical systems [3]. Indeed, Herbal-based remedies are still used as the major form of drugs by about 80% of the world's population. These medications are not usually regulated and some would debate that natural ingredients are safe to health. Indeed, any medication, herbal or synthetic, should follow thorough scientific investigation via screening, validation, preclinical and clinical procedures for the test of their efficacy and toxicity levels [14].

Around one quarter of the used drugs at present are of herbal origin and comprise at least one herbal-derived active compound or chemically modified herbal phytochemicals to produce a pharmaceutically active drug. Active phytochemical components can be used as good alternatives in combinatorial drug industry for the production of drugs that target diabetes. In this respect, many plant extracts were screened and have shown antidiabetic effects in animal test models and in clinical studies [14]. Polysaccharides, such as Anoectochilus roxburghii polysaccharides (ARP), Artemisia sphaerocephala Krasch seed polysaccharide (ASKP), Acanthopanax senticosus polysaccharide (ASP), and Coptis chinensis Franch (Ranunculaceae) polysaccharide-1 (CCPW-1), present in orchids, Astaraceae, Siberian ginseng, and the Chinese goldthreadare are used in the treatment of diabetes [23]. Terpenoids (also called isoprenoids) account for more than 40,000 individual compounds of both primary and secondary metabolisms. Terpenoids are specifically present in many herbal plants [24]. Polyphenols are a large set of compounds that include flavonoids, non-ketone polyhydroxy-polyphenols found in black tea, blueberries, citrus, cocoa, peanut and parsley; phenols, found in berries, chili peppers, oregano, sesame seeds and others; tannins, which conjugate to and precipitate proteins, present in berries, chocolate, legumes, nuts, and pomegranates. Alkaloids are nitrogen-containing organic phytochemicals and include morphine, quinine, ephedrine, etc., present in thyme and the Mediterranean saltbush. Saponins have foaming properties, and are found in basil, fenugreek, and the Mediterranean saltbush. Vitamins are also important components of plants and are known for their action as cofactors of central metabolic processes, e.g., biotin, thiamine, folate and niacin [14].

3. Strategies for the Glycemic Control

Main body organs/tissues that are involved in controlling the homeostasis of serum glucose have direct effect on signaling cascades involved in glycemic control. This relationship is shown in Figure 3.

To achieve serum glucose homeostasis around a "set point" (~5-5.5 mM) [25], balance should be maintained between the rate of glucose entering the bloodstream (glucose appearance) and glucose removed from the circulation (glucose disappearance). Glucose depletion takes place in direct routes such as glycolysis, tricarboxylic acid cycle, and the pentose phosphate pathway [26]. Indirect consumption and regeneration of glucose occur through glycogen and lipid metabolism [25]. The major hormones to regulate plasma glucose levels are insulin (which controls glucose disappearance), glucagon (which regulates glucose appearance in the liver) [27, 28], and epinephrine (regulating glucose

appearance in the muscle). Less significant contributions take place by cortisol, norepinephrine, and growth hormones [29]. Alternatively, *local* regulation of glucose levels takes place via allosteric mechanisms, among others.

Two global pathways are key contributors in regulating serum glucose homeostasis. The first introduces insulin-dependent mechanisms for the uptake of glucose and induces protein kinase B (PKB; also known as Akt) signaling [30]. The other signaling pathway is insulin-independent that induces AMP–activated protein kinase (AMPK) signaling cascades [31]. In the insulin-dependent glucose uptake, insulin binds to cell surface receptors, e.g., the insulin receptor-related receptor (IRS) and the insulin-like growth factor (IGF)-I receptor [32]. In the liver tissue, regulation of glucose and fat metabolism is the key effector in maintaining glucose homeostasis. Due to insulin-induced signaling, gluconeogenesis is inhibited; e.g., the activity promoter region of the glucose-6-phosphatase (key enzyme of gluconeogenesis) gene is attenuated and the expression gets reduced [33]. Additionally, glycogen storage is enhanced via dephosphorylating glycogen synthase (GS) and inhibiting glycogen synthase kinase 3 (GSK3), responsible for the phosphorylation of GS; among others [34]. On the other hand, due to AMPK signaling, regulation of fat metabolism at the level of gene expression as well as protein activity follows. Here, the sterol regulatory-element binding protein, SREBP-1 is inhibited and the expression of lipogenic genes is repressed [35]. At the protein level, the activity of acetyl-coA carboxylase is attenuated. As a result, biosynthesis of fatty acid is suppressed and the β-oxidation of fatty acids gets stimulated [36]. In skeletal muscles, insulin binding or contraction-induced molecular signaling (via Ca^{+2} and NOS in the proximal region as well as SNARE and Rab-GTPase proteins of the cytoskeleton in the distal region) enhances the expression and translocation of GLUT4 transporters for glucose uptake [37–40]. In adipose tissues, elevated levels of serum glucose induce lipid synthesis and inhibit adipose tissue differentiation. Here, the CCAAT-enhancer binding protein (C/EBP) is enhanced and the gene expression of transcription factors follows, e.g., C/EBP-α and the peroxisome proliferation-activity receptor, PPAR- γ [41]. Insulin and glucagon are mutually synergistic and act to induce opposite routes of phosphorylating active/inactive states of the key enzymes of sugar metabolism, which aids in shifting the equilibrium towards the directions of glucose clearance or production, respectively [42].

Local control of glucose homeostasis takes place in liver, muscle, and adipose tissues via the allosteric pool of enzymes, proteins, and other macromolecules [32]. Liver tissue has an unparalleled proficiency for "intuiting and buffering" glucose levels in plasma. In this respect, many hepatic isozymes and protein homologues have their singularities in the hepatic tissue. One example is the coded glucose transporters in the liver tissues (GLUT2, found also only in pancreatic and kidney cells, K_m = 17-20 mM) controlling glucose influx at high blood glucose concentrations [43]. Additionally, the isozymic form of liver hexokinase (glucokinase) has many folds higher K_m to glucose than most other hexokinase isoforms. Moreover, feedback inhibition by the product Glucose-6-phosphate does not happen for the liver isoform, but rather in other tissues [44]. The liver pyruvate kinase activity is noticed at very high plasma levels of glucose [45]. Glucose 6-phosphatase buffers glucose to the blood only from the liver and, to a lesser extent, the kidney [46].

Yet, carbohydrate elimination after a carbohydrates-rich meal is managed by the insulin-sensitive GLUT4 transporters, mainly allocated in muscle tissues and in adipose tissues [47, 48]. Upon exposure to high levels of plasma glucose, muscle tissues store their needs of glycogen [47]. Nonetheless, experiments on knockout mice in muscle glucose transporters showed normal mice in terms of glucose tolerance. Still, knockout experiments on glucose transporters of adipose tissues showed impaired glucose tolerance in muscle, liver, and adipose tissues, which suggests a regulatory role of adipose tissues beyond their glucose absorption capabilities [49].

The kidney, eye, some nerve tissues, erythrocytes, and leukocytes are nearly not responsive to insulin concentration in the blood. Nonetheless, they have a role in glucose depletion for their energy needs, which cannot stand the high levels of glucose in diabetic patients [12].

In summary, the mechanism of action for the antidiabetic activity of plants falls into several routes: increased pancreatic secretion of insulin by the augmentation of the pancreas; inhibition of glucose production in the liver and enhanced glucose uptake in the muscle and adipose tissues; inhibition of glucose absorption by the intestinal; the inhibition of diabetes-related complications. We have studied these mechanisms in detail previously [14]. Diabetes, metabolism disorders, and the involvement of medicinal plants were previously reviewed by us [50]. We summarize below the five aforementioned mechanisms and additionally introduce here the inhibition of epigenetic marks associated with diabetes (see next Section 4).

Increased pancreatic secretion of insulin-augmentation of the pancreas and increased insulin sensitivity: Type II DM is characterized by insulin resistance, reduced insulin production, or the failure of pancreatic β-cells. Drugs that target the pancreas aim to increase the size of pancreatic islands and the number of cells. Insulin levels can also be augmented through the ATP-dependent K-channels in the pancreatic cells, or drugs that mimic insulin action.

Inhibition of glucose production in the liver and the enhanced glucose uptake in the muscle and adipose tissues: Liver is the most crucial organ in regulating serum glucose levels. Liver utilizes the enzymes of glycolysis, gluconeogenesis, and glycogen metabolism to balance blood glucose levels. In addition to the liver tissue, muscle and adipose tissues respond to insulin and increase glucose transporter-4 (GLUT4) in the plasma membrane as a response to insulin secretion (in non-diabetic subjects). As a result, drugs that target liver metabolism and GLUT4 transporters are of interest to pharmacological research.

Inhibition of glucose absorption: The inhibition of digestive enzymes that hydrolyze complex polysaccharides and disaccharides into smaller fragments of monosaccharaides is a direct route for the inhibition of their escape to the bloodstream. Such monomer components can be absorbed through the intestinal walls to the bloodstream and absorbed

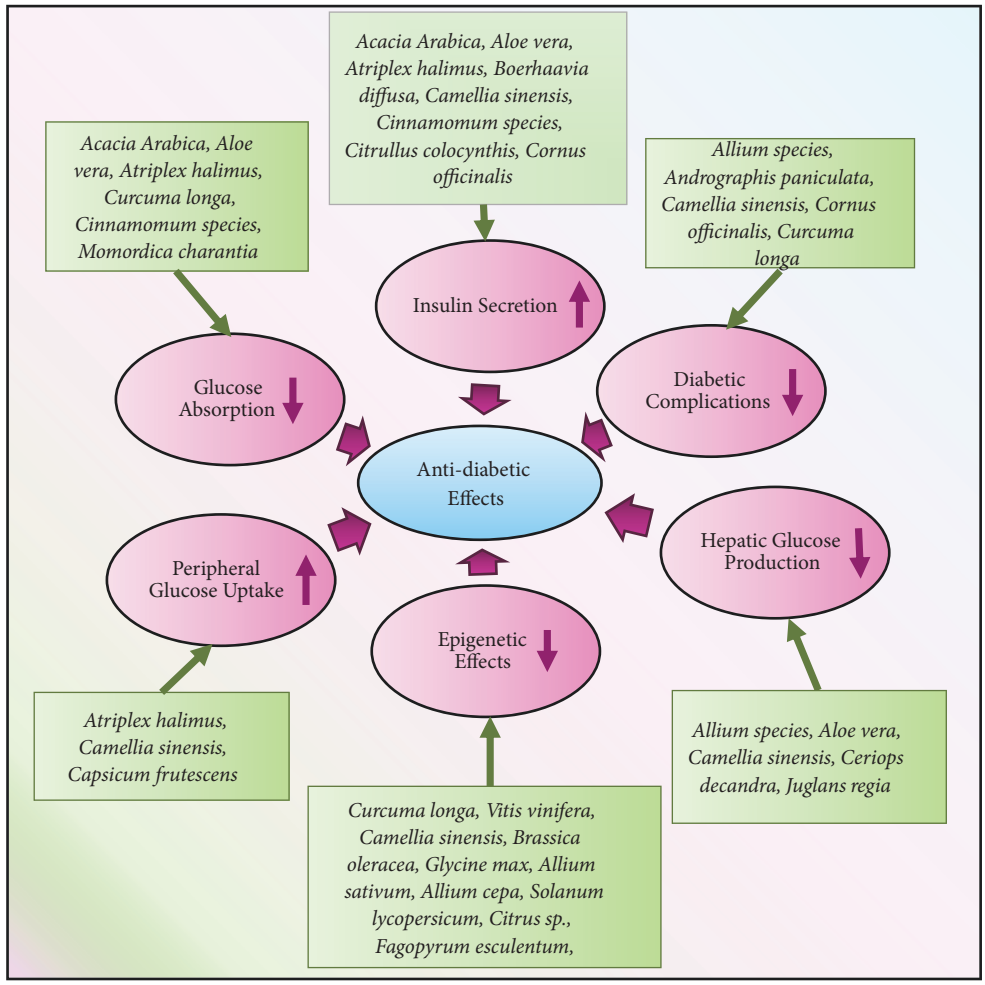


Figure 4: *Antidiabetic medicinal plants and their mechanisms of action of.* Plants work via different modes of action to alleviate diabetes. Some medicinal plants are summarized and their mechanisms of actions are mentioned.

by the liver, muscle, and fat tissues. An example of this group of inhibitors is the mammalian α-glucosidase inhibitor.

Inhibition of diabetes-related complications: The inflammatory complications of diabetes (e.g., nephropathy, neuropathy, and retinopathy) result from the oxidative damage. Treatment of diabetes alone in most cases does not reverse the disorders, which introduces the need for drugs to alleviate these disease states.

Figure 4 introduces some medicinal plants that work to target diabetes and alleviate the symptoms via the aforementioned mechanisms. For a detailed view on the mechanisms through which plants exert their effects, the readers are directed to [14, 50].

4. Epigenetics of Diabetes and Epidrugs

Although there is no uniform definition of epigenetics, it has been described as heritable changes in gene expression and downstream activity that does not target DNA sequence. Epigenetic modifications can be passed from one cell generation to the next (mitotic inheritance) and between generations of a species (meiotic inheritance). Such changes include, for example, DNA methylation, histone methylation, and acetylation. The nucleosome is composed of DNA wrapped around a histone octamer, composed of four histone protein units, two H2A-H2B-dimers, and an H3-H4 tetramer. Between two consecutive nucleosomal cores is a DNA sequence connected with a single molecule of histone H1. Chromatin modifiers at the epigenetic level usually target the amino acids of the *N*-terminal tails of histones and either enable or hamper transcription factors and other DNA binding proteins. At the level of DNA methylation, the 3D structure of chromatin and the minute supercoiling are affected by the methylation status. Changes to the structure of chromatin targets gene expression by either inactivating genes, when the chromatin is closed (heterochromatin), or by activating them when the chromatin is open (euchromatin) [51]. These changes are heritable and regulate gene expression and activity during development and differentiation as well as in response to environmental stimuli, such as nutritional life style. Such changes can nonetheless be also reversible. Thus, they are potential targets for therapeutic drugs.

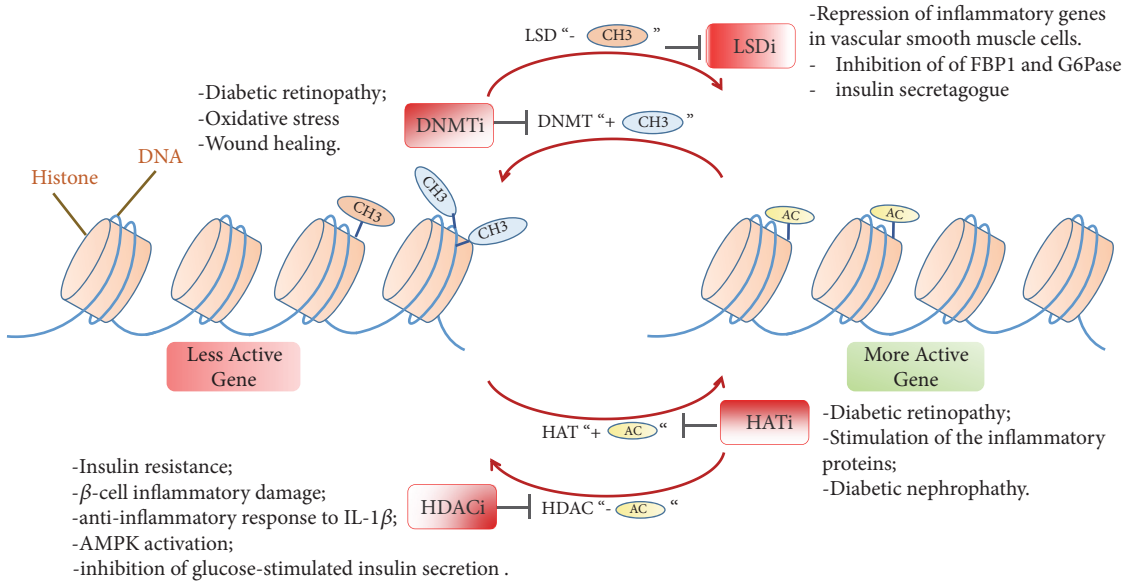


FIGURE 5: *An overview of the main epigenetic mechanisms and their association with diabetes.* Inhibitors/activators of the several epigenetic marks were shown to alleviate the effects or symptoms of diabetes via several routes. Details for such mechanisms are in the main text. LSDi: lysine-specific demethylase inhibitor; DNMTi: DNA-methyltransferases inhibitor; HATi: histone Acetyltransferase inhibitor; HDACi: histone deacetylase inhibitor; CH_3: methyl group; AC: acetyl group.

Epigenetic drugs, or "epidrugs", are an emerging field or class of drugs that target epigenetic changes to treat a wide variety of diseases. These include the metabolic disorders, e.g., diabetes and obesity and a wide variety of many other disorders, including cancer [52] and neurodegenerative diseases [53]. Many epigenetic drugs are now already in the phase of clinical trials for the treatment of diabetes. Some of the targets of epigenetic drugs include DNA methyltransferases, histone-modifying enzymes, e.g., histone deacetylases, histone acetyltransferases, protein arginine methyltransferases, histone methyltransferases, and histone demethylases. Normally, epidrugs serve as inhibitors for such targets, but they can also serve as potential activators in other contexts. Since epidrugs work on the 3D architecture of chromatin, they expectedly target a large network of biological molecules in signaling and metabolic pathways [52]. A brief overview of epigenetic mechanisms, their association with diabetes and the potential epidrugs is introduced in Figure 5.

DNA methylation of cytosine residues takes place at the C-5 position to yield 5-methylcytosine. DNA methyltransferases induce de novo (DNMT3a and DNMT3b) and maintenance (DNMT1) methylation of DNA. This process is accomplished with the aid of an S-adenosyl-methionine, which acts as a methyl donor for methylation [54]. Regulation by DNA methyltransferases has a role in the progression of diabetes, especially in the mitochondrial DNA [55]. One complication of hyperglycemia is the development of diabetic retinopathy, which is suggested to have a metabolic memory phenomenon after hyperglycemia is alleviated [56]. In this respect, DNA is dynamic, but its memory can last for several years. DNA methylation was found to be correlated with the metabolic memory of diabetic retinopathy, where the DNA methyltransferase DNMT1 is highly expressed. In this respect, the introduction of DNMT inhibitors (DNMTi), e.g., Aza, during the reversal period from hyperglycemia, could alleviate the symptoms of retinopathy [57]. Recently, diabetes was found to induce the damage in the wound healing process in a DNMT1-dependent mechanism. This study was applied on hematopoietic stem cells (HSCs) during their differentiation towards macrophages in type II diabetic mice. The process includes a Nox-2-dependent escalation of the oxidant stress in HSCs and a consequent decline in microRNA let-7d-3p. This resulted in the upregulation of DNMT1, which induced hypermethylation of *Notch1*, *PU.1*, and *Klf4*. As a result, the number of wound macrophages drastically decreased [58]. Other DNMTi also exist, e.g., procainamide and hydralazine, which are DNMTi that underwent *in silico* drug prediction and are presently in clinical trials for the treatment of diabetes [59, 60].

Histone deacetylase inhibitors (HDACi) are a new class of drugs. Some of these drugs target genes and proteins associated with diabetes. These drugs were found to efficiently manage insulin resistance in type II diabetes mellitus in preclinical and clinical trials [61]. Some drugs affect β-cell functions, prevent β-cell inflammatory damage, and relieve insulin resistance. HDACi also induce β-cell proliferation and differentiation and might alleviate late diabetic microvascular complications [62]. HDACi show also likely anti-inflammatory properties to IL-1β [62]. IL-1β is secreted from mononuclear cells and it inhibits β-cell function and induces β-cell death after prolonged exposure [63]. Among HDACis, valproic acid and sodium phenylbutyrate (PBA) are already in clinical trials for the treatment of diabetes [64, 65]. VPA is an activator of AMP-activated protein kinase (AMPK). Incubation of primary mouse hepatocytes with VPA resulted in higher than normal levels of phosphorylated AMPK and

acetyl-CoA carboxylase (ACC), a key enzyme in glucose metabolism. Valproic acid was also found to reduce hepatic fat accumulation, liver mass, and serum glucose in obese mice [66]. Phenylbutyrate (PBA) protects against cardiac injury [65]. PBA also enhances palmitate-induced inhibition of glucose-stimulated insulin secretion [67].

One more class of drugs that treats diabetes targets the histone acetyltransferase (HAT). One such aberrant epigenetic chromatin event that results from hyperglycemia is a significant increase in histone acetylation in retinas from the diabetic rats, suggested to have a metabolic memory [68]. This acetylation was proposed to contribute to the hyperglycemia-induced upregulation of proinflammatory causative proteins for the diabetic retinopathy. To this aim, inhibitors of histone acetyltransferase (garcinol and antisense against the histone acetylase, p300) and activators of histone deacetylase (resveratrol and theophylline) are introduced to lessen both the acetylation and stimulation of the inflammatory proteins [68]. New evidence has shown that HATs and HDACs inhibitors serve also to cure diabetic nephropathy in cellular and animal models [69].

Histone H3 lysine 4 dimethylation (H3K4me2) is a main chromatin mark associated with open chromatin and active gene expression. Lysine-specific demethylase1 (LSD1) regulates H3K4 methylation negatively and reduces its occupancy at gene promoters. Chromatin immunoprecipitation experiments revealed that the promoters of two inflammatory genes, namely, the monocyte chemoattractant protein-1 and interleukin-6, are highly enriched with H3K4me2 in vascular smooth muscle cells (VSMCs) of diabetic mice. Protein levels of LSD1 were, in contrast, drastically diminished [70]. Silencing of LSD1 gene promoted inflammatory gene expression in nondiabetic VSMCs. On the other hand, overexpression of LSD1 in diabetic VSMCs repressed the expression of inflammatory genes [70]. Other studies on HepG2 cells presented the inclusion of LSD1 in the activation of gluconeogenesis pathways, thus leading to an increase in serum glucose levels and a decrease in intracellular glycogen. LSD1 was found to allocate in the promoters of FBP1 and G6Pase, two key enzymes of gluconeogenesis, and to regulate their H3K4 dimethylation levels [71]. Thus, drugs that target LSD1 must be targeted in tissue- and context-specific manners. Tranylcypromine is an FDA-approved drug used to treat major depressive disorder. It is now recognized as a histone demethylase inhibitor of lysine-specific demethylases (LSD1 and LSD2). Tranylcypromine was described as an effective insulin secretagogue and hypoglycemic agent [72].

4.1. Herbal-Derived Anti-Diabetes Epidrugs. Recently, natural compounds, such as resveratrol, curcumin, and epigallocatechin gallate (EGCG), have been shown to alter epigenetic mechanisms, which may lead to the increased sensitivity of cancer cells to conventional agents and the inhibition of tumor growth [73].

Resveratrol is a natural polyphenol found in grapes and chocolate. Over a decade, resveratrol was found to activate sirtuin 1 (SIRT1), an NAD-dependent HDAC whose administration to insulin-resistant animals improves glucose homeostasis and regulates insulin sensitivity [74–76]. Until recently, however, only few clinical trials exist that have tested the health benefits of resveratrol in humans with metabolic deficiency [77]. Curcumin is an inhibitor of HATs, HDACs, and DNMTs. It also serves as inhibitor or activator of several miRNAs [78]. Epigallocatechin gallate (EGCG) is the most abundant green tea catechin. Epigenetic mechanism of action for this drug involves histone acetylation-deacetylation and DNA methylation, where EGCG upregulates the anti-inflammatory activity of regulatory T cells [79].

Other polyphenols also exist that have epigenetic targets. One such example is sulforaphane of broccoli, which is an epigenetic drug that was found to inhibit DNMT1 expression, reduce promoter methylation, and inhibit HDACs [80–82]. Cell culture, *in vivo* studies, and analysis of coexpression networks and genetic data of the liver tissue showed that sulforaphane can inhibit glucose production through mechanisms of nuclear translocation of nuclear factor erythroid 2-related factor 2 (NRF2) and the inhibition of gene expression of crucial enzymes of gluconeogenesis [83]. Genistein is a polyphenol of soy beans which reverses hypermethylation and induces active histone modifications in many tumors [84]. Genistein seems to modulate on diabetes via direct effects on β-cell proliferation, glucose-stimulated insulin secretion, and protection against apoptosis. This is suggested to involve cAMP/PKA signaling cascades and to modulate via epigenetic mechanisms [85]. Organosulfur compounds of garlic and allium also have anti-diabetic effects. These natural products were also found to modulate via the induction of histone acetylation in several malignancies [86]. Lycopene is a phytochemical present in tomatoes with a potent antioxidant effect. Some studies expected a beneficial outcome in using this phytochemical to relieve the oxidative stress of diabetic patients [87]. This drug was found to function via gene methylation modes [88]. Quercetin is another epidrug present in citrus fruits and buckwheat. This drug acts as a DNMT1 inhibitor (via the repression of TNF-induced NFkappa transcription factor) and promotes Fas ligand-related apoptosis via histone H3 acetylation and potential HDAC inhibition [89–91]. Quercetin was shown to be involved in the stimulation of glucose uptake through MAPK insulin-dependent mechanism. This is accomplished in the muscle via the translocation of GLUT4 transporters and in the liver via the downregulation of key gluconeogenesis enzymes [92, 93].

5. Protein Targets for the Treatment of Diabetes

Computer-aided drug design is nowadays used to screen the phytochemical "lead compounds" which can be antidiabetic. Quantitative structure-activity/property relationships help us filer drugs that can be administered to the human biological system with high efficiency and less side effects. Nonetheless, once the feasible phytochemicals are selected, the modes of interaction with the biological targets aid in establishing the decision for their effectiveness as antidiabetic agents. Docking studies, molecular dynamic simulations, and free energy calculations predict the detailed picture for the action mechanisms as well as interactions involved between the lead

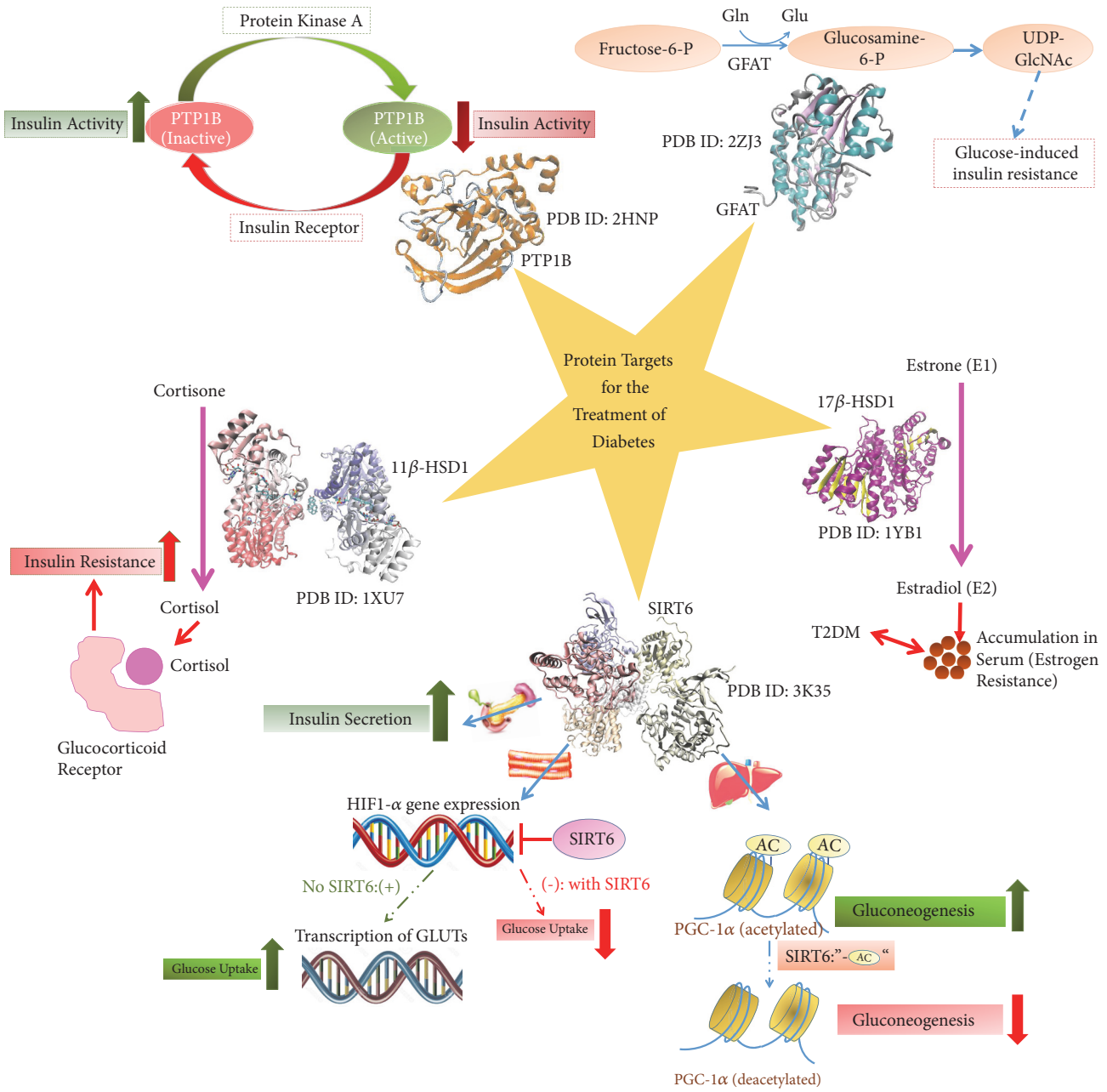


FIGURE 6: *Protein targets for antidiabetic drugs and their mechanisms of action.* Details for the targeted effect on these enzymes by antidiabetic phytochemicals are discussed in the main text. PDB: protein data bank file; PTP1B: protein tyrosine phosphatase 1B; GFAT: glutamine:fructose-6-phosphate aminotransferase; GlcNAc: N-acetyl-glucosamine; 17β-HSD1: 17β-hydroxysteroid dehydrogenase 1; 11β-HSD1: 11β-hydroxysteroid dehydrogenase 1; T2DM: type II diabetes mellitus; SIRT6: sirtuin 6; GLUTs: glucose transporters; HIF1-α: hypoxia-inducible factor 1-alpha.

and target in the process of drug design. Below, we list the common protein targets for the treatment of diabetes and discuss some phytochemicals that are expected to directly affect the activity of these targets. Figure 6 introduces the link between these protein targets and diabetes. Additionally, phytochemicals that target these proteins are briefed.

5.1. 11β-Hydroxysteroid Dehydrogenase. 11β-hydroxysteroid dehydrogenase (11β-HSDs) is a member of the short-chain reductases (SDR). It catalyzes the interconversion between the active glucocorticoids (corticosterone, cortisol) and the inert 11-keto forms (cortisone, 11-dehydrocorticosterone). 11β-HSD has several isoforms in humans, which are available in the liver, brain, adipocytes, lung, and other tissues. The 11β-HSD1 is NADPH-dependent active isoform, which is mainly expressed in the liver as well as the adipose tissue [94]. It is responsible for maintaining a sufficient exposure of relatively low affinity glucocorticoid receptors to their ligand [95]. The

11β-HSD1 faces the ER lumen, and this compartmentalization is crucial for its regulation. The cofactor NADPH that is regenerated by hexose-6-phosphate dehydrogenase is also located in the ER lumen [96].

Cortisol plays a pivotal role in diabetes. Indeed, the abnormal regulation of glucocorticoid metabolism was linked to type II diabetes [97]. The antagonism of hepatic glucocorticoid receptor was found to reduce glucose levels in the serum of diabetic mice and to ameliorate insulin resistance [98]. Thus, 11β-HSD is a potent therapeutic target whose inhibition might serve in the treatment of type II diabetes [99, 100].

The crystal structure of 11β-HSD1 was first resolved in 2005 for *Mus musculus* [101]. There exist so far 11 structures for the human isozyme, two murine structures, and a single structure for guinea pig in the RCSB database [102]. Hosfield and colleagues provided the structure of human 11β-HSD1 in both the open and closed conformations [103]. 11β-HSD1 is a tetramer composed of two dimers. The overall topology of 11β-HSD1 includes, as in other SDR enzymes, central 6-stranded, all-parallel β-sheets that are sandwiched by three α-helices. The active site is found in the region where the NADP+ and the steroidal detergent CHAPS molecules are located. The substrate binding induces the reorientation of the variable β6-α6 insert that is specific to 11β-HSD1 in order to provide the hydrophobic interface needed for binding and exclude the bulk solvent. The center of the tetramer has a Pro-Cys motif, which forms reversible disulfide bridges that can change the enzyme activity. Structural changes at the enzyme active site were found to be coupled with conformational flexibility at the tetramerization interface, which suggests a mechanism for the enzymatic activity [103]. This motif is located at the C-terminus and caps the active site of the next subunit in the tetramer. On the other hand, the N-terminal portion is responsible for orienting the enzyme in the ER membrane [104]. Of the crystal structures resolved for the human protein, several studies served to investigate the binding of 11β-HSD to its inhibitors, such as sulfonamide and triazole, which showed competitive and mixed inhibitions, respectively. Triazole interacts closely to the NADP+ cofactor and is assumed to change the NADP+ binding [105].

Hintzpeter and colleagues investigated the plausible inhibition of 11β-HSD1-mediated cortisone reduction upon the introduction of dried leaves of green tea to human microsomes, which turned out to be positive [106]. Subsequently, polyphenolic compounds were extracted from green tea and tested. Amongst the phytochemicals in green tea, (-)-epigallocatechin gallate (EGCG) exhibited the strongest inhibition of 11β-HSD1 (IC50=57.99 μM for reduction; IC50=131.2 μM for oxidation). Competitive inhibition was proposed to be the mode of action for EGCC. Docking studies showed the allocation of EGCG in the active site of 11β-HSD1, where it hydrogen bonds with Lys187 [106]. Ginger is also known for its anti-diabetic activity. Three gingerol derivative compounds are [6]-paradol, (E)-[6]-Shogaol, and (5R)-Acetoxy-[6]-Gingerol shown, known for their inhibitory activities against human and mouse 11β-HSD1 [107].

In a study by Tsang and colleagues [108], the administration of pomegranate juice to human volunteers was tested for its possible inhibition of 11β-hydroxysteroid dehydrogenase type 1. The volunteers consumed 500 ml of pomegranate juice, and a negative control group consumed 500 ml of a placebo drink containing the same levels of energy for 4 weeks. Measurements were performed for cortisol/cortisone ratio in the urine, and it was to be significantly lowered in the group of volunteers who have taken pomegranate juice for the whole time when compared to the placebo control [108].

Licorice is another plant that was also found to inhibit 11β-hydroxysteroid dehydrogenase type 1 [109]. Gumy and colleagues investigated the effect of the leave extracts of loquat in transfected HEK-293 cells and found that the extracts were capable of inhibiting 11β-HSD1. Additionally, extracts of roasted but not native coffee beans were found to exhibit the same effects [110].

5.2. 17β-Hydroxysteroid Dehydrogenase. 17β-hydroxysteroid dehydrogenases (17β-HSDs) play key roles in the first step of the degradation of androgen and estrogen as well as the last step in their activation. The weaker estrone (E1) can be synthesized from the more potent estrogen, estradiol (E2) by oxidative 17β-HSDs [111]. 17β-HSD2 catalyzes the production of E1 using NAD as a cofactor (Km =0.35±0.09 μM) [112]. Conversely, E1 can be converted to E2 by reductive 17β-HSDs, including the highly active 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1). The 17β-HSD1 isoform has a higher specific activity than the 17β-HSD2 enzyme [113]. The 17β-HSD1 has a dual function, as it is also slightly involved in catalyzing the conversion of active androgens, such as 4-androstenedione, to inactive ones, e.g., testosterone [114]. 17β-HSD1 performs its catalytic activity with the help of NADPH as a cofactor (Km value of 0.03±0.01 μM) [115].

In humans, 17β-HSD1 is expressed in endometrium, ovary, placenta, and breast. Indeed, the enzyme was found to be more profusely expressed than 17β-HSD2 in estrogen-dependent breast cancer cells [116, 117]. Additionally, Zhang and colleagues [118] showed that the expression of 17β-HSD1 is crucial in determining the [E2]/[E1] ratio in breast cancer cells. E2 was found to induce metabolic homeostasis. Thus, accumulation of the circulating E2 in serum might be indicative of estrogen resistance. This indeed might be linked to metabolic deficiency and T2DM [119]. As a result, 17β-HSD1 might provide a good therapeutic target for inhibitory drugs for the treatment of diabetes. Nonetheless, most inhibitors of this steroid-converting enzyme are estrogen analogues, which poses an obstacle in the clearance of their estrogenic activities.

Amongst the human steroid-converting enzymes, the crystal structure of 17β-HSD1 was the first to be resolved. The enzyme was crystallized in the presence of NADP-, β-octylglucoside, glycerol, and polyethylene glycol. Unlike other structures of the short-chain dehydrogenases (SDRs), this reductase enzyme was found to have an insertion of two helix-turn-helix motifs, suggested to contribute to the substrate specificity and membrane integration [120]. 17β-HSD1 is 327-amino acid long and exists as a homodimer [121, 122]. Recombinant human 17β-HSD1 in complex with

estradiol were captured at room temperature, and the structure was resolved at 1.7 Å [123]. The enzyme-estradiol interactions include a hydrophobic core of the steroid and nine residues in the enzyme binding pocket as well as hydrogen bonds, which contribute to the enzyme specificity. The three hydrogen bonds involve the side chains of Ser142, Tyr155, and His221. Additionally, Glu282 contributes to the binding process at the interface. C-19 steroids bind to 17β-HSD1 in both normal and reverse orientations, which induces an inhibition of the most potent androgen dihydrotestosterone (DHT). The mechanism involves the 3β-reduction of DHT into 5-androstane-3,17-diol (3β-diol) and 17β-oxidation of DHT into A-dione [124].

The structure of the ternary complex of 17β-HSD1 with the cofactor $NADP^+$ and equilin (an estrogen used in estrogen replacement therapy, 3.0-Å resolution) was solved by Sawicki and colleagues [125]. Equilin was found to inhibit the 17β-HSD1-catalyzed reduction of E_1 to E_2, and the crystal structure showed that the equilin molecule is bound at the active site in a similar fashion to the substrate [125].

Complex phenols in olive oil were investigated for their inhibitory action on both reductive and oxidative 17beta-hydroxysteroid dehydrogenase activity in human hepatic microsomes. Dihydroxybenzoic acid, gallic acid, hydroxytyrosol, and oleuropein glycoside could inhibit the reductive 17beta-HSD activity but not the oxidative one. Rather, gallic acid stimulated the activity by approximately 30% [126].

5.3. Glutamine Fructose-6-Phosphate Amidotransferase (GFAT). Glutamine-F-6-P amidotransferase (GFAT) is an enzyme that shifts the flow of the incoming glucose into the hexosamine biosynthesis pathway. This pathway is a minor branch in the glycolysis pathway; it is nonetheless crucial for the glycosylation of proteins and lipids. GFAT is a rate-limiting enzyme that converts fructose-6-phosphate to glucosamine-6-phosphate. First, acetyl-coenzyme (CoA) is derived from either glucose metabolism or fatty acid β-oxidation, and it transfers its acetyl group to glucosamine-6-phosphate to yield N-acetylglucosamine-6-phosphate [127, 128]. In a second step, the main end-product of the pathway, UDP-N-acetylglucosamine (UDP-GlcNAc), is produced, where a uridine nucleotide (UDP) is added to the glucosamine. UDP-GlcNAc is employed in N- and O-linked glycosylation. Glucose-induced insulin resistance is a possible consequence of this shift to the hexosamine pathway and the resultant glucose toxicity [129–131]. Crystal structures of GFAT with UDP-GlcNAc could offer strategies to derive lead compounds that target type 2 diabetes for treatment.

To help investigate the changes in enzymatic activity of GFAT, Traxinger and Marshall [132] found that the treatment of isolated rat adipocytes with insulin or glucose alone (or in combination) failed to reduce cytosolic GFAT activity after 4 h treatment. The combined treatment with insulin, glucose, and glutamine altogether caused a dramatic loss (70%) of GFAT activity in less than 2 h. Extensive treatment of the adipocytes with glucosamine (360 μM), which is a part of the hexosamine pathway, elicited a 55% loss of GFAT activity after 4 hours [132].

Glutamine analogs were used to assess the role of glutamine in the expression of glucose-induced desensitization of the insulin-responsive glucose transport systems (GTS). 0-diazoacetyl-L-serine (azaserine) and 6-diazo-5-0~0- norleucine are glutamine analogs that could irreversibly inactivate glutamine-requiring enzymes, such as glutamine:fructose-6-phosphate amidotransferase (GFAT). Both azaserine and 6-diazo-5-0~0- norleucine were found to inhibit the desensitization in 18-h treated cells without affecting maximal insulin responsiveness in control cells [132].

Human GFAT enzyme exists in two isoforms and a splice variant, GFAT1, GFAT2, and GFAT1L [133, 134]. GFAT1 was found to be highly expressed in striated muscles and adipose tissues, and to a lesser extent in the liver [135–137], which are major targets for the treatment of diabetes and obesity. GFAT has two distinct domains. The first is an N-terminal glutaminase domain (27 kDa), responsible for converting glutamine to glutamate and ammonia. The other domain is a C-terminal isomerase domain (40 kDa), which includes the active site that exploits ammonia to transform fructose-6P into glucosamine-6P [138]. Nakaishi and colleagues resolved the first crystal structure of human isomerase domain of GFAT ([139] PDB ID: 2ZJ3). The conformation of the active site is rigid, and it is composed of two analogous subdomains (residues 313–493 and 494–508). Each subdomain is composed of five parallel β sheets surrounded by α helices.

In a study by Shetty and Salimath, diabetic mice that were fed with starch diet exhibited an increase in the activity of GFAT when compared to the control group. The addition of fenugreek dramatically controlled this increase in GFAT activity, which indicates the inhibitory activity of fenugreek phytochemicals against GFAT [140].

The active phytochemical linalool in *C. sativum* was docked in one study to the protein GFAT (PDB: 2JZ3) [141]. The docking studies showed van der Waal interaction with Ala674, Cys373, Thr425, Gly374, Ser376, Thr375, Ser473, Ser676, Val471, Lys675, and Glu560 [141].

5.4. Protein Tyrosine Phosphatase 1B (PTP1B). The covalent addition of a phosphate (PO_4) group by kinase enzymes into a protein is a posttranslational modification that is biologically significant. It is involved in regulating metabolic and signal transduction processes. This modification results in the downstream inhibition or activation of the target receptor proteins and enzymes. In eukaryotes, serine, threonine, and tyrosine are the amino acids normally targeted for phosphorylation [142]. Ushiro and Cohen identified tyrosine phosphorylation as a result of epidermal growth factor-activated protein kinase [143]. The process of tyrosine phosphorylation is involved in cell proliferation and differentiation [144, 145]. Phosphorylation and dephosphorylation of tyrosine are mediated via protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), respectively. Binding of type 1 insulin-like growth factor (IGF-1) to its tetrameric receptor induces auto-phosphorylation of the receptor and the downstream activation of PKB and MAPK pathways [146, 147]. This indeed is involved in the translocation of GLUT4 transporter to the plasma membrane. PTPs constitute a huge

family of enzymes with a conserved 11-residue sequence motif of PTPs and dual specificity phosphatases. Arginine and cysteine in this motif are indispensable for the recognition and catalytic removal of phosphates. PTP1B is the first isolated PTP, which is involved in regulation the insulin-signaling pathway [148]. Barfold and colleagues resolved the first crystal structure of PTP1B in 1994 [149] with 321 residues. In this structure, the phosphate recognition domain is located in a loop at the N-terminal side. On the other hand, the catalytic site is located at the base of a shallow cleft. The cysteine is involved as a nucleophile in the cleavage.

Inhibitors of PTP1B are designed to alleviate type 2 diabetes mellitus and manage insulin resistance. These inhibitors were found to stably bind in the two binding sites and show their effect at nanomolar concentrations [148]. Nonetheless, searching for inhibitors that bind in the active site might be not the most effective as the positively charged active site is highly conserved among the proteins in the family of tyrosine phosphatases. As such, a better alternative strategy is to search for an allosteric site for inhibition. Jin and colleagues [150] used molecular docking, binding free energy calculations, and molecular dynamics simulations, and they found an allosteric site that is less conserved and more hydrophobic. The inhibitor used in their study was lupane triterpenes, which proved to inhibit PTP1B in cell culture studies. The computational studies were followed by two enzymatic assays for validation [150].

Jiang and colleagues [151] reviewed the natural products that possess inhibitory activities against PTP1B. Thus, this protein seems to be a hot target in phytochemical screening. Phytochemicals that target PTP1B include phenolics, terpenes, steroids, N- or S- containing compounds, and miscellaneous phytochemicals [151].

Papaver somniferum-derived papaverine is a member of isoquinoline alkaloids that has a high structural similarity to berberine, a known inhibitor PTP1B in human. Docking studies on papaverine showed a low energy orientation and a good fit in the binding pocket of PTP1B. This was strengthened by *in vitro* studies that show inhibitory action against PTP1B as well as *in vivo* studies that showed a significant reduction in fasting glucose levels in diabetic mice upon the administration of this phytochemical [152].

5.5. Mono-ADP-Ribosyltransferase-Sirtuin-6 (SIRT6). As we previously stated, SIRTs are targeted by anti-diabetes epidrugs. SIRT6 has NAD^+-dependent deacetylase activity [153] as well as mono-ADP-ribosyltransfease activity [154]. Drugs that target SIRT6 were found to exhibit either inhibitory or activating mechanisms. On one hand, the absence of SIRT6 was shown to be concomitant with increased tissue glucose uptake and decreased serum glucose levels [155]. SIRT6 was proposed to perform its epigenetic effect via inhibiting the expression of the transcription factor hypoxia inducible factor-1α (HIF1-α), which is involved in the transcription of glucose transporters [156]. Thus, inhibitors were used that target SIRT6 leading to the decreased uptake of glucose by peripheral tissues. On the other hand, SIRT6 was suggested to perform its epigenetic action via the deacetylation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), which robustly stimulates glucose production in the liver. Thus, activators of SIRT6 were designed that help repress hepatic gluconeogenesis and decrease blood glucose levels [157].

The resolved structure of SIRT6 showed variation from the structures of other sirtuin proteins in the class. The structures showed an extended zinc-binding domain. Additionally, the helix set that connects the Rossmann fold domain to the zinc-binding motif is absent in SIRT6. Furthermore, due to the absence of the conserved NAD(+)-binding loop, SIRT6 is suggested to be exclusively able to bind NAD(+) in the absence of an acetylated substrate. Indeed, kinetic studies ($K(d) = 27 \pm 1\ \mu M$) proved this hypothesis [158].

Le [159] has performed docking studies on the six main components of ginger ([4]-gingerol, [6]-gingerol, [8]-gingerol, [10]-gingerol, [6]-shogaol, and β- bisabolol) with all of the above-mentioned protein targets. β-bisabolol and 10-gingerol were found to have low activity and a high metabolization rate. He concluded that the effect of the phytochemicals in ginger is rather synergistic; thus the mixture of all components should be administered as plausible drugs [159].

The effect of *Euphorbia thymifolia* Linn. extracts on mice models was studied, and the plant was found to induce antihyperglycemic effects. To investigate the action mechanism and molecular interactions existing between the bioactive phytochemicals in *E. thymifolia* and protein targets of Type 2 DM, molecular docking, and bond scanning were performed on the interaction between 20 ligands and four of the above target proteins: 11-β HSD1, GFAT, PTP1B, and SIRT6. In the next step. Energy calculations indicated strong affinity (< -8.0 kcal/mol) of seven lead compounds to the targeted proteins. Additionally, the molecules had hydrophobic interactions and hydrogen bonds with the active site of the four target proteins. The bioactive phytochemicals are β-amyrine, corilagin, cosmosiin, quercetin-3-galactoside, quercitrin, taraxerol, 1-*O*-and galloyl-β-d-glucose [160].

6. Conclusion

Phytochemicals are hot plausible drugs that are thoroughly investigated for their antidiabetic effects. Studies on signaling cascades and local pool of enzymes and proteins showed the phytochemicals can target such routes to alleviate high serum glucose levels. The current perspective for the effect exerted by phytochemicals to treat diabetes suggests epigenetic modulation, where phytochemicals target central epigenetic marks. Recent interest in epigenetics has focused on phytochemicals aimed at modifying diabetes-specific gene/protein expression. Several major classes of epigenetic agents include drugs/phytochemicals already in the marketplace as well as several in various stages of preclinical as well in clinical investigations. These classes include HDACi, HATi, PRMTis, DNMTis, HDMis, and SIRTis. In this review, we discuss drugs/phytochemicals with epigenetic properties that have been identified as potential therapeutic agents in the treatment of diabetes. Further modeling and cheminformatics studies are current topics in drug discovery applied in drug

design for diabetes, and it is expected that the scope for the treatment of diabetes will be transferred into a new era.

Abbreviations

11β-HSD1: 11β-Hydroxysteroid dehydrogenase 1
17β-HSD1: 17β-Hydroxysteroid dehydrogenase 1
AC: Acetyl group
ACC: Acetyl-CoA carboxylase
Akt: Protein Kinase B
AMPK: AMP-activated protein kinase
ARP: Anoectochilus roxburghii polysaccharides
ASKP: Artemisia sphaerocephala Krasch seed polysaccharide
ASP: Acanthopanax senticosus polysaccharide
CCPW-1: Coptis chinensis Franch (Ranunculaceae) polysaccharide-1
CEBP: Ccaat-enhancer binding protein
CH3: Methyl group
CoA: Acetyl-coenzyme
DM: Diabetes mellitus
DNMT: DNA methyltransferase
DNMTi: DNA-methyltransferases inhibitor
EGCG: Epigallocatechin gallate
ER: Endoplasmic reticulum
FBP1: Fructose bisphosphatase 1
G6Pase: Glucose-6-phosphatase
G6Pase: Glucose-6-phosphatase
GFAT: Glutamine:fructose-6-phosphate aminotransferase
GlcNAc: N-Acetyl-glucosamine
GLUT: Glucose transporter
GLUT: Glucose transporter
GLUTs: Glucose transporters
GS: Glycogen synthase
GS: Glycogen synthase
GSK: Glycogen synthase kinase
GSK3: Glycogen synthase kinase 3
GTS: Glucose transport systems
H3K4me2: Histone H3 lysine 4 dimethylation
HATi: Histone Acetyltransferase inhibitor
HDACi: Histone deacetylase inhibitor
HDL: High-density lipoprotein
HIF1-α: Hypoxia-inducible factor 1-alpha
HSCs: Hematopoietic stem cells
IGF: Insulin-like growth factor
IRS: Insulin receptor-related receptor
LDL: Low-density Lipoprotein
LSD1: Lysine-specific demethylase1
LSDi: Lysine-specific demethylase inhibitor
NRF2: Nuclear factor erythroid 2-related factor 2
PBA: Phenylbutyrate
PDB: Protein data bank file
PGC-1α: Peroxisome proliferator-activated receptor-γ coactivator 1α
PKB: Protein kinase B
PTKs: Protein tyrosine kinases
PTP1B: Protein tyrosine phosphatase 1B
PTPs: Protein tyrosine phosphatases
SDR: Short-chain reductases
SIRT1: Sirtuin 1
SREPB-1: Sterol regulatory element binding protein 1
T2DM: Type II Diabetes Mellitus
UDP: Uridine nucleotide
VSMC: Vascular smooth muscle cells
WHO: World Health Organization.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Siba Shanak identified sections in the review body, wrote the review text, and prepared figures. Bashar Saad identified the introductory sections in the review, revised the whole text, and added a summary figure. Hilal Zaid revised the text, introduced key ideas, and is the corresponding author.

Acknowledgments

The authors would like to acknowledge the German Federal Ministry of Education and Research (PALGER), the Arab American University-Palestine (AAUP), and Al-Qasemi Research Foundation for their financial support.

References

[1] H. Zaid, C. N. Antonescu, V. K. Randhawa, and A. Klip, "Insulin action on glucose transporters through molecular switches, tracks and tethers," *Biochemical Journal*, vol. 413, no. 2, pp. 201–215, 2008.

[2] A. Shirwaikar, K. Rajendran, C. D. Kumar, and R. Bodla, "Antidiabetic activity of aqueous leaf extract of Annona squamosa in streptozotocin-nicotinamide type 2 diabetic rats," *Journal of Ethnopharmacology*, vol. 91, no. 1, pp. 171–175, 2004.

[3] O. Said, S. Fulder, K. Khalil, H. Azaizeh, E. Kassis, and B. Saad, "Maintaining a physiological blood glucose level with glucolevel, a combination of four anti-diabetes plants used in the traditional arab herbal medicine," *Evidence-Based Complementary and Alternative Medicine*, vol. 5, no. 4, pp. 421–428, 2008.

[4] W. Kerner and J. Brückel, "Definition, classification and diagnosis of diabetes mellitus," *Experimental and Clinical Endocrinology & Diabetes*, vol. 122, no. 7, pp. 384–386, 2014.

[5] H. Zaid, B. Saad, A. A. Mahdi, A. K. Tamrakar, P. S. Haddad, and F. U. Afifi, "Medicinal plants and natural active compounds for diabetes and/or obesity treatment," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 469762, 2 pages, 2015.

[6] R. J. McCrimmon and R. S. Sherwin, "Hypoglycemia in type 1 diabetes," *Diabetes*, vol. 59, no. 10, pp. 2333–2339, 2010.

[7] A. Soliman, V. Desanctis, M. Yassin, R. Elalaily, and N. Eldarsy, "Continuous glucose monitoring system and new era of early diagnosis of diabetes in high risk groups," *Indian Journal of Endocrinology and Metabolism*, vol. 18, no. 3, pp. 274–282, 2014.

[8] D. M. Nathan, "Long-term complications of diabetes mellitus," *The New England Journal of Medicine*, vol. 328, no. 23, pp. 1676–1685, 1993.

[9] D. Bonnefont-Rousselot, J. P. Bastard, M. C. Jaudon, and J. Delattre, "Consequences of the diabetic status on the oxidant/antioxidant balance," *Diabetes & Metabolism*, vol. 26, no. 3, pp. 163–176, 2000.

[10] J. W. Baynes, "Role of oxidative stress in development of complications in diabetes," *Diabetes*, vol. 40, no. 4, pp. 405–412, 1991.

[11] W. S. Duke-Elder, "Changes in refraction in diabetes mellitus," *British Journal of Ophthalmology*, vol. 9, no. 4, pp. 167–187, 1925.

[12] O. Marsenic, "Glucose control by the kidney: an emerging target in diabetes," *American Journal of Kidney Diseases*, vol. 53, no. 5, pp. 875–883, 2009.

[13] L. Sommese, A. Zullo, F. P. Mancini, R. Fabbricini, A. Soricelli, and C. Napoli, "Clinical relevance of epigenetics in the onset and management of type 2 diabetes mellitus," *Epigenetics*, vol. 12, no. 6, pp. 401–415, 2017.

[14] B. Saad, H. Zaid, S. Shanak, and S. Kadan, *Anti-diabetes and Anti-obesity Medicinal Plants and Phytochemicals*, Springer, 2017.

[15] S. L. Aronoff, K. Berkowitz, B. Shreiner, and L. Want, "Glucose metabolism and regulation: beyond insulin and glucagon," *Diabetes Spectrum*, vol. 17, no. 3, pp. 183–190, 2004.

[16] A. Poradzka, J. Wroński, M. Jasik, W. Karnafel, and P. Fiedor, "Insulin replacement therapy in patients with type 1 diabetes by isolated pancreatic islet transplantation," *Acta Poloniae Pharmaceutica. Drug Research*, vol. 70, no. 6, pp. 943–950, 2013.

[17] E. Bonora, G. Formentini, F. Calcaterra et al., "HOMA-estimated insulin resistance is an independent predictor of cardiovascular disease in type 2 diabetic subjects: prospective data from the Verona Diabetes Complications Study," *Diabetes Care*, vol. 25, no. 7, pp. 1135–1141, 2002.

[18] R. A. DeFronzo, "Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009," *Diabetologia*, vol. 53, no. 7, pp. 1270–1287, 2010.

[19] M. Kitada, Z. Zhang, A. Mima, and G. L. King, "Molecular mechanisms of diabetic vascular complications," *Journal of Diabetes Investigation*, vol. 1, no. 3, pp. 77–89, 2010.

[20] K. Hanhineva, R. Törrönen, I. Bondia-Pons et al., "Impact of dietary polyphenols on carbohydrate metabolism," *International Journal of Molecular Sciences*, vol. 11, no. 4, pp. 1365–1402, 2010.

[21] F. A. van de Laar, P. L. Lucassen, R. P. Akkermans, E. H. Van De Lisdonk, G. E. Rutten, and C. van Weel, "α-glucosidase inhibitors for patients with type 2 diabetes: results from a Cochrane systematic review and meta-analysis," *Diabetes Care*, vol. 28, no. 1, pp. 154–163, 2005.

[22] K. A. Deans and N. Sattar, ""Anti-inflammatory" drugs and their effects on type 2 Diabetes," *Diabetes Technology & Therapeutics*, vol. 8, no. 1, pp. 18–27, 2006.

[23] J. Wu, S. Shi, H. Wang, and S. Wang, "Mechanisms underlying the effect of polysaccharides in the treatment of type 2 diabetes: A review," *Carbohydrate Polymers*, vol. 144, pp. 474–494, 2016.

[24] T. Kawada, T. Goto, N. Takahashi, and S. Hirai, "Various Terpenoids derived from herbal and dietary plants function as PPAR modulators and regulate carbohydrate and lipid metabolism," *PPAR Research*, Article ID 483958, 9 pages, 2010.

[25] M. König, S. Bulik, and H.-G. Holzhütter, "Quantifying the contribution of the liver to glucose homeostasis: A detailed kinetic model of human hepatic glucose metabolism," *PLoS Computational Biology*, vol. 8, no. 6, 2012.

[26] W. Sacks, "Cerebral metabolism of doubly labeled glucose in humans in vivo," *Journal of Applied Physiology*, vol. 20, no. 1, pp. 117–130, 1965.

[27] P. E. Cryer, "Glucose counterregulation: Prevention and correction of hypoglycemia in humans," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 264, no. 2, pp. E149–E155, 1993.

[28] M. Farivar, J. R. Wands, K. J. Isselbacher, and N. L. R. Bucher, "Effect of insulin and glucagon on fulminant murine hepatitis," *The New England Journal of Medicine*, vol. 295, no. 27, pp. 1517–1519, 1976.

[29] J. Gerich, P. Cryer, and R. Rizza, "Hormonal mechanisms in acute glucose counterregulation: The relative roles of glucagon, epinephrine, norepinephrine, growth hormone, and cortisol," *Metabolism - Clinical and Experimental*, vol. 29, no. 11, pp. 1164–1175, 1980.

[30] S. S. Bae, H. Cho, J. Mu, and M. J. Birnbaum, "Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B.," *The Journal of Biological Chemistry*, vol. 278, no. 49, pp. 49530–49536, 2003.

[31] A. Krook, H. Wallberg-Henriksson, and J. R. Zierath, "Sending the signal: Molecular mechanisms regulating glucose uptake," *Medicine & Science in Sports & Exercise*, vol. 36, no. 7, pp. 1212–1217, 2004.

[32] A. R. Saltiel and C. R. Kahn, "Insulin signalling and the regulation of glucose and lipid metabolism," *Nature*, vol. 414, no. 6865, pp. 799–806, 2001.

[33] C. Mues, J. Zhou, K. N. Manolopoulos et al., "Regulation of glucose-6-phosphatase gene expression by insulin and metformin," *Hormone and Metabolic Research*, vol. 41, no. 10, pp. 730–735, 2009.

[34] N. Horike, H. Sakoda, A. Kushiyama et al., "AMP-activated protein kinase activation increases phosphorylation of glycogen synthase kinase 3 beta and thereby reduces cAMP-responsive element transcriptional activity and phosphoenolpyruvate carboxykinase C gene expression in the liver," *The Journal of Biological Chemistry*, vol. 283, no. 49, pp. 33902–33910, 2008.

[35] Y. Li, S. Xu, M. M. Mihaylova et al., "AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice," *Cell Metabolism*, vol. 13, no. 4, pp. 376–388, 2011.

[36] C. L. Carlson and W. W. Winder, "Liver AMP-activated protein kinase and acetyl-CoA carboxylase during and after exercise," *Journal of Applied Physiology*, vol. 86, no. 2, pp. 669–674, 1999.

[37] E. A. Richter and M. Hargreaves, "Excercise, GLUT4, and skeletal muscle glucose uptake," *Physiological Reviews*, vol. 93, no. 3, pp. 993–1017, 2013.

[38] F. Szekeres, A. Chadt, R. Z. Tom et al., "The Rab-GTPase-activating protein TBC1D1 regulates skeletal muscle glucose metabolism," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 303, no. 4, pp. E524–E533, 2012.

[39] J.-F. St-Denis and S. W. Cushman, "Role Of snare's in the glut4 translocation response to insulin in adipose cells and muscle," *Journal of Basic and Clinical Physiology and Pharmacology*, vol. 9, no. 2-4, pp. 153–166, 1998.

[40] Q. H. Wang, R. Somwar, P. J. Bilan et al., "Protein kinase B Akt participates in GLUT4 translocation by insulin in L6 myoblasts," *Molecular and Cellular Biology*, vol. 19, no. 6, pp. 4008–4018, 1999.

[41] J.-H. Lee, T. Kim, J.-J. Lee et al., "The herbal medicine KBH-1 inhibits fat accumulation in 3T3-L1 adipocytes and reduces

high fat diet-induced obesity through regulation of the AMPK pathway," *PLoS ONE*, vol. 10, no. 12, 2015.

[42] F. Q. Nuttall, A. Ngo, and M. C. Gannon, "Regulation of hepatic glucose production and the role of gluconeogenesis in humans: Is the rate of gluconeogenesis constant?" *Diabetes/Metabolism Research and Reviews*, vol. 24, no. 6, pp. 438–458, 2008.

[43] B. Thorens, "Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 270, no. 4, pp. G541–G553, 1996.

[44] S. Aiston, K. Y. Trinh, A. J. Lange, C. B. Newgard, and L. Agius, "Glucose-6-phosphatase overexpression lowers glucose 6-phosphate and inhibits glycogen synthesis and glycolysis in hepatocytes without affecting glucokinase translocation. Evidence against feedback inhibition of glucokinase," *The Journal of Biological Chemistry*, vol. 274, no. 35, pp. 24559–24566, 1999.

[45] T. Ferre, E. Riu, F. Bosch, and A. Valera, "Evidence from transgenic mice that glucokinase is rate limiting for glucose utilization in the liver," *The FASEB Journal*, vol. 10, no. 10, pp. 1213–1218, 1996.

[46] F. Chatelain, J.-P. Pégorier, C. Minassian et al., "Development and regulation of glucose-6-phosphatase gene expression in rat liver, intestine, and kidney: In vivo and in vitro studies in cultured fetal hepatocytes," *Diabetes*, vol. 47, no. 6, pp. 882–889, 1998.

[47] S. Huang and M. P. Czech, "The GLUT4 glucose transporter," *Cell Metabolism*, vol. 5, no. 4, pp. 237–252, 2007.

[48] A. Klip and M. R. Pâquet, "Glucose transport and glucose transporters in muscle and their metabolic regulation," *Diabetes Care*, vol. 13, no. 3, pp. 228–243, 1990.

[49] E. D. Abel, O. Peroni, J. K. Kim et al., "Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver," *Nature*, vol. 409, no. 6821, pp. 729–733, 2001.

[50] H. Zaid, A. K. Tamrakar, M. S. Razzaque, and T. Efferth, "Diabetes and metabolism disorders medicinal plants: a glance at the past and a look to the future 2018," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 5843298, 3 pages, 2018.

[51] D. Rodenhiser and M. Mann, "Epigenetics and human disease: translating basic biology into clinical applications," *Canadian Medical Association Journal*, vol. 174, no. 3, pp. 341–348, 2006.

[52] L. Altucci and M. G. Rots, "Epigenetic drugs: from chemistry via biology to medicine and back," *Clinical Epigenetics*, vol. 8, no. 1, article no. 56, pp. 1–3, 2016.

[53] I. A. Qureshi and M. F. Mehler, "Advances in epigenetics and epigenomics for neurodegenerative diseases," *Current Neurology and Neuroscience Reports*, vol. 11, no. 5, pp. 464–473, 2011.

[54] J. G. Herman and S. B. Baylin, "Gene silencing in cancer in association with promoter hypermethylation," *The New England Journal of Medicine*, vol. 349, no. 21, pp. 2042–2054, 2003.

[55] M. Mishra and R. A. Kowluru, "Epigenetic modification of mitochondrial DNA in the development of diabetic retinopathy," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 9, pp. 5133–5142, 2015.

[56] Q. Zhong and R. A. Kowluru, "Role of histone acetylation in the development of diabetic retinopathy and the metabolic memory phenomenon," *Journal of Cellular Biochemistry*, vol. 110, no. 6, pp. 1306–1313, 2010.

[57] M. Mishra and R. A. Kowluru, "The role of DNA methylation in the metabolic memory phenomenon associated with the continued progression of diabetic retinopathy," *Investigative Ophthalmology & Visual Science*, vol. 57, no. 13, pp. 5748–5757, 2016.

[58] J. Yan, G. Tie, S. Wang et al., "Diabetes impairs wound healing by Dnmt1-dependent dysregulation of hematopoietic stem cells differentiation towards macrophages," *Nature Communications*, vol. 9, no. 1, 2018.

[59] N. Singh, A. Dueñas-González, F. Lyko, and J. L. Medina-Franco, "Molecular modeling and molecular dynamics studies of hydralazine with human DNA methyltransferase 1," *ChemMedChem*, vol. 4, no. 5, pp. 792–799, 2009.

[60] A. N. Mohamed, A. M. Abdelhady, D. Spencer, K. M. Sowinski, J. E. Tisdale, and B. R. Overholser, "Pharmacokinetic modeling and simulation of procainamide and n-acetylprocainamide in a patient receiving continuous renal replacement therapy: A novel approach to guide renal dose adjustments," *American Journal of Kidney Diseases*, vol. 61, no. 6, pp. 1046–1048, 2013.

[61] S. Sharma and R. Taliyan, "Histone deacetylase inhibitors: Future therapeutics for insulin resistance and type 2 diabetes," *Pharmacological Research*, vol. 113, pp. 320–326, 2016.

[62] D. P. Christensen, M. Dahllöf, M. Lundh et al., "Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus," *Molecular Medicine*, vol. 17, no. 5-6, pp. 378–390, 2011.

[63] T. Mandrup-Poulsen, "The role of interleukin-1 in the pathogenesis of IDDM," *Diabetologia*, vol. 39, no. 9, pp. 1005–1029, 1996.

[64] M. Göttlicher, S. Minucci, P. Zhu et al., "Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells," *EMBO Journal*, vol. 20, no. 24, pp. 6969–6978, 2002.

[65] C. Daosukho, Y. Chen, T. Noel et al., "Phenylbutyrate, a histone deacetylase inhibitor, protects against Adriamycin-induced cardiac injury," *Free Radical Biology & Medicine*, vol. 42, no. 12, pp. 1818–1825, 2007.

[66] L. B. Avery and N. N. Bumpus, "Valproic acid is a novel activator of amp-activated protein kinase and decreases liver mass, hepatic fat accumulation, and serum glucose in obese mice," *Molecular Pharmacology*, vol. 85, no. 1, pp. 1–10, 2014.

[67] S.-E. Choi, Y.-J. Lee, H.-J. Jang et al., "A chemical chaperone 4-PBA ameliorates palmitate-induced inhibition of glucose-stimulated insulin secretion (GSIS)," *Archives of Biochemistry and Biophysics*, vol. 475, no. 2, pp. 109–114, 2008.

[68] C. S. R. Kadiyala, L. Zheng, Y. Du et al., "Acetylation of retinal histones in diabetes increases inflammatory proteins: effects of minocycline and manipulation of histone acetyltransferase (HAT) and histone deacetylase (HDAC)," *The Journal of Biological Chemistry*, vol. 287, no. 31, pp. 25869–25880, 2012.

[69] X. Li, C. Li, and G. Sun, "Histone acetylation and its modifiers in the pathogenesis of diabetic nephropathy," *Journal of Diabetes Research*, vol. 2016, Article ID 4065382, 11 pages, 2016.

[70] M. A. Reddy, L. M. Villeneuve, M. Wang, L. Lanting, and R. Natarajan, "Role of the lysine-specific demethylase 1 in the proinflammatory phenotype of vascular smooth muscle cells of diabetic mice," *Circulation Research*, vol. 106, no. 6, pp. E9–E9, 2009.

[71] D. Pan, C. Mao, and Y.-X. Wang, "Suppression of gluconeogenic gene expression by LSD1-mediated histone demethylation," *PLoS ONE*, vol. 8, no. 6, 2013.

[72] R. Bressler, M. Vargas-Cord, and H. E. Lebovitz, "Tranylcypromine - a potent insulin secretagogue and hypoglycemic agent," *Diabetes*, vol. 17, no. 10, pp. 617–624, 1968.

[73] Y. Li, D. Kong, Z. Wang, and F. H. Sarkar, "Regulation of microRNAs by natural agents: an emerging field in chemoprevention and chemotherapy research," *Pharmaceutical Research*, vol. 27, no. 6, pp. 1027–1041, 2010.

[74] K. Sakamoto, "Silencing metabolic disorders by novel SIRT1 activators," *Cell Metabolism*, vol. 7, no. 1, pp. 3-4, 2008.

[75] J. Yu and J. Auwerx, "The role of sirtuins in the control of metabolic homeostasis," *Annals of the New York Academy of Sciences*, vol. 1173, no. 1, pp. E10–E19, 2009.

[76] C. B. Brachmann, J. M. Sherman, S. E. Devine, E. E. Cameron, L. Pillus, and J. D. Boeke, "The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell-cycle progression, and chromosome stability," *Genes & Development*, vol. 9, no. 23, pp. 2888–2902, 1995.

[77] S. Timmers, M. K. C. Hesselink, and P. Schrauwen, "Therapeutic potential of resveratrol in obesity and type 2 diabetes: New avenues for health benefits?" *Annals of the New York Academy of Sciences*, vol. 1290, no. 1, pp. 83–89, 2013.

[78] S. Reuter, S. C. Gupta, B. Park, A. Goel, and B. B. Aggarwal, "Epigenetic changes induced by curcumin and other natural compounds," *Genes & Nutrition*, vol. 6, no. 2, pp. 93–108, 2011.

[79] J.-M. Yun, I. Jialal, and S. Devaraj, "Effects of epigallocatechin gallate on regulatory T cell number and function in obese v. lean volunteers," *British Journal of Nutrition*, vol. 103, no. 12, pp. 1771–1777, 2010.

[80] M. Traka, A. V. Gasper, J. A. Smith, C. J. Hawkey, Y. Bao, and R. F. Mithen, "Transcriptome analysis of human colon Caco-2 cells exposed to sulforaphane," *Journal of Nutrition*, vol. 135, no. 8, pp. 1865–1872, 2005.

[81] S. M. Meeran, S. N. Patel, and T. O. Tollefsbol, "Sulforaphane causes epigenetic repression of hTERT expression in human breast cancer cell lines," *PLoS ONE*, vol. 5, no. 7, Article ID e11457, 2010.

[82] A. Pledgie-Tracy, M. D. Sobolewski, and N. E. Davidson, "Sulforaphane induces cell type-specific apoptosis in human breast cancer cell lines," *Molecular Cancer Therapeutics*, vol. 6, no. 3, pp. 1013–1021, 2007.

[83] A. S. Axelsson, E. Tubbs, B. Mecham et al., "Sulforaphane reduces hepatic glucose production and improves glucose control in patients with type 2 diabetes," *Science Translational Medicine*, vol. 9, no. 394, 2017.

[84] S. Majid, A. A. Dar, V. Shahryari et al., "Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-cell translocation gene 3 in prostate cancer," *Cancer*, vol. 116, no. 1, pp. 66–76, 2010.

[85] E. R. Gilbert and D. Liu, "Anti-diabetic functions of soy isoflavone genistein: mechanisms underlying its effects on pancreatic beta-cell function," *Food & Function*, vol. 4, no. 2, pp. 200–212, 2013.

[86] N. Druesne, A. Pagniez, C. Mayeur et al., "Diallyl disulfide (DADS) increases histone acetylation and p21(waf1/cip1) expression in human colon tumor cell lines," *Carcinogenesis*, vol. 25, no. 7, pp. 1227–1236, 2004.

[87] M. A. Valero, A. Vidal, R. Burgos et al., "Meta-analysis on the role of lycopene in type 2 diabetes mellitus," *Nutrición Hospitalaria*, vol. 26, no. 6, pp. 1236–1241, 2011.

[88] A. King-Batoon, J. M. Leszczynska, and C. B. Klein, "Modulation of gene methylation by genistein or lycopene in breast cancer cells," *Environmental and Molecular Mutagenesis*, vol. 49, no. 1, pp. 36–45, 2008.

[89] S. Tan, C. Wang, C. Lu et al., "Quercetin is able to demethylate the p16INK4a gene promoter," *Chemotherapy*, vol. 55, no. 1, pp. 6–10, 2008.

[90] P. A. Ruiz, A. Braune, G. Hölzlwimmer, L. Quintanilla-Fend, and D. Haller, "Quercetin inhibits TNF-induced NF-kappa B transcription factor recruitment to proinflammatory gene promoters in murine intestinal epithelial cells," *Journal of Nutrition*, vol. 137, no. 5, pp. 1208–1215, 2007.

[91] W.-J. Lee, Y.-R. Chen, and T.-H. Tseng, "Quercetin induces FasL-related apoptosis, in part, through promotion of histone H3 acetylation in human leukemia HL-60 cells," *Oncology Reports*, vol. 25, no. 2, pp. 583–591, 2011.

[92] S. Chen, H. Jiang, X. Wu, and J. Fang, "Therapeutic effects of quercetin on inflammation, obesity, and type 2 diabetes," *Mediators of Inflammation*, vol. 2016, Article ID 9340637, 5 pages, 2016.

[93] H. M. Eid, A. Nachar, F. Thong, G. Sweeney, and P. S. Haddad, "The molecular basis of the antidiabetic action of quercetin in cultured skeletal muscle cells and hepatocytes," *Pharmacognosy Magazine*, vol. 11, no. 41, pp. 74–81, 2015.

[94] A. K. Agarwal, C. Monder, B. Eckstein, and P. C. White, "Cloning and expression of rat cDNA encoding corticosteroid 11β-dehydrogenase," *The Journal of Biological Chemistry*, vol. 264, no. 32, pp. 18939–18943, 1989.

[95] J. R. Seckl and B. R. Walker, "Minireview: 11 beta-hydroxysteroid dehydrogenase type 1 - A tissue-specific amplifier of glucocorticoid action," *Endocrinology*, vol. 142, no. 4, pp. 1371–1376, 2001.

[96] A. Odermatt, A. G. Atanasov, Z. Balazs et al., "Why is 11 beta-hydroxy steroid dehydrogenase type 1 facing the endoplasmic reticulum lumen? Physiological relevance of the membrane topology of 11 beta-HSD1," *Molecular and Cellular Endocrinology*, vol. 248, no. 1-2, pp. 15–25, 2006.

[97] T. M. Stulnig and W. Waldhäusl, "11β-Hydroxysteroid dehydrogenase Type 1 in obesity and Type 2 diabetes," *Diabetologia*, vol. 47, no. 1, pp. 1–11, 2004.

[98] B. Zinker, A. Mika, P. Nguyen et al., "Liver-selective glucocorticoid receptor antagonism decreases glucose production and increases glucose disposal, ameliorating insulin resistance," *Metabolism - Clinical and Experimental*, vol. 56, no. 3, pp. 380–387, 2007.

[99] B. Davani, N. Portwood, G. Bryzgalova et al., "Aged transgenic mice with increased glucocorticoid sensitivity in pancreatic beta-cells develop diabetes," *Diabetes*, vol. 53, no. Supplement 1, pp. S51–S59, 2004.

[100] N. M. Morton, "Obesity and corticosteroids: 11beta-hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease," *Molecular and Cellular Endocrinology*, vol. 316, no. 2, pp. 154–164, 2010.

[101] J. D. Zhang, T. D. Osslund, M. H. Plant et al., "Crystal structure of murine 11 beta-hydroxysteroid dehydrogenase 1: An important therapeutic target for diabetes," *Biochemistry*, vol. 44, no. 18, pp. 6948–6957, 2005.

[102] H. Berman, K. Henrick, and H. Nakamura, "Announcing the worldwide Protein Data Bank," *Nature Structural & Molecular Biology*, vol. 10, no. 12, p. 980, 2003.

[103] D. J. Hosfield, Y. Wu, E. J. Skene et al., "Conformational flexibility in crystal structures of human 11 beta-hydroxysteroid dehydrogenase type I provide insights into glucocorticoid interconversion and enzyme regulation," *The Journal of Biological Chemistry*, vol. 280, no. 6, pp. 4639–4648, 2005.

[104] D. Ogg, B. Elleby, C. Norström et al., "The crystal structure of guinea pig 11β-hydroxysteroid dehydrogenase type 1 provides a model for enzyme-lipid bilayer interactions," *The Journal of Biological Chemistry*, vol. 280, no. 5, pp. 3789–3794, 2005.

[105] H. Tu, J. P. Powers, J. Liu et al., "Distinctive molecular inhibition mechanisms for selective inhibitors of human 11 beta-hydroxysteroid dehydrogenase type 1," *Bioorganic & Medicinal Chemistry*, vol. 16, no. 19, pp. 8922–8931, 2008.

[106] J. Hintzpeter, C. Stapelfeld, C. Loerz, H.-J. Martin, and E. Maser, "Green tea and one of its constituents, epigallocatechine-3-gallate, are potent inhibitors of human 11β-hydroxysteroid dehydrogenase type 1," *PLoS ONE*, vol. 9, no. 1, 2014.

[107] T. Feng, J. Su, Z.-H. Ding et al., "Chemical constituents and their bioactivities of "tongling White Ginger" (Zingiber officinale)," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 21, pp. 11690–11695, 2011.

[108] C. Tsang, N. F. Smail, S. Almoosawi, I. Davidson, and E. A. S. Al-Dujaili, "Intake of polyphenol-rich pomegranate pure juice influences urinary glucocorticoids, blood pressure and homeostasis model assessment of insulin resistance in human volunteers," *Journal of Nutritional Science*, vol. 1, pp. 1–9, 2012.

[109] P. Namita and R. Mukesh, "Medicinal plants used as antimicrobial agents," *International Research Journal of Pharmacy*, vol. 3, 2013.

[110] C. Gumy, C. Thurnbichler, E. M. Aubry et al., "Inhibition of 11β-hydroxysteroid dehydrogenase type 1 by plant extracts used as traditional antidiabetic medicines," *Fitoterapia*, vol. 80, no. 3, pp. 200–205, 2009.

[111] M. Meier, G. Möller, and J. Adamski, "Perspectives in Understanding the Role of Human 17β-Hydroxysteroid Dehydrogenases in Health and Disease," *Annals of the New York Academy of Sciences*, vol. 1155, no. 1, pp. 15–24, 2009.

[112] M.-L. Lu, Y.-W. Huang, and S.-X. Lin, "Purification, reconstitution, and steady-state kinetics of the trans-membrane 17β-hydroxysteroid dehydrogenase 2," *The Journal of Biological Chemistry*, vol. 277, no. 25, pp. 22123–22130, 2002.

[113] A. Jansson, C. Gunnarsson, and O. Stål, "Proliferative responses to altered 17β-hydroxysteroid dehydrogenase (17HSD) type 2 expression in human breast cancer cells are dependent on endogenous expression of 17HSD type 1 and the oestradiol receptors," *Endocrine-Related Cancer*, vol. 13, no. 3, pp. 875–884, 2006.

[114] P. Lukacik, K. L. Kavanagh, and U. Oppermann, "Structure and function of human 17 beta-hydroxysteroid dehydrogenases," *Molecular and Cellular Endocrinology*, vol. 248, no. 1-2, pp. 61–71, 2006.

[115] J.-Z. Jin and S.-X. Lin, "Human estrogenic 17β-hydroxysteroid dehydrogenase: Predominance of estrone reduction and its induction by NADPH," *Biochemical and Biophysical Research Communications*, vol. 259, no. 2, pp. 489–493, 1999.

[116] M. M. Miettinen, M. V. J. Mustonen, M. H. Poutanen, V. V. Isomaa, and R. K. Vihko, "Human 17β-hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured cells and characteristic cell- and tissue-specific expression," *Biochemical Journal*, vol. 314, no. 3, pp. 839–845, 1996.

[117] Y. Laplante, C. Rancourt, and D. Poirier, "Relative involvement of three 17β-hydroxysteroid dehydrogenases (types 1, 7 and 12) in the formation of estradiol in various breast cancer cell lines using selective inhibitors," *Molecular and Cellular Endocrinology*, vol. 301, no. 1-2, pp. 146–153, 2009.

[118] C.-Y. Zhang, J. Chen, D.-C. Yin, and S.-X. Lin, "The contribution of 17beta-hydroxysteroid dehydrogenase type 1 to the estradiol-estrone ratio in estrogen-sensitive breast cancer cells," *PLoS ONE*, vol. 7, no. 1, 2012.

[119] F. Mauvais-Jarvis, "Is estradiol a biomarker of type 2 diabetes risk in postmenopausal women?" *Diabetes*, vol. 66, no. 3, pp. 568–570, 2017.

[120] S. X. Lin, D. W. Zhu, A. Azzi et al., "Studies on the three-dimensional structure of estrogenic 17 beta-hydroxysteroid dehydrogenase," *Journal of Endocrinology*, vol. 150, pp. 113–S20, 1996.

[121] H. Peltoketo, V. Isomaa, O. Mäentausta, and R. Vihko, "Complete amino acid sequence of human placental 17β-hydroxysteroid dehydrogenase deduced from cDNA," *FEBS Letters*, vol. 239, no. 1, pp. 73–77, 1988.

[122] S. X. Lin, F. Yang, J. Z. Jin et al., "Subunit identity of the dimeric 17-beta-hydroxysteroid dehydrogenase from human placenta," *Journal of Biological Chemistry*, vol. 267, no. 23, pp. 16182–16187, 1992.

[123] S. Lin, Q. Han, A. Azzi, D. Zhu, A. Gongloff, and R. Campbell, "3D-structure of human estrogenic 17β-HSD1: binding with various steroids," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 69, no. 1-6, pp. 425–429, 1999.

[124] A. Gangloff, R. Shi, V. Nahoum, and S. Lin, "Pseudo-symmetry of C19-steroids, alternative binding orientations and multi-specificity in human estrogenic 17 beta-hydroxysteroid dehydrogenase," *The FASEB Journal*, vol. 17, no. 2, pp. 274–276, 2003.

[125] M. W. Sawicki, M. Erman, T. Puranen, P. Vihko, and D. Ghosh, "Structure of the ternary complex of human 17 beta-hydroxysteroid dehydrogenase type 1 with 3-hydroxyestra-1,3,5,7-tetraen-17-one (equilin) and NADP(+)," *Proceedings of the National Acadamy of Sciences of the United States of America*, vol. 96, no. 3, pp. 840–845, 1999.

[126] I. Stupans, G. Stretch, and P. Hayball, "Olive oil phenols inhibit human hepatic microsomal activity," *Journal of Nutrition*, vol. 130, no. 9, pp. 2367–2370, 2000.

[127] G. Boehmelt, I. Fialka, G. Brothers et al., "Cloning and characterization of the murine glucosamine-6-phosphate acetyltransferase EMeg32. Differential expression and intracellular membrane association," *The Journal of Biological Chemistry*, vol. 275, no. 17, pp. 12821–12832, 2000.

[128] G. Boehmelt, A. Wakeham, A. Elia et al., "Decreased UDP-GIcNAc levels abrogate proliferation control in EMeg32-deficient cells," *EMBO Journal*, vol. 19, no. 19, pp. 5092–5104, 2000.

[129] K. Y. Trinh, R. M. O'Doherty, P. Anderson, A. J. Lange, and C. B. Newgard, "Perturbation of fuel homeostasis caused by overexpression of the glucose-6-phosphatase catalytic subunit in liver of normal rats," *The Journal of Biological Chemistry*, vol. 273, no. 47, pp. 31615–31620, 1998.

[130] M. G. Buse, "Hexosamines, insulin resistance, and the complications of diabetes: current status," *American Journal of Physiology-Renal Physiology*, vol. 290, no. 1, pp. 1–8, 2006.

[131] S. Marshall, V. Bacote, and R. R. Traxinger, "Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose-transport system - role of hexosamine biosynthesis in the induction of insulin resistance," *The Journal of Biological Chemistry*, vol. 266, no. 8, pp. 4706–4712, 1991.

[132] R. R. Traxinger and S. Marshall, "Coordinated regulation of glutamine:fructose-6-phosphate amidotransferase activity by

insulin, glucose, and glutamine: Role of hexosamine biosynthesis in enzyme regulation," *The Journal of Biological Chemistry*, vol. 266, no. 16, pp. 10148–10154, 1991.

[133] T. Oki, K. Yamazaki, J. Kuromitsu, M. Okada, and I. Tanaka, "cDNA cloning and mapping of a novel subtype of glutamine:fructose-6- phosphate amidotransferase (GFAT2) in human and mouse," *Genomics*, vol. 57, no. 2, pp. 227–234, 1999.

[134] M. Niimi, T. Ogawara, T. Yamashita et al., "Identification of GFAT1-L, a novel splice variant of human glutamine: Fructose-6-phosphate amidotransferase (GFAT1) that is expressed abundantly in skeletal muscle," *Journal of Human Genetics*, vol. 46, no. 10, pp. 566–571, 2001.

[135] J. E. DeHaven, K. A. Robinson, B. A. Nelson, and M. G. Buse, "A Novel Variant of Glutamine Fructose-6-Phosphate Amidotransferase-1 (GFAT1) mRNA Is Selectively Expressed in Striated Muscle," *Diabetes*, vol. 50, no. 7-12, pp. 2419–2424, 2001.

[136] M.-J. Lee, D.-W. Gong, B. F. Burkey, and S. K. Fried, "Pathways regulated by glucocorticoids in omental and subcutaneous human adipose tissues: A microarray study," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 300, no. 3, pp. E571–E580, 2011.

[137] G. Veerababu, J. Tang, R. T. Hoffman et al., "Overexpression of glutamine:fructose-6-phosphate amidotransferase in the liver of transgenic mice results in enhanced glycogen storage, hyperlipidemia, obesity, and impaired glucose tolerance," *Diabetes*, vol. 49, no. 12, pp. 2070–2078, 2000.

[138] S. Milewski, "Glucosamine-6-phosphate synthase - The multifacets enzyme," *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, vol. 1597, no. 2, pp. 173–192, 2002.

[139] Y. Nakaishi, M. Bando, H. Shimizu et al., "Structural analysis of human glutamine:fructose-6-phosphate amidotransferase, a key regulator in type 2 diabetes," *FEBS Letters*, vol. 583, no. 1, pp. 163–167, 2009.

[140] A. K. Shetty and P. V. Salimath, "Reno-protective effects of fenugreek (Trigonella foenum greacum) during experimental diabetes," *e-SPEN Journal*, vol. 4, no. 3, pp. e137–e142, 2009.

[141] P. Paarakh, "In silico antidiabetic activity of linalool isolated from coriandrum sativum linn fruit," *International Journal of Cancer Cellular Biology Research*, vol. 2, 2017.

[142] J. Cieśla, T. Fraczyk, and W. Rode, "Phosphorylation of basic amino acid residues in proteins: important but easily missed," *Acta Biochimica Polonica*, vol. 58, no. 2, pp. 137–148, 2011.

[143] H. Ushiro and S. Cohen, "Identification of phosphotyrosine as a product of epidermal growth factor-activated protein-kinase in A-431 cell-membranes," *The Journal of Biological Chemistry*, vol. 255, no. 18, pp. 8363–8365, 1980.

[144] J. Behrens, L. Vakaet, R. Friis et al., "Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the e-cadherin beta-catenin complex in cells transformed with a temperature-sensitive V-SRC gene," *The Journal of Cell Biology*, vol. 120, no. 3, pp. 757–766, 1993.

[145] D. Guo, Q. Jia, H.-Y. Song, R. S. Warren, and D. B. Donner, "Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains: Association with endothelial cell proliferation," *The Journal of Biological Chemistry*, vol. 270, no. 12, pp. 6729–6733, 1995.

[146] R. Baserga, "The contradictions of the insulin-like growth factor 1 receptor," *Oncogene*, vol. 19, no. 49, pp. 5574–5581, 2000.

[147] Y. Wang and Y. Sun, "Insulin-like growth factor receptor-1 as an anti-cancer target: Blocking transformation and inducing apoptosis," *Current Cancer Drug Targets*, vol. 2, no. 3, pp. 191–207, 2002.

[148] J. Zhou, J. L. Neidigh, R. Espinosa III, M. M. LeBeau, and D. A. McClain, "Human glutamine: fructose-6-phosphate amidotransferase: characterization of mRNA and chromosomal assignment to 2p13," *Human Genetics*, vol. 96, no. 1, pp. 99–101, 2004.

[149] D. Barford, A. J. Flint, and N. K. Tonks, "Crystal structure of human protein tyrosine phosphatase 1B," *Science*, vol. 263, no. 5152, pp. 1397–1404, 1994.

[150] T. Jin, H. Yu, and X.-F. Huang, "Selective binding modes and allosteric inhibitory effects of lupane triterpenes on protein tyrosine phosphatase 1B," *Scientific Reports*, vol. 6, 2016.

[151] C.-S. Jiang, L.-F. Liang, and Y.-W. Guo, "Natural products possessing protein tyrosine phosphatase 1B (PTP1B) inhibitory activity found in the last decades," *Acta Pharmacologica Sinica*, vol. 33, no. 10, pp. 1217–1245, 2012.

[152] Y. Bustanji, M. O. Taha, I. M. Al-masri, and K. Mohammad, "Docking simulations and in vitro assay unveil potent inhibitory action of papaverine against protein tyrosine phosphatase 1B," *Biological & Pharmaceutical Bulletin*, vol. 32, no. 4, pp. 640–645, 2009.

[153] H. Jiang, S. Khan, Y. Wang et al., "SIRT6 regulates TNF-alpha secretion through hydrolysis of long-chain fatty acyl lysine," *Nature*, vol. 496, no. 7443, pp. 110–113, 2013.

[154] X. Zhang, S. Khan, H. Jiang et al., "Identifying the functional contribution of the defatty-Acylase activity of SIRT6," *Nature Chemical Biology*, vol. 12, no. 8, pp. 614–620, 2016.

[155] R. Mostoslavsky, K. F. Chua, D. B. Lombard et al., "Genomic instability and aging-like phenotype in the absence of mammalian SIRT6," *Cell*, vol. 124, no. 2, pp. 315–329, 2006.

[156] L. Zhong, A. D'Urso, D. Toiber et al., "The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1 alpha," *Cell*, vol. 140, no. 2, pp. 280-93, 2010.

[157] J. E. Dominy Jr., Y. Lee, M. P. Jedrychowski et al., "The Deacetylase Sirt6 Activates the Acetyltransferase GCN5 and Suppresses Hepatic Gluconeogenesis," *Molecular Cell*, vol. 48, no. 6, pp. 900–913, 2012.

[158] P. W. Pan, J. L. Feldman, M. K. Devries, A. Dong, A. M. Edwards, and J. M. Denu, "Structure and biochemical functions of SIRT6," *The Journal of Biological Chemistry*, vol. 286, no. 16, pp. 14575–14587, 2011.

[159] L. Le, "Computational study of antidiabetic activities of bioactive compounds in zingiber officinale," *World Journal of Pharmacy and Pharmaceutical Sciences*, vol. 3, 2014.

[160] T. H. Nguyen Vo, N. Tran, D. Nguyen, and L. Le, "An in silico study on antidiabetic activity of bioactive compounds in Euphorbia thymifolia Linn," *SpringerPlus*, vol. 5, no. 1, 2016.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 2371784, 9 pages
https://doi.org/10.1155/2019/2371784

Research Article

A Validated HPLC-MS/MS Method for Simultaneous Determination of Militarine and Its Three Metabolites in Rat Plasma: Application to a Pharmacokinetic Study

Hui-Yuan Sun,[1,2] Lin Zheng,[1] Zi-Peng Gong,[1] Yue-Ting Li,[1] Chang Yang,[1] Jie Pan,[3] Yong-Lin Wang,[1] Ai-Min Wang,[3] Yong-Jun Li,[3] and Yong Huang[1]

[1]*Guizhou Provincial Key Laboratory of Pharmaceutics, State Key Laboratory of Functions and Applications of Medicinal Plants, Guizhou Medical University, Guiyang 550004, China*
[2]*School of Pharmacy, Guizhou Medical University, Guiyang 550004, China*
[3]*Guizhou Provincial Engineering Research Center for the Development and Application of Ethnic Medicine and TCM, Guizhou Medical University, Guiyang 550004, China*

Correspondence should be addressed to Yong-Jun Li; liyongjun026@126.com and Yong Huang; mailofhy@126.com

Hui-Yuan Sun and Lin Zheng contributed equally to this work.

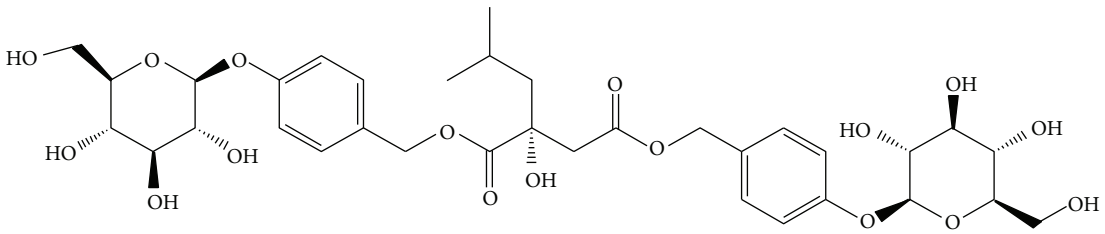
Received 20 December 2018; Accepted 9 April 2019; Published 2 May 2019

Academic Editor: Lucindo Q. Júnior

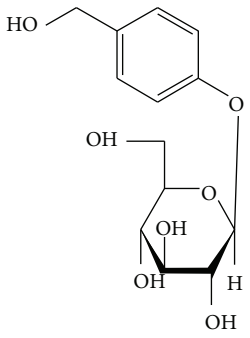
A rapid, reliable, and sensitive HPLC-electrospray ionization-tandem mass spectrometry (HPLC-MS/MS) method was established and validated for simultaneous determination of militarine and its three metabolites (gastrodin, α-isobutylmalic acid, and gymnoside I) in rat plasma. Plasma was acidified with formic acid, and protein was precipitated with methanol. MS/MS with ESI and multiple reaction monitoring at m/z 725.3⟶457.3, 457.1⟶127, 304.3⟶107.2, 189⟶129, and 417.1⟶267.1 was used for determination of militarine, gastrodin, α-isobutylmalic acid, gymnoside I, and puerarin (internal standard), respectively. Chromatographic separation was conducted using an ACE UltraCore SuperC18 (2.1 × 100 mm, 2.5 μm) column with gradient mobile phase (0.1% formic acid in water and acetonitrile). The lower limits of quantitation for militarine, gastrodin, α-isobutylmalic acid, and gymnoside I were 1.02, 2.96, 1.64, and 0.3 ng/mL, respectively. The relative standard deviations of intra- and interday measurements were less than 15%, and the method accuracy ranged from 87.4% to 112.5%. The extraction recovery was 83.52%-105.34%, and no matrix effect was observed. The three metabolites (gastrodin, α-isobutylmalic acid, and gymnoside I) were synchronously detected at 0.83 h, suggesting that militarine was rapidly transformed to gastrodin, α-isobutylmalic acid, and gymnoside I. Moreover, the area under the curve (AUC) and C_{max} of militarine were significantly lower than those of gastrodin and α-isobutylmalic acid, showing that militarine was largely metabolized to gastrodin and α-isobutylmalic acid *in vivo*. The studies on pharmacokinetics of militarine and its three metabolites were of great use for facilitating the clinical application of militarine and were also highly meaningful for the potential development of militarine.

1. Introduction

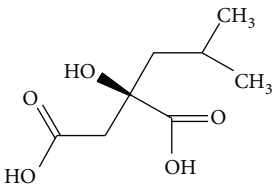
Militarine (Figure 1), a natural glucosyloxybenzyl 2-isobutylmalate containing 2-isobutylmalate as a parent nucleus, is widely distributed in orchids (Orchidaceae) [1], such as *Coelogloss*um *viride (L.)* Hartm. var. *bracteatum* [2] and *Bletilla striata* (Thunb.) Reichb. f. [3]. Recent pharmacological studies have shown that glucosyloxybenzyl 2-isobutylmalates had antiaging and neuroprotective effects; additionally, they could improve intelligence, learning ability, and memory and prevent senile dementia [4–8]. Militarine is the most abundant active component in *B. striata* (Thunb.) Reichb. f. [9–12]. *B. striata* (Thunb.) Reichb. f. has been widely used for the treatment of traumatic bleeding, hemoptysis, and hematemesis owing to its astringent and antihemorrhagic effects. In addition, it has been applied topically to relieve sores, ulcers, chapped skin, and swelling owing to its tissue regenerative capabilities [13–17]. *B. striata* is a commonly



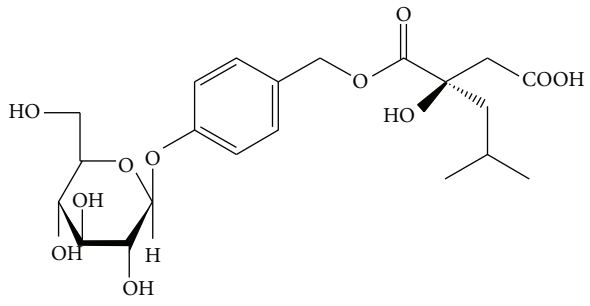
Militarine



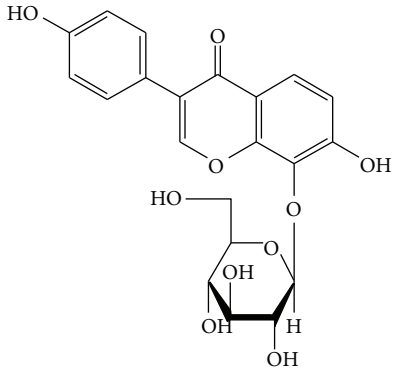
Gastrodin



α-Isobutylmalic acid



Gymnoside I



Puerarin

Figure 1: The chemical structures of gastrodin, α-isobutylmalic acid, gymnoside I, militarine, and puerarin (IS).

used traditional Chinese medicine (TCM) [18, 19]. Moreover, the vasodilator and learning and memory-improving effects of militarine have been verified [20, 21]. However, only few studies have investigated the potential effects of militarine at present.

To better understand and elucidate the relationship between the pharmacological actions and biotransformation of militarine *in vivo*, a good knowledge of the ADME processes of this constituent is getting more essential. A rat study on the pharmacokinetics of militarine (21.51 mg/kg, i.g.) suggested that the plasma concentration (C_{max} = 30.3 ± 8.61 ng/mL, $AUC_{0\text{-}t}$ = 74.3 ± 37.68 h∗ng/mL) of militarine was low [18]. The militarine was therefore speculated to be intensely metabolized in vivo in this study. However, the metabolism of militarine has not been reported in the literature as far as we know. It has been shown that militarine can be metabolized into gastrodin, α-isobutylmalic acid, and gymnoside I in our previous studies. Therefore, to understand the pharmacokinetics of militarine and its metabolites *in vivo*, a quantitative method was established to determine the dynamic changes of militarine and its metabolites *in vivo*.

To simultaneously determine militarine and its metabolites in rat plasma, a sensitive bioanalytical method should be employed. In addition, the analytical method should be selective to avoid interference from endogenous substances in the plasma. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with multiple reaction monitoring (MRM) mode utilizes two stages of

Table 1: Mass spectra properties of the analytes and IS.

Analytes	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (DP, V)	Entrance potential (EP, V)	Collision energy (CE, V)	Cell exit potential (CXP, V)
gastrodin	304.3	107.2	57	8	23	18
α-isobutylmalic acid	189.0	129.0	-73	-10	-23	-13
gymnoside I	457.1	127.0	-77	-5	-32	-6
militarine	725.3	457.3	-43	-9	-22	-40
puerarin (IS)	417.1	267.1	110	7	35	6

mass filtering, which can specifically measure the relative and absolute analyte concentrations [22]. Thus, HPLC-MS/MS could be used for quantitative analysis of drugs and their metabolites.

In this study, a rapid, reliable, and sensitive HPLC-MS/MS method was developed and validated for the simultaneous determination of militarine and its three metabolites (gastrodin, α-isobutylmalic acid, and gymnoside I) in rat plasma to study the pharmacokinetics of militarine after single oral administration to rats. To the best of our knowledge, this is the first study to determine the pharmacokinetics of militarine and its three metabolites, which could be highly meaningful for the potential development of militarine.

2. Materials and Methods

2.1. Reagents and Chemicals. Militarine (purity ≥ 98%) was purchased from Chengdu Push Bio-Technology Co., Ltd. (Chengdu, China). Gastrodin (purity: 97.6%) and puerarin (IS, purity: 95.4%) were obtained from the National Institutes for Food and Drug Control (Beijing, China). Gymnoside I (purity ≥ 95%) and α-isobutylmalic acid (purity ≥ 95%) were isolated from *B. striata* (Thunb.) Reichb. f. in our laboratory. The structure and purity of these compounds were confirmed using IR, ^{1}H nuclear magnetic resonance (NMR), MS, and HPLC-UV. Their chemical structures are shown in Figure 1. Methanol, formic acid, and acetonitrile of HPLC-grade were purchased from Merck KGaA Co. (Darmstadt, Germany). Deionized water was obtained using an EPED superpurification system (EPED, Nanjing, China). Other reagents and chemicals were of chromatographic grade.

2.2. Animals. Sprague-Dawley rats (250 ± 20 g) were supplied by Changsha Tianqin Biotechnology Co., Ltd. (Changsha, China, certificate No. SCXK (Xiang) 2014-0010). All studies were approved by the Animal Ethics Committee at Guizhou Medical University.

2.3. Instrumentation and Analytical Conditions of HPLC-MS/MS. An Acquity HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a Q-Trap ® 5500 triple quadruple mass spectrometer (AB Sciex, Foster, CA, USA) was used for HPLC-ESI-MS/MS. Applied Biosystems Analyst software version 1.6.2 was used for data acquisition. The four analytes and the IS (puerarin) were chromatographically separated using an ACE UltraCore SuperC18 (2.1 × 100 mm, 2.5 μm) column, and the column temperature was set at 45°C. The mobile phase was a binary solvent system, consisting of 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.2 mL/min. Gradient elution was used as follows: 10-10% B at 0-1 min, 10-30% B at 1-2 min, 30-40% B at 2-6 min, 40-90% B at 6-6.1 min, 90-90% B at 6.1-8.1 min, 90-10% B at 8.1-8.2 min, and 10-10% B at 8.2-12 min. The injection volume was 1 μL.

Detection of the analytes was carried out simultaneously with electrospray negative ionization (ESI^-) and electrospray positive ionization (ESI^+), and high-purity nitrogen served as both the nebulizing and drying gas. The optimized parameters were as follows: curtain gas (CUR), 0.28 MPa; ion source gas 1 (GS1), 0.38 MPa; ion source gas 2 (GS2), 0.38 MPa; source temperature, 500°C; and spray voltages, 5500 V (ESI^+) and 4500 V (ESI^-). The optimized MS parameters are listed in Table 1.

2.4. Preparation of Calibration Standards and QC Sample. The stock solutions of militarine, gastrodin, α-isobutylmalic acid, and gymnoside I were separately weighed and dissolved in methanol to obtain final concentrations of 1.8, 1, 2.9, and 1.1 mg/mL, respectively. Four series of standard mixture working standard solutions were obtained by mixing and diluting the respective stock solutions with methanol. An appropriate amount of puerarin was dissolved in methanol and diluted to obtain the IS solution (20 ng/mL). The mixture working standard solution (50 μL) and IS solution (20 μL) were added to blank rat plasma (100 μL) to prepare the calibration standard solutions at final concentrations of 1.02-97.92, 2.96-1136.64, 1.64-629.76, 0.3-57.6, and 4 ng/mL for militarine, gastrodin, α-isobutylmalic acid, gymnoside I, and the IS, respectively. The QC plasma samples, containing militarine (2.04, 32.64, and 97.92 ng/mL), gastrodin (5.92, 378.88, and 1136.64 ng/mL), α-isobutylmalic acid (3.28, 209.92, and 629.76 ng/mL), and gymnoside I (0.6, 19.2, and 57.6 ng/mL), were prepared in the same manner.

2.5. Sample Preparation. An aliquot (100 μL) of rat plasma, 20 μL of IS solution (20 ng/mL), and 40 μL of 1% formic acid solution were added into a 1.5-mL centrifuge tube and vortexed for 30 s. The mixture was extracted with 400 μL of methanol by shaking for 2 min using a vortex mixer. After centrifugation at 13225 ×g for 10 min at 4°C, the supernatant was quantitatively transferred to a clean centrifuge tube and evaporated to dryness under a stream of nitrogen at 37°C. The residue was redissolved with 200 μL of 50% methanol and

Table 2: Calibration curves, linear ranges, correlation coefficients, and LLOQ of gastrodin, α-isobutylmalic acid, gymnoside I, and militarine in rat plasma (n=3).

Analytes	Linear regression equation	R^2	Linear range (ng /mL)	LLOQ (ng /mL)
militarine	Y=0.0461X - 0.0924	0.9997	1.02 - 97.92	1.02
gastrodin	Y=0.4226X + 0.0112	0.9996	2.96 - 1136.64	2.96
α-isobutylmalic acid	Y=0.2270X + 0.0174	0.9994	1.64 - 629.76	1.64
gymnoside I	Y=0.0436X - 0.0093	0.9996	0.3 - 57.6	0.3

centrifuged at 13225 $\times g$ for 10 min. Then, the supernatant (1 μL) was used for analysis.

2.6. Method Validation. Before using the proposed method to determine militarine, gastrodin, α-isobutylmalic acid, and gymnoside I in plasma samples, the method was fully validated for specificity, selectivity, linearity, LLOQ, precision, accuracy, extraction recovery, matrix effect, and stability according to the nonclinical drug pharmacokinetic study technical guideline (China Food And Drug Administration 2014) and the Bioanalytical Method Validation Guideline (Chinese Pharmacopoeia 2015, Vol. 4).

2.6.1. Specificity and Selectivity. Specificity and selectivity were assessed by comparing the chromatograms of blank plasma from six different rats, blank plasma spiked with militarine, gastrodin, α-isobutylmalic acid, gymnoside I and the IS, and plasma samples obtained after oral administration of militarine at 60 mg/kg.

2.6.2. Linearity and LLOQ. Calibration curves were constructed, as described in Section 2.4. Linearity was evaluated by plotting the peak area ratio (y) of analytes to IS versus the nominal concentration (x) of analytes by using $1/x^2$ weighted least squares linear regression. The LLOQ should satisfy the analytical requirement of a signal-to-noise ratio (S/N) of approximately 10.

2.6.3. Precision and Accuracy. Precision and accuracy were determined by analyzing the QC samples in five replicates at three concentration levels (namely, low, medium, and high) on the same day (intraday) and on three consecutive validation days (interday).

2.6.4. Extraction Recovery and Matrix Effect. The extraction recovery of the analytes was evaluated by comparing the peak area ratios of pretreated QC samples at low, medium, and high concentrations with those of post-extracted supernatants spiked with the pure reference standards at the same concentrations. The matrix effect was assessed by comparing the peak areas of the analytes in the post-extracted spiked samples with those of the analytes dissolved in methanol at the same concentrations. Analysis of the QC samples was performed in five replicates.

2.6.5. Stability. The stability of analytes was determined using the QC samples at low, medium, and high concentrations (n = 5) under various conditions: post-preparation stability (24°C in an autosampler for 6 h) and freeze-thaw stability (three times, -20°C to 20°C) on three consecutive days.

2.7. Pharmacokinetic Study. In the pharmacokinetic experiment, the validated method was used to determine the plasma concentrations of militarine and its three metabolites (gastrodin, α-isobutylmalic acid, and gymnoside I) in six healthy SD rats weighing 250 ± 20 g. Rats were fasted with free access to water for 12 h before the experiment. They received a single oral dose (60 mg/kg) of militarine. Blood samples of 300 μL were collected from the external right jugular vein into heparinized tubes at the designated time points (0, 0.033, 0.083, 0.167, 0.33, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, and 36 h). The heparinized blood samples were centrifuged at 3306 $\times g$ for 10 min at 4°C, and the supernatant was transferred into clean centrifuge tubes and stored at -20°C until analysis.

2.8. Pharmacokinetic Data Analysis. The pharmacokinetic parameters of the four analytes were calculated by Phoenix WinNonlin version 6.4 software (Pharsight Corporation, Mountain View, USA) using noncompartmental analysis. These parameters included the AUC from 0 h to the time of last measurable concentration ($AUC_{0\text{-}t}$), AUC from 0 to infinity ($AUC_{0\text{–}\infty}$), $MRT_{0\text{–}t}$, MRT to infinity ($MRT_{0\text{-}\infty}$), C_{max}, and time to C_{max} (T_{max}). All results were expressed as the means ± standard deviations (SD).

3. Results and Discussion

3.1. Method Validation

3.1.1. Specificity and Selectivity. The chromatograms of blank rat plasma, blank rat plasma spiked with standard solutions and IS, and rat plasma after oral militarine administration are shown in Figure 2. The retention times of gastrodin, α-isobutylmalic acid, gymnoside I, militarine, and the IS (puerarin) were 1.52, 4.96, 5.4, 5.65, and 4.45 min, respectively. No significant interference from endogenous substances with the analytes and IS was observed, compared to the chromatogram of blank rat plasma sample.

3.1.2. Linearity and Lower Limit of Quantitation (LLOQ). Table 2 shows the typical calibration curves, linearity ranges, coefficients of correlation, and LLOQ for militarine, gymnoside I, gastrodin, and α-isobutylmalic acid. All the four analytes exhibited good linearity with correlation coefficients within the range of 0.9994-0.9997.

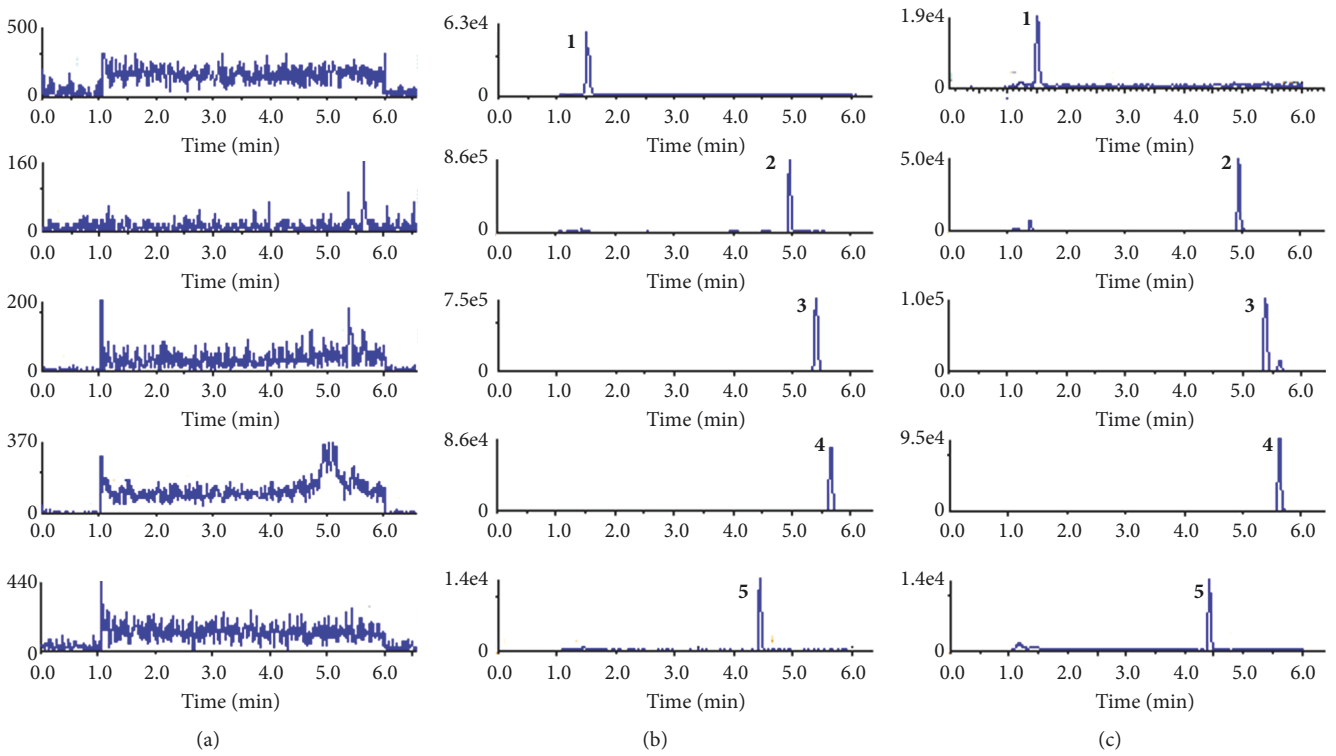


Figure 2: The chromatograms of the analytes and puerarin (IS) in rat plasma. (a) A blank rat plasma sample; (b) a blank rat plasma sample spiked with gastrodin (378.88 ng/mL), α-isobutylmalic acid (209.92 ng/mL), gymnoside I (19.2 ng/mL), militarine (32.64 ng/mL) and IS (4 ng/mL); (c) a plasma sample at 30 min after oral administration militarine to rats. (1) Gastrodin, (2) α-isobutylmalic acid, (3) gymnoside I, (4) militarine, and (5) puerarin (IS).

Table 3: Summary of precision and accuracy of the four analytes in rat plasma (n=5).

Analytes	Spiked Concentration (ng/mL)	Intra-day			Inter-day		
		Calculated concentration (ng/mL)	Precision (RSD, %)	Accuracy (%)	Calculated concentration (ng/mL)	Precision (RSD, %)	Accuracy (%)
militarine	2.04	2.22 ± 0.15	6.9	108.9	1.87 ± 0.21	11.4	91.5
	32.64	31.56 ± 2.37	7.5	96.7	36.73 ± 1.87	5.1	112.5
	97.92	105.10 ± 4.67	4.4	107.3	87.36 ± 5.97	6.8	89.2
gastrodin	5.92	6.48 ± 0.69	10.6	109.5	6.51 ± 0.63	9.7	109.9
	378.88	341.90 ± 18.12	5.3	90.2	335.42 ± 25.02	7.5	88.5
	1136.64	1253.71 ± 44.13	3.5	110.3	1051.16 ± 128.45	12.2	92.5
α-isobutylmalic acid	3.28	3.05 ± 0.38	12.5	93.0	3.59 ± 0.37	10.3	109.4
	209.92	230.72 ± 13.11	5.7	109.9	217.31 ± 27.23	12.5	103.5
	629.76	672.08 ± 49.73	7.4	106.7	575.98 ± 51.20	8.9	91.5
gymnoside I	0.6	0.58 ± 0.05	8.3	96.6	0.52 ± 0.05	10.1	87.4
	19.2	16.99 ± 1.97	11.6	88.5	17.53 ± 2.32	13.2	91.3
	57.6	52.70 ± 2.82	5.4	91.5	61.33 ± 5.70	9.3	106.5

3.1.3. Precision and Accuracy. The intra- and interday precision and accuracy were evaluated in five replicate analyses of quality control (QC) samples at three concentrations. Results are summarized in Table 3. The relative standard deviations of intra- and interday measurements were less than 15%, and the RSD (%) values of accuracy of three analytes were within the range of 87.4-112.5%. These results showed that the method was acceptable.

3.1.4. Extraction Recovery and Matrix Effect. The mean extraction recovery and matrix effect of the four analytes are shown in Table 4. The extraction recoveries of the four

Table 4: Summary of recovery and matrix effect of the four analytes in rat plasma (n=5).

Analytes	Spiked Concentration (ng/mL)	Extraction Recovery		Matrix effect	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
militarine	2.04	105.34 ± 7.91	7.5	102.82 ± 8.53	8.3
	32.64	92.40 ± 10.65	11.5	87.61 ± 11.49	13.1
	97.92	89.92 ± 8.99	10.0	92.52 ± 4.75	5.1
gastrodin	5.92	85.71 ± 6.01	7.0	88.80 ± 9.22	10.4
	378.88	92.49 ± 5.40	5.8	85.43 ± 4.63	5.4
	1136.64	87.58 ± 8.20	9.4	92.76 ± 6.78	7.3
α-isobutylmalic acid	3.28	93.14 ± 3.56	3.8	93.48 ± 8.07	8.6
	209.92	89.10 ± 10.88	12.2	84.42 ± 10.91	12.9
	629.76	95.71 ± 10.17	10.6	89.30 ± 10.13	11.3
gymnoside I	0.6	93.02 ± 3.53	3.8	85.41 ± 3.42	4.0
	19.2	90.40 ± 12.08	13.4	90.14 ± 8.92	9.9
	57.6	83.52 ± 2.86	3.4	88.30 ± 10.78	12.2

Table 5: Stability of the four analytes in rat plasma under various storage conditions (n = 5).

Analytes	Spiked Concentration (ng/mL)	Post-preparation stability			Freeze-thaw stability		
		Calculated concentration (ng/mL)	Precision (RSD, %)	Accuracy (%)	Calculated concentration (ng/mL)	Precision (RSD, %)	Accuracy (%)
militarine	2.04	1.85 ± 0.14	7.3	90.6	2.09 ± 0.21	9.9	102.6
	32.64	33.47 ± 2.22	6.6	102.5	35.09 ± 1.41	4.0	107.5
	97.92	96.57 ± 5.42	5.6	98.6	95.68 ± 7.57	7.9	97.7
gastrodin	5.92	6.29 ± 0.60	9.6	106.2	6.11 ± 0.59	9.6	103.2
	378.88	357.97 ± 11.85	3.3	94.5	410.63 ± 26.77	6.5	108.4
	1136.64	1080.94 ± 52.32	4.8	95.1	1087.88 ± 31.11	2.9	95.7
α-isobutylmalic acid	3.28	3.03 ± 0.15	4.9	92.4	3.21 ± 0.27	8.5	97.9
	209.92	213.51 ± 6.62	3.1	101.7	213.32 ± 13.89	6.5	101.6
	629.76	647.02 ± 27.63	4.3	102.7	607.66 ± 32.21	5.3	96.5
gymnoside I	0.60	0.62 ± 0.05	8.6	103.6	0.63 ± 0.05	8.5	104.6
	19.2	18.65 ± 1.34	7.2	97.1	20.14 ± 1.49	7.4	104.9
	57.6	54.29 ± 3.67	6.8	94.3	54.50 ± 1.91	3.5	94.6

analytes were within the range of 83.52-105.34%, showing that the recovery of analytes was consistent and reproducible. The matrix effects ranged from 84.42% to 102.82%. The results indicated that no endogenous substances significantly influenced the quantification of all the analytes.

3.1.5. Stability. Table 5 shows the stability data of the four analytes. Results showed that the relative standard deviations (RSD) of post-preparation stability (24°C in an autosampler for 6 h) and freeze-thaw stability (three times, -20 to 20°C) on consecutive three days of all analytes were ≤ 9.6% and ≤ 9.9%, respectively. These results indicated that the four analytes were stable and applicable within the acceptable limit.

3.2. Pharmacokinetics. The validated HPLC-MS/MS method was successfully applied for simultaneous determination of militarine and its three metabolites after single oral administration of militarine at 60 mg/kg to six healthy rats. The primary pharmacokinetic parameters calculated using noncompartmental analysis are summarized in Table 6. The mean plasma concentration-time curves of militarine and its three metabolites are shown in Figure 3.

Two minutes after administration, militarine could be detected in plasma, indicating that it was rapidly absorbed. Militarine reached the peak plasma concentration (C_{max} = 62.31 ± 10.01 ng/mL) at approximately 0.21 h and was rapidly eliminated with a mean retention time to the last sampling time (MRT_{0-t}) of 3.29 ± 0.36 h. The three metabolites (gastrodin, α-isobutylmalic acid, and gymnoside I) were synchronously detected at 0.83 h, suggesting that militarine was rapidly transformed to gastrodin, α-isobutylmalic acid, and gymnoside I. Moreover, the area under the curve (AUC) and C_{max} of militarine were significantly lower than those of gastrodin and α-isobutylmalic acid, showing that militarine was largely metabolized to gastrodin and α-isobutylmalic acid *in vivo*. The AUC and C_{max} of gymnoside I were comparable to that of militarine. These results suggested

Table 6: The pharmacokinetic parameters of militarine and its three metabolites after the single oral dose (60 mg/kg) of militarine to rats (n = 6, mean ± SD).

Parameters	Unit	Analytes			
		militarine	gastrodin	α-isobutylmalic acid	gymnoside I
T_{max}	h	0.21 ± 0.08	1.38 ± 0.25	7.50 ± 1.00	1.88 ± 0.25
C_{max}	ng/mL	62.31 ± 10.01	620.50 ± 75.98	466.86 ± 96.49	32.82 ± 5.92
AUC_{0-t}	h∗ng/mL	123.41 ± 9.95	2077.40 ± 358.66	6285.32 ± 1319.90	122.27 ± 9.54
$AUC_{0-\infty}$	h∗ng/mL	140.34 ± 10.02	2116.92 ± 384.37	6316.32 ± 1327.54	123.73 ± 9.81
MRT_{0-t}	h	3.29 ± 0.36	2.66 ± 0.50	8.71 ± 0.67	3.28 ± 0.39
$MRT_{0-\infty}$	h	5.03 ± 0.88	2.88±0.63	8.87 ± 0.68	3.41 ± 0.39

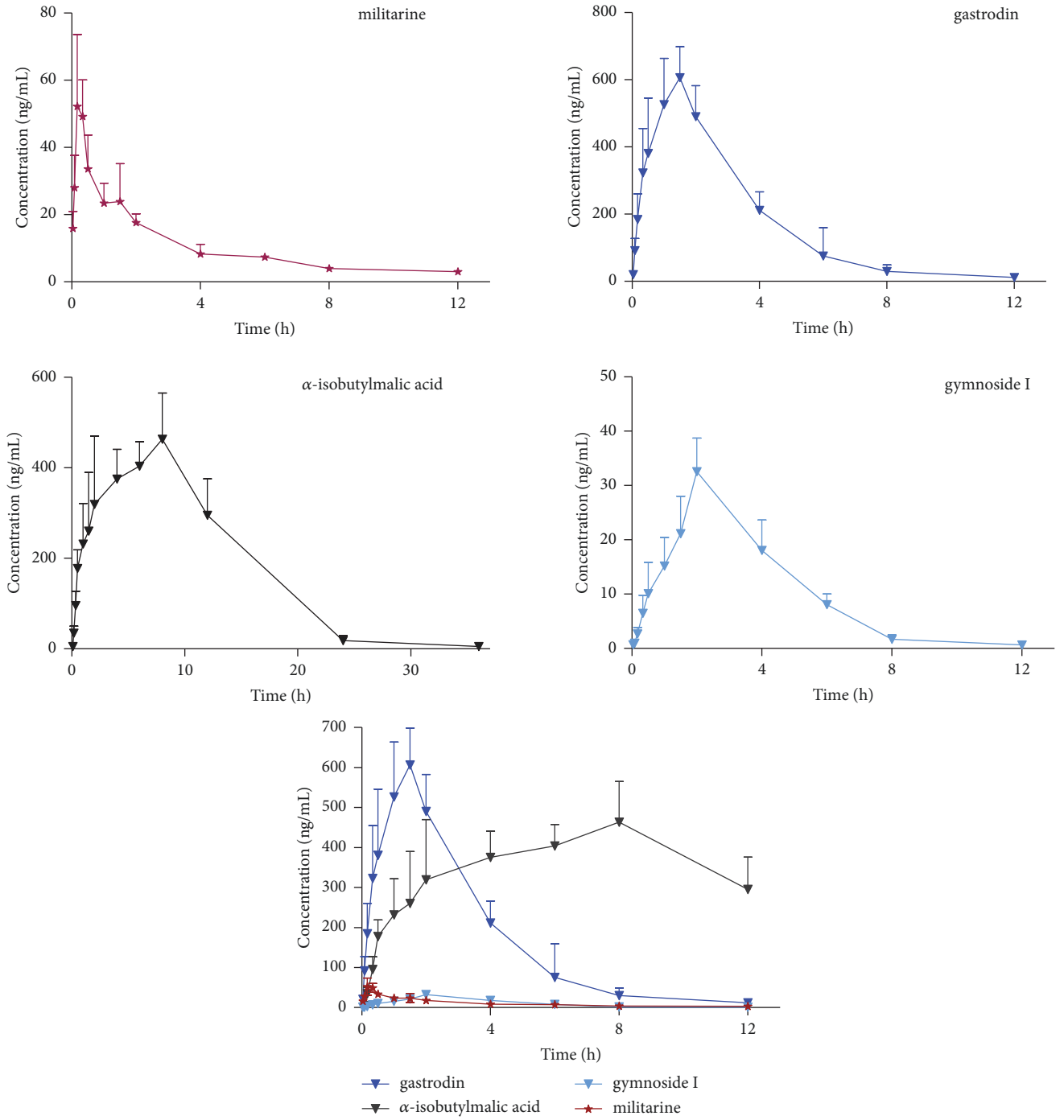


Figure 3: Mean plasma concentration-time curves of militarine and its three metabolites after the single oral dose (60 mg/kg) of militarine to rats (n=6).

that the cleavage of ester bonds in militarine could produce gymnoside I and gastrodin, and then the cleavage of the ester bonds in gymnoside I could produce gastrodin and α-isobutylmalic acid, which could account for the high AUC and C_{max} values of gastrodin and α-isobutylmalic acid, compared to those of militarine and gymnoside I. The order of AUC values was α-isobutylmalic > gastrodin > militarine > gymnoside I, suggesting that militarine could be adequately transformed to the three metabolites *in vivo*, and the most abundant constituents in the blood were α-isobutylmalic acid and gastrodin.

A drug candidate should have a favorable pharmacokinetic behavior to allow new drug development [23]. Militarine was shown to improve white matter lesions (WMLs) and cognitive impairment in rat chronic hypoperfusion model; additionally, it showed a dose-dependent and endothelium-dependent relaxing effect in rat isolated thoracic aorta rings [20, 21]. However, our results showed that the blood concentration of militarine was very low after oral administration, and gastrodin and α-isobutylmalic acid were the main components detected in the plasma after single oral administration (60 mg/kg) of militarine to rats. Gastrodin, the main bioactive ingredient in the widely known Chinese medicine "Tianma" (*Rhizoma Gastrodia*), has been widely used in China to treat central nervous system (CNS) diseases, such as neuralgia, vertigo, neurasthenia, epilepsy, insomnia, and headache [24–28]. Additionally, it has been shown to improve learning and memory and to exhibit vasodilator effects [29, 30]. α-Isobutylmalic acid exhibited longer MRT and higher C_{max} than those of militarine, indicating that α-isobutylmalic acid might exert therapeutic effects *in vivo*. In addition, glucosyloxybenzyl 2-isobutylmalates, such as gymnoside I, could improve intelligence and prevent senile dementia [1, 31]. Therefore, further studies are required to verify these effects of gymnoside I. The *in vivo* effectiveness of bioactive constituents depends to a great extent on their blood concentration, as well as the activity of their metabolites. Thus, the effects of militarine *in vivo* might be, in part, attributed to the activity of its metabolites. We believe that the results of this pharmacokinetic study of militarine after its oral administration in rats could provide the basis for the development of militarine and its clinical applications.

4. Conclusions

In this study, a sensitive, rapid, and convenient HPLC-MS/MS method to determine militarine and its three metabolites (gastrodin, α-isobutylmalic acid, and gymnoside I) in rat plasma was successfully established and validated. The validated method was successfully used in a preclinical pharmacokinetic study in rats after oral administration of militarine. This study determined the pharmacokinetic behavior of militarine and its metabolites. The current pharmacokinetic study of militarine not only provided significant and valuable clinical guidance toward the application of this constituent but also could predict the druggability of bioactive metabolites, which is essential in drug development.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (No. U1812403/81860323/81460630); Guizhou Science and Technology Department (No. [2017] 5601, [2016]5613/5677); Guiyang Science and Technology Bureau (No. [2017]30-29); and Guizhou Education Department (No. KY[2018]050).

References

[1] M. Li, *Studies on The Chemical Constituents and Quantitative Determination of Glucosyloxybenzyl 2-Isobutylmalates in The Tubers of Gymnadenia Conopsea*, eking Union Medical College, Beijing, China, 2007.

[2] S.-Y. Huang, J.-G. Shi, Y.-C. Yang, and S.-L. Hu, "Studies on the chemical constituents of Coeloglossum viride (L.) Hartm. var. bracteatum (Willd.) Richter," *Acta Pharmaceutica Sinica*, vol. 37, no. 3, pp. 199–203, 2002.

[3] G. X. Han, L. X. Wang, W. D. Zhang, M. L. Wang, H. T. Liu, and J. H. Xiao, "Studies on chemical constituents of Bletilla striata II," *Academic Journal of Second Military Medical University*, vol. 23, no. 9, pp. 1029–1031, 2002.

[4] J. Wang, C. Y. Yu, and J. J. Zhang, "Improvement of Coeloglossum viride var. bracteatum extract in IBO-induced AD rats," *Journal of Basic and Clinical Medicine*, vol. 30, no. 8, pp. 831–835, 2010.

[5] C.-Y. Yu and J.-J. Zhang, "Effect of the extract from Coeloglossum viride (L.) Hartm. Var. bracteatum (Willd.) Richter on learning and memory in dementia rats," *Chinese Journal of New Drugs*, vol. 18, no. 1, pp. 63–66, 2009.

[6] D. Zhang and J.-J. Zhang, "Effect of Coeloglossum. viride var. bracteatum extract on oxidation injury in sub-acute senescent model mice," *Acta Academiae Medicinae Sinicae*, vol. 27, no. 6, pp. 729–733, 2005.

[7] D. Zhang and J.-J. Zhang, "Effects of Coeloglossum viride (L.) Hartm. var. bracteatum (Willd.) Richter extract on memory and apoptosis-related protein expression of subacute aging mice," *Chinese Journal of Pharmacology and Toxicology*, vol. 19, no. 4, pp. 259–262, 2005.

[8] D. Zhang, G. Liu, J. Shi, and J. Zhang, "Coeloglossum viride var. bracteatum extract attenuates D-galactose and $NaNO_2$ induced memory impairment in mice," *Journal of Ethnopharmacology*, vol. 104, no. 1-2, pp. 250–256, 2006.

[9] L. Chen, C. X. Liu, X. L. He, and X. H. Zhang, "Simultaneous determination of militarine, protocatechuic acid and caffeic acid in bletilla striata by LC-MS/MS," *China Pharmacist*, vol. 18, no. 2, pp. 230–232, 2013.

[10] A.-M. Wang, Y. Yan, B. Lan, S.-G. Liao, Y.-L. Wang, and Y.-J. Li, "Simultaneous determination of nine chemical markers

of Bletillae Rhizoma by ultra performance liquid chromatography," *China Journal of Chinese Materia Medica*, vol. 39, no. 11, pp. 2051–2055, 2014.

[11] L. Y. Huang, S. S. Mao, and L. Chen, "Determination of Militarine in Bletilla striata from the Different Habitat by HPLC," *Research and Practice of Chinese Medicines*, vol. 27, no. 5, pp. 16–19, 2013.

[12] X. He, A. M. Wang, Y. J. Li, Y. L. Wang, and S. Y. Zhou, "Determination of militarine Rhizom a Bletillae by HPLC," *China Journal of Chinese Materia Medica*, vol. 34, no. 16, pp. 2076–2078, 2009.

[13] F. F. Zhao, N. C. Lin, X. Yang et al., "Effects of a non-polysaccharide fraction of Bletilla striata on platelet activation and hemorheology in rats," *Chinese Traditional Patent Medicine*, vol. 39, no. 2, pp. 244–249, 2017.

[14] F.-F. Zhao, X. Yang, D. Xu et al., "Hemostatic effect and mechanism of a non-polysaccharide fraction of bletilla striata," *Chinese Pharmacological Bulletin*, vol. 32, no. 8, pp. 1121–1126, 2016.

[15] A. J. Sun, S. Q. Pang, and G. Q. Wang, "Advances of chemical constituents and pharmacological activities of Bletilla striata," *Global Traditional Chinese Medicine*, vol. 9, no. 4, pp. 507–511, 2016.

[16] L. F. Zhang, J. H. Hu, and Y. Q. Zhang, "Rsearch progress on pharmacological action of Bletilla rhizoma," *Modern Chinese Medicine*, vol. 16, no. 1, pp. 83–86, 2014.

[17] X. He, X. Wang, J. Fang et al., "Bletilla striata: medicinal uses, phytochemistry and pharmacological activities," *Journal of Ethnopharmacology*, vol. 195, pp. 20–38, 2017.

[18] Y. Wang, B. Zhang, J. Zhang et al., "Qualitative and quantitative analysis of Yifei Tongluo granules to identify main bioactive components using LC–DAD/MS and pharmacokinetic studies," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 163, pp. 130–136, 2019.

[19] L. Huang, H. L. Chen, and L. L. Li, "Determination of militarine in Weikangling capsules by HPLC," *Drug Standards of China*, vol. 19, no. 4, pp. 261–264, 2018.

[20] Y. Wang, X. Y. Zhang, X. Y. Li, S. Li, and J. J. Zhang, "Militarine alleviates white matter damage and cognitive impairment in rats with chronic cerebral hypoperfusion," *Acta Pharmaceutica Sinica*, vol. 51, no. 5, pp. 738–742, 2016.

[21] X. X. Xu, G. T. Xu, X. P. Li, X. Zhong, H. W. Jian, and Z. Xu, "Effect and mechanism of militarine in relaxing isolated thoracic aorta rings in rats," *Chinese Archives of Traditional Chinese Medicine*, vol. 33, no. 3, pp. 617–620, 2015.

[22] K. Xiong, T. Gao, T. Zhang, Z. Wang, and H. Han, "Simultaneous determination of gentiopicroside and its two active metabolites in rat plasma by LC–MS/MS and its application in pharmacokinetic studies," *Journal of Chromatography B*, vol. 1065-1066, pp. 1–7, 2017.

[23] X. Zhang, S. Liu, Z. Pi, Z. Liu, and F. Song, "Simultaneous quantification method for comparative pharmacokinetics studies of two major metabolites from geniposide and genipin by online mircrodialysis-UPLC–MS/MS," *Journal of Chromatography B*, vol. 1041-1042, pp. 11–18, 2017.

[24] Z. Zhang, P. Ma, Y. Xu et al., "Preventive effect of gastrodin on cognitive decline after cardiac surgery with cardiopulmonary bypass: a double-blind, randomized controlled study," *Journal of Huazhong University of Science and Technology (Medical Science)*, vol. 31, no. 1, pp. 120–127, 2011.

[25] J. Xu and S. Guo, "Retrospect on the research of the cultivation of Gastrodia elata Bl, a rare traditional Chinese medicine," *Chinese Medical Journal*, vol. 113, no. 8, pp. 686–692, 2000.

[26] R. Zhang, Z. Peng, H. Weng et al., "Gastrodin ameliorates depressive-like behaviors and up-regulates the expression of BDNF in the hippocampus and hippocampal-derived astrocyte of rats," *Neurochemical Research*, vol. 39, no. 1, pp. 172–179, 2014.

[27] Y. Jia, X. Li, H. Xie et al., "Analysis and pharmacokinetics studies of gastrodin and p-hydroxybenzyl alcohol in dogs using ultra fast liquid chromatography-tandem mass spectrometry method," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 99, no. 10, pp. 83–88, 2014.

[28] M.-T. Hsieh, C.-R. Wu, and C.-F. Chen, "Gastrodin and p-hydroxybenzyl alcohol facilitate memory consolidation and retrieval, but not acquisition, on the passive avoidance task in rats," *Journal of Ethnopharmacology*, vol. 56, no. 1, pp. 45–54, 1997.

[29] Z. H. Liu, H. Ma, W. P. Wang et al., "Structure-activity relationship of gastrodin and parishins on learning and memory deficits induced by scopolamine," *Acta Pharmaceutica Sinica*, vol. 51, no. 5, pp. 743–748, 2016.

[30] Y. Zhang, T. Yu, J. Xu, X. Bian, J. Xu, and Y. Liu, "Effect and mechanism of gastrodin in relaxing isolated thoracic aorta rings in rats," *China Journal of Chinese Materia Medica*, vol. 37, no. 14, pp. 2135–2138, 2012.

[31] J. Liu, *Study on The Mass Spectrometric Analytical Method of an Active Fraction from Gymnadenia Conopsea R. BR. and Its Metabolism*, Peking and Union Medical College, Beijing, China, 2010.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 6037137, 9 pages
https://doi.org/10.1155/2019/6037137

Research Article

Comparative Phytochemical Analysis and Antioxidant Activities of Tamalakyadi Decoction with Its Modified Dosage Forms

Jeevani Maheshika Dahanayake,[1] Pathirage Kamal Perera,[1] Priyadarshani Galappatty,[2] Hettiarachchige Dona Sachindra Melshandi Perera,[3] and Liyanage Dona Ashanthi Menuka Arawwawala[3]

[1]*Department Ayurveda Pharmacology and Pharmaceutics, Institute of Indigenous Medicine, University of Colombo, Sri Lanka*
[2]*Department of Pharmacology, Faculty of Medicine, University of Colombo, Sri Lanka*
[3]*Research and Development Complex, Industrial Technology Institute, Malabe, Sri Lanka*

Correspondence should be addressed to Pathirage Kamal Perera; drkamalperera@yahoo.com

Received 1 February 2019; Revised 22 March 2019; Accepted 16 April 2019; Published 2 May 2019

Guest Editor: Jesus R. R. Amado

Background and Objective. Tamalakyadi decoction (TD) is a classical formulation mentioned in authentic traditional medicine text Sarasankshepaya under nasal diseases and used as a remedy for allergic rhinitis. It consists of 12 plant ingredients. Decoction preparations are widely used in Sri Lankan traditional system and considered effective and safe for treating many disorders. However, decoctions have to be used only in fresh state due to shorter shelf life and loss of stability. This decoction preparation method leads to decreasing the patient compliance and is also time consuming. Hence, the objective of the present study was to convert TD to consumer friendly novel dosage form, namely, freeze dried, spray dried, and traditional ganasara forms. *Methodology*. Therefore, we compared the phytochemical constituents and antioxidant activities of TD with its modified dosage forms. The chemical comparison of four dosage forms comprises phytochemical screening, TLC and HPTLC fingerprint profiles and the antioxidant activities by DPPH free radical scavenging activity, Ferric reducing antioxidant power (FRAP), total polyphenol content (TPC), and total flavonoid content (TFC). *Results*. Phytochemical screening revealed the presence of alkaloids, saponins, tannins, steroids, flavonoids, phenols, and terpenoids in all dosage forms. However, the saponins, alkaloids, flavonoids, terpenoids, and steroids were more prominent in TD and freeze dried preparation than the other two preparations. HPTLC fingerprint pattern of freeze dried dosage was more similar with HPTLC fingerprint pattern of TD in terms of number of peaks and their intensity compared to that of spray dried and ganasara dosage forms. Antioxidant activities such as DPPH, FRAP, TPC, and TFC were higher in decoction and freeze dried preparation than in spray dried and ganasara preparation. *Conclusion*. Freeze dried TD is the most suitable ready to use preparation having similar chemical properties and antioxidant activities to TD.

1. Introduction

Tamalakyadi decoction (TD) is an effective herbal decoction used for allergic rhinitis since long time. It is mentioned in authentic traditional medicine text, Sarasankshepaya, under nasa roga (nasal diseases) [1]. Allergic rhinitis is an IgE mediate immune response of the nasal mucosa against inhaled allergens and defined as symptoms of sneezing, rhinorrhea, nasal congestion, and itching of the nose and eyes. It is commonly defined as seasonal or perennial, depending upon whether symptoms are manifested at defined yearly intervals or throughout the year, respectively [2]. This condition is the most common allergic disorder and the prevalence of allergic rhinitis is estimated in the range from 9 % to 42 % [3]. The symptoms of allergic rhinitis may significantly affect a patient's quality of life and can be associated with conditions such as fatigue, headache, cognitive impairment, and sleep disturbances. Appropriate management of allergic

Table 1: Ingredients of Tamalakyadi decoction.

	Plant name	Family	Sinhala name used in Sri Lanka	Sanskrit name	Used part
1	*Phyllanthus niruri* L.	Phyllanthaceae	Pitawakka	Tamalaki	Whole plant
2	*Terminalia chebula* Retz.	Combretaceae	Aralu	Haritaki	Fruit cover
3	*Premna herbacea* Roxb.	Lamiaceae	Siritekku	Bharangi	Roots
4	*Piper retrofractum* Vahl	Piperaceae	Siviya	Chavya	Roots
5	*Piper longum* L.	Piperaceae	Tippili	Pippali	Fruits
6	*Solanum trilobatum* L.	Solanaceae	Wel Tibbatu	Vallikantakarika	Whole plant
7	*Tinospora cordifolia* (Thunb.) Miers	Menispermaceae	Rasakinda	Guduchi	Stem
8	*Zingiber officinale* Roscoe	Zingiberaceae	Inguru	Shunti	Dried Rhizome
9	*Piper nigrum* L.	Piperaceae,	Gammiris	Maricha	Fruits
10	*Solanum melongena* L.	Solanaceae	Elabatu	Vruhati	Roots
11	*Solanum xanthocarpum* L.	Solanaceae	Katuwelbatu	Kantakari	Whole plant
12	*Justicia adhatoda* L.	Acanthaceae	Adathoda	Vasa	Whole plant

rhinitis is an important component in effective management of coexisting or complicated respiratory conditions such as asthma, sinusitis, and sleep apnea [4].

In Ayurveda system of medicine, allergic rhinitis is described as Apeenasa or Peenasa and the concept of allergy is explained under "Asatmyaja vyadhi" (allergic disorders), while its effects are explained in hereditary, Viruddhahara (incompatible foods) and Dushivisha (polluted substances or allergic agents) and Ritu sandhi (seasonal changes) [5].

Effective therapeutic methods for allergic rhinitis including internal as well as external treatments are described in Sri Lankan traditional system of medicine and in Ayurveda medicine. TD is one of the effective decoctions used for allergic rhinitis. It includes 12 ingredients which are mentioned in Table 1. Among those 12 ingredients, *Clerodendrum serratum* (L.) Moon plant and *Solanum indicum* L. plant were replaced by *Premna herbacea* Roxb. and *Solanum melongena* L. plants, respectively, for many years.

Decoction is a basic Ayurveda dosage form which is one of the most commonly used and considered as very effective dosage form in system of traditional medicine. However, the decoction preparations have some drawbacks such as unpleasant taste and have to be used only in fresh state due to loss of stability [6]. Therefore, patients on treatments with decoctions need to prepare it every day which causes difficulties in their busy lifestyles. Hence in this study an approach was made to prepare ready to use user friendly decoction powder by using novel technology and choose the most similar ready to use preparation when compared with the decoction in terms of phytochemical constituents and antioxidant activity.

2. Materials and Methods

2.1. Collection and Authentication of Plant Materials. The plants used in TD were collected from Colombo city (6° 55' 54.98" N x 79° 50' 52.01" E) Western province, Sri Lanka, between July and August 2018 and authenticated by the Curator of National Herbarium of Peradeniya, Sri Lanka. The contaminants of the raw materials were removed manually, washed with water, and shade dried. Then dried raw materials were crushed to a coarse powder separately and stored in tightly closed containers.

2.2. Preparation Method of Tamalakyadi Decoction (TD). TD was prepared according to the traditional decoction preparation method [7]. Five grams was taken from each ingredient of the formulation and boiled with 1920 ml of water under mild flame to reduce the volume up to 240 ml. Then the decoction was filtered through a single folded cotton cloth and collected to a separate vessel. Same procedure was repeated for eight times and pooled decoction (240 ml ×8) was divided equally (240 ml × 2) into four portions. The fist portion was labeled as TD and others were subjected to prepare modified dosage forms.

2.3. Preparation of Freeze Dried Form of Tamalakyadi Decoction (FDF-TD). TD (240 ml × 2) was freeze dried using a freeze dryer (Telstar LyoBeta) with the temperature – 45°C to 40°C and kept in a refrigerator (at 4°C) until used.

2.4. Preparation of Spray Dried Form of Tamalakyadi Decoction (SDF-TD). TD (240 ml × 2) was spray dried using a spray dryer (Mini Spray drier B-290 BUCHI) with 180°C inlet temperature, 102°C outlet temperature, and 50 kg of feed pressure.

2.5. Preparation of Ganasara Form of Tamalakyadi Decoction (GSF-TD). TD (240 ml × 2) was subjected to mild heat, converted to semisolid form, and oven dried (at 105°C) to prepare the GSF-TD [8].

2.6. Phytochemical Screening. Phytochemical screening was carried out according to the methods described by Goveas [9] and Joanne and coworkers [10] with some modifications. In brief, freshly prepared TD (240 ml × 2) and the FDF-TD, SDF-TD, and GSF-TD samples dissolved in hot water (240 ml × 2) separately were subjected to phytochemical screening studies as follows.

2.7. Test for Saponins. Five milliliters of extract and 2.5 ml of water were added to a test tube, shaken vigorously, and kept

for 10 minutes. Then the froth was mixed with 3 drops of olive oil and shaken vigorously and the formation of emulsion was observed. The presence of stable froth indicates that saponins are found in the extract.

2.8. Tests for Tannins

(a) Ferric chloride test: five drops of $Fecl_3$ was added to each extract and mixed well. Appearance of a black precipitate indicates the presence of tannins.

(b) Lead acetate test: three drops of $Pb(OAc)_2$ was added to 5 ml of extract and mixed well. Formation of a yellow precipitate is indicative of tannins.

(c) Vanillin test: few drops of 10 % vanillin in ethyl alcohol and conc. HCl were added to each extract and mixed well. Appearance of red color indicates the presence of tannins.

2.9. Test for Phenols

(a) Vanillin test: few drops of 10 % vanillin in ethyl alcohol and conc. HCl were added to 2 ml of extract. Appearance of red color indicates the presence of phenols.

(b) Lead acetate test: three drops of $Pb(OAc)_2$ was added to 5 ml of extract and mixed well. Formation of yellow precipitate indicates the presence of phenols.

2.10. Test for Alkaloids

(a) Picric acid test: few drops of picric acid was added to 5 ml of extract and mixed well. Formation of a yellow color crystalline precipitate indicates the presence of alkaloids.

(b) Tannic acid test: few drops of tannic was added to 5 ml of extract and mixed well. Formation of a yellow color crystalline precipitate indicates the presence of alkaloids.

(c) Wagner reagent test: two drops of Wagner reagent was added to 2 ml of extract and mixed well. Appearance of a reddish color indicates the presence of alkaloids.

2.11. Test for Flavonoids

(a) Five milliliters of dilute ammonia solution was added to 5 ml of extract followed by the addition of conc. H_2SO_4. Appearance of yellow color indicates the presence of flavonoids.

(b) Five milliliters of extract was added to a test tube containing piece of metallic mg and 3 drops of conc. HCl and heated. Flavonoids give a red-orange color.

2.12. Test for Terpenoids

(a) Salkowski test: extract (5 ml) was mixed with 2 ml of chloroform in a test tube and 3 ml of conc. H_2SO_4 was added along the sides of the test tube. Formation of reddish brown color is an indicative of presence of terpenoids.

(b) Test for sesquiterpenes: one milliliter of conc. H_2SO_4 was added to 2 ml of extract and mixed well. A reddish brown color indicates the presence of terpenoids.

2.13. Test for Steroids

(a) Five milliliters of acetic anhydride and 5 ml of conc. H_2SO_4 were added to the 5 ml of extract and mixed well. A color change from violet to blue or green color indicates the presence of steroids.

(b) Lieberman Burchard test reaction: two milliliters of acetic anhydride and 2 ml of conc. H_2SO_4 were added to 2 ml of extract and mixed well. Formation of a dark bluish green color indicates the presence of steroids.

2.14. Test for Cardiac Glycosides. One milliliter of glacial acetic acid was added to 3 ml of extract and con. H_2SO_4 acid was introduced to the bottom of the tube. A reddish brown or violet brown ring at the interface of the two liquids indicates the presence of cardiac glycosides.

2.15. Development of Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) Fingerprints. Freshly prepared TD (100 ml) and FDF-TD, SDF-TD and GSF-TD dosage forms dissolved in hot water (100 ml from each) were added separately to a separating funnel containing 50 ml of dichloromethane, mixed well and kept for 20 min. After that, dichloromethane layer was separated. This was done thrice and collected dichloromethane fractions were pooled and evaporated to dryness. Dried dichloromethane fractions of TD, FDF-TD, SDF-TD, and GSF-TD dosage forms were redissolved in 5 ml of dichloromethane separately and spotted on a TLC plate. TLC fingerprint profile was developed for all fractions using dichloromethane, ethyl acetate, and cyclohexane in a ratio of 3:0.5:1.5 v/v. The plate was visualized under UV radiation (both 254 nm and 366 nm) and HPTLC fingerprint patterns were observed by using CAMAG - HPTLC scanner.

2.16. Extracts for In Vitro Antioxidant Assays. The powders obtained from freeze drying, spray drying, and ganasara methods were dissolved in methanol to prepare methanolic extracts. Liquid form decoction was dried by evaporation using rotary evaporator and redissolved in methanol.

2.17. Antioxidants Assay. The antioxidant activities of these four preparations were assessed by using DPPH free radical scavenging activity, Ferric reducing antioxidant power (FRAP), total polyphenol content (TPC), and total flavonoid content (TFC).

2.18. DPPH Free Radical Scavenging Activity. The DPPH free radical scavenging assay was performed according to the method described by Blois, [11] with some modifications in 96-well microplates. Reaction mixture of 200 μl, containing 150 μl of DPPH solution and 50 μl of each extract (dissolved

in methanol) of decoction or freeze dried or spray dried or ganasara was incubated at room temperature (25 ± 2°C) for 10 minutes in dark and the absorbance was recorded at 517 nm. Five different concentrations of Trolox (2.5, 5, 10, 20, 30 μg/ml) were used to construct the standard curve. Results were expressed as IC_{50}; μg/ml.

2.19. Ferric Reducing Antioxidant Power (FRAP). The assay was carried out according to the Benzie and Strain [12] with some modifications in 96-well microplates. The working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3.6H_2O$ (10:1:1 v/v/v) just before use and incubated at 37°C for 8 minutes. Reaction mixtures of 200 μl containing 150 μl FRAP reagent, 30 μl of acetate buffer, and 20 μl of four extracts (120 μg/ml) were incubated at room temperature (25 ± 2°C) for 8 minutes and the absorbance was recorded at 600 nm. Six different concentrations of Trolox (10.3125, 20.625, 41.25, 83.5, 167 μg /ml) were used to construct the standard curve. Results were expressed as mg TE/g of extract.

2.20. Total Polyphenol Content (TPC). Total polyphenol content of four extracts was determined by the Folin-Ciocalteu spectrophotometric method adopted from Singleton and Rossi [13] by using gallic acid as standard phenolic compound using 96-well microplates. Twenty microliters of four extracts, each dissolved in distilled water (150 μg /ml), were added to 110 μl of ten times diluted freshly prepared Folin-Ciocalteu reagent and incubated with 70 μl of 10 % sodium carbonate solution at room temperature (25 ± 2°C) for 30 minutes and the absorbance was recorded at 765 nm. Five different concentrations of gallic acid (0.78, 1.562, 3.125, 6.25, 12.5, 25, and 50 mg/ml) were used to construct the standard curve. Total Polyphenol Content was expressed as mg Gallic Acid Equivalents (GAF)/g of extract.

2.21. Total Flavonoid Content of (TFC). Total flavonoid content of four samples was determined by Aluminium chloride method [14]. One hundred microliters of 2 % Aluminium chloride in methanol solution was incubated with 100 μl of four samples dissolved in methanol (120 μg/ml) at room temperature (25 ± 2°C) for 10 minutes and absorbance was recorded at 415 nm. Six different concentrations of Quercetin (1, 2, 4, 8, 16, 32 μg/ml) were used to construct the standard curve. Total Flavonoid Content was expressed as mg Quercetin Equivalents (QE)/g of extract.

2.22. Statistical Analysis. All the assays were performed four times and the absorbance was presented as Mean ± SEM. Analysis of variance was performed using SPSS procedures. The level of significance was used for comparison at 0.05 levels. SPSS t – test was used for testing significance level between in other.

3. Results and Discussion

In Ayurveda system of medicine we can identify various medicinal preparations mentioned under Bhaishajya Kalpana [15]. Decoctions (kashaya), vati (pills), powders (churna), oils (taila), and arishta-asava (fermented preparations) are few examples for them. These drug preparations can be classified into two: primary preparations and secondary preparations. Panchavidha Kashaya Kalpana is considered as primary preparations which include five types of liquid preparations that are therapeutically effective. These primary preparations are commonly used as the initial dosage forms in treatment and as the base for the different medicinal preparations.

Decoction is one of the effective dosage forms widely used in Ayurveda treatment and the shelf life of this preparation is 24 hours, which means, in the treatment, patient should prepare the decoction everyday [16]. If we are able to develop novel products from decoctions having long shelf life, that would be convenient for people. However, in order to fulfill this requirement, potency of the preparation should be same as the traditional formulation. Potency of a medicine is critical for its efficacy. When modifying the preparation to an easy to use dosage form with appropriate shelf life, active principles or phytochemicals of the drug have to be protected as the traditional preparation.

In this study qualitative phytochemical analysis was done to detect and compare the chemical constituents of TD and its modified dosage forms. Most of the phytochemicals including saponins, alkaloids, flavonoids, phenols, terpenoids, tannins, and steroids were present in all four types of preparations (Table 2). However, saponins, alkaloids, flavonoids, terpenoids, and steroids were more prominent in both traditional TD and FDF-TD than the SDF-TD and GSF-TD. Plant secondary metabolites such as phenols, flavonoids, tannins, and saponins are responsible for many activities including antioxidants, anti-inflammatory, antibacterial, antiasthmatic, immunomodulatory actions etc. [17]. Prolonged administration of saponin from *Clerodendrum serratum* plant has been reported to exhibit antihistaminic and antiallergic activity [18, 19]. *C. serratum* is one of the ingredients in TD and high content of saponins was found in both TD and FDF-TD. This factor helps to prove the effectiveness of TD and FDF-TD in the treatment of allergic rhinitis which is characterized by nasal congestion, watery nasal discharge, itching of the nose, and sneezing [20]. Therefore, the above properties of the drug could overcome the symptoms of allergic rhinitis. Further this is the first attempt taking place to screen possible phytochemicals present in TD.

TLC and HPTLC techniques are used for quality assessment in Ayurvedic preparations. These methods are widely employed in pharmaceutical industry in process of identification, development and quality control of herbal products [21]. However, HPTLC technique is more advanced than TLC and used for quantification purpose. When considering the TLC fingerprint patterns almost similar TLC profiles were observed in all four dosage forms bearing R_f values of 0.12, 0.32, 0.43, 0.59, 0.70, and 0.93 (at 245 nm). However, one additional spot was observed in TD bearing R_f value of 0.26 (at 366 nm) (Figure 1).

Table 2: Phytochemical screening of Tamalakyadi decoction and its modified dosage forms.

Phyto constituents	Test	TD	FDF-TD	SDF-TD	GSF-TD
Saponins	Frothing test	+++ High	+++ High	+ Present	++ Moderate
Tannins	$FeCl_3$ test	+++ (Blue black precipitate)	+++ (Blue black precipitate)	+++ (Blue black precipitate)	+++ (Blue black precipitate)
	Vanillin test	Negative			
	$Pb(OAc)_2$ test	+++ (Yellow precipitate)	++ (Yellow precipitate)	+++ (Yellow precipitate)	++ (Yellow precipitate)
Phenols	Vanillin test	Negative			
	$Pb(OAc)_2$ test	+++ (Yellow precipitate)	++ (Yellow precipitate)	+++ (Yellow precipitate)	++ (Yellow precipitate)
Alkaloids	Tannic acid test	++ (yellow precipitate)	++ (yellow precipitate)	+ (yellow precipitate)	++ (yellow precipitate)
	Picric acid test	+++ (yellow precipitate)	+++ (yellow precipitate)	+ (yellow precipitate)	++ (yellow precipitate)
	Wagner test	+++ (red colour)	+++ (red colour)	++ (red colour)	++ (red colour)
Flavonoids	Test (a)	+++ (Yellow colour)	+++ (Yellow colour)	++ (Yellow colour)	++ (Yellow colour)
	Test (b)	++ (Orange colour)	+++ (Orange colour)	+ (Orange colour)	++ (Orange colour)
Terpenoids	Salkowski test	+++ (reddish brown colour)	+++ (reddish brown colour)	++ (reddish brown colour)	++ (reddish brown colour)
	Sesquiterpenes test	++ (reddish brown colour)	+++ (reddish brown colour)	+ (reddish brown colour)	++ (reddish brown colour)
Steroids	Test (a)	+++ (violet colour)	+++ (violet colour)	++ (violet colour)	++ (violet colour)
	Liebermann Burchard Test	+++ (Dark bluish green colour)	+++ (Dark bluish green colour)	++ (Dark bluish green colour)	++ (Dark bluish green colour)
Cardiac glycosides		Reddish brown ring formed	Reddish brown ring formed	Reddish brown ring formed	Reddish brown ring formed

-ve: negative, +: positive in low level, ++: positive in moderate level, +++: positive in high level.
TD: Tamalakyadi Decoction, *FDF-TD*: Freeze Dried Form of Tamalakyadi Decoction, *SDF-TD*: Spray Dried Form of Tamalakyadi Decoction, *GSF-TD*: Ganasara Form of Tamalakyadi Decoction.

HPTLC study was carried out to compare the area and intensity of the spots appeared in TLC profiles of four preparations. HPTLC fingerprint pattern of TD was similar to that of FDF-TD in terms of number of peaks and their intensity compared to that of SDF-TD and GSF-TD (Figure 2). This may be due to the temperature and time which affect chemical constituents of plant materials during drug preparation. Decomposition of chemical constituents or change in chemical structure or reduction of chemical constituents occurred when increasing the temperature and time [22–26]. TD is the traditional preparation and all the modified dosage forms are made out of it. Therefore, FDF-TD, SDF-TD, and GSF-TD initially subjected to 105°C. However, FDF-TD will not be exposed more than 105°C as we used the freeze drying process while SDF-TD will be exposed to 180°C during the preparation of modifies dosage form. When preparation of GSF-TD will not be exposed more than 105°C but it has to keep prolong time in 105°C. Therefore, heat labile compound/s in both SDF-TD and GSF-TD may be decomposed during the preparation of modified dosage forms. This may be the reason that the chemical profile of FDF-TD was similar to that of TD. In contrast, research

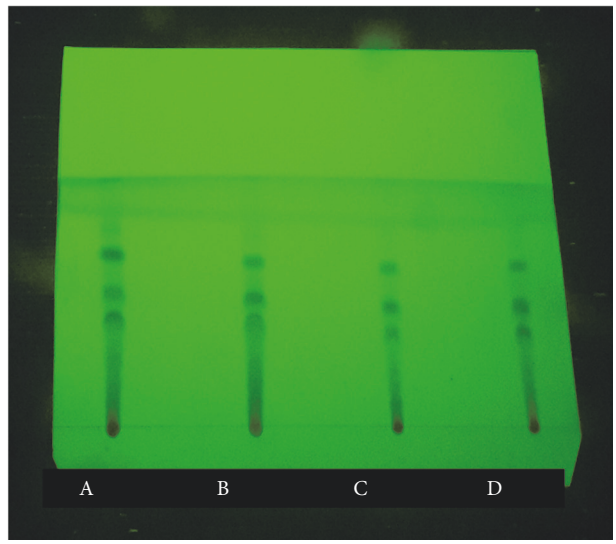


Figure 1: TLC fingerprint profiles of Tamalakyadi Decoction and its modified dosage forms. A: Tamalakyadi decoction (TD), B: Freeze Dried Form of Tamalakyadi Decoction (FDF-TD), C: Spray Dried Form of Tamalakyadi Decoction (SDF-TD), D: Ganasara Form of Tamalakyadi Decoction (GSF-TD).

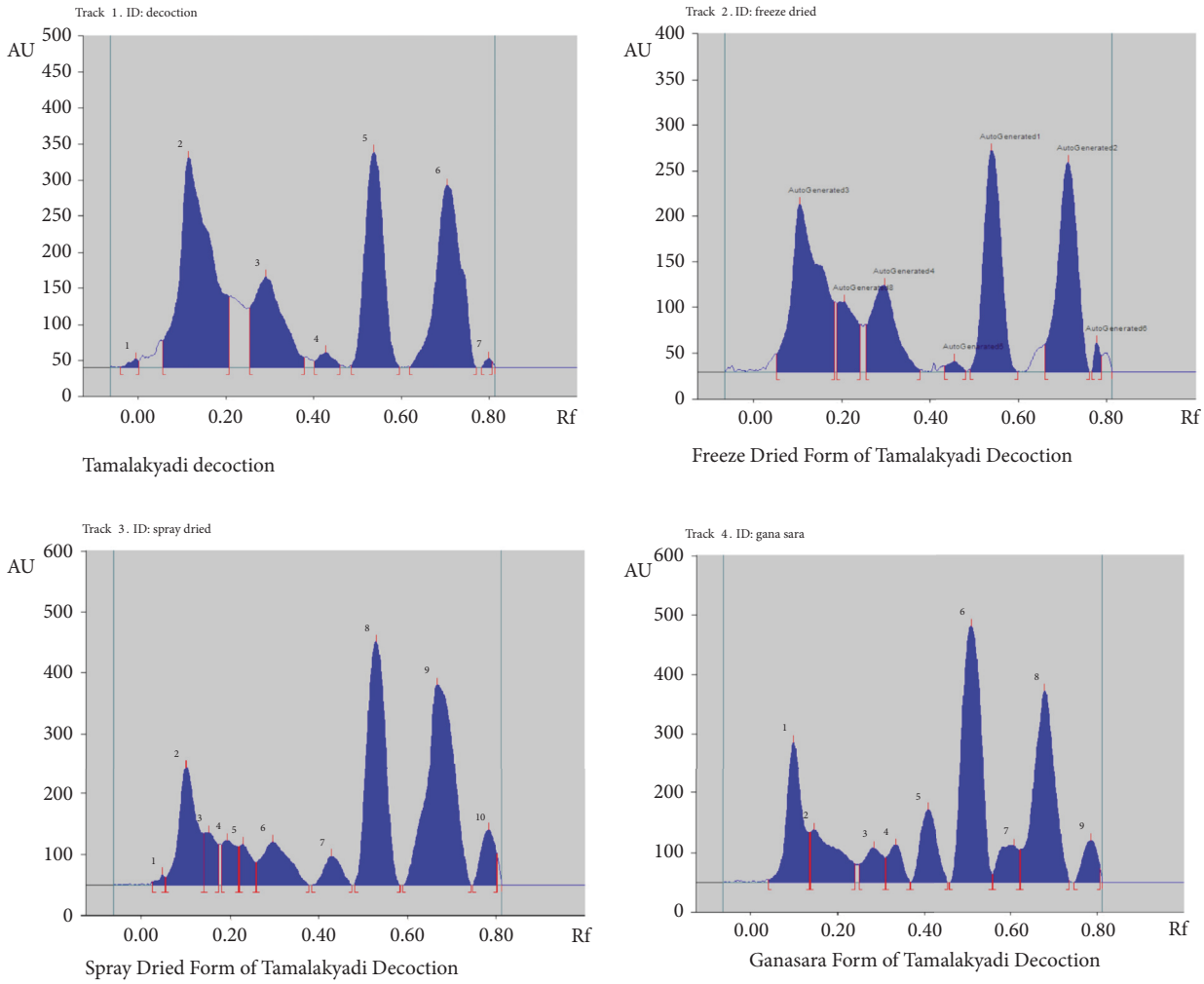


Figure 2: HPTLC fingerprint profiles of Tamalakyadi decoction and its modified dosage forms.

Table 3: Antioxidant activities of Tamalakyadi decoction and its modified dosage forms.

Extract	DPPH free radical scavenging activity (IC_{50}; μg/mL)	FRAP (mg TE/g of extract)	TPC (mg GAE/g of extract)	TFC (mg QE/g of extract)
Tamalakyadi Decoction	8.2 ± 0.1^{a}	572.5 ± 2.3^{aa}	206.0 ± 2.3^{aa1}	8.2 ± 0.5^{aa2}
Freeze Dried Form of Tamalakyadi Decoction	10.6 ± 0.3^{b}	634.2 ± 1.3^{bb}	148.2 ± 0.7^{bb1}	6.2 ± 0.3^{bb2}
Spray Dried Form of Tamalakyadi Decoction	20.8 ± 0.3^{c}	154.8 ± 1.9^{cc}	63.8 ± 2.0^{cc1}	2.9 ± 0.1^{cc2}
Ganasara Form of Tamalakyadi Decoction	17.9 ± 0.6^{d}	222.4 ± 1.0^{dd}	69.5 ± 0.4^{dd1}	2.9 ± 0.1^{dd2}
Trolox (standard)	5.35 ± 0.25^{e}	-	-	-

Results are presented as Mean±SEM (n=4).
Values with the different scripts are significantly different P≤ 0.05 from each other.
TE: Trolox Equivalents, *GAE*: Gallic Acid Equivalents, *QE*: Quercetin Equivalents.

findings of Singh and coworkers [27] showed that spray dried form of Lodhradi Kashaya was chemically similar to that of conventional dosage form which was prepared according to the classical method mentioned in Sharangadhara Samhita. Different temperatures used in spray drying in different studies may have accounted for this. The freeze dried dosage form is exposed to low temperature (- 40°C to 40°C) which may cause less damage to phytoconstituents. Research reports revealed, when compared to air drying/oven drying methods, freeze drying improved the retention of phytochemicals during processing and in some cases it even increased the concentration of phytochemicals blue berry and raspberry [28, 29].

Antioxidants are compounds that inhibit or delay onset of oxidation and may be classified as natural or synthetic [30]. There is an increasing demand for natural antioxidants for curing and prevention of diseases indicating that compounds in natural formulations are more active than their isolated form [31]. During the recent years many changes have occurred in the management of allergic rhinitis by using medicines in various traditional medicinal systems [32, 33]. A number of scientific investigations have proven the association between antioxidants and allergic diseases and antioxidant intake seems to have a protective effect on allergic diseases like rhinitis [34–36]. Hence in this study we had examined the antioxidant activities of four preparations to detect most similar antioxidant activity with the TD (Table 3). TD exhibited highest antioxidant activity in terms of capability of scavenging DPPH radicals, ferric reducing antioxidant power, and total phenolic and flavonoid contents (Table 3). Further, among the modified dosage forms, FDF-TD showed the best antioxidant activity and more closer to that of TD. The reason for the reduction of antioxidant activities of SDF-TD and GSF-TD may be due to the temperature used in the drug processing. Recent research findings highlighted that the temperature had an influence on antioxidant activities of the plant materials and degradation of heat sensitive compounds reported to be minimized at the low temperature [37]. Further, the temperature had an effect on total flavonoids and phenolic contents of the plant which have major contribution to the radical scavenging activity [38–40]. Among the four preparations, total flavonoids and phenolic contents are high in TD and therefore it indicated more DPPH radical scavenging activity than the three modified preparations. Also antioxidant assay results showed that the FRAP value is highest in FDF-TD. Similar results were observed in freeze dried samples of leaves and berries of *Cayratia trifolia* [41] and spearmint leaves [42]. This may be due to the formation of ice crystals within the tissue matrix during the freeze drying process which can rupture the cell structure, which allows the exit of cellular components and the access of solvent [43]. Hence it can perform more antioxidant potency in the media.

4. Conclusion

Phytochemical studies, HPTLC patterns, and antioxidant studies showed that the FDF- TD is more similar to TD which was prepared according to traditional method. Therefore, FDF-TD can be used as a novel dosage form to treat allergic rhinitis. However, clinical evaluation is needed for further confirmation.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This project was funded by the University Grant Commission, Sri Lanka (UGC/VCDRIC/PG2016(II)/IIM/02, 02.02.2017).

References

[1] A. Kumarasinghe, *Vaidyaka Sarasankshepaya of Rajaguru Sri Chandra*, Department of Ayurveda, Sri Lanka, 1984.

[2] D. P. Skoner, "Allergic rhinitis: Definition, epidemiology, pathophysiology, detection, and diagnosis," *The Journal of Allergy and Clinical Immunology*, vol. 108, no. 1, pp. S2–S8, 2001.

[3] H. A. Sampson, "Epidemiology of allergic rhinitis throughout the world," in *Global atlas of allergic rhinitis and chronic rhino sinusitis*, pp. 62-63, European academy of allergy and clinical immunology, 2015.

[4] P. Small, P. K. Keith, and H. Kim, "Allergic rhinitis," *Allergy, Asthma & Clinical Immunology*, vol. 14, no. S2, pp. 31–41, 2018.

[5] S. Tripathi, *Yogarathnakara*, Chaukhambha Ayurveda Series, India, 1998.

[6] A. Verma, H. Janani, S. Yadav, Galib., and P. K. Prajapati, "Pharmaceutical and analytical studies on guduchi kwatha prepared by varying proportions of water," *Journal of Ayurvedic and Herbal Medicine*, vol. 2, no. 4, pp. 125–130, 2016.

[7] "Ayurveda Aushadha Sangrahaya," Department of Ayurveda, Sri Lanka, 1976.

[8] V. K. Singh and K. R. C. Reddy, "Standardization of modified dosage form of Lodhradi Kashaya prepared by two different techniques," *Asian Journal of Pharmaceutics*, vol. 10, no. 2, pp. S134–S138, 2016.

[9] S. W. Goveas and A. Abraham, "Extraction and secondary metabolite analysis of coscinium fenestratum (Gaertn.) Colebr: an important medicinal plant of western ghats," *International Journal of Pharmaceutical Sciences and Research*, vol. 5, no. 8, pp. 3484–3489, 2014.

[10] T. J. Joanne, H. A. Ashwini, and K. G. Singh, "Qualitative and quantitative phytochemical analysis of ethnomedical folklore plant Clerodendrum colebrooianum," *Journal of Global Biosciences*, vol. 5, no. 1, pp. 3559–3566, 2016.

[11] M. S. Blois, "Antioxidant determination by use of stable free radical," *Nature*, vol. 181, no. 4617, pp. 1199-1200, 1958.

[12] I. F. F. Benzie and Y. T. Szeto, "Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 2, pp. 633–636, 1999.

[13] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventós, "Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent," *Methods in Enzymology*, vol. 299, pp. 152–178, 1999.

[14] A. Meda, C. E. Lamien, M. Romito, J. Millogo, and O. G. Nacoulma, "Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity," *Food Chemistry*, vol. 91, no. 3, pp. 571–577, 2005.

[15] B. T. Sharangdhar, *Sharangadhar Samhita*, Chaukhambha Surbharati Prakashan, Varanasi, 2010.

[16] K. R. C. Reddy, *Ocean of Ayurvedic Pharmaceutics*, Chaukhambha Sanskrit, Bhawan, India, 2007.

[17] D. Lin, M. Xiao, J. Zhao et al., "An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes," *Molecules*, vol. 21, no. 10, article 1374, 2016.

[18] S. S. Gupta, "Development of antihistamine and anti-allergic activity after prolonged administration of a plant saponin from Clerodendron serratum," *Journal of Pharmacy and Pharmacology*, vol. 20, no. 10, pp. 801-802, 1968.

[19] L. C. Mishra, "Allergic reactions," in *Scientific Basis for Ayurvedic Therapies*, pp. 203–207, CRC Press, USA, 2004.

[20] B. Walker, N. R. Colledge, S. Ralston, and I. Penman, *Davidson's Principles and Practice of Medicine*, Churchill Livingstone, New York, NY, USA, 22nd edition, 2014.

[21] K. Soni and T. Navad, "HPTLC-its application in herbal drug industry," *The Pharma Review*, vol. 4, pp. 112–117, 2010.

[22] F. Namjooyan, H. Hejazi, and Z. Ramezani, "Evaluation of drying process on the composition of black pepper ethanolic extract by high performance liquid chromatography with diode array detector," *Jundishapur Journal of Natural Pharmaceutical Products*, vol. 7, no. 4, pp. 163–167, 2012.

[23] H. Mahanom, A. H. Azizah, and M. H. Dzulkifly, "Effect of different drying methods on concentrations of several phytochemicals in herbal preparation of 8 medicinal plants leaves," *Malaysian Journal of Nutrition*, vol. 5, pp. 47–54, 1999.

[24] G. Rajkumar, S. Shanmugam, M. D. S. Galvâo et al., "Comparative evaluation of physical properties and volatiles profile of cabbages subjected to hot air and freeze drying," *LWT- Food Science and Technology*, vol. 80, pp. 501–509, 2017.

[25] Y. Li, Y. Hong, Y. Han, Y. Wang, and L. Xia, "Chemical characterization and antioxidant activities comparison in fresh, dried, stir-frying and carbonized ginger," *Journal of Chromatography B*, vol. 1011, pp. 223–232, 2016.

[26] A. Schieber, P. Keller, and R. Carle, "Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography," *Journal of Chromatography A*, vol. 910, no. 2, pp. 265–273, 2001.

[27] V. K. Singh and K. R. C. Reddy, "Validation of Lodhradi Kashaya (Ayurvedic formulation) made by traditional method and contemporary spray drier method through FTIR spectroscopy," *International Journal of Pharmacy and Analytical Research*, vol. 4, no. 3, pp. 386–390, 2015.

[28] S. S. Sablani, P. K. Andrews, N. M. Davies, T. Walters, H. Saez, and L. Bastarrachea, "Effects of Air drying and freeze drying on phytochemical content of conventional and organic berries," *Drying Technology*, vol. 29, no. 2, pp. 205–216, 2011.

[29] D. Bernard, A. Kwabena, O. Osei, G. Daniel, S. Elom, and A. Sandra, "The effect of different drying methods on the phytochemicals and radical scavenging activity of Ceylon cinnamon (*Cinnamomum zeylanicum*) plant parts," *European Journal of Medicinal Plants*, vol. 4, no. 11, pp. 1324–1335, 2014.

[30] F. Shahidi and Y. Zhong, "Novel antioxidants in food quality preservation and health promotion," *European Journal of Lipid Science and Technology*, vol. 112, no. 9, pp. 930–940, 2010.

[31] S. Alok, S. K. Jain, A. Verma, M. Kumar, A. Mahor, and M. Sabharwal, "Herbal antioxidant in clinical practice: A review," *Asian Pacific Journal of Tropical Biomedicine*, vol. 4, no. 1, pp. 78–84, 2014.

[32] J. A. Lee, S. Jang, J. H. Jun et al., "Herbal medicine (Bojungikki – tang) for allergic rhinitis. A protocol for systematic review of controlled trials," *Medicine*, vol. 97, no. 3, Article ID e9551, 2018.

[33] X. Zhang, F. Lan, Y. Zhang, and L. Zhang, "Chinese herbal medicine to treat allergic rhinitis: Evidence from a meta-analysis," *Allergy, Asthma & Immunology Research*, vol. 10, no. 1, pp. 34–42, 2018.

[34] C. Cingi, N. Bayar Muluk, and G. K. Scadding, "Will every child have allergic rhinitis soon?" *International Journal of Pediatric Otorhinolaryngology*, vol. 118, pp. 53–58, 2019.

[35] J.-H. Seo, S.-O. Kwon, S.-Y. Lee et al., "Association of antioxidants with allergic rhinitis in children from Seoul," *Allergy, Asthma & Immunology Research*, vol. 5, no. 2, pp. 81–87, 2013.

[36] S. Patel, C. S. Murray, A. Woodcock, A. Simpson, and A. Custovic, "Dietary antioxidant intake, allergic sensitization and allergic diseases in young children," *Allergy: European Journal of Allergy and Clinical Immunology*, vol. 64, no. 12, pp. 1766–1772, 2009.

[37] S. Chou and K. Chua, "New hybrid drying technologies for heat sensitive foodstuffs," *Trends in Food Science & Technology*, vol. 12, no. 10, pp. 359–369, 2001.

[38] A. Wojdyło, J. Oszmiański, and R. Czemerys, "Antioxidant activity and phenolic compounds in 32 selected herbs," *Food Chemistry*, vol. 105, no. 3, pp. 940–949, 2007.

[39] Y. Cheng, Q. Xu, J. Liu, C. Zhao, F. Xue, and Y. Zhao, "Decomposition of five phenolic compounds in high temperature water," *Journal of the Brazilian Chemical Society*, vol. 25, no. 11, pp. 2102–2107, 2014.

[40] K. Sharma, E. Y. Ko, A. D. Assefa et al., "Temperature-dependent studies on the total phenolics, flavonoids, antioxidant activities, and sugar content in six onion varieties," *Journal of Food and Drug Analysis*, vol. 23, no. 2, pp. 243–252, 2015.

[41] M. S. Rabeta and S. P. Lin, "Effects of different drying methods on the antioxidant activities of leaves and berries of Cayratia trifolia," *Sains Malaysiana*, vol. 44, no. 2, pp. 275–280, 2015.

[42] A. Orphanides, V. Goulas, and V. Gekas, "Effect of drying method on the phenolic content and antioxidant capacity of spearmint," *Czech Journal of Food Sciences*, vol. 31, no. 5, pp. 509–513, 2013.

[43] V. Goulas and G. A. Manganaris, "The effect of postharvest ripening on strawberry bioactive composition and antioxidant potential," *Journal of the Science of Food and Agriculture*, vol. 91, no. 10, pp. 1907–1914, 2011.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 6021209, 23 pages
https://doi.org/10.1155/2019/6021209

Review Article

Phytomedicines Used for Diabetes Mellitus in Ghana: A Systematic Search and Review of Preclinical and Clinical Evidence

Michael Buenor Adinortey,[1] **Rosemary Agbeko**,[2] **Daniel Boison**,[1] **William Ekloh**,[1,3] **Lydia Enyonam Kuatsienu**,[4] **Emmanuel Ekow Biney**,[1] **Obed O. Affum**,[1] **Jeffery Kwarteng**,[1] **and Alexander Kwadwo Nyarko**[5]

[1]*Department of Biochemistry, School of Biological Sciences, University of Cape Coast, Cape Coast, Ghana*
[2]*Department of Molecular Biology and Biotechnology, School of Biological Sciences, University of Cape Coast, Cape Coast, Ghana*
[3]*West Africa Centre for Cell Biology of Infectious Disease and Pathogens, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, Ghana*
[4]*Department of Pharmacology, School of Pharmacy, Princefield University College, Ho, Ghana*
[5]*Department of Pharmacology and Toxicology, School of Pharmacy, University of Ghana, Legon, Ghana*

Correspondence should be addressed to Michael Buenor Adinortey; madinortey@ucc.edu.gh

Received 22 January 2019; Accepted 19 March 2019; Published 18 April 2019

Guest Editor: José C. T. Carvalho

Background. Available data indicate that diabetes mellitus leads to elevated cost of healthcare. This imposes a huge economic burden on households, societies, and nations. As a result many Ghanaians, especially rural folks, resort to the use of phytomedicine, which is relatively less expensive. This paper aims at obtaining information on plants used in Ghana to treat diabetes mellitus, gather and present evidence-based data available to support their uses and their mechanisms of action, and identify areas for future research. *Method*. A catalogue of published textbooks, monographs, theses, and peer-reviewed articles of plants used in Ghanaian traditional medicine between 1987 and July 2018 for managing diabetes mellitus was obtained and used. *Results*. The review identified 76 plant species belonging to 45 families that are used to manage diabetes mellitus. Leaves were the part of the plants frequently used for most preparation (63.8%) and were mostly used as decoctions. Majority of the plants belonged to the Euphorbiaceae, Lamiaceae, Asteraceae, and Apocynaceae families. Pharmacological data were available on 23 species that have undergone *in vitro* studies. Forty species have been studied using *in vivo* animal models. Only twelve plants and their bioactive compounds were found with data on both preclinical and clinical studies. The records further indicate that medicinal plants showing antidiabetic effects did so via biochemical mechanisms such as restitution of pancreatic β-cell function, improvement in insulin sensitivity by receptors, stimulating rate of insulin secretion, inhibition of liver gluconeogenesis, enhanced glucose absorption, and inhibition of G-6-Pase, α-amylase, and α-glucosidase activities. *Conclusion*. This review contains information on medicinal plants used to manage diabetes mellitus, including their pharmacological properties and mechanisms of action as well as models used to investigate them. It also provides gaps that can form the basis for further investigations and development into useful medications for effective treatment of diabetes mellitus.

1. Introduction

Diabetes mellitus is a metabolic and/or hormonal condition that is usually described by persistent hyperglycemia, as a result of defects in insulin secretion by pancreatic β-cells, and reduced sensitivity of cell surface receptors to insulin or both [1]. There are four main types of diabetes mellitus: type-1, type-2, gestational diabetes mellitus, and "other specific types of diabetes mellitus" [2]. Inadequate management or uncontrolled hyperglycemia manifests into signs and symptoms that can also be referred to as acute complications. When these signs and symptoms are overlooked or not detected

early, they lead to the development of chronic complications such as hypertension, stroke, blindness, erectile dysfunction, and kidney malfunction [3].

This metabolic disorder, which is on the ascendancy all over the world, is a progressive one that is found among all age groups. The prevalence of diabetes mellitus is estimated to rise to 592 million by the year 2035 [4]. Whiting and colleagues in 2011 [5] also reported a prevalence rate of the disease in Ghana to be 4.1 % in 2011 and projected a rate of 5.0 % by 2030 to be one of the highest in the West African subregion. According to the International Diabetes Federation, diabetes is one of the highest causes of mortality in low- and middle-income countries [5]. Peer and colleagues reported in 2014 that noncommunicable diseases would outdo infectious diseases as the foremost cause of death in Africa in the next 20 years [6]. This is alarming and more attention needs to be channeled towards diabetes mellitus and its complications. The high morbidity and mortality rate seen in this condition stems from factors such as rapid rise in unhealthy lifestyles in diet and lack of exercise, urbanization, and aging [7].

Management of this chronic disease involves the use of pharmacotherapy, exercise, and dietary therapy. Different classes of antidiabetic pharmacotherapeutic agents have been discovered and their selection for use in management depends on the type of diabetes mellitus, age of individual, response of the person, and other factors. Generally, pharmacotherapy used includes (i) drugs that stimulate or facilitate the release of insulin from the pancreatic β-islet cells, (ii) those that increase the sensitivity of receptors to insulin or reduce insulin resistance, (iii) those that reduce the rate at which glucose is absorbed, and (iv) those that inhibit protein glycation.

Currently, the different classes of orthodox drugs used to manage diabetes mellitus include insulin, biguanides, sulfonylureas, inhibitors of α-glucosidase and α-amylase, aldose reductase inhibitors, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, carbamoylmethyl benzoic acid and insulin-like growth factor, Selective sodium-glucose cotransporter-2 (SGLT-2) inhibitors, glucagon-like peptide-1 receptor agonists, and amylin analogues [8]. A brief narrative of the classes of antihyperglycemic drugs with examples is as follows:

Insulin: (Several generics).

Sulfonylureas: Examples include Glutril, Tolbutamide, Glibenclamide, Gliclazide, Glibenese, Glurenorm, and Glimepiride.

Biguanide: Examples include Phenformin and Dimethylbiguanide.

α-Glucosidase inhibitors: Examples include Acarbose, Voglibose, Miglitol, Emiglitate, and Precose.

Aldose reductase inhibitor: Tolrestat, Epslstat, Alredase, Kinedak, Imirestat, Opolrestat, etc.

Thiazolidinediones: Examples include Rosiglitazone, Troglitazone, Englitazone, and Pioglitazone.

Carbamoylmethyl benzoic acid: Repaglinide.

Insulin-like growth factor: IGF-1.

Glucagon-like peptide-1 receptor agonists: Liraglutide.

Amylin analogues: Pramlintide.

Selective sodium-glucose cotransporter-2 (SGLT-2) inhibitors: remogliflozin, etabonate (known as 189075; GSK), and Sergliflozin.

Dipeptidyl peptidase-4 (DPP-4) inhibitors: Sitagliptin and vildagliptin.

Most of these orthodox drugs used are either bedeviled with many side effects such as hypoglycemia, weakness, diarrhea, shortness of breath, fatigue, nausea, dizziness, lactic acidosis, weight gain, increase in LDL-cholesterol levels, hepatotoxicity and kidney toxicity, and lactic acid intoxication or are relatively expensive [9, 10]. The high cost of managing diabetes mellitus compels many Ghanaians to patronize herbal medicine that is less expensive. This calls for intensive research to provide needed information, including the efficacy and safety of these medicinal plants. Managing diabetes mellitus using herbal remedies is not uncommon in Ghanaian rural and urban communities [11]. According to WHO, the number of people that choose traditional herbal medicines in most African countries is driven by amalgamation of factors which include economic hardships, difficult geographical approachability of the populace to conventional antidiabetics, inadequacy of healthcare systems, ease of accessibility of herbal medicine, and indigenous knowledge of the people in addition to the role of traditional healers [12].

Ghana is endowed with a rich floral diversity and likewise rich plant ethnomedicinal tradition. Several herbal preparations have been used in folklore for the management of diabetes mellitus that are purported to possess hypoglycemic effect. This assertion has heightened the interest in plant medicines as alternatives for orthodox medicines. Despite the progress made in the development of plant-based antihyperglycemic agents by many countries [13], Ghana is yet to fully harness its plant biodiversity for this purpose. Though Ghana has a rich history of herbal medicine usage, pharmacological efficacy data on these plants are highly fragmented, which underscores the need for compilation of evidence-based data on the subject. Proper documentation of these traditional medicinal plants used in managing diabetes mellitus constitutes an important task.

This review aimed to compile ethnopharmacological data on medicinal plants found in Ghana and brings together findings available on their bioactive compounds, efficacy, safety, and clinical trials. Information gathered is expected to assist in preserving indigenous knowledge and biodiversity and enhance awareness on medicinal plants and consequently access to information on medicinal plants to serve as a resource to facilitate the development of new and standardized herbal-based drugs. The availability of one-stop data on antihyperglycemic plants in Ghana is crucial for identifying gaps in knowledge and stimulating research that could lead to identification of lead compounds. This piece thus focuses on medicinal plants used in Ghana to manage diabetes mellitus and synthesizes research findings on the bioactive phytoconstituents and efficacy of medicinal plants concerned.

Table 1: Summary of studies included in this review.

Characteristics of paper used	Number of articles	source
Ethnomedicinal report	11	Table 2
In vitro mechanism	26	Table 3
In vivo mechanism	53	Table 4
Clinical studies	12	Table 5
Bioactive compounds with anti-diabetic activity	12	Table 6

One study can fall into more than one grouping.

2. Method and Literature Search Strategy

A catalogue of textbooks, monographs, published theses, and published peer-reviewed articles of plants used in Ghanaian traditional medicine was sourced [14–16, 19, 21, 21, 23, 24, 26, 26]. Electronic databases, namely, ScienceDirect, Scopus, PubMed, Springer Nature, Web of Science, and Google Scholar were used to gather information. English language articles were the sole source of information for this review. The key terms that were used in the search were "Antidiabetic", "Hypoglycemic effect", "Ethnomedicine", "Traditional medicine", and "Ghana". All retrieved articles were reviewed to obtain the needed information on Ghanaian antidiabetic medicinal plants. For each plant material identified to be used to treat diabetes mellitus in Ghana, peer-reviewed or published theses between 1987 and July 2018 were scrutinized of which the active components attributed/reported to possess antidiabetic effect were considered. Synthesized or isolated active compounds of respective plants that have been used to carry out antidiabetic studies with significant success were considered. In addition, plants with information on preclinical and clinical studies were the ones that were expatiated on. All articles accessed were evaluated for information on methods employed by investigators in their studies such as preclinical *in vitro*, *in vivo* (rodents and human) data or clinical trials and mechanism of action. Plants that did not show any marked antidiabetic effects experimentally were not included in the study. Bibliographies of ultimately used articles were appraised for other relevant information to the type of plant extract, scientific names, plant part used, active principles, category of diabetes mellitus, and disease animal model. Only peer-reviewed or published theses were used as sources for this piece. A summary of the main sources consulted is shown in Table 1.

3. Results

3.1. Overview of Characteristics of Studies Included in This Review. A summary of source of materials used for the review process is shown in Table 1. A major barrier to understanding the diversity and uses of medicinal plants in Ghana has been the lack of research and available data on these plants. In this review, efforts have been made to gather information regarding herbs used to manage diabetes in Ghana. About ten identified plants with data on preclinical and clinical trials met inclusion criteria and have been discussed. Information gathered is summarized in Tables 2–6. Table 2 presents information on reported plants used in Ghana for the management of diabetes mellitus. Tables 3–5 depict data for *in vitro*, *in vivo*, and clinical trials for medicinal plants used in managing diabetes mellitus. Table 6 provides information on plants with their corresponding bioactive ingredients responsible for the hypoglycemic effect. Figure 1 shows chemical structures of bioactive compounds isolated from plants experimentally shown to possess antidiabetic property. Also evidence-based data relating to *in vitro*, animal, and human studies aside from their bioactive compounds have been described in this piece.

3.2. Preclinical and Clinical Data on Plants with Antidiabetic Effects. Phytomedicines have been used for the management of different ailments, and many populations in the world depend entirely on plants medicines for their healthcare needs such as management of diabetes mellitus. From the ethnobotanical studies, many plants found to possess antidiabetic activities were used for dietary purposes with no comprehension of their proper functions and active principles. This practice may have continued due to their fewer side effects compared to orthodox drugs.

Although many orthodox synthetic drugs have been developed to manage diabetes mellitus, few of them are available for use in managing diabetes mellitus. Over 200 pure compounds from plants shown to possess antihyperglycemic effects [118] in different classes of natural products such as flavonoids and alkaloids are available.

This report presents information on plants that possess antidiabetic properties from which bioactive compounds have been isolated and tested. The families of plants showing some level of potency with regard to their hypoglycemic effects include Passifloraceae, Liliaceae, Asphodelaceae, Meliaceae, Cucurbitaceae, Fabaceae, Lauraceae, Costaceae, Anacardiaceae, Scrophulariaceae, and Zingiberaceae.

Allium cepa. *Allium cepa*, commonly known as onion, is a plant grown in Ghana. The bulb and leaves, which are used for cooking, possess nutritional and medicinal benefits [18]. It serves as a rich source of protein, fibre, fat, folic acid, sodium, vitamin C, vitamin B_6, and many other micronutrients [119]. The health benefits of onion include management of a number of diseases including diabetes mellitus. Jung and colleagues in a study involving streptozotocin-induced diabetic rats showed that onion peel extract improves glucose control and insulin resistance associated with type-2-diabetes mellitus [120]. Additionally, research work carried out by Ojieh and colleagues [56] demonstrated the hypoglycemic

Table 2: Ethnomedicinal reports of plants used in Ghana for managing diabetes mellitus.

Scientific name	Family	Common name	Plant part	Preparation	Reference
Abelmoschus esculentus	Malvaceae	Okra	Fruit	Decoction	[14]
Adenia lobata Engl	Passifloraceae	Snake Rope	Stem	Decoction	[15]
Ageratum conyzoides L	Asteraceae	Goat weed	Whole plant/Leaf	Decoction	[16–18]
Allium cepa L.	Amaryllidaceae	Onion	Bulb	Mastication	[17, 18]
Allium sativum L.	Liliaceae	Garlic	Bulb	Mastication	[17, 19]
Acalypha wilkesiana	Euphorbiaceae	Copper leaf	Leaf	Decoction	[19]
Aloe barbadensis	Asphodelaceae	Aloe vera	Leaf	Decoction	[14]
Alstonia boonei	Apocynaceae	Stool wood	Leaf, Stem bark	Tincture	[14, 20]
Amaranthus viridis L.	Amaranthaceae	Green amaranth	Leaf	Decoction	[16]
Anogeissus leiocarpus	Combretaceae	African birch	Leaf, Stem bark	Decoction	[18]
Annona muricata L.	Annonaceae	soursop	leaf	Decoction	[21]
Azadirachta indica A. Juss	Meliaceae	Indian Lilac tree	Leaf	Decoction	[17, 21]
Bauhinia rufescens Lam.	Fabaceae	Silver butterfly tree	Leaf	Decoction	[16]
Bridelia ferruginea Benth	Euphorbiaceae	Bridelia	Leaf	Decoction	[17, 18]
Boerhavia diffusa L	Nyctaginaceae	spreading hogweed	whole plant	Decoction	[21]
Bombax buonopozense	Bombacaceae	Gold coast Bombax	Leaf	Infusion	[14],
Carica papaya L.	Caricaceae	Pawpaw	Leaf	Decoction	[18, 21]
Cassia siamea	Caesalpiniaceae	Cassia tree	Leaf	Decoction	[17]
Cassia auriculata L.	Fabaceae	Tanner's cassia	Flowers, Root, Seed	Decoction	[16]
Catharanthus roseus (L.)	G. Don Apocynaceae	Madagascar periwinkle	Leaf	Powder	[16]
Cinnamomum zeylanicum	Lauraceae	Cinnamon	Bark	Mastication	[17]
Clausena anisata	Rutaceae	Clausena	Root	Decoction	[17]
Costus afer Ker-Gawl	Costaceae	Bush cane	Whole plant	Decoction	[22]
Costus schlechteri	Costaceae	Hairy ginger lily	Whole plant	Decoction	[23]
Cyperus esculentus	Cyperaceae	Tiger nut	Fruit	Mastication	[22]
Ehretia cymosa	Boraginaceae	Ehretia Cymosa	Leaf	Decoction	[24]
Emilia coccinea	Asteraceae	Emelia	Entire plant	Decoction	[19]
Euphorbia hirta L.	Phyllanthaceae	asthma plant	Leaf	Decoction	[21]
Euphorbia prostrate Aiton	Euphorbiaceae	Prostrate sandmat	Whole plant	Decoction	[16]
Fleurya ovalifolium	Moraceae	Sand paper leaf	Stinging nettle	Decoction	[19]
Garcinia afzelii	Guttiferae	Bitter cola	Leaf	Decoction	[18]
Glyphaea brevis	Tiliaceae	Masquerade stick	Leaf	Decoction	[18]
Gongronema latifolium	Asclepiadaceae	Bush buck	Leaf	Decoction	[16]
Guiera senegalensis	Combretaceae	Moshi medicine	Leaf	Decoction	[18]
Harungana madagascariensis	Hypericaceae	Dragon's blood tree	Stem bark	Decoction	[18]
Hoslundia opposita	Lamiaceae	Orange bird berry	Root	Decoction	[18]
Hyptis suaveolens (L.) Poit	Lamiaceae	Pignut	Leaf	Decoction	[16]
Indigofera arrecta	Papilionoideae	African indigo	Leaf	Decoction	[18]
Ipomoea sepiaria Roxb.	Convolvulaceae	Purple Heart Glory	Leaf	Decoction	[16]
Jatropha curcas	Euphorbiaceae	Barbados	Leaf	Decoction	[18]
Khaya senegalensis	Meliaceae	African mahogany	Stem bark	Decoction	[17]
Kigelia Africana (Lam) Benth	Bignoniaceae	Sausage Tree	leaf, stem bark, fruit and roots	Decoction	[19]
Launaea taraxacifolia	Asteraceae	African Lettuce	Leaf	Decoction	[14, 18, 21]

Table 2: Continued.

Scientific name	Family	Common name	Plant part	Preparation	Reference
Lagerstroemia speciosa	Lythraceae	Giant crepe-myrtle	Leaf	Decoction	[19]
Mangifera indica L.	Anacardiaceae	Mango	Leaf Stem bark	Decoction	[18, 21]
Mimosa pudica L.	Fabaceae	Touch- Me-Not	Leaf	Tincture	[16]
Mollugo nudicaulis Lamk.	Molluginaceae	Naked- stem carpetweed	Whole plant	Decoction	[16]
Mitragyna inermis O. Kuntze	Rubiaceae	Not known	leaf	Decoction	[21]
Morinda citrifolia L.	Rubiaceae	Noni	Fruit	Decoction	[21]
Morinda lucida	Rubiaceae	Brimstone tree	Root	Decoction	[18]
Moringa oleifera	Moringaceae	Moringa	Leaf	Decoction	[17]
Momordica charantia	Cucurbitaceae	bitter gourd	Whole plant	Infusion	[14, 17, 18, 21]
Myrianthus arboreus P. Beauv	Urticaceae	Monkey fruit	Stem bark	Decoction	[23]
Newbouldia laevis	Bignoniaceae	Sesemasa	Leaf	Decoction	[19]
Ocimum canum Sim	Lamiaceae	Basil	Leaf	Decoction	[23]
Ocimum gratissimum	Lamiaceae	clove basil	leaf	Decoction	[23, 25]
Phyllanthus amarus Schum.	Euphorbiaceae	Stonebreaker or seed-under-leaf	Leaf	Decoction	[16, 17]
Paullinia pinnata Griseb	Sapindaceae	Tietie	Leaves	Decoction	[21]
Phyllanthus fraternus	Euphorbiaceae	Quinine weed	Leaves	Decoction	[17]
Rauvolfia vomitoria	Apocynaceae	poison devil's-pepper	Leaf	Decoction	[19]
Scoparia dulcis	Scrophulariaceae	Sweet broom	Dried leaves	Decoction	[17, 18]
Securinega virosa	Euphorbiaceae	Snow berry tree	Leaves	Decoction	[18]
Senna siamea (Lam) H.S.	Fabaceae	Yellow cassia	Leaves Root	Infusion decoction	[17, 21]
Senna occidentalis	Fabaceae	Coffee weed	Stem bark, Leaves	Infusion	[16, 19, 26]
Sida acuta Burm. f	Malvaceae	broomweed	Leaves	Decoction	[16, 18]
Sesamum indicum L.	Pedaliaceae	Sesame	seed	powder	[21]
Solanum torvum	Solanaceae	Turkey berry	fruit	Decoction	[19]
Saccharum officinarum L.	Poaceae	sugar cane	stem	Decoction	[21]
Stachytarpheta indica	Verbenaceae	Blue vervain	Leafy stem, Leaves, Flowers	Decoction	[19]
Strychnos spinosa Lam.	Loganiaceae	Monkey orange	Leaves	Decoction	[18]
Tapinanthus banguensis	Loranthaceae	Mistletoe	Young stems, leaf	Decoction	[19]
Trema orientalis	Ulmaceae	Charcoal tree	Leaves	Decoction	[14, 17]
Talinum triangulare	Portulacaceae	Water leaf	Leaf	Decoction	[19]
Vernonia amygdalina Delile.	Asteraceae	Bitter leaf	Leaves and Root	Decoction	[14, 16, 17, 21]
Vernonia conferta	Asteraceae	Cabbage tree	Root and bark	Decoction	[14, 18]
Zingiber officinale	Zingiberaceae	Ginger	Root	Mastication	[14, 17]

Table 3: Reported *in vitro* studies of plants used for the management of diabetes mellitus in Ghana.

Scientific Name	Part used	Mode of action	Reference
Abelmoschus esculentus	Okra pod	α-amylase inhibitory activity	[27]
Abelmoschus esculentus	Peel and Seed	α-amylase and α- glucosidase inhibitory activity	[28]
Alstonia boonei	Stem bark, flower	α- glucosidase, α- amylase inhibitory activity	[29]
Anogeissus leiocarpus	Leaves	α-amylase and α- glucosidase inhibitory activity	[30]
Cassia auriculata	Seed, Whole plant	α-amylase and α- glucosidase inhibitory activity	[31]
Cassia siamea	Leaves	α-glucosidase inhibitory activity	[32]
Catharanthus roseus	Leaves	alkaloid (vindolicine) exerted high hypoglyceamic activity	[33]
Clausena anisata	Leaves	inhibition on α-amylase and G-6-Pase activity	[34, 35]
Costus afer Ker-Gawl	Leaf, Stem and Rhizome	α-amylase and α- glucosidase inhibitory activity	[36]
Cyperus esculentus	Tuber	α-amylase and α- glucosidase inhibitory activity	[37]
Ehretia cymosa	Leaves	competitive and non-competitive inhibition on α-amylase and α- glucosidase respectively	[38]
Ipomoea sepiaria Koenig Ex. Roxb	Leaves	anti-hyperglycemic property	[39]
Launaea taraxacifolia	Leaves	α- glucosidase inhibitory activity	[40]
Mangifera indica	Leaves	Exerts insulin like action	[41]
Mangifera indica	Leaves	α-amylase and α- glucosidase inhibitory activity	[42]
Mimosa pudica	Aerial part	α-amylase and α- glucosidase inhibitory activity	[43]
Mimosa pudica	Whole Plant	α-amylase inhibitory activity	[44]
Moringa oleifera	Leaves	α-amylase and α- glucosidase inhibitory activity	[45]
Myrianthus arboreus	Stem bark	α-amylase and α- glucosidase inhibitory activity	[46]
Ocimum canum	Leaves	increase insulin release from β-islet cells	[47]
Scoparia dulcis	Ariel part	α-amylase and α- glucosidase inhibitory activity	[48]
Securinega Virosa	Root	anti-hyperglyceamic activity	[49]
Sida acuta	Leaves	α-amylase inhibitory activity	[50]
Strychnos spinosa	Leaves	α- glucosidase inhibitory activity	[40]
Khaya senegalensis	Stem bark, Root and Leaves	α-amylase and α- glucosidase inhibitory activity	[51]
Zingiber officinale	Rhizome	α-amylase and α- glucosidase inhibitory activity	[52]

Table 4: Reported *in vivo* studies of medicinal plants used for the management of diabetes mellitus in Ghana.

Scientific Name	Part used	Method	Observation	Reference
Abelmoschus esculentus	Peel and Seed	Streptozotocin induced	exert blood glucose normalization and lipid profiles lowering activity	[28]
Adenia lobata	Stem	Streptozotocin induced	provide protective mechanism against reactive oxygen species associated with chronic hyperglyceamia and diabetic complications	[15]
Ageratum conyzoides	Leaves	Glucose induced	exert extra pancreatic action by stimulating insulin secretion	[53]
Ageratum conyzoides	Leaves	Streptozotocin induced	possess blood glucose lowering effect	[54]
Allium cepa	Bulb	Alloxan-induced	Stimulates insulin release and action to enhance glucose cellular uptake and utilization	[55]
Allium cepa	Bulb	Streptozotocin induced	Ameliorate possible complications associated with diabetes mellitus.	[56]
Allium sativum	Bulb	Streptozotocin-induced	Restores delayed insulin response by reacting with endogenous thiol containing molecules	[57]
Aloe barbadensis	Leaves	Alloxan induced	useful and safe agent in reducing hyperglycemia induced by alloxan	[58]
Alstonia boonei	Leaves	Alloxan induced	exert significant antidiabetic activity	[59]
Alstonia boonei	Stem bark	Streptozotocin-induced	inhibit the activity of glucogenic enzymes	[60]
Amaranthus viridis	Leaves	Streptozotocin-induced	inhibit the activity of glucogenic enzymes and restore β-cell function	[61]
Amaranthus viridis	Stem	Streptozotocin-induced	protective potential against glucogenic enzymes	[62]
Amaranthus viridis	Whole plant	Streptozotocin-induced	increased uptake of glucose for glycogen synthesis	[63]
Bauhinia rufescens	Leaves	Alloxan induced	exert significant antidiabetic activity	[64]
Bridelia ferruginea	Leaves	Sucrose-induced	Improves insulin sensitivity	[65]
Cassia auriculata	Flower	Streptozotocin induced	extract enhances the utilization of glucose through increased glycolysis	[66]
Cassia auriculata	Leaves	Streptozotocin induced	exert insulinogenic action	[67]
Cassia auriculata	Whole plant	Streptozotocin induced	exert significant antidiabetic activity	[31]
Cassia auriculata	Flower	Alloxan induced	ethanolic extract possesses hypoglycemic activity	[68]
Cassia occidentalis	Whole plant	Alloxan induced	exert significant antidiabetic activity	[69]
Carica papaya	Leaves	Streptozotocin induced	Restores pancreatic islet cell function	[70]
Catharanthus roseus	Leaves, Stem Root, flower	Alloxan induced	aqueous stem extract depicted best hypoglyceamic activity	[71]
Catharanthus roseus	Leaves	Streptozotocin induced	increase insulin sensitivity	[72]

Table 4: Continued.

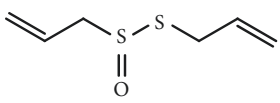
Scientific Name	Part used	Method	Observation	Reference
Catharanthus roseus	Leaves	Alloxan	restores pancreatic β-cell function	[73]
Clausena anisata	Root	Streptozotocin induced	secondary metabolites responsible for the hypoglycemic effect	[74]
Costus afer Ker-Gawl	Stem, leaf	Alloxan induced	hypoglycemic, protective potential and regenerative effect on pancreas	[75, 76]
Ehretia Cymosa	Whole plant	Streptozotocin induced	hypoglycemic effects	[24]
Glyphaea brevis	Leaves	Oral starch tolerance	α-amylase inhibitory properties coupled to control of body weight	[77]
Gongronema latifolium	Leaves	Alloxan	induce pancreatic cell regeneration	[78]
Gongronema latifolium	Leaves	Alloxan	ameliorate oxidative stress associated with diabetes mellitus	[79]
Guiera senegalensis	Leaves	Glucose induced	stimulate insulin production and glucose utilization	[80]
Hoslundia opposita	Leaves	Alloxan	ameliorative effect on Type 2 diabetic patients and associated complication	[81]
Hyptis suaveolens (L.) Poit	Leaves	Streptozotocin induced	exerts additive hypoglycemic effect with antioxidant	[82]
Indigofera arrecta	Leaves	Streptozotocin induced	insulinotropic effect	[83]
Ipomoea sepiaria Roxb.	Leaves	Streptozotocin induced	restore glucose levels to near normal level	[39]
Mangifera indica	Leaves	Alloxan induced	Insulin like effect by Inhibiting hepatic gluconeogenesis or glucose absorption in muscles or adipose tissues	[84]
Mangifera indica	Leaves	Streptozotocin induced	α-amylase and α- glucosidase inhibitory activity	[85]
Mimosa pudica L	Leaves	Alloxan induced	exerts hypoglycemic effect	[86]
Mimosa pudica L	whole plant	Streptozotocin induced	stimulates insulin secretion by the regeneration of pancreatic β-cells	[87]
Mollugo nudicaulis	whole plant	Alloxan induced	increase release of insulin from Pancreatic β -cells	[88]
Morinda Lucida	Leaves	Streptozotocin induced	glucose lowering property	[89]
Momordica charantia	Fruit	Streptozotocin induced	stimulates insulin secretion by the regeneration of pancreatic β-cells	[90, 91]
Myrianthus arboreus P. Beauv	Stem bark	Streptozotocin induced	exerts hypoglycemic effect	[92]
Ocimum canum Sim	Leaves	C57BL/KsJ db/db	enhanced insulin release genetically modified from pancreatic β –cells diabetic animal	[47]
Ocimum gratissimum	Leaves	Streptozotocin induced	exerts hypoglycemic effect	[25]
Pergularia daemia	Leaves	Alloxan induced	restores pancreatic β –cells function	[93]
Phyllanthus amarus	Whole plant	Alloxan	exerts hypoglycemic effect	[94]
Phyllanthus fraternus	Whole plant	Alloxan induced	possess antidiabetic and antioxidant activity	[95]
Scoparia dulcis	Ariel part	Streptozotocin-induced	possess antidiabetic and antioxidant activity	[48]
Securinega virosa	Leaves	Streptozotocin-induced	hypoglycemic activity	[96]
Trema orientalis	Stem bark	Streptozotocin induced	Sensitize insulin receptors or stimulate β cells of the Islet of Langerhans in the pancreas	[97]
Zingiber officinale	Bulb	Streptozotocin and alloxan induced diabetes mellitus	exhibits hypoglycemic activity in both normal and diabetic rats	[98, 99]

Table 5: Clinical studies on medicinal plants used in Ghana for the management of diabetes mellitus.

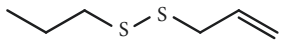
Scientific name	Part/form	Disease type	Observation	Reference
Allium cepa	Aqueous extract	Type 2	regulates blood glucose and lipids levels to normal	[100]
Allium sativum	Bulb (Garlic tablet)	Type 2	Inhibits insulin inactivation by thiol groups as well as advance glycation end products	[101]
Allium sativum	Capsule	Type 2	significant effect on improvement of glycemic status with lowering fasting blood glucose level and postprandial blood glucose level	[102]
Allium sativum	Aqueous extract	Type 2	regulates blood glucose and lipids levels to normal	[100]
Aloe barbadensis	Pulp	Types 1&2	*Aloe vera* treatment with glibenclamide depicted significant decrease in glucose level	[103]
Cinnamomum zeylanicum	Bark	Type 1	Improves insulin potentiating activity	[104]
Guiera senegalensis	Aqueous extract	Type 2	regulates blood glucose and lipids levels to normal	[100]
Indigofera arrecta	Aqueous leaves extract	Types 1&2	significant change in fasting blood glucose level	[105]
Momordica charantia	Vegetable (V-insulin)	Idiopathic Type	hypoglyceamic effect in only diabetic patients	[106, 107]
Zingiber officinale	Root	Type 2	Increase insulin receptors and enhance β- cell function to decrease insulin resistance	[98]
Zingiber officinale	Ginger powder	Type 2	Promotes glucose clearance in insulin responsive peripheral tissues	[108]

Table 6: Plant bioactive constituents used experimentally in diabetes mellitus.

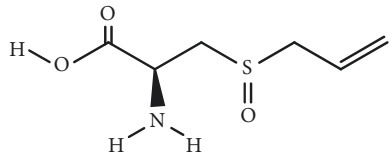
Scientific name	Part used	Active ingredient	Reference
Adenia lobata	Stem bark	Palmitic acid	[109]
Allium cepa	Bulb	Allyl propyl disulphide	[17]
Allium sativum	Bulbs	Allyl propyl disulphide, allicin	[110]
Aloe barbadensis	Leaf	Lophenol, 24-methyl lophenol 24-methylene cycloartenol, Cycloartenol, 24-ethyl lophenol	[111]
Azadirachta indica	Leaves, flowers & seed	Nimbidin, β-sitosterol	[17]
Cassia auriculata	Flower	β-sitosterol	[67, 112]
Cinnamomum zeylanicum	Bark	Cinnamaldehyde, eugenol	[113]
Costus afer Ker Gawl	Whole plant	Diosgenin	[114]
Mangifera indica	Leaf, stem bark, fruit	Mangiferin	[85]
Momordica charantia	Leaves, whole plant, fruit	Charantin, momordicin, Oleanolic acid, vicine	[115]
Scoparia dulcis	Whole plant	Apigenin, luteolin, scoparic acid D coxicol, glutinol	[17, 116]
Zingiber officinale	Bulb	Gingerol	[117]



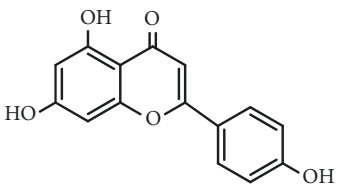
Allicin



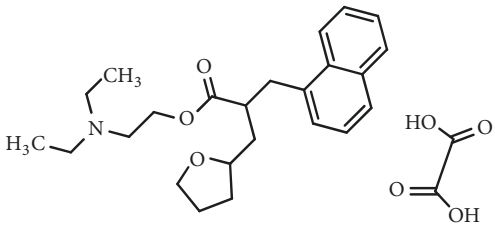
Allyl propyl disulphide



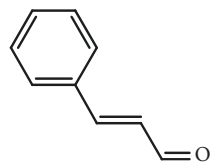
S-Allylcysteine Sulphoxide



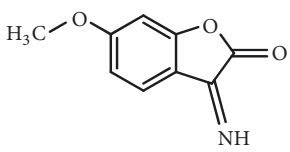
Apigenin



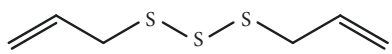
Charantin



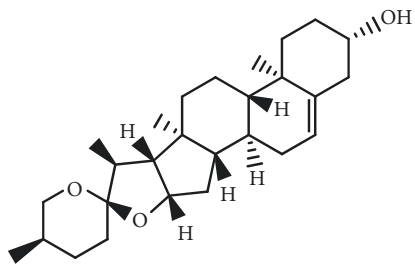
Cinnamaldehyde



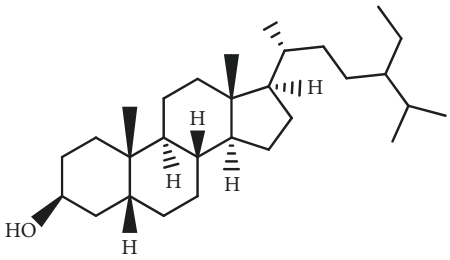
Coxicol



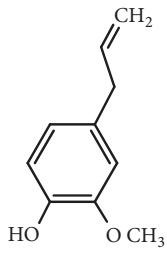
Diallyl trisulphide



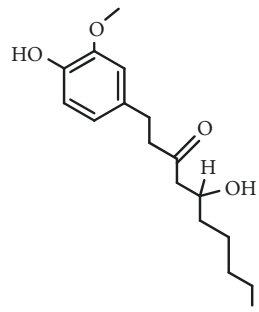
Diosgenin



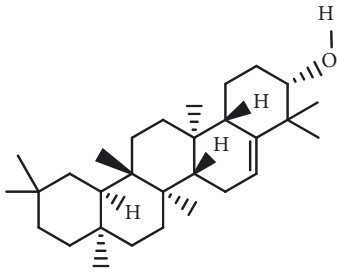
24-ethyl lophenol



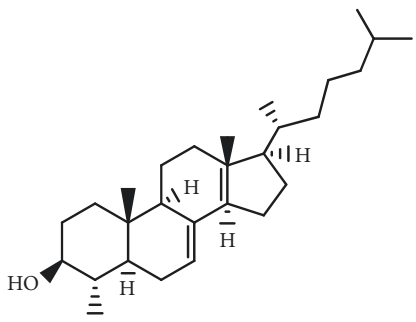
Eugenol



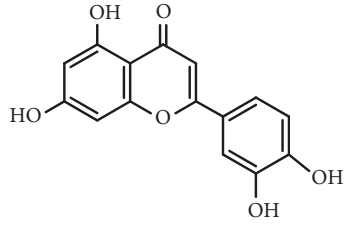
Gingerol



Glutinol

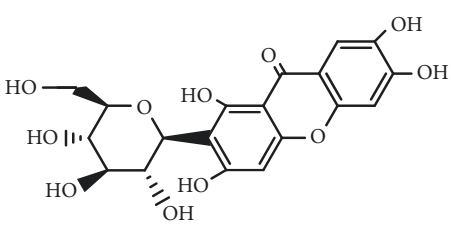


Lophenol

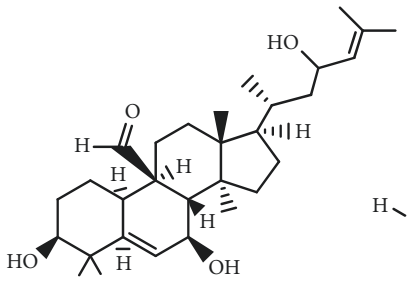


Luteolin

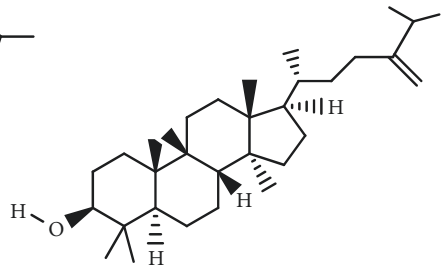
Figure 1: Continued.



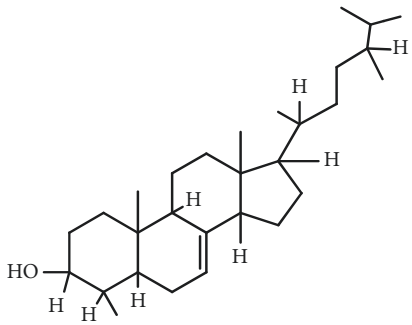
Mangiferin



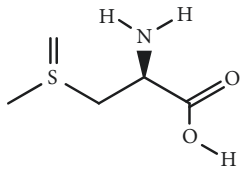
Momordicin



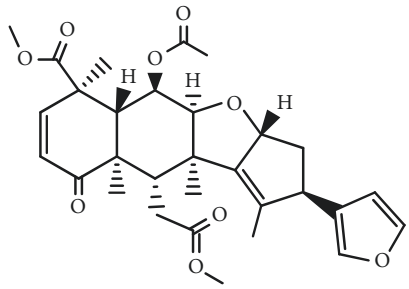
24-Methylenecycloartanol



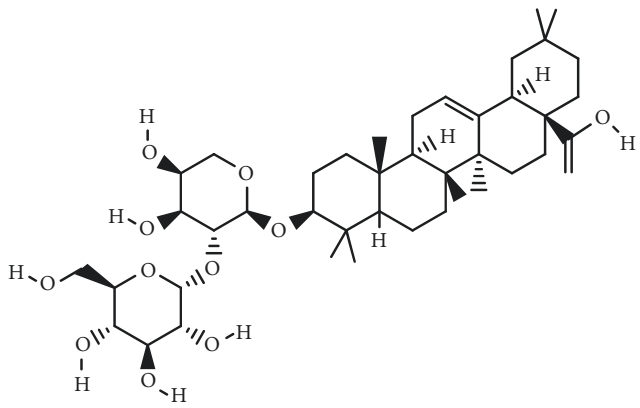
24-Methyl Lophenol



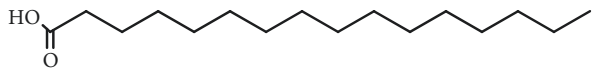
S-Methylcysteine Sulphoxide



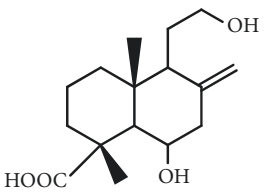
Nimbidin



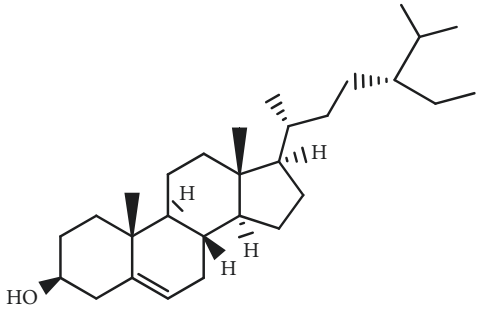
Oleanolic acid



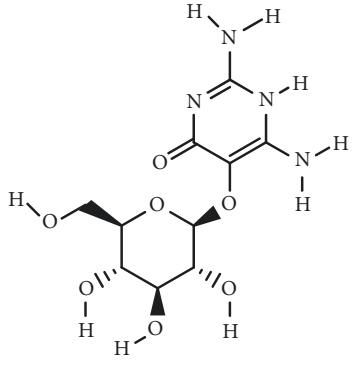
Palmitic acid



Scoparic acid D



Beta-sitosterol



Vicine

Figure 1: Chemical structures of isolated compounds listed in Table 6.

effects of *Allium cepa* and its ability to ameliorate complications associated with diabetes mellitus. Babu and Srinivasan [121] also reported that feeding onion powder-containing diet to diabetic animals produces marked reduction in their hyperglycaemic status. Petroleum ether extract of onion was demonstrated to reduce blood glucose levels in normal rabbits. Prolonged addition of freeze-dried onion powder in the diet of STZ-diabetic rats produced antihyperglycemic, hypolipidemic, and antioxidant effects [122]. Kelkar and colleagues also reported a higher hypoglycemic potential of onion callus cultures over natural onion bulb [123]. Onion juice administered to alloxan induced diabetic rats for a period of one month showed characteristics of antihyperglycemia [124].

The presence of quercetin, allyl propyl disulphide oxide (dipropyl disulphide oxide), S-methylcysteine sulphoxide, and S-allyl cysteine sulphoxide in onion is reported to be responsible for the drop in glucose level and lipid profile. Allyl propyl disulphide oxide also aids in insulin secretion [14, 120]. S-allyl cysteine sulphoxide from onion also markedly decreased blood glucose level of diabetic rats [125]. Daily oral administration of about 200 mg of S-methylcysteine sulphoxide for 45 days to alloxan diabetic rats controlled their blood glucose and lipid levels. The same study also reports improvement in the activities of liver glucose-6-phosphatase, hexokinase, and HMG CoA reductase. The observed effect of S-methylcysteine sulphoxide was analogous to that of insulin and glibenclamide [126]. Oral administration of S-methyl cysteine sulphoxide to alloxan diabetic rats for one-month period ameliorated hyperglycaemia and was similar to animals treated with glibenclamide and insulin [121].

In a clinical study of individuals with diabetes mellitus administered with juice of onion bulb, a decrease in blood glucose concentration was observed [127]. Our search did not find any published work on any reported case of adverse toxicity associated with the consumption of onion. Meanwhile there are reports of unfavorable effects of excessive intake such as abdominal bloating, heartburn, hypotension, allergies, and bad breath [128].

Allium sativum. *Allium sativum* commonly known as garlic is one of the oldest known medicinal spices in existence. It is cultivated all over Ghana. It is used to manage many disorders, which include diabetes mellitus. The bulb is washed, dried, and chewed as required for the management of diabetes mellitus [18]. The cloves of the plant are reported to possess a sulphur-containing chemical compound called allicin that is also responsible for its pungent smell [128]. The bulb is reported to contain other principles such as S-allyl cysteine sulphoxide, allicin, Bis (allixinato) oxovanadium (IV), vitamins C and B_6, and manganese [128, 129].

Administration of extract of garlic orally to normal and STZ-diabetic rats daily for 5 weeks controlled hyperglycemia [130]. A study by Kumar et al. [101] found that garlic plus metformin treatment in patients with type-2-diabetes mellitus for a duration of 12 weeks produced a drastic decline in blood glucose level. In alloxan induced diabetic rabbits, different solvent extracts produced antihyperglycemic effect [131]. Alloxan induced diabetic rats put on a diet containing garlic for a period of 15 days recorded a significant reduction in blood glucose as compared to the control group [132]. Oral administration of diet containing ajoene (obtained from garlic) for two months was also reported to reduce blood glucose in genetically transformed diabetic mice [133]. Garlic oil and diallyl trisulfide given for 3 weeks to diabetic rats markedly elevated the basal insulin levels and also increased its sensitivity [134]. Oral administration of S-allyl cysteine sulphoxide isolated from garlic to alloxan diabetic rats for one month ameliorated hyperglycaemia in treated rats, which was comparable to glibenclamide and insulin treated rats [125]. Furthermore, S-allyl cysteine sulphoxide was reported to significantly stimulate insulin secretion from beta cells isolated from healthy rats [135]. Intraperitoneal injection and oral administration of Bis (allixinato) oxovanadium (IV) to type-1-diabetic mice showed potential as a potent antidiabetic agent [136]. According to Mathew et al. [137], oral administrations of 0.25 mg of allicin to mild diabetic rabbits exhibited pronounced antihyperglycemic effect.

A clinical study has confirmed that garlic improves glycemic status by decreasing fasting blood glucose concentration and postprandial blood glucose level in humans [102]. According to Miron et al. [110], allicin acts to restore delayed insulin response by reacting with endogenous thiol molecules and to lower insulin resistance in diabetic patients. It has the ability to freely permeate through phospholipid bilayers of membranes and this enhances its intracellular interaction with thiols. Toxicity studies have shown that excessive intake of garlic is considered toxic due to the sulphone hydroxyl ion constituent. This constituent is capable of penetrating the blood-brain barrier and can cause damage to brain cells. A study by Johnson et al. [138] on alloxan induced diabetic Wistar rats demonstrated that high doses of garlic extract greater than 400-mg/kg body weight per day induced morphological changes that presented severe threats to the heart, kidney, and liver of Wistar rat. However, low doses of 250-350 mg/kg body weight/day had no deleterious effects on the organs mentioned. Raw garlic is reported to also promote botulism, inhibit blood clotting, and trigger allergic reactions by the skin and mucous membrane [17].

Aloe vera (Aloe barbadensis). This plant is commonly referred to as Aloe. *Aloe barbadensis*: the plant is widely distributed, cultivated, and used in many homes in Ghana for several purposes. It is believed to have originated from Sudan. The sap consists largely of D-glucose, D-mannose, tannins, steroids, phytosterols [lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartenol, and 24-methylene-cycloartanol], amino acids, vitamins, and minerals. Fresh aloe juice from the inner leaf parenchyma contains 96 % water.

Dry sap of the plant produced conspicuous antihyperglycemic response in alloxan induced diabetic albino mice [139]. *Aloe vera* leaf pulp extract showed antihyperglycemic activity on both types of diabetes in rat models, with the outcome enhanced in type-2-diabetes compared with the positive control-glibenclamide [140]. Extracts of aloe vera orally administered produced antihyperglycemic activity in

oral glucose fed and STZ-diabetic rats [141]. Oral administration of ethanolic extract to diabetic rats for three weeks resulted in a conspicuous decrease in fasting blood glucose along with enhanced plasma insulin levels [142]. Oral administration of aloe vera gel extract for three weeks to diabetic rats ensued in a substantial reduction of blood glucose and improved the plasma insulin level [141]. Aqueous leaf extract of *Aloe vera* was reported to be useful and safe for reducing blood glucose levels in alloxan induced diabetes mice [58]. Administration of some phytosterols isolated from aloe vera to type-2-diabetic mice for 28 days resulted in a reduction in blood glucose levels [111, 143]. A clinical study reported that oral administration of aloe vera was beneficial in lowering blood glucose concentration in diabetic patients [104, 144]. It could be adduced that the antihyperglycemic effect of aloe vera and its principles may be through stimulating synthesis and/or release of insulin from the beta cells.

Momordica charantia. This plant commonly referred to as bitter gourd is an annual climber grown in Ghana for use as vegetable. It has a wide array of medicinal uses; however it is widely known for its use in the management of diabetes in Ghana. In Ghanaian traditional medicine, the aerial part is crushed and boiled and the strained liquid drunk as required [18]. Research has shown that [115, 145] bitter gourd extracts from the fruit, seeds, and leaves contain several bioactive compounds that have hypoglycemic activity in both diabetic rats and humans. The hypoglycemic ameliorative effects of the fruit extract of the plant are reported to be closely linked to the increase in hepatic glycogen, peripheral tissue's glucose transporter (GLUT-4) expression, and higher insulin sensitivity through downregulating the expression of suppressor of cytokine signaling 3 (SOCS-3) and c-Jun N-terminal kinase (JNK) [90]. Fruit aqueous extract administered orally for 6 weeks with exercising decreased blood glucose of type-2 diabetic rats [146]. Blood glucose level dropped when about 4000 mg of *Momordica charantia* fruit extract was orally used to treat alloxan diabetic rats for 8 weeks [147]. Seed aqueous extract showed conspicuous decrease in blood glucose, glycated haemoglobin, glucose-6-phosphatase, lactate dehydrogenase, fructose-1, 6-bisphosphatase, and glycogen phosphorylase coupled with a rise in glycogen content, hexokinase, and glycogen synthase activities [148]. Ethanolic extract of *Momordica charantia* also produced antihyperglycemic effects in streptozotocin diabetic rats [149].

Bioactive principles reported to be found in *Momordica charantia* are charantin, oleanolic, vicine, and momordicin [115]. Charantin, a sterol isolated from *Momordica charantia* seeds, induced hypoglycemic effect by stimulating the release of insulin [150]. *Momordica charantia* has also been reported to inhibit gluconeogenesis [151]. Its antidiabetic effect is similar to sulfonylurea-like medicines. According to Matsuda et al. [152], an experiment conducted using rat intestine showed that oleanolic acid and momordin from the plant exhibit antihyperglycemic activity through inhibition of glucose transport in the intestine. These compounds could be considered for use as dietary supplements for people with diabetes mellitus. In a clinical study of people with diabetes mellitus, polypeptide-p obtained from fruit, seed, and tissue exhibited antihyperglycemic effects with no adverse reactions [153].

Data available shows that extracts and the main isolated bioactive compounds [charantin, vicine, polypeptide-p, and momordicin] from *Momordica charantia* are considered to produce their antidiabetic effects through diverse physiological and biochemical processes [145].

The ethanolic extract of the fruit is reported to be safe in Sprague-Dawley rats at 2000 mg and below, whereas doses higher than 2000 mg could pose safety problems to delicate organs like the liver [154]. The seeds have been shown to decrease fertility in male Wistar rats and also produce side effects such as fever and coma. *Momordica charantia* is also reported to induce abortion in pregnant women [155]; thus care must be taken in usage.

Cinnamomum zeylanicum. Cinnamon is a spice derived from the stems of the *C. zeylanicum* tree. It is widely used in food preparations as a spice particularly in baking and for culinary purposes. The plant is not only used for making food taste better, but also used as home remedies and medicines. The dried bark has golden-yellow colour with pungent taste and scent due to the active constituent cinnamaldehyde and eugenol [113]. Cinnamon is reported to reduce blood glucose through decrease of insulin resistance and upsurge in the rate of hepatic glycogenesis [156, 157]. Cinnamaldehyde possesses antioxidant and antidiabetic properties. Moreover, cinnamaldehyde demonstrated antihyperglycemic and antihyperlipidemic effects in rodent models [158]. Cinnamaldehyde is also reported to markedly and dose-dependently decrease plasma glucose concentration in streptozotocin-induced diabetic rats [113]. All these evidence supports the fact that cinnamaldehyde from cinnamon extract is a potential antidiabetic agent and thus more research is needed in that direction.

Clinical investigation shows that cinnamon is useful in the management of both type-1 and type-2 diabetes mellitus [159]. Daily consumption of cinnamon regulates high triglyceride or cholesterol levels tremendously. It also aids in controlling elevated glucose level in type-2 diabetic patients. Toxicity studies conducted with ethanolic extracts of C. zeylanicum bark did not exert any observable adverse effects. Although the ethanolic extracts of C. zeylanicum bark have no reported acute or chronic oral toxicity in mice, it has been reported to cause reduction in liver weight as well as haemoglobin levels [160].

Costus afer Ker-Gawl. *Costus afer* Ker-Gawl (bush cane or ginger lilly) is herbaceous monocot, tropical plant with creeping rhizome commonly found in moist and shady forest of West and Tropical Africa. It is often planted in home gardens. The leaves are edible and the rhizome is sometimes used as a spice. In Ghana, all parts of the plant are used in traditional medicine, but the stem is the part mostly used for treatment of diabetes [75]. In an alloxan induced rat, there was a marked reduction in the blood glucose level when *Costus afer* aqueous leaf extract with concentrations 375, 750, and 1125 mg/kg and control drug (glibendamide

(5 mg/kg)) were orally given [76]. A dose of 375 mg/kg of the extract had a preservative effect on β-cells [161]. This report is consistent with work by ThankGod et al. [162] that also reported on the regeneration of islet cells on administration of *Costus afer* stem extract to streptozotocin-induced rats. Moreover, the oral administration of 750 and 1125 mg/kg of *Costus afer* extract produced a more prominent regeneration of pancreatic islet cells and exocrine cell [163]. This therefore indicates that *C. afer* extract has a pancreatic (islet cells) curative property, which could help manage type I diabetes mellitus. This was consistent with the histopathological study of *Costus afer* extract on damaged pancreatic cells as reported by Ezejiofor et al. [161]. When stem extract was orally given to streptozotocin-induced rat, there was a marked drop in blood glucose level at extract dosage of 500, 1000, and 1500 mg in a concentration dependent manner [164]. In an *in vitro* study of the effect of solvents [hexane, ethyl acetate, methanol, and water] extracts of *Costus afer* stem, leaf, and rhizome on the activity of α-glucosidase and α-amylase activity, there was a significant inhibition of the enzymes. Ethyl acetate rhizome and methanolic leaf extract showed the highest inhibitory effect of the activity of the aforementioned enzymes with an IC_{50} value of 0.10 and 5.99 mg/mL [36].

The stem and seeds are reported to contain several steroids and sapogenins; thus, diosgenin, saponins aferosides A-C, diosin, parphyllin c, flavonoid, and glycoside with diosgenin are the most potent [75]. Diosgenin ameliorates insulin resistance by increasing glucose usage and intracellular glycogen synthesis [165]. This is achieved by restoring pancreatic β-cell function, alteration of hepatic enzymes, enhancement of adipocyte differentiation, inhibition of macrophage infiltration into adipose tissue, and decreased expression of inflammatory genes [165]. It is also reported to decrease the expression of the C/EBP homologous protein (CHOP) leading to reduced stress of endoplasmic reticulum in pancreatic β-cells.

Also the effectiveness of diosgenin as an antidiabetic agent was evident by its effect on the renal antioxidant system and oxidative markers such as myeloperoxidase and lipid peroxidation. Diosgenin is reported to exhibit a protective effect on the kidney of diabetic rats and therefore serves as a potential candidate for treatment of diabetes mellitus with renal associated problems [165, 166]. There is no reported toxic effect of diosgenin isolated from *Costus afer* in liver. Meanwhile a study conducted by Ezejiofor et al. [167] to investigate the subchronic toxic effect of the aqueous extract of *Costus afer* leaves on the liver and kidney of albino rats reported that it may be toxic to the liver but not to the kidney. This implies that much work needs to be done to provide more information on its toxicological effects.

Mangifera indica. Mango is a delicious and succulent fruit that has immense health benefits. It is popular in every part of the world including Ghana due to its delicious fruit. It is the major traditional fruit that is exported from the country. The leaf is traditionally used to treat diabetes in Ghana. Traditionally, a decoction of the leaves is drunk after meals [17]. Ganogpichayagrai et al. [42] demonstrated that leaf extract of mango tree possesses alpha amylase and alpha glucosidase inhibitory activity. Intraperitoneal administration of aqueous extract of stem bark produced a marked antihyperglycemic effect in streptozotocin-induced diabetic rats in a dose dependent manner. The oral administration of peel extract to streptozotocin-induced diabetic rats exhibited a significant antidiabetic effect [168].

Bioactive compound, mangiferin (MGF), mostly found in the leaves is reported to have alpha amylase and alpha glucosidase inhibitory effects [85]. Furthermore, mangiferin is reported to have antidiabetic as well as hypolipidemic potential effects in type-2-diabetic model rats. MGF inhibits anaerobic metabolism of pyruvate to lactate but enhances pyruvate oxidation suggesting that one of the targets of MGF is pyruvate dehydrogenase [169]. These observations highlight the therapeutic potential of activation of carbohydrate utilization in the correction of metabolic syndrome and emphasize the potential of MGF to serve as a model compound that can elicit fuel-switching effects. Mangiferin, a polyphenol isolated from M. indica, significantly prevents progression of diabetic nephropathy and improves renal function in diabetic nephropathy rat model and cultured rat mesangial cells [170]. Implicitly mangiferin is likely to possess beneficial effects in the management of type-2-diabetes mellitus with hyperlipidemia.

Scoparia dulcis. Scoparia dulcis, commonly referred to as sweet broomweed, is an annual erect herb with many medicinal uses. It is a rich source of flavones, terpenes, and steroids. Some compounds found include coxicol, glutinol, scoparic acid D, luteolin, and apigenin; they are the main constituents found in the leaves and they have various pharmacological activities. The whole plant is used as a remedy for many ailments including diabetes mellitus. The fresh or dried leaves are used to manage hypertension and diabetes mellitus [171]. In Ghanaian traditional medicine, the dried leaves are boiled with water and strained and the decoction is drunk when needed [17].

An *in vitro* study performed to assess the α-amylase and α-glucosidase inhibitory potentials of the plant extract showed that the methanol extract of *Scoparia dulcis* effectively reduces postprandial glucose levels [172]. Investigation of the effect of the aqueous extract of *Scoparia dulcis* on streptozotocin-induced diabetes mellitus showed that the plant extract-mediated reduction in blood glucose was significant and was similar to that of glibenclamide [173]. According to Latha et al. [174], *Scoparia dulcis* possesses insulin-secretagogue activity. The administration of an aqueous extract of *Scoparia dulcis* to streptozotocin diabetic rats at a dose of 200 mg markedly reduced the blood glucose with significant increase in plasma insulin level during a 15-day period of treatment. The mechanisms of action of *Scoparia dulcis* plant extracts possessing antidiabetic effect have also been reported. According to Latha et al. [174], the antidiabetic activity of the aqueous extracts of S. dulcis may be attributable to its insulin-secretagogue activity. Also, S. dulcis imparts its antidiabetic effects via altering the levels of many antioxidant enzymes and enzymes of the polyol pathway. In fact, Latha et al. showed, using streptozotocin-induced diabetic rats, that the aqueous extract of S. dulcis significantly decreases the

level of sorbitol dehydrogenase while increasing the levels of the antioxidant enzymes [173]. Beh et al. [175] demonstrated using L6 rat myoblasts (CRL-1458) that the TLC fraction seven of the aqueous extract of S. dulcis possesses glucose uptake activity comparable to that of insulin.

Luteolin, a flavonoid isolated from *Scoparia dulcis*, is reported to inhibit alpha glucosidase better than acarbose, a standard drug. Luteolin, an active constituent in the leaves of *Scoparia dulcis*, is reported to improve hepatic insulin sensitivity by suppressing expression of sterol regulatory element-binding transcription protein 1 (SREBP1) that modulates insulin receptor substrate 2 (Irs2) expression through its negative feedback and gluconeogenesis. Scoparic acid D has also been reported to possess antidiabetic effects [176].

The data supports the traditional use of *Scoparia dulcis* as an antidiabetic medicinal plant. Furthermore, luteolin and apigenin, flavonoids of *Scoparia dulcis*, have been shown to influence glucose metabolism by activating the transcription factor FOX O1 (forkhead-box gene O1) in human cells [177, 178].

Zingiber officinale. Ginger is one of the most ancient spices cultivated for its edible rhizome. The rhizome serves a variety of purposes including culinary and medicinal applications. Medicinal properties attributed to ginger include hypolipidemic, hypocholesterolemic, and antidiabetic effects. In a study based on STZ induced diabetic rat model reported, oral administration of ethanolic extract of ginger markedly reduced blood glucose level [179]. Another study demonstrated that there is a substantial blood glucose lowering effect of ginger juice in diabetic animals [180]. Ahmed and Sharma have also shown that administration of ginger extract in rats recorded a significant hypoglycemic effect [181].

Several constituents are reported to be present in ginger that include terpenes and oleoresin, which are generally called ginger oil. Ginger also contains volatile oils and nonvolatile pungent components such as oleoresin [182]. The major identified components from terpene are sesquiterpene hydrocarbons and phenolic compounds, which are gingerol and shogaol.

The major bioactive constituent reported to be present in ginger is gingerol. Studies on ginger show that it increases glucose uptake through promotion of GLUT-4 translocation via adenosine monophosphate-activated protein kinase (AMPK) activation in L6 myocytes. It has been reported that gingerol protects pancreatic β-cells from oxidative stress, increases insulin receptors sensitivity, and enhances β-cell function to decrease insulin resistance [117]. Gingerol has also been shown to regulate *in vivo* hepatic gene expression of enzymes involved in glucose metabolism, leading to a decrease in glucose production and an increase in glycogen synthesis, which contributes to the antihyperglycemic effect of gingerol. Studies have shown that gingerol could provide therapeutic as well as prophylactic benefit for type-2 diabetes individuals [117]. Ginger has no known reported toxic dose. However overconsumption can cause some minor side effects. For example, some people have experienced side effects including heartburn, diarrhea, and general stomach discomfort following consumption of ginger. High dose of ginger can also interact with certain drugs such as warfarin used in the treatment of heart condition and increase their effect to result in symptoms such as irregular pulse, palpitations, confusion, loss of appetite, diarrhea, nausea, and vomiting. Large doses may also cause dizziness and minor sedation and increase the risk of bleeding in women as well [17].

4. Discussion

The adoption of a Western lifestyle and urbanization is cited as a major cause for the tremendous increase in metabolic diseases such as diabetes mellitus in Africa, including Ghana [183]. Currently, there is no known cure for diabetes mellitus despite the availability of various classes of pharmacological agents for management of diabetes mellitus. Currently, issues related to efficacy, safety, and affordability of existing pharmacological agents for management of diabetes are driving patients to turn to complementary and alternate medicine (CAM), including plant medicines for the management of diabetes mellitus. Indeed, it has been estimated that up to one-third of diabetic patients use CAM to manage their condition. A growing number of phytomedicines and their chemical constituents have been studied in the treatment of diabetes mellitus. Despite the increased use of phytomedicines, with over 70% of the world's population using some form of it, according to WHO [184] many still lack thorough experimental investigation data to support their use.

Plant medicine remains an important means by which humans have treated ailments, prevented diseases, and maintained health for centuries. Traditional knowledge and use of plant-based medicines remain important in Ghana because Traditional Medical Practice (TMP) is readily available and is affordable to rural communities in Ghana. Various plants are used for managing diabetes mellitus in Ghanaian Traditional Medicine Practice [14, 17, 18, 21, 185] but not much is known about the plants used.

Of the plants discussed *Aloe vera* has the highest evidence supporting its use in diabetes mellitus, with multilateral level of support from *in vitro*, animal, and clinical studies and elucidation of active principle and testing in an animal model [103, 111, 143, 185]. Other findings also support its use in the treatment of various complications that arise from diabetes mellitus demonstrating broad clinical utility. Thus *Aloe vera* remains the hallmark of phytomedicine for diabetes mellitus though there are minor concerns over toxicity. *Momordica charantia* and *Zingiber officinale* offer the next most extensive evidence for use in managing diabetes mellitus with preclinical studies in animal models, with human studies showing clinical efficacy.

In this review, information on Ghanaian medicinal plants used for diabetes mellitus has been compiled (Tables 2 and 3). The information gathered demonstrates that some of these plants and/or their preparations show promise in managing diabetes mellitus. The review provides information on pharmacological mechanisms of some of the plants. The study shows that some of the plants and their bioactive compounds

(Figure 1) act by reducing glucose absorption through inhibition of the action of enzymes such as sucrase, α-glucosidase, and maltase. Others act through cellular mechanisms such as regeneration of pancreatic β-cell by inhibiting the atrophy of pancreatic islet tissue. Some medicinal plants have also been shown to suppress accumulation of fat and dyslipidemia through the enhancement of energy expenditure enzymes such as carnitine palmitoyl-transferase1 and acyl CoA oxidase and also attenuating enzymes involved in fatty acid synthesis that occurs in the liver. Furthermore, some of the plants have antioxidant and anti-inflammatory potentials and thus may be playing a central role in acting against diabetes associated with metabolic disorders of liver and kidney. Others reduce hepatic glucose output and enhance glycolysis, glycogenesis, and reduction in glycogen breakdown and gluconeogenesis.

This review has also identified various experimental studies that have examined the efficacy of antidiabetic medicinal plants. Results obtained from clinical trials revealed that using medicinal plants notably improves levels of biochemical indices of people with diabetes. Moreover, some principles isolated from these plants indicated antidiabetic activity with better efficacy than orthodox oral hypoglycemic agents. This piece provides scientific evidence of the effectiveness and efficacy of phytomedicines in the management of diabetes mellitus. Most of these studies did not reveal any major adverse effects consequent to the use of these medicinal plants suggesting that they are generally safe.

5. Concluding Remarks and Future Direction

Ghana is bestowed with abundance of plant biodiversity; several are used in managing diabetes mellitus in Traditional Medicine Practice. This review indicates that there is substantial preclinical evidence and some clinical data to support the usefulness of some of these herbs as antihyperglycaemic agents. The provision of information on medicinal plants used for the management of diabetes mellitus in Ghana in this narrative can serve to promote a more rational medicinal use of these plants. These can also offer evidence-based data for clinical development of many of these potential medicinal plants. Further phytochemical elucidation and pharmacological research should be carried out on many ethnomedicinal plants used in Ghana to standardize these traditional medicines with definite antidiabetic or antihyperglycaemic activity. Ultimately, in giving credibility to the preclinical data, clinical trial studies ought to be carried out in order to validate their medicinal usefulness in people with diabetes mellitus. It is believed that, this way, the pharmacotherapeutic potential of these plants could be harnessed towards a possible all-inclusive integration into the healthcare system.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

[1] P. Zimmet, K. G. M. M. Alberti, and J. Shaw, "Global and societal implications of the diabetes epidemic," *Nature*, vol. 414, no. 6865, pp. 782–787, 2001.

[2] M. B. Adinortey, "Biochemicophysiological Mechanisms Underlying Signs and Symptoms Associated with Diabetes mellitus," *Advances in Biological Research*, vol. 11, no. 6, pp. 382–390, 2017.

[3] M. B. Adinortey, B. E. Gyan, J. Adjimani et al., "Dyslipidaemia associated with type 2 diabetics with micro and macrovascular complications among Ghanaians," *Indian Journal of Clinical Biochemistry*, vol. 26, no. 3, pp. 261–268, 2011.

[4] L. Guariguata, D. Whiting, I. Hambleton, J. Beagley, U. Linnenkamp, and J. Shaw, "Global estimates of diabetes prevalence for 2013 and projections for 2035," *Diabetes Research and Clinical Practice*, vol. 103, no. 2, pp. 137–149, 2014.

[5] D. R. Whiting, L. Guariguata, C. Weil, and J. Shaw, "IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030," *Diabetes Research and Clinical Practice*, vol. 94, no. 3, pp. 311–321, 2011.

[6] N. Peer, A.-P. Kengne, A. Y. Motala, and J. C. Mbanya, "Diabetes in the Africa Region: an update," *Diabetes research and clinical practice*, vol. 103, no. 2, pp. 197–205, 2014.

[7] F. B. Hu, "Globalization of diabetes: the role of diet, lifestyle, and genes," *Diabetes Care*, vol. 34, no. 6, pp. 1249–1257, 2011.

[8] H. P. Raing, M. M. Dale, and J. M. Ritter, "Pharmacology," in *The Endocrine Pancreas and the Control of Blood Glucose*, pp. 389–398, Churchill Livingstone, New York, NY, USA, 4th edition, 2000.

[9] S. Akkati, K. G. Sam, and G. Tungha, "Emergence of promising therapies in diabetes mellitus," *Clinical Pharmacology and Therapeutics*, vol. 51, no. 6, pp. 796–804, 2011.

[10] J. Philippe and D. Raccah, "Treating type 2 diabetes: how safe are current therapeutic agents?" *International Journal of Clinical Practice*, vol. 63, no. 2, pp. 321–332, 2009.

[11] S. Akanbonga, *Knowledge of traditional herbalists on diabetes mellitus and the effect of herbal medicine on Glycaemic control [Ph.D. thesis]*, 2015.

[12] World Health Organization, "WHO Traditional Medicine Strategy: 2014–2023," 2016, https://www.who.int/medicines/publications/traditional/trm_strategy14_23/en/rightanglebracket.

[13] World Health Organization, "National policy on traditional medicine and regulation of herbal medicines: Report of a WHO global survey," 2005.

[14] A. A. Boadu and A. Asase, "Documentation of herbal medicines used for the treatment and management of human diseases by some communities in southern Ghana," *Evidence-Based Complementary and Alternative Medicine*, vol. 2017, Article ID 3043061, 12 pages, 2017.

[15] J. A. Sarkodie, T. C. Fleischer, D. A. Edoh et al., "Antihyperglycaemic activity of ethanolic extract of the stem of Adenia lobata Engl (Passifloraceae)," *International Journal of Pharmaceutical Sciences and Research*, vol. 4, no. 4, pp. 1370–1377, 2013.

[16] C. Larbie, D. Torkornoo, and J. Dadson, "Anti-diabetic and hypolipidaemic effect of botanicals: A review of medicinal weeds on KNUST campus, Kumasi," *Journal of Applied Pharmaceutical Science*, vol. 4, no. 10, pp. 97–104, 2014.

[17] K. Busia, Ed., *Ghana Herbal Pharmacopoeia*, Science and Technology Policy Research Institute, Council for Scientific and Industrial Research, 2007.

[18] N. R. Mshana, D. K. Abbiw, I. Addae-Mensah et al., "Traditional Medicine and Pharmacopoeia, Contribution to the revision of ethnobotanical and Floristic Studies in Ghana," OAU. STRC Tech. Rep 67, 2000.

[19] A. K. Bonsu, *Healing with simple plants*, Radiant Health Publications, New Delhi, India, 2012.

[20] J. P. K. Adotey, G. E. Adukpo, Y. O. Boahen, and F. A. Armah, "A review of the ethnobotany and pharmacological importance of Alstonia boonei De Wild (Apocynaceae)," *ISRN Pharmacology*, vol. 2012, Article ID 587160, 9 pages, 2012.

[21] A. Asase and D. T. Yohonu, "Ethnobotanical study of herbal medicines for management of diabetes mellitus in Dangme West District of southern Ghana," *Journal of Herbal Medicine*, vol. 6, no. 4, pp. 204–209, 2016.

[22] M. M. Iwu, *Handbook of African Medicinal Plants*, CRC Press, Boca Raton, Fla, USA, 2014.

[23] H. E. N. R. Y. Asare-Anane, *In vitro assay for the anti-diabetic effect of Ocimum canum and other medicinal plants [P.hD. thesis]*, University of Ghana, 1997.

[24] J. A. Sarkodie, S. A. Squire, I. A. Kretchy et al., "The antihyperglycemic, antioxidant and antimicrobial activities of Ehretia cymosa," *Journal of Pharmacognosy and Phytochemistry*, vol. 4, no. 3, pp. 105–111, 2015.

[25] N. I. Oguanobi, C. P. Chijioke, and S. I. Ghasi, "Effects of aqueous leaf extract of Ocimum gratissimum on oral glucose tolerance test in type-2 model diabetic rats," *African Journal of Pharmacy and Pharmacology*, vol. 6, no. 9, pp. 630–635, 2012.

[26] I. K. Amponsah, A. Y. Mensah, E. K. Ampofo et al., "Pharmacognostic studies of the leaves and seeds of Cassia occidentalis (Linn.) (Leguminosae)," *Journal of Pharmacognosy and Phytochemistry*, vol. 5, no. 3, pp. 250–255, 2016.

[27] M. N. Chalse, V. Bondre, and S. Wahule, "Alpha amylase inhibition activity of okra mucilage," *International Journal of Advance Research*, vol. 5, no. 4, pp. 686–691, 2017.

[28] V. Sabitha, S. Ramachandran, K. R. Naveen, and K. Panneerselvam, "Antidiabetic and antihyperlipidemic potential of Abelmoschus esculentus (L.) Moench. in streptozotocin-induced diabetic rats," *Journal of Pharmacy and Bioallied Sciences*, vol. 3, no. 3, pp. 397–402, 2011.

[29] B. L. N. Y. Nkono, S. D. Sokeng, N. Y. Nicolas et al., "Effects of alstonia boonei aqueous extract in isolated intestinal and diaphragm glucose uptake in vitro," *International Journal of Diabetes Research*, vol. 5, no. 2, pp. 35–40, 2016.

[30] S. A. Adefegha, G. Oboh, O. S. Omojokun, T. O. Jimoh, and S. I. Oyeleye, "In vitro antioxidant activities of African birch (Anogeissus leiocarpus) leaf and its effect on the α-amylase and α-glucosidase inhibitory properties of acarbose," *Journal of Taibah University Medical Sciences*, vol. 11, no. 3, pp. 236–242, 2016.

[31] S. G. Jyothi, S. C. S. Chavan, and B. V. Somashekaraiah, "In vitro and in vivo antioxidant and antidiabetic efficacy of Cassia auriculata L. Flowers," *Global Journal of Pharmacology*, vol. 6, no. 1, pp. 33–40, 2012.

[32] H. Tanty and T. Herlina, "Antidiabetic activity test for leaves extract of Cassia siamea. lamk," *MATTER: International Journal of Science and Technology*, vol. 3, no. 3, pp. 339–348, 2018.

[33] S. H. Tiong, C. Y. Looi, H. Hazni et al., "Antidiabetic and antioxidant properties of alkaloids from Catharanthus roseus (L.) G. Don," *Molecules*, vol. 18, no. 8, pp. 9770–9784, 2013.

[34] A. T. Yakoob, N. B. Tajuddin, M. I. M. Hussain, S. Mathew, A. Govindaraju, and I. Qadri, "Antioxidant and hypoglycemic activities of clausena anisata (Willd.) Hook F. ex benth. root mediated synthesized silver nanoparticles," *Pharmacognosy Journal*, vol. 8, no. 6, pp. 579–586, 2016.

[35] M. A. Mogale, H. M. Mkhombo, S. L. Lebelo et al., "The effects of Clausena anisata (Wild) Hook leaf extracts on selected diabetic related metabolizing enzymes," *Journal of Medicinal Plants Research*, vol. 6, no. 25, pp. 4200–4207, 2012.

[36] A. D. Tchamgoue, L. R. Y. Tchokouaha, P. A. Tarkang, J.-R. Kuiate, and G. A. Agbor, "Costus afer possesses carbohydrate hydrolyzing enzymes inhibitory activity and antioxidant capacity in vitro," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 987984, 10 pages, 2015.

[37] S. Sabiu, E. O. Ajani, T. O. Sunmonu, and A. O. T. Ashafa, "Kinetics of modulatory role of Cyperus esculentus L. on the specific activity of key carbohydrate metabolizing enzymes," *African Journal of Traditional, Complementary, and Alternative Medicines*, vol. 14, no. 4, pp. 46–53, 2017.

[38] A. Ogundajo and A. T. Ashafa, "Phytochemical compositions and in vitro assessments of antioxidant and antidiabetic potentials of fractions from Ehretia cymosa Thonn," *Pharmacognosy Magazine*, vol. 13, no. 51, pp. S470–S480, 2017.

[39] J. Senthil, M. V. Rameashkannan, and P. Mani, "Phytochemical profiling of ethanolic leaves extract of Ipomoea sepiaria (Koenig Ex. Roxb)," *International Journal of Innovative Research in Science, Engineering and Technology*, vol. 5, no. 3, pp. 3140–3147, 2016.

[40] M. B. Adinortey, J. K. Sarfo, C. A. Adinortey et al., "Inhibitory effects of Launaea taraxacifolia and Strychnos spinosa leaves extract on an isolated digestive enzyme linked to type-2-diabetes mellitus," *Journal of Biology and Life Science*, vol. 9, no. 2, pp. 52–65, 2018.

[41] J. Bhuvaneshwari, S. Khanam, and K. Devi, "In-vitro enzyme inhibition studies for antidiabetic activity of mature and tender leaves of Mangifera indica var. Totapuri," *Journal of Microbiology And Biotechnology*, vol. 3, no. 3, pp. 36–41, 2014.

[42] A. Ganogpichayagrai, C. Palanuvej, and N. Ruangrungsi, "Antidiabetic and anticancer activities of Mangifera indica cv. Okrong leaves," *Journal of Advanced Pharmaceutical Technology & Research*, vol. 8, no. 1, pp. 19–24, 2017.

[43] T. S. Tunna, I. S. M. Zaidul, Q. U. Ahmed et al., "Analyses and profiling of extract and fractions of neglected weed Mimosa pudica Linn. traditionally used in Southeast Asia to treat diabetes," *South African Journal of Botany*, vol. 99, pp. 144–152, 2015.

[44] P. Muthumani, R. Meera, P. Devi et al., "Phytochemical investigation and enzyme inhibitory activity of Mimosa pudica Linn," *Journal of Chemical and Pharmaceutical Research*, vol. 2, no. 5, pp. 108–114, 2010.

[45] W. Khan, R. Parveen, K. Chester, S. Parveen, and S. Ahmad, "Hypoglycemic potential of aqueous extract of Moringa oleifera leaf and in vivo GC-MS metabolomics," *Frontiers in Pharmacology*, vol. 8, article 577, 2017.

[46] B. K. Harley, R. A. Dickson, and T. C. Fleischer, "Antioxidant, glucose uptake stimulatory, α-glucosidase and α-amylase inhibitory effects of myrianthus arboreus stem bark," *Natural Products Chemistry and Research*, vol. 5, no. 4, pp. 1–7, 2017.

[47] A. K. Nyarko, H. Asare-Anane, M. Ofosuhene, and M. E. Addy, "Extract of Ocimum canum lowers blood glucose and facilitates insulin release by isolated pancreatic β-islet cells," *Phytomedicine*, vol. 9, no. 4, pp. 346–351, 2002.

[48] M. R. Mishra, A. Mishra, D. K. Pradhan, A. K. Panda, R. K. Behera, and S. Jha, "Antidiabetic and antioxidant activity of

Scoparia dulcis linn," *Indian Journal of Pharmaceutical Sciences*, vol. 75, no. 5, pp. 610–614, 2013.

[49] M. J. Moshi, M. C. Kapingu, F. C. Uiso, Z. H. Mbwambo, and R. L. A. Mahunnah, "Some pharmacological properties of an aqueous extract of Securinega virosa roots," *Pharmaceutical Biology*, vol. 38, no. 3, pp. 214–221, 2000.

[50] K. F. Akinwunmi, A. A. Ajiboye, and O. O. Ojo, "Evaluation of α-Amylase Inhibitory Potentials of Sida acuta, Tithonia diversifolia and Chromolaena odorata Leaf Extracts," *Journal of Advances in Biology and Biotechnology*, vol. 14, no. 4, pp. 1–9, 2017.

[51] M. A. Ibrahim, N. A. Koorbanally, and M. S. Islam, "Antioxidative activity and inhibition of key enzymes linked to type-2 diabetes (α-glucosidase and α-amylase) by Khaya senegalensis," *Acta Pharmaceutica*, vol. 64, no. 3, pp. 311–324, 2014.

[52] A. O. A. S. A. Adefegha, "Inhibitory effects of aqueous extract of two varieties of ginger on some key enzymes linked to type-2 diabetes in vitro," *Journal of Food and Nutrition Research*, vol. 49, no. 1, pp. 14–20, 2010.

[53] K. S. Doh and C. B. Aké, "Effect of aqueous extract of Ageratum conyzoides leaves on the glycaemia of rabbits," *The Pharma Innovation*, vol. 2, no. 8, article 1, 2013.

[54] N. Nyunaï, N. Njikam, E. H. Abdennebi, J. T. Mbafor, and D. Lamnaouer, "Hypoglycaemic and antihyperglycaemic activity of Ageratum conyzoides L. in rats," *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 6, no. 2, pp. 123–130, 2009.

[55] J. E. Eyo, J. C. Ozougwu, and P. C. Echi, "Hypoglycaemic effects of Allium cepa, Allium sativum and Zingiber officinale aqueous extracts on alloxan-induced diabetic Rattus novergicus," *Medical Journal of Islamic World Academy of Sciences*, vol. 19, no. 3, pp. 121–126, 2011.

[56] A. E. Ojieh, E. C. Adegor, A. C. Okolo, E. O. Lawrence, I. P. Njoku, and C. U. Onyekpe, "Hypoglycemic and hypolipidaemic effect of allium cepa in streptozotocin-induced diabetes," *International Journal of Science and Engineering*, vol. 6, no. 10, pp. 23–29, 2015.

[57] A. Eidi, M. Eidi, and E. Esmaeili, "Antidiabetic effect of garlic (Allium sativum L.) in normal and streptozotocin-induced diabetic rats," *Phytomedicine*, vol. 13, no. 9-10, pp. 624–629, 2006.

[58] J. John, "Evaluation of hypoglycemic effect of Aloe vera on allaxon induced diabetic rats," *International Journal of Information Research and Review*, vol. 4, no. 3, pp. 3865–3868, 2017.

[59] O. J. Owolabi, I. M. Arhewoh, S. O. Innih, O. N. Anaka, and C. F. Monyei, "The ethanol leaf extract of Alstonia boonei (Apocynaceae) reduces hyperglycemia in alloxan-induced diabetic rats," *Nigerian Journal of Pharmaceutical Research*, vol. 13, no. 1, pp. 12–21, 2014.

[60] O. A. Akinloye, R. T. Oshilaja, O. A. Okelanfa, D. I. Akinloye, and O. M. O. Idowu, "Hypoglyceamic activity of Alstonia boonei stem bark extract in mice," *Agriculture and Biology Journal of North America*, vol. 4, no. 1, pp. 1–5, 2013.

[61] K. Girija, K. Lakshman, C. Udaya, G. Sabhya Sachi, and T. Divya, "Anti–diabetic and anti–cholesterolemic activity of methanol extracts of three species of Amaranthus," *Asian Pacific Journal of Tropical Biomedicine*, vol. 1, no. 2, pp. 133–138, 2011.

[62] R. Pandhare, S. Balakrishnan, P. Mohite, and S. Khanage, "Antidiabetic and antihyperlipidaemic potential of Amaranthus viridis (L.) Merr. in streptozotocin induced diabetic rats," *Asian Pacific Journal of Tropical Disease*, vol. 2, no. 1, pp. S180–S185, 2012.

[63] S. Uddin, M. M. Islam, M. M. Hassan, A. Bhowmik, and B. Rokeya, "Amaranthus viridis modulates anti-hyperglycemic pathways in hemi-diaphragm and improves glycogenesis liver function in rats," *Journal of Pharmacognosy and Phytotherapy*, vol. 8, no. 10, pp. 73–181, 2016.

[64] B. I. Aguh, I. H. Nock, I. S. Ndams, A. Agunu, and C. A. Ukwubile, "Hypoglycaemic activity and nephro-protectective effect of Bauhinia rufescens in alloxan-induced diabetic rats," *International Journal of Advances in Pharmacy, Biology and Chemistry*, vol. 2, no. 1, pp. 249–255, 2013.

[65] D. Njamen, B. N. Nkeh-Chungag, E. Tsala, Z. T. Fomum, J. C. Mbanya, and G. F. Ngufor, "Effect of bridelia ferruginea (euphorbiaceae) leaf extract on sucrose-induced glucose intolerance in rats," *Tropical Journal of Pharmaceutical Research*, vol. 11, no. 5, pp. 759–765, 2012.

[66] M. Latha and L. Pari, "Antihyperglycaemic effect of Cassia auriculata in experimental diabetes and its effects on key metabolic enzymes involved in carbohydrate metabolism," *Clinical and Experimental Pharmacology and Physiology*, vol. 30, no. 1-2, pp. 38–43, 2003.

[67] S. Gupta, S. B. Sharma, U. R. Singh, S. K. Bansal, and K. M. Prabhu, "Elucidation of mechanism of action of cassia auriculata leaf extract for its antidiabetic activity in streptozotocin-induced diabetic rats," *Journal of Medicinal Food*, vol. 13, no. 3, pp. 528–534, 2010.

[68] B. C. Hatapakki, H. M. Suresh, V. Bhoomannavar, and S. I. Shivkumar, "Effect of Cassia auriculata Linn flowers against alloxan-induced diabetes in rats," *Journal of Natural Remedies*, vol. 5, no. 2, pp. 132–136, 2005.

[69] L. Verma, A. Khatri, B. Kaushik, U. K. Patil, and R. S. Pawar, "Antidiabetic activity of *Cassia occidentalis* (Linn) in normal and alloxan-induced diabetic rats," *Indian Journal of Pharmacology*, vol. 42, no. 4, pp. 224–228, 2010.

[70] A. A. Omonkhua, I. O. Onoagbe, A. F. Ajileye et al., "Long term anti-diabetic, anti-hyperlipidaemic and anti-atherogenic effects of Carica papaya leaves in streptozotocin diabetic rats," *European Journal of Medicinal Plants*, vol. 3, no. 4, pp. 508–519, 2013.

[71] E. Vega-Ávila, J. L. Cano-Velasco, F. J. Alarcón-Aguilar, M. D. C. Fajardo Ortíz, J. C. Almanza-Pérez, and R. Román-Ramos, "Hypoglycemic activity of aqueous extracts from Catharanthus roseus," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 934258, 7 pages, 2012.

[72] K. Rasineni, R. Bellamkonda, S. R. Singareddy, and S. Desireddy, "Antihyperglycemic activity of Catharanthus roseus leaf powder in streptozotocin-induced diabetic rats," *Pharmacognosy Research*, vol. 2, no. 3, pp. 195–201, 2010.

[73] B. Aruljothi and S. S. Samipillai, "Antidiabetic activity of Catharanthus roseus in alloxan induced diabetic rats," *International Journal of Modern Research and Reviews*, vol. 4, no. 1, pp. 1094–1097, 2016.

[74] J. A. O. Ojewole, "Hypoglycaemic effect of Clausena anisata (Willd) Hook methanolic root extract in rats," *Journal of Ethnopharmacology*, vol. 81, no. 2, pp. 231–237, 2002.

[75] A. F. Uwah, E. G. Ewere, and J. I. Ndem, "Hypoglycemic and haematologic effects of crude stem juice of costus afer on alloxaninduced diabetic wistar rats," *American Journal of Ethnomedicine*, vol. 2, no. 4, pp. 2348–9502, 2015.

[76] A. N. Ezejiofor, C. N. Orish, and O. E. Orisakwe, "Morphological changes in the pancreas and glucose reduction of the aqueous extract of Costus afer leaf on alloxan-induced diabetic

rats," *Journal of Basic and Clinical Physiology and Pharmacology*, vol. 26, no. 6, pp. 595–601, 2015.

[77] W. Dakam, D. Kuate, B. Azantsa, and J. E. Oben, "Inhibitory effects Of Glyphaea Brevis (spreng.) monach on a-amylase activity: impact on postprandial blood glucose and weight control in rats: p27-23," *Annals of Nutrition and Metabolism*, vol. 55, article 274, 2009.

[78] P. A. Akah, S. U. Uzodinma, and C. E. Okolo, "Antidiabetic activity of aqueous and methanol extract and fractions of Gongronema latifolium (Asclepidaceae) leaves in alloxan diabetic rats," *Journal of Applied Pharmaceutical Science*, vol. 1, no. 9, pp. 99–102, 2011.

[79] C. O. Ibegbulem and P. C. Chikezie, "Hypoglycemic properties of ethanolic extracts of Gongronema latifolium, Aloe perryi, Viscum album and Allium sativum administered to alloxan-induced diabetic albino rats (Rattus norvegicus)," *Pharmacognosy Communications*, vol. 3, no. 2, pp. 12–16, 2013.

[80] C. Houacine, A. O. Elkhawad, and S. M. H. Ayoub, "A comparative study on the anti-diabetic activity of extracts of some Algerian and Sudanese plants," *Journal of Diabetes and Endocrinology*, vol. 3, no. 3, pp. 25–28, 2012.

[81] N. O. Muhammad, J. O. Akolade, L. A. Usman, and O. B. Oloyede, "Haematological parameters of alloxan-induced diabetic rats treated with leaf essential oil of Hoslundia opposita (Vahl)," *EXCLI Journal*, vol. 11, pp. 670–676, 2012.

[82] S. B. Mishra, A. Verma, A. Mukerjee, and M. Vijayakumar, "Anti-hyperglycemic activity of leaves extract of Hyptis suaveolens L. Poit in streptozotocin induced diabetic rats," *Asian Pacific Journal of Tropical Medicine*, vol. 4, no. 9, pp. 689–693, 2011.

[83] A. K. Nyarko, A. A. Sittie, and M. E. Addy, "The basis for the antihyperglycemic activity of Indigofera arrecta in the rat," *Phytotherapy Research*, vol. 7, no. 1, pp. 1–4, 1993.

[84] D. P. Basha, K. P. Kumar, B. B. Teja, and M. Subbarao, "Antidiabetic activity on extracts of Mangifera indica in alloxan monohydrate induced diabetic rats," *Drug Invention Today*, vol. 3, no. 7, pp. 165–168, 2011.

[85] B. Dineshkumar, A. Mitra, and M. Manjunatha, "Studies on the anti-diabetic and hypolipidemic potentials of mangiferin (xanthone glucoside) in streptozotocin-induced type 1 and type 2 diabetic model rats," *International Journal of Advances in Pharmaceutical Sciences*, vol. 1, no. 1, pp. 75–85, 2010.

[86] N. G. Sutar, U. N. Sutar, and B. C. Behera, "Antidiabetic activity of the leaves of Mimosa pudica Linn. in albino rats," *Journal of Herbal Medicine and Toxicology*, vol. 3, no. 1, pp. 123–126, 2009.

[87] P. Yupparach and A. Konsue, "Hypoglycemic and hypolipidemic activities of ethanolic extract from Mimosa pudica L. in normal and streptozotocin-induced diabetic rats," *Pharmacognosy Journal*, vol. 9, no. 6, pp. 834–837, 2014.

[88] T. Sindhu, S. Rajamanikandan, P. Ragavendran, D. Sophia, P. Meenakshi, and D. Durga, "Antidiabetic activity of Mollugo nudicaulis against alloxan induced diabetic rats," *International Journal of Applied Biology and Pharmaceutical Technology*, vol. 1, no. 3, pp. 511–519, 2010.

[89] O. A. Olajide, S. O. Awe, J. M. Makinde, and O. Morebise, "Evaluation of the anti-diabetic property of Morinda lucida leaves in streptozotocin-diabetic rats," *Journal of Pharmacy and Pharmacology*, vol. 51, no. 11, pp. 1321–1324, 1999.

[90] C. Ma, H. Yu, Y. Xiao, and H. Wang, "Momordica charantia extracts ameliorate insulin resistance by regulating the expression of socs-3 and jnk in type 2 diabetes mellitus rats," *Pharmaceutical Biology*, vol. 55, no. 1, pp. 2170–2177, 2017.

[91] M. F. Mahmoud, F. E. Z. Z. El Ashry, N. N. El Maraghy, and A. Fahmy, "Studies on the antidiabetic activities of Momordica charantia fruit juice in streptozotocin-induced diabetic rats," *Pharmaceutical Biology*, vol. 55, no. 1, pp. 758–765, 2017.

[92] R. A. Dickson, B. K. Harley, D. Berkoh et al., "Antidiabetic and haematological effect of myrianthus Arboreus p. beauv. stem bark extract in streptozotocin-induced diabetic rats," *International Journal of Pharmaceutical Sciences and Research*, vol. 7, no. 12, pp. 4812–4826, 2016.

[93] A. O. Sijuade, O. O. Omotayo, and O. A. Oseni, "Hypoglyceamic effect of methanolic extract of Pergularia daemia in alloxan-induced diabetic mice," *British Journal of Pharmaceutical Research*, vol. 4, no. 22, pp. 2614–2621, 2014.

[94] P. Lawson-Evi, K. Eklu-Gadegbeku, A. Agbonon, K. Aklikokou, E. Creppy, and M. Gbeassor, "Antidiabetic activity of Phyllanthus amarus schum and thonn (Euphorbiaceae) on alloxan induced diabetes in male wistar rats," *Journal of Applied Sciences*, vol. 11, no. 16, pp. 2968–2973, 2011.

[95] M. Garg, V. J. Dhar, and A. N. Kalia, "Antidiabetic and antioxidant potential of Phyllanthus fraternus in alloxan induced diabetic animals," *Pharmacognosy Magazine*, vol. 4, no. 14, pp. 138–143, 2008.

[96] Y. Tanko, M. A. Okasha, G. M. Magaji et al., "Anti-diabetic properties of Securinega virosa (Euphorbiaceae) leaf extract," *African Journal of Biotechnology*, vol. 7, no. 1, pp. 022–024, 2008.

[97] T. Dimo, F. T. Ngueguim, P. Kamtchouing, E. Dongo, and P. V. Tan, "Glucose lowering efficacy of the aqueous stem bark extract of Trema orientalis (Linn) Blume in normal and streptozotocin diabetic rats," *Die Pharmazie*, vol. 61, no. 3, pp. 233–236, 2006.

[98] W. C. Haas, "The role of ginger in type 2 diabetes mellitus," *Integrative Medicine Alert*, vol. 18, no. 8, pp. 85–87, 2015.

[99] S. A. Jafri, S. Abass, and M. Qasim, "Hypoglycemic effect of ginger (Zingiber officinale) in alloxan induced diabetic rats (Rattus norvagicus)," *Pakistan Veterinary Journal*, vol. 31, no. 2, pp. 160–162, 2011.

[100] K. E. Gaber, U. Singhal, and O. Daowd, "Hypoglycemic and hypolipidaemic effects of some common plants extract in Type 2 diabetic patients at Eldabba area (North Sudan)," *IOSR Journal of Pharmacy and Biological Sciences*, vol. 8, no. 6, pp. 38–43, 2013.

[101] R. Kumar, S. Chhatwal, S. Arora et al., "Antihyperglycemic, antihyperlipidemic, anti-inflammatory and adenosine deaminase–lowering effects of garlic in patients with type 2 diabetes mellitus with obesity," *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, vol. 6, pp. 49–56, 2013.

[102] M. S. J. Shoshi and H. Akter, "Effects of Garlic (Allium sativum) on blood Glucose Level in Type 2 Diabetes Mellitus Patients treated with Metformin," *Journal of Enam Medical College*, vol. 7, no. 3, pp. 151–155, 2017.

[103] N. Bunyapraphatsara, S. Yongchaiyudha, V. Rungpitarangsi, and O. Chokechaijaroenporn, "Antidiabetic activity of Aloe vera L. juice II. Clinical trial in diabetes mellitus patients in combination with glibenclamide," *Phytomedicine*, vol. 3, no. 3, pp. 245–248, 1996.

[104] J. A. Altschuler, S. J. Casella, T. A. MacKenzie, and K. M. Curtis, "The effect of cinnamon on A1C among adolescents with type 1 diabetes," *Diabetes Care*, vol. 30, no. 4, pp. 813–816, 2007.

[105] M. E. Addy and A. K. Nyarko, "Diabetic patients' response to oral administration of aqueous extract of Indigofera arrecta," *Phytotherapy Research*, vol. 2, no. 4, pp. 192–195, 1988.

[106] C. P. Ooi, Z. Yassin, and T.-A. Hamid, "Momordica charantia for type 2 diabetes mellitus," *Cochrane Database of Systematic Reviews*, vol. 8, pp. 1–43, 2012.

[107] V. S. Baldwa, C. M. Bhandari, A. Pangaria, and R. K. Goyal, "Clinical trial in patients with diabetes mellitus of an insulin-like compound obtained from plant source," *Upsala Journal of Medical Sciences*, vol. 82, no. 1, pp. 39–41, 1977.

[108] F. Shidfar, A. Rajab, T. Rahideh, and etall, "The effect of ginger (Zingiber officinale) on glycemic markers in patients with type 2 diabetes," *Journal of Complementary and Integrative Medicine*, vol. 12, no. 2, pp. 165–170, 2015.

[109] J. Thode, H. A. Pershadsingh, J. H. Ladenson, R. Hardy, and J. M. McDonald, "Palmitic acid stimulates glucose incorporation in the adipocyte by a mechanism likely involving intracellular calcium," *Journal of Lipid Research*, vol. 30, no. 9, pp. 1299–1305, 1989.

[110] T. Miron, A. Rabinkov, D. Mirelman, M. Wilchek, and L. Weiner, "The mode of action of allicin: Its ready permeability through phospholipid membranes may contribute to its biological activity," *Biochimica et Biophysica Acta*, vol. 1463, no. 1, pp. 20–30, 2000.

[111] M. Tanaka, E. Misawa, Y. Ito et al., "Identification of five phytosterols from aloe vera gel as anti-diabetic compounds," *Biological & Pharmaceutical Bulletin*, vol. 29, no. 7, pp. 1418–1422, 2006.

[112] A. Noor, V. S. Bansal, and M. A. Vijayalakshmi, "Current update on anti-diabetic biomolecules from key traditional Indian medicinal," *Current Science Association*, vol. 104, no. 6, pp. 721–727, 2013.

[113] A. A. Hosni, A. A. Abdel-Moneim, E. S. Abdel-Reheim, S. M. Mohamed, and H. Helmy, "Cinnamaldehyde potentially attenuates gestational hyperglycemia in rats through modulation of PPARγ, proinflammatory cytokines and oxidative stress," *Biomedicine & Pharmacotherapy*, vol. 88, pp. 52–60, 2017.

[114] G. N. Anyasor, O. Funmilayo, O. Odutola, A. Olugbenga, and E. M. Oboutor, "Chemical constituents in n-butanol fractions of Castus afer ker Gawl leaf and stem," *Journal of Intercultural Ethnopharmacology*, vol. 3, no. 2, pp. 78–84, 2014.

[115] L. Harinantenaina, M. Tanaka, S. Takaoka et al., "Momordica charantia constituents and antidiabetic screening of the isolated major compounds," *Chemical and Pharmaceutical Bulletin*, vol. 54, no. 7, pp. 1017–1021, 2006.

[116] G. Pamunuwa, D. N. Karunaratne, and V. Y. Waisundara, "Antidiabetic Properties, bioactive constituents, and other therapeutic effects of scoparia dulcis," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 8243215, 11 pages, 2016.

[117] M. J. Son, Y. Miura, and K. Yagasaki, "Mechanisms for antidiabetic effect of gingerol in cultured cells and obese diabetic model mice," *Cytotechnology*, vol. 67, no. 4, pp. 641–652, 2015.

[118] C.-S. Jiang, L.-F. Liang, and Y.-W. Guo, "Natural products possessing protein tyrosine phosphatase 1B (PTP1B) inhibitory activity found in the last decades," *Acta Pharmacologica Sinica*, vol. 33, no. 10, pp. 1217–1245, 2012.

[119] M. Ashwini, J. Balaganesh, S. Balamurugan, S. B. Murugan, and R. Sathishkumar, "Antioxidant activity in in Vivo and in Vitro cultures of onion varieties (Bellary and CO3)," *Journal of Food and Nutrition Sciences*, vol. 4, no. 9, pp. 918–923, 2013.

[120] J. Y. Jung, Y. Lim, M. S. Moon, J. Y. Kim, and O. Kwon, "Onion peel extracts ameliorate hyperglycemia and insulin resistance in high fat diet/streptozotocin-induced diabetic rats," *Nutrition and Metabolism*, vol. 8, no. 18, pp. 1–8, 2011.

[121] P. Suresh Babu and K. Srinivasan, "Influence of dietary capsaicin and onion on the metabolic abnormalities associated with streptozotocin induced diabetes mellitus," *Molecular and Cellular Biochemistry*, vol. 175, no. 1-2, pp. 49–57, 1997.

[122] K. E. Campos, Y. S. Diniz, A. C. Cataneo, L. A. Faine, M. J. Q. F. Alves, and E. L. B. Novelli, "Hypoglycemic and antioxidant effect of onion, Allium cepa: diatary onion addition, antioxidant activity and hypoglycic effect on diabetic rats," *International Journal of Food Sciences and Nutrition*, vol. 54, no. 3, pp. 241–246, 2003.

[123] S. M. Kelkar, G. S. Kaklij, and V. A. Bapat, "Determination of antidiabetic activity in Allium cepa (onion) tissue cultures," *Indian Journal of Biochemistry and Biophysics*, vol. 38, no. 4, pp. 277–279, 2001.

[124] F. M. El-Demerdash, M. I. Yousef, and N. I. A. El-Naga, "Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats," *Food and Chemical Toxicology*, vol. 43, no. 1, pp. 57–63, 2005.

[125] C. G. Sheela, K. Kumud, and K. T. Augusti, "Anti-diabetic effects of onion and garlic sulfoxide amino acids in rats," *Planta Medica*, vol. 61, no. 4, pp. 356-357, 1995.

[126] K. Kumari, B. C. Mathew, and K. T. Augusti, "Antidiabetic and hypolipidemic effects of S-methyl cysteine sulfoxide isolated from Allium cepa Linn.," *Indian Journal of Biochemistry and Biophysics*, vol. 32, no. 1, pp. 49–54, 1995.

[127] P. T. Mathew and K. T. Augusti, "Hypoglycemic effects of onion, Allium cepa Linn. on diabetes mellitus-a preliminary report," *Indian Journal of Physiology and Pharmacology*, vol. 19, no. 4, pp. 213–217, 1975.

[128] M. Corzo-Martínez, N. Corzo, and M. Villamiel, "Biological properties of onions and garlic," *Trends in Food Science & Technology*, vol. 18, no. 12, pp. 609–625, 2007.

[129] S. Ziegler and O. Sticher, " HPLC of *S* -Alk(en)yl- L -cysteine Derivatives in Garlic including Quantitative Determination of (+)- *S* -Allyl- L -cysteine Sulfoxide (Alliin) ," *Planta Medica*, vol. 55, no. 04, pp. 372–378, 1989.

[130] C. T. Musabayane, P. T. Bwititi, and J. A. O. Ojewole, "Effects of oral administration of some herbal extracts on food consumption and blood glucose levels in normal and streptozotocin-treated diabetic rats," *Methods and Findings in Experimental and Clinical Pharmacology*, vol. 28, no. 4, pp. 223–228, 2006.

[131] R. C. Jain and C. R. Vyas, "Garlic in alloxan induced diabetic rabbits," *American Journal of Clinical Nutrition*, vol. 28, no. 7, pp. 684-685, 1975.

[132] G. A. Jelodar, M. Maleki, M. H. Motadayen, and S. Sirus, "Effect of fenugreek, onion and garlic on blood glucose and histopathology of pancreas of alloxan-induced diabetic rats," *Indian Journal of Medical Sciences*, vol. 59, no. 2, pp. 64–69, 2005.

[133] A. Hattori, N. Yamada, T. Nishikawa, H. Fukuda, and T. Fujino, "Antidiabetic effects of ajoene in genetically diabetic KK-Ay mice," *Journal of Nutritional Science and Vitaminology*, vol. 51, no. 5, pp. 382–384, 2005.

[134] C.-T. Liu, H. Hse, C.-K. Lii, P.-S. Chen, and L.-Y. Sheen, "Effects of garlic oil and diallyl trisulfide on glycemic control in diabetic rats," *European Journal of Pharmacology*, vol. 516, no. 2, pp. 165–173, 2005.

[135] K. T. Augusti and C. G. Sheela, "Antiperoxide effect of S-allyl cysteine sulfoxide, an insulin secretagogue, in diabetic rats," *Experientia*, vol. 52, no. 2, pp. 115–119, 1996.

[136] Y. Adachi, J. Yoshida, Y. Kodera, A. Katoh, J. Takada, and H. Sakurai, "Bis (allixinato) oxovanadium (IV) Complex Is

a Potent Antidiabetic Agent: Studies on Structure-Activity Relationship for a Series of Hydroxypyrone– Vanadium Complexes," *Journal of Medicinal Chemistry*, vol. 49, no. 11, pp. 3251–3256, 2006.

[137] P. T. Mathew and K. T. Augusti, "Studies on the effect of allicin (diallyl disulphide-oxide) on alloxan diabetes. I. Hypoglycemic action and enhancement of serum insulin effect and glycogen synthesis," *Indian Journal of Biochemistry and Biophysics*, vol. 10, no. 3, pp. 209–212, 1973.

[138] O. R. Johnson, O. P. Emeka, and B. P. Femi, "Comparative effect of daily administration of allium sativum and allium cepa extracts on alloxan induced diabetic rats," *Journal of Biotechnology and Biochemistry*, vol. 1, no. 2, pp. 26–31, 2015.

[139] N. Ghannam, M. Kingston, I. A. Al-Meshaal, M. Tariq, N. S. Parman, and N. Woodhouse, "The antidiabetic activity of aloes: Preliminary clinical and experimental observations," *Hormone Research in Paediatrics*, vol. 24, no. 4, pp. 288–294, 1986.

[140] A. Okyar, A. Can, N. Akev, G. Baktir, and N. Sütlüpinar, "Effect of Aloe vera leaves on blood glucose level in type I and type II diabetic rat models," *Phytotherapy Research*, vol. 15, no. 2, pp. 157–161, 2001.

[141] S. Rajasekaran, K. Sivagnanam, K. Ravi, and S. Subramanian, "Hypoglycemic effect of aloe vera gel on streptozotocin-induced diabetes in experimental rats," *Journal of Medicinal Food*, vol. 7, no. 1, pp. 61–66, 2004.

[142] S. Rajasekaran, K. Sivagnanam, and S. Subramanian, "Modulatory effects of Aloe vera leaf gel extract on oxidative stress in rats treated with streptozotocin," *Journal of Pharmacy and Pharmacology*, vol. 57, no. 2, pp. 241–246, 2005.

[143] E. Misawa, M. Tanaka, K. Nomaguchi et al., "Administration of phytosterols isolated from Aloe vera gel reduce visceral fat mass and improve hyperglycemia in Zucker diabetic fatty (ZDF) rats," *Obesity Research & Clinical Practice*, vol. 2, no. 4, pp. 239–245, 2008.

[144] S. Yongchaiyudha, V. Rungpitarangsi, N. Bunyapraphatsara, and O. Chokechaijaroenporn, "Antidiabetic activity of Aloe vera L. juice. I. Clinical trial in new cases of diabetes mellitus," *Phytomedicine*, vol. 3, no. 3, pp. 241–243, 1996.

[145] F. E. Wehash, I. I. Abpo-Ghanema, and R. M. Saleh, "Some physiological effects of Momordica charantia and Trigonella foenum-graecum extracts in diabetic rats as compared with cidophage®," *World Academy of Science, Engineering and Technology*, vol. 64, pp. 1206–1214, 2012.

[146] T. Miura, Y. Itoh, N. Iwamoto, M. Kato, and T. Ishida, "Suppressive activity of the fruit of Momordica charantia with exercise on blood glucose in type 2 diabetic mice," *Biological & Pharmaceutical Bulletin*, vol. 27, no. 2, pp. 248–250, 2004.

[147] Y. Srivastava, H. Venkatakrishna-Bhatt, and Y. Verma, "Effect of Momordica charantia linn. Pomous aqueous extract on cataractogenesis in murrin alloxan diabetics," *Pharmacological Research Communications*, vol. 20, no. 3, pp. 201–209, 1988.

[148] D. S. Sekar, K. Sivagnanam, and S. Subramanian, "Antidiabetic activity of Momordica charantia seeds on streptozotocin induced diabetic rats," *Die Pharmazie*, vol. 60, no. 5, pp. 383–387, 2005.

[149] B. A. Shibib, L. A. Khan, and R. Rahman, "Hypoglycemic activity of Coccinia indica and Momordica charantia in diabetic rats: depression of the hepatic gluconeogenic enzymes glucose-6-phosphatase and fructose-1, 6-bisphosphatase and elevation of both liver and red-cell shunt enzyme glucose-6-phosphate dehydrogenase," *Biochemical Journal*, vol. 292, no. 1, pp. 267–270, 1993.

[150] T. B. Ng, C. M. Wong, W. W. Li, and H. W. Yeung, "Insulin-like molecules in Momordica charantia seeds," *Journal of Ethnopharmacology*, vol. 15, no. 1, pp. 107–117, 1986.

[151] J. C. Chen, C. Bik-San Lau, J. Y. W. Chan et al., "The antigluconeogenic activity of cucurbitacins from Momordica charantia," *Planta Medica*, vol. 81, no. 4, pp. 327–332, 2015.

[152] H. Matsuda, Y. Li, T. Murakami, N. Matsumura, J. Yamahara, and M. Yoshikawa, "Antidiabetic principles of natural medicines. III. Structure-related inhibitory activity and action mode of oleanolic acid glycosides on hypoglycemic activity," *Chemical & Pharmaceutical Bulletin*, vol. 46, no. 9, pp. 1399–1403, 1998.

[153] P. Khanna, S. C. Jain, A. Panagariya, and V. P. Dixit, "Hypoglycemic activity of polypeptide-p from a plant source," *Journal of Natural Products*, vol. 44, no. 6, pp. 648–655, 1981.

[154] R. N. Husna, A. Noriham, H. Nooraain, A. H. Azizah, and O. F. Amna, "Acute oral toxicity effects of Momordica charantia in sprague dawley rats," *International Journal of Bioscience, Biochemistry and Bioinformatics*, vol. 3, no. 4, pp. 408–410, 2013.

[155] G. M. Shah, M. A. Khan, M. Ahmad, M. Zafar, and A. A. Khan, "Observations on antifertility and abortifacient herbal drugs," *African Journal of Biotechnology*, vol. 8, no. 9, pp. 1959–1964, 2009.

[156] B. Qin, K. S. Panickar, and R. A. Anderson, "Cinnamon: Potential role in the prevention of insulin resistance, metabolic syndrome, and type 2 diabetes," *Journal of Diabetes Science and Technology*, vol. 4, no. 3, pp. 685–693, 2010.

[157] K. Couturier, B. Qin, C. Batandier et al., "Cinnamon increases liver glycogen in an animal model of insulin resistance," *Metabolism - Clinical and Experimental*, vol. 60, no. 11, pp. 1590–1597, 2011.

[158] J. Li, T. Liu, L. Wang et al., "Antihyperglycemic and antihyperlipidemic action of cinnamaldehyde in C57blks/j Db/db mice," *Journal of Traditional Chinese Medicine*, vol. 32, no. 3, pp. 446–452, 2012.

[159] P. Subash Babu, S. Prabuseenivasan, and S. Ignacimuthu, "Cinnamaldehyde-A potential antidiabetic agent," *Phytomedicine*, vol. 14, no. 1, pp. 15–22, 2007.

[160] A. H. Shah, A. H. Al-Shareef, A. M. Ageel, and S. Qureshi, "Toxicity studies in mice of common spices, Cinnamomum zeylanicum bark and Piper longum fruits," *Plant Foods for Human Nutrition*, vol. 52, no. 3, pp. 231–239, 1998.

[161] A. N. Ezejiofor, Z. N. Igweze, N. A. Udowelle, and O. E. Orisakwe, "Histopathological and biochemical assessments of Costus afer stem on alloxan-induced diabetic rats," *Journal of Basic and Clinical Physiology and Pharmacology*, vol. 28, no. 4, pp. 383–391, 2017.

[162] K. T. G. Nwauche, C. C. Monago, and F. C. Anacletus, "Antihyperglycemic activity of the aqueous extract of Costus afer stem alone and in combination with metformin," *European Journal of Biotechnology and Bioscience*, vol. 1, no. 5, pp. 19–25, 2014.

[163] A. N. Ezejiofor, C. N. Orish, and O. E. Orisakwe, "Cytological and biochemical studies during the progression of alloxan-induced diabetes and possible protection of an aqueous leaf extract of Costus afer," *Chinese Journal of Natural Medicines*, vol. 12, no. 10, pp. 745–752, 2014.

[164] S. Momoh, O. W. Yusuf, M. M. Adamu, C. O. C. Agwu, and F. O. Atanu, "Evaluation of the phytochemical composition and hypoglycemic activity of methanolic leaves extract of Costus afer in albino rats," *British Journal of Pharmaceutical Research*, vol. 1, no. 1, pp. 1–8, 2011.

[165] M. Jesus, A. P. J. Martins, E. Gallardo, and S. Silvestre, "Diosgenin: recent highlights on pharmacology and analytical methodology," *Journal of Analytical Methods in Chemistry*, vol. 2016, Article ID 4156293, 16 pages, 2016.

[166] D. M. Kanchan, G. S. Somani, V. V. Peshattiwar, A. A. Kaikini, and S. Sathaye, "Renoprotective effect of diosgenin in streptozotocin induced diabetic rats," *Pharmacological Reports*, vol. 68, no. 2, pp. 370–377, 2016.

[167] A. N. Ezejiofor, C. N. Orish, and O. E. Orisakwe, "Effect of aqueous leaves extract of Costus afer Ker Gawl (Zingiberaceae) on the liver and kidney of male albino Wistar rat," *Ancient Science of Life*, vol. 33, no. 1, article 4, 2013.

[168] H. S. Parmar and A. Kar, "Possible amelioration of atherogenic diet induced dyslipidemia, hypothyroidism and hyperglycemia by the peel extracts of Mangifera indica, Cucumis melo and Citrullus vulgaris fruits in rats," *BioFactors*, vol. 33, no. 1, pp. 13–24, 2008.

[169] P. Apontes, Z. Liu, K. Su et al., "Mangiferin stimulates carbohydrate oxidation and protects against metabolic disorders induced by high-fat diets," *Diabetes*, vol. 63, no. 11, pp. 3626–3636, 2014.

[170] X. Li, X. Cui, X. Sun, X. Li, Q. Zhu, and W. Li, "Mangiferin prevents diabetic nephropathy progression in streptozotocin-induced diabetic rats," *Phytotherapy Research*, vol. 24, no. 6, pp. 893–899, 2010.

[171] A. H. M. Zulfiker, F. A. Ripa, M. M. Rahman et al., "Antidiabetic and antioxidant effect of Scoparia dulcis in alloxan induced albino mice," *International Journal of PharmTech Research*, vol. 2, no. 4, pp. 2527–2534, 2010.

[172] Q. Liu, Q.-M. Yang, H.-J. Hu et al., "Bioactive diterpenoids and flavonoids from the aerial parts of Scoparia dulcis," *Journal of Natural Products*, vol. 77, no. 7, pp. 1594–1600, 2014.

[173] M. Latha and L. Pari, "Effect of an aqueous extract of Scoparia dulcis on blood glucose, plasma insulin and some polyol pathway enzymes in experimental rat diabetes," *Brazilian Journal of Medical and Biological Research*, vol. 37, no. 4, pp. 577–586, 2004.

[174] M. Latha, L. Pari, S. Sitasawad, and R. Bhonde, "Insulin-secretagogue activity and cytoprotective role of the traditional antidiabetic plant Scoparia dulcis (Sweet Broomweed)," *Life Sciences*, vol. 75, no. 16, pp. 2003–2014, 2004.

[175] J. E. Beh, J. Latip, M. P. Abdullah, A. Ismail, and M. Hamid, "Scoparia dulcis (SDF7) endowed with glucose uptake properties on L6 myotubes compared insulin," *Journal of Ethnopharmacology*, vol. 129, no. 1, pp. 23–33, 2010.

[176] M. Latha, L. Pari, K. M. Ramkumar et al., "Antidiabetic effects of scoparic acid D isolated from Scoparia dulcis in rats with streptozotocin-induced diabetes," *Natural Product Research*, vol. 23, no. 16, pp. 1528–1540, 2009.

[177] K. Tobe, R. Suzuki, M. Aoyama et al., "Increased expression of the sterol regulatory element-binding protein-1 gene in insulin receptor substrate-2–/– mouse liver," *The Journal of Biological Chemistry*, vol. 276, no. 42, pp. 38337–38340, 2001.

[178] C. Bumke-Vogt, M. A. Osterhoff, A. Borchert et al., "The flavones apigenin and luteolin induce FOXO1 translocation but inhibit gluconeogenic and lipogenic gene expression in human cells," *PLoS ONE*, vol. 9, no. 8, article e104321, 2014.

[179] J. A. O. Ojewole, "Analgesic, antiinflammatory and hypoglycaemic effects of ethanol extract of Zingiber officinale (Roscoe) rhizomes (Zingiberaceae) in mice and rats," *Phytotherapy Research*, vol. 20, no. 9, pp. 764–772, 2006.

[180] M. Sharma and S. Shukla, "Hypoglycaemic effect of ginger," *Journal of Research in Indian Medicine, Yoga and Homeopathy*, vol. 12, pp. 127–130, 1977.

[181] R. S. Ahmed and S. B. Sharma, "Biochemical studies on combined effects of garlic (Allium sativum Linn) and ginger (Zingiber officinale Rosc) in albino rats," *Indian Journal of Experimental Biology*, vol. 35, no. 8, pp. 841–843, 1997.

[182] S. M. Zick, Z. Djuric, M. T. Ruffin et al., "Pharmacokinetics of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and conjugate metabolites in healthy human subjects," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 17, no. 8, pp. 1930–1936, 2008.

[183] F. L. Bowling, J. Paterson, and A. Ndip, "Applying 21st century imaging technology to wound healing: an Avant-Gardist approach.," *Journal of Diabetes Science and Technology*, vol. 7, no. 5, pp. 1190–1194, 2013.

[184] Organizacion Mundial de la Salud and World Health Organization, "WHO guidelines on good agricultural and collection practices [GACP] for medicinal plants," World Health Organization, 2003.

[185] H. C. Voon, R. Bhat, and G. Rusul, "Flower extracts and their essential oils as potential antimicrobial agents for food uses and pharmaceutical applications," *Comprehensive Reviews in Food Science and Food Safety*, vol. 11, no. 1, pp. 34–55, 2012.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 8104951, 10 pages
https://doi.org/10.1155/2019/8104951

Research Article

Thirteen-Week Oral Toxicity Study of HVC1 in Rats

Kyungjin Lee and Ho-Young Choi

Department of Herbal Pharmacology, College of Korean Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea

Correspondence should be addressed to Ho-Young Choi; hychoi@khu.ac.kr

Received 17 February 2019; Accepted 31 March 2019; Published 11 April 2019

Guest Editor: José C. T. Carvalho

Studies on the safety of herbal medicine are essential for the development of new drugs. The aim of this study was to evaluate the no-observed-adverse-effect-level (NOAEL) of HVC1 (Gamisamhwangsasim-tang, a 30% ethanol extract of a mixture of Pruni Cortex, Scutellariae Radix, Coptidis Rhizoma, and Rhei Rhizoma) and identify its target organs after oral administration to Sprague-Dawley (SD) rats repeatedly for 13 weeks. Three test groups were treated with HVC1 at a dose of either 500 (low-dose), 1,000 (middle-dose), or 2,000 (high-dose) mg/kg/day. Another group received high-dose HVC1 and was observed for 4 weeks following treatment to examine recovery from the effects of the extract. All treatment groups were compared to a vehicle control group. During the study, mortality, clinical signs, body weight changes, food consumption, abnormal lesions in the eye, urinary parameters, hematological parameters, blood coagulation time, blood biochemical parameters, changes in organ weight, gross findings, and histopathological changes were examined. No systemic toxicity related to HVC1 was observed in any group, and it was concluded that the NOAEL of HVC1 was 2,000 mg/kg/day. No target organ was identified.

1. Introduction

Scutellariae Radix (SR), Coptidis Rhizoma (CR), Rhei Rhizoma (RR), and Pruni Cortex (PC) have been commonly used for several thousand years in traditional Chinese, Japanese, and Korean medicine. SR has been used to treat dampness-heat, stuffiness and fullness, jaundice, high fever, thirst, hematemesis, epistaxis, and threatened abortion [1], and it has been reported to have hypotensive [2] and antihyperlipidemic effects [3]. Moreover, SR components wogonin and baicalin have vasorelaxant activities [4, 5]. CR has been used to treat dampness-heat, stuffiness and fullness, acid regurgitation, jaundice, high fever, hyperactive heart fire, inability to sleep, palpitations, hematemesis, and epistaxis [1], and it has been reported to have hypocholesterolemic activity [6] and antihyperglycemic effects. RR produces antihyperlipidemic effects [7] and has been used to treat constipation, hematemesis, epistaxis, amenorrhea, intestinal abscess, and blood stasis [1]. The herbal prescription Samhwangsasim-tang (San-Huang-Xie-Xin-Tang in Chinese), containing SR, CR, and RR, produces hypotensive effects [8] and neuroprotective effects [9, 10]. PC has been used to treat cough, urticaria, pruritus, dermatitis, asthma, and measles, and we showed that PC has a potent vasorelaxant effect [11]. These reports show that SR, CR, RR, and PC could be useful as clinical therapies for the treatment of hypertension and hyperlipidemia. Safety and efficacy studies of herbal medicines and prescriptions are necessary for the effective use of traditional medicines and new drug developments. During the process of obtaining Investigational New Drug (IND) approval of these herbal medicines, the Ministry of Food and Drug Safety of Korea demands efficacy data and toxicity data including acute, subacute (4-week), and repeated dose (13-week) oral toxicity. Therefore, we prepared an herbal extract (Gamisamhwangsasim-tang, abbreviated HVC1) of SR, CR, RR, and PC and investigated the hypotensive and hypolipidemic effects of this preparation and the results have already been reported [12–15]. In addition, we have also evaluated acute and subacute toxicity studies and reported safety results [15]. This study was conducted to assess the cumulative toxicity of HVC1 when orally administered once daily to SD rats for a period of 13 weeks at doses of 500, 1,000, and 2,000 mg/kg/day. The reversibility of any effects during a 4-week recovery phase was also examined.

Table 1: Preparation of HVC1.

Herb	Used part	Amount (kg)	Company of purchase	Source
Pruni Cortex	Stem bark	6.7	DWD	Korea
Rhei Rhizoma	Root	6.7	DWD	China
Coptidis Rhizoma	Root	3.3	DYH	China
Scutellariae Radix	Root	3.3	DYH	Korea
Total amount		20.0		
Extract yield		4.6		

DWD: Dongwoodang Co., Ltd. (Yeongcheon city, Kyungpook, Republic of Korea).
DYH: Dong Yang Herb Co., Ltd. (Seoul, Republic of Korea).

2. Materials and Methods

2.1. Compliance with Ethical and Procedural Standards. This study was performed at Korea Conformity Laboratories (KCL, Incheon, Korea) with the approval of KCL's Institutional Animal Care and Use Committees (IA14-00150). The procedures used in this study were in compliance with KCL's ethical standards, in accordance with Good Laboratory Practice (GLP) of the Ministry of Food and Drug Safety (MFDS, No. 2013-40), and in line with the applicable guidelines for repeated-dose 90-day oral toxicity studies in rodents (OECD guideline No. 408) and the testing guidelines for safety evaluation of drug (MFDS guideline No. 2014-06).

2.2. Preparation of HVC1. A decoction of HVC1 was produced by Kolmar Korea Co., Ltd. (Seojeong, Korea) from a mixture of chopped crude herbs (Table 1). Professor Hocheol Kim of Kyung Hee University identified the materials. The herbal mixture (20 kg) was extracted with 30% ethanol for 2 h in a reflux apparatus. After reflux and filtration, the solvent was removed under reduced pressure at 80–95°C to yield 4.6 kg of crude extract (1 g extract = about 4.35 g raw/dried herb).

2.3. Test Substance and Dosing Solution. HVC1 was dissolved in distilled water and dosing solutions were prepared daily. The dose volume was 10 mL/kg/day for all animals. To maintain a constant dose, the dose volume was adjusted to the latest recorded body weight for each individual rat.

2.4. High-Performance Liquid Chromatography (HPLC) Conditions. The HPLC analysis was performed at room temperature, at a flow rate of 0.5 mL/min, and for a duration of 100 min using the same equipment used in the previous study [16]. The injection volume was 10 μL. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (HPLC grade, J.T. Baker®, Avantor Performance Materials, Inc., Center Valley, PA, USA) (B) in a ratio specified by the following binary gradient with linear interpolation: 0 min, 20% B; 60 min, 30% B; 70 min, 60% B; and 100 min, 70% B. The column eluent was monitored at 250 nm.

2.5. Animals. Male and female pathogen-free SD rats (approximately 5-week-old) were obtained from Orientbio Inc. (Sungnam, Gyeonggi province, Korea). On arrival, the animals were examined for signs of ill health and were allowed to acclimatize for 6 days prior to start of the study. One day before the start of oral administration, the animals were divided into 4 main groups and 2 recovery groups according to body weight. The rats were housed in stainless steel cages with a barrier system to control the light-dark cycle (08–20 h), air exchange (10–15 changes/h), temperature (23.1 ± 1.0°C), and relative humidity (54.2 ± 5.6 %) during the study. Animals were provided Teklad certified global 18% protein rodent diet (Harlan Co., Ltd., Madison, WI, USA) and filtered municipal tap water. Food and water were provided *ad libitum*.

Table 2: Calibration parameters of HPLC analysis for the 9 tested compounds.

Compound	Regression equation	R^2
1	$y = 4699.07x - 32464.02$	0.9950
2	$y = 17643.82x - 1925.72$	0.9998
3	$y = 54479.75x - 146134.56$	0.9996
4	$y = 29573.31x - 72978.81$	0.9999
5	$y = 55396.74x + 6805.61$	0.9995
6	$y = 48543.21x - 108301.56$	0.9999
7	$y = 17086.68x - 57907.43$	0.9994
8	$y = 8381.04x - 14106.11$	0.9999
9	$y = 104880.25x - 190133.26$	0.9997

Compounds: 1, sennoside A; 2, genistein-7-O-β-glucopyranoside; 3, coptisine; 4, baicalin; 5, prunetin-5-O-β-glucopyranoside; 6, berberine; 7, baicalein; 8, wogonin; 9, prunetin.

2.6. Experimental Design

2.6.1. Experimental Groups and Administration. In previous studies using rats, the effective dose of HVC1 was 250 mg/kg/day. We calculated the pharmacologically active dose in humans according to guidelines on converting animal doses to human-equivalent doses [17]: 250 mg/kg/day/6.17 (conversion factor for rats)/10 (safety factor) × 60 (mean weight of a person in kg) = 243.1 mg/kg/day. Therefore, the recommended daily dose of HVC1 for humans is 250 mg/person/day. In a previous dose selection study (repeated dosing of 0, 500, 1000, or 2000 mg/kg/day HVC1 for 4 weeks), treatment-related toxicity was not observed. Therefore, in the present 13-week study, the dose range of 0, 500, 1,000, and 2000 mg/kg/day HVC1 was chosen.

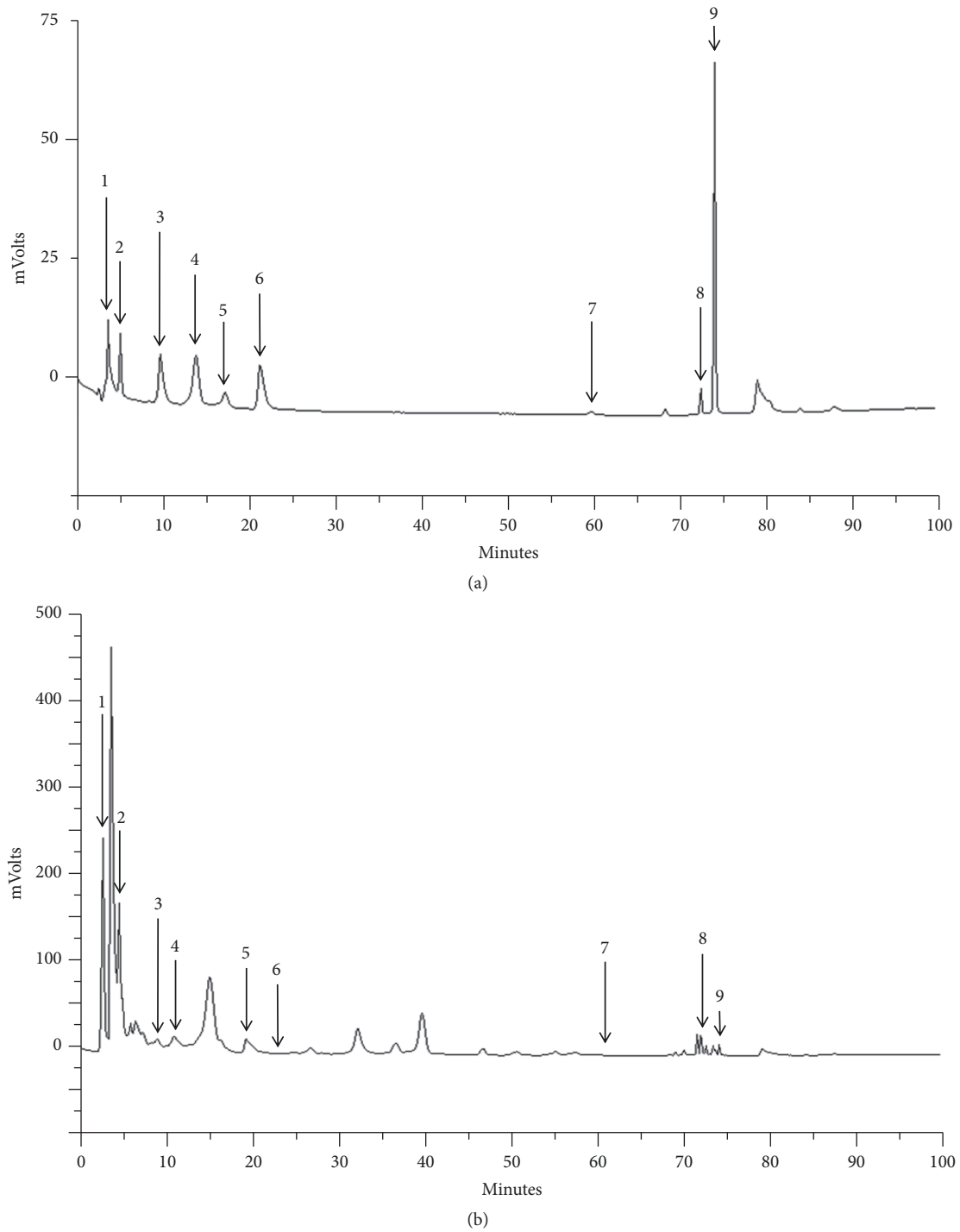


Figure 1: HPL chromatogram of standard mixtures (a) and HVC1 (b). Compounds: 1, sennoside A; 2, genistein-7-O-β-glucopyranoside; 3, coptisine; 4, baicalin; 5, prunetin-5-O-β-glucopyranoside; 6, berberine; 7, baicalein; 8, wogonin; and 9, prunetin.

The study included 4 main groups with 10 males and 10 females in each group and 2 recovery groups with 5 males and 5 females in each group. The main groups were a vehicle (distilled water) control group and 3 treatment groups receiving 500, 1,000, or 2,000 mg/kg/day HVC1 for 13 weeks. The recovery groups (a vehicle-treated control group and a group treated with high-dose (2,000 mg/kg/day) HVC1) received treatment for 13 weeks, followed by 4 weeks without further treatment. The test substances and vehicle were orally administered once daily into the stomach by gavage (using a stainless steel catheter attached to a disposable syringe).

2.6.2. Clinical Sign Observation and Body Weight. During the course of the experiment, all animals were observed

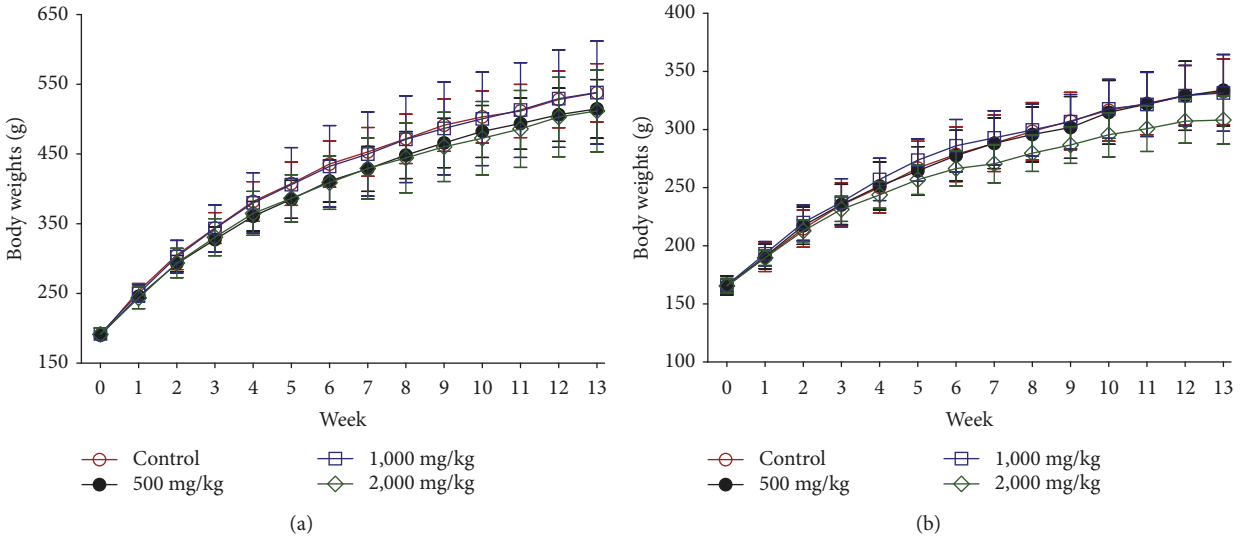


Figure 2: Body weight of male (a) and female (b) rats treated with 0 (control), 500, 1,000, or 2,000 mg/kg HVC1 for 13 weeks. Values are expressed as mean ± SD.

daily for clinical signs. Body weight and food consumption were measured weekly just before dosing and on the day of necropsy.

2.6.3. Ophthalmoscopy and Urinalysis. Ophthalmoscopic examinations of all rats were conducted prior to the start of the treatment and prior to necropsy by using an ophthalmoscope (Vantage plus, Keeler Ltd., Windsor, UK) and a digital fundus camera (Genesis, Kowa Co., Ltd., Nagoya, Japan).

Urine was collected for urinalysis from 5 rats in each group prior to necropsy. Urinalysis was performed to assess urine volume, specific gravity, pH, glucose, bilirubin, ketone bodies, protein, urobilinogen, nitrates, leukocytes, cast, occult blood, red blood cells, white blood cells, and epithelial cells using a urine analyzer (CliniTek 50, Siemens, Munich, Germany).

2.6.4. Hematology and Serum Biochemistry. Blood samples were collected from an abdominal artery and analyzed to determine white blood cell count, red blood cell count, hemoglobin concentration, hematocrit, neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, large unstained cell count, reticulocyte count, platelet count, percentage of neutrophils, percentage of lymphocytes, percentage of monocytes, percentage of eosinophils, percentage of basophils, percentage of large unstained cells, mean corpuscular volume, mean corpuscular hemoglobin concentration, red cell width distribution, and mean platelet volume using an automatic hematological analyzer (ADVIA 2120, Siemens, Munich, Germany). In addition, blood samples were treated with 3.2% sodium citrate and analyzed for prothrombin time and activated partial thromboplastin time using an automatic coagulation time meter (ACL 7000, Instrumentation Laboratory, Bedford, MA, USA).

Serum biochemistry analysis was performed to assess the levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma(γ)-glutamyl transferase, lactate dehydrogenase, blood urea nitrogen, creatinine, glucose, total cholesterol, total protein, creatine phosphokinase, albumin, total bilirubin, triglycerides, uric acid, calcium, inorganic phosphorus, chloride, magnesium, sodium, and potassium, as well as albumin/globulin ratio, by using an automatic serum biochemical analyzer (Hitachi 7180, Hitachi, Tokyo, Japan).

2.6.5. Gross Observation and Measurement of Organ Weights. After exsanguination from the abdominal aorta, macroscopic examination of the full external surface, cranial cavity, and organs of the thoracic and abdominal cavities was performed. The absolute weights of the spleen, liver, adrenal glands, kidney, heart, lung, brain, pituitary, thymus, testis, prostate, ovary, and uterus were measured, and their relative organ weights (organ-to-body weight ratios) were calculated.

2.6.6. Histopathology. Histopathological analysis of the brain, pituitary gland, heart, lung, liver, kidney, urinary bladder, mesenteric lymph node, thymus, spleen, parathyroid gland, adrenal gland, esophagus, aorta, spinal cord, sciatic nerve, skeletal muscle, skin, mammary gland, eye, cecum, colon, rectum, femur, sternum, trachea, tongue, prostate gland, testes, epididymis, pancreas, salivary gland, submandibular lymph node, thyroid gland, stomach, duodenum, jejunum, ileum, seminal vesicle, ovary, uterus, and vagina was performed. All organs were preserved in 10% neutral buffer formalin except the testes (preserved in Bouin's solution) and eyes (preserved in Davison's solution).

2.7. Statistical Analysis. Statistical analyses were performed using SPSS v.12.0 statistical analysis software (IBM Corp.,

Table 3: Urinalysis results of HVC1-treated male and female rats.

Sex	Test item (grade)	Treatment group (mg/kg/day)			
		Control	500	1,000	2,000
Males	Glucose (0–4)	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.5	1.2 ±0.4*
	Ketone body (0–4)	0.6 ± 0.9	1.8 ±0.4**	2.0 ±0.0**	2.6 ±0.5**
	Leukocyte (0–4)	0.8 ± 0.4	1.0 ± 1.7	4.0 ±0.0**	4.0 ±0.0**
Females	Ketone body (0–4)	0.0 ± 0.0	1.2 ±0.8*	1.6 ±0.5*	1.6 ±0.5*
	Leukocyte (0–4)	0.0 ± 0.0	1.6 ±2.2**	0.8 ±1.8**	1.6 ±2.2**

Values are expressed as mean ± SD ($n = 5$). * $p < 0.05$, ** $p < 0.01$ compared with the control group.
Grades: 0 (negative), 1 (trace), 1+, 2, 2+, 3, 3+, 4.

Table 4: Hematological and plasma coagulation values of HVC1-treated male rats.

Test item (unit)	Treatment group (mg/kg/day)			
	Control	500	1,000	2,000
WBC (K/μL)	9.86 ± 1.77	9.01 ± 2.41	11.78 ±2.54*	11.71 ±3.27*
Neutrophil (K/μL)	1.64 ± 0.68	1.47 ± 0.42	1.92 ± 0.49	1.34 ± 0.38
Lymphocyte (K/μL)	7.83 ± 1.65	7.12 ± 2.05	9.35 ± 2.13	9.92 ± 2.82
Monocyte (K/μL)	0.21 ± 0.07	0.24 ± 0.08	0.29 ± 0.08	0.25 ± 0.10
Eosinophil (K/μL)	0.08 ± 0.03	0.07 ± 0.02	0.08 ± 0.03	0.07 ± 0.04
Basophil (K/μL)	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
LUC (K/μL)	0.10 ± 0.04	0.10 ± 0.04	0.14 ± 0.08	0.13 ± 0.06
NEP (%)	16.8 ± 6.2	16.8 ± 3.6	16.4 ± 3.9	11.5 ±1.5*
LYP (%)	79.2 ± 5.8	78.6 ± 3.3	79.2 ± 3.9	84.6 ±2.0**
MOP (%)	2.2 ± 0.9	2.6 ± 0.6	2.4 ± 0.5	2.1 ± 0.6
EOP (%)	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.2	0.6 ± 0.3
BAP (%)	0.0 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
LUP (%)	1.0 ± 0.4	1.1 ± 0.4	1.2 ± 0.5	1.0 ± 0.3
RBC (K/μL)	8.81 ± 0.28	8.80 ± 0.44	8.72 ± 0.42	8.69 ± 0.43
Hemoglobin (g/dL)	15.6 ± 0.2	15.2 ± 0.6	15.2 ± 0.6	15.5 ± 0.8
Hematocrit (%)	45.1 ± 1.7	44.2 ± 1.8	44.1 ± 1.8	45.3 ± 2.7
MCV (fL)	51.3 ± 1.8	50.2 ± 1.7	50.6 ± 1.5	52.1 ± 1.6
MCH (pg)	17.8 ± 0.5	17.2 ±0.6*	17.4 ± 0.4	17.9 ± 0.5
MCHC (g/dL)	34.7 ± 1.0	34.3 ± 0.3	34.4 ± 0.5	34.3 ± 0.7
RDW (%)	12.8 ± 1.0	12.2 ± 0.9	12.6 ± 0.5	12.6 ± 0.5
Platelet (K/μL)	1068 ± 119	1015 ± 102	1111 ± 104	1108 ± 113
MPV (fL)	6.4 ± 1.3	6.6 ± 1.4	6.5 ± 1.4	6.5 ± 1.5
Reticulocyte (%)	2.05 ± 0.80	1.60 ± 0.59	1.85 ± 0.55	1.94 ± 0.33
PT (sec)	9.89 ± 0.71	9.60 ± 0.63	9.39 ± 0.21	9.40 ± 1.01
APTT (sec)	13.7 ± 2.7	16.0 ± 0.9	15.9 ± 1.3	15.7 ± 1.9

WBC: white blood cell; LUC: large unstained cell; NEP: percent of neutrophil; LYP: percent of lymphocyte; MOP: percent of monocyte; EOP: percent of eosinophil; BAP: percent of basophil; LUP: percent of large unstained cell; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; MPV: mean platelet volume; PT: prothrombin time; APTT: active partial thromboplastin time. Values are expressed as mean ± SD ($n = 10$). * $p < 0.05$, ** $p < 0.05$ compared with the control group.

Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used for the statistical analyses of body weight, food consumption, urine volume, hematology, plasma coagulation, serum biochemistry, and organ weight. The chi-square test and Kruskal-Wallis test were used for the analyses of specific gravity, glucose, protein, leukocyte, ketone, occult blood, bilirubin, nitrite, and urobilinogen. Results were considered statistically significant for p values less than 0.05.

3. Results

3.1. Calibration Curve and Limits of Detection and Quantification. Calibration curves were calculated for the 9 reference compounds using linear regression derived from the peak

Table 5: Hematological and plasma coagulation values of HVC1-treated female rats.

Test item (unit)	Treatment group (mg/kg/day)			
	Control	500	1,000	2,000
WBC (K/μL)	5.47 ± 2.32	6.19 ± 2.46	5.94 ± 1.46	7.17 ± 3.34
Neutrophil (K/μL)	0.80 ± 0.37	0.79 ± 0.31	0.72 ± 0.15	0.87 ± 0.37
Lymphocyte (K/μL)	4.39 ± 2.03	5.10 ± 2.17	4.95 ± 1.42	5.97 ± 2.91
Monocyte (K/μL)	0.14 ± 0.07	0.15 ± 0.06	0.15 ± 0.10	0.16 ± 0.07
Eosinophil (K/μL)	0.08 ± 0.05	0.08 ± 0.04	0.07 ± 0.03	0.08 ± 0.05
Basophil (K/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
LUC (K/μL)	0.06 ± 0.06	0.06 ± 0.04	0.05 ± 0.03	0.08 ± 0.05
NEP (%)	15.5 ± 7.2	13.3 ± 4.3	12.8 ± 4.6	12.6 ± 2.3
LYP (%)	79.3 ± 7.3	81.8 ± 4.4	82.6 ± 4.4	82.5 ± 3.6
MOP (%)	2.5 ± 0.7	2.5 ± 0.7	2.5 ± 1.1	2.3 ± 0.9
EOP (%)	1.6 ± 1.1	1.3 ± 0.6	1.2 ± 0.7	1.4 ± 1.3
BAP (%)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
LUP (%)	1.1 ± 0.7	1.0 ± 0.4	0.8 ± 0.3	1.1 ± 0.3
RBC (K/μL)	7.85 ± 0.38	8.01 ± 0.39	7.78 ± 0.64	8.14 ± 0.32
Hemoglobin (g/dL)	14.7 ± 0.7	15.2 ± 0.7	14.9 ± 0.7	15.2 ± 0.6
Hematocrit (%)	43.3 ± 2.7	44.7 ± 2.7	43.3 ± 3.0	44.5 ± 0.9
MCV (fL)	55.2 ± 2.3	55.8 ± 1.9	55.7 ± 1.8	54.7 ± 1.4
MCH (pg)	18.8 ± 0.6	19.0 ± 0.5	19.2 ± 1.0	18.7 ± 0.4
MCHC (g/dL)	34.0 ± 1.1	34.0 ± 1.2	34.4 ± 1.6	34.1 ± 1.0
RDW (%)	11.5 ± 0.5	11.6 ± 0.4	11.6 ± 1.3	11.2 ± 0.4
Platelet (K/μL)	1097 ± 138	1038 ± 165	965 ± 367	1122 ± 131
MPV (fL)	8.6 ± 1.0	8.5 ± 0.7	8.9 ± 1.3	8.6 ± 0.7
Reticulocyte (%)	2.01 ± 0.26	2.03 ± 0.26	2.76 ± 2.31	1.90 ± 0.39
PT (sec)	8.78 ± 0.61	8.87 ± 0.47	8.84 ± 0.41	8.73 ± 0.53
APTT (sec)	14.9 ± 3.5	16.2 ± 1.3	15.1 ± 2.5	15.9 ± 1.2

WBC: white blood cell; LUC: large unstained cell; NEP: percent of neutrophil; LYP: percent of lymphocyte; MOP: percent of monocyte; EOP: percent of eosinophil; BAP: percent of basophil; LUP: percent of large unstained cell; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; MPV: mean platelet volume; PT: prothrombin time; APTT: active partial thromboplastin time. Values are expressed as mean ± SD ($n = 10$).

area. The regression equations (correlation coefficient, R^2) of the reference compounds are shown in Table 2. The calibration curves had good linearity.

3.2. HPLC Analysis of HVC1. Nine compounds in HVC1 were measured via HPLC (Figure 1). The contents of sennoside A, genistein-7-O-β-glucopyranoside, coptisine, baicalin, prunetin-5-O-β-glucopyranoside, berberine, baicalein, wogonin, and prunetin in HVC1 were 14.12 ± 3.81, 1.71 ± 0.08, 0.85 ± 0.01, 25.89 ± 0.00, 16.89 ± 3.09, 3.58 ± 0.45, 0.40 ± 0.00, 7.13 ± 0.03, and 0.71 ± 0.00 mg/g, respectively.

3.3. Mortality, Clinical Signs, and Body Weight. Throughout the 13-week study period, no signs of ill health or mortality related to HVC1 treatment were observed. There were no significant differences in body weight between the control and HVC1-treated groups (Figure 2). There were no significant differences in feed intake between the control and HVC1-treated groups (data not shown). There were no significant differences in clinical observations, body weight, and feed intake during the recovery period (data not shown).

3.4. Ophthalmoscopy and Urinalysis. Ophthalmoscopic examination did not reveal changes after HVC1 treatment (data not shown).

Urinalysis showed significant differences in glucose level, ketone body level, and leukocyte count between the control and HVC1-treated male rats. Significant differences in ketone body level were observed between the control and HVC1-treated female rats. No other significant differences between the control and HVC1-treated male and female groups were observed (Table 3).

3.5. Hematology and Serum Biochemistry. In male rats, white blood cell levels significantly increased in the groups treated with 1,000 and 2,000 mg/kg/day HVC1 ($p < 0.05$), and the percentage of lymphocytes in the group treated with 2,000 mg/kg/day HVC1 also significantly increased ($p < 0.01$). The mean corpuscular hemoglobin level in the group treated with 500 mg/kg/day HVC1 and the percentage of neutrophils in the group treated with 2,000 mg/kg/day HVC1 significantly decreased ($p < 0.05$) in comparison with those of the control group. No other significant differences in hematological and

Table 6: Serum biochemical values in HVC1-treated male rats.

Test item (unit)	Treatment group (mg/kg/day)			
	Control	500	1,000	2,000
AST (IU/L)	113 ± 22	113 ± 34	109 ± 24	125 ± 47
ALT (IU/L)	30 ± 4	31 ± 8	32 ± 10	41 ± 25
ALP (IU/L)	275 ± 39	280 ± 51	263 ± 38	233 ± 37
GGT (IU/L)	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3
Lactate dehydrogenase (IU/L)	1441 ± 585	1295 ± 651	1123 ± 351	1164 ± 340
Blood urea nitrogen (mg/dL)	18.0 ± 1.8	16.6 ± 2.0	17.2 ± 2.1	15.7 ± 1.1
Creatinine (mg/dL)	1.08 ± 0.84	1.29 ± 0.76	1.04 ± 0.50	0.87 ± 0.64
Glucose (mg/dL)	179 ± 20	180 ± 27	192 ± 24	176 ± 26
Total cholesterol (mg/dL)	64 ± 9	57 ± 7	59 ± 7	59 ± 13
Total protein (g/dL)	6.2 ± 0.2	6.0 ± 0.2	6.1 ± 0.2	6.0 ± 0.4
Creatine phosphokinase (U/L)	644 ± 261	576 ± 284	480 ± 166	522 ± 147
Albumin (g/dL)	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.1 ± 0.2
Total bilirubin (mg/dL)	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.03	0.06 ± 0.04
Albumin/Globulin ratio	0.51 ± 0.03	0.52 ± 0.04	0.49 ± 0.03	0.53 ± 0.04
Triglyceride (mg/dL)	48 ± 19	39 ± 18	39 ± 13	36 ± 13
Uric acid (mg/dL)	1.9 ± 0.5	1.9 ± 0.4	2.2 ± 0.3	2.6^{*} ± 0.7
Calcium (mg/dL)	9.3 ± 0.4	9.4 ± 0.4	9.6 ± 0.4	9.8 ± 0.5
Inorganic phosphorus (mg/dL)	9.4 ± 0.6	9.2 ± 0.6	9.8 ± 0.6	10.7^{**} ± 1.1
Chloride (mmol/L)	106 ± 1	107 ± 2	107 ± 2	107 ± 1
Magnesium (mg/dL)	2.6 ± 0.2	2.6 ± 0.2	2.8 ± 0.2	2.9^{*} ± 0.3
Sodium (mmol/L)	144 ± 1	145 ± 1	144 ± 2	144 ± 1
Potassium (mmol/L)	5.0 ± 0.7	5.1 ± 0.6	5.4 ± 0.3	6.3^{**} ± 1.0

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma(γ)-glutamyl transferase. Values are expressed as mean ± SD ($n = 10$). $^{*}p < 0.05$, $^{**}p < 0.05$ compared with the control group.

plasma coagulation values were observed between the control and HVC1-treated male and female groups (Tables 4 and 5).

In male rats treated with 2,000 mg/kg/day HVC1, levels of uric acid ($p < 0.05$), inorganic phosphorus ($p < 0.01$), magnesium ($p < 0.05$), and potassium ($p < 0.01$) significantly increased. No other significant differences in serum biochemical values were observed between the control and HVC1-treated male and female groups (Tables 6 and 7).

3.6. Gross Observations and Organ Weights. No treatment-related changes in gross findings were observed in any of the treated animals. Absolute spleen weight in the male rats treated with 500 or 2,000 mg/kg/day HVC1 significantly decreased ($p < 0.05$), whereas absolute spleen weight significantly increased in the female rats treated with 1,000 mg/kg/day HVC1, and absolute kidney weight of both kidneys significantly increased in the female rats treated with 1,000 or 2,000 mg/kg/day HVC1 ($p < 0.05$) (Table 8). The relative weight of the left kidney in the male rats treated with 1,000 or 2,000 mg/kg/day HVC1 significantly increased ($p < 0.05$). The relative weight of the spleen in the female rats treated with 1,000 mg/kg/day HVC1 ($p < 0.05$) and the relative weight of both the kidneys in the female rats treated with 1,000 or 2,000 mg/kg/day HVC1 significantly increased ($p < 0.01$) (Table 9). No other significant differences in absolute or relative organ weights were observed between the control and HVC1-treated male and female groups (data not shown).

3.7. Histopathological Findings. No abnormal histopathological changes were observed in any animals (data not shown).

4. Discussion

In the present study, no signs of ill health or mortality were observed after oral administration of HVC1. Although salivation was sporadically observed in the male and female rats treated with 1,000 and 2,000 mg/kg/day, it was concluded that this symptom was temporarily stimulated by administration and did not have any toxicological significance. There were no significant differences in body weight and feed intake between the control and HVC1-treated groups.

Ophthalmoscopy revealed no abnormal lesions in any of the animals examined. The urinalysis showed significant differences in glucose, ketone body level, and leukocyte level in HVC1-treated male rats and in ketone body level in HVC1-treated female rats, in comparison with the control group. The color of the urine changed in a dose-dependent manner in animals of both sexes in all dosing groups and tended to have a reddish color (amber, orange, or dark red). The change in urine color was thought to be caused by the test substance. No changes were observed in biochemical parameters related to the kidneys, such as blood urea nitrogen and creatinine levels, and histopathological examination revealed no kidney-related abnormalities. In addition, no differences in urine color were observed during the 4-week recovery period, and

Table 7: Serum biochemical values in HVC1-treated female rats.

Test item (unit)	Treatment group (mg/kg/day)			
	Control	500	1,000	2,000
AST (IU/L)	153 ± 85	138 ± 57	136 ± 71	121 ± 27
ALT (IU/L)	44 ± 25	40 ± 21	34 ± 22	33 ± 14
ALP (IU/L)	110 ± 21	135 ± 30	114 ± 27	136 ± 49
GGT (IU/L)	0.0 ± 0.0	0.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
Lactate dehydrogenase (IU/L)	1307 ± 848	1381 ± 838	1300 ± 598	1340 ± 407
Blood urea nitrogen (mg/dL)	20.6 ± 3.6	18.6 ± 2.1	21.9 ± 6.2	21.9 ± 6.5
Creatinine (mg/dL)	0.69 ± 0.08	0.69 ± 0.09	0.70 ± 0.17	0.72 ± 0.15
Glucose (mg/dL)	154 ± 29	167 ± 35	167 ± 41	152 ± 33
Total cholesterol (mg/dL)	85 ± 18	82 ± 15	81 ± 14	79 ± 19
Total protein (g/dL)	7.1 ± 0.4	6.9 ± 0.3	7.0 ± 0.4	7.0 ± 0.3
Creatine phosphokinase (U/L)	571 ± 347	592 ± 351	624 ± 323	542 ± 137
Albumin (g/dL)	2.9 ± 0.2	2.8 ± 0.2	2.9 ± 0.3	2.8 ± 0.2
Total bilirubin (mg/dL)	0.10 ± 0.04	0.08 ± 0.03	0.12 ± 0.13	0.07 ± 0.02
Albumin/Globulin ratio	0.69 ± 0.02	0.67 ± 0.05	0.68 ± 0.05	0.68 ± 0.04
Triglyceride (mg/dL)	49 ± 30	56 ± 31	45 ± 21	40 ± 25
Uric acid (mg/dL)	1.8 ± 0.4	1.7 ± 0.4	1.9 ± 0.4	1.7 ± 0.4
Calcium (mg/dL)	10.1 ± 0.5	10.1 ± 0.4	10.3 ± 0.6	10.2 ± 0.5
Inorganic phosphorus (mg/dL)	8.3 ± 1.3	8.4 ± 0.7	9.0 ± 0.8	9.3 ± 1.0
Chloride (mmol/L)	106 ± 3	105 ± 1	106 ± 1	105 ± 2
Magnesium (mg/dL)	2.9 ± 0.2	2.9 ± 0.2	2.9 ± 0.3	3.1 ± 0.2
Sodium (mmol/L)	143 ± 1	143 ± 1	143 ± 1	143 ± 1
Potassium (mmol/L)	5.2 ± 0.9	5.2 ± 0.6	5.6 ± 0.9	5.6 ± 0.5

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma(γ)-glutamyl transferase. Values are expressed as mean ± SD ($n = 10$).

Table 8: Absolute organ weights (g) in HVC1-treated male and female rats.

Sex	Organ	Treatment group (mg/kg/day)			
		Control	500	1,000	2,000
Males	Spleen	0.991 ± 0.180	0.805 ±0.124*	0.924 ± 0.147	0.842 ±0.115*
Females	Spleen	0.580 ± 0.090	0.565 ± 0.042	0.812 ±0.412*	0.598 ± 0.086
	Kidney (left)	0.950 ± 0.105	0.969 ± 0.096	1.095 ±0.154*	1.076 ±0.113*
	Kidney (right)	0.983 ± 0.125	1.001 ± 0.010	1.126 ±0.157*	1.118 ±0.110*

Values are expressed as mean ± SD ($n = 10$). * $p < 0.05$ compared with the control group.

Table 9: Relative organ weights (g) in HVC1-treated male and female rats.

Sex	Organ	Treatment group (mg/kg/day)			
		Control	500	1,000	2,000
Males	Kidney (left)	0.297 ± 0.034	0.299 ± 0.023	0.325 ±0.026*	0.327 ±0.030*
Females	Spleen	0.186 ± 0.029	0.182 ± 0.020	0.261 ±0.127*	0.208 ± 0.031
	Kidney (left)	0.305 ± 0.041	0.310 ± 0.027	0.352 ±0.043**	0.374 ±0.038**
	Kidney (right)	0.315 ± 0.045	0.320 ± 0.022	0.363 ±0.052**	0.389 ±0.039**

Values are expressed as mean ± SD ($n = 10$). * $p < 0.05$, ** $p < 0.01$ compared with the control group.

changes in urine color were thus not considered a toxic result of HVC1 treatment.

According to hematological testing, the male rats in HVC1-treated groups showed increased white blood cell count and lymphocyte percentage, as well as decreased mean corpuscular hemoglobin level and neutrophil percentage. However, these changes were within the range of natural biological variation and were not observed during the recovery period. No other significant differences in hematological parameters and plasma coagulation values were observed.

Analysis of serum biochemistry revealed increased levels of uric acid, inorganic phosphorus, magnesium, and potassium in the male rats treated with 2,000 mg/kg HVC1. However, these changes were within the range of natural biological variation. These differences were not observed during the recovery period.

The absolute spleen weights in male rats decreased, while the absolute and relative weights of the spleen and both kidneys in females, as well as the relative weight of the left kidney in males, increased. However, these changes in organ weight were not dose-dependent and were not observed during the recovery period, and no spleen- or kidney-related abnormalities were observed in the biochemical and histopathological analyses. No abnormal histopathological changes related to HVC1 oral administration were observed in any animals.

5. Conclusion

In this study, no systemic toxicity related to HVC1 was observed in any of the primary treatment or recovery groups after repeated dosing for 13 weeks. Therefore, the NOAEL (no-observed-adverse-effect-level) of HVC1 was established as 2,000 mg/kg/day, and no target organ of HVC1 toxicity was identified. This result suggested that there are no toxicity and side effects from repeated administration of HVC1. For the clinical trial, we have obtained IND approval from this result and previous hypolipidemia efficacy results and toxicity results in Korea. We are going to conduct phase II clinical trials. Like this, many studies are currently being carried out actively to discover new efficacies of traditional herbal prescriptions and to develop new prescriptions in Korea. If many clinical trials based on ethnopharmacological studies are carried out, it is expected that many new drugs using herbal medicine could be developed.

Abbreviations

HVC1: A 30% ethanol extract of a mixture comprising Pruni Cortex, Scutellariae Radix, Coptidis Rhizoma, and Rhei Rhizoma.
SHR: Spontaneous hypertensive rat.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (B110081).

References

[1] State Pharmacopoeia Committee, *Pharmacopoeia of the Peoples Republic of China*, Pharmacopoeia of the People's Republic of China, Peoples Medical Publishing House, Beijing, China, 2010.

[2] L. A. Usov, "Scutellaria baicalensis on experimental hypertension," *Farmakol Toksikol*, vol. 21, no. 2, pp. 31–34, 1958.

[3] K. H. Song, S. H. Lee, B.-Y. Kim, A. Y. Park, and J. Y. Kim, "Extracts of *Scutellaria baicalensis* reduced body weight and blood triglyceride in db/db mice," *Phytotherapy Research*, vol. 27, no. 2, pp. 244–250, 2013.

[4] H.-C. Shih and L.-L. Yang, "Relaxant effect induced by wogonin from Scutellaria baicalensis on rat isolated uterine smooth muscle," *Pharmaceutical Biology*, vol. 50, no. 6, pp. 760–765, 2012.

[5] Y. Huang, S. Y. Tsang, X. Yao, C. W. Lau, Y. L. Su, and Z. Y. Chen, "Baicalin-induced vascular response in rat mesenteric artery: role of endothelial nitric oxide," *Cliniclal and experimetal pharmacology physiology*, vol. 29, no. 8, pp. 721–724, 2002.

[6] Y. Cao, W. Bei, Y. Hu et al., "Hypocholesterolemia of Rhizoma Coptidis alkaloids is related to the bile acid by up-regulated CYP7A1 in hyperlipidemic rats," *Phytomedicine*, vol. 19, no. 8-9, pp. 686–692, 2012.

[7] W. Xie, Y. Zhao, and L. Du, "Emerging approaches of traditional Chinese medicine formulas for the treatment of hyperlipidemia," *Journal of Ethnopharmacology*, vol. 140, no. 2, pp. 345–367, 2012.

[8] H.-H. Tsai, I.-J. Chen, and Y.-C. Lo, "Effects of San-Huang-Xie-Xin-Tang on U46619-induced increase in pulmonary arterial blood pressure," *Journal of Ethnopharmacology*, vol. 117, no. 3, pp. 457–462, 2008.

[9] Y.-C. Lo, Y.-T. Shih, Y.-T. Tseng, and H.-T. Hsu, "Neuroprotective effects of San-Huang-Xie-Xin-Tang in the MPP(+)/MPTP models of parkinson's disease in vitro and in vivo," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 501032, 10 pages, 2012.

[10] Y.-T. Shih, I.-J. Chen, Y.-C. Wu, and Y.-C. Lo, "San-Huang-Xie-Xin-Tang protects against activated microglia- and 6-OHDA-induced toxicity in neuronal SH-SY5Y cells," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 429384, 11 pages, 2011.

[11] K. Lee, I. Ham, G. Yang et al., "Vasorelaxant effect of *Prunus yedoensis* bark," *BMC Complementary and Alternative Medicine*, vol. 13, article 31, 2013.

[12] S. Cheon, K. Chung, K. Lee et al., "HVC1 ameliorates hyperlipidemia and inflammation in LDLR−/− mice," *BMC Complementary and Alternative Medicine*, vol. 17, article 222, 2017.

[13] C.-Y. Kim, K.-S. Chung, S.-Y. Cheon et al., "Hypolipidemic effects of HVC1 in a high cholesterol diet-induced rat model of hyperlipidemia," *Molecular Medicine Reports*, vol. 14, no. 4, pp. 3152–3158, 2016.

[14] K. Lee, B. Kim, H. Hur, K. S. Chinannai, I. Ham, and H.-Y. Choi, "Antihypertensive Effect of the GaMiSamHwangSaSimTang in Spontaneous Hypertensive Rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 802368, 7 pages, 2015.

[15] K. Lee, B. Kim, H. Hur, K. S. Chinannai, I. Ham, and H. Choi, "Experimental research of hypotensive and hypolipidemic effects with modified Sanhuang Xiexin decoction," *Chinese Journal of Integrative Medicine*, pp. 1–7, 2016.

[16] K. Lee, M. S. Shin, I. Ham, and H.-Y. Choi, "Investigation of the mechanisms of Angelica dahurica root extract-induced

vasorelaxation in isolated rat aortic rings," *BMC Complementary and Alternative Medicine*, vol. 15, article 395, 2015.

[17] Food and Drug Administration, *Guidance for Industry and Reviewers: Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers*, Food and Drug Administration, Rockville, MD, USA, 2002.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 6087509, 25 pages
https://doi.org/10.1155/2019/6087509

Research Article

The Use of Medicinal Plants in the Riverside Community of the Mazagão River in the Brazilian Amazon, Amapá, Brazil: Ethnobotanical and Ethnopharmacological Studies

Rosângela do Socorro Ferreira Rodrigues Sarquis,[1,2] Ícaro Rodrigues Sarquis,[2] Iann Rodrigues Sarquis,[3] Caio Pinho Fernandes,[4] Gabriel Araújo da Silva,[5] Raullyan Borja Lima e Silva,[6] Mário Augusto Gonçalves Jardim,[7] Brenda Lorena Sánchez-Ortíz,[2] and José Carlos Tavares Carvalho[1,2]

[1]*Graduate Program in Biodiversity and Biotechnology, Federal University of Amapá (UNIFAP), Brazil*
[2]*Laboratory of Pharmaceutical Research, Pharmacy Course, Department of Biological Sciences and Health, Federal University of Amapá (UNIFAP), Rodovia Juscelino Kubitscheck, Km 02, 68902-290 Macapá, AP, Brazil*
[3]*Biochemistry Laboratory, Nursing Course, Department of Health Sciences, Faculdade Estácio de Macapá, Brazil*
[4]*Phytopharmaceutical Nanobiotechnology Laboratory, Pharmacy Course, Department of Biological and Health Sciences, Federal University of Amapá (UNIFAP), Brazil*
[5]*Laboratory of Organic Chemistry and Biochemistry, Collegiate Degree in Chemistry, State University of Amapá (UEAP), Brazil*
[6]*Center of Biodiversity, Institute for Scientific and Technological Research of Amapá (IEPA), Brazil*
[7]*Paraense Emílio Goeldi Museum (MPEG), Coordination of Botany, Brazil*

Correspondence should be addressed to José Carlos Tavares Carvalho; farmacos@unifap.br

Received 5 November 2018; Revised 15 February 2019; Accepted 4 March 2019; Published 10 April 2019

Academic Editor: Filippo Fratini

The inhabitants of the floodplain of the Mazagão River in the State of Amapá in the Brazilian Amazon have inherited from indigenous African and Cabocla cultures indications for the use and forms of preparation of medicinal plants to cure diseases of the body and spirit. This study aimed to perform an ethnopharmacological survey of medicinal plants used by the riparian community of the floodplains of the Mazagão River, in the State of Amapá. In this study, we chose semistructured interviews with socioeconomic, ethnopharmacological, and ethnobotanical aims. The collection of medicinal plants occurred during guided tours. The Use Value (UV), Informant Consensus Factor (ICF), Correction Factor (CF), and Fidelity level (FL) were calculated. There were 130 species of medicinal plants, distributed in 116 genera and 57 families; Fabaceae (16), Lamiaceae (14), Euphorbiaceae (7), and Arecaceae (6) include 33.33% of the total species sampled. All 95 native species of floodplain forests were previously described, and 35 are exotic species. The species with the highest UV (≥ 0.5) at the mouth of the Mazagão River were *Carapa guianensis* (0.91), *Pentachlethra macrol*oba (0.83), *Dalbergia subcymosa* (0.77), *Uncaria tomentosa* (0.75), *Otacanthus azureus* (0.62), *Virola surinamensis* (0.62), *Hura crepitans* (0.58), *Euterpe oleracea* (0.56), and *Arrabidaea chica* (0.51). These species were also the ones that presented the highest ICF among the informants and 100% in FL for a specific therapeutic use. The study is comprised of 16 categories of therapeutic use, of which the majority of the plants used are related to diseases such as microbial infections (20.67%, 73 species), gastrointestinal disorders (13.31%), and inflammation (11.61%). The results showed that knowledge about the use of medicinal plants along the rivers and streams that form the mouth of the Mazagão River is evenly distributed. Most of the interviewees present diversified knowledge about the medicinal resources because they have a close relationship with the floodplain forest. Native species of this forest predominate among the most commonly used medicinal plants as subsidies for future pharmacological studies.

1. Introduction

The floodplain forest is characterized by flood-prone areas influenced by white water rivers with high nutrient-rich sediment loads of Andean or pre-Andean origin, which occupy approximately 2/3 of the flood-prone areas in the Amazon [1]. These forests are inhabited by riparian people, human populations who live on the flood-prone river banks and who produce their food through vegetable extractivism, logging, fishing, handicrafts, and shrimp trapping [2, 3]. The floodplain has always played a central role in the development of the Amazon region, playing different roles in the food and economic survival of riparian dwellers at each period of their history [4, 5].

The riverside communities of the tropical floodplains coexist with a great diversity of natural resources, and they develop some exploration techniques for their own survival, aiming for the establishment of their own management systems that allows them to achieve their needs with low environmental damage, all of this based on their experiences [6–8].

The use of medicinal plants for the treatment of diseases is related to human evolution itself; its use has been reported in all time periods, in all social strata, and for almost all of humanity. The use of medicinal plants for therapeutical purposes in developing countries such as Brazil can be used as an alternative treatment [9, 10]. Phytotherapy and the use of medicinal plants are traditionally part of popular medicine based on the knowledge of different populations, users, and practitioners. It is an effective form of primary health care for the lower income population. Medicinal plants have strongly contributed to the development of new therapeutic strategies through the isolation and identification of its secondary metabolites. These are known to act directly or indirectly through several molecular and cellular targets [11].

The use of medicinal plant-based medicines and the popular knowledge itself implies the need to implement basic research to clarify and confirm information about the actions of the plants, minimizing side, and toxicological effects so that their use is reliable and safe [12].

Historically, the city of Mazagão was founded in the 16th century in North Africa, then it was transferred in the eighteenth century to Portuguese America. It is located on the banks of the Mutuacá River in the State of Amapá, at the Brazilian Amazon rainforest [13]. The New Village of Mazagão, lost in the banks of the Amazon rainforest, was a point of overlapping of cultures, dualities, and conflicts. The memory of the city of the Moroccan coast was diluted and adapted to the new conditions of Portuguese America, building a new urbanity [14]. At that time, the African Mazagans had to live with the outbreak of malaria, a tropical disease endemic to the region; and in order to survive they used traditional knowledge of the use of medicinal plants of the riverside and indigenous communities [15].

Though cultural aspects such as the religious festivals in praise of St. James were preserved and, to this day in July, the battle waged between the Moors and Christians on the coast of Africa is remembered, new practices also emerged from the cultural synthesis of the Mazans, with indigenous people, slaves, and riparians [16, 17]. In the floodplain forest regions of the Eastern Amazon, medicinal plants represent the main form of disease treatment for most of the riparian populations living in this ecosystem, as they live geographically isolated from the urban centers, which causes barriers between them and the public services, especially health service and basic sanitation [18, 19]. These riverside communities have a very peculiar way of interacting with the environment where they live; in most of the houses, septic tanks are still used, and they overflow periodically when the river floods. This water is collected for consumption, several domestic tasks and even for the preparation of the açaí wine (*Euterpe oleracea*), which is the main food consumed in the locality, but it does not undergo adequate treatment to be considered potable, making parasites and stomach diseases a recurring problem in the community [18, 20].

The human populations that settled in floodplain regions started to have a very strong connection with the forest, knowing and exploring it, which allowed a greater contact with the vectors of tropical diseases such as malaria, Chagas disease, and tegumentary leishmaniasis, which are endemic diseases of these regions. To treat and control these diseases, they started to use medicinal plants, a knowledge that is acquired through both experience and verbal cultural transmission, which refers to the past knowledge of one generation to another and plays an important historical role as it facilitates human survival through these generations [19]. Therefore, the riverside inhabitants of the floodplain forest possess important collections of plants that are used for therapeutic purposes as they inherited the use and forms of preparation of medicinal plants for the cure of their diseases of the body and spirit from the indigenous African and Cabocla cultures [19]. However, this knowledge is usually restricted and little studied. Undoubtedly, many plants native to floodplains with therapeutic potential remain unknown and will become extinct because logging is reducing forests and decharacterizing traditional communities in the region.

Few ethnobotanical studies have been performed in the area; for instance, Silva [20] surveyed plants used in Carvão District, Mazagão, AP, where 218 different plant species distributed over 69 families were reported. In the riverside communities of Mazagão Velho, Maracá, and Ajurixi Nascimento [21] it has been reported 73 different plant species distributed over 37 families and their medicinal uses. Overall, these ethnobotanical and ethnopharmacological studies indicate an excellent target to pharmacological researches in the north of Brazil [19, 22, 23].

The State of Amapá has rich plant biodiversity, in addition to a great ethnic and cultural diversity, favoring the accumulation of empirical knowledge about medicinal uses of these plants. Considering the importance to rescue this knowledge from traditional communities and systematize it, this study aimed to perform an ethnobotanical and ethnopharmacological survey of medicinal plants used by riverside communities at the mouth of Mazagão River, in the State of Amapá.

2. Materials and Methods

2.1. Study Area. The municipality of Mazagão occupies an area of 13,131 km^2, it has a population of 17,032 inhabitants

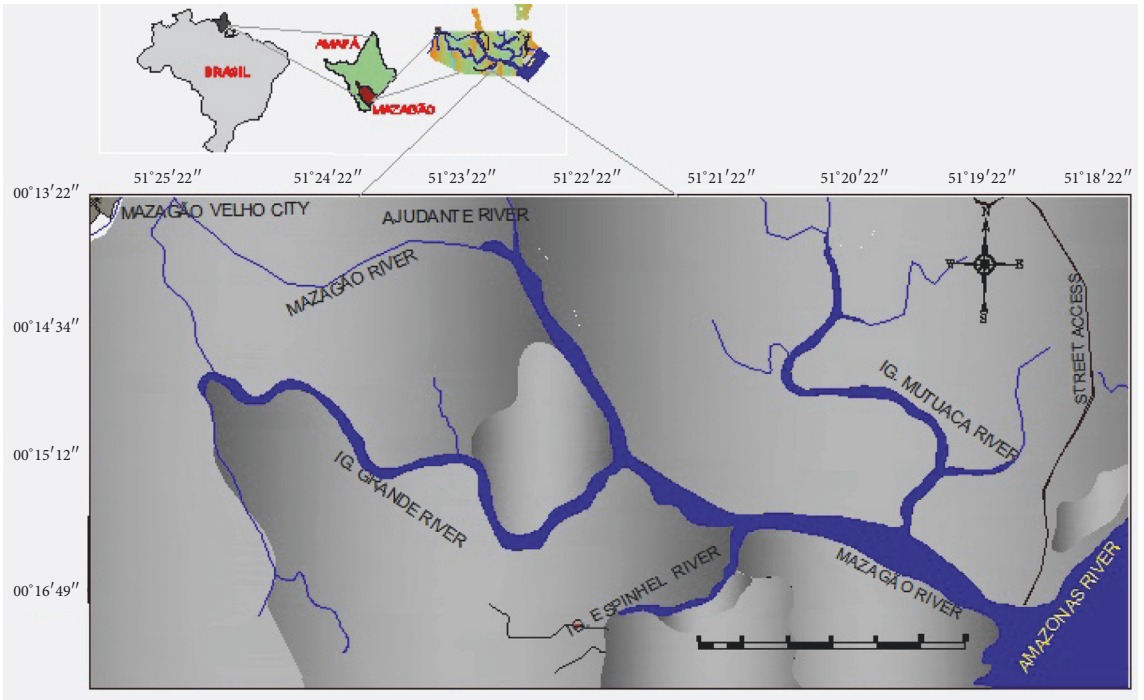


Figure 1: Location map of the mouth of the Mazagão River, Amapá State, Brazilian Amazon.

and a population density of 1.30 hab/km^2 [24], it is 36 km long from the capital Macapá, and it is located at the right margin of the Vila Nova River, south of the State of Amapá under the geographic coordinates 00° 06′ 54″ S and -51° 17′ 20″ W (Figure 1). Data collection occurred at the eastern boundary of the municipality of Mazagão in a riparian community that lives at the mouth of the Mazagão River, which has the Mutuacá Mirim, Espinhel, Grande and Ajudante rivers as tributaries. The community at the mouth lives along the Mazagão River, which is situated to the east of the Amazon River and to the west of the Old Mazagão Village, according to Carim et al. [25], the predominant climate type Ami, based on the Köppen classification, with a minimum temperature of 23°C and a maximum temperature of 33°C (annual average of 27°C), with relative humidity above 80%, high rainfall from 2,000 mm/year to 2,500 mm/year, and the Haplic Gleisol soil with a very clayey texture.

2.2. Research Authorization. At the beginning of the study, a meeting was organized with the community to present the project and its objectives to obtain community consent for the development of the study. Subsequently, according to the Resolutions of the National Commission for Research Ethics involving Humans and the National Health Council/Ministry of Health, the project was submitted to the Ethics Research Committee of the Faculdade Estácio de Macapá. To do so, the project was registered in the Brazil Platform at http://aplicacao.saude.gov.br/plataformabrasil/login.jsf, in which the following was inserted: interview forms (socioeconomic, ethnobotanical and ethnopharmacological), Community Consent Statement, and the terms of Free and Informed Consent, according to Resolution 466/12 of the National Health Council. This study was approved on 04/13/2016 under opinion number 14.94.994.

2.3. Area of Study's Typical Vegetation. Floodplain forests have about 25,000 km^2 in the area of the Amazonian estuary; it represents about 4.85% from the State of Amapá area and about 15.46% of the coastal estuarine sector [26]. These forests are the flooded ecosystem with the highest biodiversity in the world and have more than 1,000 different species of trees [27, 28]. The local vegetation is cataloged as Alluvial Dense Ombrophilous Forest [29], and the most representative plant families are Fabaceae, Malvaceae, Meliaceae, and Rubiaceae [30]. There is a significant number of palm trees such as *Astrocaryum murumuru* Mart., *Manicaria saccifera* Gaertn., *Mauritia flexuosa* L. f., *Attalea excelsa* (Aubl.) Mart., and *Euterpe oleracea* Mart.; and emerging trees such as *Carapa guianensis* Alblet, *Virola surinamensis* (Rol. ex Rottb.) Warb., *Mora paraensis* (Ducke) Ducke, *Calycophyllum spruceanum* (Benth.) Hook. f. ex K. Schum., *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg., *Cedrela odorata* L., and *Pentaclethra macroloba* (Willd.) Kuntze [30, 31].

The soil under estuarine floodplain forests of Mazagão, AP is, shallow and continuously subjected to flood. In this area, the soil is cataloged as typical Eutrophic Ta Melanic Gleysol [32].

2.4. Selection of Informants. In the community, there are 128 houses, all of which were visited, but only the families living in the community for at least 10 years were considered in the interviews. In the selected family, the person who

everyone agreed was knowledgeable about home remedies (healer) was the one who was always approached because he/she cultivates and holds knowledge about local plants that are medicinal. A total of 93 forms were applied, representing 72.65% of the total families living in the community.

The study area was investigated to get information from local traditional healers having practical knowledge of medicinal plants; they were interviewed in two villages during April 2016 to May 2018. During the course of the study, six field trips were carried out in the study, and a total 30 days were spent with their local traditional healers. Methods of selecting informants depended upon the distribution of local people having sound knowledge.

All participants agreed to sign the terms of Informed Consent. The interviews were conducted with semistructured forms with open and closed questions about the socioeconomic aspects and the identification of respondents (age, ethnicity, schooling, sex, length of time living in the community, religion, food consumption, family income, and participation in any social project of the government) and information about the medicinal plants used (popular name, part used, indications, and preparation) were recorded. In this work, we considered both plants that are used for diseases well known and treatable by traditional medicine and those indicated for cultural diseases such as "panemeira" (a condition in which the individual is unlucky or has bad luck), bewitching, evil eye, or jinx (the individual is haunted by evil spirits). Other conditions included "ferida brava" (Leishmaniosis ulcer), ringworm (individual with itching in the body), bubo (inflammation in the lymph nodes), "úra" or "fly eye" (fly larvae that lodges in the dermis and causes inflammation in the skin), catarrh in the chest (secretion in the lungs), and "nascida" (a tumor that swells in the skin of any part of the body).

2.5. Collection of Botanical Material. The collections were carried out using the guided tour technique [33], which consisted of exploratory walks conducted by the interviewees in the backyard of their residence and in the interior of the forest to identify and collect botanical material from the medicinal plants mentioned during the interview. The referenced species were collected and herborized according to the usual methodology of Fidalgo and Bononi [34]. The plant material was identified by comparison to the Embrapa Eastern Amazon Herbarium; IAN collection; and specialized bibliography [35] and when necessary, via consultations with specialists. Exsiccating of the collected species was included in the IAN of the Embrapa Amazônia Oriental and Herbário Amapaense (HAMAB) of the Institute for Scientific and Technological Research of the State of Amapá. The species mentioned only with the vegetative part were identified, when possible, through specialized literature and using virtual herbarium images for comparison. The spelling and authors of the scientific names were verified in the Missouri Botanical Garden database at www.tropicos.org, and the families were determined according to the classification system proposed by the Angiosperm Phylogenetic Group III [36]. The geographical origin of the species was verified in a specialized bibliography [35, 37].

2.6. Data Analysis. The medicinal plants listed by the informants were organized according to the scientific name, popular name, part used, method of preparation, and indications for medicinal use. The reported diseases and symptoms were grouped into 16 categories of therapeutic use according to the indicated body systems. Data were analyzed statistically and described in percentages using Graphpad Prism software (version 6.0). To analyze the relative importance of a species for its ethnomedicinal use, quantitative data (frequency of use and therapeutic indication) were calculated using the Index of Use Value (UV), Loyalty Level (LL), Informant Consensus Factor (ICF), and Correction Factor (CF), according to the mathematical formulas below.

2.6.1. Use Value (UV). It is a quantitative index that expresses the therapeutic importance of each species. It is calculated by the following formula: $UV = \Sigma^{Ui}/n$ [38], where UV is equivalent to the Use Value of a species, Ui is the number of therapeutic uses reported for each species of plant, and n is the total number of respondents interviewed [39]. The UV parameter helps determine which plant is most frequently used for specific purposes. UV is high when the plant is mentioned by a large number of informants and low when there are few cited uses [40].

2.6.2. Informant Consensus Factor (ICF). The proposal by Troter and Logan [41] aims to identify the body systems or categories of diseases that have greater relative importance in the site of the study. The ICF is calculated by obtaining the number of citations of uses in each category (N_{ur}) minus the number of species used (N_t), divided by the number of use citations in each category minus 1. The maximum value a category can achieve is 1, which would indicate that there is a well-defined criterion for selecting medicinal plants in the community and/or that use information is shared among the people.

$$\mathrm{ICF} = \frac{(N_{ur} - Nt)}{(N_{ur} - 1)} \tag{1}$$

2.6.3. Fidelity Level (FL). The FL measures the species most frequently used by informants in the study area for a specific therapeutic treatment [42]. FL was calculated using the following formula:

$$\mathrm{FL}\,(\%) = \frac{N_p}{N} \times 100 \tag{2}$$

where Np is the number of citations for therapeutic use given by an informant indicating a species for the highest therapeutic use and N is the total number of informants who cited the species for some use. FL = 100% means that all informants use the species for a therapeutic application, while values below this value mean that the species is used for different purposes [40].

2.6.4. Correction Factor (CF). The CF determines the difference in the number of informants who cited uses for each species. The CF is calculated by the following formula: CF = N/ICEMC [43], where N is the total number of informants who cited uses for the species and ICEMC is the number of citations of the most frequent species. To extract the importance values related to the species most cited by the interviewees, the following formula was used: Pcusp = LL x CF [44], where Pcusp is the corrected concordance use index, NF is the loyalty level of use, and CF is the correction factor for each species.

3. Results and Discussion

3.1. Sociocultural Characteristics of Informants. The riverside community at the mouth of the Mazagão River lives on the banks of a floodplain forest; the family income is based on the extractive management of the açaí fruit (*Euterpe oleracea*) and regional shrimp (*Macrobrachium amazonicum*) fishery. In this study, 93 residents were interviewed, ranging in age from 18 to 70 years. The oldest informants with ages ranging from 51 to 70 years were responsible for 51% of citations for use of the medicinal plants; informants ranging from 31 and 50 years old for 38.7% of citations; and informants ranging from 18 to 30 years for 10.3%. When comparing the number of plants cited with the age of the interviewees, it can be seen that the oldest female subjects between 55 and 70 years of age presented the highest number of citations for medicinal plant use. Other ethnobotanical studies also point to the fact of older women knowing more about the medicinal use of plant species [6, 45]. The prevalence is likely higher in older women due to the household duties of caring for the children, the house and the yard, and the place around the house where "girais" (wood artifact, a type of raised bed garden that reaches 3 meters from the ground) are built for the planting of medicinal and food plants. Additionally, the riverside inhabitants live on the edge of the forest, which is periodically flooded, and there is a practice of passing on traditional knowledge about the use of medicinal plants to future generations by word of mouth.

With regard to schooling, 20.4% of the older people were never literate, and those with elementary school education until the 5th grade represent 41% of the community; elementary school through the 8th grade, 20%; high school, 8.6%; incomplete high school, 9%; and higher education, 1%. According to Elisabetsky [46], what makes traditional knowledge of interest to science is the verbal communication of the systematic observation of biological phenomena, made by people who are often illiterate but are certainly insightful regarding the observations of the environment where they live. In this study, it is possible to verify that the older interviewees and those with less education in general are able to recognize a greater number of medicinal plants than are the younger interviewees. This pattern is also observed in other studies, such as those by Hanazaki et al. [47], Pinto [6], and Negrelle et al. [48].

3.2. Diversity of Medicinal Plants. A total of 130 medicinal species were identified, which were distributed in 116 genera and 57 families. Fabaceae (16 species), Lamiaceae (14), Euphorbiaceae (7), Arecaceae (6), Asteraceae (4), Rubiaceae (4), Rutaceae (4), Amaranthaceae (4), and Anacardiaceae (4) accounted for the greatest number of species, totaling 48.83% of the sampled species, and 22.5% of the families were represented by only one species. In total, 95 native species of floodplain forest and 35 exotic or introduced species were mentioned (Table 1).

Inventories in Amazonian floodplain forests have shown that Fabaceae has the greatest diversity of species [1, 5, 25, 49, 50]. This fact is demonstrated in this study and is corroborated by Guarado Neto and Moraes [51], who state that when human populations use the native forest for medicinal purposes, the family that has the largest number of species used is the most representative family of the forest.

A study performed by Vásquez et al. [52] at riverside communities of Manacapuru, in the state of Amazonas, reported that 82.7% of the plants used were cultivated, and the family Lamiaceae was the most representative, while in our study the most representative plant family regarding the frequency of citations was Fabaceae. Of the plants cited, 73.07% of them were taken from the forest while 26.93% were cultivated, showing that the people from this community enter the forest to search for the plants.

The "Use" criterion of a species is in the versatility of being mentioned for several therapeutic indications in the community [53, 54]. According to Alexiades [55], the most reliable medicinal uses are those already used by informants, relatives or acquaintances. The species with the highest levels of use agreement and frequency were *Carapa guianensis, Pentaclethra macroloba, Dalbergia monetaria, Uncaria tomentosa, Otacanthus azureus, Virola surinamensis, Hura crepitans* and *Euterpe oleracea*. In other studies, carried out in the Brazilian Amazon, the species *Carapa guianensis, Pentaclethra macroloba, Uncaria tomentosa,* and *Virola surinamensis* were also the most cited in terms of therapeutic use, demonstrating their regional value and the certainty that these plants may become targets in pharmacological research in the region [56, 57].

3.3. Used Plant Parts. A total of 170 therapeutic preparations were mentioned, and bark, aerial parts, latex, rhizome, leaf, seed, root, flower, inflorescence, and fruit were the parts of the plant used in the preparations. The most used parts were leaf (40%), bark (32.95%), fruits (7.64%), root (4.7%), and inflorescence (4.7%). In the community, parts of the same plant may be used for distinct indications, such as in *Carapa guianensis, Pentaclethra macroloba,* and *Virola surinamensis,* whose oil extracted from the seed is used topically in the case of inflammatory processes of the skin, as a repellent and for the healing of wounds, and the leaves and barks are used in oral preparations by decoction for inflammation of the digestive, urinary, and reproductive systems.

The leaves are vegetative structures that stand out in the methods of ethnomedicinal preparations by decoction, maceration, and infusion in traditional communities in Brazil [50, 58, 59] and in other regions of the world [39, 60–63]. One of the reasons is the ease of collection [64] and

Table 1: Vegetal species used as medicinal plants by residents at the mouth of the Mazagão River, State of Amapá, Brazilian Amazonia, and the use value.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(1) ***Acmella oleracea* (L.) R.K. Jansen** (Asteraceae), IAN 195948	Jambú	Native	Leaves and inflorescences	Pain, inflammation, Anemia and malaria.	Decoction; 1 cup 3x a day for 7 days	0,09
(2) ***Acrocomia aculeata* (Jacq.) Lodd. ex Mart.** (Arecaceae), IAN 195943	Mucajá	Native	Root and leaves	Catarrh in the chest (secretion in the lung) and cough.	Decoction; 1 spoon 4 x a day for 7 days.	0,02
(3) ***Adenocalymna alliaceum* Miers** (Bignoniaceae), IAN 196009	Cipó d'alho	Native	Leaves and bark	Cold, inflammation of the throat and fever.	Decoction; 1 scoop 2 x daily and inhale 2x steam for 7 days.	0,17
(4) ***Aeollanthus suaveolens* Matt. ex Spreng.** (Lamiaceae), IAN 195989	Caatinga de mulata	Introduced	Leaves	Irritation of the nerves and strong headache.	Dyeing and infusion; bathe the body 2 x in the day for 10 days.	0,46
(5) ***Allamanda cathartica* L.** (Apocynaceae), IAN 196010	Buiuçú	Native	Exsudate and leaves	Elimination of lice and antihemintics.	Decoction; 1 spoon 2 x a day for 7 days and soak hair 1 x a day for 10 minutes for 3 days.	0,01
(6) ***Allium sativum* L.** (Liliaceae), HAMAB 8938	Alho	Introduced	Leaves	Flu, pain in the stomach and antihemintics	Decoction; 1 cup 3 x daily for 7 days.	0,03
(7) ***Aloe vera* (L.) Burm.** (Asphodelaceae), IAN 194110	Babosa	Native	Leaves	Healing.	Cataplasm; spend 3 x a day until the wound is dry.	0,10
(8) ***Alstroemeria brasiliensis* Spreng.** (Alstroemeriaceae), IAN 196011	Carajurú, cajuru	Native	Bark and root	Malaria.	Decoction; 1 cup 3 x daily for 7 days.	0,01
(9) ***Alternanthera brasiliana* (L.) Kuntze** (Amarathaceae), IAN 194099	Perpétua do mato, sempre-viva.	Native	Leaves	Elimination of kidney stone, antitumor, infections of the liver and bladder.	Decoction; leaves of *Alternanthera brasiliana*, *Alternanthera dentata* and *Alternanthera ficoidea*, ingest 1 cup 4 x a day for 20 days.	0,20
(10) ***Alternanthera dentata* (Moench) Stuchlík ex R.E. Fr.** (Amaranthaceae), IAN 195934	Cibalena	Native	Leaves	Elimination of kidney stone, infections of the liver, stomach pains.	Decoction; leaves of *Alternanthera brasiliana*, *Alternanthera dentata* and *Alternanthera ficoidea*, ingest 1 cup 4 x a day for 20 days.	0,01
(11) ***Alternanthera ficoidea* (L.) Sm.** (Amaranthaceae), IAN 195952	Piriquitinho verde	Native	Leaves	Elimination of kidney stone, inflammation of the uterus and ovary.	Decoction; leaves of *Alternanthera brasiliana*, *Alternanthera dentata* and *Alternanthera ficoidea*, ingest 1 cup 4 x a day for 20 days.	0,07
(12) ***Anacardium occidentale* L.** (Anacardiaceae), IAN 195977	Caju	Native	Leaves	Diarrhea, uterine cramps.	Decoction; 1 cup 3 x daily for 7 days.	0,16

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(13) ***Annona muricata* L.** (Annonaceae), IAN 195945	Graviola	Native	Leaves	Diarrhea and skin sore.	Decoction and infusion; 1 cup 3 x daily for 7 days.	0,03
(14) ***Arrabidaea chica* (Bonpl.) B. Verl.** (Bignoniaceae), IAN 194107	Pariri	Native	Leaves	Anemia, diarrhea, washing of wounds, yellow skin and swollen body of pregnant woman.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,51
(15) ***Astrocaryum murumuru* Mart.** (Arecaceae), IAN 196007	Murumuru	Native	Fruit	Dog eye inflammation and irritation in human eyes.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,01
(16) ***Bactris maraja* Mart.** (Arecaceae), IAN 196006	Marajá	Native	Root	Diarrheal, inflammation of the uterus.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,02
(17) ***Bauhinia splendens* Kunth.** (Fabaceae), IAN 196005	Escada de jabuti	Native	Bark	Joint pain, rheumatism and syphilis.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,03
(18) ***Bixa orellana* L.** (Bixaceae), IAN 196001	Urucum	Native	Fruit	Repellent, burn, diarrhea and asthma.	Syrup and decoction; 1 cup 3 x daily for 7 days. Mix with *Carapa guianensis* Aubl. oil and pass on the body 1 x a day.	0,08
(19) ***Brassica oleracea* L.** (Brassicaceae), IAN 195942	Couve manteiga	Introduced	Leaves	Anemia and cholesterol.	Decoction; 1 cup 2 x daily for 7 days.	0,03
(20) ***Bryophyllum pinnatum* (Lam.) Oken** (Crassulaceae), IAN 195954	Pirarucu	Introduced	Leaves	Irritation of the skin.	Decoction; pass on the skin 2 x in the day for 7 days.	0,03
(21) ***Libidibia ferrea* (Mart. ex Til.) L.P. Queiroz** (Fabaceae), IAN 195940	Jucá	Native	Fruit	Bleeding, inflammation in general, washing of wounds and swelling of the legs.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,01
(22) ***Caladium humboldtii* Schott** (Araceae), IAN 194115	Brasileirinho	Native	Leaves	Fever, pain in the uterus and inflammation in general.	Decoction; 1 cup 3 x daily for 7 days.	0,07
(23) ***Calophyllum brasiliense* Cambess.** (Clusiaceae), IAN 195937	Jacareúba	Native	Exsudate and Bark	Joint pain, rheumatism and skin sore.	Exudate; pass on the skin 4 x a day for 7 days.	0,01
(24) ***Calycophyllum spruceanum* (Benth.) Hook. f. ex K. Schum.** (Rubiaceae), IAN 195980	pau mulato	Native	Bark	Itching, inflammation in general and insect stings.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(25) ***Carapa guianensis* Aubl.** (Meliaceae), IAN 194102	Andiroba	Native	Seed	Inflammation in general, massage the pregnant belly to put the baby in place.	Oil; ingest 1 teaspoon 4 x daily for 10 days, topical use 2 x daily for 7 days.	0,91

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(26) ***Cecropia pachystachya* Trécul** (Urticaceae), IAN 195956	Embaúba vermelha	Native	Leaves	Skin irritation and verminoses.	Decoction; 1 teaspoon 4x a day for 7 days.	0,08
(27) ***Cedrela odorata* L.** (Meliaceae), IAN 195971	Cedro	Native	Bark	Verminose, skin irritation, intestinal infections.	Decoction and maceration; 1 cup 4 x daily for 7 days.	0,08
(28) ***Cereus jamacaru* DC.** (Cactaceae), IAN 195976	Mandacarú.	Native	Branches and root	Pulmonary problems, skin infections, sores and kidney stones.	Decoction; 1 teaspoon 4x a day for 10 days.	0,01
(29) ***Chenopodium ambrosioides* L.** (Amaranthaceae), IAN 195978	Mastruz	Introduced	Leaves	Pain, inflammation in general and rheumatism.	Decoction; 1 teaspoon 4x a day for 7 days.	0,11
(30) ***Cichorium intybus* L.** (Asteraceae), IAN 195979	Chicória	Introduced	Leaves and root	Inflammation in general, pain and fever.	Decoction; 1 cup 2 x daily for 7 days.	0,10
(31) ***Cinnamomum zeylanicum* Blume.** (Lauraceae), IAN 195982	Canela	Introduced	Leaves and bark	Flu, diarrhea, inflammation of the uterus and wounds in the mouth.	Syrup and infusion; 1 cup 2 x daily for 7 days.	0,02
(32) ***Cissus verticillata* (L.) Nicolson & C.E. Jarvis** (Vitaceae), IAN 195983	Cipó-urumucá, cipó-pucá	Native	Leaves	High blood pressure, stroke, anemia, tremors and epileptic seizures.	Decoction; 1 cup 2 x daily for 7 days.	0,03
(33) ***Citrus aurantium* L.** (Rutaceae), IAN 195960	Laranja da terra	Introduced	Bark	Swelling of a pregnant woman, inflammation in general, and itching of the foot.	Maceration; 1 cup 2 x a day at dawn and dusk for 7 days.	0,07
(34) ***Citrus limon* (L.) Burm.** (Rutaceae), IAN 195988	Limão galego	Introduced	Fruit	Rheumatism and inflammation of the kidneys.	Decoction; 1 cup 2 x daily for 7 days.	0,02
(35) ***Citrus limonum* L.** (Rutaceae), IAN 195989	Limão	Introduced	Fruit	Rheumatism and inflammation of the kidneys.	Syrup and infusion; 1 cup 3 x daily and inhale the steam 2 x daily for 7 days.	0,29
(36) ***Cocos nucifera* L.** (Arecaceae), IAN 195990	Côco	Native	Fruit and root	Hydration of child and verminoses.	Decoction of the root 1 cup 2 x a day for 7 days and fruit water will during the day for 7 days.	0,02
(37) ***Copaifera* sp.** (Fabaceae), IAN 195991	Copaiba	Native	Bark and oil	Inflammation of the uterus and ovary, and diseases of the stomach.	Decoction; 1 cup 2 x daily for 7 days.	0,02
(38) ***Costus spicatus* (Jacq.) Sw.** (Costaceae), IAN 195993	Canafiche, canarana	Native	Aerial part	Inflammation of the uterus and ovary, vaginal discharge and syphilis.	Decoction; 1 cup 3 x daily for 7 days.	0,03

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(39) ***Crescentia cujete* var. *puberula* Bureau & K. Schum.** (Bignoniaceae), IAN 195959	Cuieiro	Native	Leaves and bark	Malaria, fever and rheumatism.	Decoction; 1 cup 3 x daily for 7 days.	0,05
(40) ***Croton cajucara* Benth.** (Euphorbiaceae), IAN 195994	Sacaca	Native	Leaves and bark	Diarrhea, diabetes, inflammation of the liver, kidneys and bladder.	Decoction; 1 cup 2 x daily for 7 days.	0,01
(41) ***Croton urucurana* Baill.** (Euphorbiaceae), IAN 195996	Forsangue	Native	Bark	Hemorrhagic and inflammation of the uterus.	Decoction; 1 cup 3 x daily for 7 days.	0,18
(42) ***Cucumis anguria* L.** (Cucurbitaceae), IAN 195998	Maxixe	Introduced	Fruit	Eliminate kidney stones, hemorrhoids, inflammation of the kidneys, vomiting.	Decoction; 1 cup 3 x daily for 10 days.	0,01
(43) ***Cymbopogon citratus* (DC.) Stapf** (Poaceae), IAN 195953	Capim marinho	Introduced	Leaves	Inflammation of the uterus and ovary and abdominal pain.	Decoction; 1 cup 3 x daily for 7 days.	0,10
(44) ***Dalbergia monetaria* L. f.** (Fabaceae), IAN 195964	Verônica	Native	Bark	Inflammation, diarrhea and intestinal infection.	Decoction and maceration ingest 1 cup 3 x daily for 7 days.	0,77
(45) ***Dimorphandra gardneriana* Tul.** (Fabaceae), IAN 195995	Faveira	Native	Fruit	High pressure.	Decoction; 1 cup 3 x daily for 7 days.	0,02
(46) ***Eleutherine bulbosa* (Mill.) Urb.** (Iridaceae), IAN 195997	Marupá, Marupázinho	Introduced	Rhizome	Verminoses, wound healing on the skin.	Decoction; 1 cup 3 x daily for 7 days.	0,18
(47) ***Erythrina fusca* Lour.** (Fabaceae), IAN 194108	Assacurana	Native	Bark	Pain in body and head, inflammation and verminoses.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(48) ***Eucalyptus* sp.** (Myrtaceae), IAN 196000	Eucalipto	Introduced	Leaves	Inflammation in general.	Decoction; 1 cup 2 x daily for 7 days.	0,04
(49) ***Eupatorium triplinerve* Vahl.** (Asteraceae), IAN 195946	Japana	Introduced	Aerial parts	Flu	Syrup and infusion; ingest 1 cup 3 x daily for 7 days.	0,09
(50) ***Euterpe oleracea* Mart.** (Arecaceae), HAMAB 10919	Açaí	Native	Bark	Diarrhea and intestinal inflammation.	Decoction; 1 cup 3 x daily for 7 days.	0,56

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(51) ***Genipa americana* L.** (Rubiaceae), IAN 196001	Desinflama	Native	Leaves and bark	Diarrhea, sores on the skin, pains and phlegm in the lung.	Decoction; 1 cup 3 x daily for 7 days.	0,03
(52) ***Gossypium hirsutum* L.** (Malvaceae), IAN 195690	Algodão	Introduced	Leaves	Fever of infections.	Decoction; 1 cup 3 x daily for 7 days.	0,21
(53) ***Gustavia augusta* L.** (Lecythidaceae), IAN 196003	Geniparana	Native	Bark, leaves and flowers	Brave wound (leishmaniasis).	Poultry, decoction and maceration, ingest 1 x 2 x daily for 10 days. Topical use 2 x in day for 7 days.	0,04
(54) ***Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.** (Euphorbiaceae), IAN 196068	Seringueira	Native	Exsudate	Brave wound (leishmaniasis).	Exudate as poultice, topical use 2 x in day for 7 days.	0,03
(55) ***Himatanthus sucuuba* (Spruce ex Müll. Arg.) Woodson** (Apocynaceae), IAN 194107	Sucuúba	Native	Bark and exsudate	Verminoses, inflammation and pain in the uterus, malaria and rheumatism.	Cataplasm; topical use 2 x in day for 7 days.	0,04
(56) ***Hura crepitans* L.** (Euphorbiaceae), IAN 195981	Assacú	Native	Bark	Cancer.	Decoction; 1 cup 2 x daily for 10 days.	0,58
(57) ***Hymenaea oblongifolia* Huber** (Fabaceae), IAN 196009	Jatobá da várzea	Native	Bark and fruit	Diarrhea, pain and inflammation in the body.	Decoction and maceration; 1 cup 2 x daily for 7 days.	0,17
(58) ***Hypericum perforatum* L.** (Hyperaceae), IAN 194100	Dipirona, hipericão, erva de são João	Introduced	Leaves and casca	Pain, depression, nervous irritation.	Decoction; 1 cup 3 x daily for 10 days.	0,04
(59) ***Scutellaria agrestis* A. St. Hil. ex Benth.** (Lamiaceae), IAN 195992	Trevo rocho	Native	Leaves and inflorescences	Headache, itchy skin.	Infusion and poultice; topical use 3 x daily for 7 days.	0,01
(60) ***Jatropha curcas* L.** (Euphorbiaceae), IAN 195944	Pião branco	Introduced	Leaves, seed and bark	Headaches, catarrh in the chest, flu, stroke and scarring.	Dyeing and infusion; 1 cup 2 x daily for 10 days.	0,38
(61) ***Jatropha gossypiifolia* L.** (Euphorbiaceae), IAN 195949	Pião rocho	Introduced	Leaves	Broken and evil eye.	Infusion; 1 cup 2 x daily for 7 days.	0,08
(62) ***Centratherum punctatum* Cass.** (Asteraceae), IAN 195987	Anador, melhoral	Native	Leaves	Bleeding and inflammation of the kidneys.	Decoction; 1 cup 2 x daily for 10 days.	0,21
(63) ***Justicia pectoralis* Jacq.** (Acanthaceae), IAN 194100	Anador, melhoral	Native	Leaves	Headache and joint pain.	Decoction; 1 cup 2 x daily for 7 days.	0,21

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(64) ***Lecythis pisonis* Cambess.** (Lecytidaceae), IAN 196010	Sapucaia	Native	Bark, leaves and flowers	Diarrhea and inflammation in general.	Decoction; 1 cup 2 x daily for 10 days.	0,01
(65) ***Licania macrophylla* Benth.** (Chrysobalanaceae), IAN 194104	Anoerá ou anuerá	Native	Bark	Diarrhea and bleeding.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,20
(66) ***Lippiaalba* (Mill.) N.E. Br. ex Britton & P. Wilson** (Verbenaceae), IAN 194109	Camelitana, erva cidreira	Native	Leaves and flowers	Pain and catarrh in the chest.	Decoction; 1 cup 3 x daily for 7 days.	0,01
(67) ***Luffa operculata* (L.) Cogn.** (Cucurbitaceae), IAN 196013	Buchinha ou cabacinha	Native	Leaves, bark and fruit	Flu, abortion and verminosis.	Decoction; 1 cup 3 x daily for 7 days.	0,02
(68) ***Machaerium lunatum* (L.) Ducke** (Fabaceae), IAN 195973	Aturiá	Native	Leaves	Rheumatism.	Decoction; 1 cup 3 x daily for 7 days.	0,03
(69) ***Macrolobium acaciifolium* (Benth.) Benth.** (Fabaceae), IAN 196011	Faveira-araparí	Native	Bark	Healing.	Maceration; 1 x 3 x daily and topical use 2 x daily for 7 days.	0,04
(70) ***Mangifera indica* L.** (Anacardiaceae), IAN 195965	Manga	Introduced	Leaves and bark	Diarrhea and intestinal infections.	Decoction; 1 cup 3 x daily for 7 days.	0,47
(71) ***Manicaria saccifera* Gaerth.** (Arecaceae), IAN 195984	Bussú	Native	Fruit	Inflammation in the stomach and born in the body (furunculosis).	Maceration; 1 cup 3 x daily for 10 days.	0,04
(72) ***Maquira calophylla* (Poepp. & Endl.) C.C. Berg** (Moraceae), IAN 196012	Muiratingarana	Native	Bark	Cancer.	Decoction; 1 cup 3 x daily for 20 days.	0,04
(73) ***Maquira coriacea* (H. Karst) C.C. Berg** (Moraceae), IAN 196013	Muiratinga	Native	Bark	Malaria.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(74) ***Melissa officinalis* L.** (Lamiaceae), IAN 195947	Cidreira, erva-cidreira	Introduced	Leaves and inflorescences	Headaches, digestive problems, anxiety and nervousness.	Infusion; 1 cup 3 x daily for 7 days.	0,03

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(75) ***Mentha* × *piperita* L.** (Lamiaceae), IAN 196014	Hortelânzinho	Introduced	Leaves	Verminoses and inflammations of the kidney, bladder and liver.	Infusion; 1 cup 3 x daily for 7 days.	0,26
(76) ***Mentha arvensis* L.** (Lamiaceae), IAN 196015	Vick	Introduced	Leaves	Eliminate phlegm from the chest, headache and flu.	Syrup and infusion; 1 cup 3 x daily and inhale the steam 2 x daily for 7 days.	0,08
(77) ***Mentha pulegium* L.** (Lamiaceae), IAN 196016	Hortelân grande	Introduced	Leaves	Flu and inflammation of the kidney.	Syrup and infusion; 1 cup 3 x daily and inhale the steam 2 x daily for 7 days.	0,04
(78) ***Mikania hirsutissima DC.*** (Asteraceae), IAN 195691	Cipó-Sucuriju	Native	Bark	Diarrhea and intestinal infections.	Decoction; 1 cup 3 x daily for 7 days.	0,03
(79) ***Minquartia guianensis* Aubl.** (Olacaceae), HAMAB 6910	Acapú	Native	Bark	Verminoses.	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,04
(80) ***Montrichardia linifera* (Arruda) Schott** (Araceae), IAN 194103	Aninga	Native	Leaves and inflorescences	Healing and general inflammation.	Poultry, topical use 2 x daily for 8 days.	0,09
(81) ***Mora paraensis* Ducke** (Fabaceae), IAN 195702	Pracuúba branca	Native	Bark	Diarrhea, inflammation of the body and irritation of the skin.	Decoction; 1 cup 3 x daily for 7 days.	0,17
(82) ***Musa balbisiana* Colla.** (Musaceae), IAN 194111	Bananeira	Native	Leaves	Hemorrhage and high blood pressure.	Decoction; 1 cup 3 x daily for 7 days.	0,06
(83) ***Ocimum basilicum* L.** (Lamiaceae), IAN 195957	Alfavaquinha	Introduced	Leaves	Cough and catarrh in the chest.	Decoction; 1 cup 3 x daily for 7 days.	0,03
(84) ***Ocimum campechianum* Mill.** (Lamiaceae), IAN 195974	Alfavacão	Introduced	Leaves	Cough and catarrh in the chest.	Syrup and infusion; 1 cup 3 x daily and inhale the steam 2 x daily for 7 days.	0,03
(85) ***Ocimum micranthum* Willd.** (Lamiaceae), IAN 195969	Esturaque	Native	Leaves	Flu, fever and intestinal gas.	Syrup and infusion; 1 cup 3 x daily and inhale the steam 2 x daily for 7 days.	0,25
(86) ***Ocimum selloi* Benth.** (Lamiaceae), IAN 196017	alfavava cheirosa de anis	Native	Leaves and inflorescences	Intestinal gas, inflammation in the stomach, vomiting, flu and fever.	Decoction; 1 cup 3 x daily for 7 days.	0,02

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(87) ***Ocotea cymbarum* Kunth** (Lauraceae), IAN 196018	louro-mamorim	Native	Bark	Inflammation in the body and muscle pain.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(88) ***Origanum vulgare* L.** (Lamiaceae), IAN195970	Majerona da angola	Introduced	Leaves and inflorescences	Pains in the body, eliminate phlegm, flu and fever.	Infusion; 1 cup 3 x daily for 8 days.	0,02
(89) ***Otacanthus azureus* (Linden) Ronse** (Plantaginaceae), IAN 195972	Copaibinha	Native	Leaves	Brave wound (leishmaniasis) and inflammation.	Cataplasm, decoction and maceration; 1 cup 2 x daily for 10 days. Topical use 2 x in day for 7 days.	0,62
(90) ***Ouratea hexasperma* (St. Hill.) Benth.** (Ochnaceae), IAN 194112	Barbatimão	Native	Bark	Rheumatism and inflammation in the stomach.	Decoction; 1 cup 3 x daily for 7 days.	0,07
(91) ***Parahancornia amapa* (Huber) Ducke** (Apocynaceae), IAN 196019	Leite do amapá	Native	Exsudate	Infections.	Exudate; 1 cup 2 x daily for 7 days.	0,03
(92) ***Parkia pendula* (Willd.) Benth. ex Walp.** (Fabaceae), IAN 196010	Comadre de azeite- visgo	Native	Bark and inflorescences	Skin wounds and bleeding from the skin.	Decoction and maceration; topical use 2 x daily for 7 days.	0,01
(93) ***Passiflora edulis* Sims** (Passifloraceae), IAN 195936	Maracujá grande	Native	Leaves and bark	Soothing, nervous irritation and inflammation of the stomach.	Decoction; 1 cup 3 x daily for 7 days.	0,24
(94) ***Passiflora tholozanii* Sacco** (Passifloraceae), IAN 196004	Maracujá do mato	Native	Leaves and bark	Brave wound (leishmaniasis), inflammation and soothing.	Decoction; 1 cup 2 x daily for 7 days.	0,02
(95) ***Pedilanthus tithymaloides* Poit.** (Euphorbiaceae), IAN 196021	Coromina, melhoral	Introduced	Bark	Inflammation in the body and verminoses.	Decoction; 1 cup 2 x daily for 10 days.	0,04
(96) ***Pentachletra macroloba* (Willd.) Kuntze** (Fabaceae), IAN 195962	Pracaxi	Native	Leaves, inflorescences and bark	Inflammation in the body and verminoses.	Oil; ingest 1 teaspoon 3x a day for 7 days.	0,83
(97) ***Peperomia pellucida* (L.) Kunth** (Piperaceae), IAN 195961	Erva de jaboti	Native	Leaves	Elimination of kidney stones, cough, sore throats and itchy skin.	Decoction; 1 cup 2 x daily for 10 days.	0,01
(98) ***Petiveria alliaceae* L.** (Phytolaccaceae), IAN 196022	Mucuracaa	Native	Leaves and root	Malaria, pain and phlegm elimination from the chest.	Decoction; 1 cup 2 x daily for 7 days.	0,36

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(99) ***Phyllanthus amarus*** **Schumach. & Thonn.** (Phyllanthaceae), IAN 195955	Quebra pedra	Native	Aerial parts	Urinary tract pain (Urinary tract infection).	Decoction; 1 cup 2 x daily for 7 days.	0,03
(100) ***Physalis angulata*** **L.** (Solanaceae), IAN 195985	Camapú	Native	Root	Anemia and malária.	Decoction; 1 cup 2 x daily for 7 days.	0,07
(101) ***Platymiscium ulei*** **Harms** (Fabaceae), IAN 196023	Macacaúba	Native	Bark	Elimination of kidney stones, cough and urinary inflammation.	Decoction; 1 cup 2 x daily for 7 days.	0,01
(102) ***Plectranthus ornatus*** **Codd.** (Lamiaceae), IAN 194114	Boldo, boldinho	Introduced	Leaves	Flu, acidity and inflammation of the stomach.	Infusion; 1 cup 2 x daily for 7 days.	0,26
(103) ***Pogostemon heyneanus*** **Benth.** (Lamiaceae), IAN 196024	Oriza	Introduced	Leaves and inflorescences	Flu, phlegm elimination in the chest and cough.	Syrup and infusion; 1 cup 2 x daily for 10 days. Topical use 2 x in day for 7 days.	0,01
(104) ***Portulaca pilosa*** **L.** (Portulacaceae), IAN 194098	Amor crescico	Introduced	Leaves	Pain in urine (urinary tract infection) and verminoses.	Infusion; 1 cup 2 x daily for 7 days.	0,21
(105) ***Pourouma guianensis*** **Aubl.** (Urticaceae), IAN 196025	Embaubarana	Native	Bark	Brave wound (leishmaniasis).	Decoction and maceration ingest 1 cup 3 x daily for 7 days Use topic 2 x on day for 7 days.	0,04
(106) ***Pouteria procera*** **(Mart.) T.D. Penn.** (Sapotaceae), IAN 196026	Maparajuba	Native	Bark	Verminoses and wounds on the skin.	Decoction ingest 1 cup 2 x daily for 7 days.	0,04
(107) ***Psidium guajava*** **L.** (Myrtaceae), IAN 195950	Goiaba	Native	Leaves	Diarrhea, intestinal and renal infections.	Decoction; 1 cup 2 x daily for 7 days.	0,39
(108) ***Quassia amara*** **L.** (Simaroubaceae), IAN 195951	Quina	Native	Leaves	Malaria.	Decoction; 1 cup 2 x daily for 7 days.	0,24
(109) ***Ruta graveolens*** **L.** (Rutaceae), IAN 194106	Arruda	Introduced	Leaves	Ear pain (ear infection), inflammation in the uterus and skin irritations.	Decoction ingest 1 cup 2 x daily for 7 days.	0,23

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(110) ***Sambucus australis* Cham. & Schltdl.** (Adoxaceae), IAN 195958	Sabugueiro	Native	Leaves and bark	Measles and chicken pox.	Cataplasm and decoction; 1 cup 2 x daily for 7 days. Topical use 2 x a day for 7 days.	0,02
(111) ***Schinus terebinthifolia* Raddi** (Anacardiaceae), IAN 194105	Aroiera branca	Native	Bark	Healing and inflammation.	Decoction; 1 cup 3 x daily for 7 days. Topical use 2 x a day for 7 days.	0,05
(112) ***Senna reticulata* (Willd.) H.S. Irwin & Barneby** (Fabaceae), IAN 196027	Pacapeá, barajo.	Native	Bark	Elimination of liver stone and rheumatism.	Decoction; 1 cup 3 x daily for 7 days.	0,18
(113) ***Simaba multiflora* A. Juss.** (Simaroubaceae), IAN 196028	Cajurana, Marupaúba.	Native	Bark	Malaria.	Decoction; 1 cup 2 x daily for 7 days.	0,05
(114) ***Siparuna guianensis* Aubl.** (Siparunaceae), IAN 195941	Capitiú	Native	Leaves and bark	Elimination of kidney stones, headache and muscle aches.	Decoction; 1 cup 3 x daily for 7 days.	0,01
(115) ***Spondias mombin* L.** (Anacardiaceae), IAN 195999	Tapereba	Native	Leaves	Inflammation of the mouth and throat and local massages.	Decoction; 1 cup 2 x daily for 7 days.	0,44
(116) ***Stryphnodendron adstringens* Mart.** (Fabaceae), IAN 194113	Barbatimão	Native	Bark	Urinary Tract Pain (Kidney Infections).	Decoction and maceration; 1 cup 3 x daily for 7 days.	0,06
(117) ***Symphonia globulifera* L. f** (Clusiaceae), IAN 194101	Anani	Native	Bark	Anemia and malaria.	Decoction; 1 cup 3 x daily for 7 days.	0,11
(118) ***Syzygium cumini* (L.) Skeels** (Myrtaceae), IAN 194097	Ameixa	Introduced	Bark	Diarrhea and bleeding.	Decoction; 1 cup 3 x daily for 7 days.	0,07
(119) ***Terminalia catappa* L.** (Combretaceae), IAN 195975	Castanhola	Introduced	Bark	Malaria.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(120) ***Theobroma cacau* L.** (Malvaceae), IAN 195967	Cacau	Native	Leaves and seed	Cleaning of body wounds and verminoses.	Decoction and maceration; 1 cup 2 x daily for 7 days.	0,06

Table 1: Continued.

Scientific Name (Family) Voucher n°	Local name	Distribution	Plant part used	Traditional uses	Preparation method and use form	UV
(121) ***Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum.** (Malvaceae), IAN 195966	Cupuaçú	Native	Bark and fruit	High and fortifying body pressure.	Decoction; 1 cup 3 x daily for 7 days.	0,03
(122) ***Triplaris surinamensis* Cham.** (Polygonaceae), IAN 195939	Tachí da várzea	Native	Bark	Rheumatism.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(123) ***Uncaria guianensis* (Aubl.) J.F. Gmel.** (Rubiaceae), IAN 196011	Jupindá vermelho	Native	Leaves and bark	Diarrhea, hemorrhage and cancer.	Decoction; 1 cup 3 x daily for 7 days.	0,01
(124) ***Uncaria tomentosa* (Willd.) DC.** (Rubiaceae), IAN 195935	Jupindá	Native	Leaves and bark	Diarrhea, hemorrhage and cancer.	Decoction; 1 cup 3 x daily for 7 days.	0,75
(125) ***Unonopsis floribunda* Diels** (Annonaceae) IAN 195938	Envira-sangue	Native	Bark	Rheumatism and hemorrhage.	Decoction; 1 cup 3 x daily for 7 days.	0,04
(126) ***Vatairea guianensis* Aubl.** (Fabaceae), IAN 196002	Fava de bolacha	Native	Bark and seed	Irritation of the skin.	Decoction; 1 cup 3 x daily for 7 days.	0,05
(127) ***Virola surinamensis* (Rol.) Warb.** (Myristicaceae), IAN 195963	Virola	Native	Bark and fruit	Born in the body (furunculosis) and intestinal infections.	Decoction and exudate as poultice; topical use 2 x a day for 7 days.	0,62
(128) ***Vismia macrophylla* Kunth** (Clusiaceae), IAN 195986	Lacre da folha grande	Native	Bark	Brave wound (leishmaniasis) and skin irritation.	Decoction; 1 cup 2 x daily for 10 days.	0,04
(129) ***Vitex agnus-castus* L.** (Lamiaceae), IAN 195968	Alecrim de angola, Pau de angola	Introduced	Leaves	Cough and phlegm elimination from the chest.	Syrup; 1 teaspoon 3 times a day for 10 days.	0,04
(130) ***Zingiber officinale* Roscoe** (Zingiberaceae), HAMAB 10286	Gengibre	Introduced	Rhizome	Sore throat (inflammation of the throat) and inflammation in the joints.	Decoction; 1 cup 2 x daily for 7 days.	0,01

Native Species: plant that is natural, own of the region of forest of low várzea.
Species Introduced: a species that is established beyond its natural range, after being transported and introduced intentionally or accidentally by man.

Table 2: Ethnopharmacological indications of medicinal plants cited by the residents of the river mouth of the Mazagão River.

Category of therapeutic use	Number of plants used	Cited uses	ICF
Microbial Infections	73	532	0,86
Gastrointestinal disorders	47	412	0,89
Inflammatory disorders	41	321	0,88
Pain, fever, cold	25	167	0,86
Malaria	25	90	0,73
Cardiovascular disorders	24	79	0,71
Parasites	23	66	0,66
Respiratory Infections	23	111	0,86
Rheumatism	23	49	0,54
Disorders of the Nervous System	11	45	0,77
Renal, hepatic and biliary disorders	9	30	0,72
Healing	8	41	0,83
Leishmaniasis	8	74	0,9
Cancer	5	58	0,93
Gynecological disorders	5	12	0,64
Metabolic Diseases	3	16	0,87

the production of secondary metabolites present mainly in the leaves [65]. Phytochemical studies with leaves contained flavonoids, tannins, saponins, steroids, and triterpenoids [66, 67]. According to Matos and Matos [68], flavonoids have direct action on capillaries and potentiate ascorbic acid, whose hemorrhagic action and anti-inflammatory action are similar to cortisone. Tannins are used in the treatment of burns, in the recomposition of exposed tissue proteins and in the formation of slightly antiseptic coating [66]. Steroids have important therapeutic properties (cardiotonic, anabolic, contraceptive, and anti-inflammatory) [69]. According to Robbers et al. [70], the triterpenoids have antimicrobial and antitumor action, but some are very toxic to the human body.

3.4. Forms of Ethnomedicinal Preparations. The preparation forms were classified as decoction, infusion, syrup, maceration, oil, hydration water, latex, tincture, and poultice (Table 1). Decoction was the most used form of preparation (59.4%), followed by maceration (11.5%) and infusion (9.7%), and the other six forms of preparation combined corresponded to 19.4%. According to Amorozo and Gély [71] and Lisboa [72], decoction is the most common way of administration home remedies in Amazonian communities. Decoction is performed so that the home remedy is stored in a refrigerator and has more use lifetime. Teas obtained by decoction or infusion are consumed orally and can be used in baths for various types of diseases, including the cultural diseases reported in this work such as "nascida", "panemeira", bewitching, evil eye, "úra", and bubo.

In the community, women collect the fruits of *Carapa guianensis* (andiroba) and *Pentaclethra macroloba* (pracaxi), which are dispersed in the water, for the production of medicinal oil, a common management practice to commercialized them in the city of Macapá.

Maceration is a preparation that uses barks, leaves, branches and roots that are immersed in water and/or alcohol, are indicated for inflammation, diarrhea and other intestinal disorders, and are consumed by mouth. In the community, the latex that flows from the trunk of medicinal tree species is applied to wounds, and in this study, it is also collected in medicinal bottles for oral use. According to Viega and Scudeller [73], the use of bark, root, and latex in the preparation of home remedies is known as medicinal bottle, and it is a preparation that is widely disseminated throughout the Amazon and other areas of the world.

3.5. Therapeutic Indications. The riverside residents reported 2103 phytotherapeutic uses for medicinal plants, which were grouped into 16 categories of therapeutic use. For this, the type of body system involved was related to the disease and symptoms, as well as the medicinal species used in the community. It was observed that most of the plants used are related to diseases such as microbial infections (20.67%, 73 species), gastrointestinal disorders (13.31%, 47 species) and inflammations (11.61%, 41 species), emphasizing that 1 specie can be cited for several diseases (Table 2). Studies carried out in traditional communities in Brazil often point to several plant species for problems of the gastrointestinal system, including parasitic diseases and infections caused by microbial agents [57, 74, 75]. These results corroborate studies carried out in communities that do not have basic sanitation in developing countries alongside Latin America [38, 57, 76–79], and this ranking may therefore be related to economic conditions and regional habits as the riparian region studied does not present basic sanitation, and the community uses septic tanks and collects water directly from the river for their essential needs.

Leão et al. [80] performed an ethnobotanical survey in Santa Barbara, state of Pará. Similarly to this study, the authors observed that the major diseases and symptoms treated by the community were gastrointestinal disorders,

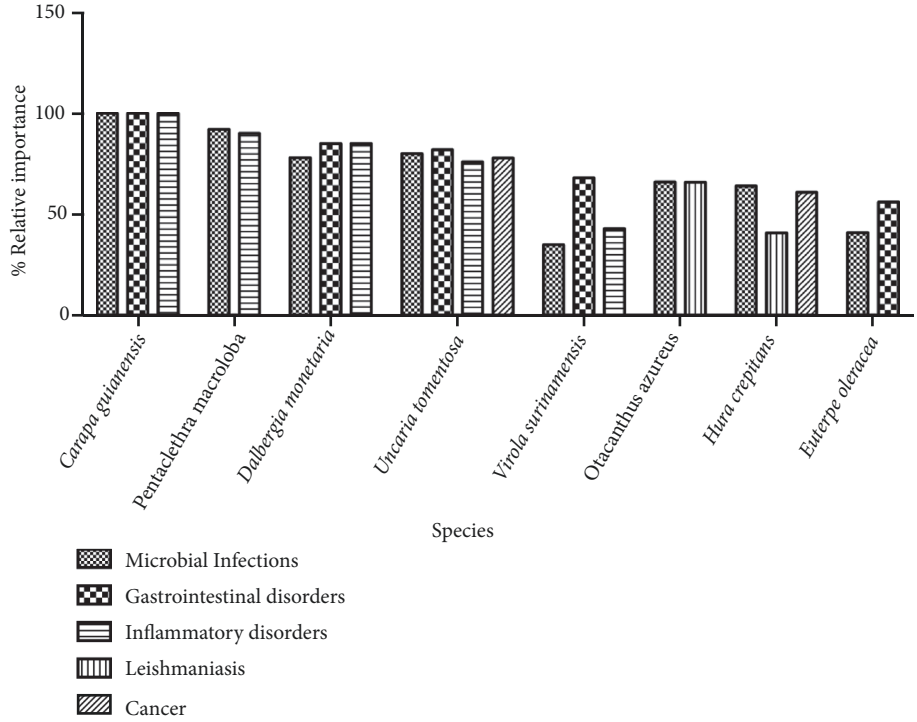


Figure 2: Relative importance of the most cited species in relation to main categories of therapeutic use in the community.

microbial infections, and inflammations. This was also reported in other study by Amorozo [58].

In this study, the species *Carapa guianensis* (100%), *Pentaclethra macroloba* (90-92%), *Dalbergia monetaria* (78-85%), *Uncaria tomentosa* (80-82%), *Virola surinamensis* (43-68%), *Otacanthus azureus* (66%), *Hura crepitans* (41-64%), and *Euterpe oleracea* (41-56%) (Figure 2) are widely used by the riverside community to treat microbial infections, gastrointestinal disorders, inflammatory conditions, leishmaniasis, and cancer, as seen by the use agreement index (Table 3). According to Vendruscolo and Mentz [53], this index indicates the most promising species to perform pharmacological researches according to their use.

Ethnopharmacological studies performed with the species *C. guianensis, P. macroloba*, *D. monetaria,* and *U. tomentosa* show that species are popularly used to treat abscesses, asthma, skin diseases, infectious diseases, deep wounds, gastritis, inflammations, gastric ulcer [16, 23, 44, 80]. Currently, this is being confirmed experimentally in studies with the oil from the seeds of *C. guianensis* that show its anti-inflammatory activity [35, 80], antiallergic activity [81–83], and wound-healing activity [84, 85]. Also, phytochemical studies attribute anti-inflammatory and antiallergic activity to the tetranortriterpenoids, main molecules of *C. guianensis* oil [86]. The same occurs with *U. tomentosa*, whose extract is reported to have antimicrobial, anti-inflammatory and anticancer activity *in vitro* and *in vivo* [87]; this is due to the presence of alkaloids, triterpenic heterosides, and polyphenols, mainly tannins [88–90].

The species *P. macroloba* has some medicinal application against snakebites. Triterpenic saponins isolated from its fruit were reported to be effective against snake venom [91]. Also, the essential oil of *O. azureus* has bactericidal, antioxidant [92], antifungal [93], and leishmanicidal activity [94]. However, the species *D. monetaria, V. surinamensis, O. azureus*, *H. crepitans,* and *E. oleracea* still need further *in vitro* and *in vivo* pharmacological research to corroborate their use in folk medicine.

Of the informants, 100% believe in the efficacy of medicinal plants in common diseases such as fever, diarrhea, and infections and prefer to use them because they understand that there are no side effects and because it is a therapeutic resource free of cost and easily obtainable in the community; however, when serious complications result from malaria and heart disease, they prefer drugs from the pharmacy because they works faster.

3.6. Comparison of the Different Indices. In this study, UVs between 0.91 and 0.56 were from native medicinal plants that are frequently used as an ethnomedicinal resource by the riparians: *Carapa guianensis* (0.91), *Pentachletra macroloba* (0.83), *Dalbergia monetaria* (0.77), *Uncaria tomentosa* (0.75), *Otacanthus azureus* (0.62), *Virola surinamensis* (0.62), *Hura crepitans* (0.58), and *Euterpe oleracea* (0.56). These species were the most versatile in the therapeutic preparations and obtained a use index ranging from 100% to 56%; they also were the most frequent in the study area, with a loyalty index of 100% for a specific therapeutic use (Table 1). The most important species for a community are those that have the highest Use Value, and they should be prioritized for conservation [95].

Table 3: Category of therapeutic use and use agreement index (Pcusp) of the medicinal plants cited by the residents of the river mouth of the Mazagão river.

Category of therapeutic use	Scientific Name	NF	FC	Pcusp
Microbial Infections	*Carapa guianensis*	100	1	100
	Hura crepitans	100	0,64	64
	Otacanthus azureus	96,7	0,68	66
	Uncaria tomentosa	97,2	0,82	80
	Pentaclethra macroloba	100	0,92	92
	Copaifera sp.	100	1	100
	Dalbergia monetaria	92,3	0,85	78
Gastrointestinal disorders	*Euterpe oleracea*	89,8	0,62	56
	Carapa guianensis	100	1	100
	Uncaria tomentosa	100	0,82	82
	Spondias mombin	100	0,48	48
	Dalbergia monetaria	100	0,85	85
	Virola surinamensis	100	0,68	68
Inflammatories disorders	*Carapa guianensis Aubl.*	100	1	100
	Uncaria tomentosa	92,1	0,82	76
	Pentachletra macroloba	97,5	0,92	90
	Dalbergia monetaria	100	0,85	85
	Virola surinamensis	79,3	0,54	43
	Copaifera sp.	100	1	100
Pain, Fever, Cold	*Plectranthus ornatus*	92,1	0,82	76
	Eleutherine bulbosa	97,5	0,92	90
	Origanum vulgare	100	0,85	85
	Petiveria alliacea	79,3	0,54	43
	Jatropha curcas	92,1	0,82	76
Malaria	Symphonia globulifera	100	0,85	85
	Parkia pendula	97,5	0,92	90
	Petiveria alliacea	100	0,68	68
	Maquira coriacea	92,1	0,82	76
	Arrabidaea chica	100	0,85	85
	Quassia amara	100	0,68	68
	Sambucus australis	92,1	0,82	76
Cardiovascular disorders	*Minquartia guianensis*	92,1	0,82	76
	Licania macrophylla	97,5	0,92	90
	Croton urucurana	100	0,85	85
	Arrabidaea chica	79,3	0,54	43
	Jatropha curcas	92,1	0,82	76
Parasites	*Portulaca pilosa*	92,1	0,82	76
	Symphonia globulifera	97,5	0,92	90
	Cedrela odorata	100	0,85	85
	Eleutherine bulbosa	79,3	0,54	43
	Arrabidaea chica	77,1	0,44	34
Respiratory Infections	*Gossypium hirsutum*	92,1	0,82	76
	Adenocalymna alliaceum	97,5	0,92	90
	Mentha pulegium	100	0,85	85
	Eupatorium triplinerve	79,3	0,54	43
	Citrus limonum	96,4	0,32	31
	Ocimum campechianum	92,3	0,28	26
	Chenopodium ambrosioides	78,6	0,13	10

Table 3: Continued.

Category of therapeutic use	Scientific Name	NF	FC	Pcusp
Rheumatism	*Justicia pectoralis*	60	0,14	8
	Carapa guianensis	100	1	100
	Siparuna guianensis	92,1	0,82	76
	Ocotea cymbarum	97,5	0,92	90
	Petiveria alliacea	100	0,85	85
	Copaifera sp.	100	1	100
Disorders of the Nervous System	*Aeollanthus suaveolens*	92,1	0,82	76
	Genipa americana	97,5	0,92	90
	Hypericum perforatum	100	0,85	85
	Cítrus aurantium	79,3	0,54	43
	Passiflora tholozanii	100	0,02	2
	Passiflora edulis	95,8	0,27	26
	Eleutherine bulbosa	70,6	0,14	10
Renal, hepatic and biliary disorders	*Alternanthera brasiliana*	92,1	0,82	76
	Senna reticulata	97,5	0,92	90
	Arrabidaea chica	100	0,85	85
	Phyllanthus amarus	79,3	0,54	43
	Croton cajucara	33,3	0,01	0
Healing	*Portulaca pilosa*	92,1	0,82	76
	Montrichardia linifera	97,5	0,92	90
	Macrolobium acaciifolium	100	0,85	85
	Eleutherine bulbosa	79,3	0,54	43
	Jatropha curcas	16,7	0,07	1
Leishmaniasis	*Hura crepitans*	79,6	0,51	41
	Otacanthus azureus	96,7	0,68	66
	Pourouma guianensis	92,1	0,82	76
	Gustavia augusta	97,5	0,92	90
	Vismia macrophylla	100	0,85	85
	Passiflora tholozanii	79,3	0,54	43
	Eleutherine bulbosa	70,6	0,14	10
Cancer	*Alternanthera brasiliana*	52,6	0,12	6
	Hura crepitans	94,7	0,64	61
	Uncaria tomentosa	97,1	0,8	78
	Uncaria guianensis	92,1	0,82	76
	Maquira coriacea	97,5	0,92	90
	Passiflora tholozanii	100	0,85	85
Gynecological disorders	*Hura crepitans*	79,3	0,54	43
	Copaifera sp.	100	1	100
	Uncaria tomentosa	97,1	0,8	78
	Uncaria guianensis	50	0,01	1
	Carapa guianensis	100	1	100
	Pentachletra macroloba	97,5	0,92	90
	Dalbergia monetaria	100	0,85	85
Metabolic Diseases	*Euterpe oleracea*	81,1	0,51	41
	Arrabidaea chica	100	0,85	85
	Croton cajucara	79,3	0,54	43

The species *Carapa guianensis* and *Uncaria tomentosa* have been among the most phytochemically studied species in recent years. Studies show that *C. guianensis* (andiroba) oil has anti-inflammatory [35, 95], antiparasitic [96], antispasmodic [97], repellent, antiallergic, antirheumatic and healing properties [98]. Pharmacological tests performed *in vitro* and *in vivo* with *U. tomentosa* (jupindá) showed antioxidant [99], anticancer [100], anti-inflammatory [101], antimicrobial [102], antiherpetic [103] and antidiabetic [104] activities. In turn, *Pentaclethra macroloba, Virola surinamensis*, *Dalbergia monetaria*, *Otacanthus azureus*, *Virola surinamensis*, *Hura crepitans,* and *Euterpe oleracea* are little known pharmacologically.

Eight floodland forest native species are mentioned for the first time in an ethnobotanical study in the region, and no pharmacological studies have been found for *Allamanda cathartica* (0.01), which is used for the treatment of intestinal parasites*; Astrocaryum murumuru* (0.01), used for the treatment of eye infections of dogs and skin irritations of other animals; *Calophyllum brasiliense* (0.01), used for joint inflammations and skin ulcers; *Passiflora tholozanii* (0.02), used for leishmaniasis ulcers, cancer, depression and soothing; *Manicaria saccifera* (0.04), used for gastritis; *Pourouma guianensis* (0.04), used for leishmaniasis joint inflammation and ulcers; *Triplaris surinamensis* (0.04), used for the treatment of joint inflammation; and *Unonopsis floribunda* (0.04), used for joint and stomach inflammation. Although many species are reported for the treatment of the diseases mentioned, these species deserve attention because the region is going through a rural exodus, and people with this knowledge are decreasing in number and have no successors of that knowledge, which is traditionally passed by word of mouth.

The Informant Consensus Factor (ICF) was calculated for 16 categories of therapeutic uses (Table 2). Leishmaniasis is endemic in the riverside area and recorded an ICF value of 0.9, followed by cancer (0.93), gastrointestinal disorders such as diarrhea, vomiting, and gastritis (0.89), inflammation in the uterus and burns (0.88), diabetes and albumin (0.87), and microbial, respiratory infections and pain, fever and cold symptoms (0.86); these include diseases such as itching, uterine infections, body aches, insect bites, flu, catarrh in the chest and cough. Nervous system disorders such as epilepsy, convulsions and depression had ICF values of 0.77; malaria, 0.73; renal and bile calculi, 0.72; infarction, bleeding, and high blood pressure, 0.71; followed by verminoses, 0.66; gynecological problems after childbirth, 0.64; and joint inflammation, 0.54.

High ICF values clearly showed that the community uses medicinal plants for their health problems and that there are well-defined choice criteria, which are shared orally [48, 105]. A total of 73 medicinal plants were used for microbial infections, followed by 47 that are referred for the gastrointestinal system and 41 for general inflammation in the body. According to the interviewees, the therapeutic use of plants in the community is the first alternative for the treatment of health problems, and in many cases, it constitutes the only immediate resource for this purpose, as there are difficulties in accessing allopathic medicines because they live far from urban centers, have small boats as transportation means, which are called "rabetas", and do not have public health units for simple clinical care for diarrhea, headaches and infections. According to Lima et al. [106], the rich traditional knowledge of communities living in isolation in the Amazon arises from the need to have an alternative therapeutic treatment, caused by the limited access to the public health network and the great cultural influence of these peoples.

In the study, the floodland forest native species with the highest values of relative importance, for the treatment of the most frequent diseases in the community, are as follows (Table 3): *Carapa guianensis, Hura crepitans, Otacanthus azureus Uncaria tomentosa, Uncaria guianensis, Pentaclethra macroloba, Copaifera sp., Dalbergia monetaria, Spondias mombin, Virola surinamensis, Symphonia globulifera*, *Parkia pendula*, *Maquira coriacea, Croton urucurana, Quassia amara, Genipa americana, Vismia macrophylla, Gustavia augusta,* and *Passiflora tholozanii.* They are the most versatile because they can be used in various therapeutic treatments.

4. Conclusion

This is a pioneer study of the riparian community in flooded areas of Mazagão, and it shows that knowledge about plants and their medicinal uses is diverse and widespread in the community, likely because of the high incidence of tropical diseases such as malaria and leishmaniasis as well as the difficulty in accessing medicines distributed by the government and easy access to local plants. The riparians listed 130 ethnospecies, of which 95 are mostly native trees of lowland forest. In the study, the residents showed that they use conservation practices such as not annealing the individuals when collecting bark/bast; in addition, after extracting medicinal oil from the copaiba trunk, they always allow one year for the plant to recover, and if needed, they search for another trunk so as to not to exhaust the resource. They also collect *Carapa guianensis* (andiroba) and *Pentachletra macroloba* (pracaxi) fruits, which are dispersed in the water, for extraction of the medicinal oil, which is marketed in urban areas. Residents report that they have a potential consumer market for these medicinal oils and that organization is lacking to make the production of this oil profitable. Therefore, native medicinal species should be prioritized for conservation, as riparians depend on the collection of these plants as the main drugs for the region's endemic and cultural diseases; furthermore, these plants may be used for future pharmacological studies. Studies of this nature reinforce the importance of the relationship between the community and biodiversity, valuing, and bringing visibility to the ethnobotanical and ethnopharmacological knowledge they possess. The systematization of knowledge about these resources rescues popular knowledge, contributing not only to the conservation of diversity but also to the preservation of a rich and important cultural heritage.

Data Availability

All data generated and analyzed to support this study are included in this published article. The project was submitted to the Ethics Research Committee of the Faculdade Estácio de Macapá (http://aplicacao.saude.gov.br/plataformabrasil/login.jsf, under the opinion no. 14.94.994). And the complete information of the individual interviews could be requested from the corresponding authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors would like to thank CAPES (no. 3292/2013 AUXPE) and CNPq Proc. 407768/2013-0 (RAPBioFar) for the financial support.

References

[1] F. Wittmann, J. Schongart, J. M. Brito et al., *Manual of Trees from Central Amazonian Várzea Floodplains: Taxonomy, Ecology and Use*, INPA, 2010.

[2] S. S. Almeida, D. D. Amaral, and A. S. Silva, "Análise florística e estrutura de florestas de várzea no estuário amazônico," *Acta Amazonica*, vol. 34, no. 4, pp. 513–524, 2004.

[3] J. A. L. Queiroz and S. Mochiutti, "Cultivo de açaizeiros e manejo de açaizais para a produção de frutos," *Embrapa Amapá*, 2001.

[4] A. G. Martins, D. L. Rosário, M. N. Barros, and M. A. G. Jardim, "Levantamento etnobotânico de plantas medicinais, alimentares e tóxicas da ilha do Combu, município de Belém, estado do Pará, Brasil," *Revista Brasileira de Farmácia*, vol. 86, no. 1, pp. 21–30, 2005.

[5] L. M. B. Rodrigues, A. U. S. Lira, F. A. Santos, and M. A. G. Jardim, "Composição florística e uso das espécies vegetais em dois ambientes de florestas de várzea," *Revista Brasileira de Farmácia*, vol. 87, no. 2, pp. 45–48, 2006.

[6] E. P. P. Pinto, M. C. M. Amorozo, and A. Furlan, "Conhecimento popular sobre plantas medicinais em comunidades rurais de mata atlântica - Itacaré, BA, Brasil," *Acta Botanica Brasilica*, vol. 20, no. 4, pp. 751–762, 2006.

[7] U. P. Albuquerque and L. D. H. C. Andrade, "Conhecimento botânico tradicional e conservação em uma área de caatinga no estado de Pernambuco, Nordeste do Brasil," *Acta Botanica Brasilica*, vol. 16, no. 3, pp. 273–285, 2002.

[8] A. S. Ribeiro, M. d. Palha, M. M. Tourinho, C. Whiteman, and A. d. Silva, "Utilização dos recursos naturais por comunidades humanas do Parque Ecoturístico do Guamá, Belém, Pará," *Acta Amazonica*, vol. 37, no. 2, pp. 235–240, 2007.

[9] F. G. S. Oliveira and C. R. Lehn, "Riscos e perspectivas na utilização de fitoterápicos no Brasil," *Opará: Etnicidades, Movimentos Sociais e Educação, Paulo Afonso*, vol. 3, no. 4, pp. 35–44, 2015.

[10] L. N. Pinto, A. S. O. Flor, and W. L. R. Barbosa, "Fitoterapia popular na Amazônia Paraense: uma abordagem no município de Igarapé-Mirí, estado do Pará nos anos de 2000 e 2008," *Revista Ciências Farmacêuticas Básica Aplicada*, vol. 35, no. 2, pp. 305–311, 2011.

[11] J. B. Calixto, "Twenty-five years of research on medicinal plants in Latin America: a personal view," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 131–134, 2005.

[12] W. D. C. Firmo, V. A. M. Menezes, C. E. D. C. Passos et al., "Contexto histórico, uso popular e concepção científica sobre plantas medicinais," *Caderno de Pesquisa*, vol. 18, no. especial, pp. 90–95, 2011.

[13] B. Véronique, "Passado Português, presente negro e indizibilidade ameríndia: o caso de MazagãoVelho, Amapá," *Religião & Sociedade*, vol. 28, no. 2, pp. 11–29, 2008.

[14] P. de Assunção, "Mazagão: cidades em dois continentes," in *Revista eletrônica de Arquitetura e Urbanismo*, vol. 1, pp. 22–55, Universidade São Judas Tadeu, São Paulo, Brazil, 2009.

[15] A. C. D. Silva, E. S. Custódio, and E. D. L. S. Foster, "Intangible cultural heritage and religiosity: Celebrations in the Mazagão Velho, in the state of Amapá," *Estudos Teológicos*, vol. 55, no. 2, pp. 388–403, 2015.

[16] V. Laurent, *Mazagão a cidade que atravessou o Atlântico: do Marrocos à Amazônia, (1769-1783)*, Martins Fontes, São Paulo, Brazil, 2008.

[17] D. A. C. Sant'Ana, *O Mito Moderno da Natureza Intocada*, Hucitec NAPUB USP, São Paulo, Brazil, 2008.

[18] R. M. Lomba, "Modos de vida ribeirinho na comunidade Foz do Rio Mazagão – Mazagão (AP/Brasil)," *Ateliê Geográfico*, vol. 11, no. 1, pp. 257–276, 2017.

[19] M. S. de Mendonça, M. A. D. de Souza, M. F. Cassino et al., *Plantas medicinais usadas por comunidades ribeirinhas do médio Rio Solimões, Amazonas: identificação, etnofarmacologia e caracterização estrutural*, Universidade Federal do Amazonas, 2014.

[20] R. B. L. Silva, *Diversidade, uso e manejo de quintais agroflorestais no Distrito do Carvão, Mazagão-AP, Brasil [PhD thesis]*, Universidade Federal do Pará, Belém, Brazil, 2010.

[21] E. S. Nascimento, *Levantamento dos conhecimentos etnobotânicos de comunidades ribeirinhas do estuário amapaense*, Universidade Federal do Amapá, Macapá, Brasil, 2011.

[22] J. P. G. dos Santos-Silva and P. C. de Oliveira, "Ethnobotany of Medicinal Plants in the Low Land Community Igarapé do Costa, SantarémPará, Brazil," *Ambiente y Sostenibilidad*, vol. 2016, no. 6, pp. 136–151, 2016.

[23] S. P. F. Vásques, M. S. Mendonça, S. N. Noda, S. P. F. Vásques, and M. S. Mendonça, "Etnobotânica de plantas medicinais em comunidades ribeirinhas do Município de Manacapuru, Amazonas, Brasil," *Acta Amazônica*, vol. 44, no. 4, pp. 457–472, 2014.

[24] IBGE, *Instituto Brasileiro de Geografia e Estatística*, censo populacional, 2010, http://www.ibge.gov.br.

[25] M. J. V. C. Carim, M. A. G. Jardim, and T. D. S. Medeiros, "Composição florística e estrutura da floresta de várzea no município de Mazagão, Estado do Amapá, Brasil," *Scientia Forestalis*, vol. 36, no. 79, pp. 191–201, 2008.

[26] A. C. Pinto, A. P. S. C. Simas, A. T. Tardin et al., "Macrodiagnóstico do Estado do Amapá: Primeira aproximação do zoneamento econômico ecológico," in *Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá*, Macapá, Brasil, 2002.

[27] F. Wittmann, J. Schöngart, J. C. Montero et al., "Tree species composition and diversity gradients in white-water forests across the Amazon Basin," *Journal of Biogeography*, vol. 33, no. 8, pp. 1334–1347, 2006.

[28] F. Wittmann, J. Schöngart, and W. J. Junk, "Phytogeography, species diversity, community structure and dynamics of Central Amazonian floodplain forests," *Amazonian Floodplain Fororest*, vol. 210, pp. 61–102, 2010.

[29] IBGE, "Instituto Brasileiro de Geografia e Estatística," in *Manual técnico da vegetação brasileira*, IBGE, Rio de janeiro, Brazil, 2012.

[30] J. V. M. Carim, M. F. K. Wittmann, M. T. F. Piedade, J. R. S. Guimarães, and L. C. L. Tostes, "Composition, diversity, and structure of tidal "Várzea" and "Igapó" floodplain forests in eastern Amazonia, Brazil," *Brazilian Journal of Botany*, vol. 40, no. 1, pp. 115–124, 2017.

[31] B. V. Rabelo, *Mazagão: Realidades Que Devem Ser Conhecidas*, IEPA, Macapá, Brasil, 2005.

[32] E. R. Pinto, *Solos, hidrologia e estrutura populacional de pracuubeiras em florestas de várzea do estuário amazônico*, Universidade Federal do Amapá, Macapá, Brazil, 2014.

[33] U. P. Albuquerque and R. F. P. Lucena, "Methods and techniques in the etnobotany research," in *Livro Rápido/Núcleo de Publicações em Ecologia e Etnobotânica Aplicada*, Pernambuco, Recife, Brazil, 2004.

[34] O. Fidalgo and L. R. Bononi, *Técnica de coleta, preservação e herborização de material botânico*, Instituto de Botânica, São Paulo, Brasil, 1989.

[35] H. Lorenzi and F. J. A. Matos, *Plantas Medicinais no Brasil: nativas e exóticas cultivadas*, Instituto Plantarum, Nova Odessa, Brasil, 2008.

[36] Angiosperm Phylogeny Group (APG), "An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV," *Botanical Journal of the Linnean Society*, vol. 181, pp. 1–20, 2016.

[37] A. M. Carneiro and B. E. Irgang, "Origem e distribuição geográfica das espécies ruderais da Vila de Santo Amaro, General Câmara, Rio Grande do Sul," *Iheringia, Série Botânica*, vol. 60, no. 2, pp. 175–188, 2005.

[38] O. Phillips and A. H. Gentry, "The useful plants of Tambopata, Peru: I. Statistical hypotheses tests with a new quantitative technique," *Economic Botany*, vol. 47, no. 1, pp. 15–32, 1993.

[39] J. E. Morvin Yabesh, S. Prabhu, and S. Vijayakumar, "An ethnobotanical study of medicinal plants used by traditional healers in silent valley of Kerala, India," *Journal of Ethnopharmacology*, vol. 154, no. 3, pp. 774–789, 2014.

[40] M. Kichu, T. Malewska, K. Akter et al., "An ethnobotanical study of medicinal plants of Chungtia village, Nagaland, India," *Journal of Ethnopharmacology*, vol. 166, pp. 5–17, 2015.

[41] R. T. Troter and M. H. Logan, "Informant consensus: a new approach for identifying potentially effective medicinal plants," in *Plants in Indigenous Medicine and Diet*, N. L. Etkin, Ed., pp. 91–112, Redgrave Publishing Company, New York, NY, USA, 1986.

[42] J. Friedman, Z. Yaniv, A. Dafni, and D. Palewitch, "A preliminary classification of the healing potential of medicinal plants, based on a rational analysis of an ethnopharmacological field survey among Bedouins in the Negev Desert, Israel," *Journal of Ethnopharmacology*, vol. 16, no. 2-3, pp. 275–287, 1986.

[43] V. A. Da Silva, L. D. Andrade, and U. P. De Albuquerque, "Revising the Cultural Significance Index: The case of the Fulnio in Northeastem Brazil," *Field Methods*, vol. 18, no. 1, pp. 98–108, 2016.

[44] M. C. Pasa and G. d. Ávila, "Ribeirinhos e recursos vegetais: a etnobotânica em Rondonópolis, Mato Grosso, Brasil," *Interações (Campo Grande)*, vol. 11, no. 2, pp. 195–204, 2010.

[45] B. G. Brasileiro, V. R. Pizziolo, D. S. Matos, A. M. Germano, and C. M. Jamal, "Plantas medicinais utilizadas pela população atendida no "Programa de Saúde da Família", Governador Valadares, MG, Brasil," *Revista Brasileira de Ciências Farmacêuticas*, vol. 44, no. 4, pp. 629–636, 2008.

[46] E. Elisabetsky, "Etnofarmacologia," *Ciência e Cultura. Campinas*, vol. 55, no. 3, pp. 35-36, 2003.

[47] N. Hanazaki, J. Y. Tamashiro, H. F. Leitão-Filho, and A. Begossi, "Diversity of plant use in two caiçara communities from the Atlantic Forest coast, Brazil," *Biodiversity and Conservation*, vol. 9, no. 5, pp. 597–615, 2000.

[48] R. R. B. Negrelle and K. R. C. Fornazzari, "Estudo etnobotânico em duas comunidades rurais (Limeira e Ribeirão Grande) de Guaratuba (Paraná, Brasil)," *Revista Brasileira de Plantas Medicinais*, vol. 9, no. 2, pp. 36–54, 2007.

[49] J. A. L. Queiroz, S. Mochiutti, A. S. Machado, and F. Galvão, "Composição florística e estrutura de floresta em várzea alta estuarina amazônica," *Revista Floresta*, vol. 35, no. 1, pp. 41–56, 2005.

[50] L. C. Ming and J. A. Amaral, "Aspectos etnobotânicos de plantas medicinais na reserva extrativista "Chico Mendes"," *The New York Botanical Garden*, 2015, http://www.nybg.org/bsci/acre/www1/medicinal.html.

[51] G. Guarim Neto and R. G. Morais, "Recursos medicinais de espécies do Cerrado de Mato Grosso: um estudo bibliográfico," *Acta Botanica Brasilica*, vol. 17, no. 4, pp. 561–584, 2003.

[52] S. P. Vásquez, M. S. Mendonça, and S. d. Noda, "Etnobotânica de plantas medicinais em comunidades ribeirinhas do Município de Manacapuru, Amazonas, Brasil," *Acta Amazonica*, vol. 44, no. 4, pp. 457–472, 2014.

[53] G. S. Vendruscolo and L. A. Mentz, "Estudo da concordância das citações de uso e importância das espécies e famílias utilizadas como medicinais pela comunidade do bairro Ponta Grossa, Porto Alegre, RS, Brasil," *Acta Botanica Brasilica*, vol. 20, no. 2, pp. 367–382, 2006.

[54] J. P. G. Santos-Silva and P. C. Oliveira, "Ethnobotany of Medicinal Plants in the Low Land Community Igarapé do Costa, Santarém–Pará, Brazil," *Revista del Doctorado Interinstitucional en Ciencias Ambientales: Ambiente y Sostenibilidad*, vol. 6, pp. 136–151, 2016.

[55] M. N. Alexiades and J. W. Sheldon, *Selected Guidelines for Ethnobotanical Research: A Field Manual*, Institute of Economic Botany, The New York Botanical Garden, Bronx, NY, USA, 1996.

[56] E. M. Miranda, J. A. Sousa, and R. C. A. Pereira, "Caracterização e avaliação de populações nativas de unha-de-gato [Uncaria tomentosa (Willd.) D.C. e Uncaria guianensis (Aubl.) Gmel.] no vale do rio Juruá-AC," *Revista Brasileira de Plantas Medicinais*, vol. 5, no. 2, pp. 41–46, 2003.

[57] A. S. Filocreão, A. G. Galindo, and T. D. Dos Santos, "Fitoterapia na Amazônia: a experiência do estado do Amapá-Brasil," *Desenvolvimento e Meio Ambiente*, vol. 40, 2017.

[58] M. C. Amorozo, "Uso e diversidade de plantas medicinais em Santo Antonio do Leverger, MT, Brasil," *Acta Botanica Brasilica*, vol. 16, no. 2, pp. 189–203, 2002.

[59] G. Guarim Neto and R. G. Morais, "Recursos medicinais de espécies do Cerrado de Mato Grosso: Um estudo Bibliografico," *Acta Botanica Brasilica*, vol. 17, no. 4, pp. 561–584, 2003.

[60] M. Sadat-Hosseini, M. Farajpour, N. Boroomand, and F. Solaimani-Sardou, "Ethnopharmacological studies of indigenous medicinal plants in the south of Kerman, Iran," *Journal of Ethnopharmacology*, vol. 199, pp. 194–204, 2017.

[61] M. Ayyanar and S. Ignacimuthu, "Ethnobotanical survey of medicinal plants commonly used by Kani tribals in Tirunelveli hills of Western Ghats, India," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 851–864, 2011.

[62] E. Amri and D. P. Kisangau, "Ethnomedicinal study of plants used in villages around Kimboza forest reserve in Morogoro, Tanzania," *Journal of Ethnobiology and Ethnomedicine*, vol. 8, no. 1, 2012.

[63] Y. Gidey, T. Mekonen, and K. Mezgebe, "Survey of medicinal plants used to treat human ailments in Hawzen district, Northern Ethiopia," *International Journal of Biodiversity and Conservation*, vol. 3, no. 13, pp. 709–714, 2011.

[64] M. Giday, Z. Asfaw, and Z. Woldu, "Medicinal plants of the Meinit ethnic group of Ethiopia: an ethnobotanical study," *Journal of Ethnopharmacology*, vol. 124, no. 3, pp. 513–521, 2009.

[65] A. Ghorbani, "Studies on pharmaceutical ethnobotany in the region of Turkmen Sahra, north of Iran (part 1): general results," *Journal of Ethnopharmacology*, vol. 102, no. 1, pp. 58–68, 2005.

[66] A. Braca, C. Sortino, M. Politi, I. Morelli, and J. Mendez, "Antioxidant activity of flavonoids from *Licania licaniaeflora*," *Journal of Ethnopharmacology*, vol. 79, no. 3, pp. 379–381, 2002.

[67] M. G. de Carvalho, L. F. de Oliveira Cândido, P. M. Da Costa, and V. M. Rumjanek, "Chromones from Licania arianeae (Chrysobalanaceae)," *Natural Product Research (Formerly Natural Product Letters)*, vol. 19, no. 1, pp. 7–12, 2005.

[68] J. M. D. Matos and M. E. O. Matos, *Farmacognosia Curso teórico-prático*, UFC, Edições, Fortaleza, Brazil, 1989.

[69] B. Mendes, C. M. N. VenΓncio, M. A. G. Jardim, J. C. da Silva, and C. M. N. Venâncio, "Informações fitoterápicas e composição química de Mikania lindleyana DC. (Asteraceae)," *Revista Brasileira de Farmácia*, vol. 83, no. 1, pp. 27–29, 2002.

[70] J. E. Robbers, M. K. Speedie, and V. E. Tyler, "Farmacognosia Biotecnologia," *Editorial Premier. São Paulo*, 1997.

[71] M. C. M. Amorozo and A. L. Gély, "Uso de plantas medicinais por caboclos do baixo Amazonas, Barcarena, PA, Brasil," *Boletim do Museu Paraense Emílio Goeldi. Nova Série Botânica*, vol. 4, no. 1, pp. 47–131, 1998.

[72] P. L. B. Lisboa, *Caxiuanã: Populações Tradicionais, Meio Físico e Diversidade Biológica*, Museu Paraense Emílio Goeldi, Belém, PA, Brazil, 2002.

[73] J. Veiga and V. Scudeller, "Etnobotânica e medicina popular no tratamento de malária e males associados na comunidade ribeirinha Julião – baixo Rio Negro (Amazônia Central)," in *Revista Brasileira de Plantas Medicinais*, vol. 17, pp. 737–747, Campinas, 2015.

[74] M. F. Medeiros, V. S. Fonseca, and R. H. Andreata, "Plantas medicinais e seus usos pelos sitiantes da Reserva Rio das Pedras, Mangaratiba, RJ, Brasil," *Acta Botanica Brasilica*, vol. 18, no. 2, pp. 391–399, 2004.

[75] N. R. Bueno, R. O. Castilho, R. B. da Costa et al., "Medicinal plants used by the kaiowá and guarani indigenous populations in the caarapó reserve, Mato Grosso do Sul, Brazil," *Acta Botanica Brasilica*, vol. 19, no. 1, pp. 39–44, 2005.

[76] M. F. Silva-Almeida and M. C. M. Amorozo, "Medicina popular no Distrito de Ferraz, Município de Rio Claro, Estado de São Paulo," *Brazilian Journal of Ecology*, vol. 2, pp. 36–46, 1998.

[77] M. Heinrich, A. Ankli, B. Frei, C. Weimann, and O. Sticher, "Medicinal plants in Mexico: healers' consensus and cultural importance," *Social Science & Medicine*, vol. 47, no. 11, pp. 1859–1871, 1998.

[78] B. C. Bennett and G. T. Prance, "Introduced plants in the indigenous pharmacopoeia of northern South America," *Economic Botany*, vol. 54, no. 1, pp. 90–102, 2000.

[79] E. C. Fernandez, Y. E. Sandi, and L. Kokoska, "Ethnobotanical inventory of medicinal plants used in the bustillo province of the potosi department," *Bolivia1*, vol. 4, pp. 35–72, 2003.

[80] R. B. A. Leão, M. R. Coelho-ferreira, M. A. G. Jardim, and R. B. A. Leão, "Levantamento de plantas de uso terapêutico no município de Santa Bárbara do Pará, Estado do Pará, Brasil," *Revista Brasileira de Farmácia*, vol. 88, no. 1, pp. 21–25, 2007.

[81] C. Penido, F. P. Conte, M. S. S. Chagas, C. A. B. Rodrigues, J. F. G. Pereira, and M. G. M. O. Henriques, "Anti-allergic effects of natural tetranortriterpenoids isolated from *Carapa guianensis* Aublet on allergen-induced vascular permeability and hyperalgesia," *Inflammation Research*, vol. 54, no. 7, pp. 295–303, 2005.

[82] F. K. Ferraris, K. H. Moret, A. B. C. Figueiredo, C. Penido, and M. O. das Graças Henriques, "Gedunin, a natural tetranortriterpenoid, modulates T lymphocyte responses and ameliorates allergic inflammation," *International Immunopharmacology*, vol. 14, no. 1, pp. 82–93, 2012.

[83] C. Penido, K. A. Costa, M. F. D. S. Costa, J. D. F. G. Pereira, A. C. Siani, and M. D. G. M. D. O. Henriques, "Inhibition of allergen-induced eosinophil recruitment by natural tetranortriterpenoids is mediated by the suppression of IL-5, CCL11/eotaxin and NFκB activation," *International Immunopharmacology*, vol. 6, no. 2, pp. 109–121, 2006.

[84] B. S. Nayak, J. Kanhai, D. M. Milne et al., "Investigation of the wound healing activity of Carapa guianensis L. (Meliaceae) bark extract in rats using excision, incision, and dead space wound models," *Journal of Medicinal Food*, vol. 13, no. 5, pp. 1141–1146, 2010.

[85] B. S. Nayak, J. Kanhai, D. M. Milne, L. P. Pereira, and W. H. Swanston, "Experimental evaluation of ethanolic extract of carapa guianensis L. leaf for its wound healing activity using three wound models," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 419612, 2011.

[86] F. K. Ferraris, R. Rodrigues, V. P. Da Silva, R. Figueiredo, C. Penido, and M. D. G. M. O. Henriques, "Modulation of T lymphocyte and eosinophil functions in vitro by natural tetranortriterpenoids isolated from Carapa guianensis Aublet," *International Immunopharmacology*, vol. 11, no. 1, pp. 1–11, 2011.

[87] Q. Zhang, J. J. Zhao, J. Xu, F. Feng, and W. Qu, "Medicinal uses, phytochemistry and pharmacology of the genus Uncaria," *Journal of Ethnopharmacology*, vol. 173, pp. 48–80, 2015.

[88] C. Pavei, S. Kaiser, G. L. Borré, and G. G. Ortega, "Validation of a LC method for polyphenols assay in cat's claw (Uncaria tomentosa)," *Journal of Liquid Chromatography & Related Technologies*, vol. 33, no. 17, pp. 1551–1561, 2010.

[89] C. Pavei, S. Kaiser, S. G. Verza, G. L. Borre, and G. G. Ortega, "HPLC-PDA method for quinovic acid glycosides assay in Cat's claw (Uncaria tomentosa) associated with UPLC/Q-TOF-MS analysis," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 62, pp. 250–257, 2012.

[90] Y. Sheng, C. Åkesson, K. Holmgren, C. Bryngelsson, V. Giamapa, and R. W. Pero, "An active ingredient of Cat's Claw water extracts: Identification and efficacy of quinic acid," *Journal of Ethnopharmacology*, vol. 96, no. 3, pp. 577–584, 2005.

[91] J. O. da Silva, R. S. Fernandes, F. K. Ticli et al., "Triterpenoid saponins, new metalloprotease snake venom inhibitors isolated from *Pentaclethra macroloba*," *Toxicon*, vol. 50, no. 2, pp. 283–291, 2007.

[92] L. J. Reddy, J. Beena, G. Spandana, and D. J. Reshma, "Evaluation of antibacterial and antioxidant activities of the leaf essential oil and leaf extracts of Otacanthus caerulus (Linden) Ronse," *International Journal of Pharmacy and Pharmaceutical Science Research*, vol. 2, pp. 20–25, 2012.

[93] E. Houël, A. M. S. Rodrigues, A. Jahn-Oyac et al., "In vitro antidermatophytic activity of *Otacanthus azureus* (Linden) Ronse essential oil alone and in combination with azoles," *Journal of Applied Microbiology*, vol. 116, no. 2, pp. 288–294, 2014.

[94] E. Houël, G. Gonzalez, J.-M. Bessière et al., "Therapeutic switching: From antidermatophytic essential oils to new leishmanicidal products," *Memórias do Instituto Oswaldo Cruz*, vol. 110, no. 1, pp. 106–113, 2015.

[95] P. Shanley and N. A. Rosa, "Conhecimento em erosão: um inventário etnobotânico na fronteira de exploração da Amazônia Oriental," *Boletim do Museu Paraense Emílio Goeldi*, vol. 1, pp. 147–171, 2005.

[96] J. H. Costa-Silva, C. R. Lima, E. J. R. Silva et al., "Acute and subacute toxicity of the *Carapa guianensis* Aublet (Meliaceae) seed oil," *Journal of Ethnopharmacology*, vol. 116, no. 3, pp. 495–500, 2008.

[97] J. S. Prophiro, M. A. N. da Silva, L. A. Kanis, B. M. da Silva, J. E. Duque-Luna, and O. S. da Silva, "Evaluation of time toxicity, residual effect, and growth-inhibiting property of *Carapa* guianensis and Copaifera sp. in *Aedes aegypti*," *Parasitology Research*, vol. 110, no. 2, pp. 713–719, 2012.

[98] O. J. D. Santos, O. Malafaia, J. M. Ribas-Filho, N. G. Czeczko, R. H. P. Santos, and R. A. P. Santos, "Efeito de Schinus terebinthifolius Raddi (aroeira) e Carapa guianensis Aublet (andiroba) na cicatrização de gastrorrafias," *Arquivos Brasileiros de Cirurgia Digestiva*, vol. 26, no. 2, pp. 84–91, 2013.

[99] A. A. Dreifuss, A. L. Bastos-Pereira, and I. A. Fabossi, "*Uncaria tomentosa* exerts extensive anti-neoplastic effects against the walker-256 tumour by modulating oxidative stress and not by alkaloid activity," *PLos One*, vol. 8, no. 2, Article ID e54618, pp. 1–14, 2013.

[100] R. Pilarski, B. Filip, J. Wietrzyk, M. Kuraś, and K. Gulewicz, "Anticancer activity of the *Uncaria tomentosa* (Willd.) DC. preparations with different oxindole alkaloid composition," *Phytomedicine*, vol. 17, no. 14, pp. 1133–1139, 2010.

[101] R. Rojas-Duran, G. González-Aspajo, C. Ruiz-Martel et al., "Anti-inflammatory activity of mitraphylline isolated from *Uncaria tomentosa* bark," *Journal of Ethnopharmacology*, vol. 143, no. 3, pp. 801–804, 2012.

[102] R. A. Ccahuana-Vasquez, S. S. Ferreira dos Santos, C. Y. Koga-Ito, and A. O. Cardoso Jorge, "Antimicrobial activity of Uncaria tomentosa against oral human pathogens," *Brazilian Oral Research*, vol. 21, no. 1, pp. 46–50, 2007.

[103] T. Caon, S. Kaiser, C. Feltrin et al., "Antimutagenic and antiherpetic activities of different preparations from Uncaria tomentosa (cat's claw)," *Food and Chemical Toxicology*, vol. 66, pp. 30–35, 2014.

[104] A. Domingues, A. Sartori, M. A. Golim et al., "Prevention of experimental diabetes by Uncaria tomentosa extract: Th2 polarization, regulatory T cell preservation or both?" *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 635–642, 2011.

[105] S. Baydoun, L. Chalak, H. Dalleh, and N. Arnold, "Ethnopharmacological survey of medicinal plants used in traditional medicine by the communities of Mount Hermon, Lebanon," *Journal of Ethnopharmacology*, vol. 173, pp. 139–156, 2015.

[106] R. A. Lima, A. S. Magalhães, and M. R. A. Santos, "Levantamento etnobotânico de plantas medicinais utilizadas na cidade de Vilhena , Rondônia," *Revista Pesquisa & Criação*, vol. 10, no. 2, pp. 165–179, 2011.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7475151, 13 pages
https://doi.org/10.1155/2019/7475151

Review Article

Traditional Chinese Medicine Yimucao Injection Combined with Western Medicine for Preventing Postpartum Hemorrhage after Cesarean Section: A Systematic Review and Meta-Analysis

Shichun Chen[1], **Baocheng Xie**[1], **Hao Tian**[1], **Shaobo Ding**[2] and **Chengyu Lu**[1,3]

[1]*Department of Pharmacology, Guangdong Medical University, Dongguan 523808, China*
[2]*Department of Pharmacy, Dongguan People's Hospital, Dongguan, 523000, China*
[3]*Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical University, 524023 Zhanjiang, China*

Correspondence should be addressed to Chengyu Lu; gdmclu@qq.com

Received 2 October 2018; Accepted 17 January 2019; Published 9 April 2019

Guest Editor: Caio P. Fernandes

Objective. Yimucao injection combined with several contraction uterus drugs is in use for preventing postpartum hemorrhage after cesarean section. The present study is a meta-analysis comparing the efficacy and safety of these drugs. *Methods*. PubMed, Cochrane Library, Embase, the China National Knowledge Infrastructure (CNKI), the Chinese Biomedical Database (CBM), VIP, and Wanfang database were searched until June 2018. We selected RCTs of Yimucao injection combined with western medicine for preventing postpartum hemorrhage and study quality was assessed using the revised Cochrane risk of bias tool. Forty-eight RCTs are comprised of 7,330 participants. *Results*. The overall response rate of Yimucao injection combined with western medicine as a class (OR=4.19, 95%CI=2.83, 6.20, $P<0.00001$) was found to be significantly improved than western medicine alone. Yimucao injection combined with western medicine group could significantly reduce blood loss in intraoperative (SMD= -1.15, 95%CI= -1.43, -0.87, $P<0.00001$), compared with control group. The treatment group could significantly reduce postpartum blood loss within 2 hours (SMD= -1.73, 95%CI= -2.01, -1.46, $P<0.00001$) and had a significantly lower blood loss within 24 hours (SMD= -1.92, 95%CI= -2.21, -1.63, $P<0.00001$) than control group. Additionally, in terms of the safety, Yimucao injection group reduced the risk of adverse events in the course of prevention than the western medicine group. *Conclusions*. This study demonstrated that Yimucao injection combined with western medicine may be more effective for preventing postpartum hemorrhage after cesarean section. However, high-quality and large multicenter randomized clinical trials will be needed to prove the consequence in the further.

1. Introduction

Postpartum hemorrhage (PPH) is one of most common diseases of maternal death worldwide and severe morbidity during pregnancy [1]. Uterine atony, injury of birth canal, abnormal placenta, and dysfunction of blood coagulation were the leading cause of PPH in pregnant women with cesarean section, and the average blood loss during cesarean section is significantly higher than that during vaginal delivery [2, 3]. Currently, western medicine such as oxytocin [4], carboprost tromethamine [5], and misoprostol [6] can play an important role in addressing the issue of PPH during and after cesarean section.

Oxytocin is an effective method to prevent uterine atony and PPH during cesarean section, and it is generally regarded that oxytocin is the first-line drug proposed by the world health organization (WHO) and other international guidelines [7]. However, the main characteristic of oxytocin is that the effect of contractile uterus was quick and short duration during the treatment of PPH, and it could be discontinues to contract uterine in certain dosage range and results in some side effects, such as tachycardia, hypotension, and arrhythmia [8].

A large number of studies confirmed that Yimucao injection as a traditional Chinese medicine is more effective for clinical prevention of postpartum hemorrhage. The main

active components of Yimucao injection were alkaloids, and it has uterine contraction effects [9]. In addition, Yimucao injection combined with western medicine (oxytocin, carboprost tromethamine, and misoprostol) can not only prevent PPH during cesarean section and improve the curative effect, but also decrease the rate of adverse events. Even though several studies assessing the effect of Yimucao injection combined with western medicine increased dramatically in the decade, it is still lack of comprehensive systematic review to guidance. Therefore, our study included 48 RCTs with a total of 7330 pregnant women with cesarean section in order to investigate the effect of western medicine (oxytocin, carboprost tromethamine, and misoprostol) alone or combined with Yimucao injection in women after cesarean section, and we also performed subgroup analyses in order to acquire high-quality evidence, which comprehensive systematic review to assess the efficacy and safety of Yimucao injection as adjuvant treatment for preventing PPH during and after cesarean section.

2. Methods

2.1. Search Strategy. We systematically searched Medical databases, including PubMed, Cochrane Library, Embase, the China National Knowledge Infrastructure (CNKI), the Chinese Biomedical Database (CBM), VIP database, and Wanfang for RCTs examining the effect of Yimucao injection combined with western medicine (oxytocin, carboprost tromethamine, and misoprostol) for preventing postpartum hemorrhage after cesarean section, from their inception until June 2018. The searched the terms of medical keywords: (1) "yimucao", "yimucao injection", "leonurus japonicus injection"connected with "OR"; (2) "cesarean section", "postpartum hemorrhage", "cesarean section", "abdominal delivery" connected with "OR"; (3) "randomized controlled" or "Clinical Trials". Then, the above search terms of (1), (2), and (3) were connected with "AND". We manually searched all research studies that are the references of the original and review articles for possible related studies.

2.2. Study Selection. This systematic review included 48 clinical studies that met the following criteria: (1) studies reported patients with cesarean section, (2) studies compared the effectiveness and safety of Yimucao injection combined with western medicine, (3) studies selected as randomized controlled trials (RCTs), and (4) studies included clinical outcomes which the estimated blood loss.

2.3. Data Abstraction. We collected relevant information including: study characteristics (publication year and sample size), participant characteristic (average age and cesarean section), interventions (type of administration, dose, treatment protocol, and duration of treatment), and outcomes (intraoperative blood loss, blood loss within 2 hours, blood loss within 24 hours, and adverse events). We assessed the clinical efficacy and safety of Yimucao injection combined with western medicine for preventing PPH according to the guideline on the information of extraction.

2.4. Quality Assessment. For eligible studies, data were extracted independently by the two authors and carried out a quality assessment process according to the predefined inclusion criteria. Disagreements were resolved by consensus or discussion with a third author. The methodological quality assessed the risk of bias of RCTs by the Cochrane risk of bias tool. We considered random sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome reporting, and other potential sources of bias. The modified instrument removed the "Low risk" option and added "Unclear risk" and "High risk".

2.5. Statistical Analysis. In this meta-analysis, RevMan 5.3 software provided by the Cochrane Collaboration was used to perform the data analysis. The analyses of dichotomous data were presented as the risk ratios (RR) or odds ratios (OR), and the continuous data were presented as mean difference (MD) or standardized mean difference (SMD) with 95% CI. All clinical studies were measured by the chi-square test, with the I^2 test. If the I^2 was less than 50%, we considered that the heterogeneity among studies was small, and we used the fixed effects model for data analysis. If heterogeneity was detected (I^2 value >50%), we suggest severe heterogeneity between the studies by considering possible factors, such as the dose of medicine, treatment course, and disease type. If the heterogeneity was still significant, we chose the random effects model or used only qualitative descriptions.

3. Results

3.1. Search Results. Through the seven medical database searches, we found 632 citations from all searches and excluded 353 duplicates. After screening the titles and abstracts, we retrieved 279 full texts for further assessment. Of these, 231 were excluded for the following reasons: duplicate publication as conference abstract, animal experimentation, basic studies of cells, trial without a control arm, the treatment of Yimucao injection combined with other medicine, the vaginal delivery of study, graduation thesis, and narrative reviews; 48 trials involving 7330 women delivered were finally included (Figure 1).

3.2. Study Characteristics. In the 48 trials included, a total of 7330 pregnant women participated: the treatment group of Yimucao injection combined with oxytocin and the control group with oxytocin (32 studies); the treatment group of Yimucao injection combined with carboprost tromethamine and the control group with carboprost tromethamine (12 studies); the treatment group of Yimucao injection combined with misoprostol and the control group with misoprostol (4 studies). All the studies were conducted in China and were published in Chinese. Baseline characteristics were summarized in Table 1.

3.3. Quality Assessment. The methodological quality of included studies was estimated according to the bias risk assessment tools provided by the Cochrane Collaboration. All of the included trials mentioned randomized allocation and allocation concealment were unclear. Forty-six studies

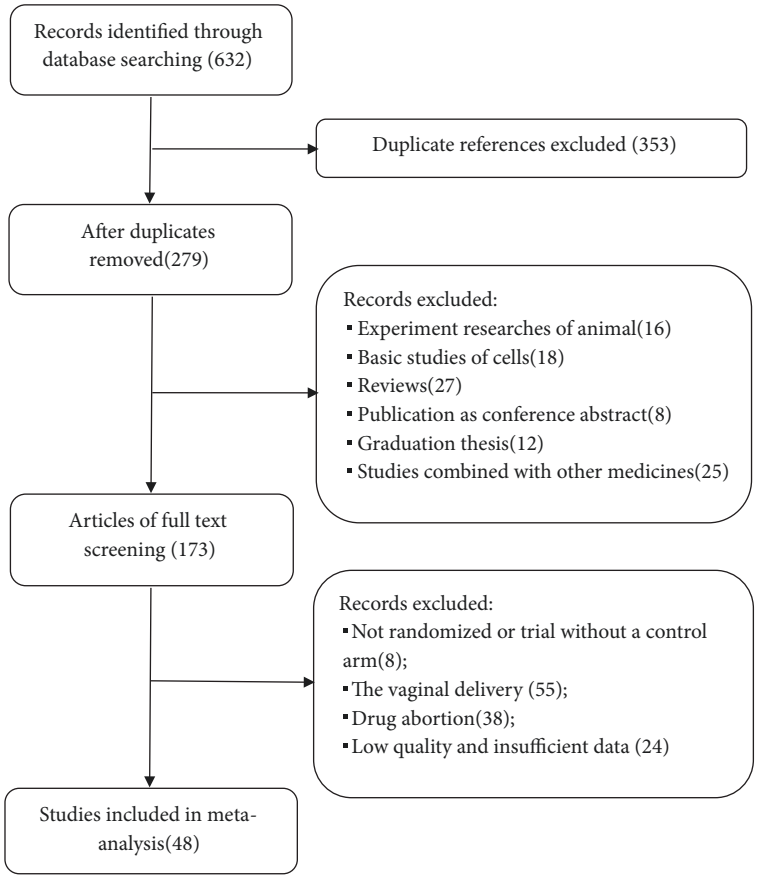


Figure 1: Flowchart and strategy of the meta-analysis.

were at an unclear risk of bias for blinding of participants and personnel, 12 trials [10–21] described in detail the method of random number table, and only 2 trials [12, 19] described as double-blind. All trials reported methods with a low risk of incomplete outcome data and thirty-eight studies were at low risk of bias. These results are summarized in Figure 2.

3.4. Major Outcomes

3.4.1. The Total Effective Rate. The total effective rate was reported in seven studies [10, 14, 17, 22–25] in which 630 patients in the treatment group and 627 patients in the control group. The meta-analysis was conducted, as shown in Figure 3, Yimucao injection combined with western medicine showed a better effect on the prevention of PPH compared with the western medicine alone (OR=4.19, 95%CI=2.83, 6.20, $P<0.00001$).

3.4.2. Intraoperative Blood Loss. In total, thirty-three studies [10–14, 16–18, 22–46] reported the intraoperative blood loss during cesarean section. Decreased intraoperative blood loss in response to Yimucao injection combined with western medicine (oxytocin, carboprost tromethamine, and misoprostol) group than the western medicine group alone was observed by our analysis (SMD= -1.15, 95%CI= -1.43, -0.87, $P<0.00001$). The result of this study further revealed that the treatment group performed better than the control group in improving PPH during cesarean section (Figure 4).

3.4.3. Blood Loss within 2 Hours after Delivery. Forty-six RCTs [10–27, 29–39, 41–57] (n=7042) reported the outcome of blood loss within 2 hours after delivery. The incidence of blood loss in the Yimucao injection combined with western medicine group significantly decreases compared to that in the control group (SMD= -1.73, 95%CI= -2.01, -1.46, $P<0.00001$) (Figure 5).

3.4.4. Blood Loss within 24 Hours after Delivery. In our analysis, there were forty-seven studies [10–25, 27–57] providing the data of blood loss within 24 hours after delivery. Our meta-analysis showed a significant difference in blood loss within 24 hours after delivery which was witnessed between the treatment group and control group (SMD= -1.92, 95%CI= -2.21, -1.63, $P<0.00001$). The result exhibited a significant efficacy in PPH in Yimucao injection combined with western medicine compared to the western medicine alone (Figure 6).

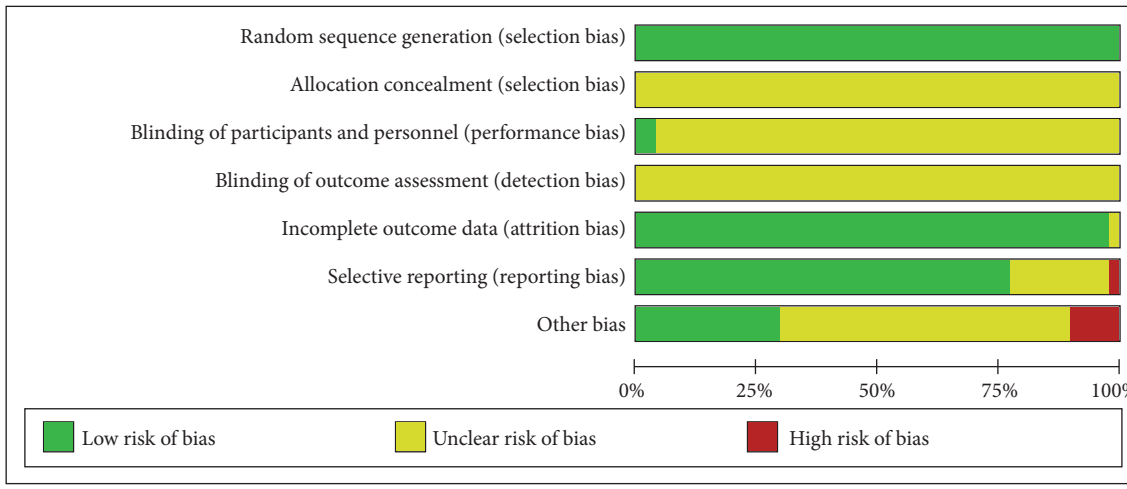
3.5. Subgroup Analysis

3.5.1. Yimucao Injection + Oxytocin versus Oxytocin. Participants with PPH were treated with Yimucao injection and

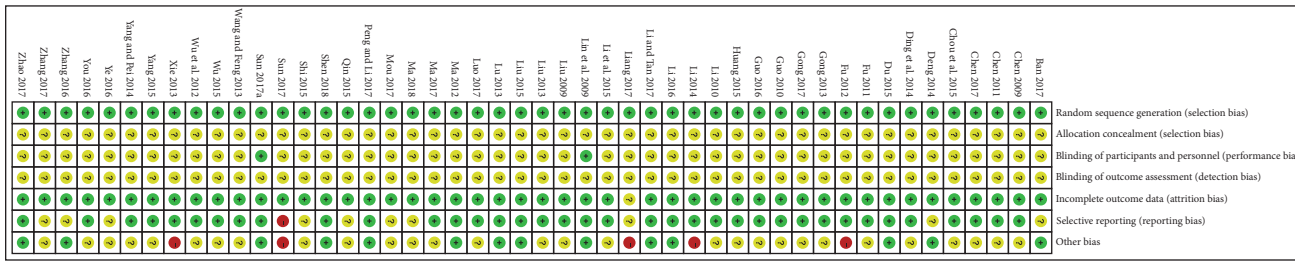
Table 1: Characteristics of included articles.

Study	Sample (T/C)	Age (T/C)	Intervention		Follow-up	Evaluation
			T	C		
Peng and Li, 2017 [22]	250/250	28.32/27.45	YM+OT	OT	NR	①②③④
Ban, 2017 [26]	64/64	28.45/27.56	YM+OT	OT	4d	②③④
Sun, 2017 [47]	40/40	34.98/35.41	YM+OT	OT	3d	③④
Li et al., 2015 [27]	75/75	25.2	YM+OT	OT	NR	②③④
Du, 2015 [10]	80/80	29.6/29.7	YM+OT	OT	3d	①②③④
Shi, 2015 [28]	34/34	NR	YM+OT	OT	3d	②④
Ma, 2012 [11]	256/256	24.46/24.18	YM+OT	OT	NR	②③④
Chen, 2011 [29]	100/100	29	YM+OT	OT	3d	②③④
Fu, 2011 [30]	63/63	NR	YM+OT	OT	NR	②③④
Lin et al., 2009 [12]	144/149	NR	YM+OT	OT	NR	②③④
Shen, 2018 [13]	57/57	27.95/27.86	YM+CT	CT	NR	②③④
Li and Tan, 2017 [14]	90/87	28.7/29.2	YM+CT	OT	3d	①②③④
Ye, 2016 [48]	98/98	28.89/29.56	YM+CT	CT	2d	③④
Yang, 2015 [49]	70/70	28.8/29.0	YM+CT	CT	2d	③④
Chou et al., 2015 [50]	52/52	26.6/27.3	YM+CT	OT	NR	③④
Ding et al., 2014 [15]	60/60	NR	YM+CT	OT	NR	③④
You, 2016 [31]	50/50	28	YM+OT	OT	NR	②③④
Zhang, 2016 [51]	37/37	26.3/27.9	YM+OT	OT	NR	③④
Li, 2016 [16]	45/45	24.9/24.7	YM+OT	OT	NR	②③④
Liu, 2015 [23]	50/50	28.11/27.81	YM+OT	OT	NR	①②③④
Qin, 2015 [52]	59/59	28.3/28.5	YM+OT	OT	NR	③④
Deng, 2014 [53]	68/68	28.4/29.3	YM+OT	OT	3d	③④
Xie, 2013 [32]	100/100	NR	YM+OT	OT	3d	②③④
Lu, 2013 [45]	129/129	27.5/27.9	YM+OT	OT	NR	②③④
Liu, 2009 [33]	200/200	NR	YM+OT	OT	NR	②③④
Chen, 2009 [44]	100/100	NR	YM+OT	OT	NR	②③④
Ma, 2018 [54]	30/30	29.26/28.95	YM+CT	CT	2d	③④
Wang and Feng, 2013 [34]	40/40	25.2/27.4	YM+MS+OT	MS+OT	NR	②③④
Liu, 2013 [35]	65/65	25/25.2	YM+MS	OT	NR	②③④
Li, 2010 [36]	100/100	NR	YM+OT	OT	NR	②③④
Wu et al., 2012 [37]	30/30	NR	YM+OT	OT	NR	②③④
Yang and Pei, 2014 [24]	50/50	28.09/28.16	YM+OT	OT	NR	①②③④
Liang, 2017 [17]	50/50	29.6/29.3	YM+OT	OT	NR	①②③④
Luo, 2017 [38]	60/60	29.7/29.1	YM+OT	OT	NR	②③④
Guo, 2010 [39]	50/50	27.0/30.5	YM+OT	OT	NR	②③④
Guo, 2016 [25]	60/60	30.2/30.5	YM+OT	OT	NR	①②③④
Huang, 2015 [46]	36/36	27.2	YM+OT	OT	NR	②③④
Gong, 2013 [40]	110/110	NR	YM+OT	OT	NR	②④
Wu, 2015 [41]	103/101	24.56	YM+OT	OT	NR	②③④
Zhang, 2017 [42]	100/100	22/23	YM+OT	OT	NR	②③④
Zhao, 2017 [18]	46/46	28.23/28.62	YM+OT	OT	NR	②③④
Sun, 2017 a[19]	53/53	28.09/28.21	YM+CT	CT	2d	③④
Mou, 2017 [43]	52/52	31.62/31.59	YM+CT	CT	NR	②③④
Ma, 2017 [20]	40/40	28.13/28.46	YM+CT	CT	NR	③④
Li, 2014 [55]	40/40	NR	YM+CT	CT	NR	③④
Gong, 2017 [21]	51/51	29.03/28.47	YM+CT	CT	2d	③④
Fu, 2012 [56]	46/46	26.6	YM+MS	OT	NR	③④
Chen, 2017 [57]	84/84	24.3/24.7	YM+MS	MS	3d	③④

Note. T: treatment group; C: control group; d: days; NR: not report; YM: Yimucao injection; OT: oxytocin; CT: carboprost tromethamine; MS: misoprostol; ①: the total effective rate; ②: intraoperative blood loss; ③: blood loss within 2 hours; ④: blood loss within 24 hours.



(a) Summary of RCTs quality showing the percentage of RCTs satisfying each risk of bias graph



(b) Detailed item-by-item analysis of the risk of bias summary

Figure 2

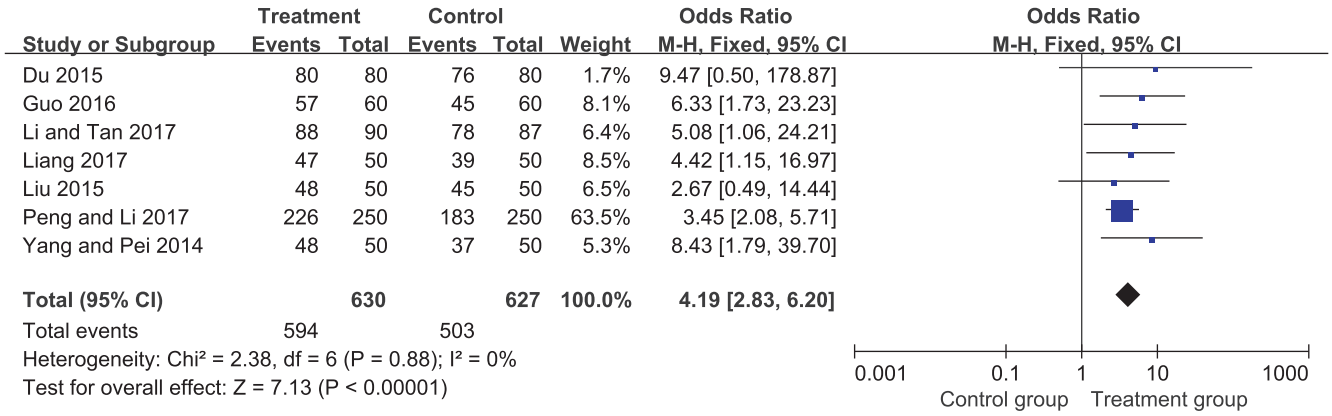


Figure 3: Forest plot of the meta-analysis with the total effective rate.

oxytocin in the treatment group and with oxytocin in the control group. Results of subgroup analysis showed that Yimucao injection combined with oxytocin may be effectively reduces the intraoperative blood loss (SMD= -1.06, 95%CI= -1.34, -0.77, P<0.00001), blood loss within 2 hours (SMD= -1.33, 95%CI= -1.60, -1.06, P<0.00001), and blood loss within 24 hours after delivery (SMD= -1.46, 95%CI= -1.74, -1.19, P<0.00001) for preventing PPH compared with the oxytocin alone.

3.5.2. Yimucao Injection + Carboprost Tromethamine versus Carboprost Tromethamine. Patients with PPH were treated with Yimucao injection and carboprost tromethamine in the treatment group and with carboprost tromethamine in the control group. Results of subgroup analysis demonstrated that the treatment group could significantly reduce the intraoperative blood loss (SMD= -1.28, 95%CI= -2.78, 0.22, P=0.09), blood loss within 2 hours (SMD= -2.50, 95%CI= -3.23, -1.76, P<0.00001), and blood loss within 24 hours

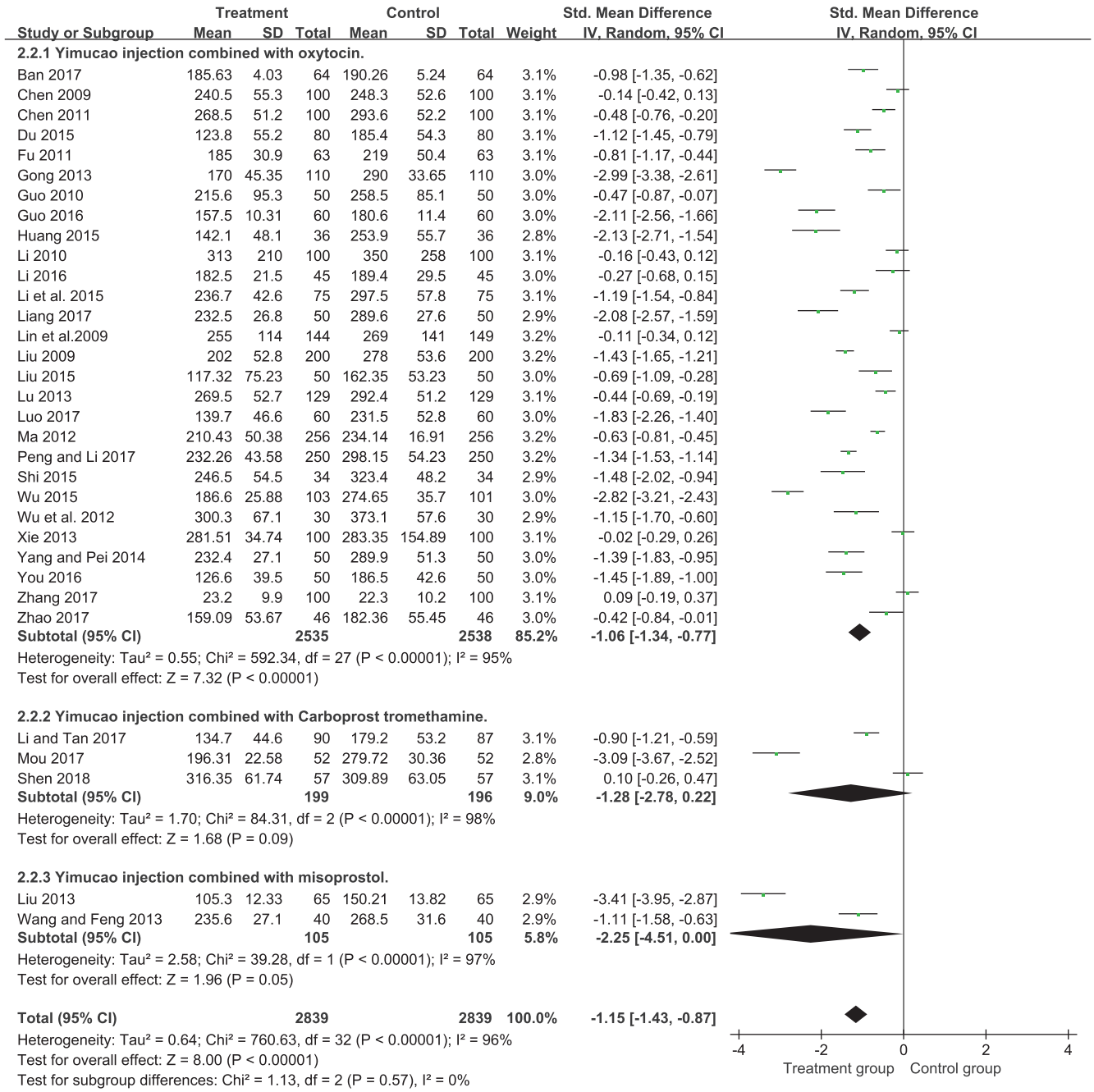


Figure 4: Forest plot of the meta-analysis of effects of Yimucao injection combined with western medicine versus western medicine alone for the prevention of PPH on the intraoperative blood loss.

(SMD= -2.64, 95%CI= -3.31, -1.97, P<0.00001) compared to the control group. Although carboprost tromethamine appeared to decrease intraoperative blood loss, the difference was not statistically significant.

3.5.3. Yimucao Injection + Misoprostol versus Misoprostol. Parturient women with PPH were treated with Yimucao injection and misoprostol in the treatment group and with misoprostol in the control group. The results of subgroup analysis indicated that Yimucao injection combined with misoprostol therapy was significantly decrease compared to misoprostol alone in the intraoperative blood loss (SMD= -2.25, 95%CI= -4.51, 0.00, P=0.05), blood loss within 2 hours (SMD= -2.52, 95%CI= -3.42, -1.62, P<0.00001), and blood loss within 24 hours (SMD= -3.37, 95%CI= -5.14, -1.60, P=0.0002).

3.6. Heterogeneity and Publication Bias. During the meta-analysis, we found high heterogeneity among studies. Consequently, we performed a sensitivity analysis by Egger's test

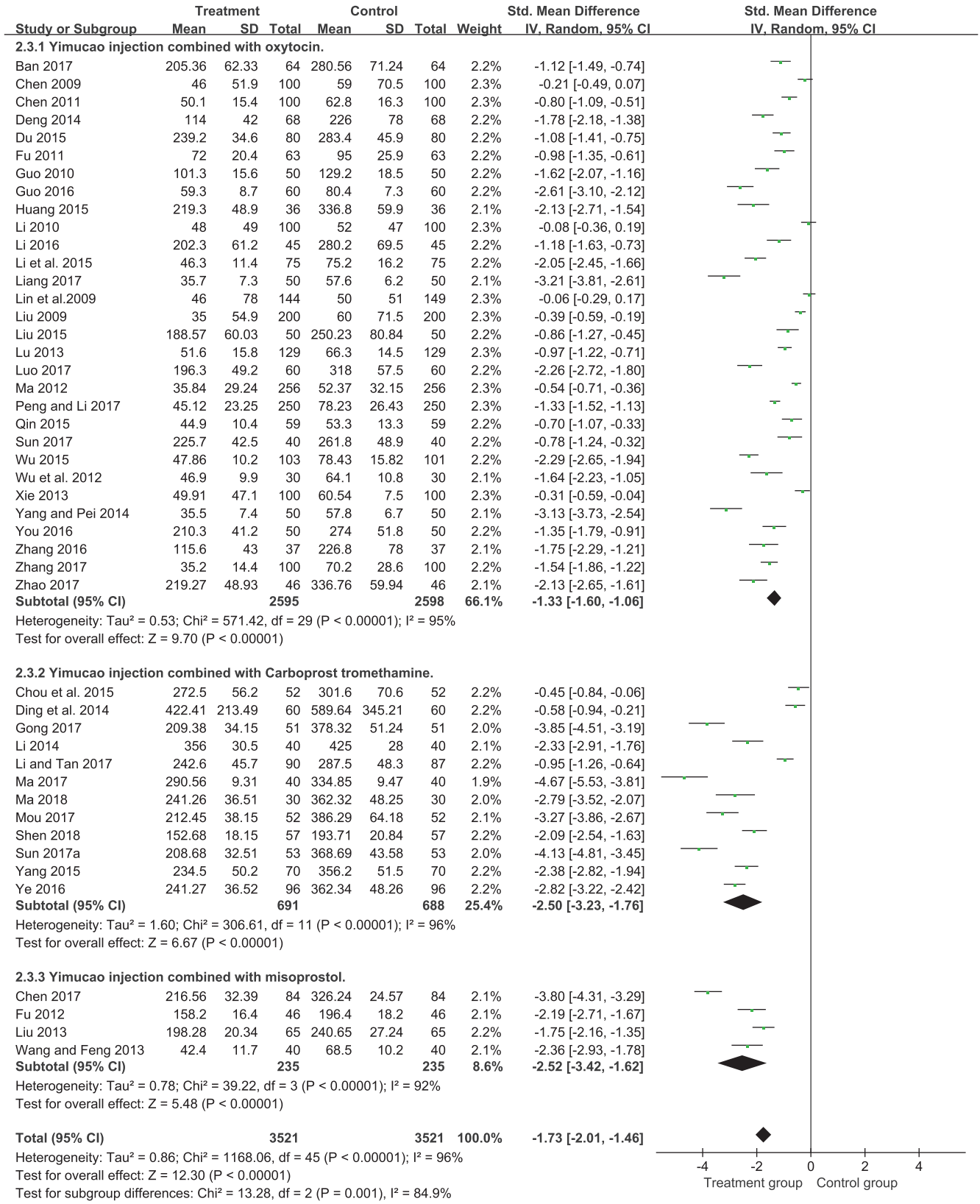


Figure 5: Forest plot of the meta-analysis of effects of Yimucao injection combined with western medicine versus western medicine alone for the prevention of PPH on the blood loss within 2 hours after delivery.

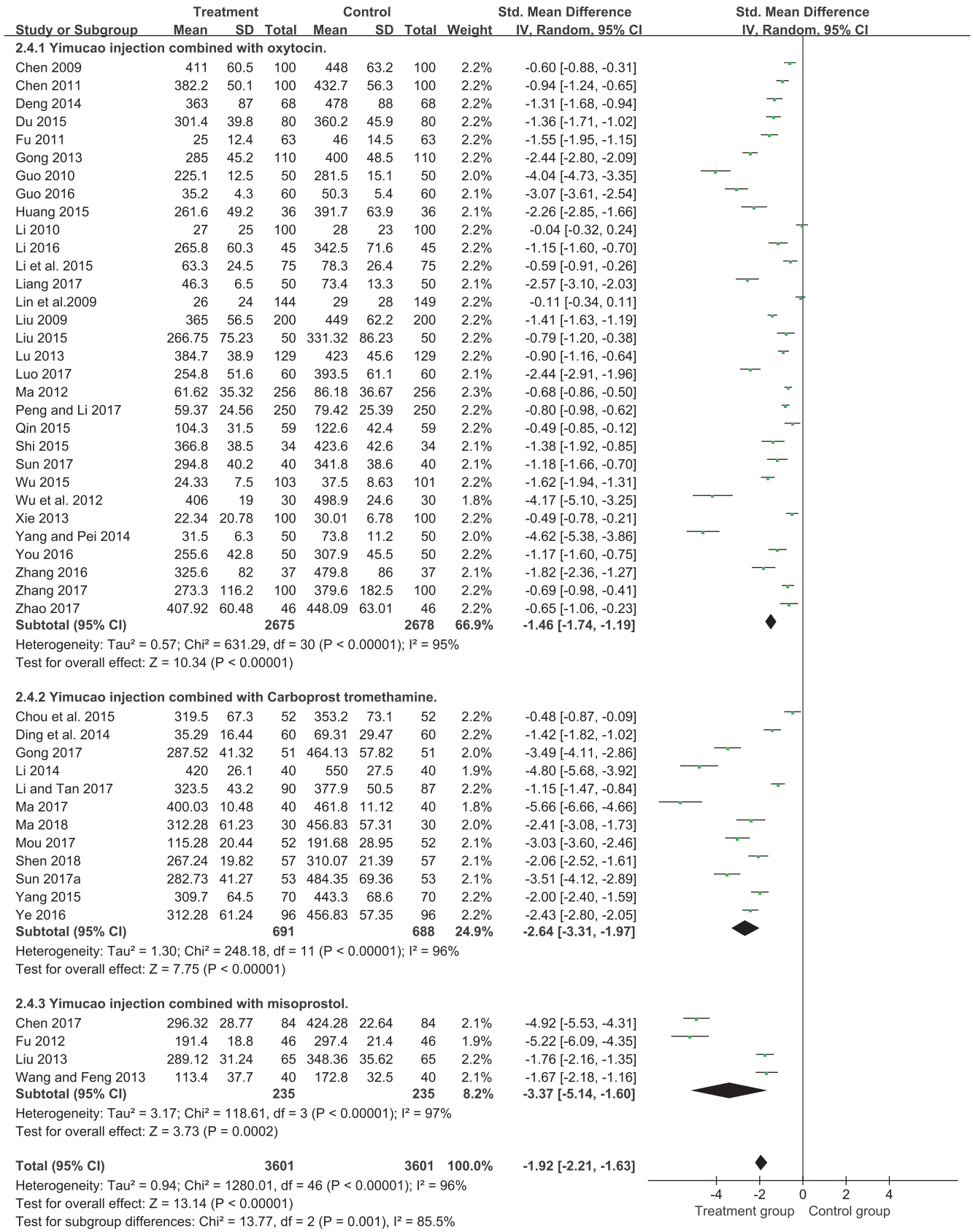


Figure 6: Forest plot of the meta-analysis of effects of Yimucao injection combined with western medicine versus western medicine alone for the prevention of PPH on the blood loss within 24 hours after delivery.

Table 2: The incidence of adverse reactions between the treatment group and control group.

	treatment group (n=1669)	control group (n=1664)
blood pressure elevation	24 (1.44%)	38 (2.23%)
facial flushing	22 (1.32%)	30 (1.80%)
nausea and vomiting	29 (1.74%)	82 (4.93%)
abdominal discomfort	5 (0.30%)	7 (0.42%)
chest discomfort	15 (0.90%)	34 (2.04%)
arrhythmia	11 (0.66%)	20 (1.20%)
allergic reaction	1 (0.06%)	0 (0.00%)

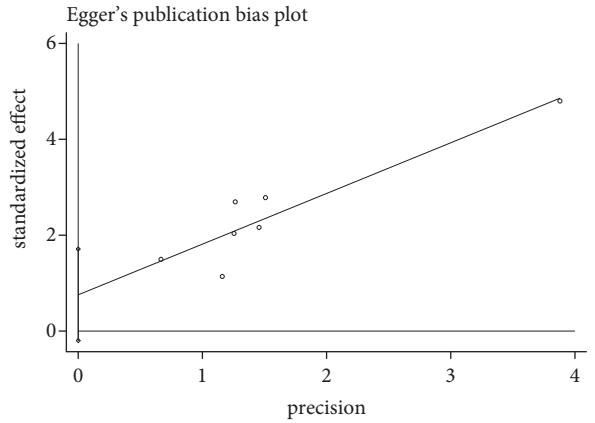


Figure 7: Egger's funnel plot of total effective rate.

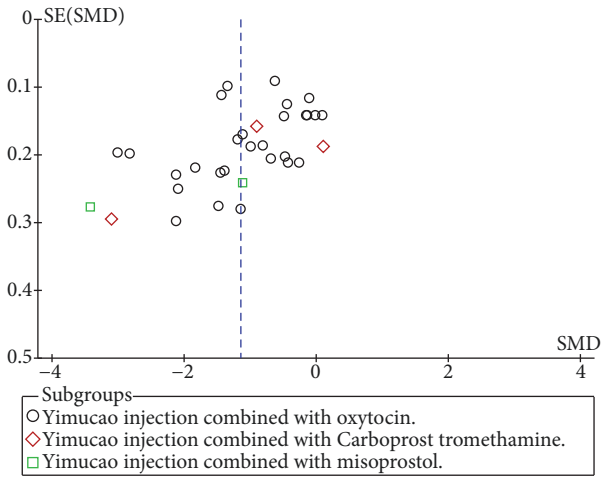


Figure 8: Funnel plot of intraoperative blood loss.

of the total effective rate (Figure 7). A significant symmetry was noted for distribution in funnel plots of the intraoperative blood loss (Figure 8). These results did not demonstrate any evidence of publication bias ($P>0.098$).

3.7. Adverse Events. In the 48 included studies, twenty-six trials [10, 13–16, 19, 22, 23, 25–30, 32–34, 36, 41, 43–45, 49–51, 53] reported on adverse events. No adverse effects of Yimucao injection combined with western medicine group were identified in 4 trials. Only one trial reported allergic reaction in the Yimucao treatment group. However, all of these twenty-two trials reported blood pressure elevation, facial flushing, nausea, and vomiting in both the control group and treatment groups, in which 3 trials reported arrhythmia, 3 studies reported abdominal discomfort, and 2 trials reported chest discomfort. The side-effect incidence rates of the treatment group and the control group of blood pressure elevation were 1.44% and 2.23%, facial flushing 1.32% and 1.80%, nausea and vomiting 1.74% and 4.93%, abdominal discomfort 0.30% and 0.42%, chest discomfort 0.90% and 2.04%, arrhythmia 0.66% and 1.20%, and allergic reaction 0.06% and 0.00%, respectively. But all of these adverse events were not severe. No serious adverse events were reported. Symptoms of patients disappeared or significantly improved in the short term without provided treatment. In our analysis of adverse events it was suggested that the treatment group has less adverse effects than the control group (Table 2).

4. Discussion

4.1. Main Outcome. Postpartum hemorrhage (PPH) continues to be the most important cause of maternal mortality and morbidity worldwide. Therefore, in women during delivery, we should give priority to the care of the prevention and treatment of PPH. Yimucao is a Chinese herbal medicine of the genus Leonurus Lamiaceae and the main active ingredient of alkaloid. It has antithrombosis, anticoagulation, improving microcirculation, antioxygen free radical, and excited uterine smooth muscle strips and maintains the stability of intracellular calcium. Yimucao injection has been widely used in the treatment of obstetric and gynecologic disease such as abnormal menstruation, promoting gestation, postpartum hemorrhage, uterine involution, and so on. In 48 RCTs in our study, a total of 7,330 women were included in order to acquire high-quality evidence for the clinical efficacy and safety of Yimucao injection therapy in PPH. The results showed that Yimucao injection which is combined with western medicine for the treatment of PPH and significantly improved the total effective rate compares control group. Our meta-analysis shows that, compared with control group, Yimucao injection combined with western medicine has the more significant effect on the blood loss during the intraoperative, delivery hours of 2 hours and 24 hours, and it effectively reduced the amount of blood loss and risk of adverse events.

4.2. Subgroup Analysis. Oxytocin is one of the naturally occurring hormones, which can initiate oxytocin receptors

and maintain adequate uterine tone resulting in rhythmic uterine contractions and thereby minimise blood loss [58]. Moreover, oxytocin is first-line uterotonics for prevent uterine atony and PPH during delivery [7]. The meta-analysis results showed that oxytocin combined with Yimucao injection was effective agent than oxytocin alone in reducing the blood loss during the intraoperative, 2 hours and 24 hours during cesarean section in our subgroup analysis.

Although oxytocin was found to be the most commonly used uterotonic agent for the prevention of PPH and has been reported to reduce blood loss during cesarean section [59]. However, it has a half-life of <10 min (short duration of action), negative inotropic, antiplatelet, and antidiuretic effects. The study [24] found that the effect of traditional Chinese medicine Yimucao contractile uterus was slow and lasted more than 6 hours. Thus, Yimucao injection and oxytocin have synergistic effect. Moreover, most of the studies have reported other uterotonic agents and also showed the prevention of PPH, including carboprost tromethamine and misoprostol [60–62].

Carboprost tromethamine is the synthetic 15-methyl analogue of prostaglandin F2α, and it was reported [5] that carboprost tromethamine for the treatment of persistent hemorrhage due to uterine atony significantly improved the effective rate by 84-96%. A study [63] reported that 237 patients were used to PPH with carboprost tromethamine, and the result showed that the improvement rate of PPH was 87.8%. This study was to compare carboprost tromethamine combined with Yimucao injection with carboprost tromethamine alone for the prevention of PPH in high-risk females undergoing cesarean delivery. Results of subgroup analysis indicated that carboprost tromethamine combined with Yimucao injection group could significantly enhance the uterine contraction for preventing PPH compared with carboprost tromethamine group.

Misoprostol is a PGE1 analogue of the strong effect on the uterus, which has been widely used for the prevention of PPH during cesarean delivery especially in developing countries [64]. Most of the studies [65–68] were suggested that misoprostol to be effective in reducing blood loss during cesarean section and it is regarded as an effective drug could substitute for other uterotonic agents. In addition, the advantages of misoprostol were inexpensive and thermostable. A few studies [34, 57] have reported the efficacy of Yimucao injection combined with misoprostol versus misoprostol alone. According to the above analysis it was demonstrated that the clinical efficacy of Yimucao injection combined with misoprostol for preventing PPH was significantly better than that of misoprostol.

This study systematically reviewed that the effect of Yimucao injection combined with western medicine for preventing PPH during cesarean section. In forty-eight RCTs in our study, a total of 26 trials reported adverse events. However, all trials did not show abnormal changes and severe adverse drug reactions during the course of treatment. Therefore, in the aspect of safety analysis, it could be judged that Yimucao injection combined with western medicine has better safety in the prevention of PPH.

4.3. Limitations of This Study. Although, we have comprehensive analysis and evaluate all studies, it still has limitations. First, the review includes 48 RCTs, with 7,330 women, which were published in Chinese and lacked relevant foreign experiments; the results of this study were regional and there may be publication bias. Secondly, most of the studies showed only the randomized trials, but no specific methods of random sequence generation, RCTs of allocation concealment, and blinding of outcome assessment. There were only two studies which reported double blinding. The methodological quality of many of the included RCTs was generally low and might have a high risk of bias. Third, we found that the outcome indicator of PPH in some trials was high heterogeneity. There was the difference in the doses of Yimucao injection combined with western medicine and western medicine alone in the treatment group and the control group. Furthermore, the control group was the use of different drugs in varied regimens. We considered different methods of administration in subgroup analyses, and there was still a very high heterogeneity. The source of high heterogeneity may be inconsistent with the method used to measure the amount of bleeding in clinical studies. We also found that the doses of drugs and methods of administration were different between study groups. In our meta-analysis, reporting bias is an important section. The funnel plot of the intraoperative blood loss showed symmetry and the quantitation of Egger's test with the total effective rate ($P>0.098$). The results indicated that publication bias was possibly low and partly reliable, but it could not show the whole publication biases.

5. Conclusions

In summary, this meta-analysis of RCTs suggested the use of Yimucao injection for the effective prevention PPH during and after cesarean section. It can effectively prevent of PPH and reduce the risk of adverse events. Subgroup analyses indicated that the clinical efficacy of Yimucao injection combined with western medicine such as oxytocin, carboprost tromethamine, and misoprostol could significantly improve PPH compared with western medicine alone. However, the methodological quality of these trials was relatively low and the significant intergroup heterogeneity in this study. Trials of larger scale or long-term, double-blind RCTs and high methodological standards are needed to provide more evidence to demonstrate the efficacy of Yimucao injection in preventing PPH during and after cesarean section.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Shichun Chen and Baocheng Xie contributed equally to this work. Shichun Chen, Baocheng Xie, and Hao Tian comprehensively searched the medical database and collected and extracted consistent randomized clinical trials; Shichun Chen, Baocheng Xie, and Hao Tian discussed and analyzed

data together; Shichun Chen and Baocheng Xie wrote papers; Hao Tian provided suggestions for writing preparation; Chengyu Lu conceived the idea for this paper and revised the paper. The final version of the article is determined after review by all authors.

References

[1] D. D. C. A. Henriquez, K. W. M. Bloemenkamp, and J. G. van der Bom, "Management of postpartum hemorrhage: how to improve maternal outcomes?" *Journal of Thrombosis and Haemostasis*, vol. 16, no. 8, pp. 1523–1534, 2018.

[2] E. F. Magann, S. Evans, M. Hutchinson, R. Collins, G. Lanneau, and J. C. Morrison, "Postpartum hemorrhage after cesarean delivery: An analysis of risk factors," *Southern Medical Journal*, vol. 98, no. 7, pp. 681–685, 2005.

[3] S. Bodur, I. Gun, O. Ozdamar, and M. A. Babayigit, "Safety of uneventful cesarean section in terms of hemorrhage," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 11, pp. 21653–21658, 2015.

[4] A. Luo and P. Mao, "Late postpartum hemorrhage due to placental and fetal membrane residuals: experience of two cases," *Clinical and Experimental Obstetrics and Gynecology*, vol. 42, no. 1, pp. 104-105, 2015.

[5] J. Bai, Q. Sun, and H. Zhai, "A comparison of oxytocin and carboprost tromethamine in the prevention of postpartum hemorrhage in high-risk patients undergoing cesarean delivery," *Experimental and Therapeutic Medicine*, vol. 7, no. 1, pp. 46–50, 2013.

[6] E. O. Afolabi, O. Kuti, E. O. Orji, and S. O. Ogunniyi, "Oral misoprostol versus intramuscular oxytocin in the active management of the third stage of labour," *Singapore Medical Journal*, vol. 51, no. 3, pp. 207–211, 2010.

[7] Ö. Tunçalp, J. P. Souza, and M. Gülmezoglu, "New WHO recommendations on prevention and treatment of postpartum hemorrhage," *International Journal of Gynecology & Obstetrics*, vol. 123, no. 3, pp. 254–256, 2013.

[8] T. Pantoja, E. Abalos, E. Chapman, C. Vera, and V. P. Serrano, "Oxytocin for preventing postpartum haemorrhage (PPH) in non-facility birth settings," *Cochrane Database of Systematic Reviews*, vol. 4, Article ID CD011491, 2016.

[9] Z. Chen, J. B. Wu, X. J. Liao, W. Yang, and K. Song, "Development and validation of an UPLC-DAD-MS method for the determination of leonurine in Chinese motherwort (Leonurus japonicus)," *Journal of Chromatographic Science (JCS)*, vol. 48, no. 10, pp. 802–806, 2010.

[10] W. Y. Du, "Curative effects and clinical safety of leonurus injection combined with oxytocin in prevention of postpartum hemorrhage after secarean section," *Chinese Journal of Family Planning*, vol. 23, no. 5, pp. 321–323, 2015.

[11] B. Ma, "Analysis to the impact of combined use of motherwort injection with oxytocin on hemorrhage in cesarean section and postoperative stage," *Journal of Traditional Chinese Medicine*, vol. 34, no. 10, pp. 888-889, 2012.

[12] J. H. Lin, Q. D. Lin, and X. H. Liu, "Multi-center study of motherwort injection to prevent postpartum hemorrhage after caesarian section," *Chinese Journal of Obstetrics and Gynecology*, vol. 44, no. 3, pp. 175–178, 2009.

[13] J. P. Shen, "Effect of motherwort injection combined with prostaglandin trometamol on prevention of cesarean section postpartum hemorrhage in high -risk pregnant women," *Chinese Journal of Family Planning Gynecotokology*, vol. 10, no. 3, pp. 69–72, 2018.

[14] Y. M. Li and B. J. Tan, "Clinical observation of leonurus artemisia injection combined with carboprost tromethamine for prevent-ing postpartum hemorrhage after cesarean section," *China Pharmacy*, vol. 28, no. 9, pp. 1197–1200, 2017.

[15] X. Y. Ding, T. Feng, H. Geng, F. Liu, and A. R. Ma, "Clinical study of carboprost tromethamine combined with motherwort injection in prevention of high-risk ce-sarean section hemorrhage," *Chinese Journal of Primary Medicine and Pharmacy*, vol. 21, no. 7, pp. 1006-1007, 2014.

[16] J. M. Li and X. Z. Wang, "Study on the efficacy of carbetocin combined with yimucao injection in cesarean section," *Maternal and Child Health Care of China*, vol. 31, no. 17, pp. 3630–3632, 2016.

[17] X. X. Liang, "Research of synergistic effect by leonurus heterophyllus injection for oxytocin in the treatment of postpartum hemorrhage in cesarean section uterine inertia," *China Practical Medical*, vol. 12, no. 3, pp. 9–11, 2017.

[18] D. H. Zhao, "The clinical effect of oxytocin combined with Yimucao injection in preventing postpartum hemorrhage in cesarean section," *Guide of China Medicine*, vol. 15, no. 32, pp. 22-23, 2017.

[19] L. Sun, "Effects of yimucao injection and oxytocin on hemorrhage and blood coagulation index after cesarean section," *China Health Care & Nutrition*, vol. 27, no. 12, pp. 24-25, 2017.

[20] W. J. Ma, "Clinical study on prevention of postpartum hemorrhage due to cesarean section by intravenous injection of pretreatment with cardioprost and tandromeuse injection," *Modern Diagnosis and Treatment*, vol. 28, no. 8, pp. 1445-1446, 2017.

[21] H. Y. Gong and M. Xia, "Effect observation of leonurus heterophyllus injection combined with carboprost tromethamine in the prevention of caesarean postpartum hemorrhage," *IMHGN*, vol. 23, no. 8, pp. 1216–1219, 2017.

[22] L. Q. Peng and L. Li, "Yimucao Injection combined with oxytocin in preventing postpartum hemorrhage after cesarean section," *Drug Evaluation and Research*, vol. 40, no. 6, pp. 820–823, 2017.

[23] S. H. Liu, "Clinical efficacy and safety of yimucao injection combined with oxytocin in preventing postpartum hemorrhage in cesarean section," *Journal of North Pharmacy*, vol. 12, no. 10, pp. 116-117, 2015.

[24] X. J. Yang and Y. S. Pei, "Observation on the application of yimucao injection and oxytocin to bleeding in and after the surgery of cesarean section," *Western journal of chinese medicine*, vol. 27, no. 7, pp. 91–93, 2014.

[25] M. X. Guo, "The leonurus injection combined with oxytocin in the treatment of intraoperative and postoperative hemorrhage in cesarean section," *China Continuing Medical Education*, vol. 8, no. 9, pp. 189–191, 2016.

[26] F. Q. Ban, "Clinical effect observation of Motherwort injection combined with Oxytocin in prevention of postpartum hemorrhage aftercesarean section," *Medical Journal of Chinese People's Health*, vol. 29, no. 17, pp. 79–81, 2017.

[27] S. Y. Li, X. C. Liu, Y. M. Zhang, G. Y. Zhang, and S. Y. Cao, "Leonurus japonicus injection in combined with oxytocin on postpartum hemorrhage after cesarean section," *Changchun University of Chinese Medicine*, vol. 31, no. 6, pp. 1243–1245, 2015.

[28] R. P. Shi, "Effect of leonurus heterophyllus injection combined with oxytocin on preventing patients from hemorrhage after

cesarean section," *Acta Medicinae Sinica*, vol. 28, no. 2, pp. 63–65, 2015.

[29] H. Chen, "Motherwort injection combined with oxytocin in the prevention of pre-and post-operative hemorrhage in cesarean section: A clinical observation," *Journal of Huaihai Medicine*, vol. 29, no. 5, pp. 406-407, 2011.

[30] Y. F. Fu, "Motherwort combined with oxytocin in the prevention of postpartum hemorrhage and the acceleration of uterine restoration," *IMHGN*, vol. 17, no. 9, pp. 1103–1105, 2011.

[31] A. P. You, "Effect of Yimucao injection and oxytocin on prevention of intraoperative and postpartum hemorrhage in cesarean section," *The World Clinical Medicine*, vol. 10, no. 15, p. 133, 2016.

[32] X. Q. Xie and J. Liu, "Treatment of 100 cases of cesarean section hemorrhage with Yimucao injection and oxytocin," *Chinese Medicine Modern Distance Education of China*, vol. 11, no. 17, p. 58, 2013.

[33] S. J. Liu, "Clinical observation on prevention of hemorrhage after cesarean section with yimucao injection," *Maternal and Child Health Care of China*, vol. 24, no. 20, pp. 2877-2878, 2009.

[34] M. Y. Wang and R. H. Feng, "Efficacy of misoprostol combined with Yimucao for preventiny postoperative bleeding of cesarean section," *China Modern Doctor*, vol. 51, no. 31, pp. 51-52, 2013.

[35] Z. F. Liu, "Clinical observation on prevention of postoperative hemorrhage after cesarean section with yimucao injection combined with misoprostol," *Hebei Journal of Traditional Chinese Medicine*, vol. 35, no. 10, pp. 1506-1507, 2013.

[36] H. M. Li and L. L. Zang, "Observation of curative effect on motherwort injection, misoprostol tablets and oxytocin in conjunction with the prevention of postpartum hemorrhage," *Chinese Journal of Medicinal Guide*, vol. 12, no. 5, pp. 819-820, 2010.

[37] X. M. Wu, Y. P. Li, C. X. Hu, and S. Jin, "Application of yimucao injection in cesarean section of twin pregnancy," *Journal of Obstetrics and Gynaecology*, vol. 28, no. 8, pp. 691-692, 2012.

[38] Y. F. Luo, "Effect of Yimucao injection and oxytocin on prevention of hemorrhage after cesarean section," *Nei Mongol Journal of Traditional Chinese Medicine*, vol. 36, no. 1, pp. 40-41, 2017.

[39] J. H. Guo, "Effects of yimucao Injection and oxytocin on postpartum hemorrhage and estrogen levels," *China Journal of Traditional Chinese Medicine and Pharmacy*, vol. 16, no. 4, pp. 40-41, 2010.

[40] J. Gong, "Therapeutic effect of yimucao injection combined with oxytocin on prevention of postpartum hemorrhage in selective cesarean section," *Chinese Journal of Modern Drug Application*, vol. 7, no. 03, pp. 66-67, 2013.

[41] H. Wu, "Effect of Yimucao injection combined with oxytotin on intraoperatve and postoperative bleeding in cesarean section," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 24, no. 11, pp. 1171–1174, 2015 (Chinese).

[42] Y. J. Zhang and J. W. Zhang, "Effect of Yimucao injection and oxytocin on cesarean section," *Shenzhen Journal of Integrated Traditional Chinese and Western Medicine*, vol. 27, no. 8, pp. 34–36, 2017.

[43] Y. Y. Mou, "Effect of Yimucao injection combined with cardoprost tromethamine on prevention of hemorrhoids caused by hysterocolic force in cesarean section," *Chinese Primary Health Care*, vol. 31, no. 2, pp. 81-82, 2017.

[44] T. M. Chen, J. H. Zhu, and H. L. Yuan, "Clinical observation on prevention of hemorrhage after cesarean section by combined use of yimucao injection and oxytocin," *Journal of Clinical Research*, vol. 26, no. 11, pp. 2126-2127, 2009.

[45] L. P. Lu, "Analysis on effect of leonurus heterophyllus injection combined oxytocin in the prevention of postpartum hemorrhage after cesarean delivery," *Chinese Journal of Modern Drug Application*, vol. 7, no. 22, pp. 12-13, 2013.

[46] L. J. Huang, "Clinical observation on prevention of postpartum hemorrhage by yimucao Injection combined with oxytocin in cesarean section," *J. hebei. Nat. Sci*, vol. 21, no. 4, pp. 647–649, 2015.

[47] K. J. Sun, "Therapeutic effect of yimucao injection combined with cardoprost tromethamine on preventing hemorrhoids caused by hysterocolic force," *Journal of North Pharmacy*, vol. 14, no. 11, pp. 128-129, 2017.

[48] X. C. Ye, "Effect of Leonurus heterophyllus injection combined with carboprost tromethamine on preventing haemorrhage after cesarean section and its significance on coagulation," *China Modern Medicine*, vol. 23, no. 16, pp. 128–130, 2016.

[49] X. S. Yang and L. Xiong, "Curative efficacy of yimucao injection combined with carboprost tromethamine in preventing cesarean section postpartum hemorrhage and on FIB and D-dimer levels," *Chinese Journal of Experimental Traditional Medical Formulae*, vol. 21, no. 18, pp. 159–162, 2015.

[50] Q. M. Chou, Z. M. Du, and S. Dai, "Therapeutic evaluation of postpartum hemorrhage by carboprost tromethamine and motherwort injection in preeclampsia," *Strait Pharmaceutical Journal*, vol. 27, no. 9, pp. 88–90, 2015.

[51] H. Zhang, "Effect of Yimucao combined with oxytocin on postpartum hemostasis," *Journal of Clinical Medical Literature*, vol. 3, no. 8, pp. 1567-1568, 2016.

[52] X. F. Qin, "Clinical efficacy of yimucao injection combined with oxytocin in the treatment of postpartum hemorrhage," *Chinese Journal of Clinical Rational Drug Use*, vol. 8, no. 6, pp. 56-57, 2015.

[53] X. Q. Deng, "Therapeutic effect of yimucao injection combined with oxytocin on prevention of postpartum hemorrhage," *Modern Journal of Integrated Traditional Chinese and Western Medicine*, vol. 23, no. 5, pp. 510-511, 2014.

[54] G. Q. Ma, "Analysis of Curative effect of yimucao injection combined with cardoprost tromethamine on prevention of postpartum hemorrhage in cesarean section," *Women's Health Research*, vol. 5, pp. 95-96, 2018.

[55] J. L. Li, "Effect of uterine body injection of carboprost tromethamine injection combined with motherwort injection in the prevention of cesarean section's postpartum hemorrhage," *Chinese Journal of New Clinical Medicine*, vol. 7, no. 7, pp. 638–640, 2014.

[56] C. H. Fu and P. J. Shi, "Effect of misoprostol combined with yimucao injection in prevention and treatment of postpartum hemorrhage in cesarean section," *Chinese Community Doctors*, vol. 14, no. 6, p. 208, 2012.

[57] H. Z. Chen and Q. Liu, "Standardized measures to assist misoprostol in combination with intrauterine injection of Yimucao in preventing cesarean section hemorrhage during pregnancy-induced hypertension and its effect on coagulation system," *Journal of Preventive Medicine of Chinese People's Liberation Army*, vol. 35, no. S1, pp. 8–10, 2017.

[58] L. S. Meshykhi, M. R. Nel, and D. N. Lucas, "The role of carbetocin in the prevention and management of postpartum haemorrhage," *International Journal of Obstetric Anesthesia*, vol. 28, pp. 61–69, 2016.

[59] M. Roach, A. Abramovici, and A. N. Tita, "Dose and duration of oxytocin to prevent postpartum hemorrhage: A review,"

American Journal of Perinatology, vol. 30, no. 7, pp. 523–528, 2013.

[60] L. L. Su, Y. S. Chong, and M. Samuel, "Carbetocin for preventing postpartum haemorrhage," *Cochrane Database Syst Rev*, vol. 4, p. D5457, 2012.

[61] D. Cordovani, M. Balki, D. Farine, G. Seaward, and J. C. A. Carvalho, "Carbetocin at elective Cesarean delivery: A randomized controlled trial to determine the effective dose," *Canadian Journal of Anesthesia*, vol. 59, no. 8, pp. 751–757, 2012.

[62] K. K. Than, Y. Mohamed, V. Oliver et al., "Prevention of postpartum haemorrhage by community-based auxiliary midwives in hard-to-reach areas of Myanmar: A qualitative inquiry into acceptability and feasibility of task shifting," *BMC Pregnancy and Childbirth*, vol. 17, no. 1, p. 146, 2017.

[63] M. A. Oleen and J. P. Mariano, "Controlling refractory atonic postpartum hemorrhage with Hemabate sterile solution," *American Journal of Obstetrics & Gynecology*, vol. 162, no. 1, pp. 205–208, 1990.

[64] E. R. Othman, M. F. Fayez, D. E. M. A. El Aal, H. S. El-Dine Mohamed, A. M. Abbas, and M. K. Ali, "Sublingual misoprostol versus intravenous oxytocin in reducing bleeding during and after cesarean delivery: A randomized clinical trial," *Taiwanese Journal of Obstetrics and Gynecology*, vol. 55, no. 6, pp. 791–795, 2016.

[65] A. M. Gülmezoglu, J. Villar, N. T. Ngoc et al., "WHO multicentre randomised trial of misoprostol in the management of the third stage of labour," *The Lancet*, vol. 358, no. 9283, pp. 689–695, 2001.

[66] S. Miller, F. Lester, and P. Hensleigh, "Prevention and treatment of postpartum hemorrhage: new advances for low-resource settings," *Journal of Midwifery & Women's Health*, vol. 49, no. 4, pp. 283–292, 2004.

[67] B. B. Nielsen, L. Høj, L. E. Hvidman, J. Nielsen, P. Cardoso, and P. Aaby, "Reduced post-partum bleeding after treatment with sublingual misoprostol: A randomized double-blind clinical study in a developing country (secondary publication)," *Ugeskrift for Læger*, vol. 168, no. 13, pp. 1341–1343, 2006.

[68] T. F. Baskett, V. L. Persad, H. J. Clough, and D. C. Young, "Misoprostol versus oxytocin for the reduction of postpartum blood loss," *International Journal of Gynecology and Obstetrics*, vol. 97, no. 1, pp. 2–5, 2007.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 4513792, 22 pages
https://doi.org/10.1155/2019/4513792

Research Article

Ethnobotanical Study of Medicinal Plants Used in Central Macedonia, Greece

Efthymia Eleni Tsioutsiou,[1] Paolo Giordani,[2] Effie Hanlidou,[3] Marco Biagi,[4] Vincenzo De Feo,[5] and Laura Cornara[6]

[1]*Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Athens, Greece*
[2]*Department of Pharmacy, University of Genoa, Viale Cembrano, 4, 16148 Genoa, Italy*
[3]*Laboratory of Systematic Botany and Phytogeography, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece*
[4]*Department of Physical Sciences, Earth and Environment, University of Siena, Via Laterina, 8, 53100 Siena, Italy*
[5]*Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano (Selerno), Italy*
[6]*Department of Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132 Genoa, Italy*

Correspondence should be addressed to Vincenzo De Feo; defeo@unisa.it

Received 14 December 2018; Accepted 19 February 2019; Published 1 April 2019

Guest Editor: José Carlos Tavares Carvalho

This work provides the ethnobotanical data concerning the traditional use of medicinal plants in Macedonia region (Northern Greece), which has, up to now, been poorly investigated. The aim of the present study was to collect, analyze, and evaluate information on the use of medicinal plants among different population groups living in Central Macedonia. The study was carried out in the area of two small cities, Edessa and Naoussa, and nearby villages. The ethnobotanical data were gathered through extensive and semistructured interviews. The informants belonged to different population groups living in the study areas and were involved, at least partially, in agriculture. Together with detailed reports on each species, data were also summarized by some indices, such as Fidelity Level (FL) and Informant Consensus Factor (F_{ic}). A group of 96 informants was interviewed and 87 plant taxa with medicinal uses were cited. Medicinal plants are used to treat a wide range of diseases, in particular ailments of the respiratory tract and skin disorders. The importance of the traditional use of plants to cure and prevent common and some uncommon diseases had been highlighted. About 55% of medicinal plants mentioned by the informants had been previously reported to be sold in Thessaloniki herbal market as traditional remedies. Medicinal uses of some endemic taxa had been reported, e.g., *Satureja montana* subsp. *macedonica,* a member of the *S. montana* group restricted to Northern Central Greece, *Origanum dictamnus*, an endemic species of Crete, and six Balkan endemics, i.e., *Achillea holosericea, Digitalis lanata, Helleborus odorus* subsp. *cyclophyllus, Sideritis scardica, Thymus sibthorpii,* and *Verbascum longifolium*. Several differences in Traditional Ethnobotanical Knowledge (TEK) were observed in relation to social and cultural components of the population. Only 7 species (*Crataegus monogyna*, *Hypericum perforatum*, *Matricaria chamomilla*, *Rosa canina*, *Sambucus nigra*, *Sideritis scardica,* and *Tilia platyphyllos*) were commonly reported by all population groups, whereas 30 out of 87 taxa (34%) were exclusively mentioned by a single group. All groups are incorporated in the local society and do not identify themselves as members of different ethnic groups, although they try to preserve their distinctiveness by keeping their traditions and dialects. Nevertheless, our data show that the knowledge regarding the medicinal plant use was rarely accompanied by preservation of linguistic diversity concerning the plant names. This work contributes to improve the knowledge on the traditional use of plants in the folk medicine of a region like Central Macedonia where different population groups live together, partially maintaining their traditions. A part of data of this paper has been presented as posted at 112° Congress of Italian Botanical Society (IPSC), Parma 20-23 September 2017.

1. Introduction

The rural regions of southeastern Europe represent a unique social and environmental context for ethnobotanical studies, owing to the occurrence of a large mountainous area that is recognized as a hotspot for both biodiversity and cultural/ethnic/religious diversities. Medicinal plants have represented, for thousands of years, the only remedy for various diseases. Phytotherapy still maintains an important role in the treatment of many diseases in Greece. Despite this fact, only a few studies have explored the use of plants in Greek folk medicine while, on the contrary, in the neighboring region of the Balkan Peninsula very intensive and highly effective ethnobotanical studies were carried out in the last decade ([1–5] and references therein). Ethnobotanical studies on traditional uses of plants and their products in Greece are relatively scarce. An old study concerning the knowledge of medicinal plants of Greece (Lawrendiadis, 1961) reported some data about most important plants used in folk medicine throughout Greece. More recent works have been mainly focused on the regions of Zagori [6, 7], Thessaloniki [8–10], Crete [11], Mt. Pelion [12], and more recently on the Greek Islands of North Aegean Region [13]. In contrast, the regions of Edessa and Naoussa (Central Macedonia), where this study was carried out, remain poorly explored from an ethnobotanical point of view, despite their high floristic and vegetation diversity.

Our study area, Central Macedonia, lies in the core of the ancient Macedonian state (500-168 B.C.) and is the homeland of Alexander the Great. It was conquered by the Romans (168-284 B.C.), then was a part of the Byzantine Empire, and subsequently became a part of Ottoman Empire (from 1430), and, finally, it was incorporated into the Greek state in 1917 [14]. Centuries of foreign dominance and migrations had shaped the area into a cultural and linguistic mosaic. Until the beginning of 20th century, Greeks, Slavs, Bulgarians, Turks, and Vlachs were living together. The current population composition was formed after the Neuilly Treaty (1919) and mainly after the Greek-Turkish War in Asia Minor (1919-1922) and the Lausanne Treaty (1923), when much of the Slav-speaking and all the Muslim populations left, and Greek populations from Asia Minor and Pontus moved in and settled [15]. Nowadays, the population consists mainly of five groups: (a) the Dopioi, i.e., the local people that remained after the migrations of the first quarter of 20th century. Many of them (Slavophones or Slavo-Macedonians) speak a local Slavic-based dialect while others are Greek-speaking; (b) the Pontians, which come from the Greek population lived in the shores of the Black Sea since antiquity. Under the subsequent Ottoman rule, they survived relatively intact, preserving their customs and dialect (Pontian Greek), which is related to ancient Greek, until they were forced to leave their homeland (Day et al., 2002); (c) the Mikrasiates or prosfyges (meaning refugees), descendants of the Greeks of Asia Minor (Mikra Asia in Greek); (d) the Vlachs or Aromanians, people mostly living in montane region and occupied in animal husbandry, who speak a Latin-based language, having a long history of settlement in the study area. According to some historians, they are Latinized indigenous populations (Greeks, Illyrians, Thracians or Dardanias), due to the historical presence of the Roman military in the territory [16]. Many Romanian historians claim that the Aromanians were part of a Daco-Romanian migration from the north of the Danube [17, 18]; (e) others, who recently moved to the region from various parts of Greece.

One of the most prominent differences of above populations groups is in their dialects, which are still used, particularly by the older people, as a second language after contemporary Greek. All groups are incorporated in the local society and do not identify themselves as members of different ethnic groups (REF). However, they define themselves based on their origin and try to preserve their distinctiveness by keeping, in a large degree, their customs, music, dances, dialects, and cuisine [19–23], (Winnfrith, 2001). Taking into account the lack of any published ethnobotanical information as well as the great cultural diversity of Central Macedonia, our aim is to survey medicinal plants and their uses and to find if significant differences concerning the use of medicinal plants still persist among the groups of inhabitants of the area.

2. Materials and Methods

2.1. Study Area. Our survey was conducted in two cities, Edessa and Naousa, each with a population of c. 18.000 inhabitants and their nearby rural small villages (Figure 1). The inhabitants are mostly occupied in agriculture and stock raising. The area is located at the foothills of Mt Vermio and Mt Voras. The two mountains, which are part of the Natura 2000 Network (GR1210001 and GR1240008, respectively), are characterized by a rich and diverse flora (> 1000 taxa were recorded on Vermio, and > 1500 on Voras), including several Greek and Balkan endemic species [24, 25].

2.2. Methods. The fieldwork was conducted during spring and summer in 2016 and 2017. The ethnobotanical data were gathered through extensive interviews, aimed to create open informal and semistructured interviews. Snowball sampling techniques were used to recruit 96 informants (37 men, 59 women). In snowball sampling, the first contact with the community is selected as a well-known expert; in a subsequent phase, the expert indicates another expert, and so on, until all the specialists in the community are covered [26]. The informants were selected proportionally to the occurrence of five groups within the local population: (a) Dopioi (28 persons), DO (b) Pontians (22 persons), PO (c) Mikrasiates (17 persons), MI (d) Vlachs (10 persons), VL (e) others (19 persons), OTH.

In particular, in the Naoussa municipality a total of 24 informants were selected (8 OTH, 3 MI, 9 PO; 2 DO and 4 VL), 20 coming from the town and 4 from the villages. In the Edessa municipality, there were a total of 72 (11 OTH; 14 MI; 13 PO; 26 DO and 6 VL), 15 of which come from the town and 57 from 16 villages. The subdivision of the inhabitants of the area in various groups was mainly based on the different dialects that are still spoken in the two municipalities. In addition, it is worth mentioning that the various groups are diversified from each other due to different cultural characteristics such

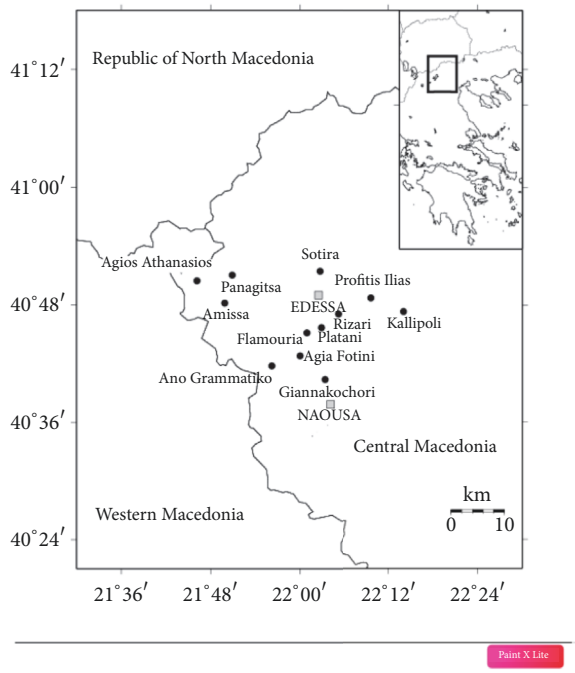


Figure 1: Map of Edessa, Naoussa, and the nearby villages in Central Macedonia (Greece).

as traditional costumes, music, and folk dances. Starting from Seventies the modern way of living has prevailed. However, at the same time, there was a turning towards folklore in every county town of Greece, including Central Macedonia. Cultural societies are formed with folklore dance groups and a recovery of the traditional music is still in progress [27]. Also in the study area several different folk traditions still persist among the group population, as shown by the presence of many Folklore Museums spreading in Central Macedonia (e.g., Vlach Folklore Museum, Folklore Museum of Edessa, History and Folklore Museum of Naoussa).

For every informant we recorded personal information about age, gender, education level, profession, and population group. This distinction helped to note differences and similarities between citations based on different factors. The informants had personal experience in self-medication using herbs and had ethnobotanical knowledge because of family tradition or personal interest. Their age ranged between 24 and 94 years (mean = 59 years), educational levels included primary (30%), secondary (41%), and higher education (29%), jobs included employees (e.g., public employees, civil engineers), farmers, workers, and people involved in humanistic occupations (e.g., painter, teachers). All information was obtained after receiving an oral prior informed consent from the participants, according to the ISE (International Society of Ethnobiology) Code of Ethics. During the interviews, the informants were requested to indicate vernacular names of plants, parts of the plant used, association with other plants, folk uses, and preparation procedures. In many cases, data on specific recipes and their sources were included. Quite often the interviews took place in the village square or in the houses of the informants (Figure 2) where they also showed us traditional remedies that are currently used. Specimens of the plants were either given to us by the informants or collected from the wild, according to their instructions. The information collected refers to wild and cultivated species. The taxa were identified using standard Floras [24, 25, 28, 29]. Nomenclature is according to Dimopoulos et al. [30]. Voucher specimens are deposited at the Herbarium of the Aristotle University of Thessaloniki (TAU).

The data gathered on plant uses were organized in citations and each citation coincided with a single row in a database created using Microsoft Excel. Then data were analyzed and compared with several ethnobotanical references, primarily based on a search in Scopus database, using the search string "ethnobotany and Greece". Following recent recommendations for reporting ethnobotanical field studies, primary data are presented in an unaltered form, allowing direct comparison between other similar researches [31]. In order to compare our results with the list of the most common medicinal plants used in Central Macedonia, in Table 1 we reported the species sold in the herbal market of the regional capital Thessaloniki [8]. Several ethnobotanical indices were adopted for interpreting the large quantity of information.

2.3. Quantitative Indices and Statistical Analysis. To estimate the use variability of the species, we adopted the Informant Consensus Factor [32], which was calculated for each medicinal category. This index was calculated as follows: number of citations in a subcategory (n_{ur}) minus the number of taxa used in the same subcategory (n_t), divided by the number of citations minus one [33, 34]:

$$F_{ic} = \frac{(n_{ur} - n_t)}{(n_{ur} - 1)} \quad (1)$$

Figure 2: Informant interviewed during the ethnobotanical study.

The value of this factor ranges from 0 to 1. A high F_{ic} value indicates an agreement among the informants on the use of taxa within a medicinal subcategory. The F_{ic} reflects homogeneity of information provided by different informants.

The Fidelity Level index (FL) was also considered to indicate the informants' choice for a potential plant species to treat a given disease. It was calculated by the following formula [35]:

$$\text{FL } (\%) = \left(\frac{\text{Np}}{\text{N}}\right) * 100 \quad (2)$$

where Np is number of use reports for a given species reported to be used for a particular ailment category and N is total number of use reports cited for any given species.

Descriptive statistical analyses were carried out in R environment (vers. 3.4.0, R Core Team 2017) adopting a circlize package (vers. 0.4-2, [36]) and Venn Diagram package (vers. 1.6-19, [37]).

3. Results and Discussion

3.1. Medicinal Plants. Our results showed that 87 medicinal plant species belonging to 48 families are used in the study area to treat several ailments. The surveyed species are listed in Table 1, where plant families and species within each family are cited in alphabetical order. In this table, for each taxon reported, data on scientific name, family, local name, part of plant used, medicinal use, and number of citations and if they are wild or cultivated are included. In the last column, it is also reported if the species has been previously cited in the study of Hanlidou et al. [8] on the herbal market in Thessaloniki (representing ca. 55% of the taxa cited in our work).

Noteworthy, the knowledge regarding the medicinal plant use was rarely accompanied by preservation of linguistic diversity concerning the plant names. In fact, only 6 out of 87 taxa were cited also under their dialect names. Accordingly, also in previous works on ethnobotanical use of plants in folk medicine in Greece, dialect names were seldomly reported [6]. Lawrendiadis (1961), speaking about common names of medicinal plants in Greece, referred that they are usually related to the part of the body on which they have curative effects or to the disease against which they can be used. For example, *Tussilago farfara* was called "vychion" from the Greek word "vyx" (cough) or *Origanum dictamnus* "stomachochorton" from the Greek words "stomachos" (stomach) and "chorton" (herb). More recently, Hanlidou et al. [8], referring to medicinal plants sold in the Thessaloniki market, reported for most taxa both commercial names and names in Dioscurides.

3.1.1. Most Cited Species. Data show that Lamiaceae (21%) and Asteraceae (13%) are the most represented families, followed by Rosaceae (6%). Among the taxa (species and subspecies) recorded, 23 are cultivated, either grown in the study area or purchased from the local market. However, most of the taxa (64) were collected from the wild. Among them, there is a Greek endemic taxon, *Satureja montana* subsp. *macedonica,* a member of the *S. montana* group restricted to Northern Central Greece, and six Balkan endemics (some of them extending to Italy or Anatolia), i.e., *Achillea holosericea, Digitalis lanata, Helleborus odorus* subsp. *cyclophyllus, Sideritis scardica, Thymus sibthorpii, and Verbascum longifolium.* The

Table 1: Ethnobotanical uses of plants in the regions of Edessa and Naoussa (Central Macedonia, Greece).

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] *
				OTH	MI	PO	DO	VL			
Adoxaceae											
Sambucus nigra L. (TAU 60500)	Zampouko	Inf	MED-RES: decoction against inflammation of the respiratory tract, cough or as expectorant, (also combined with Le. of *Alcea rosea* or with Se of *Linum usitatissimum*, Ap of *Verbascum longifolium* and *Thymus vulgaris*)	5	1	3	7	2	21	W	+
			MED-SKI: stems' bark with wax and olive oil applied on cuts, bruises, burns, and wounds	1	0	2	1	0			
Amaryllidaceae											
Allium cepa L.	Kremidi	Bu	MED-SKI: used as cataplasm with salt or wine or olive oil against skin burns, bruises, and edemas	0	2	0	1	0	3	C	+
Allium sativum L.	Skordo	Bu	MED-CAR: eaten raw or cooked to prevent and treat hypertension	0	0	1	3	0	4	C	+
Anacardiaceae											
Pistacia lentiscus L. var. chia	Masticha	Re	MED-GAS: chewed against gastric ulcers and ailments of the digestive system	0	0	1	0	0	1	C	+
Apiaceae											
Apium graveolens L.	Selino	Le	MED-CAR: decoction against hypertension	1	0	0	0	0	2	C	
			MED-RES: boiled with milk to treat the symptoms of common cold	1	0	0	0	0			
Aquifoliaceae											
Ilex aquifolium L. (TAU 60501)	Ou	Le	MED-MUS: decoction to treat joint and muscular pains	0	0	0	1	0	1	W	
Araceae											
Arum italicum Mill. (TAU 60502)	Drakontia/ Smiina bilka (DO)	Le, Ro	MED-RES: decoction against cough, bronchial catarrh, and sore throat	0	0	1	1	0	2	W	
Arum maculatum L. (TAU 60503)	Drakontia	Le, Ro	MED-RES: decoction against cough, bronchial catarrh, and sore throat	0	0	1	1	0	2	W	
Araliaceae											
Hedera helix L. (TAU 60504)	Kissos	Le	MED-RES: decoction against cough	2	0	0	1	0	3	W	+

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] *
				OTH	MI	PO	DO	VL			
Asparagaceae											
Ruscus aculeatus L. (TAU 60505)	Agathi	Ap	MED-GEN: decoction for the treatment and prevention of prostatitis and as diuretic (combined in the decoction with Fr. of *Paliurus spina-christi*)	0	0	0	3	0	3	W	+
Asphodelaceae											
Aloe vera (L.) Burm. f.	Aloi	Le	MED-SKI: parenchymatic gel used as poultice applied on wounds, sunburns, and insect bites	0	0	0	1	0	1	C	
Asteraceae											
Achillea holosericea Sm. (TAU 60506)	Kitrini Achillia	Inf	MED-RES: decoction against inflammation of the respiratory tract and cough or as expectorant	0	0	0	0	2	4	W	
			MED-GEN: decoction against cystitis	0	0	0	0	1			
			MED-SKI: water of maceration as cataplasm against hemorrhoids	1	0	0	0	0			
Achillea millefolium L. (TAU 60507)	Lefki Achillia	Inf	MED-RES: decoction against inflammation of the respiratory tract and cough	0	0	3	0	2	17	W	+
			MED-GEN: decoction against cystitis, urogenital inflammation, and regulation of menstrual cycle (with *Capsella bursa-pastoris*, cinnamon bark and orange peel)	4	0	5	0	1			
			MED-SKI: water of maceration as cataplasm against hemorrhoids; used fresh as cataplasm against acne	1	0	1	0	0			
		Ro	FOOD: eaten raw as depurative	1	0	2	0	0			
Arctium lappa L. (TAU 60508)	Arktio	Le	MED-MUS: decoction for joint pain and inflammation	2	1	2	0	0	11	W	+
		Le	MED-SKI: decoction used as poultice applied on wounds and furuncles	1	0	2	0	0			
Cirsium eriophorum (L.) Scop. (TAU 60509)	Kirsio	Fl	MED-MET: decoction for detoxification and treatment of liver diseases	0	2	0	0	0	2	W	
Helianthus tuberosus L. (TAU 60510)	Kolokasi	Tu	FOOD: eaten boiled as salad to improve intestinal function	0	0	3	0	0	6	C	
			MED-GAS: decoction against constipation	0	0	3	0	0			
Lactuca serriola L. (TAU 60511)	Agriomarulo	Le, Fl	MED-MET: decoction against hypercholesterolemia	2	0	0	1	0	3	W	
Matricaria chamomilla L. (TAU 60512)	Chamomili	Inf	MED-NER: decoction or infusion against insomnia and otitis	1	6	1	5	1	33	W	+
			MED-GAS: decoction against abdominal pain	3	3	4	1	0			
			MED-GEN: washing to treat vaginitis	3	0	1	0	0			
			MED-RES: oily extract or decoction against cough		1						
			MED-SKI: oily extract applied for wound healing	3	0	0	0	0			
Pulicaria dysenterica (L.) Bernh.	Pulicaria	Ap, Fl	MED-GAS: decoction for constipation and intestinal problems	1	0	0	0	0	1	W	
Silybum marianum (L.) Gaertn. (TAU 60513)	Gaiduragatho	Fl, Fr	MED-MET: decoction for detoxification and treatment of liver diseases	3	0	0	2	0	5	W	+
Taraxacum sp. (TAU 60514)	Taraxaco	Ap	MED-NER: decoction against headache and insomnia	1	0	1	0	1	4	W	+
			MED-MET: to decrease triglyceride and cholesterol levels	1	0	0	0	0			
Tussilago farfara L. (TAU 60515)	Vihio/ Flomos	Ap	MED-RES: decoction against cough and inflammation of the respiratory tract	2	0	0	1	0	3	W	+

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] *
				OTH	MI	PO	DO	VL			
Boraginaceae											
Cynoglossum creticum Mill.	Kynoglosso/ Aritsovotano	Fr	MED-SKI: eaten triturated for the treatment of warts	4	0	0	0	0	4	W	
Caprifoliaceae											
Valeriana officinalis L. (TAU 60516)	Valeriana	Ro	MED-NER: tincture against insomnia and as calmative (combined with *Capsella bursa-pastoris*)	1	0	4	0	0	5	W	+
Caryophyllaceae											
Saponaria officinalis L. (TAU 60517)	Sapunochorto	Fl	MED-SKI: cataplasm applied on skin against eczema and dermatitis	2	0	0	1	0	3	W	+
Convolvulaceae											
Cuscuta campestris Yunck. (TAU 60518)	Kitrino parasito	Ap	MED-SKI: stems of the aerial part rubbed on bee stings	0	0	0	3	0	3	W	
Cornaceae											
Cornus spp.	Krana	Fr	MED-GAS: alcoholic extract drunk against abdominal pain		1				1	W	
Cucurbitaceae											
Cucurbita pepo L.	Kolokithi	Se	MED-GEN: oily extract drunk to cure and prevent prostatitis	0	0	0	1	0	2	C	
			MED-MUS: oily extract topically applied against joint pain	0	0	0	1	0			
Momordica charantia L. (TAU 60519)	Kanturi	Fr	MED-SKI: oily extract against wounds, burns, calluses; drunk against gastric ulcers.	5	2	0	6	1	30	C	
			VET: oily extract used on goats and pigs to cicatrize wounds	5	2	0	6	0			
Cupressaceae											
Juniperus communis L. (TAU 60520)	Mavros Kedros	Ga	MED-CAR: eaten raw or used as flavoring for meat to prevent cardiovascular diseases	0	0	2	0	0	2	W	
Juniperus oxycedrus L. (TAU 60521)	Kedros	Ga	FOOD-MED: flavoring for meat and digestive	0	0	1	1	0	2	W	+
Dennstaedtiaceae											
Pteridium aquilinum (L.) Kuhn (TAU 60522)	Fteri	Le	DOM: wrap up raw meat to keep it fresh	0	1	0	4	0	7	W	
			MED-GEN: decoction as diuretic and to remove kidney stones		1			1			
Dioscoreaceae											
Tamus communis L.	Riza tou Adam	Ro	MED-MUS: alcoholic extract of sliced root (forms a cream) against muscular pain and inflammation	1	0	2	0	0	3	W	
Equisetaceae											

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] ∗
				OTH	MI	PO	DO	VL			
Equisetum arvense L. (TAU 60523)	Ekuiseto/ Polikombi/Ura tu alogu	Ap	MED-GEN: decoction against urogenital diseases and for the treatment of prostatitis as diuretic	0	0	0	10	0	10	W	+
Ericaceae											
Vaccinium myrtillus L. (TAU 60524)	Mirtillo	Fr	MED-NER: eaten raw to improve vision MED-SKI: pulped against gums' inflammation	0	0 1	2	0	0	3	W	+
Fabaceae											
Robinia pseudoacacia L. (TAU 60525)	Akakia	Fl	MED-MUS: decoction against joint pains and rheumatisms (also combined with Fl. of *Cercis siliquastrum*)	0	0	0	5	0	5	W	
Cercis siliquastrum L. (TAU 60526)	Kutsupia	Fl	MED-MUS: decoction against joint pains and rheumatisms (also combined with Fl. of *Robinia pseudacacia* and Le. of *Alcea rosea*)	0	0	0	7	0	7	W	
Fagaceae											
Castanea sativa Mill. (TAU 60527)	Kastania	Fl	MED-GAS: decoction against diarrhea	6	0	0	0	0	6	C	+
Grossulariaceae											
Ribes uva-crispa L. (TAU 60528)	Fragostaphilo	Ap	MED-MET: decoction or tincture to increase iron levels and as tonic	1	0	2	0	0	3	W	
Hypericaceae											
Hypericum perforatum L. (TAU 60529)	Valsamochorto/ Rumana/ Spathochorto	Inf	MED-SKI: oily or alcoholic extract applied on wounds, burns; mixed with wax and Re. of *Pistacia lentiscus* var. *chia* and applied on deep sores MED-MUS: as massage to alleviate arthritis symptoms and joint pain MED-GAS: drunk to treat gastric ulcers and gastrointestinal disturbs (1 spoon/day) MED-NER: decoction against insomnia and as antidepressant	8 0 1 0	7 1 4 0	14 3 6 1	6 0 1 1	9 0 3 0	65	W	+
Iridaceae											
Crocus sativus L.	Krokos	Fl	MED-MET: water of maceration of stigmas as immunostimulant or for the prevention of common colds and flu	0	0	1	0	0	1	C	+

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] *
				OTH	MI	PO	DO	VL			
Lamiaceae											
Hyssopus officinalis L. (TAU 60530)	Issopos	Ap	MED-NER: decoction against headache	1	0	2	0	0	3	C	
Lavandula angustifolia Mill. (TAU 60531)	Levanta	Inf	MED-NER: decoction as bland sedative MED-CAR: against hypertension if combined with Le. of *Mentha spicata*	1 1	1 1	1 1	0 0	0 0	6	C	+
Melissa officinalis subsp. *altissima* (Sm.) Arcang. (TAU 60532)	Melissochorto	Le	MED-NER: decoction or infusion as sedative; used as decoction in combination with Ap. of *Crataegus monogyna* Jacq. for the prevention of cardiovascular diseases MED-MET: decoction or infusion against hypercholesterolemia	4 1	0 0	0 0	2 4	0 0	6	W	+
Mentha spicata L. (TAU 60533)	Menta	Le, Ap	MED-CAR: cardiovascular problems and hypertension MED-GAS: decoction in combination with Inf. of *Hypericum perforatum* for the treatment of gastric ulcers, nausea and flatulence MED-GAS: decoction against nausea and flatulence	1 1	0 2 2	0 1	4 4	0 0 1	18	W	+
Mentha spp.	Menta/Dyosmos	Le	MED-RES: decoction against inflammation of the respiratory tract and cough		2 1				6	W	
Micromeria juliana (L.) Rchb. (TAU 60534)	Kiparissaki	Ap	MED-GEN: decoction as diuretic and for the prevention and treatment of prostatitis (also in combination with Se of *Rosa canina* and Ap. of *Equisetum arvense*)	1	1	6	0	0	8	W	+
Ocimum basilicum L. (TAU 60535)	Vasilikos	Le	MED-NER: used fresh as decoction or infusion against insomnia and to improve memory and concentration	0	1	0	2	0	3	C	+
Origanum dictamnus L.	Diktamo	Ap	MED-RES: decoction against cough and symptoms of common cold		1				1	C	+
Origanum majorana L. (TAU 60536)	Matzurana	Ap	MED-GAS: decoction as digestive, against nausea and gastric disturbs MED-GEN: decoction against menstrual pain	1	2 1	3	1	0	8	C	+
Origanum vulgare subsp. *hirtum* (Link) Ietswaart (TAU 60537)	Rigani	Ap	MED-MET: decoction or water of maceration as blood depurative MED-GAS: eaten raw to improve digestion	0	0	0	2	0 1	3	W	+
Origanum vulgare subsp. *viridulum* (Martrin-Donos) Nyman (TAU 60538)	Tourkiko tsai	Inf	MED-RES: decoction with honey and butter against allergic cough	2	0	0	0	0	2	W	
Rosmarinus officinalis L. (TAU 60539)	Dentrolivano	Ap	MED-MET: decoction against hypercholesterolemia	0	4	0	0	0	4	C	+

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] ∗
				OTH	MI	PO	DO	VL			
Salvia officinalis L. (TAU 60540)	Faskomilo	Le	MED-NER: decoction or water of maceration against insomnia and anxiety (in combination with Inf. of *Aloysia citrodora*)	2	1	0	3	0	6	W	
Satureja montana subsp. *macedonica* (Formànek) Baden (TAU 60541)	Thymari/ Thympiron (PO)	Inf	MED-RES: decoction against inflammation of the respiratory tract and for cough MED-MET: decoction against hypercholesterolemia MED-NER: decoction to alleviate symptoms of tinnitus	0 0 0	0 1 0	4 0 3	0 0 0	0 0 0	8	W	
Sideritis montana subsp. *remota* (d'Uvr.) P.W. Ball (TAU 60542)	Aspro tsai	Inf, Ap	MED-RES: decoction against inflammation of the respiratory tract and cough	2	0	1	0	2	5	W	
Sideritis scardica Griseb. (TAU 60543)	Tsai tu vunu	Inf, Ap	MED-RES: decoction against inflammation of the respiratory tract and cough	6	11	7	2	4	30	W	+
Teucrium capitatum L. (TAU 60544)	Tefkrio	Fl	MED-GAS: decoction against abdominal pain	0	0	1	2	0	3	W	
Thymus sibthorpii Benth. (TAU 60545)	Materina	Inf, Le	MED-GAS: decoction against abdominal pain or gastrointestinal disturbs	3	0	0	0	1	4	W	
Linaceae											
Linum usitatissimum L.	Linari	Se	MED-GAS, decoction against constipation MED-RES: decoction or infusion as expectorant or to alleviate cough (also in combination with Inf. of *Sambucus nigra* and Ap. of *Verbascum longifolium*. and *Thymus vulgaris*) MED-SKI: as cataplasm to treat wounds and furuncles	0 1	0 3 1	3 5	0 0	0 0	13	C	+
Loranthaceae											
Loranthus europaeus Jacq. (TAU 60546)	Parasito	Ap	MED-CAR: decoction of twigs to treat hypertension, varicose veins and to improve blood circulation	2	0	0	5	0	7	W	
Malvaceae											
Alcea rosea L. (TAU 60547)	Althea	Le	MED-RES: decoction or infusion against cough in combination with Inf. of *Sambucus nigra*; decoction against rheumatisms and musculoskeletal pain in combination with Fl. of *Robinia pseudacacia* and of *Cercis siliquastrum*	1	0	0	3	0	4	W	
Malva sylvestris L. (TAU 60548)	Molocha	Fl	MED-RES: decoction or infusion as expectorant and against cough	6	4	2	1	0	14	W	+

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] ∗
				OTH	MI	PO	DO	VL			
Tilia platyphyllos Scop. (TAU 60549)	Tilios/Flamuri	Inf	MED-GAS: decoction against intestinal pain MED-RES: decoction for cough and bronchial catarrh MED-NER: decoction as sedative	0 3 0	2 5 0	2 6 0	1 6 1	0 1 0	26	W	+
Oleaceae											
Fraxinus ornus L. (TAU 60550)	Fraksos	Ap	VET: bark of stems into chickens' water to prevent their illnesses	0	0	1	1	0	2	W	+
Onagraceae											
Epilobium angustifolium L. (TAU 60551)	Epilovio	Le	MED-GEN: decoction to treat and prevent prostatitis or as diuretic	3	1	0	1	1	6	W	
Epilobium parviflorum Schreb.	Epilovio	Le	MED-GEN: decoction to treat and prevent prostatitis and cystitis or as diuretic	1	0	0	0	0	1	W	
Orchidaceae											
Dactylorhiza sambucina (L.) Soó (TAU 60552)	Salepi	Bu	MED-RES: decoction as expectorant against cough and common cold	5	0	2	0	1	8	W	
Papaveraceae											
Papaver rhoeas L. (TAU 60553)	Paparuna	Se	MED-NER: infusion or decoction as sedative	0	0	2	0	0	2	W	
Passifloraceae											
Passiflora sp. (TAU 60554)	Passiflora	Fl	MED-NER: decoction as sedative	0	0	0	2	0	2	C	+
Piperaceae											
Piper nigrum L.	Piperi	Se	MED-RES: alcoholic extract used as cataplasm against cough		1				1	W	+
Plantaginaceae											
Plantago media L. (TAU 60555)	Pentanevro/ Tigavits (DO)	Le	MED-SKI: used fresh, rubbed on wounds, furuncles and hemorrhoids MED-GEN: decoction or eaten as salad to treat or prevent urogenital ailments (prostatitis) and improve kidneys' function	3 2	1 0	1 0	3 0	0 0	10	W	
Digitalis lanata Ehrh. (TAU 60556)	Digitalis	Le	MED-CAR: decoction to improve blood circulation and prevent cardiovascular diseases	1	0	0	0	0	1	W	
Poaceae											
Elytrigia repens (L.) Nevski (syn. *Agropyron repens* (L.) P. Beauv.)	Agriada	Ap	MED-MUS: decoction or infusion against joint and muscular pains	1	1	0	0	0	2	W	
Zea mays L.	Kalampoki	StyFl	MED-NER: decoction or water of maceration as sedative MED-GEN: decoction in combination with fruit stalksof *Prunus avium* as diuretic or for the prevention and treatment of urogenital disturbs	1 2	1 1	0 0	1 0	0 0	6	W	+
Portulacaceae											
Portulaca oleracea L. (TAU 60557)	Glistrida/ Adrakla	Ap	MED-MET: eaten raw or boiled against hypercholesterolemia MED-NER: eaten raw against toothache and to remove tartar MED-CAR: eaten raw to prevent cardiovascular diseases, hypertension, obstructed arteries	0 0 0	0 0 0	1 0 1	1 1 2	0 0 0	6	W	
Ranunculaceae											
Helleborus odorus subsp. *cyclophyllus* (A. Braun) Maire & Petitm. (TAU 60558)	Elevoros/ Spres (DO)	Ro	MED-MUS: decoction of fragmented root (1 part of root in 2 liters of water) to treat joint pain; toxic plant VET: decoction of fragmented root with salt to treat joint pain in goats, cows and pigs	0 0	0 0	0 0	2 2	0 0	4	W	
Rhamnaceae											

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] ∗
				OTH	MI	PO	DO	VL			
Paliurus spina-christi Mill. (TAU 60559)	Paliuri/ Fluri	Fr	MED-GEN,: decoction for urinary tract and urogenital diseases and the treatment of prostatitis as diuretic	3	0	1	6	0	11	W	+
			MED-MET: decoction against hypercholesterolemia	0	1	0	0	0			
Rosaceae											
Agrimonia eupatoria L. (TAU 60560)	Agrimonio	Ap	MED-SKI: infusion applied as cataplasm for the cicatrization of wounds	1	0	2	0	0	3	W	+
Crataegus monogyna Jacq. (TAU 60561)	Krategos	Ap, (Fl, Fr, LeSh)	MED-CAR: decoction (Ap) or alcoholic extract (Fr) for the prevention of cardiovascular diseases and for the treatment of hypertension	1	4	2	3	3	13	W	+
Prunus avium L. (L.) (TAU 60562)	Kerasia	FrPed, Se	MED-MUS: decoction against arthritis and joint pain (in combination with StyFl. of *Zea mays*); as diuretic	1	0	0	1	1	3	C	+
Rosa canina L. (TAU 60563)	Agriotriantafilia/ Kynorodo/ Masura (PO)	Fl, Fr	FOOD: marmalade; tonic liquor (made of petals)	0	0	0	2	0	24	W	+
		Se Fr	MED-MUS: against rheumatisms in combination with Fl. of *Cercis siliquastrum*; to treat osteoarthritis; eaten pulverized against arthritis pain	0	1	0	1	0			
		Fr	MED-GEN: decoction helps to remove kidney stones, against prostatitis in combination with StyFl. of *Zea mays* and Ap. of *Micromeria juliana*	0	0	1	0	0			
		Fl, Fr	MED-MET: eaten raw as antioxidant, for the prevention of cardiovascular diseases; decoction, syrup, marmalade or liquor as immunostimulant (to prevent cold and flu)	3	3	4	7	2			
Rosa x damascena Herrm. (TAU 60564)	Palia triantafyllia	Fl	MED-MUS: oily extract of petals applied to treat musculoskeletal pain/also used to treat children's joint pain while they grow up	0	0	0	2	0	2	C	

Table 1: Continued.

Families/Species	Local names	Parts used	Use categories, preparation, and ailment treated	No. citations per population group					No. of total reports	WS/CS	Sold in Thessaloniki market [8] ∗
				OTH	MI	PO	DO	VL			
Rutaceae											
Citrus limon (L.) Osbeck	Lemoni	Le	MED-MET: decoction against hypercholesterolemia	0	0	2	0	0	2	C	
Scrophulariaceae											
Verbascum longifolium Ten. (TAU 60565)	Verbasco/ Lupusiou (VL)	Ap	MED-RES: decoction to treat cough	3	0	3	1	0	10	W	
		Ap	MED-GEN: decoction to treat and prevent prostatitis	0	0	2	0	0			
		Le	MED-GAS: decoction against abdominal pain	0	0	2	0	0			
Solanaceae											
Alkekengi officinarum Moench. (syn. *Physalis alkekengi* L.) (TAU 60566)	Fanaraki	Fr	MED-MET: eaten raw as immunostimulant and tonic	1	0	0	1	0	2	W	
Nicotiana tabacum L. (TAU 60567)	Kapnos	Le	MED-SKI: used fresh, applied on wounds as cicatrizing, hemostatic, disinfectant	0	0	2	0	0	2	C	
Urticaceae											
Urtica dioica L. (TAU 60568)	Tsouknida	Le	MED-SKI: decoction for greasy hair	0	0	2	0	0	19	W	+
			MED-MUS: infusion or decoction against rheumatisms	3	0	0	1	0			
			MED-GEN: decoction against prostatitis and as diuretic	3	0	2	0	0			
			MED-MET: decoction or eaten raw as salad as blood depurative and against anemia	3	0	2	3	0			

Legends: *OTH*= others; *MI*= Mikrasiates; *PO*= Pontians; *DO*= Dopioi; *VL*=Vlachs. *CS*=cultivated species; *WS*= wild species; *Ap*=aerial part; *Bu*= bulb; *Ga*=galbula; *Fl*=flowers; *Fr*=fruits; *FrPed*= fruit pedicels; *Inf*= inflorescences; *Le*= leaves; *LeSh*=leaf shoots; *Re*=resin; *Ro*= roots; *Se*=seeds; *StFl*=styles of female flowers; *Tu*=tubers. *DOM*=domestic; *FOOD*=for human nutrition and health; *MED-CAR*=cardiovascular diseases; *MED-GAS*=gastrointestinal diseases; *MED-GEN*=genitourinary diseases; *MED-MET*=metabolic diseases; *MED-MUS*=muscular-skeletal diseases; *MED-NER*=nervous-sensorial diseases; *MED-RES*= respiratory diseases; *MED-SKI*=skin diseases. ∗ Reported in Hanlidou et al. [8] for at least one of the uses cited in the present study.

Table 2: Informant consensus factor.

Disease categories	Citations	Taxa	FIC
Respiratory diseases	154	24	0,85
Skin diseases	104	19	0,83
Cardiovascular diseases	39	9	0,79
Gastrointestinal diseases	75	18	0,77
Genitourinary diseases	65	17	0,75
Nervous-sensorial diseases	58	16	0,74
Metabolic diseases	59	18	0,71
Muscular-skeletal diseases	42	15	0,66
Total	596	136	

remaining are more widespread plants, with a European (14 taxa), Mediterranean (16), Eurasiatic, or Cosmopolitan (23) distribution [30].

As shown in Table 2, the highest number of plants was used to treat respiratory and skin diseases followed by cardiovascular, gastrointestinal, genitourinary, nervous-sensorial, metabolic, and muscular-skeletal diseases. The most used parts of the plants were flowers and inflorescences (40%), followed by aerial parts (22%), leaves (16%), fruits and seeds (15%), and underground parts (8%). Principal methods of herbal preparations included infusion or decoction (76%), maceration in alcohol or oil (12%), used raw (7%), poultice (3%), and other (2%). The most cited species were *Hypericum perforatum*, *Matricaria chamomilla*, the endemic *Sideritis scardica, Tilia platyphyllos,* and *Sambucus nigra.*

About 48% of the medicinal plants mentioned by the informants had been previously reported to be sold in Thessaloniki market as traditional remedies [8]. Seventeen of the 22 most relevant species, with at least 8 citations and high Fidelity Level (Table 3), are among them. The traditional shops and the street markets of this city include interesting information mainly derived from the inherited knowledge of the herbal sellers. Lamiaceae, the most frequently recorded family both in our study area and in the market of Thessaloniki, includes the greatest number of species mentioned for treating digestive, nervous, and respiratory diseases [10].

In many cases, the most common species reported for Central Macedonia for medicinal purposes are in agreement with those cited from other regions of Balkan Peninsula. For example, *H. perforatum*, *M. chamomilla*, *S. nigra*, *R. canina*, *U. dioica,* and *C. monogyna* were frequently cited by informants from Bosnia-Herzegovina [1] and from south Kosovo [4]. In Northern Macedonia Albanian informants reported *H. tuberosum* as a remedy for improving heart contractility, in addition to the use as human food [3]. In the same area, *Sideritis scardica* was reported mainly for treatment of respiratory diseases.

3.1.2. Plants Used for Respiratory Diseases. In our study *S. scardica* is one of the most important (FL = 100%) plants cited for respiratory diseases, followed by *Tilia platyphyllos*, *Sambucus nigra*, *Malva sylvestris,* and *Dactylorhiza sambucina*. *S. scardica* is an aromatic plant very popular in Greece, Bulgaria, Albania, and North Macedonia where it is largely used in local cuisines. The species, together with several related *Sideritis* species, is known as "mountain tea", and in folk medicine of the Balkan countries, it is used for the preparation of decoctions mainly indicated to aid digestion, strengthen the immune system, and treat cold, flu, and allergies [6, 38]. It is also employed against shortness of breath, sinus congestion, and even pain and mild anxiety [5, 38, 39]. It is worth noting that tubers of *Dactylorhiza sambucina* (as well as other Orchidaceae species) are collected from the wild and used to prepare a beverage, called "salepi", used as a cough remedy popular in several eastern Mediterranean countries [40]. Similar uses were reported for another Orchidacea, *Orchis morio* L., in south Kosovo where the tuber infusion is indicated as a remedy for influenza, stomach disorders, and wound healing [4].

3.1.3. Plants Used for Skin Diseases. Among plants used to treat skin diseases *Momordica charantia* shows the highest FL (94%), while *Hypericum perforatum* (Figure 3(a)) reaches the highest number of citations (44). *M. charantia* or bitter melon is a tropical vegetable extensively used in Indian folk medicine as a remedy for diabetes. In Ayurveda, the fruit is considered as tonic, stomachic, stimulant, emetic, antibilious, and laxative. In addition, the fruit juice and/or a leaf tea is employed for malaria, colic, sores, wounds, and other skin diseases [41]. *M. charantia* fruit powder, in the form of an ointment (10% w/w dried powder in a simple ointment base), showed a statically significant response in terms of wound healing in rats [42]. In addition, recent studies showed that a *M. charantia* extract improves and accelerates the process of wound healing in rabbits in comparison with conventional creams used therapeutically [43]. It is a surprising fact that this tropical species has been incorporated in the traditional herbal medicine of the study area. In Macedonia we have previously reported the traditional preparation of an ointment made from the fragmented fruits of the plant that are immersed in olive oil and placed in the sun for 30–40 days. This ointment is indicated for the treatment of human and animal wounds [44]. The same use was referred by Turks living in south Kosovo, but in this case internal uses were also cited, including antidiabetic and anticancer [4].

A particular use of *Cuscuta campestris* (Figure 3(b)) was reported as a topic remedy against bee sting. Some of the aromatic plants used for the treatment of gastrointestinal

Table 3: Results of quantitative analysis for the most relevant species (≥ 8 citations). Disease categories with max FL are reported in bold.

	N informants citing	Total N citations	Cardiovascular diseases	Gastrointestinal diseases	Genitourinary diseases	Metabolic diseases	Muscular-skeletal diseases	Nervous-sensorial diseases	Respiratory diseases	Skin diseases	Max FL
Hypericum perforatum L.	47	66		15			4	2	1	**44**	67
Matricaria chamomilla L.	29	34		11	4			**14**	2	3	41
Sideritis scardica Griseb.	30	30							**30**		100
Tilia platyphyllos Scop.	23	27		4				1	**22**		81
Sambucus nigra L.	24	24							**20**	4	83
Rosa canina L.	16	20			1	**17**	2				85
Urtica dioica L.	11	19			5	**8**	4			2	42
Mentha spicata L.	13	18	5	**8**		5					44
Achillea millefolium L.	12	17			**10**				5	2	59
Momordica charantia L.	16	17		1						**16**	94
Malva silvestris L.	14	16		1		1			**14**		88
Crataegus monogyna Jacq.	12	13	**13**								100
Dactylorhiza sp.	13	13							**13**		100
Linum usitatissimum L.	9	13		3					**9**	1	69
Paliurus spina-christi Mill.	11	11			**10**	1					91
Equisetum arvense L.	7	10			**5**		**5**				50
Plantago media L.	9	10			2					**8**	80
Verbascum longifolium Ten.	8	10		2	2				**6**		60
Arctium lappa L.	5	8					**5**			3	63
Micromeria juliana (L.) Rchb.	7	8			**8**						100
Origanum majorana L.	8	8		**7**	1						88
Satureja montana L. *subsp. macedonica* (Formanek) Baden	8	8				1		3	**4**		50

(a) (b) (c) (d)

Figure 3: Plant species used to treat various ailments in the regions of Edessa and Naoussa: (a) *Hypericum perforatum,* (b) *Cuscuta campestris,* (c) *Origanum majorana, and* (d) *Paliurus spina-christi.*

problems are *Origanum majorana* (Figure 3(c)), *Matricaria chamomilla,* and *Mentha spicata*. It is interesting to note that this latter species is included within plant taxa sold as "mint" in the market of Thessaloniki, where it is mostly recommended for common cold and cough [9]. *O. majorana* is widely used to treat colds and rhinitis [45, 46], but it is also quoted for its antiulcer effect [47]. In addition, *Castanea sativa* catkins are widely used as a decoction for the treatment of diarrhea.

3.1.4. Plants Used for Cardiovascular Diseases. Two species were found to be the most quoted (FL = 100%) for the treatment of cardiovascular diseases: *Crataegus monogyna* and *Loranthus europaeus*. *C. monogyna* is popularly known for its cardioprotective action reducing cardiovascular risk factors, such as hypertension and hypercholesterolemia [48, 49]. In Macedonia this species is used as an infusion prepared with flowers, fruits, or leaves, but also as an alcoholic extract prepared from the fruits. *L. europaeus* (yellow-berried mistletoe) is a hemiparasite plant usually found on the branches of trees. The popular use of this plant for the treatment of cardiovascular problems has also been reported in Bosnia and Herzegovina [1]. In addition, a recent study on the ethnobotany of mistletoes species also cited the frequent use of this plant to treat cardiovascular disorders [50].

3.1.5. Plants Used for Nervous Diseases. The use of *Melissa officinalis* and *Valeriana officinalis* to cure nervous system problems is well known [51, 52], while that of *Salvia officinalis* is less common. In the traditional medicine of many European countries this species has been used mainly to treat mild dyspepsia, excessive sweating, and throat and skin inflammations [53], but the use in age-related brain disorders has also been reported [54]. In addition, some informants reported an uncommon use of *Taraxacum officinale* for treating nervous diseases, i.e., headache and insomnia. This use has been previously reported in an ethnobotanical survey of Zagori, Epirus, Greece [6].

3.1.6. Plants Used for Genitourinary Diseases. Different plant parts of a large variety of species are used for genitourinary diseases, as diuretic and in prevention and treatment of prostatitis, mainly in the form of a decoction. Among them, the most quoted are *Micromeria juliana* (8 citations, FL.= 100%), *Paliurus spina-christi* (10 citations, FL= 91%; Figure 3(d)), and *Achillea* spp.

3.1.7. Plants Used for Musculoskeletal Diseases. A large number of species are used for musculoskeletal diseases, in particular against joint pains and rheumatism, such as *Equisetum arvense* (aerial parts), *Arctium lappa* (root), *Cercis siliquastrum* and *Robinia pseudoacacia* (flowers). The alcoholic extract of the sliced root of *Tamus communis* is also employed to treat muscular pain and inflammation. This species shows toxicity due to its content of calcium oxalates, saponins, tannins, and other substances; nevertheless, its anti-inflammatory and analgesic properties are well known [55] and its use in the treatment of rheumatisms has also been reported in the traditional medicine of Turkey [56] and Portugal [57].

3.1.8. Plants Used for Metabolic Diseases. The most quoted plant for the treatment of metabolic diseases is *Rosa canina* (17 citations, FL = 85%), followed by *Urtica dioica* and *Silybum marianum*. In particular, *R. canina* is used as a decoction of fruits and flowers, syrup of boiled fresh fruits but also eaten raw as an immunostimulant, and for the prevention of the cardiovascular diseases.

3.2. Plants with Unusual Medicinal Uses or Used Only by a Single Population Group. Some medicinal uses of plants seem to be particularly interesting because they have been infrequently or never reported. In addition, some of these uses concern toxic plants and rare or endemic species. *Cuscuta campestris* stems (Figure 3(b)) are applied topically against bee stings; Dopioi informants have exclusively reported this use. There are only a few ethnobotanical uses that have been detailed for this species: as purgative and against constipation in Saudi Arabia [58] and for its analgesic effect in the treatment of rheumatisms and headaches in Nepal [59].

In the present study, the use of a toxic species, *Cynoglossum creticum,* which contains pyrrolizidine alkaloids, has been cited [60]. The fruits of this plant have been reported to be eaten to treat a skin disease called *"myrmecia"* (Greek "myrmigkia"= ant), a word used allover Greece for the common skin warts caused by HPV (Human Papilloma Virus). The disease is characterized by the presence of small skin warts apparently similar to anthills. In the traditional medicine of India the aerial portion of *Cynoglossum* spp. is used against wounds, burns, ear infections, and cough for its antibacterial properties [61] and against fungal skin infections [62]. In Sardinia the root is used to prepare an emollient cream against burns [63], while leaves are directly applied as a cicatrizant or as a poultice to treat eczema [64]. In this study only informants from the group "others" have cited this particular use of *C. creticum* for the treatment of *myrmecia*.

Satureja montana subsp. *macedonica*, in addition to the common use of the flower infusion for the treatment of flu and cough, is also used in the study area by Pontians informants to relieve tinnitus and improve hearing. Generally, *Satureja* spp. have been used since ancient times as flavorings for food and for the treatment of various diseases; their essential oils have been documented for antimicrobial, antidiarrheal, fungicidal, and antioxidant activities [65]. However, the specific use for tinnitus treatment and for hearing improvement is particularly unusual and, to our knowledge, has never been previously reported.

Hyssopus officinalis is an aromatic plant commonly used in the traditional medicine of the Balkan Peninsula for its antiseptic, carminative, and spasmolytic properties [66]. The aerial parts of the species are used against chronic bronchitis and asthma [67, 68]. Notably, the use of the decoction from the aerial parts as an analgesic to treat headache, cited in the present study, has never been previously reported.

Helianthus tuberosus is a naturalized species, native to North America, which was introduced in Europe during the XVII century. It was used at first as animal feed and subsequently as human food. Its tuber contains inulin as its main polysaccharide and is therefore indicated in the diet of diabetics. In the Carpathian basin it is also used in the treatment of asthma and heart problems [69]. Although it had not been highly reported in traditional medicine of the Mediterranean region, in the present survey, the decoction of the fragmented tuber was indicated against constipation. Similar uses have been described in Austria [70]. Also in south Kosovo the use of fresh tubers of *H. tuberosum* as human food was recently reported [4].

Finally, *Equisetum arvensis* (10 citations) and *Robinia pseudoacacia* (4 citations) were used by Dopioi only, while *Castanea sativa* (6 citations) by the group "others". Moreover 7 out of 8 citations concerning *Satureja montana* came from Pontians.

3.3. Quantitative Analyses

3.3.1. Traditional Ethnobotanical Knowledge among Different Population Groups. The group "others" (i.e., informants who come from various parts of Greece) and Dopioi reported the highest number of species (53), followed by Pontians (42) and Mikrasiates (23 species). Vlach informants only cited 10 species. Only 7 species (*Crataegus monogyna*, *Hypericum perforatum*, *Matricaria chamomilla*, *Rosa canina*, *Sambucus nigra*, *Sideritis scardica,* and *Tilia platyphyllos*) were commonly reported by all population groups (Figure 4), whereas 30 out of 87 taxa (34%) were exclusively mentioned by a single group: 11 by Dopioi, 8 by Pontians, 7 by the group "others", and 4 by Mikrasiates. The species reported by Mikrasiates and Vlachs were mostly or fully shared with one or more of the groups. Notably, only 12% of taxa were referred with more than one name and only 6% were known with different dialectal names by different population groups (Table 1). It was demonstrated that in different Balkan areas that share similar flora but have different cultural or linguistic heritage, medicinal plants are used in very distinct ways [71–73]. For example, Mustafa et al. [4] found that Albanian, Bosniak/Gorani, and Turkish communities of Kosovan villages shared 22% of the taxa used for food and medicine, suggesting a hybrid character of the Kosovar plant knowledge. However, 42% of the plant species were only cited by a single ethnic group.

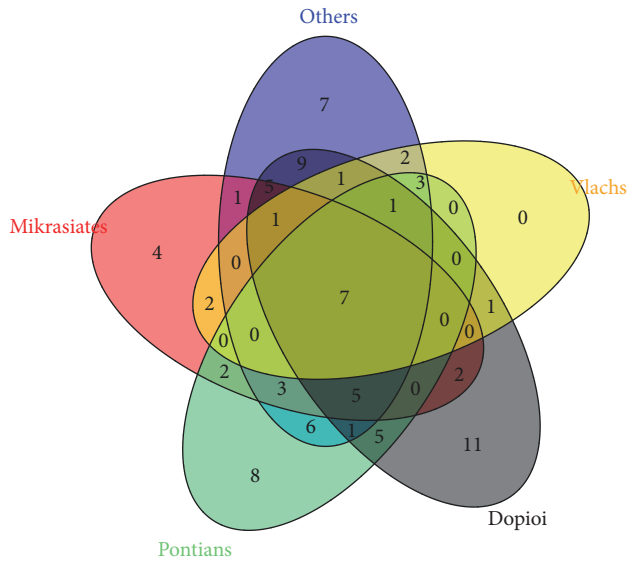


Figure 4: Venn diagram representing an overlap of Traditional Ethnobotanical Knowledge of the 5 population groups. Numbers are the species in common for each combination of overlapping (including nonoverlapping subsets, i.e., species reported by a single population group).

3.3.2. Plant Knowledge according to Sociologic Variables. The diagrams in Figure 5 report the most important remedies-informant groups associations (> 20% of the total information reported for each disease category) observed, as a function of the main social categorizations of informants interviewed in our survey. When analyzing the distribution of disease categories in relation to population groups, some significant differences have been observed (Figure 5(a)). The knowledge of less numerous groups (Vlachs and Mikrasiates) was equally distributed among disease categories, but less relevant with respect to that provided by the other groups. Plant species used against skin and respiratory diseases were highly cited by other informant groups, such as Pontians, Dopioi, and "others". For these latter about 50% of the information is related to these diseases. Dopioi showed important contribution in four remedy categories, being highly prevalent in the citations of treatments for muscular disorders. Similarly, the information delivered by Pontians represented nearly half of the total knowledge about the treatments for gastrointestinal diseases.

Although both male and female informants cited species that are used for different healing purposes, the ethnobotanical knowledge of females was more equally distributed in all use categories, whereas men mainly reported information on respiratory, genitourinary, and skin diseases (Figure 5(b)).

Remedies for respiratory diseases were highly cited by informants, independently from their educational level, whereas the knowledge of medicinal plants used for treating nervous, skin, and gastrointestinal diseases was mainly reported by informants with a secondary level of education. Graduate informants had a major degree of knowledge about the treatment of metabolic disorders (Figure 5(c)).

Independently from their job, many informants reported treatments of respiratory diseases (Figure 5(d)). Moreover, in contrast with other job groups, the information reported by farmers was largely distributed among nearly all disease categories. Unexpectedly, no relevant differences were observed in relation to the age of the informants: both younger and older groups equally cited plant remedies for most of the diseases (data not shown). However, some associations were observed: persons < 40 years old frequently cited remedies for skin diseases, whereas plants used for treating genitourinary disorders were mainly cited by informants between 60 and 70 years old.

4. Conclusions

Our study highlights the importance of reporting the TEK typical of areas of Europe that have until now been poorly investigated. In fact, despite the large number of papers dealing with ethnobotany in the Balkans and southeastern Europe [74, 75], Greece is still scarcely explored from an ethnobotanical point of view although it is characterized by a high floristic diversity. This is particularly true for regions like Central Macedonia where different population groups live together, maintaining most of their traditions. Information on species used in folk medicine of this region showed that most of them were wild and cultivated plants well known in the European ethnobotany for their healthcare and curative properties. Nevertheless some unusual uses were found, in particular concerning several toxic plants and rare or endemic species. Despite growing erosion of existing European tradition ethnobotanical knowledge, population groups in this region maintain some exclusive folk remedies such as the use of *Cuscuta campestris* against bee stings reported only by Dopioi informants and that of *Satureja montana* subsp. *macedonica* cited by Pontians to relieve tinnitus and improve hearing. Such local knowledge is culturally significant and can provide information for developing future

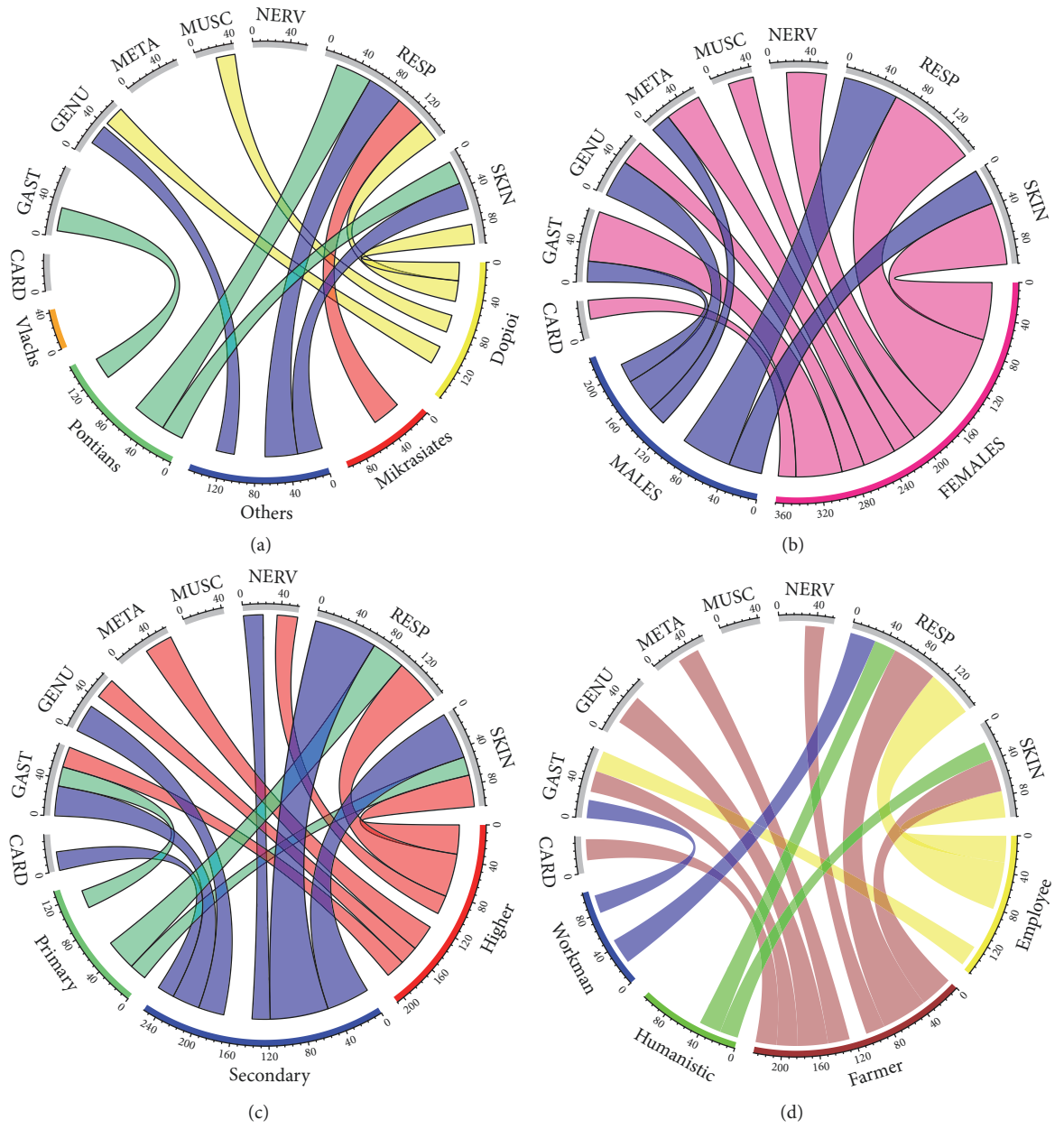


Figure 5: Circular plots showing the relationships among referred disease categories and the informants grouped on the basis of different social factors: (a) origin; (b) gender; (c) education; (d) job. Color bands summarize main relationships (> 20% of the total information) between each disease category and population groups. Numbers below the external segments in the graphs represent the total numbers of citations for each disease category and population group (e.g., in Figure 5(a), 40 citations were overall reported for plants used as a remedy for muscular-skeletal diseases, whereas a total of 132 citations were provided by Dopioi).

researches and promoting ethnopharmacological advances. When taking in account differences among the studied population groups, only 7 plant species were commonly reported by all groups, whereas 34% of plants were exclusively mentioned by a single group. This observation supports the idea that some differences are maintained among population groups, although the knowledge on the medicinal plant use is not accompanied by preservation of linguistic diversity concerning the plant names. Furthermore our analysis of plant knowledge according to sociologic variables contributes to a better understanding of factors that affect changes in plant uses and perceptions in different sociocultural contexts.

Data Availability

The ethnobotanical data used to support the findings of this study have been deposited in the Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, National and Kapodistrian University of Athens, Athens.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Special thanks are due to all informants, who generously shared their knowledge regarding local plants and their uses and especially to Aikaterini Tsitsi for her contribution, help, and passion about traditional medicine. We thank SiFit Lab of Italian Society of Phytotherapy for the partial economic support to this study.

References

[1] B. Šarić-Kundalić, C. Dobeš, V. Klatte-Asselmeyer, and J. Saukel, "Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina," *Journal of Ethnopharmacology*, vol. 131, no. 1, pp. 33–55, 2010.

[2] A. Pieroni and C. L. Quave, *Ethnobotany and Biocultural Diversities in the Balkans: Perspectives on Sustainable Rural Development and Reconciliation*, Springer, 2014.

[3] B. Rexhepi, B. Mustafa, A. Hajdari, J. Rushidi-Rexhepi, C. L. Quave, and A. Pieroni, "Cross-cultural ethnobotany of the Sharr Mountains (Northwestern Macedonia)," in *Ethnobotany and Biocultural Diversities in the Balkans. Perspectives on Sustainable Rural Development and Reconciliation*, pp. 67–86, Springer, New York, NY, USA, 2014.

[4] B. Mustafa, A. Hajdari, A. Pieroni, B. Pulaj, X. Koro, and C. L. Quave, "A cross-cultural comparison of folk plant uses among Albanians, Bosniaks, Gorani and Turks living in south Kosovo," *Journal of Ethnobiology and Ethnomedicine*, vol. 11, no. 1, 2015.

[5] A. Pieroni, "Traditional uses of wild food plants, medicinal plants, and domestic remedies in Albanian, Aromanian and Macedonian villages in South-Eastern Albania," *Journal of Herbal Medicine*, vol. 9, pp. 81–90, 2017.

[6] D. Vokou, K. Katradi, and S. Kokkini, "Ethnobotanical survey of Zagori (Epirus, Greece), a renowned centre of folk medicine in the past," *Journal of Ethnopharmacology*, vol. 39, no. 3, pp. 187–196, 1993.

[7] M. Malamas and M. Marselos, "The tradition of medicinal plants in Zagori, Epirus (northwestern Greece)," *Journal of Ethnopharmacology*, vol. 31, pp. 197–203, 1992.

[8] E. Hanlidou, R. Karousou, V. Kleftoyanni, and S. Kokkini, "The herbal market of Thessaloniki (N Greece) and its relation to the ethnobotanical tradition," *Journal of Ethnopharmacology*, vol. 91, no. 2-3, pp. 281–299, 2004.

[9] R. Karousou, M. Balta, E. Hanlidou, and S. Kokkini, ""Mints", smells and traditional uses in Thessaloniki (Greece) and other Mediterranean countries," *Journal of Ethnopharmacology*, vol. 109, no. 2, pp. 248–257, 2007.

[10] V. Kleftoyanni and S. Kokkini, "The Labiatae plants used traditionally in Thessaloniki," *Bocconea*, vol. 16, pp. 1117–1121, 2003.

[11] M. Skoula, C. Dal Cin D'Agata, and A. Sarpaki, "Contribution to the ethnobotany of Crete, Greece," *Bocconea*, vol. 23, pp. 479–487, 2009.

[12] D. E. Brussell, "Medicinal plants of Mt. Pelion, Greece," *Economic Botany*, vol. 58, pp. S174–S202, 2004.

[13] E. Axiotis, M. Halabalaki, and L. A. Skaltsounis, "An ethnobotanical study of medicinal plants in the greek islands of north aegean region," *Frontiers in Pharmacology*, vol. 9, 2018.

[14] M. V. Sakelariou, Ed., *Macedonia, 4000 Years of Greek History and Civilization*, Ekdotike Athenon, Athens, Greece, 1982.

[15] E. Kontogiorgi, "Slavophone and refugees, social, demographic and ethnological aspects of the Macedonian issue during the interwar period," *Balkanika Symmeikta*, vol. 10, pp. 197–224, 1998 (Greek).

[16] A. Tanner, *The Vlachs - A contested identity. The Forgotten Minorities of Eastern Europe: The History and Today of Selected Ethnic Groups in Five Countries*, East-West Books, 2004.

[17] T. J. Winnifrith, *Badlands - Borderlands. A History of Southern Albania / Northern Epirus*, Duckworth, London, UK, 2002.

[18] T. J. Winnifrith, "The vlachs," in *Minorities in Greece. Aspects of a Plural Society*, R. Clogg, Ed., pp. 112–121, Hurst Publishers, London, UK, 2002.

[19] P. Fann, "Pontic Performance: Minority Theater vs. Greek Ideology," *Journal of Modern Greek Studies*, vol. 9, no. 1, pp. 107–122, 1991.

[20] A. James, "Memories of Anatolia: generating Greek refugee identity," http://journals.openedition.org/balkanologie/720, 2001.

[21] C. Kaltsoudas, *Taking stock of ethnic group identity in a town in Greek Macedonia [Masters thesis]*, Concordia University, 2003.

[22] E. Kirtsoglou and D. Theodossopoulos, "Fading Memories, Flexible Identities: The Rhetoric about the Self and the other in a Community of 'Christian' Refugees from Anatolia," *Journal of Mediterranean Studies*, vol. 11, pp. 395–415, 2001.

[23] M. Zografou, "The politics of dance: The incorporation of the pontic refugees in modern greek culture through the manipulation of dancing practices in a Northern Greek village," *Journal of Mediterranean Studies*, vol. 17, no. 1, pp. 1–21, 2007.

[24] A. Strid, Ed., *Mountain Flora of Greece*, vol. 1, Cambridge University Press, Cambridge, UK, 1986.

[25] A. Strid and K. Tan, Eds., *Mountain Flora of Greece*, vol. 2, Edinburgh University Press, Edinburgh, Scotland, 1991.

[26] U. P. Albuquerque, R. F. P. Lucena, and L. V. F. C. Cunha, "Métodos e Técnicas na Pesquisa Etnobiológica e Etnoecológica," in *Métodos e Técnicas Para Coleta De Dados Etnobiológicos*, U. P. Albuquerque, R. F. P. Lucena, and N. L. Alencar, Eds., pp. 41–64, Nupeea, Recife, Brazil, 2010.

[27] C. Zalios, "The popular musicians and the companies of Naoussa 1870-1970," in *Proceedings of the 20th International Symposium for the Survey of the Dance Athens*, pp. 1–25, 2006.

[28] T. G. Tutin, V. H. Heywood, N. A. Burges et al., *Flora Europaea*, vol. 2-5, Cambridge University Press, Cambridge, UK, 1968-1980.

[29] T. G. Tutin, N. A. Burges, A. O. Chater et al., *Flora Europaea*, vol. 1, Cambridge University Press, Cambridge, UK, 1993.

[30] P. Dimopoulos, T. Raus, E. Bergmeier et al., "Vascular plants of Greece: An annotated checklist. Supplement," *Willdenowia*, vol. 46, no. 3, pp. 301–347, 2016.

[31] C. S. Weckerle, H. J. de Boer, R. K. Puri, T. van Andel, R. W. Bussmann, and M. Leonti, "Recommended standards for conducting and reporting ethnopharmacological field studies," *Journal of Ethnopharmacology*, vol. 210, pp. 125–132, 2018.

[32] R. T. Trotter and M. H. Logan, "Informant consensus: a new approach for identifying potentially effective medicinal plants," in *Plants in Indigenous Medicine and Diet*, L. N. Etkin, Ed., pp.

91–112, Redgrave Publishing Company, Bedford Hill, NY, USA, 1986.

[33] S. Collins, X. Martins, A. Mitchell, A. Teshome, and J. T. Arnason, "Quantitative ethnobotany of two East Timorese cultures," *Economic Botany*, vol. 60, no. 4, pp. 347–361, 2006.

[34] A. G. Singh, A. Kumar, and D. D. Tewari, "An ethnobotanical survey of medicinal plants used in Terai forest of western Nepal," *Journal of Ethnobiology and Ethnomedicine*, vol. 8, article 19, 2012.

[35] J. Friedman, Z. Yaniv, A. Dafni, and D. Palewitch, "A preliminary classification of the healing potential of medicinal plants, based on a rational analysis of an ethnopharmacological field survey among Bedouins in the Negev Desert, Israel," *Journal of Ethnopharmacology*, vol. 16, no. 2-3, pp. 275–287, 1986.

[36] Z. Gu, L. Gu, R. Eils, M. Schlesner, and B. Brors, "Circlize implements and enhances circular visualization in R," *Bioinformatics*, vol. 30, no. 19, pp. 2811-2812, 2014.

[37] H. Chen and P. C. Boutros, "VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R," *BMC Bioinformatics*, vol. 12, 2011.

[38] I. Yaneva and V. Balabanski, "History of the uses of Pirin mountain tea (Sideritis scardica Griseb) in Bulgaria," *Bulgarian Journal of Public Health*, vol. 5, pp. 48–57, 2013.

[39] European Medicines Agency, 1995, http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2016/10/WC500215565.pdf, 2016.

[40] A. Kreziou, H. de Boer, and B. Gravendeel, "Harvesting of salep orchids in north-western Greece continues to threaten natural populations," *Oryx*, vol. 50, no. 3, pp. 393–396, 2016.

[41] D. S. Kumar, K. V. Sharathnath, P. Yogeswaran et al., "A medicinal potency of Momordica charantia," *International Journal of Pharmaceutical Sciences Review and Research*, vol. 1, pp. 95–100, 2010.

[42] J. Sankaranarayanan and C. I. Jolly, "Phytochemical, antibacterial and pharmacological investigations on Momordica charantia Linn. Emblica officinalis gaertn. and Curcuma longa Linn.," *Indian Journal of Pharmaceutical Sciences*, vol. 55, no. 1, pp. 6–13, 1993.

[43] A. Pişkin, B. Z. Altunkaynak, G. Tümentemur, S. Kaplan, Ö. B. Yazici, and M. Hökelek, "The beneficial effects of Momordica charantia (bitter gourd) on wound healing of rabbit skin," *Journal of Dermatological Treatment*, vol. 25, no. 4, pp. 350–357, 2014.

[44] E. E. Tsioutsiou, E. Miraldi, P. Governa, M. Biagi, P. Giordani, and L. Cornara, "Skin wound healing: from mediterranean ethnobotany to evidence based phytotherapy," *Athens Journal of Sciences*, vol. 4, no. 3, pp. 199–212, 2017.

[45] D. Bown, *New Encyclopedia of Herbs and their Uses. The Royal Horticultural Society*, vol. 296, The Royal Horticultural Society, New York, NY, USA, 2002.

[46] G. V. Rao, T. Mukhopadhyay, T. Annamalai, N. Radhakrishnan, and M. R. Sahoo, "Chemical constituents and biological studies of Origanum vulgare Linn," *Pharmacognosy Research*, vol. 3, no. 2, pp. 143–145, 2011.

[47] T. Al-Howiriny, A. Alsheikh, S. Alqasoumi, M. Al-Yahya, K. ElTahir, and S. Rafatullah, "Protective effect of Origanum majorana L. 'Marjoram' on various models of gastric mucosal injury in rats," *American Journal of Chinese Medicine*, vol. 37, no. 3, pp. 531–545, 2009.

[48] W.-T. Chang, J. Dao, and Z.-H. Shao, "Hawthorn: Potential roles in cardiovascular disease," *American Journal of Chinese Medicine*, vol. 33, no. 1, pp. 1–10, 2005.

[49] S. F. Nabavi, S. Habtemariam, T. Ahmed et al., "Polyphenolic composition of Crataegus monogyna jacq.: From chemistry to medical applications," *Nutrients*, vol. 7, no. 9, pp. 7708–7728, 2015.

[50] A. C. Guimarães, C. D. J. Silva, and A. C. Siani, "Ethnobotany of mistletoes species (families Loranthaceae and Viscaceae)," *Revista de Fitoterapia*, vol. 13, no. 2, pp. 137–152, 2013.

[51] V. López, S. Martín, M. P. Gómez-Serranillos, M. E. Carretero, A. K. Jäger, and M. I. Calvo, "Neuroprotective and neurological properties of *Melissa officinalis*," *Neurochemical Research*, vol. 34, no. 11, pp. 1955–1961, 2009.

[52] P. Morazzoni and E. Bombardelli, "Valeriana officinalis: Traditional use and recent evaluation of activity," *Fitoterapia*, vol. 66, no. 2, pp. 99–112, 1995.

[53] A. Ghorbani and M. Esmaeilizadeh, "Pharmacological properties of Salvia officinalis and its components," *Journal of Traditional and Complementary Medicine*, vol. 7, no. 4, pp. 433–440, 2017.

[54] M. Adams, F. Gmünder, and M. Hamburger, "Plants traditionally used in age related brain disorders—a survey of ethnobotanical literature," *Journal of Ethnopharmacology*, vol. 113, no. 3, pp. 363–381, 2007.

[55] F. Capasso, N. Mascolo, G. Autore, F. de Simone, and F. Senatore, "Anti-inflammatory and analgesic activity in alcoholic extract of Tamus communis L.," *Journal of Ethnopharmacology*, vol. 8, no. 3, pp. 321–325, 1983.

[56] E. Yeşilada, G. Honda, E. Sezik et al., "Traditional medicine in Turkey. V. Folk medicine in the inner Taurus Mountains," *Journal of Ethnopharmacology*, vol. 46, no. 3, pp. 133–152, 1995.

[57] M. Rafael, L. Barros, A. M. Carvalho, and I. C. F. R. Ferreira, "Topical anti-inflammatory plant species: Bioactivity of Bryonia dioica, Tamus communis and Lonicera periclymenum fruits," *Industrial Crops and Products*, vol. 34, no. 3, pp. 1447–1454, 2011.

[58] S. A. Youssef Randa, "Medicinal and non-medicinal uses of some plants found in the middle region of Saudi Arabia," *Journal of Medicinal Plants Research*, vol. 7, pp. 2501–2517, 2013.

[59] M. Siwakoti and S. Siwakoti, *Ethnobotanical studies of Satars of Jhapa District, Nepal: A Case Study of Haldibari VDC*, University Grants Commission, Kathmandu, Nepal, 1996.

[60] A. El-Shazly, T. Sarg, L. Witte, and M. Wink, "Pyrrolizidine alkaloids from Cynoglossum creticum," *Phytochemistry*, vol. 42, no. 4, pp. 1217–1221, 1996.

[61] K. Joshi, "Cynoglossum L.: a review on phytochemistry and chemotherapeutic potential," *Journal of Pharmacognosy and Phytochemistry*, vol. 5, pp. 32–39, 2016.

[62] T. F. Xavier, M. Kannan, and A. Auxilia, "Traditional Medicinal Plants Used in the treatment of different skin diseases," *International Journal of Current Microbiology and Applied Sciences*, vol. 4, pp. 1043–1053, 2015.

[63] M. C. Loi, L. Maxia, and A. Maxia, "Ethnobotanical comparison between the villages of Escolca and Lotzorai (Sardinia, Italy)," *Journal of Herbs, Spices & Medicinal Plants*, vol. 11, no. 3, pp. 67–84, 2005.

[64] C. Sanna, M. Ballero, and A. Maxia, "Le piante medicinali utilizzate contro le patologie epidermiche in Ogliastra (Sardegna centro-orientale)," *Atti della Società Toscana di Scienze Naturali Memorie Serie B*, vol. 113, pp. 73–82, 2006.

[65] E. Sarrou, P. Chatzopoulou, E. Lalidou, A. Koutsogianni, and S. Katsiotis, Essential oil composition and antioxidant activity of some medicinal plants used traditionally as herbal teas in N. Greece. Conference: 8th CMAPSEEC, At Durres, Albania, 2014.

[66] N. Stanković, T. Mihajilov-Krstev, B. Zlatković et al., "Antibacterial and antioxidant activity of traditional medicinal plants from the balkan peninsula," *NJAS - Wageningen Journal of Life Sciences*, vol. 78, pp. 21–28, 2016.

[67] S. Tasić, K. Šavikin-Fodulović, and N. Menković, *Guide through the World of Medicinal Plants*, World Agency Valjevac, Valjevo, Serbia, 2004.

[68] M. Mahboubi, G. Haghi, and N. Kazempour, "Antimicrobial activity and chemical composition of *hyssopus officinalis* L. essential oil," *Journal of Biologically Active Products from Nature*, vol. 1, no. 2, pp. 132–137, 2011.

[69] R. Filep, L. Balogh, V. L. Balázs et al., "Helianthus tuberosus L. agg. in the Carpathian Basin: a blessing or a curse," *Genetic Resources and Crop Evolution*, vol. 64, pp. 1–15, 2007.

[70] G. Bärwald, *Gesund Abnehmen Mit Topinambur*, Trias, Stuttgart, Germany, 2008.

[71] A. Pieroni, B. Rexhepi, A. Nedelcheva et al., "One century later: the folk botanical knowledge of the last remaining Albanians of the upper Reka Valley, Mount Korab, Western Macedonia," *Journal of Ethnobiology and Ethnomedicine*, vol. 9, no. 1, article 22, 2013.

[72] A. Pieroni, K. Cianfaglione, A. Nedelcheva, A. Hajdari, B. Mustafa, and C. L. Quave, "Resilience at the border: Traditional botanical knowledge among Macedonians and Albanians living in Gollobordo, Eastern Albania," *Journal of Ethnobiology and Ethnomedicine*, vol. 31, p. 10, 2014.

[73] A. Pieroni, A. Ibraliu, A. M. Abbasi, and V. Papajani-Toska, "An ethnobotanical study among Albanians and Aromanians living in the Rraice and Mokra areas of Eastern Albania," *Genetic Resources and Crop Evolution*, vol. 62, no. 4, pp. 477–500, 2015.

[74] C. L. Quave, M. Pardo-de-Santayana, and A. Pieroni, "Medical ethnobotany in Europe: from field ethnography to a more culturally sensitive evidence-based cam?" *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 156846, 17 pages, 2012.

[75] M. Pardo-de-Santayana, C. L. Quave, R. Sõukand, and A. Pieroni, "Medical ethnobotany and ethnopharmacology of Europe," in *Ethnopharmacolog*, M. Heinrich and A. K. Jäger, Eds., pp. 343–355, Wiley, 2015.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 5370473, 13 pages
https://doi.org/10.1155/2019/5370473

Research Article

The Effects of *Cordyceps sinensis* (Berk.) Sacc. and *Gymnema inodorum* (Lour.) Decne. Extracts on Adipogenesis and Lipase Activity *In Vitro*

Kanokwan Tiamyom, Kittipot Sirichaiwetchakoon, Tanaporn Hengpratom, Sajeera Kupittayanant, Rungrudee Srisawat, Atcharaporn Thaeomor, and Griangsak Eumkeb

School of Preclinic, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 3000, Thailand

Correspondence should be addressed to Griangsak Eumkeb; griang@sut.ac.th

Received 19 January 2019; Revised 9 February 2019; Accepted 17 March 2019; Published 1 April 2019

Academic Editor: Lucindo Q. Júnior

This study aimed to investigate the effects of *Cordyceps sinensis* extract (CSE) and *Gymnema inodorum* extract (GIE), used alone and combined, on antiadipogenesis in 3T3-L1 cells. Oil Red O staining was used to examine the effects of these extracts on inhibition of intracellular lipid accumulation in 3T3-L1 adipocytes and on lipid droplet morphology. Fourier transform-infrared (FTIR) microspectroscopy was used to examine biomolecular changes in 3T3-L1 adipocytes. The pancreatic lipase assay was used to evaluate the inhibitory effects of CSE and GIE on pancreatic lipase activity. Taken together, the results indicated that CSE, GIE, and their combination suppressed lipid accumulation. The FTIR microspectroscopy results indicated that CSE, GIE, and their combination had inhibitory effects on lipid accumulation in the adipocytes. Compared with the untreated adipocytes, the signal intensity and integrated areas of glycogen and other carbohydrates, the acyl chain of phospholipids, and the lipid/protein ratios of the CSE, GIE, alone, and combined treated adipocytes were significantly lower ($p < 0.05$). Combination treatment resulted in a synergistic effect on lipid accumulation reduction in the adipocytes. Principal component analysis of the biomolecular changes revealed six distinct clusters in the FTIR spectra of the sample cells. The pancreatic lipase assay results indicated that CSE and GIE inhibited the pancreatic lipase activity in a dose-dependent manner (mean ± standard error of the mean IC_{50} values, 2312.44 ± 176.55 μg mL^{-1} and 982.24 ± 44.40 μg mL^{-1}, resp.). Our findings indicated that FTIR microspectroscopy has potential application for evaluation of the effectiveness of medicinal plants and for the development of infrared biochemical obesity markers useful for treating patients with obesity. These results suggested that use of CSE and GIE alone and in combination may be efficacious as a complementary therapy for hyperlipidemia and obesity management. However, clinical trials in animals and humans must first be completed.

1. Introduction

The incidence of obesity has been increasing steadily in developed and developing countries worldwide. Analysis of the global burden of obesity revealed that there were 396 million adults with obesity in 2005 and that the expected number is projected to be 573 million individuals in 2030, without adjusting for secular trends [1]. Excessive fat accumulation that increases the risk of adverse health effects is one definition of obesity. Obesity is implicated as a risk factor for various diseases (e.g., hypertension, coronary heart disease, and type II diabetes) [2, 3]. Despite the unavoidable progression of this disease and the positive effects of some medications on body weight reduction and alleviation of numerous cardiometabolic complications, large numbers of approved and wholesaled antiobesity drugs have been withdrawn due to serious adverse effects [4]. Phytochemicals present in plants traditionally used for medicinal purposes have the potential for use as newer therapeutics for obesity and other metabolic diseases [5].

Combinations of phytochemicals often occur naturally and using them in combination may significantly improve bioactivity. *Cordyceps sinensis* and *Gymnema inodorum* extracts are used as traditional food and medicine in Asia; they have received intense attention by a researcher in recent years [6–8]. Triterpenoids in *G. inodorum* leaves have an inhibitory effect on glucose absorption from the intestinal tract that relies on CH_2OH production [7]. The potential hypoglycemic and renoprotective effects of *C. sinensis* solid-state fermented mycelia extract were examined in patients with type 2 diabetes mellitus [9]. Cordycepin inhibits adipocyte differentiation and accumulation of lipid in mature adipocytes. As cordycepin blocks both adipocyte differentiation and lipid accumulation, it has the potential to be an effective therapeutic agent for obesity and obesity-related disorders [10].

Synergy research has found that standardized phytodrugs have therapeutic equivalence to the standard drugs. Compared with synthetic drugs, they also have fewer or no side effects [11]. However, the synergistic effects of *C. sinensis* extract (CSE) plus *G. inodorum* extract (GIE) on antiadipogenesis in 3T3-L1 adipocytes have not been investigated. The objective of this study was to examine the synergistic effects of CSE plus GIE on antiadipogenesis in 3T3-L1 cells.

2. Materials and Methods

2.1. Reagents and Cells. All chemicals were of the highest quality available (e.g., analytical grade). Bovine calf serum and 3T3-L1 mouse embryonic fibroblasts and were purchased from the American Type Culture Collection (ATCC, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide (MTT), and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid were obtained from Gibco Invitrogen (Grand Island, NY, USA). Insulin solution (bovine), 3-isobutyl-1-methylxanthine (IBMX), 4-nitrophenyl dodecanoate (pNP laurate), porcine pancreas lipase, and simvastatin (SIM) were obtained from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO) was purchased from Carlo Erba Reagents S.r.l. (Chaussée du Vexin, Val de Reuil, USA). Dexamethasone was obtained from G Bioscience (St. Louis, USA). Oil Red O was purchased from Amresco Inc. (Solon, OH, USA).

2.2. Preparation of Plant Extracts. The *C. sinensis* extract was obtained from the Cordythai Company, Ltd., Thailand. The identity of the specimen was authenticated by a microbiologist at Kasetsart University (Bangkok, Thailand). Leaves of *G. inodorum* were collected from the Chiangda organic company garden (Chiang Mai, Thailand). These plant specimens were authenticated by Dr. Santi Wattana, a lecturer and plant biologist at the Institute of Science, Suranaree University of Technology. A specimen was deposited in the botanical garden, Suranaree University of Technology.

A 250-g dried powder of *G. inodorum* was soaked in 750 mL 95% ethanol at room temperature (25 ± 1°C) for 7 days with occasional stirring. After 7 days, the ethanol extract was filtered (Whatman no. 1 filter paper) and concentrated using a rotary evaporator at reduced pressure and <50°C. The concentrated extract was collected in a petri dish and air-dried to the point of complete evaporation of the ethanol. The blackish-green semisolid extract was kept at -20°C until use [12].

2.3. Preliminary Qualitative Phytochemical Screening Tests. The preliminary qualitative phytochemical screening analysis was performed as previously described, with some modification [13–15]. The chemical compounds of extract constituents (i.e., tannins, saponins, alkaloids, flavonoids, steroids, and glycosides) were tested.

2.4. Quantitative Estimation of Chemical Constituents

2.4.1. Total Phenolic Content. The Folin–Ciocalteu assay was used to investigate the total phenolic content as previously described by Singleton et al. [16] and Rupasinghe et al. [17], with little modification. Briefly, 100 μL 0.2 M Folin–Ciocalteu, 20 μL CSE or GIE, and various concentrations (0.0025, 0.005, 0.0075, 0.01, 0.015, 0.02, and 0.0625 mg mL^{-1} in 100% methanol) of gallic acid were added to each well of 96-well plates. Then 80 μL 7.5% (w/v) sodium carbonate was added to each well and the samples were incubated for 2 h at room temperature. The absorbance of the resulting blue color solution was measured using a spectrophotometer (wavelength, 765 nm). A gallic acid standard curve was used to determine the total phenolic content. The results were expressed as mg gallic acid equivalents (mg GAE/g) per g of dry weight.

2.4.2. Total Flavonoid Content. The total flavonoid content was measured using the aluminium chloride colorimetric assay following Chen et al.'s and Settharaksa et al.'s methods [18, 19], with little modification. Briefly, 125 μL distilled water, 25 μL standard catechin at various concentrations (0.025, 0.05, 0.1, 0.2, 0.3, and 0.4 mg mL^{-1}) or CSE or GIE, and 10 μL 5% $NaNO_2$ were mixed in each well of 96-well plates. The samples were then incubated for 6 min at room temperature and 15 μL 10% $AlCl_3$ solution was added to each well. The plates were incubated for 5 min at room temperature and then 50 μL 1 M NaOH was added. Each plate was shaken in a microplate reader spectrophotometer for 5 min before measuring absorbance (wavelength, 595 nm). A catechin standard curve was used to determine the total flavonoid content. The results were presented as mg catechin equivalents (mg CE/g) per gram of dry weight.

2.5. Cell Culture. Phitaktim et al.'s methods with some modifications were used for 3T3-L1 preadipocyte culture and differentiation [20]. The culture medium was high-glucose DMEM supplemented with 10% bovine calf serum, 1.5 mg mL^{-1} sodium bicarbonate, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin; the cells were grown until being confluent. The incubation conditions were 37°C, 5% CO_2, and 95% humidity. At 2 days after confluence was observed

(day 0), the preadipocytes were induced to differentiate into adipocytes using 48-h culture in a differentiation medium containing 10% FBS, 1.0 mM dexamethasone, 0.5 mM IBMX, and 1.0 mg mL^{-1} insulin in DMEM. At 48 h (i.e., day 2), the differentiation medium was changed to a maintenance medium (10% FBS and 1.0 mg mL^{-1} insulin in DMEM) for another 48-h culture period (day 4). Every 48 h until day 10, the medium was replaced with newly-prepared maintenance medium. The preliminary concentration interval testing of CSE and GIE alone and combined to determine that the extracts were not toxic (i.e., not significantly different from the control) was then performed. This testing found that the dosage intervals of CSE at 10–40 μg mL^{-1}, GIE at 500–2000 μg mL^{-1}, and CSE plus GIE (CSE/GIE) at 10 + 500 μg mL^{-1} were the correct intervals for the experiments. The SIM dosage was 1.67 μg mL^{-1}, which was the dosage used by Sirichaiwetchakoon et al. [21]. SIM was used as a positive control. During testing of preadipocyte differentiation, the cells were treated with the various obtained concentrations of CSE, GIE, and SIM alone, and with the CSE/GIE combination, for 48-h differentiation phase periods (i.e., at days 0, 2, 4, 6, and 8). The rates of differentiation into adipocytes were calculated on day 10.

2.6. In Vitro Cytotoxic Test (MTT Assay). A tetrazolium dye (MTT) colorimetric assay was used to examine the cytotoxic effects of exposure to CSE, GIE, SIM alone, and CSE/GIE combination on cell proliferation [22]. The 96-well plates were seeded at a density of 5×10^3 cells/well. Cell adherence was then allowed to progress for 48 h. The cells were then subjected to a 48-h exposure of various concentrations of four compounds. The medium was then removed, and the final concentration of 0.5 mg mL^{-1} MTT was added to each well. The cells were then incubated for 4 h at 37°C. DMSO was then used to dissolve the formazan crystals formed by viable cells and a microplate spectrophotometer (Bio-Rad Laboratories, Inc., USA) was used to measure the absorbance (wavelength, 540 nm). Linear regression analysis and a dose-response curve were used to calculate the extract concentration resulting in the death of 50% of the test cells (i.e., 50% lethal concentration (LC50)).

2.7. Oil Red O and Hematoxylin Staining. On day 10, Jarinyaporn et al.'s and Sirichaiwetchakoon et al.'s [21, 23] methods (including Oil Red O staining), with some modifications, were used to determine the rates of differentiation of preadipocytes into adipocytes that were associated with the amount of lipid accumulation. Briefly, adipogenesis of the 3T3-L1 preadipocytes was first induced using adipogenic medium; they were treated with concentrations similar to the established safe MTT concentration intervals of CSE at 10–40 μg mL^{-1}, GIE at 500–2000 μg mL^{-1}, SIM at 1.67 μg mL^{-1}, and CSE/GIE at 10 + 500 μg mL^{-1} for 48 h. The differentiation medium was then changed to maintenance medium that included the treatment agents; this change in maintenance medium was then performed every 48 h until day 10. PBS was then used to wash the cells (twice) and then they were fixed for 1 h using formaldehyde (10%) in PBS. Distilled water was then used to wash the cells (twice) and they were stained using a 5% Oil Red O solution in 60:40 (v/v) isopropanol:distilled water at room temperature for 30 min. The Oil Red O-stained cells were then washed with distilled water (twice) and stained with hematoxylin solution at room temperature for 10 min. An inverted fluorescence microscope (Olympus Corporation, Japan) was used to examine the stained cells. Quantification of lipid accumulation was performed after washing the Oil Red O-stained triglyceride droplets with 60% isopropanol (twice), eluting with 100% isopropanol and transferring to a new 96-well plate. A microplate spectrophotometer was used to measure absorbance (wavelength, 490 nm). The equation $(OD_{sample} - OD_{blank})/(OD_{positive\ control} - OD_{blank}) \times 100$ was used to calculate the differentiation rate (%).

The fractional effective concentration (FEC) index was calculated for each combination to measure the interaction between two agents. The FEC for each agent was calculated by dividing the concentration of the compound present in that treated group in combination where the effective concentration (EC) of the treated group showed equal or higher (or better) effect than a negative control group and those compounds treated alone. The FEC index (FECI) was calculated using the following: FEC of A compound = EC of A in combination/EC of A alone; FEC of B compound = EC of B in combination/EC of B alone; hence, FECI = FEC of A compound + FEC of B compound. When the FECI of the combination was <1.0, a combination was designated as synergistic. An FECI = 1.0 indicated "no interaction or zero-interaction" between the agents, and a value >1.0 indicated antagonism between the two compounds [24, 25].

2.8. Pancreatic Lipase Assay. The pancreatic lipase assay was used to investigate the effects of CSE and GIE on the reduction of fat digestion and absorption. Guo et al.'s and Hengpratom et al.'s [26, 27] methods, with slight modifications, were used to measure lipase activity. The supernatant of 5 mg mL^{-1} porcine pancreas lipase type 2 in distilled water was used for the assay. The reaction buffer consisted of 0.1% (w/v) pNP laurate buffer (100 mM Tris buffer pH 8.2) and 5 mM sodium acetate (pH 5.0) containing 1% Triton X-100. The buffer was heated in boiling water for 2 min and then cooled to room temperature. Ranges in preliminary concentrations of CSE, GIE were then tested to determine the concentrations appropriate for the calculation of the IC_{50} for lipase inhibitory activity. The concentration intervals of CSE at 1000–1600 μg mL^{-1} and GIE at 250–1000 μg mL^{-1} were obtained, which were almost similar to the ranges for Oil Red O staining. Fifty percent DMSO in reaction buffer was used to dissolve each test sample. The sample (20 μL) and lipases (30 mL) were added to 40 mL reaction buffer; a substrate solution (30 mL) was used to initiate the reaction. The negative control contained 50% DMSO instead of the sample. Each mixture was incubated at 37°C for 2 h. A microplate spectrophotometer (wavelength, 405 nm) was used to measure absorbance. The inhibition rate (%) was calculated as $(1 - (OD_{sample} - OD_{sample\ blank})/OD_{negative\ control}) \times 100$.

2.9. Focal Plane Array-FTIR Microspectroscopy. FTIR measurement and Siriwong et al.'s method with some modifications were used to evaluate the effects of CSE, GIE, and the CSE/GIE combination on the adipocytes [28]. The lowest concentrations of CSE (10 μg mL^{-1}) and GIE (500 μg mL^{-1}) used alone, and CSE/GIE (10 + 500 μg mL^{-1}) in combination, which the MTT assay revealed to be nontoxic and the Oil Red O staining indicated to have synergistic activity, were used to investigate biomolecular changes in the adipocytes (focal plane array-FTIR microspectroscopy). A positive control using SIM (1.67 μg mL^{-1}) at the same concentration as the MTT and Oil Red O assays was also tested. Preadipocytes, untreated adipocytes, and adipocytes treated using these agents at the above concentrations on day 10 were collected and centrifuged (4000 × g, 5 min). The cells were then washed with 0.85% NaCl (once) and recentrifuged (4000 × g, 5 min). The cell pellets were dropped onto a barium fluoride optical window 13 mm Ø × 2 mm (Crystran, Ltd.). Excess water was removed using air drying. The dried cells were kept in a desiccator until FTIR analysis. For the FTIR microspectroscopy, each sample was evaluated in triplicate. Staff at a spectroscopy facility (Synchrotron Light Research Institute, Public Organization, Thailand) recorded the FTIR spectra using a Bruker Tensor 27 spectrometer (Globar source) coupled with a Bruker Hyperion 3000 microscope (Bruker Optics Inc., Ettlingen, Germany). The microscope was equipped with a 64 × 64 element mercury cadmium telluride, focal plane array detector, which allowed simultaneous acquisition of the spectral data. The FTIR samples were examined using the 15× objective, and the results of 64 scans were recorded in transmission mode at a 4 cm^{-1} spectral resolution. An 8 × 8 binning FTIR image mosaic was constructed from the images. A spectral range of 4000–700 cm^{-1} was used to acquire the absorbance spectra; the single spectra were acquired from approximately 20 μm × 20 μm sample areas. OPUS 7.2 software (Bruker Optics Ltd., Ettlingen, Germany) was used to obtain the FTIR spectral data and to control the instrument system. Microcal TM origin 6.0 software (Microcal Software, Inc., Northampton, USA) was used to plot the mean values for the spectral data as a stacked view. Principal component analysis (PCA) was used to identify sample spectra variability and clustering (Unscrambler X 10.1 software, CAMO Software AS, Oslo, Norway). During spectral preprocessing, second derivative transformations were obtained using Savitzky-Golay algorithms (nine smoothing points) and were normalized using an extended multiplicative signal correction (spectral regions, 3000–2800 cm^{-1} and 1800–950 cm^{-1}). This method was used to identify regions of the absorption peak overlap, to reduce variation between replicate spectra and to correct for baseline shift. Score plots (2D) and loading plots were used to represent the different data classes and the associations between data set variables, respectively. The samples' signal intensities and integrated peak areas were analyzed using OPUS 7.2 software (Bruker). The results for the signal strengths and integrated peak areas of the lipids (3000–2800 cm^{-1}), nucleic acids, and C-O vibrations from glycogen and other carbohydrates (1300–950 cm^{-1}) were illustrated using a histogram.

2.10. Statistical Analysis. All results were expressed as a mean ± standard error of the mean (SEM) values. The statistically significant differences between treatment and control groups for cell viability, amounts of lipid accumulation, and biomolecular changes were analyzed using one-way analysis of variance and Tukey's honestly significant difference post-hoc test. Student's t-tests were used to determine statistically significant between-group differences in lipase inhibition activity. The results were considered statistically significant when $p < 0.05$ and were representative of at least three independent experiments [26, 29].

3. Results

3.1. Preliminary Phytochemical Analysis. Each lyophilized ethanol extract of *G. inodorum* was weighed to calculate the percentage yield of the extract. The percentage of yield obtained was 17.43% (w/w). The results of the preliminary qualitative phytochemical screening tests of *C. sinensis* and *G. inodorum* indicated that they contained flavonoids. *C. sinensis* contained alkaloids, tannins, and saponin and *G. inodorum* contained terpenoids and glycoside. Steroids were not found in extracts from either plant (Table 1). The total mean flavonoid content of *C. sinensis* and *G. inodorum* was 8.79 ± 2.46 and 4.99 ± 0.63 mg CE/g of dry weight, respectively. The total phenolic content of *C. sinensis* and *G. inodorum* was 0.14 ± 0.01 and 0.81 ± 0.01 mg GAE/g of dry weight, respectively.

3.2. 3T3-L1 Preadipocyte Viability Assay. The effects of CSE and GIE on preadipocyte viability were dose dependent (Figure 1). The LC_{50} values for the CSE and GIE antiproliferative effects were 112.23 ± 0.49 μg mL^{-1} and 3075.73 ± 274.24 μg mL^{-1}, respectively. Assessment of the viability of the 3T3-L1 cells revealed that the CSE-treated group was not significantly different from the untreated group of preadipocytes. The GIE (500–1000) and CSE/GIE combination resulted in significantly greater cell viability than the untreated preadipocyte groups ($p < 0.05$). The nontoxic concentrations of CSE, GIE, and the CSE/GIE combination were chosen for further investigation using Oil Red O staining.

3.3. Effects of CSE, GIE, and the CSE/GIE Combination on Lipid Accumulation during Adipogenesis. The effects of CSE, GIE, and the CSE/GIE combination on lipid accumulation during 10 days of preadipocyte to adipocyte differentiation were examined. The results for microscopic examination of the Oil Red O and hematoxylin-stained cells indicated that exposure to CSE and GIE resulted in decreased numbers of Oil Red O-stained droplets in mature adipocytes at 10 and 500 μg mL^{-1}, respectively (Figure 2). The adipocyte intracellular lipid accumulation for the treated groups of CSE and GIE alone and combined was significantly decreased compared with untreated adipocytes ($p < 0.05$, Figure 3). The interaction of CSE (10) plus GIE (500) was compared to treating with

Table 1: Preliminary qualitative phytochemical screening of *C. sinensis* and *G. inodorum* extracts.

Phytochemical compounds	*Cordyceps sinensis*	*Gymnema inodorum*
Alkaloids	+	-
Flavonoids	+	+
Tannins	+	-
Terpenoids	-	+
Saponin	+	-
Glycoside	-	+
Steroids	-	-

(+) = presence; (-) = absence

Table 2: Fourier transform-infrared band assignments.

Second derivative spectra band position (cm^{-1})	Band assignments
2964	CH_3 asymmetric stretch associated with membrane phospholipid methyl terminals: mainly lipid
2923	CH_2 asymmetric stretch associated with membrane phospholipid methylene groups: mainly lipids, some contribution from proteins, carbohydrates, and nucleic acids
2854	CH_2 symmetric stretch: mainly lipids, some contribution from proteins, carbohydrates, and nucleic acids
1735	C=O stretching vibration: lipids (triglycerides and cholesterol esters)
1650	Amide I: C=O (80%) and C–N (10%) stretching, N–H (10%) bending vibration: α-helix proteins
1542	Amide II: N–H (60%) bending and C–N (40%) stretching vibration: α-helix proteins
1457	CH_2 bending vibration: lipids and proteins
1399	COO-symmetric stretching and CH_3 bending vibration: lipids, proteins
1234	PO_2-asymmetric stretching vibration: RNA, DNA, and phospholipids
1084	PO_2-symmetric stretching vibration: RNA, DNA
1157	C–O vibration: glycogen and other carbohydrates
1045	C–O vibration: glycogen and other carbohydrates

only CSE or GIE. The EC for CSE (30) alone = 30 and the EC for CSE (10) in combination = 10. Therefore, the FEC for the CSE compound = 0.33. The EC for GIE (2000) alone = 2000 and the EC for GIE (500) in combination = 500. Therefore, the FEC for the GIE compound = 0.25. Thus, the FECI of the combination was 0.58 (Figure 3). These results suggested that the combination had a synergistic effect on the reduction in intracellular lipid accumulation. Intracellular fat deposit formation for SIM exposure at 1.67 μg mL^{-1} was significantly reduced by 54 ± 1.37%, compared with the untreated adipocytes ($p < 0.05$) (Figure 3).

3.4. Effects of CSE and GIE on Pancreatic Lipase Activity. Pancreatic lipase assays were performed to investigate the effects of CSE and GIE on the reduction of fat digestion and absorption. The results indicated that CSE and GIE inhibited lipase activity in a dose-dependent manner (Figure 4). Pancreatic lipase activity was also inhibited by CSE and GIE. The half-maximal inhibitory concentration (IC_{50}) values were 2312.44 ± 176.55 μg mL^{-1} and 982.24 ± 44.40 μg mL^{-1}, respectively.

3.5. FTIR Microspectroscopy. The FTIR microspectroscopy technique was used to identify biomolecular changes in groups of preadipocytes that were untreated, exposed to 1.67 μg mL^{-1} SIM, 10 μg mL^{-1} CSE, 500 μg mL^{-1} GIE, or CSE and GIE combined (CSE (10) + GIE (500)) when they were adipocytes on day 10 after differentiation (day 0). Representative FTIR spectra for samples (wavelengths, 3000–950 cm^{-1}) are presented in Figure 5. The cells' infrared spectrum profiles for the different treatments were examined for three regions: (1) lipids (3000–2800 cm^{-1}), (2) proteins (1700–1500 cm^{-1}), and (3) carbohydrates and nucleic acids (1300–950 cm^{-1}). Detection of between-cell type differences for the spectra was difficult to achieve using an examination of only the raw spectra. Therefore, they were used for a second derivative analysis (range: 3000–2800 cm^{-1} and 1800–950 cm^{-1}) that more clearly and precisely revealed the bands' peak positions (Figures 6(a) and 6(b), resp.). The results for the IR spectra band assignments for the samples are presented in Table 2. They indicated that the spectral differences were mostly in the lipid region (3000–2800 cm^{-1}). Strong peaks at 2923 cm^{-1} and 2854 cm^{-1} correspond to CH_2 asymmetry and symmetric stretching frequency, respectively; both of these were mostly associated with lipids, with some contribution from proteins, carbohydrates, and nucleic acids [30]. The signal intensity increases and increases in the areas of the peaks at 2923 cm^{-1} and 2854 cm^{-1} indicated lipoprotein

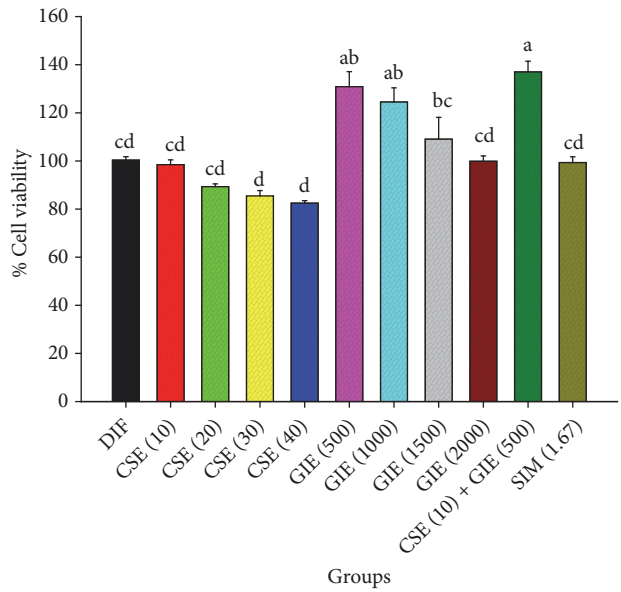


FIGURE 1: Effects of *C. sinensis* extract (CSE) and *G. inodorum* extract (GIE) used alone and combined on 3T3-L1 preadipocyte viability. DIF = differentiated adipocytes; CSE (10) = CSE at 10 μg mL^{-1}; GIE (500) = GIE at 500 μg mL^{-1}; CSE (10) + GIE (500) = CSE at 10 μg mL^{-1} plus GIE at 500 μg mL^{-1}. SIM (1.67) = simvastatin at 1.67 μg mL^{-1}. Each value is the mean ± SEM of three (replicate) experiments and is expressed as population growth. The results are expressed as mean ± SEM values. Mean values followed by the same superscript letter were not significantly different ($p < 0.05$, Tukey's test).

fractions and the small contributions from carbohydrates and nucleic acids of untreated adipocytes; they were greater than the other peaks (Figure 6(a)) [31–33]. The lipid ester C=O stretching at 1735 cm^{-1} for untreated cells had greater signal intensities and band areas than the other groups (Figure 6(b)). The peak signal intensities and areas for the preadipocytes and the SIM, CSE, and CSE/GIE combination-treated adipocytes at 1157 cm^{-1} indicated absorption peaks for C–O vibrations due to glycogen and other carbohydrates [34]; they were smaller than those for the untreated adipocytes (Figure 6(b)). The signal intensities and areas of the peaks for the preadipocytes and the SIM, CSE, and GIE and CSE/GIE combination-treated groups at 1650 cm^{-1}, 1542 cm^{-1}, and 1234 cm^{-1} indicated absorption peaks for protein amide I alpha-helix (centered at 1650 cm^{-1}), amide II (centered at 1542 cm^{-1}), and the functional group of the PO_2 stretching mode, respectively. The changes of those treated groups were mostly due to changes in nucleic acids, with some contribution from phospholipids (at 1234 cm^{-1}) and were greater than the untreated group (Figure 6(b)) [34, 35].

We evaluated changes in macromolecules for preadipocytes and untreated SIM, CSE, GIE, and CSE/GIE combination-treated adipocytes, the integrated areas of the nucleic acid regions (1313–1294 cm^{-1} and 1165–1142 cm^{-1}), the regions for glycogen and other carbohydrates (1255–1208 cm^{-1}, 1096–1073 cm^{-1}, and 1054–1000 cm^{-1}), and the ratios for the integrated areas for functional groups associated with lipids and proteins (e.g., CH_2 (2938–2907 cm^{-1})/CH_3 (2973–2954 cm^{-1}) asymmetric stretching, CH_2 asymmetric stretching (2938–2907 cm^{-1})/amide I (1673–1624 cm^{-1})) [36]. The results indicated that the integrated areas for the glycogen and other carbohydrate regions in the preadipocyte and SIM, CSE, GIE, and CSE/GIE combination-treated adipocyte groups were significantly smaller than the untreated adipocyte groups. The integrated areas of the GIE-treated adipocyte group nucleic acid regions were significantly greater than the other groups ($p < 0.05$; Figure 7(a)). The values for the ratios of the integrated areas of CH_2/CH_3 asymmetric stretching (i.e., associated with the amounts of lipid acyl chain lengths of the lipids) [36] were the greatest for the untreated adipocytes and were significantly greater than the other groups ($p < 0.05$; Figure 7(b)). The statistically significant reduction in the values of the ratios of the integrated areas of CH_2 asymmetric stretching/amide I in the preadipocytes and the SIM, CSE, GIE, and CSE/GIE combination-treated groups compared with untreated adipocytes ($p < 0.05$; Figure 7(b)) might have been due to alterations in lipid and protein concentrations in each sample. PCA was used for further analysis of the second derivative spectra obtained for the six experimental groups.

Characterization of the spectral properties of biological samples can be achieved using FTIR spectroscopy. The results provide molecular information that varies with macromolecular composition and reflects changes in the absorbance of bands in the FTIR spectrum. IR spectrum molecular fingerprint region absorbance bands in the mid-IR range (4000–400 cm^{-1}) are derived from individual structure- and conformation-associated chemical bonds. PCA was used to analyze the characteristics of the biological molecules from the FTIR spectra [29]. This statistical data-reduction method transformed the original values for the variables in the data set into a new set of uncorrected variables (i.e., PCs). We used the PCA to determine the wave numbers present in the complex FTIR spectra that had the statistically significant greatest spectral variation within and between treatment groups. Similar spectra present within the dataset were visualized using scores plots, and variables (the molecular groups within the samples were represented by the spectral bands) were identified using loading plots.

The PCA modeling revealed that distinct clustering of the spectra from the treatment groups was visualized more clearly with the two-dimensional score plot PC1 compared with PC2 (Figure 8(a)). The results from the PCA score plot indicated that the clusters associated with the untreated adipocytes and the GIE-treated group were separated from the clusters associated with the preadipocytes and the SIM- and CSE-treated adipocytes along the PC1 axis (28%). Similarly, the clusters associated with the untreated adipocytes and the SIM-treated group were separated from the GIE- and CSE-treated groups along the PC2 axis (22%). The spectral regions that most contributed to the clustering (Figure 8(a)) were identified using the PCA loading plots (Figure 8(b)). The PC1-associated discrimination was likely associated with the positive loading in the C-H stretch region (centered at 2834 cm^{-1}) and the C–O vibrations associated with

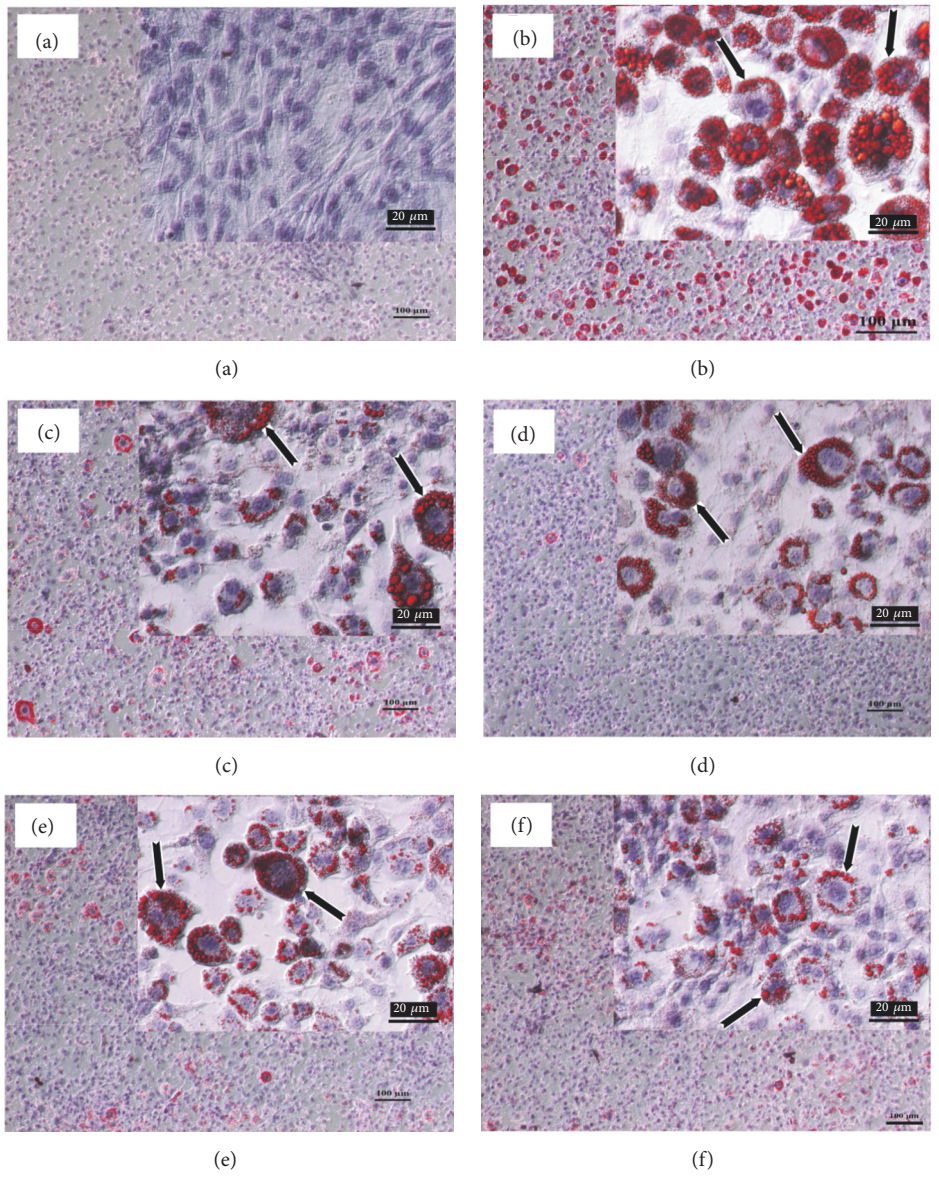


Figure 2: Results of Oil Red O and hematoxylin staining for intracellular lipid for six sample groups: (a) preadipocytes (nondifferentiated cells); (b) untreated adipocytes, (c) simvastatin at 1.67 μg mL^{-1}; (d) CSE at 10 μg mL^{-1}; (e) GIE at 500 μg mL^{-1}; (f) combination of CSE at 10 μg mL^{-1} plus GIE at 500 μg mL^{-1} (original magnification x 100, scale bar: 100 μm; *inset*: x 600, scale bar: 20 μm.)

glycogen and other carbohydrates (centered at 1064 cm^{-1}). These characteristics separated the negative score plot of the spectra of the preadipocytes and the SIM, CSE, and CSE/GIE combination-treated adipocytes from the positive score plot of the untreated and GIE-treated adipocytes. Taken together, these results indicated that untreated and GIE-treated adipocytes had greater concentrations of nucleic acids, lipoprotein, lipid acyl chains associated with membrane lipids, and carbohydrates than the preadipocytes and the SIM, CSE, and CSE/GIE combination-treated adipocytes.

The PC2 axis discrimination result between the positive score plot for the spectra associated with untreated adipocytes, SIM, and CSE/GIE combination-treated adipocytes and the negative score plot of the spectra associated with the preadipocytes and CSE and GIE-treated adipocytes can be explained by positive loading of the PC2 axis in the C–H stretch region (centered at 2935 cm^{-1} and at 1662 cm^{-1} (indicating amide I)). There was also negative loading of the PC2 axis in the C–H stretch region (centered at 2915 cm^{-1} and 2850 cm^{-1} and at 1639 cm^{-1} (indicating amide I)).

4. Discussion

The doses used in the experiments were standardized based on the results of preliminary screening of concentration intervals. The concentration intervals from the MTT assay that were not toxic and were not significantly different from

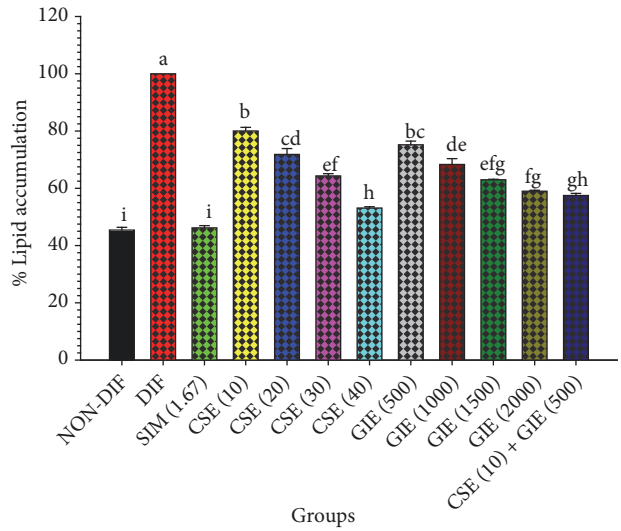


FIGURE 3: Graphical representation of the effects of CSE and GIE alone and combined on the percentage of intracellular lipid accumulation in differentiated 3T3-L1 cells, after Oil Red O staining. NON-DIF = preadipocytes (nondifferentiated cells); DIF = differentiated adipocytes (untreated adipocytes); SIM (1.67) = simvastatin at 1.67 μg mL^{-1}; CSE (10) = CSE at 10 μg mL^{-1}; GIE (500) = GIE at 500 μg mL^{-1}; the mean ± SEM values presented are for three replicates. Mean values followed by the same superscript letter were not statistically different ($p < 0.05$, Tukey's test).

the control were used in the subsequent experiments. The concentration intervals for the Oil Red O and FTIR assays were obtained from the safety concentration ranges of the MTT assay. The ranges for the lipase inhibition assay were selected from the results of preliminary testing of the tested extracts for the IC_{50} of lipase inhibition activity. The results of this study indicated that during adipocyte differentiation CSE and GIE alone and the CSE/GIE combination induced statistically significant reductions in lipid accumulation. CSE, GIE, and the CSE/GIE combination suppressed lipid accumulation without cytotoxicity. This antiadipogenic effect could have occurred via one or more molecular pathways. Any or all of them might result in lipid accumulation suppression during differentiation.

Treatment of mice with the extract of *C. sinensis* mycelia significantly reduces cholesterol and triglyceride concentrations and the synthesis of very-low-density lipoprotein (low-density lipoprotein precursor) [37, 38]. Therefore, a useful mechanism for obesity prevention might be inhibition of preadipocyte differentiation. Our results revealed that the range in CSE (10–40 μg mL^{-1}) that could reduce the lipid accumulation was much lower than the range in GIE (500–2000 μg mL^{-1}). The greater effect of CSE on lipid accumulation inhibition may be because CSE has a total flavonoid content and an alkaloid content greater than GIE. CSE also contains cordycepin, which is a main active ingredient that can have strong antiadipogenesis effects. Cordycepin significantly inhibits the biosynthesis of total cholesterol and triglycerides in HepG2 cells [8]; it reduces oleic acid-elicited intracellular lipid accumulation and increases AMPK activity [39].

In patients with diabetes, plasma insulin and glucose level imbalance result in a reduction of triglyceride-derived fatty acid membrane transport; this reduction results in increases in the half-life of triglyceride-rich lipoproteins and remnant particles [40]. There are many suggested mechanisms by which GIE induces its hypoglycemic effect. This mechanism supports the reduction of lipid accumulation [41]. In rats, GIE leaf extracts can inhibit glucose absorption in isolated intestinal tract tissue and suppress the increased blood glucose. The triterpenoid saponin in GIE extracts can suppress the high K^+-induced contraction of intestinal smooth muscle and affect the Na^+/K^+ pump. Pump suppression results in a change in the electrochemical potential of intracellular Na^+, which affects the Na^+-dependent cotransport system [7, 42]. Thus, our findings suggested that the mechanism of action of GIE may be inhibition of intestinal tract glucose absorption. The IC_{50} of GIE for the inhibition of pancreatic lipase was 982.24 ± 44.40 μg mL^{-1}; previous studies found that orlistat used as a positive control also has this inhibitory effect when used at 68.23 ± 6.67 μg mL^{-1} [21]. Under these conditions, the potential strength of orlistat on lipase activity inhibition is approximately 13.7 times greater than the GIE. Taken together, these results indicate that the inhibitory effects of GIE on pancreatic lipase activity increase in a dose-dependent manner. The enzyme pancreatic lipase has a significant role in fat digestion via hydrolysis of triglycerides to diglycerides and eventually to monoglycerides and free fatty acids [43]. Inhibition of dietary triglyceride absorption via pancreatic lipase inhibition likely represents a new approach for obesity treatment [44]. The lipase inhibition results indicated that the IC_{50} value of GIE was approximately 2.35 times lower than the IC_{50} for CSE. These results are consistent with the results of Hengpratom et al. and Sirichaiwetchakoon et al.; *Oroxylum indicum* extract and *Pluchea indica* tea have lipase inhibition activity, with IC_{50} values of 1062.04 ± 32.21 mg GAE/g and 1708.75 ± 335.85 mg GAE/g of dry weight, respectively [21, 26]. Polyphenolic compounds can inhibit enzyme lipase activity and reduce lipid absorption in the intestine [44, 45]. Hence, the lipase inhibitory effects of both extracts might be dependent on the total phenolic compound concentrations in these extracts; the total phenolic contents in GIE and CSE are 0.81 ± 0.01 mg GAE/g and 0.14 ± 0.01 mg GAE/g of dry weight, respectively.

The antiadipogenic effects of CSE, GIE, and the CSE/GIE combination in 3T3-L1 cells were confirmed by FTIR analysis, which is useful for detection of biomolecular changes in biological samples [29, 46, 47]. We used FTIR analysis to identify spectral profiles of 3T3-L1 preadipocytes and untreated and SIM-, CSE-, GIE-, and CSE/GIE combination-treated 3T3-L1 adipocytes. For the six different conditions, changes in the cells were correlated with changes in the lipid (2964, 2923, and 2854 cm^{-1}), nucleic acid (1234 cm^{-1} and 1084 cm^{-1}), and glycogen and other carbohydrate regions (1157 cm^{-1} and 1045 cm^{-1}) in the second derivative spectra. Compared with the untreated adipocytes, the signal intensities and areas of glycogen and other carbohydrates of the treated

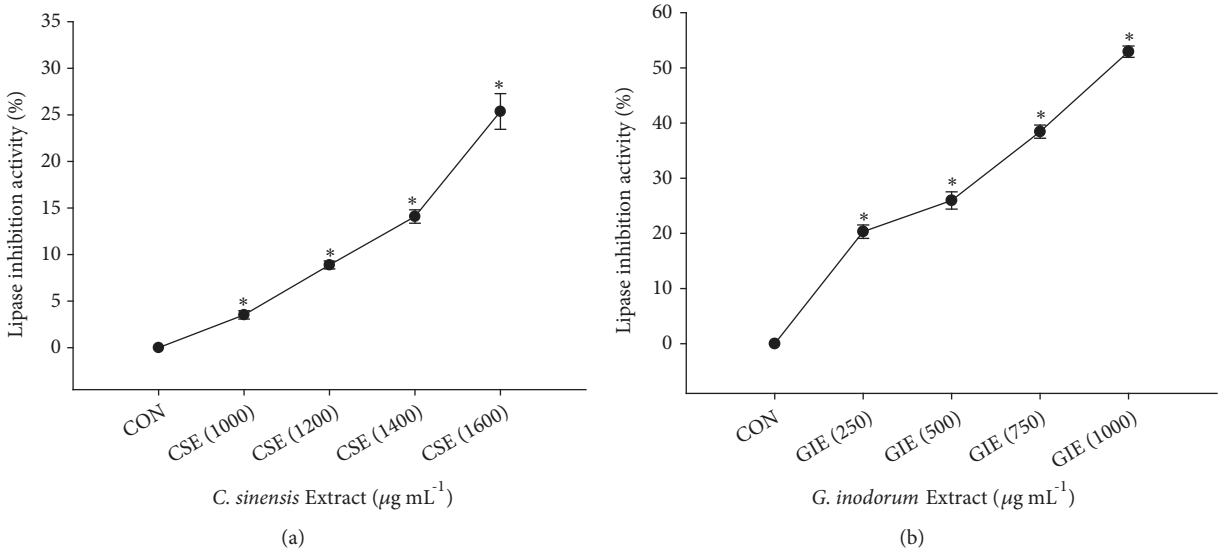


Figure 4: Inhibitory effects of CSE (a) and GIE (b) at various concentrations on pancreatic lipase activity (%). CON = control; CSE (1000) = CSE at 1000 μg mL^{-1}; GIE (250) = GIE at 250 μg mL^{-1}. Results are expressed as mean ± SEM values (three replicates). $* p < 0.05$ indicates result significantly different from the control.

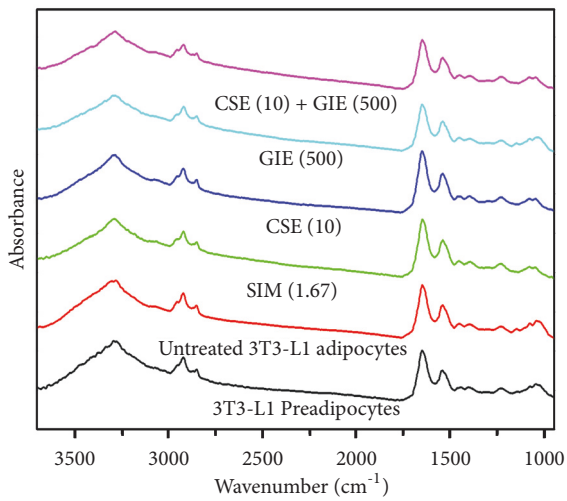


Figure 5: Representative original FTIR spectra (3000–950 cm^{-1}) obtained from 3T3-L1 cells at day 10 after differentiation. 3T3-L1 preadipocytes (n = 41); untreated 3T3-L1 adipocytes (n = 39); SIM (1.67) = simvastatin at 1.67 μg mL^{-1} (n = 49); CSE (10) = CSE at 10 μg mL^{-1} (n = 98); GIE (500) = GIE at 500 μg mL^{-1} (n = 76); CSE (10) + GIE (500) = CSE at 10 μg mL^{-1} plus GIE at 500 μg mL^{-1} (n = 97).

adipocytes were significantly smaller after CSE, GIE, or CSE/GIE exposure ($p < 0.05$). Hence, in patients with obesity, decreasing the synthesis of glycogen and other carbohydrates may reduce the rates of carbohydrate and lipid accumulation [48, 49]. The results for the CH_2/CH_3 asymmetric stretching ratio indicated that compared with the other groups the untreated adipocytes had significantly greater proportions of longer acyl chains of lipids. The increases in the CH_2/CH_3 asymmetric stretching ratio proportions might have been due to lipoprotein fractions involved in lipid storage and accumulation of free cholesterol and cholesterol esters; these fractions can be used as markers of adipogenesis from the preadipocyte to the adipocyte stages [29, 50]. The lipid storage-associated decreases in the CH_2/CH_3 asymmetric stretching ratio were also consistent with the Oil Red O staining results. The result for CH_2 asymmetric stretching/amide I indicated that the untreated adipocyte lipid/protein ratio was significantly greater than the other groups ($p < 0.05$). Taken together, these findings indicated that the lipid/protein results were virtually the same as the lipid storage results.

The classification methods used in this study included PCA, which was used to discriminate the clusters associated with the six FTIR spectra from the different treatments. The discrimination was based on between-group differences in biomolecular composition (nucleic acid, amide I protein, lipids, and glycogen and other carbohydrates). Significant differences between the spectra from the six sample groups were examined using a two-dimensional score plot obtained from the PCA (Figure 8(a)). The loading plots revealed relationships among the six sample groups for variables that contributed to clustering (Figure 8(b)). The score plot revealed that the untreated adipocyte and GIE-treated adipocyte clusters were separate from the clusters associated with the other four groups along the PC1 axis and accounted for 28% of the total variance. Twenty-two percent of the total variance was described with the PC2 score plot; the clusters associated with the untreated adipocytes were clearly separated from the clusters associated with the GIE-treated adipocytes. The discrimination along PC1 between the negative score plot for the preadipocytes, SIM, CSE, and CSE/GIE combination-treated adipocyte spectra and the positive score plot for the untreated and GIE-treated adipocyte spectra can be explained by the negative PC1 loadings for the preadipocytes and the SIM, CSE, and CSE/GIE combination centered at

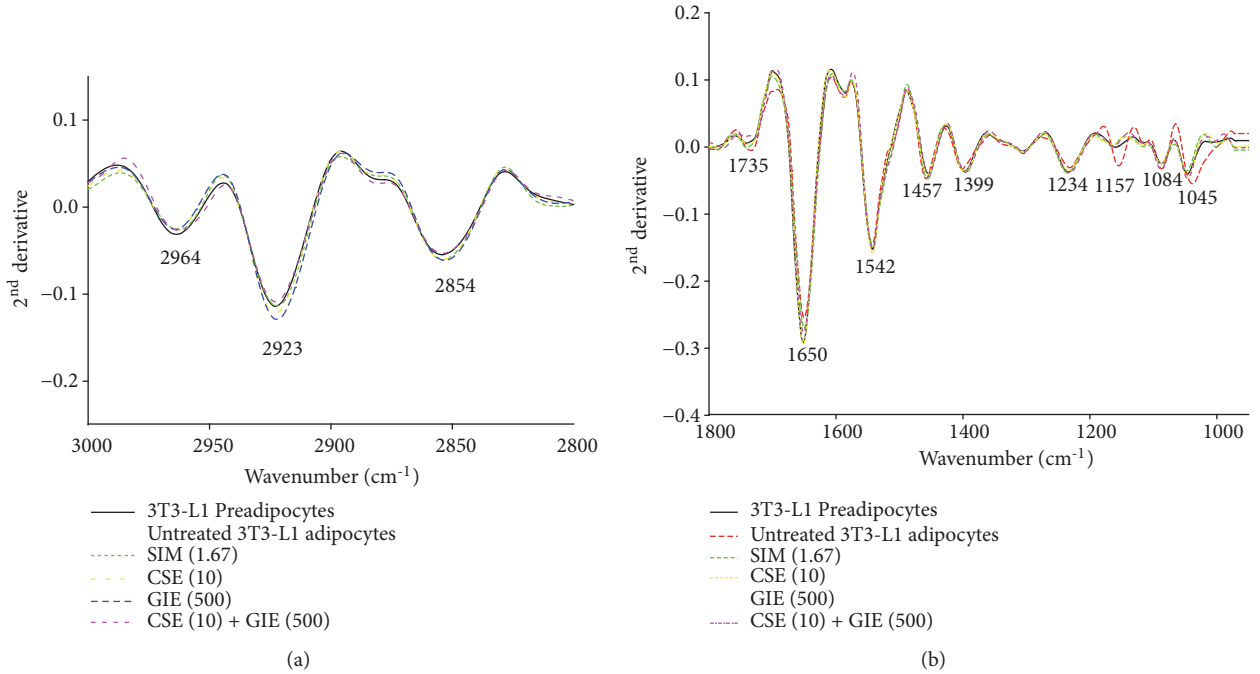


FIGURE 6: Representative secondary derivative spectra for 3T3-L1 cells. SIM (1.67) = simvastatin at 1.67 μg mL^{-1}; CSE (10) = CSE at 10 μg mL^{-1}; GIE (500) = GIE at 500 μg mL^{-1}; CSE (10) + GIE (500) = CSE at 10 μg mL^{-1} plus GIE at 500 μg mL^{-1}. The results represent two regions: (a) lipid (3000–2800 cm^{-1}); (b) protein, nucleic acid, glycogen, and other carbohydrates (1800–950 cm^{-1}).

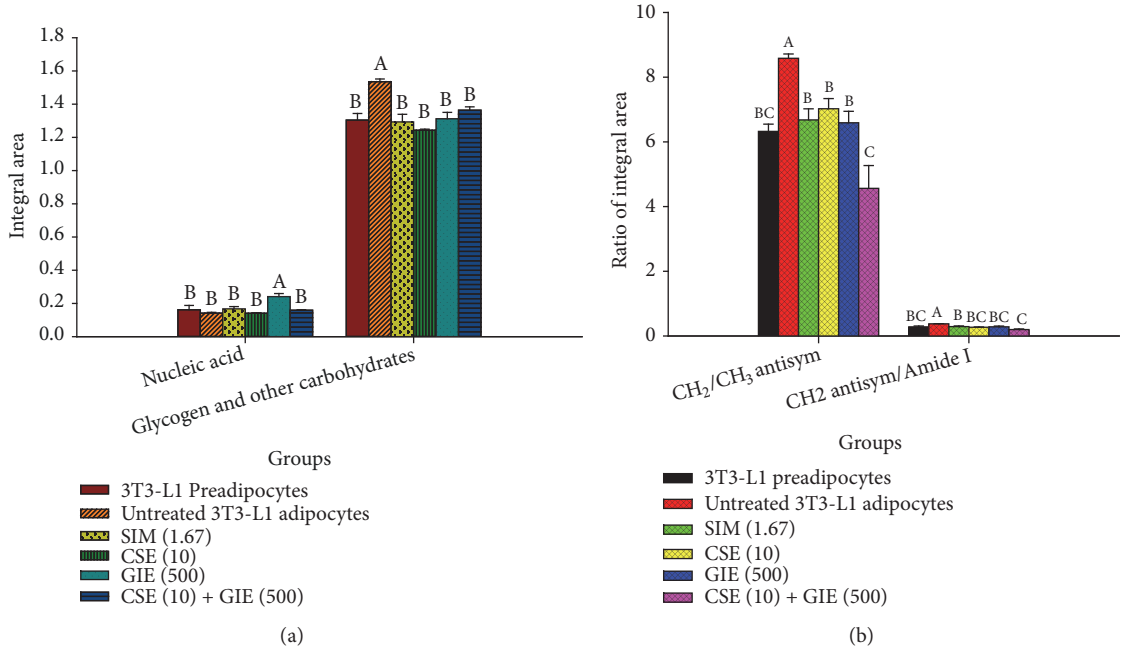


FIGURE 7: (a) Integrated areas of notable nucleic acids and regions corresponding to glycogen and other carbohydrates and (b) CH_2/CH_3 asymmetric stretching and CH_2 asymmetric stretching/amide I integrated area ratios for 3T3-L1 cells. SIM (1.67) = simvastatin at 1.67 μg mL^{-1}; CSE (10) = CSE at 10 μg mL^{-1}; GIE (500) = GIE at 500 μg mL^{-1}; CSE (10) + GIE (500) = CSE at 10 μg mL^{-1} plus GIE at 500 μg mL^{-1}; CH_2 antisym = CH_2 asymmetric stretch. The results are presented as mean ± SEM (three replicates). Mean values with the same superscript letter were not significantly different (Tukey's test, $p < 0.05$).

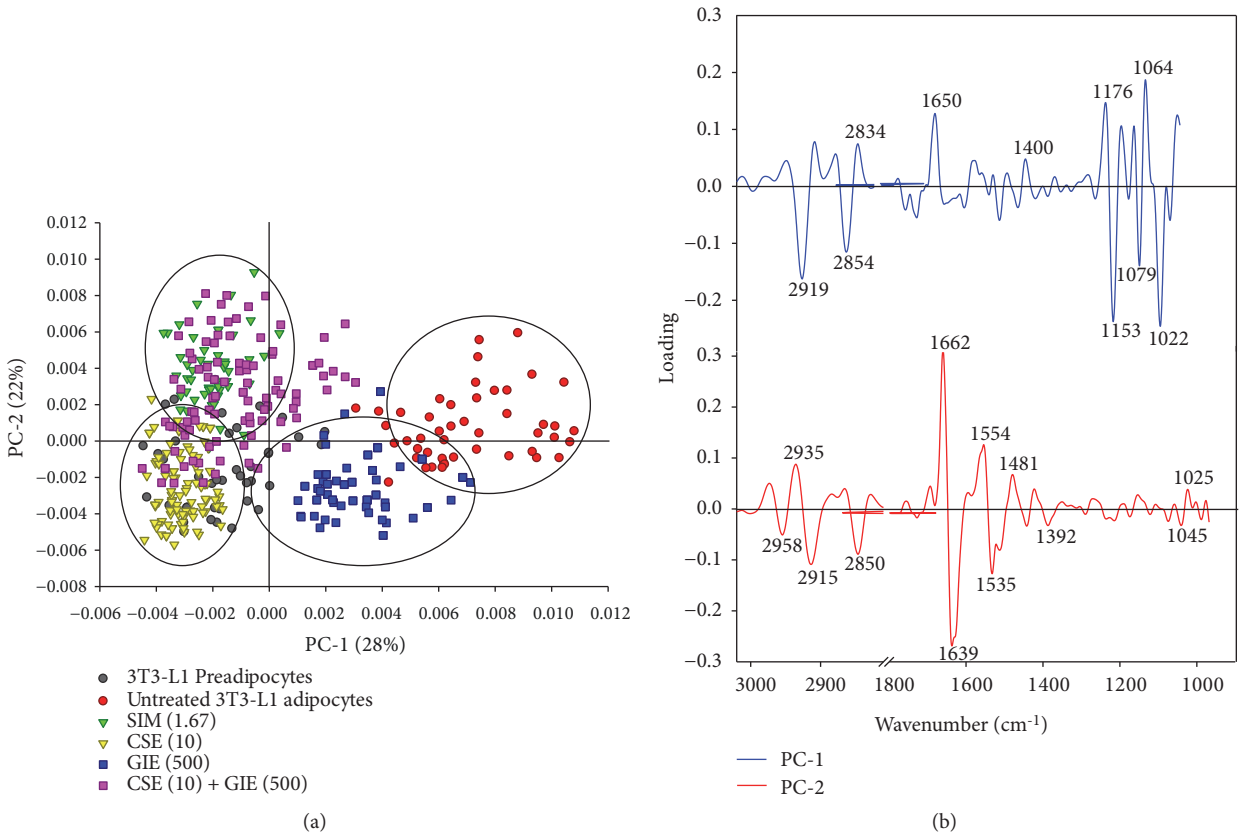


FIGURE 8: PCA of FTIR spectral ranges 3000–2800 cm^{-1} and 1800–950 cm^{-1}. PCA score plot (a) and PCA loading plot (b). Score plots revealed distinct clustering between 3T3-L1 groups. SIM (1.67) = simvastatin at 1.67 μg mL^{-1}; CSE (10) = CSE at 10 μg mL^{-1}; GIE (500) = GIE at 500 μg mL^{-1}; CSE (10) + GIE (500) = CSE at 10 μg mL^{-1} plus GIE at 500 μg mL^{-1}. The PC1 and PC2 loading plots identify biomarker differences over a spectral range of samples.

2950 cm^{-1}, 2854 cm^{-1}, 1153 cm^{-1}, and 1022 cm^{-1} and the positive loading of the other two groups centered at 2834 cm^{-1}, 1650 cm^{-1}, and 1064 cm^{-1} (Figure 8(b)). Similarly, the PC2 loading between the positive score plot centered at 2935 cm^{-1} and 1662 cm^{-1} for the untreated adipocytes and the negative score plot centered at 2915 cm^{-1}, 2850 cm^{-1}, and 1639 cm^{-1} for the GIE-treated adipocytes was apparent. Taken together, these results indicated that this approach could be adapted for the obesity biomarker development and evaluation of the efficacy of obesity drugs in patients with obesity.

5. Conclusions

In summary, the flavonoids were the important group of phytochemical compounds found in both CSE and GIE; alkaloids were found only in CSE. The viability of cells treated with CSE (40) or GIE (2000) alone or combined (CSE (10) + GIE (500)) was not significantly reduced compared with the preadipocytes ($p > 0.05$). The Oil Red O and hematoxylin staining results indicated that compared with no treatment the combination of CSE (10) and GIE (500) resulted in synergistic effects on the reduction in adipocyte lipid accumulation and significantly decreased lipid accumulation ($p < 0.05$). The FTIR assay revealed that adipocytes treated with CSE (10) plus GIE (500) had significantly lower lipid levels than the adipocytes treated with CSE (10) or GIE (500) alone and the adipocytes that were untreated ($p < 0.05$). The ratios of the integrated areas of glycogen and other carbohydrates and lipid/protein ratios in the adipocytes treated with CSE (10) or GIE (500) alone or combined were significantly lower than those for the untreated adipocytes ($p < 0.05$). These findings indicated that the use of FTIR microspectroscopy combined with the multivariate statistical PCA could be an effective approach for classification of differentiated adipocytes.

Data Availability

The datasets used and analyzed during this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Acknowledgments

The authors are indebted and grateful to the One Research One Grant scholarship from the Suranaree University of Technology for assistance with research funds support and to the Synchrotron Light Research Institute (Public Organization), Thailand, for supporting the FTIR microspectroscopy technique. The authors are also grateful to Cordythai Company, LTD, Thailand, for providing the *Cordyceps sinensis* extract.

References

[1] T. Kelly, W. Yang, C. S. Chen, K. Reynolds, and J. He, "Global burden of obesity in 2005 and projections to 2030," *International Journal of Obesity*, vol. 32, no. 9, pp. 1431–1437, 2008.

[2] S. E. Kahn, R. L. Hull, and K. M. Utzschneider, "Mechanisms linking obesity to insulin resistance and type 2 diabetes," *Nature*, vol. 444, no. 7121, pp. 840–846, 2006.

[3] A. Guilherme, J. V. Virbasius, V. Puri, and M. P. Czech, "Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 5, pp. 367–377, 2008.

[4] R. Birari, V. Javia, and K. K. Bhutani, "Antiobesity and lipid lowering effects of *Murraya koenigii* (L.) spreng leaves extracts and mahanimbine on high fat diet induced obese rats," *Fitoterapia*, vol. 81, no. 8, pp. 1129–1133, 2010.

[5] R. Zouari, K. Hamden, A. E. Feki et al., "Protective and curative effects of Bacillus subtilis SPB1 biosurfactant on high-fat-high-fructose diet induced hyperlipidemia, hypertriglyceridemia and deterioration of liver function in rats," *Biomedicine & Pharmacotherapy*, vol. 84, pp. 323–329, 2016.

[6] S. Hu, J. Wang, Z. Li, J. Fu, Y. Wang, and C. Xue, "Hpyerglycemic effect of a mixture of sea cucumber and cordyceps sinensis in streptozotocin-induced diabetic rat," *Journal of Ocean University of China*, vol. 13, no. 2, pp. 271–277, 2014.

[7] K. Shimizu, M. Ozeki, A. Iino, S. Nakajyo, N. Urakawa, and M. Atsuchi, "Structure-activity relationships of triterpenoid derivatives extracted from Gymnema inodorum leaves on glucose absorption," *Japanese Journal of Pharmacology*, vol. 86, no. 2, pp. 223–229, 2001.

[8] P. Guo, Q. Kai, J. Gao et al., "Cordycepin prevents hyperlipidemia in hamsters fed a high-fat diet via activation of AMP-activated protein kinase," *Journal of Pharmacological Sciences*, vol. 113, no. 4, pp. 395–403, 2010.

[9] W.-C. Kan, H.-Y. Wang, C.-C. Chien et al., "Effects of extract from solid-state fermented *Cordyceps sinensis* on type 2 diabetes mellitus," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 743107, 10 pages, 2012.

[10] S. Takahashi, M. Tamai, S. Nakajima et al., "Blockade of adipocyte differentiation by cordycepin," *British Journal of Pharmacology*, vol. 167, no. 3, pp. 561–575, 2012.

[11] H. Wagner, "Synergy research: approaching a new generation of phytopharmaceuticals," *Fitoterapia*, vol. 82, no. 1, pp. 34–37, 2011.

[12] S. Prabhu and S. Vijayakumar, "Antidiabetic, hypolipidemic and histopathological analysis of *Gymnema sylvestre* (R. Br) leaves extract on streptozotocin induced diabetic rats," *Biomedicine & Preventive Nutrition*, vol. 4, no. 3, pp. 425–430, 2014.

[13] S. Al-Daihan, M. Al-Faham, N. Al-shawi et al., "Antibacterial activity and phytochemical screening of some medicinal plants commonly used in Saudi Arabia against selected pathogenic microorganisms," *Journal of King Saud University - Science*, vol. 25, no. 2, pp. 115–120, 2013.

[14] N. Savithramma, M. L. Rao, and D. Suhrulatha, "Screening of medicinal plants for secondary metabolites," *Middle East Journal of Scientific Research*, vol. 8, no. 3, pp. 579–584, 2011.

[15] R. Yadav and M. Agarwala, "Phytochemical analysis of some medicinal plants," *Journal of Phytology*, vol. 3, no. 12, 2011.

[16] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventós, "[14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent," *Methods in Enzymology*, vol. 299, pp. 152–178, 1999.

[17] H. V. Rupasinghe, L. Wang, G. M. Huber, and N. L. Pitts, "Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder," *Food Chemistry*, vol. 107, no. 3, pp. 1217–1224, 2008.

[18] J.-J. Chen and X.-R. Li, "Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice," *Asia Pacific Journal of Clinical Nutrition*, vol. 16, no. S1, pp. 290–294, 2007.

[19] S. Settharaksa, F. Madaka, L. Sueree et al., "Effect of solvent types on phenolic, flavonoid contents and antioxidant activities of *Syzygium gratum* (wight) SN," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 6, no. 2, pp. 114–116, 2014.

[20] S. Phitaktim, M. Chomnawang, K. Sirichaiwetchakoon, B. Dunkhunthod, G. Hobbs, and G. Eumkeb, "Synergism and the mechanism of action of the combination of α-mangostin isolated from *Garcinia mangostana* L. and oxacillin against an oxacillin-resistant *Staphylococcus saprophyticus*," *BMC Microbiology*, vol. 16, no. 1, p. 195, 2016.

[21] K. Sirichaiwetchakoon, G. M. Lowe, K. Thumanu, and G. Eumkeb, "The Effect of *Pluchea indica* (L.) less. Tea on Adipogenesis in 3T3-L1 adipocytes and lipase activity," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 4108787, 13 pages, 2018.

[22] F. Denizot and R. Lang, "Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability," *Journal of Immunological Methods*, vol. 89, no. 2, pp. 271–277, 1986.

[23] J. Naowaboot, C. H. Chung, P. Pannangpetch et al., "Mulberry leaf extract increases adiponectin in murine 3T3-L1 adipocytes," *Nutrition Research*, vol. 32, no. 1, pp. 39–44, 2012.

[24] G. Eumkeb, S. Tanphonkrang, K. Sirichaiwetchakoon, T. Hengpratom, and W. Naknarong, "The synergy effect of daidzein and genistein isolated from *Butea superba* Roxb. on the reproductive system of male mice," *Natural Product Research (Formerly Natural Product Letters)*, vol. 31, no. 6, pp. 672–675, 2017.

[25] H. Wagner and G. Ulrich-Merzenich, "Synergy research: approaching a new generation of phytopharmaceuticals," *Phytomedicine*, vol. 16, no. 2-3, pp. 97–110, 2009.

[26] T. Hengpratom, G. M. Lowe, K. Thumanu, S. Suknasang, K. Tiamyom, and G. Eumkeb, "*Oroxylum indicum* (L.) Kurz extract inhibits adipogenesis and lipase activity in vitro," *BMC Complementary and Alternative Medicine*, vol. 18, no. 1, p. 177, 2018.

[27] X. Guo, J. Liu, S. Cai, O. Wang, and B. Ji, "Synergistic interactions of apigenin, naringin, quercetin and emodin on inhibition of 3T3-L1 preadipocyte differentiation and pancreas lipase activity," *Obesity Research & Clinical Practice*, vol. 10, no. 3, pp. 327–339, 2016.

[28] S. Siriwong, Y. Teethaisong, K. Thumanu, B. Dunkhunthod, and G. Eumkeb, "The synergy and mode of action of quercetin plus amoxicillin against amoxicillin-resistant *Staphylococcus epidermidis*," *BMC Pharmacology & Toxicology*, vol. 17, no. 1, p. 39, 2016.

[29] B. Dunkhunthod, K. Thumanu, and G. Eumkeb, "Application of FTIR microspectroscopy for monitoring and discrimination of the anti-adipogenesis activity of baicalein in 3T3-L1 adipocytes," *Vibrational Spectroscopy*, vol. 89, pp. 92–101, 2017.

[30] C. Aksoy and F. Severcan, "Role of vibrational spectroscopy in stem cell research," *Journal of Spectroscopy*, vol. 27, no. 3, pp. 167–184, 2012.

[31] V. Dritsa, "FT-IR spectroscopy in medicine," in *Infrared Spectroscopy-Life and Biomedical Sciences*, InTech, 2012.

[32] D. Krilov, M. Balarin, M. Kosović, O. Gamulin, and J. Brnjas-Kraljević, "FT-IR spectroscopy of lipoproteins—A comparative study," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 73, no. 4, pp. 701–706, 2009.

[33] S. Garip, A. C. Gozen, and F. Severcan, "Use of Fourier transform infrared spectroscopy for rapid comparative analysis of *Bacillus* and *Micrococcus* isolates," *Food Chemistry*, vol. 113, no. 4, pp. 1301–1307, 2009.

[34] J. Cao, E. S. Ng, D. McNaughton et al., "The characterisation of pluripotent and multipotent stem cells using fourier transform infrared microspectroscopy," *International Journal of Molecular Sciences*, vol. 14, no. 9, pp. 17453–17476, 2013.

[35] P. Heraud, E. S. Ng, S. Caine et al., "Fourier transform infrared microspectroscopy identifies early lineage commitment in differentiating human embryonic stem cells," *Stem Cell Research*, vol. 4, no. 2, pp. 140–147, 2010.

[36] F. Kucuk Baloglu, S. Garip, S. Heise, G. Brockmann, and F. Severcan, "FTIR imaging of structural changes in visceral and subcutaneous adiposity and brown to white adipocyte transdifferentiation," *Analyst*, vol. 140, no. 7, pp. 2205–2214, 2015.

[37] J.-H. Koh, J.-M. Kim, U.-J. Chang, and H.-J. Suh, "Hypocholesterolemic effect of hot-water extract from mycelia of *Cordyceps sinensis*," *Biological and Pharmaceutical Bulletin*, vol. 26, no. 1, pp. 84–87, 2003.

[38] J.-S. Zhu, G. M. Halpern, and K. Jones, "The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis* part I," *The Journal of Alternative and Complementary Medicine*, vol. 4, no. 3, pp. 289–303, 1998.

[39] C. Wu, Y. Guo, Y. Su et al., "Cordycepin activates AMP-activated protein kinase (AMPK) via interaction with the γ1 subunit," *Journal of Cellular and Molecular Medicine*, vol. 18, no. 2, pp. 293–304, 2014.

[40] G. Schmitz, H. Hohage, and K. Ullrich, "Glucose-6-phosphate: A key compound in glycogenosis I and favism leading to hyper- or hypolipidaemia," *European Journal of Pediatrics*, vol. 152, Supplement 1, pp. 77–84, 1993.

[41] C. Lara-Castro and W. T. Garvey, "Intracellular lipid accumulation in liver and muscle and the insulin resistance syndrome," *Endocrinology and Metabolism Clinics of North America*, vol. 37, no. 4, pp. 841–856, 2008.

[42] K. Shimizu, M. Ozeki, K. Tanaka et al., "Suppression of glucose absorption by extracts from the leaves of G*ymnema inodorum*," *Journal of Veterinary Medical Science*, vol. 59, no. 9, pp. 753–757, 1997.

[43] F. K. Winkler, A. D'Arcy, and W. Hunziker, "Structure of human pancreatic lipase," *Nature*, vol. 343, no. 6260, pp. 771–774, 1990.

[44] R. B. Birari and K. K. Bhutani, "Pancreatic lipase inhibitors from natural sources: unexplored potential," *Drug Discovery Therapy*, vol. 12, no. 19-20, pp. 879–889, 2007.

[45] B. Zhang, Z. Y. Deng, D. D. Ramdath et al., "Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on α-glucosidase and pancreatic lipase," *Food Chemistry*, vol. 172, pp. 862–872, 2015.

[46] A. Lamberti, C. Sanges, and P. Arcari, "FT-IR spectromicroscopy of mammalian cell cultures during necrosis and apoptosis induced by drugs," *Spectroscopy: An International Journal*, vol. 24, no. 5, pp. 535–546, 2010.

[47] F. Gasparri and M. Muzio, "Monitoring of apoptosis of HL60 cells by Fourier-transform infrared spectroscopy," *Biochemical Journal*, vol. 369, no. 2, pp. 239–248, 2003.

[48] P. R. Shepherd, L. Gnudi, E. Tozzo, H. Yang, F. Leach, and B. B. Kahn, "Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue," *Journal of Biological Chemistry*, vol. 268, no. 30, pp. 22243–22246, 1993.

[49] J. Wylie-Rosett, C. Segal-Isaacson, and A. Segal-Isaacson, "Carbohydrates and increases in obesity: does the type of carbohydrate make a difference?" *Obesity Research*, vol. 12, no. S11, 2004.

[50] B. Gauthier, M. Robb, and R. McPherson, "Cholesteryl ester transfer protein gene expression during differentiation of human preadipocytes to adipocytes in primary culture," *Atherosclerosis*, vol. 142, no. 2, pp. 301–307, 1999.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7621929, 45 pages
https://doi.org/10.1155/2019/7621929

Review Article

Phellodendri Cortex: A Phytochemical, Pharmacological, and Pharmacokinetic Review

Yue Sun, George Binh Lenon, and Angela Wei Hong Yang

School of Health and Biomedical Sciences, RMIT University, Victoria, Australia

Correspondence should be addressed to George Binh Lenon; george.lenon@rmit.edu.au

Received 18 November 2018; Accepted 5 February 2019; Published 26 March 2019

Guest Editor: Jesus R. R. Amado

Background. *Phellodendri Cortex* (PC) or Huang Bai. According to the scientific database of China Plant Species and Chinese pharmacopeia 2015 edition, PC has two main species which are *Phellodendron amurense Rupr* (PAR) or "Guan Huang bai" in Chinese and *Phellodendron chinense Schneid* (PCS) or "Chuan Huang bai" in Chinese. The crude drugs of PAR and PCS are also called *Phellodendri amurensis cortex* (PAC) and *Phellodendri chinense cortex* (PCC), respectively. The medicinal part of the plant is the dried trunk bark. PC has comprehensive therapeutic effects which include anti-inflammatory, antimicrobial, anticancer, hypotensive, antiarrhythmic, antioxidant, and antipyretic agents. The exact ingredients in PC and its species are not fully summarised. *Aim of the Study*. This study was designed to review and evaluate the pharmacological actions of compounds and to explore the pharmacokinetic knowledge of PC and its species and to also identify the chemical compound(s) with a potential therapeutic effect on atopic dermatitis. *Methods*. "Huang Bai" and its English, botanical, and pharmaceutical names were used as keywords to perform database search in Encyclopaedia of traditional Chinese Medicines, PubMed, EMBASE, MEDLINE, Science Direct, Scopus, Web of Science, and China Network Knowledge Infrastructure. The data selection criteria included all the studies that were related to the phytochemical, pharmacological, and pharmacokinetic perspectives of PC and its species or their active constituents. More importantly, the voucher number has been provided to ensure the genuine bark of PC used as the medicinal part in the studies. *Results*. 140 compounds were summarized from PC and its species: specifically, 18 compounds from PCC, 44 compounds from PCS, 34 compounds from PAC, and 84 compounds from PAR. Obacunone and obaculactone are probably responsible for antiatopic dermatitis effect. PC and its species possess a broad spectrum of pharmacological actions including anti-inflammatory effect, antibacterial effect, antiviral effect, antitumor effect, antigout effect, antiulcer effect, neuroprotective effect, and antiatopic dermatitis effect. PC could widely distribute in plasma, liver, spleen, kidney, and brain. Berberine may be responsible for the toxic effect on the susceptible users with hemolytic disease or in the peripartum and neonatal period. *Conclusions*. The compounds of the crude bark of PC and its subspecies have showcased a wide range of pharmacological effects. Pharmacological efficacies of PC are supported by its diverse class of alkaloid, limonoid, phenolic acid, quinic acid, lignan, and flavonoid. Obacunone and obaculactone could be the bioactive compounds for atopic dermatitis management. PC and its subspecies are generally safe to use but extra care is required for certain conditions and group of people.

1. Introduction

Phellodendri Cortex (PC) is also known as "Huang bai" in Chinese and "Obaku" in Japanese. PC is a plant grown in China, Korea, Japan, Vietnam, and Far East of Russia. The earliest record of this plant was on "Shennong's Classic of Materia Medica" [1]. According to the scientific database of China Plant Species and Chinese pharmacopeia 2015 edition, PC has two main species which are *Phellodendron amurense Rupr* (PAR) or "Guan Huang bai" in Chinese and *Phellodendron chinense Schneid* (PCS) or "Chuan Huang bai" in Chinese. The crude drugs of PAR and PCS are called *Phellodendri amurensis cortex* (PAC) and *Phellodendri chinense cortex* (PCC), respectively. PAR and PCS are naturally grown in the Northeast and Southwest part of China, respectively [2]. According to the latest information from "information system of Chinese rare and endangered plants," PAR is categorized as one of the second degrees of endangered plants. PAR and PCS could be interchangeably used in clinical application because both species contain

similar chemical constituents [3]. According to the scientific database of China Plant Species and Chinese pharmacopeia 2015 edition, PC is categorized in the family of *Phellodendron Rupr*. The medicinal part of the plant is the dried trunk bark. PC had been viewed as one of the 50 fundamental herbs in Chinese herbalism. Traditionally, its medicinal part could exert therapeutic effects in various diseases such as meningitis, cirrhosis, dysentery, pneumonia, tuberculosis, etc. [4, 5]. Nowadays, PC has comprehensive therapeutic effects which include immune modulation, anti-inflammatory, antimicrobial, antibacterial, anticancer, hypotensive, antiarrhythmic, antioxidant, antigastric ulcer, and antipyretic agents, etc. [5]. According to the Clinical Chinese Materia Medica, 2006 edition, PC has a bitter flavor and cold nature and can enter the meridian of kidney, bladder, and large intestine. PC could clear heat, dry dampness, drain fire, eliminate steam, resolve toxin, and treat sores [6]. PC and its species contain various chemical derivatives. One of the important ones is alkaloids which contain berberine and jatrorrhizine. Both compounds were proven to be effective against some type of tumours, infections, neurological diseases [7]. To further acquire the knowledge on PC, a systematic review of its phytochemical, pharmacological, and pharmacokinetic properties is required. The aim of this study is to review and evaluate the pharmacological actions of compounds as well as to explore the pharmacokinetic knowledge of the PC and its species. The chemical compounds that exert therapeutic effect for atopic dermatitis are also desired.

2. Methods

Data were searched from the following databases until May 2018: Encyclopaedia of Traditional Chinese Medicines; PubMed; EMBASE; MEDLINE; ScienceDirect; SCOPUS; Web of Science; China Network Knowledge Infrastructure. The keywords used for the literature search included: Huang Bai and its English, botanical, and pharmaceutical names. The selection criteria included process controls of the herbal substances, reporting reference standards such as authentication of reference materials and profile chromatograms, and analytical procedures and validation data. Papers in English or Chinese language are included for this review. Scientific rigidity was determined by the chemical markers of herbs through the use of strict parameters in testing, quantitative, and qualitative measures of the bioactive components, such as high-performance liquid chromatography, fingerprint spectrum, correlations differentiation, and stability evaluation, reference standards, and toxicological assessments. Plant voucher specimens are a guarantee for traceability of the plant material and data verification for other researchers or commercial purposes [60]. The chemical formulas of the compounds of PC and its species were acquired from selected studies. Chemical structures and molecular weights were extracted from *ChemDraw professional 170*.

3. Results

A total of 125 papers were identified through the literature search. Fifty-two papers were excluded based on the reasons for nonpharmacodynamic, nonphytochemistry, and nonpharmacological studies. 73 studies fitted the selection criteria. Among these articles, 32 studies are about pharmacodynamics, 38 studies are about phytochemistry, and 3 studies are about pharmacokinetics (Figure 1).

4. Bioactive Compounds

The crude barks of PC and its species contain alkaloids, isoquinoline alkaloid, limonoids, phenolic acid, quinic acid, lignans, and flavonoid, and so on. A summary of the bioactive compounds of the PC and its species reported in the included studies is presented in Table 1. Molecular formula, molecular weight, and chemical structure of the major constituted alkaloids of PC, including berberine, palmatine, and jatrorrhizine, are listed in Tables 2, 3, 4, and 5. They could exert a broad spectrum of pharmacological influence which contains antimicrobial, anti-inflammation, antitumor, antidepressant, and antiulcer effects [47]. Limonoids including limonin and obakunone play an important role in the anti-inflammatory effects of PC [11]. Phenolic acid or phenol carboxylic acids belong to aromatic acid compound substances which are characterized by a phenolic ring and an organic carboxylic acid function [61]. According to the description on Buchler quinine plant in Braunschweig, Germany, quinic acid is a natural sugary compound which can be found in multiple plants such as well-known coffee beans and tobacco leaves. Based on the description on PubChem, lignans affiliate a class of dibenzylbutane derivatives which exists in plants and in body fluids such as bile, serum, urine, etc. These compounds have anticancer potential. Quercetin belongs to flavonoids which can reduce coronary heart disease according to PubChem data. Two species of PC, PCS and PAR, can be differentiated based on the contents of the chemical compounds. Specifically, the 2005 edition of Chinese Medicinal encyclopedia described the PCS's minimal content of berberine hydrochloride and phellodendron hydrochloride to be 3.0% and 0.34%, respectively; the PAR's minimal content of berberine hydrochloride and palmatine hydrochloride is 0.60% and 0.30%, respectively [62].

5. Pharmacology

5.1. Anti-Inflammatory Effects. The PAR extract could efficiently adjust lipopolysaccharide (LPS)-induced release of nitric oxide (NO) and inducible nitric oxide sythase (iNOS) production in microglia of both BV2 cells and mice. Besides, PAR extract could also attenuate the LPS-stimulated release of tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) from microglia. More importantly, the latter mechanism is more significant than the previous one in terms of IL-1β release. The inhibition of NO suggested that the extract of PAR probably could affect NO-induced neuronal cell death [28]. Another study had also vindicated the anti-inflammatory effect of PC extract on ear swelling model of mice. Magnoflorine and phellodenrine belong to alkaloids isolated from PC that are the effective compounds against oxazolone-induced contact-delayed type hypersensitivity (DTH) reaction induced by picryl chloride. Another

Figure 1: PC study selection flowchart.

mechanism in this study is that PC could reduce myeloperoxidase (MPO) activity to its utmost by restraining leukocyte mobility and/or a secretory activity by phellodendron. In contrast, PC extract exerts no effect on phospholipase A2 (PLA_2) activity and less effect in arachidonic acid (AA) induced swelling [29]. PC showcased the inhibitory effect on the build-up of NO in LPS-stimulated macrophage Raw 264.7 cells and inhibited iNOS expression. In contrast, PC has no effect on cyclooxygenase-2 (COX-2) expression in LPS-induced RAW 264.7 cells [30]. PCC and PAC could not only shrink the size of edema, but also reduce the activity of MPO and the content of reactive oxygen species (ROS) caused by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). They can also restrain the levels of TNF-α, Il-1β, IL-6, and COX-2 in mice treated by TPA. Remarkably, there are a number of chemical compounds in these two species of PC including berberine, palmatine, and phellodendrine. And they are viewed as the anti-inflammatory active candidates. In addition, they may jointly take effect in this regard [3]. The nonalkaloid PAR extract suppressed NO production; besides, limonin and obakunone significantly downregulated NO production and iNOS gene expression via an nuclear factor-κB (NF-κB)-mediated pathway [11]. PC could reverse the airway inflammation by reducing the infiltration of inflammatory cells and releasing of inflammatory mediators into the affected lung and airways. This could vindicate its applications on the infectious pulmonary diseases [31].

5.2. Antimicrobic Effects. For the antibacterial effect, the study showed both aqueous and ethanol extracts of PAR exerted an intermediate antimicrobial effect. Besides, PAR extracts had a slightly better effect on gram-positive bacteria than gram-negative one because of different sensitivity. The most sensitive bacteria is Streptococcus pyogenes [33]. For the bacteria in the oral cavity, PC could have a strong inhibitory effect on Porphyromonas gingivalis; moderate inhibitory effect on Streptococcus mutans; partial effect on Streptococcus sanguis; no effect on Streptococcus mitis [34]. Propionibacterium acnes which are the culprit for acne is active to PC which is one of the crude herbs in the clinical trial and the second best herb in terms of antiacne activity in this study [39]; Mycoplasma hominis which causes infections on humans genital tracts and respiratory tracts is susceptive to PC, and the susceptibility rate is 93% [35]. Salmonellosis which is responsible for food poisoning is vulnerable to PC extract because it can lower the IgG levels and induce TNF-α expression in RAW264.7 cells [36]. In case of PAR, berberine could restrain the bacterial adhesion of Staphylococcus aureus and intracellular invasion into human gingival fibroblasts [37]. Moreover, berberine could attenuate the aminoglycoside resistance of P. aeruginosa, A. xylosoxidans, and B. cepacia in the MexXY-dependent manner. It also inhibits MexXY- or MexVW-mediated resistance of P. aeruginosa mutants, synergistically restrains MexXY-mediated gentamicin resistance in P. aeruginosa mutants, and enhances the synergistic effect of piperacillin and amikacin in

Table 1: Summary of chemical constituents isolated from PC and its different species (140 compounds).

Compound derivatives	Chemical compounds	Methods	References	Original species
Alkaloid	Berberine	(i) UPLC-ESI-Q-TOF-MS	(i) [8]	(i) PAC
		(ii) HPLC-DAD-ESI-MS^2	(ii) [9]	(ii) PCS and PAR
		(iii) UPLC-Q/TOF-HDMS	(iii) [10]	(iii) PAC
		(iv) HPLC-TLC- NMR-EI-MS	(iv) [11]	(iv) PAR
		(v) HPLC-DAD-MS	(v) [12]	(v) PAR
		(vi) N/A	(vi) [13]	(vi) PAC and PCS
	8,13-dioxo-14-butoxycanadine	CC	[14]	PCS
	Berberrubine	UPLC-ESI-Q-TOF-MS	[8]	PAC
	Berberastine	UPLC-Q/TOF-HDMS	[10]	PAC
	Bis-[4-(dimethylamino)phenyl]methanone	HPLC-ESI-MS/MS	[15]	PAR
	Brucine	CE	[16]	PCS
	Δ^7-dehydrosophoramine	N/A	[17]	PAC
	Dihydrocyclobuxine-D	HPLC-ESI-MS/MS	[15]	PAR
	3,4-dihydro-1-[(4-hydroxyphenyl)methyl]-7-methoxy-2-methyl-8-isoquinolinol	HPLC-ESI-MS/MS	[15]	PAR
	3,4-dihydro-1-[(4-hydroxyphenyl)methyl]-7-methoxy-2-methyl-6-isoquinolinol	HPLC-ESI-MS/MS	[15]	PAR
	7,8-dihydroxyrutaecarpine	HPLC-ESI-MS/MS	[15]	PAR
	4-dimethylamino-4 -isopropylbenzene	HPLC-ESI-MS/MS	[15]	PAR
	Evodiamine	HPLC-ESI-MS/MS	[15]	PAR
	Palmatine	(i) UPLC-ESI-Q-TOF-MS	(i) [8]	(i) PAC
		(ii) HPLC-DAD-ESI-MS^2	(ii) [9]	(ii) PCS and PAR
		(iii) UPLC-Q/TOF-HDMS	(iii) [10]	(iii) PCC
		(iv) HPLC-DAD-MS	(iv) [12]	(iv) PAR
		(v) N/A	(v) [18]	(v) PCS
	Tetrahydropalmatine	UPLC-ESI-Q-TOF-MS	[8]	PAC
	Tetrahydroberberine	HPLC-ESI-MS/MS	[15]	PAR
	Phellodendrine	(i) UPLC-ESI-Q-TOF-MS	(i) [8]	(i) PAC
		(ii) HPLC-DAD-ESI-MS^2	(ii) [9]	(ii) PCS and PAR
		(iii) UPLC-Q/TOF-HDMS	(iii) [10]	(iii) PAC
		(iv) HPLC-DAD-MS	(iv) [12]	(iv) PAR
		(v) N/A	(v) [18]	(v) PAC and PCS

Table 1: Continued.

Compound derivatives	Chemical compounds	Methods	References	Original species
	Magnocurarine	(i) HPLC-ESI-MS/MS	(i) [15]	(i) PAR
	Magnoflorine	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PCC
		(iii) HPLC-DAD-MS	(iii) [12]	(iii) PAR
		(iv) N/A	(iv) [19]	(iv) PCS
	Jatrorrhizine or Neprotin, 2,9,10-Trimethoxy-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-3-ol	(i) UPLC-ESI-Q-TOF-MS	(i) [8]	(i) PAC
		(ii) HPLC-DAD-ESI-MS^2	(ii) [9]	(ii) PCS and PAR
		(iii) HPLC-DAD-MS	(iii) [12]	(iii) PAR
		(iv) N/A	(iv) [19]	(iv) PAC
	13-methoxyjatrorrhizine	HPLC-ESI-MS/MS	[15]	PAR
	Y-Fagarine	CC	[20]	PAR
	Canthin-6-one	CC	[20]	PAR
	4-methoxy-N-methyl-2-quinolone	CC	[20]	PAR
	Oxypalmatine	CC	[20]	PAR
	Candicine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Lotusine	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PAC
	N-methylhigenamine-7-O-glucopyranoside	HPLC-ESI-MS/MS	[15]	PAR
	N-methylhigenamine-7-O-β-D-glucopyranoside	HPLC-DAD-ESI-MS^2	[9]	PAR
	(−)-Oblongine	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PCC
	Isomer-of-berberine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Isomer-of-magnoflorine	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
	Isomer-of-palmatine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Tetrahydroreticuline	HPLC-DAD-ESI-MS^2	[9]	PAR
	Tetrahydrojatrorrhizine	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
	Menisperine	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PCC
		(iii) N/A	(iii) [19]	(iii) PAC
	(+) N-methylcorydine	HPLC-DAD-ESI-MS^2	[9]	PAR
	N-methyl Tetrahydropalmatine	HPLC-ESI-MS/MS	[15]	PAR
	N-methylflindersine	HPLC-ESI-MS/MS	[15]	PAR
	Litcubine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Hydroxyl-palmatine	HPLC-DAD-ESI-MS^2	[9]	PAR
	11-hydroxylpalmatine	HPLC-ESI-MS/MS	[15]	PAR
	13-hydroxypalmatine	HPLC-ESI-MS/MS	[15]	PAR
	7-hydroxy-8-methoxydedihydrorutaecarpine	HPLC-ESI-MS/MS	[15]	PAR
	Tetrahydropalmatine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Xanthoplanine	HPLC-DAD-ESI-MS^2	[9]	PAR
	N-methylphoebine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Columbamine	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
	Dihydroxyl-jatrorrhizine	HPLC-DAD-ESI-MS^2	[9]	PAR
	Epiberberine	HPLC-DAD-ESI-MS^2	[9]	PAR

Table 1: Continued.

Compound derivatives	Chemical compounds	Methods	References	Original species
	(6aS)-1,2,10,11-tetramethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4H-dibenzo[de, g] quinolinium	UPLC-Q/TOF-HDMS	[10]	PCC
	Dasycarpamin	UPLC-Q/TOF-HDMS	[10]	PCC
	Pteleine	HPLC-UV	[21]	PAR
	(-)-(R)-platydesmin	HPLC-UV	[21]	PAR
	Noroxyhydrastinine	HPLC-UV	[21]	PAR
	Chilenine	HPLC-UV	[21]	PAR
	Rutecarpine	HPLC-ESI-MS/MS	[15]	PAR
	Skimmianine	HPLC-ESI-MS/MS	[15]	PAR
	Tembetarine	HPLC-ESI-MS/MS	[15]	PAR
	Tetramethyl-O-scutellarin	UPLC-Q/TOF-HDMS	[10]	PCC
	5,8,13,13a-Tetrahydro-2,9,10,11-tetrahydroxy-3-methoxy-7-methyl-6H-dibenzo[a,g]quinolizinium	HPLC-ESI-MS/MS	[15]	PAR
	ϒ-hydroxybutenolide derivatives II	UPLC-Q/TOF-HDMS	[10]	PCC

Table 1: Continued.

Compound derivatives	Chemical compounds	Methods	References	Original species
Isoquinoline alkaloid	Armepavine	UPLC-ESI-Q-TOF-MS	[8]	PAC
	Demethyleneberberine	UPLC-ESI-Q-TOF-MS	[8]	PAC
	8-oxoberberine	HPLC-ESI-MS/MS	[15]	PAR
	8-oxoepiberberine	HPLC-ESI-MS/MS	[15]	PAR
	8-oxopalmatine	HPLC-ESI-MS/MS	[15]	PAR
	Oxyberberine	HPLC	[20]	PAR
	Oxypalmatine	HPLC	[20]	PAR
Limonoid	Kihadanin B	N/A	[19]	PAC
	Niloticin	N/A	[18]	PAC
	Niloticin acetate	N/A	[18]	PCS
	Obaculactone or limonin	(i) UPLC-ESI-Q-TOF-MS	(i) [8]	(i) PAC
		(ii) CC	(ii) [20]	(ii) PAR
		(iii) UPLC-Q/TOF-HDMS	(iii) [10]	(iii) PCC
		(iv) HPLC-TLC- NMR- EI-MS	(iv) [11]	(iv) PAC
		(v) N/A	(v) [18]	(v) PAC
	Derivative of obaculactone	UPLC-Q/TOF-HDMS	[10]	PCC
	Obacunone or Obacunoic acid	(i) CC	(i) [20]	(i) PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PCC
		(iii) HPLC-TLC- NMR-EI-MS	(iii) [11]	(iii) PAC
		(iv) HPLC-DAD-ESI-MS^2	(iv) [9]	(iv) PAR
		(v) N/A	(v) [18]	(v) PAC
	12α-hydroxylimonin	CC	[20]	PAR
	Piscidinol A	N/A	[18]	(i) PCS
	Rutaevin	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PCC
	Coniferin	HPLC-DAD-ESI-MS^2	[9]	PAR
	Vanilloloside	HPLC-DAD-ESI-MS^2	[9]	PAR
	N-methyltetrahydrocolumbamine	UPLC-Q/TOF-HDMS	[10]	PCC
N-acyl amines	Herculin	N/A	[19]	PAC

Table 1: Continued.

Compound derivatives	Chemical compounds	Methods	References	Original species
Phenolic acid	Ferulic acid	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) HPLC-TLC- NMR- EI-MS	(ii) [11]	(ii) PAC
	Methyl ferulate	CC	[4]	PCS
	Protocatechuic acid	CC	[4]	PCS
Quinic acid	Quinic acid	UPLC-Q/TOF-HDMS	[10]	PAC
	Neo-chlorogenic acid	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
	3-O-feruloylquinic acid	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PAC
	3-O-feruloylquinic acid glucoside	UPLC-Q/TOF-HDMS	[10]	PCC
	4-O-feruloylquinic acid	HPLC	[22]	PCS
	5-O-feruloylquinic acid	HPLC	[22]	PCS
	Chlorogenic acid	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PAC
		(iii) HPLC	(iii) [23]	(iii) PAR
		(iv) HPLC-DAD-MS	(iv) [12]	(iv) PAR
	Methyl 3-*O*-feruloylquinate	HPLC-DAD-ESI-MS^2	[9]	PAR
	Methyl 5-O-feruloylquinate	NMR	[24]	PAR
	3-Feruoyl-4-caffeoylquinic acid	HPLC-DAD-ESI-MS^2	[9]	PAR
	Sanleng acid	NMR	[25]	PAC
Hydroxycinnamic acid	Caffeic Acid Methyl Ester	HPLC	[22]	PCS
Phytosterol	β-Sitosterol	N/A	[18]	PAC
Lignan	(+/-)-lyoniresinol	HPLC-DAD-ESI-MS^2	[9]	PAR
	(+/-)-5,5′-dimethoxylariciresinol-4-*O*-glucoside	(i) HPLC-DAD-ESI-MS^2	(i) [9]	(i) PCS and PAR
		(ii) UPLC-Q/TOF-HDMS	(ii) [10]	(ii) PCC
	Syringaresinol di-O-β-D-glucopyranoside	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
	(-)-Syringaresinol	CC	[4]	PCS
Flavonoid	Amurensin	N/A	[13]	PAC
	Quercetin	HPLC-TLC- NMR- EI-MS	[11]	PAC
	Phellamurin	N/A	[18]	PAC
	Phellatin	N/A	[18]	PAC
	Phellavin	N/A	[18]	PAC
	Phellodendroside	N/A	[18]	PAC
	β-anhydronoricaritin	UV	[26]	PAR
	Icariside-1	UV	[26]	PAR
	Phellamuretin	UV	[26]	PAR
	Phelloside	UV	[26]	PAR
	Dihydrophelloside	UV	[26]	PAR
	Isovaleric acid	UV	[26]	PAR
	Kaempferol	UV	[26]	PAR
	D-glucose	UV	[26]	PAR
Rutaceae	Noricariside	N/A	[18]	PAC
Stigmastane	7-Dehydrostigmasterol	N/A	[17]	PAC
Coumarin	3-acetyl-3,4-dihydro-5,6-dimethoxy-1H-2-benzopyran-1-one	CC	[27]	PCS

Table 1: Continued.

Compound derivatives	Chemical compounds	Methods	References	Original species
Monosaccharide	Syringin	NMR	[25]	PAC
	Daucosterol	NMR	[25]	PAC
Paraben	N-propyl paraben	HPLC	[5]	PC
Phenolic lactone	Phellolactone	CC	[4]	PCS
Ferulate	N-butyl Ferulate	CC	[4]	PCS
	Amurenlactone A	CC	[4]	PCS
	Amurenamide A	NMR	[25]	PAC
Hydroxybenzaldehyde	4-hydroxybenzaldehyde	CC	[4]	PCS
Phenolic aldehyde	Vanillin	CC	[4]	PCS
Glycoside	Sinapyl aldehyde-4-O-beta-D-glucopyranoside	NMR	[24]	PAR
	3,4,5-Trimetoxyphenol O-β-D-glucopyranoside	HPLC	[22]	PCS
	2-methoxy-4-(2-propenyl)phenyl-beta-D-glucopyranoside	HPLC	[22]	PCS
	2-(p-hydroxyphenyl)-ethanol-1-O-β-D-apiofuranosyl (1–6)-O-β-D-glucopyranoside	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
	2-(p-hydroxyphenyl) -ethanol-1-O-β-D-glucoside	UPLC-Q/TOF-HDMS	[10]	PCC
	3, 5-dihydroxybenzoicacid-O-xylopyranosyl-glucopyranoside	HPLC-DAD-ESI-MS^2	[9]	PCS and PAR
Phenolic glycoside	Tachioside	HPLC	[22]	PCS
Glucoside	Salidroside	HPLC	[22]	PCS
	4-hydroxybenzyl alcohol	HPLC	[22]	PCS
Phenol	Methyl syringate	HPLC	[22]	PCS
Dehydrovomifoliol	(6S)-dehydrovomifoliol	HPLC	[22]	PCS
	(6R,7aR)-epiloliolide	HPLC	[22]	PCS

PAC: phellodendri amurensis cortex
PCS: phellodendron chinense schneid
PAR: phellodendron amurense rupr.
PCC: phellodendri Chinensis cortex
UPLC-ESI-Q-TOF-MS: ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry
CC: column chromatography
CE: capillary electrophoresis
HPLC/MSD: high-performance liquid chromatography coupled with mass spectrometric detection
UPLC–Qthe -TOF-MS: ultra-performance liquid chromatography with quadrupole TOF-MS
HPLC– ESI-MS2: high-performance liquid chromatography with electrospray ionization mass spectrometry coupled with photodiode array detection
UPLC-Q/TOF-HDMS: ultra-performance liquid chromatography-quadrupole/time-of-flight high-definition mass spectrometry
UV: UV detection
EI-MS: Electron ionization mass spectrometry
NMR: Nuclear magnetic resonance
TLC: Thin-layer chromatography
HPLC-DAD-MS: high-performance liquid chromatography coupled with diode array detection and mass spectrometry

Table 2: Molecular formula, molecular weight and chemical structures of compounds derived from PCC species (18 compounds).

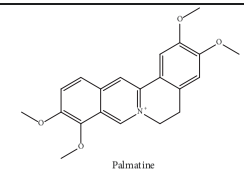
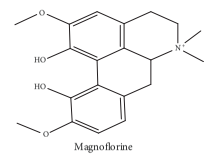
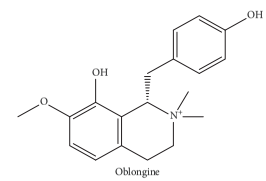
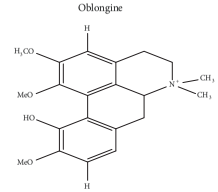
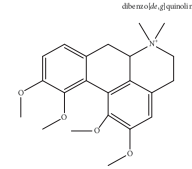
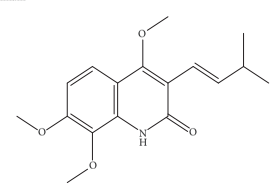
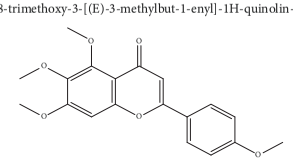
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Alkaloid	Palmatine	$C_{21}H_{22}NO_4^+$	352.41 g/mol	 Palmatine
	Magnoflorine	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 Magnoflorine
	(−)-Oblongine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 Oblongine
	Menisperine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 11-hydroxy-1,2,10-trimethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinolin-6-ium
	(6aS)-1,2,10,11-tetramethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4H-dibenzo[de, g] quinolinium	$C_{22}H_{28}NO_4$	370.47 g/mol	 (6aS)-1,2,10,11-tetramethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4H-dibenzo[de, g] quinolinium
	Dasycarpamin	$C_{17}H_{21}NO_4$	303.36 g/mol	 4,7,8-trimethoxy-3-[(E)-3-methylbut-1-enyl]-1H-quinolin-2-one
	Tetramethyl-O-scutellarin	$C_{19}H_{18}O_6$	342.35 g/mol	 5,6,7-trimethoxy-2-(4-methoxyphenyl)chromen-4-one

Table 2: Continued.

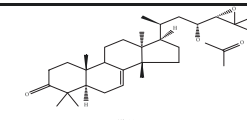
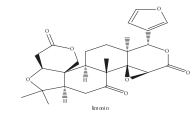
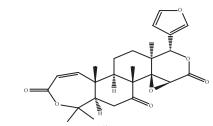
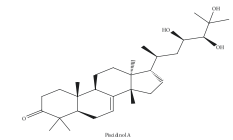
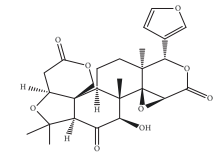
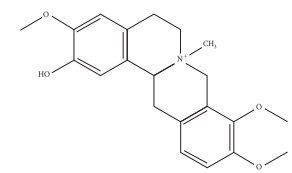
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Y-hydroxybutenolide derivatives II			N/A
Limonoid	Niloticin acetate	$C_{32}H_{50}O_4$	498.75 g/mol	
	Obaculactone or limonin	$C_{26}H_{30}O_8$	470.52 g/mol	
	Derivative of obaculactone			N/A
	Obacunone or Obacunoic acid	$C_{26}H_{30}O_7$	454.519 g/mol	
	Piscidinol A	$C_{30}H_{50}O_4$	474.73 g/mol	
	Rutaevin	$C_{26}H_{30}O_9$	486.52 g/mol	
	N–methyltetrahydrocolumbamine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 2-hydroxy-3,9,10-trimethoxy-7-methyl-5,6,7,8,13,13a-hexahydroisoquinolino[3,2-*a*]isoquinolin-7-ium

Table 2: Continued.

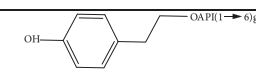
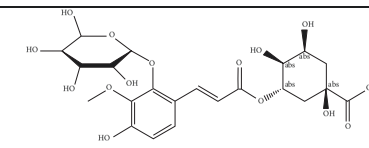
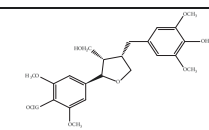
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Phenolic acid	2-(p-hydroxyphenyl) -ethanol-1-O-β-D-glucoside	$C_9H_{12}O$	136.19 g/mol	 2-(p-hydroxyphenyl)-ethanol-1-O-beta-D-apiofuranosyl (1-6)-O-beta-D-glucopyranoside
Quinic acid	3-O-feruloylquinic acid glucoside	$C_{22}H_{28}O_{15}$	532.45 g/mol	 3-O-feruloylquinic acid glucoside
Lignan	(+/-)-5,5′-dimethoxylariciresinol-4-O-glucoside	$C_{28}H_{38}O_{13}$	582.6 g/mol	 (+/-)-5,5′-dimethoxylariciresinol-4-O-glucoside

Table 3: Molecular formula, molecular weight and chemical structures of compounds derived from PCS species (44 compounds).

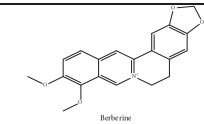
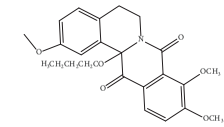
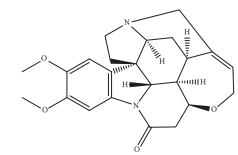
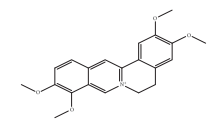
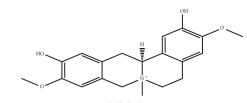
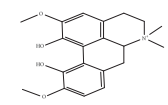
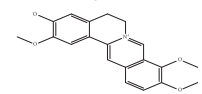
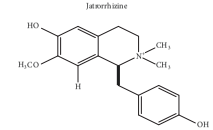
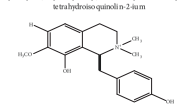
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Alkaloid	Berberine	$C_{20}H_{18}NO_4^+$	336.37 g/mol	 Berberine
	8,13-dioxo-14-butoxycanadine	$C_{24}H_{25}NO_7$	439.46 g/mol	 13a-butoxy-9,10-dimethoxy-5,13a-dihydro-6*H*-[1,3]dioxolo[4,5-*g*]isoquinolino[3,2-*a*]isoquinoline-8,13-dione
	Brucine	$C_{23}H_{26}N_2O_4$	394.47 g/mol	 Brucine
	Palmatine	$C_{21}H_{22}NO_4^+$	352.41 g/mol	 Palmatine
	Phellodendrine	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 Phellodendrine
	Magnoflorine	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 Magnoflorine
	Jatrorrhizine or Neprotin, 2,9,10-Trimethoxy-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-3-ol	$C_{20}H_{19}NO_4$	337.38 g/mol	 Jatrorrhizine
	Lotusine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 (*S*)-6-hydroxy-1-(4-hydroxybenzyl)-7-methoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolin-2-ium
	(−)-Oblongine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 (*S*)-8-hydroxy-1-(4-hydroxybenzyl)-7-methoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolin-2-ium

Table 3: Continued.

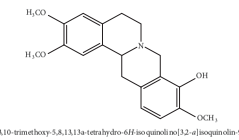
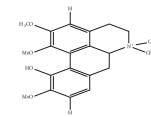
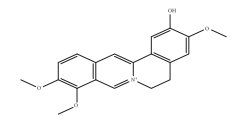
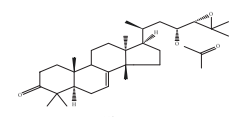
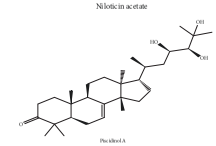
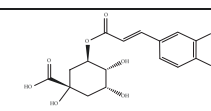
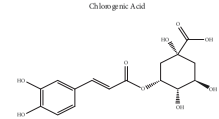
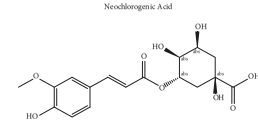
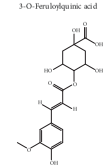
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Isomer-of-magnoflorine			N/A
	Tetrahydrojatrorrhizine	$C_{20}H_{23}NO_4$	341.41 g/mol	 2,3,10-trimethoxy-5,8,13,13a-tetrahydro-6*H*-iso-quinolino[3,2-*a*]isoquinolin-9-ol
	Menisperine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 11-hydroxy-1,2,10-trimethoxy-6*α*-dimethyl-5,6*α*,7-tetrahydro-4*H*-dibenzo[*d*,*fg*]quinolin-6-ium
	Columbamine	$C_{20}H_{20}NO_4^+$	338.38 g/mol	 2-hydroxy-3,9,10-trimethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium
	Niloticin acetate	$C_{32}H_{50}O_4$	498.75 g/mol	 Niloticin acetate
	Piscidinol A	$C_{30}H_{50}O_4$	474.73 g/mol	 Piscidinol A
Quinic acid	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31 g/mol	 Chlorogenic Acid
	Neo-chlorogenic acid	$C_{16}H_{18}O_9$	354.31 g/mol	 Neochlorogenic Acid
	3-O-feruloylquinic acid	$C_{17}H_{20}O_9$	368.34 g/mol	 3-O-Feruloylquinic acid
	4-O-feruloylquinic acid	$C_{17}H_{20}O_9$	368.34 g/mol	 (1*S*,3*S*,4*S*,5*R*)-3,4,5-trihydroxy-1-[(3-(4-hydroxy-3-methoxyphenyl)acryloyl)oxy]cyclohexane-1-carboxylic acid

Table 3: Continued.

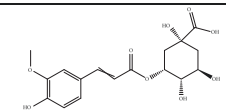
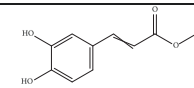
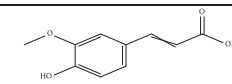
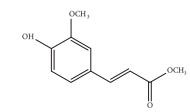
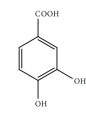
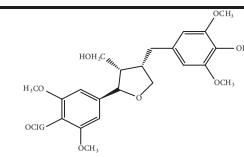
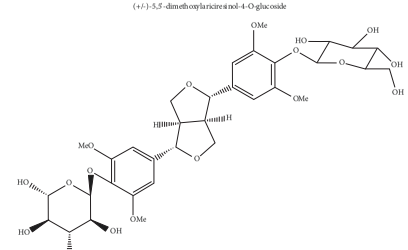
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	5-O-feruloylquinic acid	$C_{17}H_{20}O_9$	368.34 g/mol	 (1*R*,3*R*,4*S*,5*R*)-1,3,4-trihydroxy-5-{(3-(4-hydroxy-3-methoxyphenyl)acryloyl]oxy}cyclohexane-1-carboxylic acid
Hydroxycinnamic acid	Caffeic Acid Methyl Ester	$C_{10}H_{10}O_4$	194.19 g/mol	 Caffeic Acid Methyl Ester
Phenolic acid	Ferulic acid	$C_{10}H_{10}O_4$	194.19 g/mol	 Ferulic acid
	Methyl ferulate	$C_{11}H_{12}O_4$	208.21 g/mol	 methyl (*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylate
	Protocatechuic acid	$C_7H_6O_4$	154.12 g/mol	 3,4-dihydroxybenzoic acid
Lignan	(+/-)-5,5′-dimethoxylariciresinol-4-*O*-glucoside	$C_{28}H_{38}O_{13}$	582.6 g/mol	 (+/-)-5,5′-dimethoxylariciresinol-4-O-glucoside
	Syringaresinol di-O-β-D-glucopyranoside	$C_{33}H_{44}O_{18}$	728.70 g/mol	 (2*R*,3*R*,4*R*,5*S*,6*R*)-6-(4-((1*S*,3a*R*,4*S*,6a*R*)-4-(3,5-dimethoxy-4-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)tetrahydro-1*H*,3*H*-furo[3,4-*c*]furan-1-yl)-2,6-dimethoxyphenoxy)tetrahydro-2*H*-pyran-2,3,4,5-tetraol

Table 3: Continued.

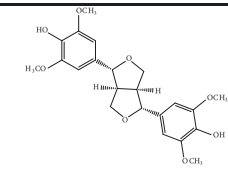
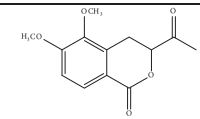
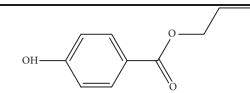
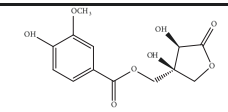
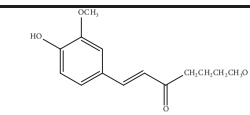
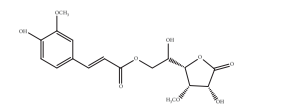
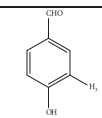
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	(-)-syringaresinol	$C_{22}H_{26}O_8$	418.44 g/mol	 4,4′-{(1*R*,3a*S*,4*R*,6a*S*)-tetrahydro-1*H*,3*H*-furo[3,4-*c*]furan-1,4-diyl}bis(2,6-dimethoxyphenol)
Coumarin	3-acetyl-3,4-dihydro-5,6-dimethoxy-1H-2-benzopyran-1-one	$C_{13}H_{14}O_5$	250.25 g/mol	 3-acetyl-5,6-dimethoxyisochroman-1-one
Paraben	N-propyl paraben	$C_{10}H_{12}O_3$	180.20 g/mol	 propyl 4-hydroxybenzoate
Phenolic lactone	Phellolactone	$C_{13}H_{14}O_8$	298.25 g/mol	 ((3*R*,4*R*)-3,4-dihydroxy-5-oxotetrahydrofuran-3-yl)methyl 4-hydroxy-3-methoxybenzoate
Ferulate	N-butyl Ferulate	$C_{14}H_{18}O_4$	250.29 g/mol	 (*E*)-4-(3,7-dioxo-7λ^6-hept-1-en-1-yl)-1-hydroxy-2-methoxybenzene
	Amurenlactone A	$C_{17}H_{20}O_9$	368.34 g/mol	 (*R*)-2-hydroxy-2-((2*S*,3*S*,4*R*)-4-hydroxy-3-methoxy-5-oxotetrahydrofuran-2-yl)ethyl (*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylate
Hydroxybenzaldehyde	4-hydroxybenzaldehyde	$C_7H_8O_2$	124.14 g/mol	 4-hydroxybenzaldehyde compound with dihydrogen (1:1)

Table 3: Continued.

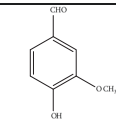
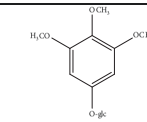
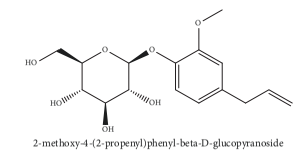
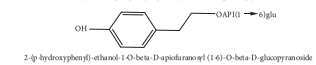
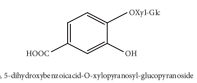
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Phenolic aldehyde	Vanillin	$C_8H_8O_3$	152.15 g/mol	 4-hydroxy-3-methoxybenzaldehyde
Glycoside	3,4,5-trimetoxyphenol O-β-D-glucopyranoside	$C_9H_{11}O_3$	167.18 g/mol	 3,4,5-Trimethoxyphenol O-ß-D-glucopyranoside
	2-methoxy-4-(2-propenyl)phenyl-beta-D-glucopyranoside	$C_{16}H_{22}O_7$	326.35 g/mol	 2-methoxy-4-(2-propenyl)phenyl-beta-D-glucopyranoside
	2-(p-hydroxyphenyl)-ethanol-1-O-β-D-apiofuranosyl (1–6)-O-β-D-glucopyranoside	$C_9H_{12}O$	136.19 g/mol	 2-(p-hydroxyphenyl)-ethanol-1-O-beta-D-apiofuranosyl (1-6)-O-beta-D-glucopyranoside
	3, 5-dihydroxybenzoicacid-O-xylopyranosyl-glucopyranoside	$C_8H_8O_3$	152.15 g/mol	 3, 5-dihydroxybenzoicacid-O-xylopyranosyl-glucopyranoside

Table 3: Continued.

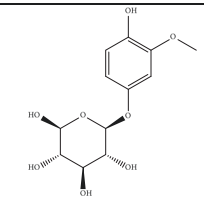
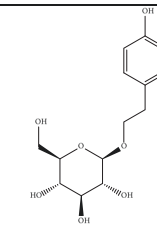
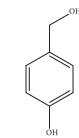
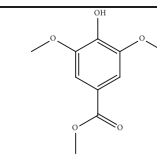
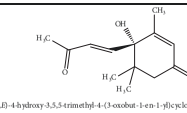
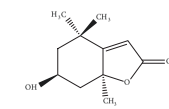
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Phenolic glycoside	Tachioside	$C_{12}H_{16}O_8$	288.25 g/mol	 (2*S*,3*S*,4*S*,5*R*,6*R*)-6-(4-hydroxy-3-methoxyphenoxy)tetrahydro-2*H*-pyran-2,3,4,5-tetraol
Glucoside	Salidroside	$C_{14}H_{20}O_7$	300.31 g/mol	 (2*R*,3*S*,4*S*,5*R*,6*R*)-2-(hydroxymethyl)-6-(4-hydroxyphenethoxy)tetrahydro-2*H*-pyran-3,4,5-triol
	4-Hydroxybenzyl alcohol	$C_7H_8O_2$	124.14 g/mol	 4-(hydroxymethyl)phenol
Phenol	Methyl Syringate	$C_{10}H_{12}O_5$	212.20 g/mol	 methyl 4-hydroxy-3,5-dimethoxybenzoate
Dehydrovomifoliol	(6S)-dehydrovomifoliol	$C_{13}H_{18}O_3$	222.28 g/mol	 (5*E*)-4-hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-en-1-one
	(6R,7aR)-epiloliolide	$C_{11}H_{16}O_3$	196.25 g/mol	 (6*S*,7a*S*)-6-hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4*H*)-one

Table 4: Molecular formula, molecular weight and chemical structures of compounds derived from PAC species (34 compounds).

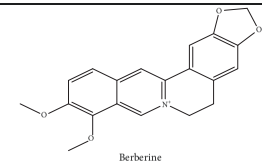
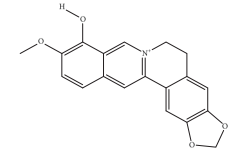
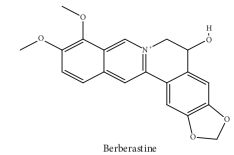
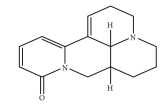
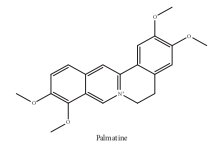
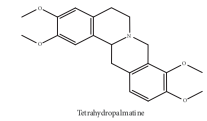
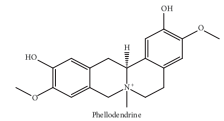
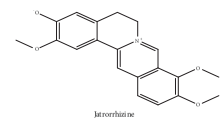
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Alkaloid	Berberine	$C_{20}H_{18}NO_4^+$	336.37 g/mol	 Berberine
	Berberrubine	$C_{19}H_{16}NO_4^+$	322.34 g/mol	 Berberrubine
	Berberastine	$C_{20}H_{18}NO_5^+$	352.37 g/mol	 Berberastine
	Δ^7-Dehydrosophoramine	$C_{15}H_{18}N_2O$	242.32 g/mol	 Delta-7-dehydrosophoramine
	Palmatine	$C_{21}H_{22}NO_4^+$	352.41 g/mol	 Palmatine
	Tetrahydropalmatine	$C_{21}H_{25}NO_4$	355.43 g/mol	 Tetrahydropalmatine
	Phellodendrine	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 Phellodendrine
	Jatrorrhizine or Neprotin, 2,9,10-Trimethoxy-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-3-ol	$C_{20}H_{19}NO_4$	337.38 g/mol	 Jatrorrhizine

Table 4: Continued.

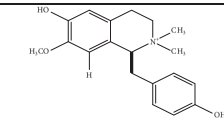
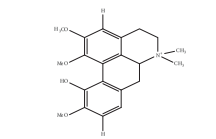
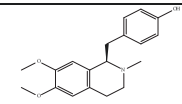
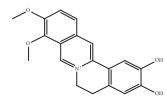
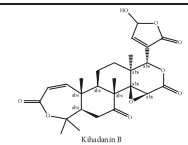
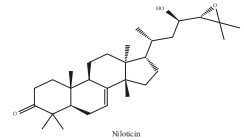
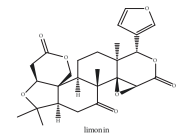
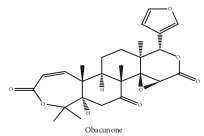
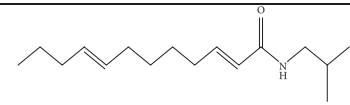
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Lotusine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 (S)-6-hydroxy-1-(4-hydroxybenzyl)-7-methoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolin-2-ium
	Menisperine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 11-hydroxy-1,2,10-trimethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinolin-6-ium
Isoquinoline alkaloid	Armepavine	$C_{19}H_{23}NO_3$	313.40 g/mol	 Armepavine
	Demethyleneberberine	$C_{19}H_{18}NO_4^+$	324.36 g/mol	 Demethyleneberberine
Limonoid	Kihadanin B	$C_{26}H_{30}O_9$	486.52 g/mol	 Kihadanin B
	Niloticin	$C_{30}H_{48}O_3$	456.71 g/mol	 Niloticin
	Obaculactone or limonin	$C_{26}H_{30}O_8$	470.52 g/mol	 limonin
	Obacunone or Obacunoic acid	$C_{26}H_{30}O_7$	454.52 g/mol	 Obacunone
N-acyl amines	Herculin	$C_{16}H_{29}NO$	251.41 g/mol	 (2E,8E)-N-(2-methylpropyl)dodeca-2,8-dienamide

Table 4: Continued.

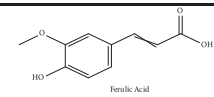
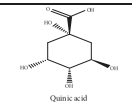
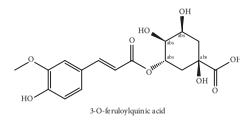
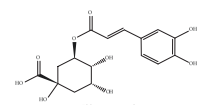
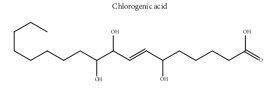
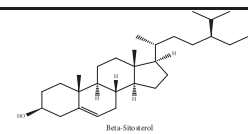
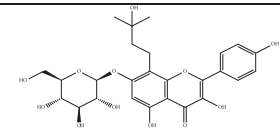
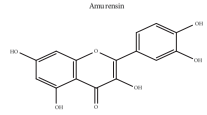
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Phenolic acid	Ferulic acid	$C_{10}H_{10}O_4$	194.19 g/mol	 Ferulic Acid
Quinic acid	Quinic acid	$C_7H_{12}O_6$	192.17 g/mol	 Quinic acid
	3-O-feruloyl quinic acid or 5-O-feruloyl quinic acid	$C_{17}H_{20}O_9$	368.34 g/mol	 3-O-feruloylquinic acid
	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31 g/mol	 Chlorogenic acid
	Sanleng acid	$C_{18}H_{34}O_5$	330.47 g/mol	 (E)-6,9,10-trihydroxyoctadec-7-enoic acid
Phytosterol	β-sitosterol	$C_{29}H_{50}O$	414.72 g/mol	 Beta-Sitosterol
Flavonoid	Amurensin	$C_{26}H_{30}O_{12}$	534.51 g/mol	 Amurensin
	Quercetin	$C_{15}H_{10}O_7$	302.24 g/mol	 Quercetin

Table 4: Continued.

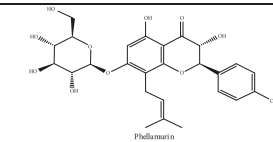
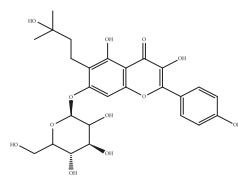
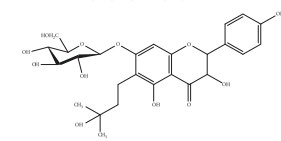
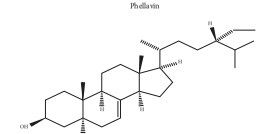
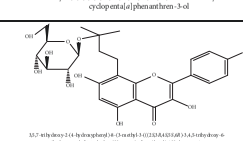
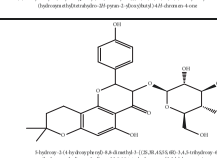
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Phellamurin	$C_{26}H_{30}O_{11}$	518.52 g/mol	 Phellamurin
	Phellatin	$C_{26}H_{30}O_{12}$	534.51 g/mol	 3,5-dihydroxy-6-(3-hydroxy-3-methylbutyl)-2-(4-hydroxyphenyl)-7-[(2*S*,4*S*,5*S*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one
	Phellavin	$C_{26}H_{32}O_{12}$	536.53 g/mol	 Phellavin
	7-dehydrostigmasterol	$C_{29}H_{50}O$	414.72 g/mol	 (3*S*,5*S*,9*R*,10*S*,13*R*,14*R*,17*R*)-17-((2*R*,5*S*)-5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,5,6,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol
Rutaceae	Noricariside	$C_{26}H_{30}O_{12}$	534.51 g/mol	 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8-(3-methyl-3-((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)butyl)-4*H*-chromen-4-one
Stigmastane	Phellodendroside	$C_{26}H_{30}O_{11}$	518.52 g/mol	 5-hydroxy-2-(4-hydroxyphenyl)-8,8-dimethyl-3-((2*R*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yloxy)-2,3,9,10-tetrahydropyrano[2,3-*b*]chromen-4-one

Table 4: Continued.

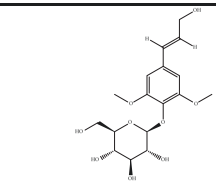
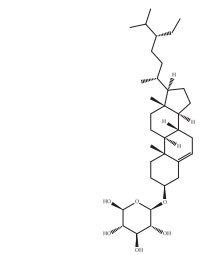
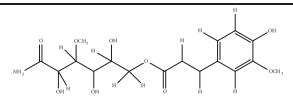
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Monosaccharide	Syringin	$C_{17}H_{24}O_9$	372.37 g/mol	 (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(hydroxymethyl)-6-{4-[(*E*)-3-hydroxyprop-1-en-1-yl]-2,6-dimethoxyphenoxy}tetrahydro-2*H*-pyran-3,4,5-triol
	Daucosterol	$C_{34}H_{58}O_6$	562.83 g/mol	 (2*S*,3*R*,4*S*,5*S*,6*R*)-6-(((3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-((2*R*,5*R*)-5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl)oxy)tetrahydro-2*H*-pyran-2,3,4,5-tetraol
Ferulate	Amurenamide A	$C_{17}H_{25}NO_9$	387.39 g/mol	 6-amino-2,3,5-trihydroxy-4-methoxy-6-oxohexyl 3-(4-hydroxy-3-methoxyphenyl)propanoate

Table 5: Molecular formula, molecular weight and chemical structures of compounds derived from PAR species (84 compounds).

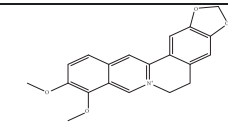
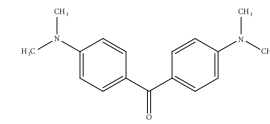
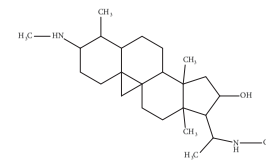
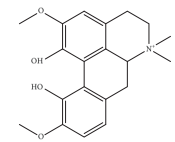
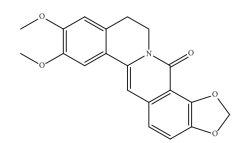
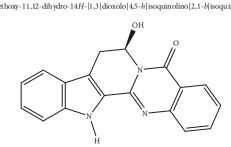
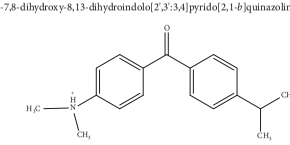
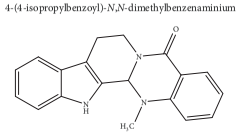
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
Alkaloid	Berberine	$C_{20}H_{18}NO_4^+$	336.37 g/mol	 Berberine
	Bis-[4-(dimethylamino)phenyl]methanone	$C_{17}H_{20}N_2O$	268.36 g/mol	 bis(4-(dimethylamino)phenyl)methanone
	Dihydrocyclobuxine-D	$C_{25}H_{44}N_2O$	388.64 g/mol	 2a,5a,8-trimethyl-9-(methylamino)-3-(1-(methylamino)ethyl)tetradecahydro-1*H*,12*H*-cyclopenta[*a*]cyclopropa[*e*]phenanthren-4-ol
	3,4-Dihydro-1-[(4-hydroxyphenyl)methyl]-7-methoxy-2-methyl-8-isoquinolinol	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 1,11-dihydroxy-2,10-dimethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinolin-6-ium
	3,4-Dihydro-1-[(4-hydroxyphenyl)methyl]-7-methoxy-2-methyl-6-isoquinolinol	$C_{20}H_{17}NO_5$	351.36 g/mol	 8,9-dimethoxy-11,12-dihydro-14*H*-[1,3]dioxolo[4,5-*h*]isoquinolino[2,1-*b*]isoquinolin-14-one
	7,8-Dihydroxyrutaecarpine	$C_{18}H_{13}N_3O_3$	319.32 g/mol	 (7*R*)-7,8-dihydroxy-8,13-dihydroindolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one
	4-Dimethylamino-4 -isopropylbenzene	$C_{18}H_{22}NO^+$	268.38 g/mol	 4-(4-isopropylbenzoyl)-*N*,*N*-dimethylbenzenaminium
	Evodiamine	$C_{19}H_{17}N_3O$	303.37 g/mol	 14-methyl-8,13,13b,14-tetrahydroindolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one

Table 5: Continued.

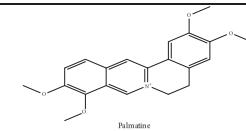
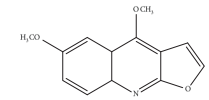
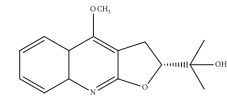
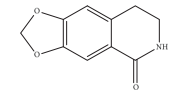
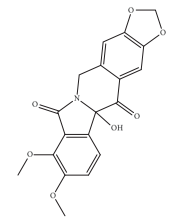
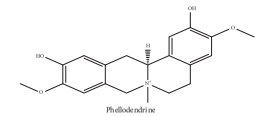
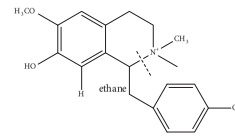
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Palmatine	$C_{21}H_{22}NO_4^+$	352.41 g/mol	 Palmatine
	Pteleine	$C_{13}H_{13}NO_3$	231.25 g/mol	 4,6-dimethoxy-4a,8a-dihydrofuro[2,3-*b*]quinoline
	(-)-(R)-platydesmin	$C_{15}H_{19}NO_3$	261.32 g/mol	 2-((2*R*)-4-methoxy-2,3,4a,8a-tetrahydrofuro[2,3-*b*]quinolin-2-yl)propan-2-ol
	Noroxyhydrastinine	$C_{10}H_9NO_3$	191.19 g/mol	 7,8-dihydro-[1,3]dioxolo[4,5-g]isoquinolin-5(6*H*)-one
	Chilenine	$C_{19}H_{15}NO_7$	369.33 g/mol	 11b-hydroxy-8,9-dimethoxy-[1,3]dioxolo[4,5-g]isoindolo[2,1-*b*]isoquinoline-7,12(5*H*,11b*H*)-dione
	Phellodendrine	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 Phellodendrine
	Magnocurarine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 7-hydroxy-1-(4-hydroxybenzyl)-6-methoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolin-2-ium

Table 5: Continued.

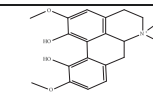
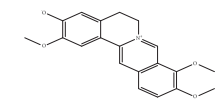
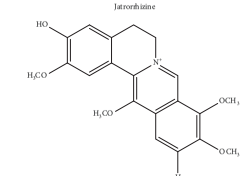
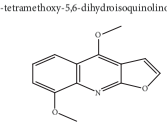
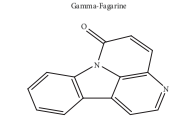
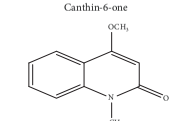
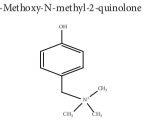
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Magnoflorine	$C_{20}H_{24}NO_4^+$	342.41 g/mol	 Magnoflorine
	Jatrorrhizine or Neprotin, 2,9,10-Trimethoxy-5,6-dihydroisoquinolino[2,1-b]isoquinolin-7-ium-3-ol	$C_{20}H_{19}NO_4^+$	337.38 g/mol	 Jatrorrhizine
	13-Methoxyjatrorrhizine	$C_{21}H_{22}NO_5^+$	368.41 g/mol	 3-hydroxy-2,9,10,13-tetramethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium
	Y-Fagarine	$C_{13}H_{11}NO_3$	229.24 g/mol	 Gamma-Fagarine
	Canthin-6-one	$C_{14}H_8N_2O$	220.23 g/mol	 Canthin-6-one
	4-Methoxy-N-methyl-2-quinolone	$C_{11}H_{11}NO_2$	189.21 g/mol	 4-Methoxy-N-methyl-2-quinolone
	Candicine	$C_{10}H_{16}NO^+$	166.24 g/mol	 1-(4-hydroxyphenyl)-*N*,*N*,*N*-trimethylmethanaminium

Table 5: Continued.

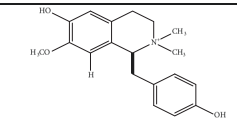
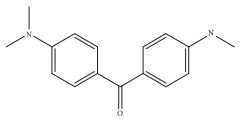
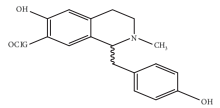
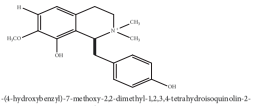
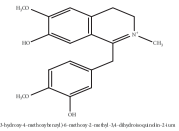
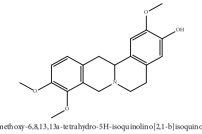
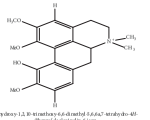
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Lotusine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 (*S*)-6-hydroxy-1-(4-hydroxybenzyl)-7-methoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolin-2-ium
	N-Methylhigenamine-7-O-glucopyranoside	$C_{17}H_{20}N_2O$	268.36 g/mol	 bis(4-(dimethylamino)phenyl)methanone
	N-methylhigenamine-7-O-β-D-glucopyranoside	N/A	N/A	 N-methylhigenamine-7-O-beta-D-glucopyranoside
	(−)-Oblongine	$C_{19}H_{24}NO_3^+$	314.40 g/mol	 (*S*)-8-hydroxy-1-(4-hydroxybenzyl)-7-methoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolin-2-ium
	Isomer-of-berberine Isomer-of-magnoflorine Isomer-of-palmatine		N/A	
	Tetrahydroreticuline	$C_{19}H_{22}NO_4^+$	328.39 g/mol	 7-hydroxy-1-(3-hydroxy-4-methoxybenzyl)-6-methoxy-2-methyl-3,4-dihydroisoquinolin-2-ium
	Tetrahydrojatrorrhizine	$C_{20}H_{23}NO_4$	341.41 g/mol	 2,9,10-trimethoxy-6,8,13,13a-tetrahydro-5H-isoquinolino[2,1-b]isoquinolin-3-ol
	Menisperine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 11-hydroxy-1,2,10-trimethoxy-6,6-dimethyl-5,6,8a,7-tetrahydro-4*H*-dibenzo[de,g]quinolin-6-ium

Table 5: Continued.

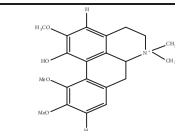
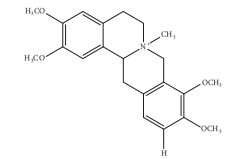
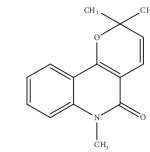
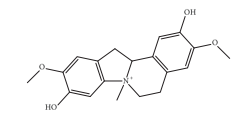
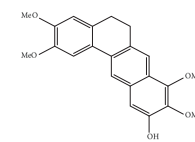
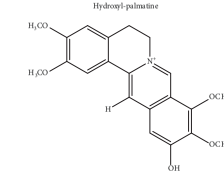
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	(+) N-methylcorydine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 1-hydroxy-2,10,11-trimethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[de,g]quinolin-6-ium
	N-Methyl Tetrahydropalmatine	$C_{22}H_{28}NO_4^+$	370.47 g/mol	 2,3,9,10-tetramethoxy-7-methyl-5,6,7,8,13,13a-hexahydroisoquinolino[3,2-*a*]isoquinolin-7-ium
	N-Methylflindersine	$C_{15}H_{15}NO_2$	241.29 g/mol	 2,2,6-trimethyl-2,6-dihydro-5*H*-pyrano[3,2-*c*]quinolin-5-one
	Litcubine	$C_{19}H_{22}NO_4^+$	328.39 g/mol	 3,10-dimethoxy-7-methyl-5,6,12,12a-tetrahydroindolo[2,3-*a*]isoquinolin-7-ium-2,9-diol Caution: A net charge appears to be present
	Hydroxyl-palmatine	$C_{22}H_{22}O_5$	366.41 g/mol	 Hydroxyl-palmatine
	11-Hydroxylpalmatine	$C_{21}H_{22}NO_5^+$	368.41 g/mol	 11-hydroxy-2,3,9,10-tetramethoxy-5,6-dihydroisoquinolino[3,2-*a*]iso-quinolin-7-ium

Table 5: Continued.

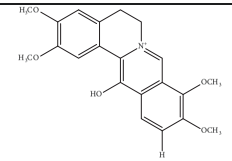
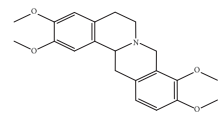
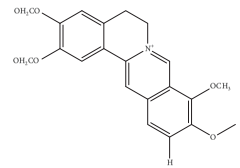
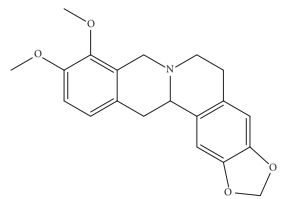
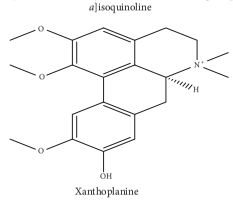
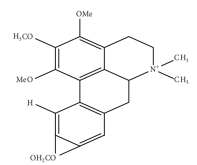
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	13-Hydroxypalmatine	$C_{21}H_{22}NO_5^+$	368.41 g/mol	 13-hydroxy-2,3,9,10-tetramethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium
	7-Hydroxy-8-methoxydedihydrorutaecarpine		N/A	
	Tetrahydropalmatine	$C_{21}H_{25}NO_4$	355.43 g/mol	 Tetrahydropalmatine
	5,8,13,13a-Tetrahydro-2,9,10,11-tetrahydroxy-3-methoxy-7-methyl-6H-dibenzo[a,g]quinolizinium	$C_{21}H_{20}NO_6^+$	382.39 g/mol	 2,3-bis((λ¹-oxidaneyl)methoxy)-9,10-dimethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium
	Tetrahydroberberine	$C_{20}H_{21}NO_4$	339.39 g/mol	 9,10-dimethoxy-5,8,13,13a-tetrahydro-6*H*-[1,3]dioxolo[4,5-*g*]isoquinolino[3,2-*a*]isoquinoline
	Xanthoplanine	$C_{21}H_{26}NO_4^+$	356.44 g/mol	 Xanthoplanine
	N-methylphoebine	$C_{22}H_{26}NO_5^+$	384.45 g/mol	 formaldehyde, 1,2,3-trimethoxy-6,6-dimethyl-5,6,6a,7-tetrahydro-4*H*-benzo[*de*]oxireno[2′,3′:4,5]benzo[1,2-*g*]quinolin-6-ium salt

Table 5: Continued.

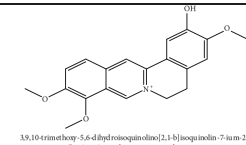
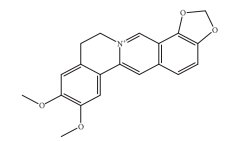
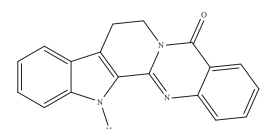
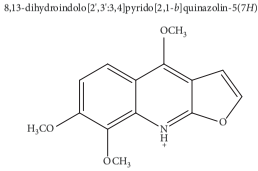
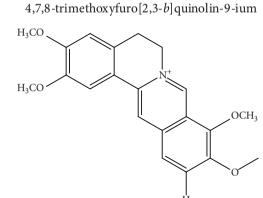
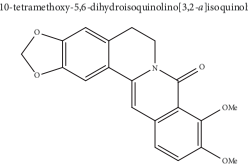
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Columbamine	$C_{20}H_{20}NO_4^+$	338.38 g/mol	 3,9,10-trimethoxy-5,6-dihydroisoquinolino[2,1-*b*]isoquinolin-7-ium-2-ol Caution: A net charge appears to be present
	Dihydroxyl-jatrorrhizine		N/A	
	Epiberberine	$C_{20}H_{18}NO_4^+$	336.37 g/mol	 Epiberberine
	Rutecarpine	$C_{18}H_{13}N_3O$	287.32 g/mol	 8,13-dihydroindolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one
	Skimmianine	$C_{14}H_{14}NO_4^+$	260.27 g/mol	 4,7,8-trimethoxyfuro[2,3-*b*]quinolin-9-ium
	Tembetarine	$C_{21}H_{22}NO_4^+$	352.41 g/mol	 2,3,9,10-tetramethoxy-5,6-dihydroisoquinolino[3,2-*a*]isoquinolin-7-ium
	8-Oxoberberine	$C_{20}H_{17}NO_5$	351.36 g/mol	 9,10-dimethoxy-5,6-dihydro-8*H*-[1,3]dioxolo[4,5-*g*]isoquinolino[3,2-*a*]isoquinolin-8-one

Table 5: Continued.

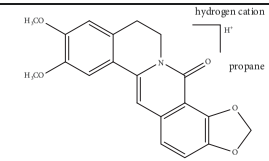
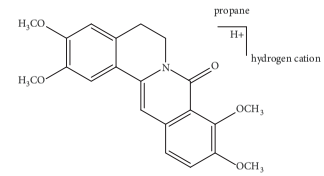
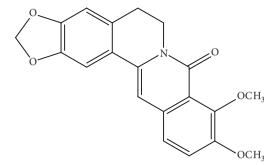
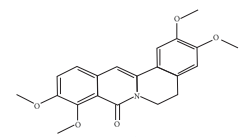
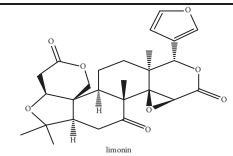
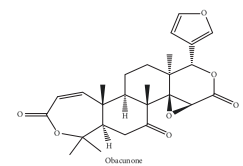
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	8-Oxoepiberberine	$C_{20}H_{17}NO_5$	351.36 g/mol	 8,9-dimethoxy-11,12-dihydro-14*H*-[1,3]dioxolo[4,5-*h*]isoquinolino[2,1-*b*]isoquinolin-14-one
	8-Oxopalmatine	$C_{21}H_{21}NO_5$	367.40 g/mol	 2,3,9,10-tetramethoxy-5,6-dihydro-8*H*-isoquinolino[3,2-*a*]isoquinolin-8-one
	Oxyberberine	$C_{20}H_{17}NO_5$	351.36 g/mol	 9,10-dimethoxy-5,6-dihydro-8*H*-[1,3]dioxolo[4,5-*g*]isoquinolino[3,2-*a*]isoquinolin-8-one
	Oxypalmatine	$C_{21}H_{21}NO_5$	367.40 g/mol	 Oxypalmatine
Limonoid	Obaculactone or limonin	$C_{26}H_{30}O_8$	470.52 g/mol	 limonin
	Obacunone or Obacunoic acid	$C_{26}H_{30}O_7$	454.52 g/mol	 Obacunone

Table 5: Continued.

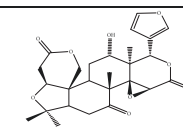
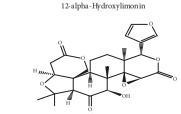
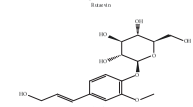
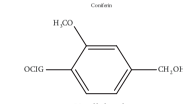
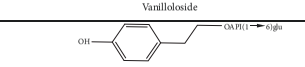
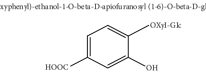
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	12α-hydroxylimonin	$C_{26}H_{30}O_9$	470.52 g/mol	 12-alpha-Hydroxylimonin
	Rutaevin	$C_{26}H_{30}O_9$	486.52 g/mol	 Rutaevin
	Coniferin	$C_{16}H_{22}O_8$	342.34 g/mol	 Coniferin
	Vanilloloside	$C_{14}H_{20}O_8$	316.31 g/mol	 Vanilloloside
Phenolic acid	2-(p-hydroxyphenyl)-ethanol-1-O-β-D-apiofuranosyl (1–6)-O-β-D-glucopyranoside	$C_9H_{12}O$	136.19 g/mol	 2-(p-hydroxyphenyl)-ethanol-1-O-beta-D-apiofuranosyl (1-6)-O-beta-D-glucopyranoside
	3, 5-dihydroxybenzoicacid-O-xylopyranosyl-glucopyranoside	$C_8H_8O_3$	152.15 g/mol	 3, 5-dihydroxybenzoicacid-O-xylopyranosyl-glucopyranoside

Table 5: Continued.

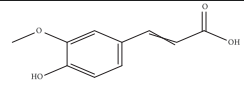
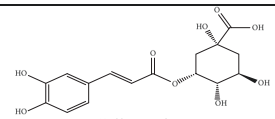
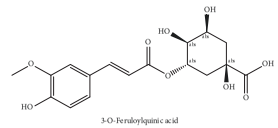
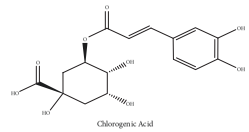
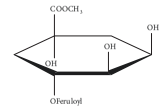
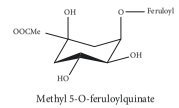
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Ferulic acid	$C_{10}H_{10}O_4$	194.19 g/mol	 Ferulic acid
Quinic acid	Neochlorogenic acid	$C_{16}H_{18}O_9$	354.31 g/mol	 Neochlorogenic Acid
	3-O-feruloyl quinic acid or 5-O-feruloyl quinic acid	$C_{17}H_{20}O_9$	368.34 g/mol	 3-O-Feruloylquinic acid
	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31 g/mol	 Chlorogenic Acid
	Methyl 3-*O*-feruloylquinate	$C_{18}H_{22}O_9$	382.37 g/mol	 Methyl 3-O-feruloylquinate
	Methyl 5-O-feruloylquinate	$C_8H_{13}O_6$	205.19 g/mol	 Methyl 5-O-feruloylquinate

Table 5: Continued.

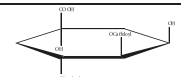
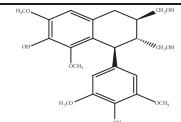
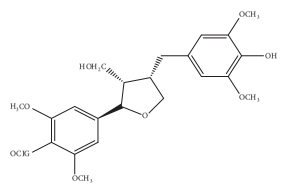
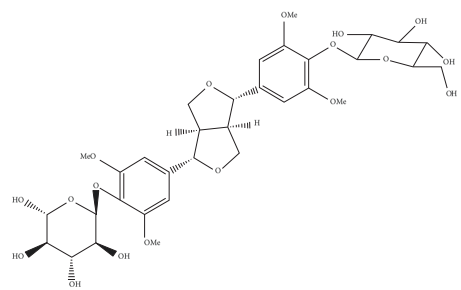
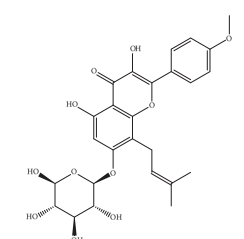
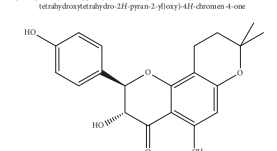
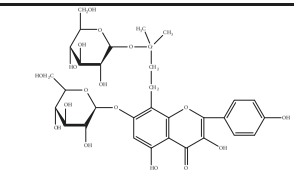
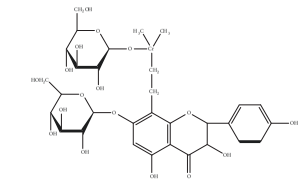
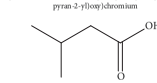
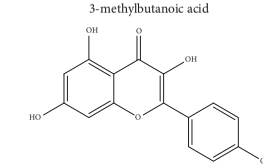
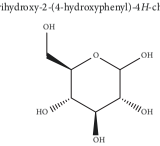
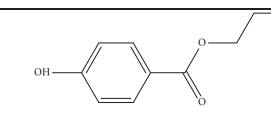
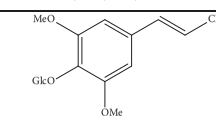
Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	3-Feruoyl-4-caffeoylquinic acid	$C_{26}H_{26}O_{12}$	530.48 g/mol	 3-Feruoyl-4-caffeoylquinic acid
Lignan	(+/-)-lyoniresinol	$C_{22}H_{28}O_8$	420.46 g/mol	 ((1*S*,2*R*,3*R*)-7-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-6,8-dimethoxy-1,2,3,4-tetrahydronaphthalene-2,3-diyl)dimethanol
	(+/-)-5,5′-dimethoxylariciresinol-4-*O*-glucoside	$C_{28}H_{38}O_{13}$	582.6 g/mol	 (+/-)-5,5'-dimethoxylariciresinol-4-O-glucoside
	Syringaresinol di-O-β-D-glucopyranoside	$C_{33}H_{44}O_{18}$	728.70 g/mol	 (2*R*,3*R*,4*R*,5*S*,6*R*)-6-(4-((1*S*,3a*R*,4*S*,6a*R*)-4-(3,5-dimethoxy-4-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)tetrahydro-1*H*,3*H*-furo[3,4-c]furan-1-yl)-2,6-dimethoxyphenoxy)tetrahydro-2*H*-pyran-2,3,4,5-tetraol
Flavonoid	β-anhydronoricaritin			N/A
	Icariside-1	$C_{26}H_{28}O_{11}$	516.50 g/mol	 3,5-dihydroxy-2-(4-methoxyphenyl)-8-(3-methylbut-2-en-1-yl)-7-(((2*R*,3*R*,4*S*,5*S*,6*S*)-3,4,5,6-tetrahydroxytetrahydro-2*H*-pyran-2-yl)oxy)-4*H*-chromen-4-one
	Phellamuretin	$C_{20}H_{20}O_6$	356.37 g/mol	 (2*R*,3*R*)-3,5-dihydroxy-2-(4-hydroxyphenyl)-8,8-dimethyl-2,3,9,10-tetrahydro-4*H*,8*H*-pyrano[2,3-*f*]chromen-4-one

Table 5: Continued.

Compound derivatives	Compound	Molecular formula	Molecular weight	Chemical structures
	Phelloside	$C_{31}H_{40}CrO_{17}$	736.64 g/mol	 (2-(3,5-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-7-(((3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-4*H*-chromen-8-yl)ethyl)dimethyl(((3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)chromium
	Dihydrophelloside	$C_{31}H_{44}CrO_{17}$	740.67 g/mol	 (2-(3,5-dihydroxy-2-(4-hydroxycyclohexa-1,3-dien-1-yl)-4-oxo-7-(((3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)chroman-8-yl)ethyl)dimethyl(((3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)chromium
	Isovaleric acid	$C_5H_{10}O_2$	102.13 g/mol	 3-methylbutanoic acid
	Kaempferol	$C_{15}H_{10}O_6$	286.24 g/mol	 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one
	D-glucose	$C_6H_{12}O_6$	180.16 g/mol	 (3*R*,4*S*,5*S*,6*R*)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2,3,4,5-tetraol
Paraben	N-propyl paraben	$C_{10}H_{12}O_3$	180.20 g/mol	 propyl 4-hydroxybenzoate
Glycoside	Sinapyl aldehyde-4-O-beta-D-glucopyranoside	$C_{13}H_{14}O_6$	266.25 g/mol	 (*E*)-2,6-dimethoxy-4-(3-oxoprop-1-en-1-yl)phenyl 2-hydroxyacetate

multimedication resistant P. aeruginosa strains. The extract of PCS significantly downregulated minimal inhibitory concentrations (MIC_s) of amikacin and gentamicin in the two multimedication resistant P. aeruginosa strains [38]. PC showed its potential on the inhibition of Propionibacterium acnes strains. Its MIC_{50} and MIC_{90} were 24 μg/ml and 190 μg/ml, respectively [39]. For fungal infections, the monomers of PC showed antifungal activity through compromising the integrity of fungal cell wall and cell membrane and increasing the expressions of energy metabolic genes. Therefore the life expectancy of Microsporum Canis is shortened. Furthermore, the mingling use of palmatine hydrochloride and berberine hydrochloride could effectively treat Microsporum Canis induced dermatomycosis in rabbit [40]. For virus infections, the ethanol extract of PAR exerted moderate effect against Herpes Simplex Virus by either interrupting virion envelope structures or disguising as indispensable viral compounds for absorption or infiltration of host cells [33]. Another study had proved that PAR has a broad spectrum of functions against virus-like VSV-GFP, PR8-GFP, NDV-GFP, HSV-GSP, H3-GFP, and EV-71 in vitro and also has effects on different strains of influenza A such as H1N1, H5N2, H7N3, and H9N2 in vivo mice model [41].

5.3. Antitumor Effects. There are 3 compounds in PCS that have been found to resist three types of tumours which included leukemic cell lines K562 and HEL, breast cancer cell line MDA, and prostate cancer cell line PC3. The compound *[(21S, 23R) epoxy-24-hydroxy-21β, 25-diethoxy] tirucalla-7-en-3-one* has a relatively strong effect as Adriamycin against four tumors with the measurement of IC_{50}; *toonaciliatin K* and *piscidinol A* have a comparably moderate effect in this regard [42]. There are 9 most effective compounds of PC for prostate cancer, namely, *magnoflorine-O-glucuronide, (p-hy-droxybenzyl)-6, 7-dihydroxy-N-methyl tetrahydro isoquinoline-7-O-p-D-glucopyranosid, magnoflorine, menisperine-O-glucuronide, menisperine, berberine, Jatrorrhizine, obaculactone*, and *obacunone* [43]. Polysaccharides from an aqueous extract of PCS act on cell-mediated stimulation and humoral immunity instead of tumor cell inhibition to exert tumoricidal activity. Specifically, some polysaccharides stimulate macrophages and NK cells via β-glucan-binding lectin site of complement receptor type 3 [44].

5.4. Antigout Effects. Si-Miao-Wan (SMW) formula had been proved to be effective for gout and gouty arthritis. In this formula, PC is the monarch herb which is the core ingredient to guide the other three herbs indicating that the alkaloids and organic acids in PC are the potential compounds for SMW. Alkaloids include candicine, oblongine, phellodendrine, tembetarine, magnoflorine, lotusine, n-methylterahydrocolumbamine, menisperine, noroxyhydrastinine, demethyleneberberine, tetrahydropalmatine, oxyberberine, armepavine, oxypalmatine, columbamine, jatrorrhizine, thalifendine, berberrubine, n-methyl canadine, palmatine, berberine, obaculactone, obacunone, and amurenlaetone B, and organic acids include neochlorogenic acid, chlorogenic acid, cryptogenic acid, cryptochlorogenin acid, caffeoyl-CH_2-O-quinic acid, 3-O-feruloylqinic acid, ferulic acid, and sanleng acid [45]. Er-Miao-Wan formula is a modified version of SMW, which had been elucidated for its chief ingredient PCS which exerts potent hypouricemic effect [46].

5.5. Antiulcer Effects. Peptic ulcers are associated with psychological stress and mental illness. The middle dosage of PC extract could significantly reduce the levels of serotonin in the brain and noradrenaline in the adrenal gland. Both serotonin and noradrenaline take effect in the mental depression. Besides, for the molecule in PC, berberine has the ability to inhibit monoamine oxidase-A and modulate the brain noradrenaline, serotonin, and dopamine levels [47]. Another study revealed that PC could protect the gastric mucosa by reinforcing the gastric mucosal barrier through endogenous sulfhydryl compounds and diethyldithiocarbamate-sensitive compounds [48].

5.6. Antioxidant Effects. The antioxidant activity of PAR is proportional to its extract's concentration. In another aspect, the ethanol extract exhibited a better antioxidant effect because of its high concentration of phenolics and flavonoids than aqueous extract [33]. Phellodendrine from PC could play an antioxidant role by modulating the AKT/NF-κB pathway in the zebrafish embryo. Besides, phellodendrine could undo the expression of AKT and NF-κB, IKK, and COX-2 [49].

5.7. Sun Screening Effects. The sun screening effects of PC have been demonstrated through an experiment which was designed for sun screening effect of 50% alcohol extracts of 100 Chinese herbal medicines. The study showed PC could absorb 91.8% of ultraviolet-C, 79.1% of ultraviolet-B, and 50.7% of ultraviolet-A. It is regarded as highly effective for sun screening if the absorption rate of ultraviolet is higher than 90%. Therefore, PC could be a strong candidate for sunproof of ultraviolet-C [50]. Also, PC could also improve skin oxidative lesion induced by ultraviolet radiation via decreasing lipid peroxidation and increasing antioxidant enzymes activities [51].

5.8. Other Effects. PC stimulates longitudinal bone growth and chondrocyte proliferation via upregulating bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor (IGF-1) expression in the growth cartilage [52]. Besides, PC could activate the fibrinogen system to take the hemostatic effect. This validated charcoaled PC could stop bleeding [53]. The extract of PC also shows neuroprotective effect through adjusting the PC-12 cell apoptosis which was induced by 1-methyl-4-phenylpyridinium (MPP^+) and hindered the release of cytochrome C into the cytosol [54]. PAR could ease the symptoms of atopic dermatitis by decreasing the numbers of mast cells, serum levels of TNF-α, and INF- γ and the expression levels of cytokines [56]. PAR could delay or even prevent the progression of diabetic nephropathy by correcting the high blood sugar state, antioxidant enzyme system, and kidney malfunction and reversing histopathological changes inflicted by diabetes on kidney [57]. To further elaborate its mechanism on the compound level,

berberine could attenuate the renal malfunction by inhibiting renal aldose reductase and decreasing oxidative stress [58]. Magnoflorine and phellodendrine could inhibit the immune response by suppressing local graft versus host reaction and induction phase of picryl chloride-induced delayed-type hypersensitivity [59]. To counter asthma attack, the extract of PCS inhibits tracheal smooth muscle contraction induced by high K^+. Meanwhile, it could also block tracheal smooth muscle concentration induced by Nifedipine [6]. The pharmacological activities of PC and its related derivatives have been listed in Table 6.

6. Pharmacokinetics

According to Chinese Pharmacopeia (Edition 2015), phellodendrine has been used as one of the evaluating indexes of PC. Phellodendrine could be rapidly absorbed in tissues such as plasma, liver, spleen, kidney, and brain. Besides, kidney is the major distribution tissue and the target organ of phellodendrine. Furthermore, the experimental study on animal suggested that this constituent has no long-term build-up effect on the tissues. Intriguingly, the extract of phellodendrine could be found in the animal brain tissue indicating that this constituent may penetrate the common medication's biggest hurdle: blood-brain barrier. The maximum concentration time is at 5 minutes after intravenous administration. The elimination half-life is no longer than 2 hours [63]. Another constituent from PC is magnoflorine which shows low bioavailability and high absorption and elimination rates after oral and intravenous administration of this constituent in pure compound form. While its bioactivity can be dramatically increased, absorption and elimination rates can be significantly decreased after oral administration of the PC decoction. Similarly, with oral administration of mixture, magnoflorine (40 mg/kg) and berberine (696.4 mg/kg, the equivalent dosage in PC decoction), the bioavailability and absorption and elimination rates have a similar trend. It suggested berberine plays an important role in the drug-drug interaction with magnoflorine in the PC decoction. On the other hand, these findings also warn us that the mingling use of berberine and magnoflorine possibly increases the risk of toxicity which possibly gives some support to the theory that herbal medicine may achieve better therapeutic effects and fewer side effects [64]. When it comes to the different type of processed PC, they have different kinds of effects. For the raw PC, it could downregulate CYP1A2 and activate CYP3A4. As for rice-wine and salt-water processed PC, they can alter the activities of cytochrome P450. Also, rice-wine processed PC alone can counter the inhibitory effect of CYP1A2 and promote the induction of CYP3A4 [65].

6.1. Toxicity and Contraindication. Several studies have been conducted regarding the toxicology of PC applications. So far, no conclusive result has been reached due to the controversial results from different studies. The common allegation for PC application is neonatal jaundice and kernicterus. Due to this concern, it even causes the complete ban on the use of related herbs including PC in Singapore since 1978. Besides, according to the latest Singaporean official regulations for health supplements guideline, PC is still on the list of prohibited or restricted ingredients. However, according to a cohort study, under the guidance of Chinese medicine practitioners, the application of PC's berberine is clinically safe even in patients who have hematological diseases with profound cytopenia and multiple comorbidities. Despite these, some precautionary measures such as bilirubin and hemoglobin monitoring are still required for the patients who have underlining hemolytic disease. On the other hand, the restriction for PC is necessary for the users in their peripartum and neonatal period due to the concern of its aggravation risk for neonatal jaundice and kernicterus [66].

6.2. Processing, Differentiation, and Authentication. Traditional Chinese medicine (TCM) processing or preparation or "Pao Zhi" in Chinese is a unique technique and process in TCM. Pao Zhi is a technique to turn the raw herbs into decoction pieces. This technique must be performed under the guidance of TCM's theory to satisfy the different requirements of medicinal materials and special production processes. The quality of Pao Zhi directly affects the therapeutic effects of herbal medicine [67]. It has a time-honored history because as early as 5 B.C. during the Jin dynasty, "Leigong Treatise on the Preparation" was composed as a book for Pao Zhi. It systematically summarized the herbal processing techniques until the Jin dynasty. Intriguingly, it stated that the right way of using the bark of PC is to remove the coarse bark [68]. In the Song dynasty, Pao Zhi had regulated a mandatory process for Chinese medicinal product [67].

In terms of PC's processing, TCM doctors in history emphasized the importance of PC's processing and recorded 16 kinds of methods for PC's processing in the books. Nowadays, the most common types of processing are raw PC, PC fried with salt, PC fried with wine, PC fried with honey, and fried-to-charcoal PC [69]. However, it still lacks official standard when it comes to quantity and quality of the processed PC and its subspecies; some of the existing pioneer studies could explain this ambiguous and abstract concept in a scientific way. Besides, for PC's quality control, the most practical approach is to use thin layer chromatography (TLC) for qualitative and high-performance liquid chromatography (HPLC) for quantitative measurement [70]. According to the results of thin layer chromatography, water percentage in the raw PC is less than 10.0%, PC fried with salt is less than 8.0%, PC fried with wine is less than 7.0%, and PC fried with honey is less than 8.0%. Total ash content in the raw PC is less than 8.0%; acid insoluble ash content is less than 0.8%; PC fried with salt is less than 8.0%; acid insoluble ash content is less than 0.6%; PC fried with wine is less than 8.0%; acid insoluble ash content is less than 0.8%; PC fried with honey is less than 8.0%; acid insoluble ash content is less than 0.4%. For alcohol-soluble extract, the raw PC is more than 16.0%, PC fried with salt is more than 20.0%, PC fried with wine is more than 16.0%, and PC fried with honey is more than 22.0%. For percentage of phellodendrine, the raw PC is more than 0.41%, and berberine is more than 3.92%; PC fried with salt is more than 0.36%, and berberine is more than 3.89%; PC fried with

Table 6: Pharmacological activities of PC and processed PC.

Pharmacological Activity	Tested Substance	*In vivo/ In vitro*	Model or sample	Active concentration	Administration (In vivo)	References
Anti-inflammatory effect	PAR extract with voucher specimens	*In vitro*	BV2 cells (Mouse microglial cell line)	100 μg/ml		[28]
	PCC extract	*In vivo, in vitro*	12-*O*-Tetradecanoylphorbol-13-acetate-induced mouse ear edema	(i) TPA and AA tests: 0.5 mg/ear (ii) TPA multiple application: 1 mg/ear (iii) DTH test: 1 mg/ear	Topical application	[29]
	PCC extract with voucher specimens	*In vivo, in vitro*	lipopolysaccharides-induced systemic inflammation mice model and macrophage RAW 264.7 cells	1,10,100 μg/mL	Oral administration	[30]
	Ethanol extract of PCC with voucher specimens	*In vivo*	12-O-tetradecanoyl-phorbol-13-acetate- induced ear edema in mice	200-400 mg/kg	Administered intragastrically	[3]
	PAR methanol extract with voucher specimens	*In vitro, in vivo*	ICR mice and male Wistar rats	IC_{50}: Methanol extract 20.9 ± 3.8 μg/mL; Non-alkaloids 22.0 μg/mL; Limonin 15.8 ± 5.2 μm; Obakunone 2.6 ± 1.1 μm	Oral administration	[11]
	PCC methanol extract with voucher specimens	*In vivo*	Lipopolysaccharides- induced acute airway inflammation on a mouse model	100, 200 and 400 mg/kg	Administrated by gavage	[31]
	Demethyleneberberine	*In vivo*	Acute colitis mice model	150,300 mg/kg	Oral administration	[32]
Anti-bacterial effect	Ethanol Extract of PAR; Aqueous extract of PAR	*In vitro*	Enterococcus faecium, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa	MIC and MBC: 3.676 mg/ml and 7.353 mg/ml for ethanol extract; 6.25 mg/ml and 50 mg/ml for aqueous extract		[33]
	PCC extract with voucher specimens	*In vitro*	Streptococcus. mitis, Streptococcus. sanguis, Streptococcus. mutans, Streptococcus. gingivalis	2.5 g/ml		[34]
	PCC extract	*In vitro*	Mycoplasma hominis	0.24-250 mg/ml		[35]

Table 6: Continued.

Pharmacological Activity	Tested Substance	*In vivo/ In vitro*	Model or sample	Active concentration	Administration (In vivo)	References
	Aqueous extract of PCC	*In vivo, In vitro*	*S.* Typhimurium 21 infected mouse model	2.5 or 5 mg/day	Administrated by gavage	[36]
	Berberine in PAR	*In vitro*	Staphylococcus aureus	32 to 128 μg/mL		[37]
	Berberine in PCS	*In vitro*	P. aeruginosa	100 ml		[38]
	PCC	*In vitro*	Propionibacterium acnes	MIC50%: 24 μg/mL MIC90%: 190 μg/mL		[39]
Anti-fungal effect	Berberine hydrochloride, palmatine hydrochloride	*In vitro, In vivo*	Microsporum Canis –induced dermatitis in rabbits	MIC_s 1 mg/ml	Administered through the sterile pipette tip	[40]
Anti-viral effect	Ethanol extract of PAR; Aqueous extract of PAR	*In vitro*	African green monkey kidney cells	6.73 ± 0.87 mg/ml for aqueous extract; 4.26 ± 0.59 mg/ml for ethanol extract		[33]
	Aqueous extract of PAR with voucher specimens	*In vitro, in vivo*	H1N1, H5N2, H7N3 or H9N2 infected BALB/c mice	0.8 μg/g in a total volume of 200 μl at 1, 3 and 5 days before infection	Oral administration	[41]
Anti-tumor effect	PCS extract with voucher specimens	*In vitro*	(i) Leukemic cell lines K562 (ii) Leukemic cell lines HEL (iii) Breast cancer cell line MDA (iv) Prostate cancer cell line PC3	(i) IC_{50} of Compound 1: 7.66 ±2.08; (ii) IC_{50} of compound 3: 14.30 ± 1.93; (iii) IC_{50} of compound 4: 11.81 ± 2.79		[42]
	PCC extract	*In vivo, in vitro*	Prostate cancer cell infested Male BALB/c-nude mice model	1.6 g/kg per day for 28 days	Administered intragastrically	[43]

Table 6: Continued.

Pharmacological Activity	Tested Substance	*In vivo*/ *In vitro*	Model or sample	Active concentration	Administration (In vivo)	References
	Aqueous extract of PCS with voucher specimens	*In vitro* *In vivo*	Sarcoma 180 ascites cells implanted Mice model	2 mg/100g; 5 mg/100g; 10 mg/100g	Injected intraperitoneally daily for 10 days	[44]
Anti-gout effect	Compounds of Si-Miao-Wan (containing PCC)	*In vivo*	Male Sprague-Dawley rats	1.0 ml/100 g	Oral administration	[45]
	PCS extract with voucher specimens	*In vivo*	Uricase inhibitor potassium oxonate induced male ICR mice model	480 mg/kg	Oral or intraperitoneal administration	[46]
Anti-ulcer effect	Ethanol extract of PCC with voucher specimens	*In vivo In vitro*	Acetic acid-induced chronic gastric ulcers on Sprague Dawley rats model	30 mg/kg/day	Administered intragastrically once a day for seven days.	[47]
	Aqueous extract of PCC	*In vivo*	Ethanol-induced gastric lesions on Male Wistar rats model	100 mg/kg	Oral administration	[48]
Anti-oxidant effect	Ethanol extract of PAR; Aqueous extract of PAR	*In vitro*	For anti-microbial Enterococcus faecium, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumonia, and Pseudomonas aeruginosa; Anti-herpes simplex virus tested on African green monkey kidney cells	25 mg/ml		[33]
	Phellodendrine isolated from PCC extract	*In vivo*	AAPH-induced oxidative stress on zebrafish embryo model	200 μg/mL	Waterborne exposure	[49]

Table 6: Continued.

Pharmacological Activity	Tested Substance	*In vivo*/ *In vitro*	Model or sample	Active concentration	Administration (In vivo)	References
Sun screening effect	PCC extract + 50% ethanol	*In vitro*	PC extract+50% ethanol	0.5 mg/ml		[50]
	PCC extract	*In vivo*	UVB lamp inflicted skin lesions on the dorsal of the rats model	200, 400 or 800 mg/kg, once daily for 11 days	Oral administration	[51]
Bone-growth effect	PCC extract	*In vivo*	72 intact 21-day-old female Sprague–Dawley rats	100 and 300 mg/kg	Oral administration	[52]
Hemostatic effect	PCC Carbonisatus-carbon dots	*In vivo In vitro*	Mouse tail amputation and liver scratch on male Kunming mice models	5, 2 and 1 mg/kg	Subcutaneous administration	[53]
Neuroprotective effect	PCC extract with voucher specimens	*In vitro*	PC-12 cells	10 and 30 μg/mL		[54]
	PCC and PAC extract with voucher specimens	*In vitro*	PC-12 cells	0.1 and 1 g/ml for 2 hours		[55]
Counter-atopic dermatitis effect	Salt processed PAC extract with voucher specimens	*In vivo*	2,4-dinitrochlorobenzene induced skin lesions on the NC/Nga mice model	200 μl	Topical administration	[56]
Counter-diabetic nephropathy effect	PAR aqueous extract	*In vivo*	Streptozotocin-induced diabetes on male Sprague-Dawley rats model	379 mg/kg	Oral administration	[57]
	Berberine	*In vivo*	Streptozotocin-induced diabetes on male Wistar rats model	200 mg/kg once a day for 12 weeks	Oral intubation	[58]
Immunity suppressing effect	Phellodendrine and cyclophosphamide isolated from PCC in saline water	*In vivo*	ddY mice, BALB/c mice, and Hartlay guinea pigs	(i) 0.1 ml/10 g for mice (ii) 1 ml for guinea pigs	Administered intraperitoneally	[59]
Anti-asthmatic effect	n-butyl alcohol extract of PCS with voucher specimens	*In vivo*	BALB/c mice asthmatic model induced by saline solution	The IC50 of n-butyl alcohol extract of PCS was 12.2 ± 1.3 μg/mL	intranasally	[6]

honey is more than 0.37%, and berberine is more than 3.90% [69].

Previously, the differentiation of PCS and PAR is based on the experiences of the herbal professionals to distinguish the minor differences from their appearances in naked eyes and microscope. Due to the increasing mixing applications and counterfeits, more efficient and accurate approaches are required. It is noticeable that the mass fractions of obaculactone and obacunone have a plunging order among raw PC, PC fried with wine, and PC fried with salt. Therefore, it is reasonable to deduce limonin and obacunone have been undergoing a series of chemical reactions during herbal processing. For differentiation of PCS and PAR, the mass fractions of limonin and obacunone have a significant difference between PCS and PAR. The mass fraction of obaculactone and obacunone in PAR is 10 times higher than in PCS. As a result, limonin and obacunone could be utilized as the differential targeted chemical constituents for PC's differentiation [71]. HPLC method demonstrates that PC and charcoaled PC have a significant disparity in terms of characteristic chromatograms and chemical constituents. The peak numbers and proportions of characteristic chromatograms are reducing with the increasing temperature of charcoaled PC. On the other hand, due to the heating effect, berberine hydrochloride will transfer into berberrubine by losing one methyl. As a result, berberrubine is vindicated to be another targeted chemical constituent for charred PC identification [72]. For authentication of PC and its subspecies, berberine hydrochloride could be used as another targeted chemical constituent except for limonin and obacunone as mentioned above [73].

7. Discussion

This review work has illustrated the diverse bioactive properties of PC and its species associated with active pharmacological actions in vitro and in vivo. Its clinical applications which are demonstrated in these experimental studies indicated the potency of the bioactive compounds and its pharmacological effects.

The diverse derivatives are the backbone for the pharmaceutical efficacies of PC and its species. The alkaloids play a very significant role in this regard not only because they account for a great proportion of constituents in the whole herb, but also because these constituents are relatively well-studied compared to other constituents in other derivatives. Berberine, magnoflorine, palmatine, and phellodendrine are viewed as the anti-inflammatory active candidates in experimental studies. Besides, palmatine and berberine could exert antimicrobial effects. Berberine also has the ability to inhibit monoamine oxidase-A and modulate the brain noradrenaline, serotonin, and dopamine for antiulcer efficacy. The nonalkaloid in PAR extract suppressed NO production; besides, limonin and obakunone significantly downregulated NO production and iNOS gene expression via an NF-κB-mediated pathway. [(21S, 23R) Epoxy-24-hydroxy-21β, 25-diethoxy] tirucalla-7-en-3-one, toonaciliatin K, and piscidinol have tumor-shrinking effects on four types of tumors. Polysaccharides from an aqueous extract of PCS act on cell-mediated stimulation and humoral immunity to exert tumoricidal activity.

Phellodendrine and magnoflorine have well absorption rate in the animal organs which could indicate their safety for human trials. However, berberine could interact with magnoflorine or other constituents which could further increase the tissue absorption rates. This should be noticed by other clinical trials or advanced studies to avoid adverse effects. It is also noticeable that different processing techniques or "Pao Zhi" could render the herb with different kind of therapeutic effects. For the raw PC, it could downregulate CYP1A2 and activate CYP3 A4. As for rice-wine and salt-water processed PC, they can alter the activities of cytochrome P450. Also, for rice-wine processed PC alone, it can counter the inhibitory effect of CYP1A2 and promote the induction of CYP3A4. For toxicology, there is no affirmative conclusion in terms of PC and its species' absolute clinical safety. Therefore, safety precautionary measures are still required for vulnerable groups of people. For the processed PC and its species, berberrubine, limonin, and obacunone became targeted chemical constituents for authentication of charred PC, PC, and its subspecies. Furthermore, obacunone and obaculactone are probably responsible for antiatopic dermatitis effect [56].

8. Conclusions

In summary, the compounds of the crude bark of PC and its species have showcased a wide range of pharmacological effects. Pharmacological efficacies of PC are supported by its diverse class of alkaloids, limonoids, phenolic acid, quinic acid, lignans, and flavonoid. Through this review, in total, 140 chemical compounds had been summarized. Among these compounds are 18 compounds from PCC, 44 compounds from PCS, 34 compounds from PAC, and 84 compounds from PAR. However, more studies are still needed to demonstrate more knowledge to allow a better understanding of this herb and its species.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] Y. Q. Dong, H. X. Hong, and G. Y. Zhong, "Research status and problems of phellodendron quality," *Research and Practice of Chinese Medicines*, vol. 3, 2007.

[2] Y. Zheng, R. H. Hui, and D. Y. Hou, "Studies on flavones and anti-oxidation effect of phellodendron," *Journal of Anshan Normal University*, 2010.

[3] Y.-F. Xian, Q.-Q. Mao, S.-P. Ip, Z.-X. Lin, and C.-T. Che, "Comparison on the anti-inflammatory effect of Cortex Phellodendri Chinensis and Cortex Phellodendri Amurensis in 12-O-tetradecanoyl-phorbol-13-acetate-induced ear edema in mice," *Journal of Ethnopharmacology*, vol. 137, no. 3, pp. 1425–1430, 2011.

[4] X. Li, W. Zhang, H. Qi, and Y. Shi, "Phenolic constituents of Phellodendron chinense bark," *Canadian Journal of Chemistry*, vol. 87, no. 9, pp. 1218–1221, 2009.

[5] J. A. Ryuk, M. S. Zheng, M. Y. Lee et al., "Discrimination of Phellodendron amurense and P. chinense based on DNA analysis and the simultaneous analysis of alkaloids," *Archives of Pharmacal Research*, vol. 35, no. 6, pp. 1045–1054, 2012.

[6] Q.-J. Jiang, W. Chen, H. Dan et al., "Cortex phellodendri extract relaxes airway smooth muscle," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 8703239, 9 pages, 2016.

[7] X. Cao, L. Sun, D. Li, G. You, M. Wang, and X. Ren, "Quality evaluation of phellodendri chinensis cortex by fingerprint chemical pattern recognition," *Molecules*, vol. 23, no. 9, article 2307, 2018.

[8] H. Wang, G. Yan, A. Zhang et al., "Rapid discovery and global characterization of chemical constituents and rats metabolites of Phellodendri amurensis cortex by ultra-performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry coupled with pattern recognition approach," *Analyst*, vol. 138, no. 11, p. 3303, 2013.

[9] Y. M. Hu, G. H. Su, S. C.-W. Sze, W. Ye, and Y. Tong, "Quality assessment of Cortex phellodendri by high-performance liquid chromatography coupled with electrospray ionization mass spectrometry," *Biomedical Chromatography*, vol. 24, no. 4, pp. 438–453, 2010.

[10] H. Sun, H. Wang, A. Zhang et al., "Chemical discrimination of cortex Phellodendri amurensis and cortex Phellodendri chinensis by multivariate analysis approach," *Pharmacognosy Magazine*, vol. 12, no. 45, pp. 41–49, 2016.

[11] A. Fujii, T. Okuyama, K. Wakame, T. Okumura, Y. Ikeya, and M. Nishizawa, "Identification of anti-inflammatory constituents in Phellodendri Cortex and Coptidis Rhizoma by monitoring the suppression of nitric oxide production," *Journal of Natural Medicines*, vol. 71, no. 4, pp. 745–756, 2017.

[12] Z. Zhang, H. Liu, B. Zhang, Y. Liao, Y. Zhang, and Z. Zhang, "Quantitative and chemical fingerprint analysis for quality evaluation of the dried bark of wild Phellodendron amurense Rupr. based on HPLC-DAD-MS combined with chemometrics methods," *Analytical Methods*, vol. 7, no. 5, pp. 2041–2049, 2015.

[13] J. Zhou, G. Xie, and X. Yan, *Encyclopedia of Traditional Chinese Medicines - Molecular Structures, Pharmacological Activities*, vol. 1, Isolated Compounds A-C, 1st edition, 2011.

[14] X. Li, J. Liang, X. Lu, and L. Tao, "A new protoberberine from the bark of Phellodendron chinense," *Chemistry of Natural Compounds*, vol. 47, no. 5, pp. 770–772, 2011.

[15] X. Xian, B. Sun, X. Ye, G. Zhang, P. Hou, and H. Gao, "Identification and analysis of alkaloids in cortex Phellodendron amurense by high-performance liquid chromatography with electrospray ionization mass spectrometry coupled with photodiode array detection," *Journal of Separation Science*, vol. 37, no. 13, pp. 1533–1545, 2014.

[16] Y.-M. Liu, S.-J. Sheu, S.-H. Chiou, H.-C. Chang, and Y.-P. Chen, "A comparative study on commercial samples of Phellodendri cortex," *Planta Medica*, vol. 59, no. 6, pp. 557–561, 1993.

[17] J. Zhou, G. Xie, and X. Yan, "Encyclopedia of Traditional Chinese Medicines - Molecular Structures, Pharmacological Activities, Natural Sources and Applications," Isolated Compounds D-G, vol. 2, 2011.

[18] J. Zhou, G. Xie, and X. Yan, *Encyclopedia of Traditional Chinese Medicines - Molecular Structures, Pharmacological Activities, Natural Sources and Applications*, vol. 4, Isolated Compounds N-S, 1st edition, 2011.

[19] J. Zhou, G. Xie, and X. Yan, *Encyclopedia of Traditional Chinese Medicines - Molecular Structures, Pharmacological Activities, Natural Sources and Applications*, vol. 3, Isolated Compounds H-M, 1st edition, 2011.

[20] Y. D. Min, H. C. Kwon, M. C. Yang et al., "Isolation of limonoids and alkaloids from Phellodendron amurense and their multidrug resistance (MDR) reversal activity," *Archives of Pharmacal Research*, vol. 30, no. 1, pp. 58–63, 2007.

[21] C. D. Li, Y. Liu, and Y. P. Shi, "Simultaneous determination of six alkaloids in Phellodendron amurense by high-performance liquid chromatography," *Acta Chromatographica*, vol. 25, no. 2, pp. 275–285, 2013.

[22] Y. P. Li, D. D. Li, L. Q. Ding, and F. Qiu, "Non-alkoloids components from Phellodendri Cortex," *Chinese Traditional and Herbal Drugs*, vol. 47, no. 15, pp. 2621–2626, 2016.

[23] Z. Zhang, Y. Zhang, Z. Zhang et al., "Comparative analysis of DNA barcoding and HPLC Fingerprint to trace species of phellodendri cortex, an Important Traditional Chinese Medicine from multiple sources," *Biological & Pharmaceutical Bulletin*, vol. 39, no. 8, pp. 1325–1330, 2016.

[24] Y. Ida, Y. Satoh, M. Ohtsuka, M. Nagasao, and J. Shoji, "Phenolic constituents of phellodendron amurense bark," *Phytochemistry*, vol. 35, no. 1, pp. 209–215, 1993.

[25] H. Zhou, D. Wang, and Z. Cui, "Ferulates, amurenlactone A and amurenamide A from traditional Chinese medicine cortex Phellodendri Amurensis," *Journal of Asian Natural Products Research*, vol. 10, no. 5, pp. 409–413, 2008.

[26] O. I. Shevchuk, N. P. Maksyutina, and V. I. Litvinenko, "Flavonoids of Phellodendron sachalinense and Ph. amurense," *Chemistry of Natural Compounds*, vol. 4, no. 2, pp. 66–70, 1968.

[27] W. Cui, J. Tian, Z. Ma, Y. Guo, J. Wang, and X. Li, "A new isocoumarin from bark of Pellodendoron chinense," *Natural Product Research*, vol. 17, no. 6, pp. 427–429, 2003.

[28] Y.-k. Park, Y. S. Chung, Y. S. Kim, O. Y. Kwon, and T. H. Joh, "Inhibition of gene expression and production of iNOS and TNF-α in LPS-stimulated microglia by methanol extract of Phellodendri cortex," *International Immunopharmacology*, vol. 7, no. 7, pp. 955–962, 2007.

[29] M. J. Cuellar, R. M. Giner, M. C. Recio, S. Manez, and J. L. Rios, "Topical anti-inflammatory activity of some Asian medicinal plants used in dermatological disorders," *Fitoterapia*, vol. 72, no. 3, pp. 221–229, 2001.

[30] Y. Y. Choi, M. H. Kim, J. M. Han et al., "The anti-inflammatory potential of cortex phellodendron in vivo and in vitro: down-regulation of NO and iNOS through suppression of NF-κB and MAPK activation," *International Immunopharmacology*, vol. 19, no. 2, pp. 214–220, 2014.

[31] Y.-F. Mao, Y.-Q. Li, L. Zong, X.-M. You, F.-Q. Lin, and L. Jiang, "Methanol extract of *Phellodendri cortex* alleviates lipopolysaccharide-induced acute airway inflammation in mice," *Immunopharmacology and Immunotoxicology*, vol. 32, no. 1, pp. 110–115, 2010.

[32] Y. Chen, R. Li, M. Shi et al., "Demethyleneberberine alleviates inflammatory bowel disease in mice through regulating NF-κB signaling and T-helper cell homeostasis," *Inflammation Research*, vol. 66, no. 2, pp. 187–196, 2017.

[33] W. Wang, Y. Zu, Y. Fu et al., "In vitro antioxidant, antimicrobial and anti-herpes simplex virus type 1 activity of Phellodendron amurense Rupr," *American Journal of Chinese Medicine*, vol. 37, no. 1, pp. 195–203, 2009.

[34] R. W. K. Wong, U. Hägg, L. Samaranayake, M. K. Z. Yuen, C. J. Seneviratne, and R. Kao, "Antimicrobial activity of Chinese medicine herbs against common bacteria in oral biofilm. A pilot

study," *International Journal of Oral and Maxillofacial Surgery*, vol. 39, no. 6, pp. 599–605, 2010.

[35] Y. Che, S. Mao, W. Jiao, and Z. Fu, "Susceptibilities of Mycoplasma hominis to herbs," *American Journal of Chinese Medicine*, vol. 33, no. 2, pp. 191–196, 2005.

[36] M. C. Yin, C. H. Chang, C. H. Su, B. Yu, and Y. M. Hsu, "Pteris multifida, Cortex phellodendri, and probiotics attenuated inflammatory status and immunity in mice with a Salmonella enterica serovar Typhimurium infection," *Bioscience, Biotechnology, and Biochemistry*, pp. 1–12, 2018.

[37] H.-H. Yu, K.-J. Kim, J.-D. Cha et al., "Antimicrobial activity of berberine alone and in combination with ampicillin or oxacillin against methicillin-resistant Staphylococcus aureus," *Journal of Medicinal Food*, vol. 8, no. 4, pp. 454–461, 2005.

[38] Y. Morita, K. Nakashima, K. Nishino et al., "Berberine is a novel type efflux inhibitor which attenuates the MexXY-mediated aminoglycoside resistance in Pseudomonas aeruginosa," *Frontiers in Microbiology*, vol. 7, article 1223, 2016.

[39] S. Higaki, M. Nakamura, M. Morohashi, Y. Hasegawa, and T. Yamagishi, "Activity of eleven Kampo formulations and eight Kampo crude drugs against Propionibacterium acnes isolated from acne patients: retrospective evaluation in 1990 and 1995," *The Journal of Dermatology*, vol. 23, no. 12, pp. 871–875, 1996.

[40] C. Xiao, Q. Ji, Q. Wei, Y. Liu, and G. Bao, "Antifungal activity of berberine hydrochloride and palmatine hydrochloride against Microsporum canis -induced dermatitis in rabbits and underlying mechanism," *BMC Complementary and Alternative Medicine*, vol. 15, article 177, 2015.

[41] J. Kim, P. Weeratunga, M. S. Kim et al., "Inhibitory effects of an aqueous extract from Cortex Phellodendri on the growth and replication of broad-spectrum of viruses in vitro and in vivo," *BMC Complementary and Alternative Medicine*, vol. 16, article 265, 2016.

[42] C. Yan, Y.-D. Zhang, X.-H. Wang et al., "Tirucallane-type triterpenoids from the fruits of Phellodendron chinense Schneid and their cytotoxic activities," *Fitoterapia*, vol. 113, pp. 132–138, 2016.

[43] X. Li, A. Zhang, M. Wang et al., "Screening the active compounds of Phellodendri Amurensis cortex for treating prostate cancer by high-throughput chinmedomics," *Scientific Reports*, vol. 7, article 46234, 2017.

[44] S.-D. Park, Y.-S. Lai, and C.-H. Kim, "Immunopontentiating and antitumor activities of the purified polysaccharides from Phellodendron chinese SCHNEID," *Life Sciences*, vol. 75, no. 25, pp. 2621–2632, 2004.

[45] J.-J. Lu, X.-W. Hu, P. Li, and J. Chen, "Global identification of chemical constituents and rat metabolites of Si-Miao-Wan by liquid chromatography-electrospray ionization/quadrupole time-of-flight mass spectrometry," *Chinese Journal of Natural Medicines*, vol. 15, no. 7, pp. 550–560, 2017.

[46] L. D. Kong, C. Yang, F. Ge, H. D. Wang, and Y. S. Guo, "A Chinese herbal medicine Ermiao wan reduces serum uric acid level and inhibits liver xanthine dehydrogenase and xanthine oxidase in mice," *Journal of Ethnopharmacology*, vol. 93, no. 2-3, pp. 325–330, 2004.

[47] L. Wang, X. Wang, X. Zhu et al., "Gastroprotective effect of alkaloids from cortex phellodendri on gastric ulcers in rats through neurohumoral regulation," *Planta Medica*, vol. 83, no. 3-4, pp. 277–284, 2017.

[48] H. Takase, O. Inoue, Y. Saito, E. Yumioka, and A. Suzuki, "Roles of sulfhydryl compounds in the gastric mucosal protection of the herb drugs composing oren-gedoku-to (a traditional herbal medicine)," *The Japanese Journal of Pharmacology*, vol. 56, no. 4, pp. 433–439, 1991.

[49] L. Li, T. Huang, C. Tian et al., "The defensive effect of phellodendrine against AAPH-induced oxidative stress through regulating the AKT/NF-κB pathway in zebrafish embryos," *Life Sciences*, vol. 157, pp. 97–106, 2016.

[50] S. Y. Cheng, D. X. Luo, Q. J. He, and Z. H. Li, *Determination of the Sun-screening Effect for 50% Alcohol Extracts of 100 Chinese Herbal Medicine Most in Use*, China Academic Journal Electronic Publishing House, 2005.

[51] H. Yan, X. Sun, S. Sun et al., "Anti-ultraviolet radiation effects of Coptis chinensis and Phellodendron amurense glycans by immunomodulating and inhibiting oxidative injury," *International Journal of Biological Macromolecules*, vol. 48, no. 5, pp. 720–725, 2011.

[52] S. H. Lee, H. J. Lee, S. H. Lee et al., "Effects of Huang Bai (Phellodendri Cortex) on bone growth and pubertal development in adolescent female rats," *Chinese Medicine*, vol. 13, no. 3, 2018.

[53] X. Liu, Y. Wang, X. Yan et al., "Novel Phellodendri Cortex (Huang Bo)-derived carbon dots and their hemostatic effect," *Nanomedicine*, vol. 13, no. 4, pp. 391–405, 2018.

[54] H. W. Jung, G.-Z. Jin, S. Y. Kim, Y. S. Kim, and Y.-K. Park, "Neuroprotective effect of methanol extract of Phellodendri Cortex against 1-methyl-4-phenylpyridinium (MPP+)-induced apoptosis in PC-12 cells," *Cell Biology International*, vol. 33, no. 9, pp. 957–963, 2009.

[55] Y.-F. Xian, Z.-X. Lin, S.-P. Ip, Z.-R. Su, J.-N. Chen, and X.-P. Lai, "Comparison the neuropreotective effect of Cortex Phellodendri chinensis and Cortex Phellodendri amurensis against beta-amyloid-induced neurotoxicity in PC12 cells," *Phytomedicine*, vol. 20, no. 2, pp. 187–193, 2013.

[56] S. Park, D. S. Kim, S. Kang, and B. K. Shin, "Synergistic topical application of salt-processed Phellodendron amurense and Sanguisorba officinalis Linne alleviates atopic dermatitis symptoms by reducing levels of immunoglobulin E and pro-inflammatory cytokines in NC/Nga mice," *Molecular Medicine Reports*, vol. 12, no. 5, pp. 7657–7664, 2015.

[57] H.-J. Kim, M.-K. Kong, and Y.-C. Kim, "Beneficial effects of Phellodendri Cortex extract on hyperglycemia and diabetic nephropathy in streptozotocin-induced diabetic rats," *Journal of Biochemistry and Molecular Biology*, vol. 41, no. 10, pp. 710–715, 2008.

[58] W. Liu, P. Liu, S. Tao et al., "Berberine inhibits aldose reductase and oxidative stress in rat mesangial cells cultured under high glucose," *Archives of Biochemistry and Biophysics*, vol. 475, no. 2, pp. 128–134, 2008.

[59] H. Mori, M. Fuchigami, N. Inoue, H. Nagai, A. Koda, and I. Nishioka, "Principle of the bark of *Phellodendron amurense* to suppress the cellular immune response," *Planta Medica*, vol. 60, no. 5, pp. 445–449, 1994.

[60] J. Kreiner, E. Pang, G. B. Lenon, and A. W. Yang, "Saposhnikoviae divaricata: a phytochemical, pharmacological, and pharmacokinetic review," *Chinese Journal of Natural Medicines*, vol. 15, no. 4, pp. 255–264, 2017.

[61] S. A. Heleno, A. Martins, M. J. Queiroz, and I. C. Ferreira, "Bioactivity of phenolic acids: metabolites versus parent compounds: a review," *Food Chemistry*, vol. 173, pp. 501–513, 2015.

[62] H. Q. Shan, L. S. Duan, X. Qi, and W. Yang, "Quality control of preparations with phellodendron chinense schneid or phellodendron amurense rupr in three editions of Chinese pharmacopoeia," *Chinese Pharmaceutical Affairs*, 2016.

[63] Y. Li, X. Liu, H. Wang et al., "Pharmacokinetic studies of phellodendrine in rat plasma and tissues after intravenous administration using ultra-high performance liquid chromatography–tandem mass spectrometry," *Journal of Chromatography B*, vol. 1029-1030, pp. 95–101, 2016.

[64] X. Tian, Z. Li, Y. Lin, M. Chen, G. Pan, and C. Huang, "Study on the PK profiles of magnoflorine and its potential interaction in Cortex phellodendri decoction by LC-MS/MS," *Analytical and Bioanalytical Chemistry*, vol. 406, no. 3, pp. 841–849, 2014.

[65] P.-P. Liu, T.-Z. Jia, S. Xu, and F. Zhang, "Application of cocktail probe drugs for detecting influences of raw and processed phellodendri cortex on cytochrome P450 isoforms," *Journal of Chinese Medicinal Materials*, vol. 38, no. 10, pp. 2065–2069, 2015.

[66] Y.-C. Linn, J. Lu, L.-C. Lim et al., "Berberine-induced haemolysis revisited: safety of Rhizoma coptidis and Cortex phellodendri in chronic haematological diseases," *Phytotherapy Research*, vol. 26, no. 5, pp. 682–686, 2012.

[67] H. W. Yao and Y. Liu, "Study of the processing of Chinese herbal medicine: status quo and development strategy," China Pharm, 2008.

[68] M. Jin, "Discussion on the easily confusing species Huang Bai and Guan Huangbai in the adjustment," in *Capital Food Medicine*, 2018.

[69] Q. Wu, C. G. Ju, X. Ai, and F. Zhang, "Study on the quality standards of cork and its different processed products from different places of purchase," *Asia-Pacific Traditional Medicine*, 2017.

[70] S. Zhou, Y. G. Liu, G. X. Zhang, and Z. L. Chen, *Research Progress on Chemical Composition and Quality Control of Corktree*, China Pharm, 2012.

[71] Z. L. Zhang, D. H. Liu, Y. C. Huang, and G. Wei, "Determination of limonin and obacunone in Phellodendri Amurensis Cortex, Phellodendri Chinensis Cortex and their processed products by HPLC," *Chinese Traditional Patent Medicine*, 2011.

[72] H. Su, L. Yue, Y. Liu et al., "Comparison on HPLC specific chromatograms between phellodendri Chinensis cortex and charred phellodendri Chinensis cortex decoction pieces," *Chinese Journal of Experimental Traditional Medical Formulae*, 2017.

[73] F. L. Liang, H. Z. Zhao, Y. C. Lian, and Z. G. Huang, "Rapid distinguishment and identification of cortex phellodendri chinense and cortex phellodendri amurensis based on TLC," *Journal of Zhaoqing University*, 2017.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7528214, 9 pages
https://doi.org/10.1155/2019/7528214

Research Article

Qiliqiangxin Capsule Improves Cardiac Function and Attenuates Cardiac Remodeling by Upregulating miR-133a after Myocardial Infarction in Rats

Huiyang Chen, Lixia Lou, Dongmei Zhang, Yizhou Zhao, Jing Zhao, Chunhong Li, Ya Huang, Keke Liu, Mingjing Zhao, and Aiming Wu

Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Key Laboratory of Chinese Internal Medicine of Ministry of Education and Beijing, Beijing 100700, China

Correspondence should be addressed to Aiming Wu; wam688@163.com

Received 28 November 2018; Revised 25 February 2019; Accepted 26 February 2019; Published 14 March 2019

Guest Editor: José Carlos Tavares Carvalho

Qiliqiangxin capsule (QLC), a natural herb recipe with therapeutic effect from China, has been widely used in clinical practice for attenuating cardiac remodeling induced by myocardial infarction (MI). However, the pharmacological mechanism of QLC on cardiac remodeling after MI is not entirely clear. The present study aims to investigate the effectiveness and mechanisms of QLC on cardiac remodeling induced by MI in rats. The animal model was established by permanently ligating the left anterior descending coronary artery in rats. Subsequently, rats with successful ligation were randomly divided into model group, captopril group, and QLC group. And the control group was operated upon in parallel except ligation, namely, the sham group. All rats were treated through the intragastric administration once a day for 4 weeks. Cardiac hemodynamics was measured after treatment. Then, the left ventricular mass index (LVMI) was examined. The pathological changes were observed by HE staining. The collagen volume fraction (CVF) was detected by Masson trichrome staining. The apoptosis index was obtained by TUNEL fluorescent staining. The miR-133a and mRNA of TGF-β1, CTGF, Caspase9, and Caspase3 were examined by real-time PCR. The protein expressions of TGF-β1, CTGF, Caspase9, Caspase3, and cleaved-Caspase3 were tested by Western blot. Compared with the model group, QLC partially improved cardiac hemodynamics and decreased LVMI. miR-133a was significantly increased in QLC group. In addition, QLC declined CVF by downregulating TGF-β1 rather than CTGF. Meanwhile, QLC decreased the apoptosis index by attenuating Caspase9, Caspase3, and cleaved-Caspase3. This study suggested that QLC could improve cardiac function and partially attenuate cardiac remodeling by attenuating fibrosis and decreasing apoptosis, which might be partially related to miR-133a, TGF-β1, Caspase9, and Caspase3.

1. Introduction

Coronary heart disease is considered as one cardiovascular disease (CVD) seriously affecting human health worldwide, which may develop into myocardial infarction (MI) or even chronic heart failure. Recently, one study from Beijing, the capital of China, showed an increase in MI patients, and the authors called for more efforts to prevent it [1]. As we all know, cardiac remodeling after MI involves complex pathological changes, which cause cardiac dysfunction [2]. With the continuous exploration of pathological mechanism, people gradually realized the important roles of miRNAs in cardiac remodeling after MI [3, 4]. miRNAs, a class of small noncoding RNAs widely existing in vivo, regulate protein-coding genes and may be a suppressor of heart diseases [5]. Among them, miR-133a seemed to a central role in the diagnosis and the treatment of cardiac remodeling [6, 7]. Traditional Chinese medicine (TCM) has long been widely applied to treat CVD [8]. Whether its therapeutic mechanisms are related to miR-133a is a meaningful issue worthy of attention and discussion. At the same time, according to the TargetScan database (online database for predicting miRNA target genes), TGF-β1, CTGF, Caspase9, and Caspase3 might be downstream genes of miR-133a, and their changes also deserved our attention.

Ethnopharmacology is of extreme importance for the conservation and enhancement of TCM. Exploring and dedicating the pharmacological effects of TCM are of great significance to develop novel drugs for treating MI. The "Qi and Blood Theory" is the characteristic theory of TCM in dealing with MI. Founded on the theory of TCM and clinical practice, under normal conditions, the "Qi" and "Blood" are filled and circulate well in the human body to maintain life activities. However, "Qi" and "Blood" are abnormal (namely "Qi Deficiency and Blood Stasis") after MI in the whole process of the disease. Benefiting Qi and activating blood circulation are the basic principles for treating MI in TCM. Qiliqiangxin capsule (QLC) is a natural herb recipe from China (Yiling Pharmaceutical Corporation), which is specially developed for benefiting Qi and activating blood circulation. And QLC has been approved by China Food and Drug Administration and widely used in clinic [9, 10]. However, the cardioprotective effects and mechanisms of this herb recipe have not been fully elucidated yet. The present study focuses on verifying the pharmacological effectiveness of QLC on cardiac remodeling after MI in rats and exploring the potential mechanisms of QLC on miR-133a, TGF-β1, CTGF, Caspase9, and Caspase3.

2. Materials and Methods

2.1. Ethics Statement. All experimental protocols and application of animals in this experiment were approved by the Standing Committee on Animals at the Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine. The SPF level of male Sprague-Dawley rats (weighted 200 ± 20 g) in this study was acquired from Beijing Vital River Laboratory Animal Technology Co. Ltd. (License number SCXK (Beijing) 2012-0001). During the experiment, all rats were raised in the animal barrier system of the Key Laboratory of Chinese Internal Medicine of the Ministry of Education and Beijing and with free access to deionized water and common feed.

2.2. Coronary Artery Ligation Surgery. The rats with MI were established by permanent left coronary artery ligation as previously described [11]. 1% pentobarbital sodium (5 ml/Kg) was used to anaesthetize rats by intraperitoneal injection. Then, in the supine position, tracheal intubation was performed in rats, and the rats were connected with the animal ventilator (ALC-V8S, Shanghai, China). Meanwhile, the twelve-lead electrocardiogram (ECG) was performed preoperatively (FX-7202, Beijing, China). Then, 2cm incision between the 3rd and 4th ribs on the left was made, and the subcutaneous tissue and muscle were separated carefully and bluntly. The left anterior descending coronary artery was ligated directly by 5/0 surgical line, while the sham operation group was assigned to the control group by only threading the surgical line without ligation. After the operations above, the thorax was tightly closed, and the ventilator was disconnected at the end of inhalation. All rats were carefully cared for until they regained sufficient consciousness to spontaneous breathing. Compared with preoperative ECG, corresponding leads showed ST-segment elevated immediately after ligation and the pathological Q-waves appeared on the second day after surgery, which indicated successful coronary artery ligation surgery. Finally, penicillin (F6062105, Hebei, China) was injected intraperitoneally for 3 days to prevent infection.

2.3. Allocation and Drug Administration. Rats with successful coronary artery ligation surgery were then randomly divided into model group, captopril group, and QLC group. Meanwhile, rats without ligation were set as the control group, namely, the sham group. Each group had 10 rats.

QLC (0.3g per capsule) consists of many natural herbs such as *Radix Astragali, Ginseng, Radix Aconiti Lateralis Preparata, Salviae Miltiorrhizae Radix, Semen Lepidii, Alismatis Rhizoma, Rhizoma Polygonati Odorati, Ramulus Cinnamomi, Carthami Flos, Cortex Periplocae, and Pericarpium Citri Tangerine*, which is the product of Shijiazhuang Yiling Pharmaceutical Co., Ltd (Shijiazhuang, China). The captopril tablet (12.5mg per table) is the product of Sino-American Shanghai Squibb Pharmaceuticals Ltd. (Shanghai, China). The drugs were thoroughly mixed with deionized water. Subsequently, according to the group, all rats were treated via intragastric administration (2.25 mg/Kg/d in the captopril group, 0.32 g/Kg/d in the QLC group, equal volume of deionized water in the model group and the sham group). The treatment lasted for 4 weeks from the second day after surgery (10 ml/Kg/d by gavage).

2.4. Measurements of Cardiac Hemodynamic. The intubation of internal left ventricular was carried out to detect cardiac hemodynamics in rats (BL-420S, Chengdu, China) [12]. 1% pentobarbital sodium (5 ml/Kg) was performed to anaesthetize rats by intraperitoneal injection after treatment. Then, after skin preparation and disinfection of the neck, the rats were positioned in supine position. The anterior cervical muscles were separated bluntly to expose the right carotid artery. A heparin-soaked PE catheter (0.05mm inner diameter) was inserted into the right carotid artery and carefully pushed to the left ventricle. The other end of the catheter was connected to the biological function acquisition system (BL-420S, Chengdu, China). After the signal stabilizes, recorded the following 4 indicators: left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), the maximal rate of increase or decrease of left ventricular pressure (±dp/dt max).

2.5. Left Ventricular Mass Index (LVMI). After removing the rat hearts, we rinsed it completely in precooled 0.9% physiological saline. Then, we trimmed unnecessary parts off the heart, like the right atrium, right ventricle, left atrial appendage, and excess blood vessels. We recorded the Left Ventricle Mass (g, LVM) and Body Mass (g, BM). The LVMI equals the ratio of LVM to BM, LVMI= LVM/BM.

2.6. Pathological Staining Experiments

2.6.1. H&E Staining. Histological samples were fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, and then made 4μm section by drum-type slicer (RM2135, LEICA, Germany). Subsequently, they were deparaffinized through xylene and rehydrated via a series of ethanol washes

(100%, 95%, 90%, 80%, and 70% ethanol). The specific steps of H&E staining were as follows: H&E Staining Kit (D006, Nanjing, China), hematoxylin staining for 5min, deionized water washing for 10s, eosin staining for 2min, hyperchromic liquid washing for 10s×2, dehydrating by 100% ethanol for 30s and washing by xylene for 15min. Finally, observed the section with the microscope (Axio Scope.A1, ZEISS, Germany).

2.6.2. Masson Trichrome Staining. Masson Trichrome Staining Kit (D026, Nanjing, China), 4-μm-thick paraffin sections, hematoxylin staining for 1min, deionized water washing for 30s, eosin staining for 40s, deionized water washing for 30s, aniline blue staining for 2.5min, deionized water washing for 10s, dehydrating by 100% ethanol for 30s and washing by xylene for 15min, and sealing with resinous mounting medium (ZLI-9516, Beijing, China) were used. Finally, we observed sections with microscope (Axio Scope.A1, ZEISS, Germany). The collagen content was analyzed by Image-Pro Plus 6.0 (American), and the collagen volume fraction (CVF) equals the ratio of collagen area to the sum of myocardial area and collagen area, and the mean value represented the CVF of the section.

2.6.3. TUNEL Assay. Prepared and pretreated 4-μm-thick paraffin sections of myocardial tissues in strict accordance with the DeadEnd™ Fluorometric TUNEL System (G3250, Promega, American). We covered the tissue with 100μl of Equilibration Buffer at room temperature (RT) for 10min. Meanwhile, 51μl rTdT incubation buffers (consist of 45μl Equilibration Buffer, 5μl Nucleotide Mix, 1μl rTdT Enzyme) were strictly prepared and temporarily stored on ice for each slice under dark conditions. Then, they were blotted around the equilibrated areas with nonfiber paper to remove the Equilibration Buffer adding 51μl of rTdT incubation buffer to the tissue. The operation of the negative control was the same as above but without rTdT Enzyme. After covering the tissue with cover glass, we incubated slides inside the humidified chamber under dark conditions for 60min at 37°C to allow the tailing reaction to occur. Next, we removed the cover glass and terminated the reactions by immersing the slides in 2X SSC for 15 min at RT. After that, we washed the slides in fresh PBS solution for 5min×3 to remove unincorporated fluorescein-12-dUTP. Finally, we added one drop of antifade solution with DAPI (S2110, Solarbio, American) on the myocardial tissue, covered the slides by cover glass, and sealed the edges with clear nail polish. Immediately we analyzed samples under a fluorescence microscope (Axio Scope.A1, ZEISS, Germany) and performed a standard fluorescein filter set to view the green fluorescence of fluorescein at 520nm, which indicated the apoptotic nucleus, and blue DAPI at 460nm, which was considered as all nucleus. The number of apoptotic nucleus and normal nucleus was counted by Image-Pro Plus 6.0 (American). We analyzed the apoptosis index of each group.

2.7. Real-Time PCR Analysis. The total RNA in the sample was extracted in strict accordance with the miRNA extraction kit (217004, QIAGEN Company, American) and Trizol regent (15596026, Thermo Fisher Scientific, American). We confirmed the ratio of OD 260/OD 280 in the range of 1.8-2.0. The primers sequences of miR-133a were obtained from Applied Biosystems (4427975, American). The primers sequences of TGF-β1, CTGF, Caspase9, and Caspase3 were synthesized by SinoGenoMax Co., Ltd. (Beijing, China). They were prepared for real-time PCR reaction system and amplified under the following conditions: miR-133a: predenatured at 94°C for 10min, denatured at 94°C for 15s, annealed at 60°C for 60s, extended at 72°C for 10s, and cycled above reaction for 45 times. The U6 was set as an internal control. Reaction conditions for TGF-β1, CTGF, Caspase9, and Caspase3 were as follows: predenatured at 95°C for 10 min, denatured at 95°C for 30s, annealed at 55°C for 30s, extended at 72°C for 20s, and circulated for 40 times. The β-actin was considered as internal control. The primer sequences were as follows:

miR-133a (F:5'-ATGGTTCGTGCGTTTGGTCCCCTTCAACC-3', R:5'-GCAGGGTCCGAGGTATTC-3'), U6 (F:5'-GCTTCGGCAGCACATATACTAAAAT-3', R:5'-CGCTTCACGAATTTGCGTGTCAT-3'). TGF-β1 (F: 5'-GCAACAACGCAATCTATGA-3', R: 5'-CAAGGTAACGCCAGGAAT-3'), CTGF (F: 5'-CTATGATGCGAGCCAACT-3', R:5'-CGGTAGGTCTTCACACTG-3'), Caspase9 (F: 5'-GCCACTGCCTCATCATCAACAA-3, R: 5'-TCGTTCTTCACCTCCACCATGA-3'), Caspase3 (F: 5'-GAATCCACGAGCAGAGTC-3', R: 5'-TCAACAAGCCAACCAAGT-3'), β-actin (F:5'-TACCCCATTGAACACGGCAT-3', R: 5'-AGGCATACAGGGACAACACA-3').

2.8. Western Blot. The myocardial tissue was lysed to obtain the supernatant. The protein concentration was detected by BCA method. Samples (30μg/lane) were electrophoresed in 10% or 12% SDS-PAGE and transferred onto 0.2μm NC membrane (A10161124, GE healthcare Life Science, American). The membranes were incubated with the primary antibodies overnight at 4°C and incubated with secondary antibody for 1 hour at RT. Strips were detected by Super ECL Plus and then analyzed with Image J.

2.9. Statistical Analysis. All values were analyzed with SPSS22.0 and presented as means ± standard deviation (SD). One-way ANOVA of LSD test was used for comparisons between multiple groups. Comparisons of nonnormal distribution data were followed by the Kruskal-Wallis method. P value < 0.05 was considered statistically significant.

3. Results

3.1. Change of QLC on Hemodynamic Assessments. In vivo, cardiac hemodynamics could directly indicate the changes of left ventricular pressure in the whole cardiac cycle, which was always used to diagnose the systolic and diastolic functions of left ventricle. As shown in (Figure 1(a)), compared with the sham group, the LVSP significantly decreased in the model group and the captopril group. Compared with the model group, the LVSP had no change in the captopril and QLC group. Compared with the sham group, the LVEDP significantly increased while the ±dp/dt max was significantly decreased in model group. Compared with the model group,

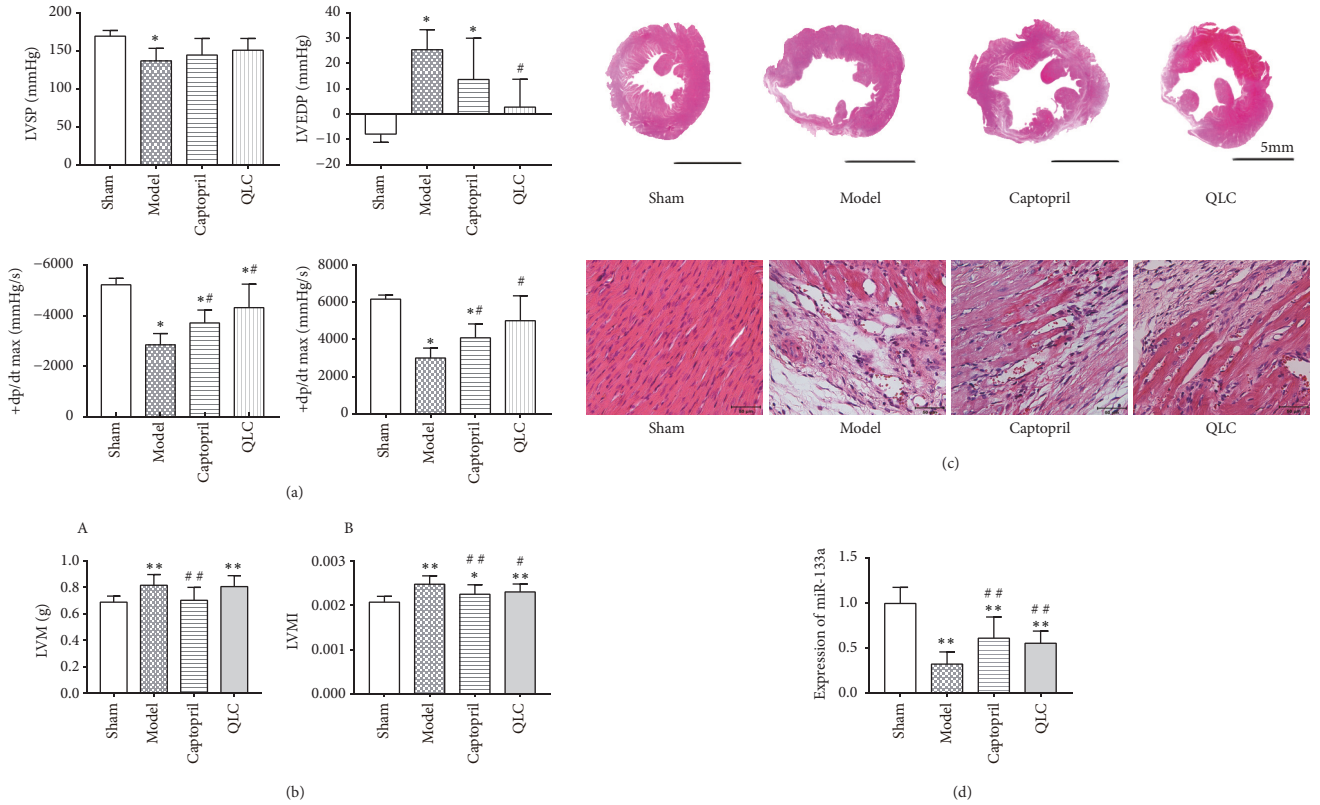


Figure 1: (a) Hemodynamic assessments (LVSP, LVEDP, and ±dp/dt max) of each group at 4 weeks after surgery. (b) H&E staining of each group at 4 weeks after surgery. (c) LVM and LVMI of each group at 4 weeks after surgery. (d) The expression level of miR-133a in the left ventricular myocardial tissue of each group at 4 weeks after surgery. The data are expressed as Mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ versus the sham group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus the model.

the LVEDP was significantly attenuated after treatment of QLC and captopril, and the ±dp/dt max was significantly improved in the captopril group and the QLC group.

3.2. Change of LVM and LVMI. LVM and LVMI were always used as indicators to roughly reflect the left ventricular remodeling. As shown in (Figure 1(b)), compared with the sham group, the LVM was significantly increased in the model group. When compared with the model group, it was partially decreased in the captopril group rather than the QLC group. When BM was considered, the results showed that the LVMI was significantly increased in the model group when compared with the sham group. Compared with the model group, the LVMI was partially decreased in the captopril group and the QLC group.

3.3. Change of QLC on Histopathology. H&E staining could partially reflect the changes of pathological structure of myocardial tissue at 4 weeks after surgery. As shown in (Figure 1(c)), when compared with the sham group, pathological tissue in the model group showed the left ventricular wall existed in a widely thinned and infarcted zone; meanwhile, the cavity of left ventricular increased obviously. These pathological changes were relatively improved in the captopril and QLC group. Similarly, under high magnification (40×), the myocardial cells in the model group were partially lost and disordered when compared with the sham group. However, after treatments of captopril and QLC, the shape of myocardial fibers in the infarct margin area was basically regular and the number of necrotic myocardial cells was decreased.

3.4. Change of miR-133a. In this part, the expression level of miR-133a was detected by real-time PCR reflected. As shown in (Figure 1(d)), the miR-133a was significantly decreased in the model group when compared with the sham group. However, compared with the model group, the expression levels of miR-133a were all increased in the captopril group and the QLC group.

3.5. Change of Myocardial Fibrosis

3.5.1. Change of CVF. Based on the Masson trichrome staining, CVF was a recommended index for evaluating fibrosis of the left ventricular. As shown in (Figure 2(a)), normal myocardial tissue in the sham group had only a small amount of collagen. However, a large number of blue collagen occupied the infarcted and interstitial area of myocardial cells in the model group. After treatment of captopril and QLC, collagen was partially decreased. Compared with the sham group, the CVF significantly increased in the model group.

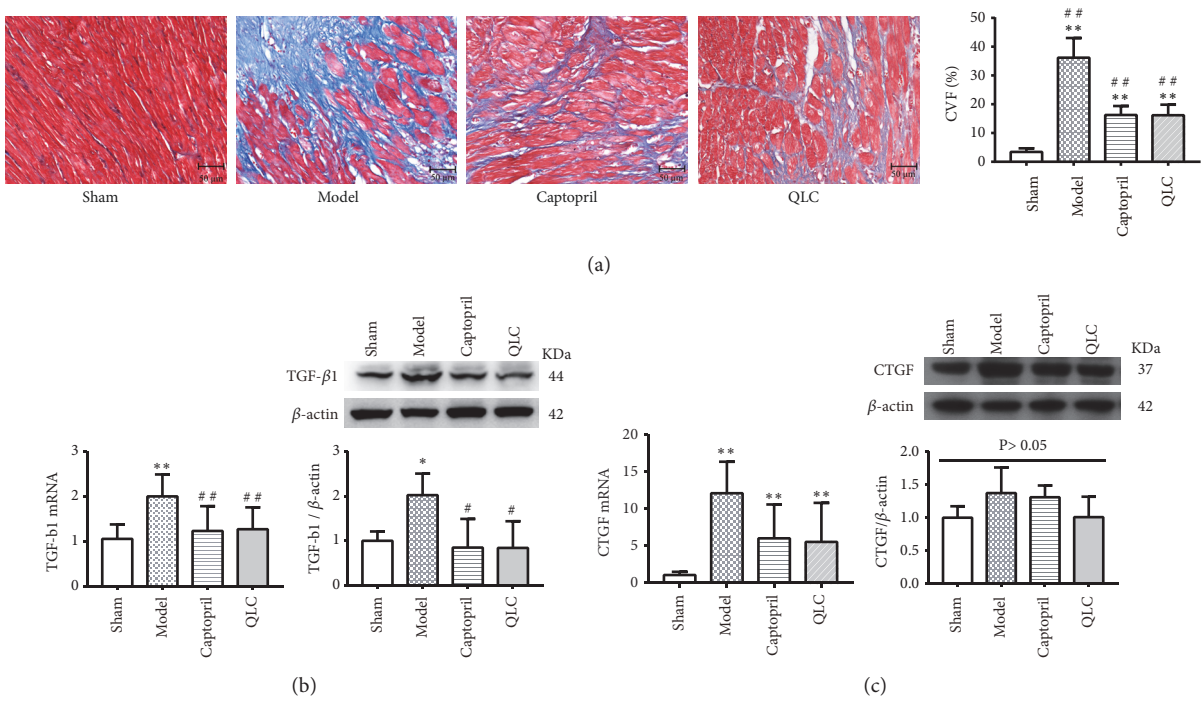


FIGURE 2: (a) Masson trichrome staining and the CVF of each group at 4 weeks after surgery. Blue represented collagen and red indicated normal myocardial tissue. (b) The mRNA and protein expression level of TGF-β1. (c) The mRNA and protein expression level of CTGF. The data are expressed as Mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ versus the sham group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus the model.

Compared with the model group, the CVF was significantly attenuated in the captopril and QLC group.

3.5.2. Changes of TGF-β1 and CTGF. TGF-β1 and CTGF were involved in the process of fibrosis, and their expressions were detected by real-time PCR and Western blot. As shown in (Figure 2(b)), compared with the sham group, the expression level of TGF-β1 mRNA was significantly increased in the model group. When compared with the model group, it was significantly decreased in the captopril and QLC group. Similarly, compared with the sham group, the protein expression level of TGF-β1 was increased in model group. When compared with the model group, it was attenuated in the captopril group and the QLC group. Compared with the sham group, the protein expression level of CTGF had no change although CTGF mRNA was increased in the model group. Meanwhile, whether at the gene or protein level, captopril and QLC showed no effect on CTGF when compared with the model group (Figure 2(c)).

3.6. Change of the Apoptotic Cardiomyocytes

3.6.1. Change of the Apoptosis Index. Since cardiomyocytes apoptosis was considered as important participants after MI. The Apoptosis Index, detected by the classical TUNEL assay, was an index for evaluating apoptosis. As shown in (Figure 3(a)), the green fluorescence indicated the broken DNA strand and blue DAPI considered as all nucleus. The model group elicited a significantly increased apoptotic nucleus in comparison with the sham group, which was reduced by captopril and QLC treatment. Precisely, compared with the sham group, the apoptosis index in model group was significantly increased. When compared with the model group, it was partially but significantly attenuated in the captopril group and the QLC group.

3.6.2. Changes of Caspase9, Caspase3, and Cleaved-Caspase3. Caspase9 and Caspase3 were both important participants in apoptosis signaling pathway, whose expressions were tested by real-time PCR and Western blot in this part. Compared with the sham group, expression levels of Caspase9 mRNA were significantly increased in the model group (Figure 3(b)), and captopril and QLC could significantly decrease the level of Caspase9 mRNA when compared with the model group. At the protein level, Caspase9 had significantly increased in the model group when compared with the sham group, while the situation was partially suppressed by treatment of captopril and QLC when compared with the model group. In addition, compared with the model group, the level of Caspase3 mRNA was upregulated in the model group. While it was downregulated by treatment of captopril and QLC when compared with the model group (Figure 3(c)). At protein level, compared with the sham group, Caspase3 and cleaved-Caspase3 significantly increased in the model group. When compared with the model group, QLC could partially attenuate the expression level of Caspase3 and cleaved-Caspase3 while captopril failed to play a regulatory role in them.

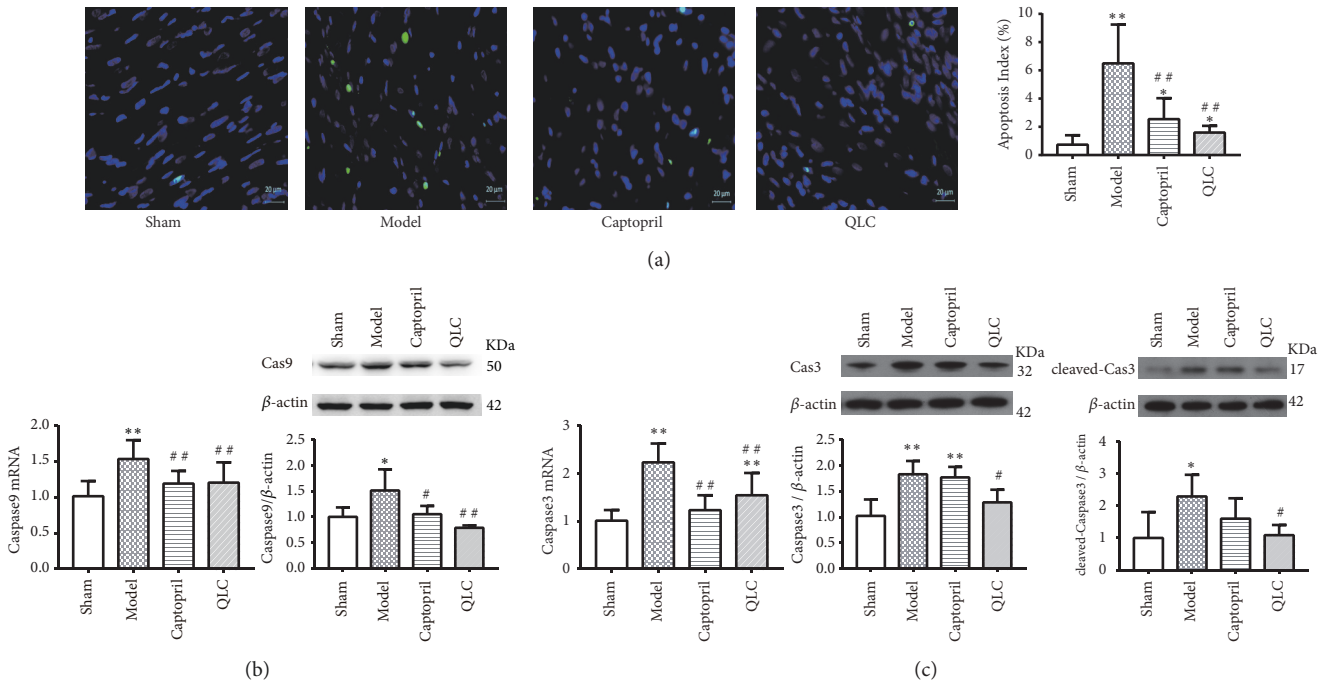


FIGURE 3: (a) TUNEL fluorescent staining and statistical analysis of apoptosis index in each group at 4 weeks after surgery. Green fluorescence indicated apoptotic nucleus and blue fluorescence indicated all nucleus. (b) The mRNA and protein expression level of Caspase9. (c) The mRNA and protein expression level of Caspase3 and the expression level of cleaved-Caspase3. The data are expressed as Mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ versus the sham group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus the model.

4. Discussion

Cardiac remodeling after MI is a complex pathophysiological process that seriously threatens the patient's life, which includes inflammation, fibrosis, apoptosis, and other pathological processes. These pathological factors contribute to the development of heart failure after MI, which indicate that it is positive to mitigate the adverse impact as soon as possible [13]. In China, TCM have been considered as an alternative and adjuvant approach to the prevention of CVD and injury induced by ischemia/reperfusion [8, 14, 15]. The present study focuses on QLC, composed of multiple natural herbs, which plays a protective role in cardiac remodeling [16]. However, its pharmacological mechanism is not entirely clear. This study revealed QLC could partially improve cardiac hemodynamics and early ventricular remodeling after MI, and these pharmacological effects might partially attribute to its ability to regulate miR-133a, TGF-β1, Caspase9, and Caspase3.

In this experiment, firstly, the cardiac hemodynamics was carried out to evaluate the effect of QLC on cardiac function in vivo. The results showed that QLC could partially improve cardiac function in rats at 4 weeks after surgery. The effect of QLC on improving cardiac hemodynamics might rely on several main constituents in the compound. Ma et al. [17] suggested that the astragalus membranaceus dramatically improved cardiac function, including the ±dp/dt max, LVSP and LVEDP. Moreover, Zheng et al. [18] claimed that Astragaloside IV can induce ischemia/reperfusion injury and improve cardiac function. In addition, the Ginsenoside Rg3, one of the principal constituents in Panax ginseng, could significantly increase the left ventricular fractional shortening and improve the left ventricular ejection fraction in rats [19]. The recent study demonstrated that tanshinone IIA, the main component of salvia miltiorrhiza, increased the ejection fraction and ±dp/dt max in rats with MI [20]. In fact, the cardiac remodeling process induces the abnormal hemodynamics and further stress upon the left ventricular wall, inciting a vicious cycle of stress and remodeling [21]. In the present study, H&E staining and LVMI generally showed the pathological changes of heart after MI, and the results suggested that QLC decreased infarct zone and the LVMI after MI. Therefore, QLC could improve cardiac function and partially attenuate pathological changes in rats after MI.

Cardiac remodeling after MI was always accompanied by the complex gene regulation. miRNAs are considered as a group of small noncoding RNAs, and there are about one-third of human genes regulated by more than 2000 miRNAs [22]. These special miRNAs can negatively regulate the translation of target genes or degrade target genes after being processed and matured. miR-133a, one of the cardiac-specific miRNAs, is associated with cardiac hypertrophy, heart failure [23, 24]. Abdullatif M [25] revealed that the expression of miR-133 in myocardial hypertrophy model was significantly downregulated and negatively related to the degree of myocardial hypertrophy. Song et al. [26] found that the expression of miR-133a in the heart of chronic heart failure from patients or rats was decreased; however, overexpression of miR-133a improved cardiac hemodynamics and cardiac remodeling in chronic heart failure rats. In the

present study, the expression level of miR-133a in infarct margin area was further detected. We found that miR-133a was significantly decreased because of MI-surgery while QLC could increase it. Therefore, upregulation of miR-133a might be one of the mechanisms of QLC on improving cardiac remodeling. After searching the database and previous studies, we found that TGF-β1, CTGF, Caspase9, and Caspase3 were closely related to miR-133a. The following work was mainly to explore and discuss their changes after MI as well as the intervention effects of QLC.

The complicated remodeling induced by MI is considered a mainly pathophysiological process consisting of multiple links, including oxygen deficit, myocardial fibrosis, and apoptosis. The fibrosis is always a key contributor during the development of cardiac dysfunction and remodeling in diverse pathological situations. In this study, QLC significantly decreased the CVF, which indicated that QLC could partially alleviate fibrosis after MI. As we all know, TGF-β1 and CTGF were recognized as cytokines that promote fibrosis. TGF-β1 could regulate extracellular matrix metabolism and initiate fibrosis [27]. Liu et al. [28] found that overexpression of miR-133 suppressed TGF-β1-mediated fibrosis. TGF-β1 might be a potential target gene for miR-133a. At the same time, Duisters et al. [29] found that miR-133 might combine with the 3' untranslated region of CTGF and thus downregulate CTGF. In addition, CTGF was also considered as the downstream mediator of the TGF-β1 pathway in cardiac fibroblasts and cardiac myocytes [30]. In this study, the results of real-time PCR and Western blot showed that QLC markedly decreased TGF-β1 while had no effect on CTGF. Therefore, the mechanism of QLC in alleviating fibrosis might be related to inhibit TGF-β1.

On the other hand, cardiomyocytes apoptosis was also involved in the pathological process of the left ventricular remodeling after MI. Early researches suggested that apoptosis had always been considered a programmed death process under physiological or pathological conditions, which were dispensable for physiological homeostasis in the normal organ [31]. However, it could also lead to excessive loss of myocardial cells that were associated with serious cardiac dysfunction in multiple pathological conditions, like the reperfusion injury, MI, and heart failure [32]. Abnormal changes or damages of mitochondria after MI were involved in apoptosis of cardiomyocytes in the infarcted area [2]. Cytochrome C and other apoptosis-promoting active proteins located in the gap between mitochondrial membranes are released into the cytoplasm, which mediated recruitment of pro-Caspase9 and eventually leaded to the activation of Caspase3 [2, 33]. Activation of Caspase3 was considered a necessary condition to initiate apoptosis. An early experiment had proved that cleavage of Caspase3 and apoptosis all decreased in Caspase9-knockout mice [34]. He et al. [35] suggested that miR-133 might inhibit apoptosis by targeting Caspase9. Meanwhile, when subjected to myocardial ischemia reperfusion injury, overexpression of Caspase3 increased infarct size and depressed cardiac function in mice [36]. Pharmacological intervention of apoptosis could diminish infarct area and improve cardiac function [37]. Song et al. [38] confirmed Caspase9 as the target of miR-133 and found the tanshinone IIA could reduce apoptosis by upregulation of miR-133 and downregulation of Caspase-9. Ginsenoside Rg3 might attenuate apoptosis through inhibiting the activation of Caspase3 in ischemia/reperfusion rats [19]. Similarly, in the rat model of myocardial ischemia, the astragalus membranaceus could enhance the myocardial cell viability via arresting the influx of Ca^{2+} to prevent apoptosis [17]. The Descurainia sophia (L.) Webb ex Prantl might improve cardiac function via regulating the balance between Bax and Bcl-2, blocking the activation of Caspase3 in the chronic heart failure rats [39]. Obviously, QLC contained many antiapoptosis components. This pharmacological effect of QLC as shown above might have a strong relationship with these components. In this study, QLC could attenuate Caspase9 and suppress Caspase3 thus protecting myocardial cells from apoptosis.

In conclusion, QLC could improve cardiac function and partially attenuate cardiac remodeling in rats after the ligation of left coronary artery. The pharmacological action of QLC on improving cardiac remodeling might be partially related to the reduction of fibrosis and apoptosis. And the underlying mechanism of QLC included increasing miR-133a, attenuating TGF-β1, Caspase9, Caspase3, and cleaved-Caspase3.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81673895) and the Outstanding Young Talents Project of Beijing University of Chinese Medicine (No. BUCM-2019-JCRC008).

References

[1] Q. Zhao, Y. Yang, Z. Chen et al., "Changes in characteristics, risk factors, and in-hospital mortality among patients with acute myocardial infarction in the capital of China over 40years," *International Journal of Cardiology*, vol. 265, pp. 30–34, 2018.

[2] N. G. Frangogiannis, "Pathophysiology of myocardial infarction," *Compr Physiol*, vol. 5, no. 4, pp. 1841–1875, 2015.

[3] S. Zhou, J. Jin, J. Wang et al., "miRNAS in cardiovascular diseases: potential biomarkers, therapeutic targets and challenges," *Acta Pharmacologica Sinica*, vol. 39, no. 7, pp. 1073–1084, 2018.

[4] A. Tuttolomondo, I. Simonetta, and A. Pinto, "MicroRNA and receptor mediated signaling pathways as potential therapeutic targets in heart failure," *Expert Opinion on Therapeutic Targets*, vol. 20, no. 11, pp. 1287–1300, 2016.

[5] B. C. Bernardo, J. Y. Y. Ooi, R. C. Y. Lin, and J. R. Mcmullen, "miRNA therapeutics: a new class of drugs with potential therapeutic applications in the heart," *Future Medicinal Chemistry*, vol. 7, no. 13, pp. 1771–1792, 2015.

[6] Y. Liu, Y. Liang, J.-F. Zhang, and W.-M. Fu, "MicroRNA-133 mediates cardiac diseases: mechanisms and clinical implications," *Experimental Cell Research*, vol. 354, no. 2, pp. 65–70, 2017.

[7] N. Li, H. Zhou, and Q. Tang, "miR-133: a suppressor of cardiac remodeling?" *Frontiers in Pharmacology*, vol. 9, p. 903, 2018.

[8] P. Hao, F. Jiang, J. Cheng, L. Ma, Y. Zhang, and Y. Zhao, "Traditional chinese medicine for cardiovascular disease: evidence and potential mechanisms," *Journal of the American College of Cardiology*, vol. 69, no. 24, pp. 2952–2966, 2017.

[9] A. Han, Y. Lu, Q. Zheng et al., "Qiliqiangxin attenuates cardiac remodeling via inhibition of TGF-β1/Smad3 and NF-κB signaling pathways in a rat model of myocardial infarction," *Cellular Physiology and Biochemistry*, vol. 45, no. 5, pp. 1797–1806, 2018.

[10] X. Li, J. Zhang, J. Huang et al., "A multicenter, randomized, double-blind, parallel-group, placebo-controlled study of the effects of qili qiangxin capsules in patients with chronic heart failure," *Journal of the American College of Cardiology*, vol. 62, no. 12, pp. 1065–1072, 2013.

[11] A. Wu, L. Lou, J. Zhai et al., "miRNA expression profile and effect of wenxin granule in rats with ligation-induced myocardial infarction," *International Journal of Genomics*, vol. 2017, Article ID 2175871, 10 pages, 2017.

[12] Y. Zhang, Q. Sun, B. He, J. Xiao, Z. Wang, and X. Sun, "Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion," *International Journal of Cardiology*, vol. 148, no. 1, pp. 91–95, 2011.

[13] M. C. Bahit, A. Kochar, and C. B. Granger, "Post-myocardial infarction heart failure," *JACC: Heart Failure*, vol. 6, no. 3, pp. 179–186, 2018.

[14] J. Y. Han, Q. Li, Z. Z. Ma, and J. Y. Fan, "Effects and mechanisms of compound Chinese medicine and major ingredients on microcirculatory dysfunction and organ injury induced by ischemia/reperfusion," *Pharmacology & Therapeutics*, vol. 177, pp. 146–173, 2017.

[15] Y. Cui, L. Yan, C. Pan et al., "The contribution of different components in qishenyiqi pills® to its potential to modulate energy metabolism in protection of ischemic myocardial injury," *Frontiers in Physiology*, vol. 9, p. 389, 2018.

[16] H. Wang, X. Zhang, P. Yu et al., "Traditional Chinese medication Qiliqiangxin protects against cardiac remodeling and dysfunction in spontaneously hypertensive rats," *International Journal of Medical Sciences*, vol. 14, no. 5, pp. 506–514, 2017.

[17] X. Ma, K. Zhang, H. Li, S. Han, Z. Ma, and P. Tu, "Extracts from Astragalus membranaceus limit myocardial cell death and improve cardiac function in a rat model of myocardial ischemia," *Journal of Ethnopharmacology*, vol. 149, no. 3, pp. 720–728, 2013.

[18] Q. Zheng, J. Z. Zhu, X. Y. Bao et al., "A preclinical systematic review and meta-analysis of astragaloside iv for myocardial ischemia/reperfusion injury," *Frontiers in Physiology*, vol. 9, p. 795, 2018.

[19] L. P. Zhang, Y. C. Jiang, X. F. Yu et al., "Ginsenoside Rg3 improves cardiac function after myocardial ischemia/reperfusion via attenuating apoptosis and inflammation," *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, Article ID 6967853, 8 pages, 2016.

[20] D. Wu, Y. Wang, X. Han et al., "Tanshinone IIA prevents left ventricular remodelling via the TLR4/MyD88/NF-κB signalling pathway in rats with myocardial infarction," *Journal of Cellular and Molecular Medicine*, vol. 22, no. 6, pp. 3058–3072, 2018.

[21] Z. K. Haque and D.-Z. Wang, "How cardiomyocytes sense pathophysiological stresses for cardiac remodeling," *Cellular and Molecular Life Sciences*, vol. 74, no. 6, pp. 983–1000, 2017.

[22] S. M. Hammond, "An overview of microRNAs," *Advanced Drug Delivery Reviews*, vol. 87, pp. 3–14, 2015.

[23] A. Carè, D. Catalucci, F. Felicetti et al., "MicroRNA-133 controls cardiac hypertrophy," *Nature Medicine*, vol. 13, no. 5, pp. 613–618, 2007.

[24] S. Gholamin, A. Pasdar, M. S. Khorrami et al., "The potential for circulating microRNAs in the diagnosis of myocardial infarction: a novel approach to disease diagnosis and treatment," *Current Pharmaceutical Design*, vol. 22, no. 3, pp. 397–403, 2016.

[25] M. Abdellatif, "The role of microRNA-133 in cardiac hypertrophy uncovered," *Circulation Research*, vol. 106, no. 1, pp. 16–18, 2010.

[26] H.-Q. Sang, Z.-M. Jiang, Q.-P. Zhao, and F. Xin, "MicroRNA-133a improves the cardiac function and fibrosis through inhibiting Akt in heart failure rats," *Biomedicine & Pharmacotherapy*, vol. 71, pp. 185–189, 2015.

[27] M. Dobaczewski, W. Chen, and N. G. Frangogiannis, "Transforming growth factor (TGF)-β signaling in cardiac remodeling," *Journal of Molecular and Cellular Cardiology*, vol. 51, no. 4, pp. 600–606, 2011.

[28] L. J. Duan, J. Qi, X. J. Kong et al., "MiR-133 modulates TGF-β1-induced bladder smooth muscle cell hypertrophic and fibrotic response: implication for a role of microrna in bladder wall remodeling caused by bladder outlet obstruction," *Cellular Signalling*, vol. 27, no. 2, pp. 215–227, 2015.

[29] R. F. Duisters, A. J. Tijsen, B. Schroen et al., "MiR-133 and miR-30 Regulate connective tissue growth factor: implications for a role of micrornas in myocardial matrix remodeling," *Circulation Research*, vol. 104, no. 2, pp. 170–178, 2009.

[30] M. M. Chen, A. Lam, J. A. Abraham, G. F. Schreiner, and A. H. Joly, "CTGF expression is induced by TGF-β in cardiac fibroblasts and cardiac myocytes: a potential role in heart fibrosis," *Journal of Molecular and Cellular Cardiology*, vol. 32, no. 10, pp. 1805–1819, 2000.

[31] N. N. Danial and S. J. Korsmeyer, "Cell death: critical control points," *Cell*, vol. 116, no. 2, pp. 205–219, 2004.

[32] R. S. Whelan, V. Kaplinskiy, and R. N. Kitsis, "Cell death in the pathogenesis of heart disease: mechanisms and significance," *Annual Review of Physiology*, vol. 72, pp. 19–44, 2010.

[33] L. A. Allan and P. R. Clarke, "Apoptosis and autophagy: regulation of caspase-9 by phosphorylation," *FEBS Journal*, vol. 276, no. 21, pp. 6063–6073, 2009.

[34] K. Kuida, T. F. Haydar, C.-Y. Kuan et al., "Reduced apoptosis and cytochrome C-mediated caspase activation in mice lacking caspase 9," *Cell*, vol. 94, no. 3, pp. 325–337, 1998.

[35] B. He, J. Xiao, A. J. Ren et al., "Role of miR-1 and miR-133a in myocardial ischemic postconditioning," *Journal of Biomedical Science*, vol. 18, p. 22, 2011.

[36] G. Condorelli, R. Roncarati, J. Ross Jr. et al., "Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function," *Proceedings of the National Acadamy of Sciences of the United States of America*, vol. 98, no. 17, pp. 9977–9982, 2001.

[37] M. Chiong, Z. V. Wang, Z. Pedrozo et al., "Cardiomyocyte death: mechanisms and translational implications," *Cell Death & Disease*, vol. 2, p. e244, 2011.

[38] T. Song, Y. Yao, T. Wang, H. Huang, and H. Xia, "Tanshinone IIA ameliorates apoptosis of myocardiocytes by up-regulation of miR-133 and suppression of Caspase-9," *European Journal of Pharmacology*, vol. 815, pp. 343–350, 2017.

[39] Y. Luo, Z. Sun, P. Hu, Y. Wu, W. Yu, and S. Huang, "Effect of aqueous extract from *descurainia sophia* (L.) webb ex prantl on ventricular remodeling in chronic heart failure rats," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 1904081, 8 pages, 2018.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7507549, 13 pages
https://doi.org/10.1155/2019/7507549

Research Article

Antibacterial and Antibiotic Modifying Potential of Crude Extracts, Fractions, and Compounds from *Acacia polyacantha* Willd. against MDR Gram-Negative Bacteria

Flora T. Mambe,[1,2,3] Jean Na-Iya,[4] Ghislain W. Fotso,[4] Fred Ashu,[1,2] Bathélémy Ngameni,[5] Bonaventure T. Ngadjui,[4] Veronique P. Beng,[2] and Victor Kuete[1]

[1]*Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon*
[2]*Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon*
[3]*Ministry of Scientific Research and Innovation, Cameroon*
[4]*Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon*
[5]*Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Medicine and Biomedical Science, University of Yaoundé I, Yaoundé, Cameroon*

Correspondence should be addressed to Victor Kuete; kuetevictor@yahoo.fr

Received 16 January 2019; Revised 12 February 2019; Accepted 24 February 2019; Published 7 March 2019

Guest Editor: José Carlos Tavares Carvalho

The present study aimed to assess the *in vitro* antibacterial and antibiotic modifying activities of methanol extracts prepared from the leaf (APL) and bark (APB) of *Acacia polyacantha*, fractions (APLa-d) and compounds isolated from APL against a panel of multidrug resistant (MDR) Gram-negative bacteria. Leaf extract was subjected to column chromatography for compounds isolation; antibacterial assays were performed on samples alone and with an efflux pump inhibitor (EPI), respectively, and several antibiotics on the tested bacteria. The phytochemical investigation of APL led to the isolation of stigmasterol (**1**), β-amyrin (**2**), 3-*O*-β-$_D$-glucopyranosylstigmasterol (**3**), 3-O-methyl-D-chiro-inositol (**4**), epicatechin (**5**), quercetin-3-*O*-glucoside (**6**), 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**7**), and 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**8**). APL and APB had minimal inhibitory concentration (MIC) values ≤ 1024 μg/mL on 73.3% and 46.7% of the tested bacteria, respectively. APLb and APLd were effective against 88.9% of tested bacterial species with compound 8 showing the highest activity inhibiting 88.9% of tested bacteria. The EPI, phenylalanine-arginine-β-naphthylamide (PAßN), strongly improved the activity of APL, APLb, APLd, and compound **8** on all tested bacteria. Synergistic effects were obtained when APL and compounds **7** and **8** were combined with erythromycin (ERY), gentamycin (GEN), ciprofloxacin (CIP), and norfloxacin (NOR). The present study demonstrates the antibacterial potential of *Acacia polyacantha* and its constituents to combat bacterial infections alone or in combination with EPI.

1. Introduction

Bacterial drug resistance constitutes a serious concern in the therapy of infectious diseases. Despite the abundance of various classes of antibiotics, the emergence of resistant strains of bacteria is increasing [1]. This phenomenon of resistance has increased the disease burden, and it has become necessary to search for new and cheaper alternatives with fewer side effects [2]. Botanicals (or crude plant extracts) and their secondary metabolites have long been used by humans for medicinal purposes. It is estimated that about 80% of the world's population uses medicinal plants as alternative for their health care [3]. Cameroon's flora is an enormous reservoir of antibacterial botanicals and phytochemicals (or plants secondary metabolites); some Cameroonian medicinal plants previously documented for their antibacterial potential include *Treculia obovoidea* [4], *Vismia laurentii* [5], *Artocarpus communis* [6], *Piper nigrum* and *Vernonia amygdalina* [7], *Cyperus esculentus* [8], and *Beilschmiedia obscura* [9]. Several botanicals have

previously been reported for their activity against multidrug resistant (MDR) bacteria and had the ability to potentiate the activity of currently used antibiotics; such plants included *Dorstenia psilurus* [10], *Combretum molle* [11], *Xanthosoma mafaffa*, *Moringa oleifera,* and *Passiflora edulis* [12], *Rubus fellatae,* and *Manihot esculenta* [13]. It is important to improve our library of botanicals and phytochemicals with promising antibacterial potential, in order to combat MDR phenotypes. In the present study, we selected another Cameroonian medicinal plant, *Acacia polyacantha* Willd. (Fabaceae). *Acacia polyacantha* is a deciduous, straight cylindrical, erect tree of about 10-15 m height found in Tropical Africa. It has a geographical distribution, ranging from Gambia to Ethiopia and southwards to Kenya and Zimbabwe [14, 15]. The plant is traditionally used to treat livestock diseases and gastrointestinal infections [16]. The plant is also used as a remedy for snakebite and as an infusion to bath children who are restless at night [14]. This is the first report on the antibacterial potential of this plant against MDR bacteria. It was found that this plant had no anthelmintic effect against a levamisole resistant strain of the nematode *Caenorhabditis elegans* [17]. Previous phytochemical investigations of the leaves of the plant led to the isolation of polyacanthoside A, oleanolic acid, stigmasterol, stigmasterol-3-*O*-β-glucopyranosyl, epicatechin quercetin-3-*O*-glucoside, 3-O-methyl-D-Chiro-inositol, and 3-*O*-[β-$_D$- galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid [15]. The present study was designed to evaluate the antibacterial activity of the leaf and bark extracts of *Acacia polyacantha* against Gram-negative bacteria expressing MDR phenotypes. The work includes the isolation and identification of the active constituents of the leaf as well as the ability of this plant and its components to potentiate the activity of commonly used antibiotics.

2. Material and Methods

2.1. General Procedure. Optical rotation was measured with a Horiba SEPA-300 polarimeter (Horiba, Kyoto, Japan). NMR spectra were recorded on Bruker DMX Avance 600 instruments equipped with an autotune probe and using the automation mode aided by the Bruker program. HREI-SMS spectra were determined on a micrOTOF-Q 98 spectrometer (Bruker-Daltonics, Bilerica, MA). For column chromatography, silica gel 60 particles size 0.04–0.063 mm (Merck, Darmstadt, Germany) and Sephadex LH-20 purchased at Sigma-Aldrich (St Louis, MO) were used. The plates were visualized using UV (254 and 366 nm) and revealed by spraying with vanillin-sulphuric acid (1% ethanolic solution of vanillin + 10% ethanolic sulphuric acid).

2.2. Plant Material and Extraction. The bark and leaf of *Acacia polyacantha* Willd. (Fabaceae) were collected on February 2016 in Kaéle, in the Far North Region of Cameroon. The plant was then identified at the National Herbarium of Cameroon by Mr. Nana Victor and a voucher specimen was deposited under the registration number 58985/SRF/CAM. Air-dried and powdered leaf and bark of *A. polyacantha* (2 kg) were extracted twice at room temperature with 4 L of methanol (MeOH) for 48 hours. The solvent was evaporated under reduced pressure to yield 229 g and 108 g of crude leaf (APL) and bark (APB) extracts, respectively. These extracts were then kept at 4°C until further use.

2.3. Isolation of the Constituents from Leaves of Acacia polyacantha. Part of APL (225 g) was dissolved in a mixture of petroleum ether/ethyl acetate (99:1) and shaken to remove a dark green extract of chlorophyll. The residue (110 g) was subjected to silica gel column chromatography (40-63 μm, 6 x 50 cm) using hexane-ethyl acetate (AcOEt) and chloroform ($CHCl_3$)-MeOH gradients as eluents. 198 sub-fractions (frs) of 300 mL each were collected as follows: sub-frs 1-13 (hexane:AcOEt, 95:5), sub-frs 14-29 (hexane:AcOEt, 90: 10), sub-frs 30-63 (hexane:AcOEt,85:15), sub-frs 64-117 (hexane:AcOEt, 80:20), sub-frs 118-122 (hexane:AcOEt, 70:30), sub-frs 123-129 (hexane:AcOEt, 60:40), sub-frs 130-140 ($CHCl_3$:MeOH, 97.5:2.5), sub-frs 141-152 ($CHCl_3$:MeOH, 95:5), sub-frs 153-166 ($CHCl_3$:MeOH, 90:10), sub-frs 167-182 ($CHCl_3$:MeOH, 85:15), sub-frs 183-190 ($CHCl_3$:MeOH, 80:20), and sub-frs 191-198 ($CHCl_3$:MeOH, 75:25). These sub-frs were then pooled on the basis of their thin layer chromatography (TLC) profiles into four fractions as follows: APLa (sub-frs 1-34); APLb (sub-frs 35-171); APLc (sub-frs 172-183); and APLd (sub-frs 184-198). Upon antibacterial testing, fractions APLa, APLb, and APLd were selected for further purification.

Fraction APLa was column chromatographed over silica gel 60 column using increasing gradient of hexane:AcOEt, mixtures as eluents. 105 subfractions of 100 mL each were collected as follows: sub-frs 1-39 (hexane:AcOEt,95:5), sub-frs 40-87 (hexane:AcOEt,90:10), and sub-frs 88-105 (hexane:AcOEt,85:15). Compounds **1** (45.9 mg) and **2** (44.1 mg) were obtained as white powders after filtration from sub-frs 15-40 and sub-frs 41-90, respectively.

Fraction APLb was submitted to column chromatography (CC) over silica gel 60 using increasing gradient hexane:AcOEt, and $CHCl_3$:MeOH, mixtures as eluents. 235 sub-fractions of 200 mL each were collected as follows: sub-frs 1-56 (hexane:AcOEt,85:15), sub-frs 57-133 (hexane:AcOEt,80:20), sub-frs 134-142 (hexane:AcOEt,70:30), sub-frs 143-155 (hexane:AcOEt,60:40), sub-frs 156-176 ($CHCl_3$:MeOH, 97.5:2.5), sub-frs 177-199 ($CHCl_3$:MeOH, 95:5), sub-frs 200-226 ($CHCl_3$:MeOH, 90:10), and sub-frs 227-235 ($CHCl_3$:MeOH, 85:15). Compound **3** (83.2 mg) was obtained as white powder after filtration from sub-frs 1-30. Subfraction 31-235 were pooled together and were further purified over Sephadex LH-20 using isocratic $CHCl_3$:MeOH, (7:3) as eluent. Sub-frs of 5 mL were collected. Sub-frs 6-11 afforded compound **4** (6.11 mg) as a white powder while compound **5** (18 mg) was isolated in sub-frs 35-50 as a red powder.

Fraction APLd was submitted to CC using silica gel 60 with increasing gradient of $CHCl_3$:MeOH, mixtures as eluents. 45 sub-frs of 100 mL each were collected as follows: sub-frs 1-21 ($CHCl_3$:MeOH, 80:20) and sub-frs 22-45 ($CHCl_3$:MeOH, 75:25). Compound **6** (30 mg) was obtained as yellow powder after filtration from sub-frs 1-13. Sub-frs

14-34 was further purified twice over Sephadex LH-20 using isocratic $CHCl_3$:MeOH, (7:3) to afford compound **7** (95.2 mg) as beige crystals. Sub-frs 35-42 was also purified similarly to sub-frs 14-34 for yield compound **8** (10.2 mg) as a white powder.

2.4. Antibacterial Assays

2.4.1. Chemicals for Antimicrobial Assay. Chemicals used included phytochemicals, reference antibiotics (RA), microbial growth indicator, and efflux pump inhibitor (EPI). Phytochemicals were stigmasterol, β-amyrin, stigmasterol-3-*O*-β-glucopyranosyl, 3-O-methyl-D-chiro-inositol, epicatechin, quercetin-3-*O*-glucoside, 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid, and 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid. They were isolated from the leaf of *Acacia polyacantha*. Their ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) spectra as well as their major chemical shifts are provided as supporting information (S1). The RA tested were chloramphenicol (CHL), ciprofloxacin (CIP), cloxacillin (CLX), doxyciclin (DOX), gentamycin (GEN), erythromycin (ERY), kanamycin (KAN), and norfloxacin (NOR) obtained from Sigma-Aldrich, St. Quentin Fallavier, France. The microbial growth indicator used was iodonitrotetrazolium ≥ 97% (INT, Sigma-Aldrich) while the EPI was phenylalanyl-arginine-β-naphthylamide (PAßN) (Sigma-Aldrich). Dimethyl sulfoxide (Sigma-Aldrich) was used to dissolve chemicals.

2.4.2. Microbial Strains and Culture Media. In this study, 15 Gram-negative bacterial strains belonging to five species were used. They included reference (from American Type Culture Collection, ATCC) and clinical (Laboratory collection) strains of *Escherichia coli* (ATCC8739, ATCC10536, AG102, and AG100Atet), *Enterobacter aerogenes* (ATCC13048, CM64, EA27 and EA289), *Klebsiella pneumoniae* (ATCC11296, KP55 and KP63), *Providencia stuartii* (ATCC29916 and NEA16), and *Pseudomonas aeruginosa* (PA01 and PA124). Bacterial features or resistance profiles previously reported [19] are shown as supporting information (Table S2). Bacterial cultures were maintained on agar plates at 4°C and subcultured on a fresh appropriate agar plates 24 h prior to any antimicrobial assay. The activation of bacteria prior to any assay was done in Mueller Hinton Agar (Sigma) meanwhile antibacterial assays were carried out using Mueller Hinton broth (MHB; Sigma) [20].

2.4.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MICs and MBCs of extract, fractions and isolated compounds against the tested bacteria were determined by microplate dilution method using the rapid INT colorimetric assay according to previously described methods [21] with some modifications [19, 22, 23]. In general, the concentrations ranges were 8-1024 μg/mL for crude extracts, 4-512 μg/mL for fractions, and 2-256 μg/mL for chloramphenicol.

The role of efflux pumps in the susceptibility of Gram-negative bacteria to the most active samples (APL, APLb, APLd, compound **8** and CHL) was evaluated by testing the studied samples in the presence of an EPI, PAβN (at 30 μg/mL) using the rapid INT colorimetric assay as earlier described [7, 19]. A preliminary study showed that the concentration of 30 μg/mL did not affect the growth of selected bacteria [23]. Nine selected bacterial strains including *E. coli* ATCC8739 and AG102, *E. aerogenes* ATCC13048 and CM64, *K. pneumoniae* KP55 and KP63, *P. aeruginosa* PA01 and PA124 and *P. stuartii* ATCC29916 were used. Increase of activity was determined as the ratio of MIC in the absence of EPI versus MIC in the presence of EPI.

To evaluate the potentiating or antibiotic resistance modulating effect of samples, a preliminary assay was performed against a problematic bacterium, *P. aeruginosa* PA124 (see supporting information S3); the selected samples were tested at various subinhibitory concentrations in combination with antibiotics. MIC/2 and MIC/4 were selected as the best subinhibitory concentrations [6, 24] and were further used for the best samples (APL, compounds **7** and **8**) in combination with antibiotics against the seven other bacteria. Briefly, the MIC was determined as described above. The 96-wells microplate rows receiving antibiotic dilutions without extracts were used for the determination of the MICs of the antibiotics. The concentrations ranges of antibiotics were generally 2-256 μg/mL. The MIC was determined as described using INT colorimetric method as earlier described [3, 19]. The modulation factor was defined as the ratio of the MIC for the antibiotic alone and that of the antibiotics in the presence of the extract (RHL). Modulation factor ≥ 2 was set as the cutoff for biological significance of antibiotic resistance modulating effects [19, 20].

3. Results

3.1. Phytochemistry. The chemical structures of compounds isolated from the leaf of *Acacia polyacantha* were determined using NMR (^{1}H and ^{13}C) data, in comparison with the literature (Figure 1). Compounds were identified as stigmasterol $C_{29}H_{50}O$ (**1;** melting point (m.p.): 134-135°C; m/z 414) [21], β-amyrin $C_{30}H_{50}O$ (**2;** m.p.: 187-190°C; m/z 426) [25], 3-*O*-β-$_D$-glucopyranosylstigmasterol $C_{35}H_{58}O_6$ (**3;** m.p.: 272-274°C; m/z 412) [26], 3-O-methyl-D-chiro-inositol $C_7H_{14}O_6$ (**4;** m.p.: 181°C; *m/z* 217; $[\alpha]_D^{25}$: +60,00) [24], epicatechin $C_{15}H_{14}O_6$ (**5;** m.p.: 345-350°C; *m/z* 270) [27], quercetin-3-*O*-glucoside $C_{21}H_{20}O_{12}$ (**6;** m.p.: 230-232°C; *m/z* 464) [28], 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid $C_{41}H_{66}O_{12}$ (**7;** m.p.: 216-217°C; *m/z* 773; $[\alpha]_D^{25}$: +23,2° (c 1,25; MeOH)) [15] and 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid $C_{42}H_{68}O_{13}$ (**8;** amorphous powder; *m/z* 803) [21]. The ^{1}H NMR, ^{13}C NMR spectra and major chemical shifts of these compounds are available as supplementary data (S2).

3.2. Antibacterial Activity. Extracts, fractions, and isolated compounds were tested for their antimicrobial activity against the studied Gram-negative bacteria. The results are

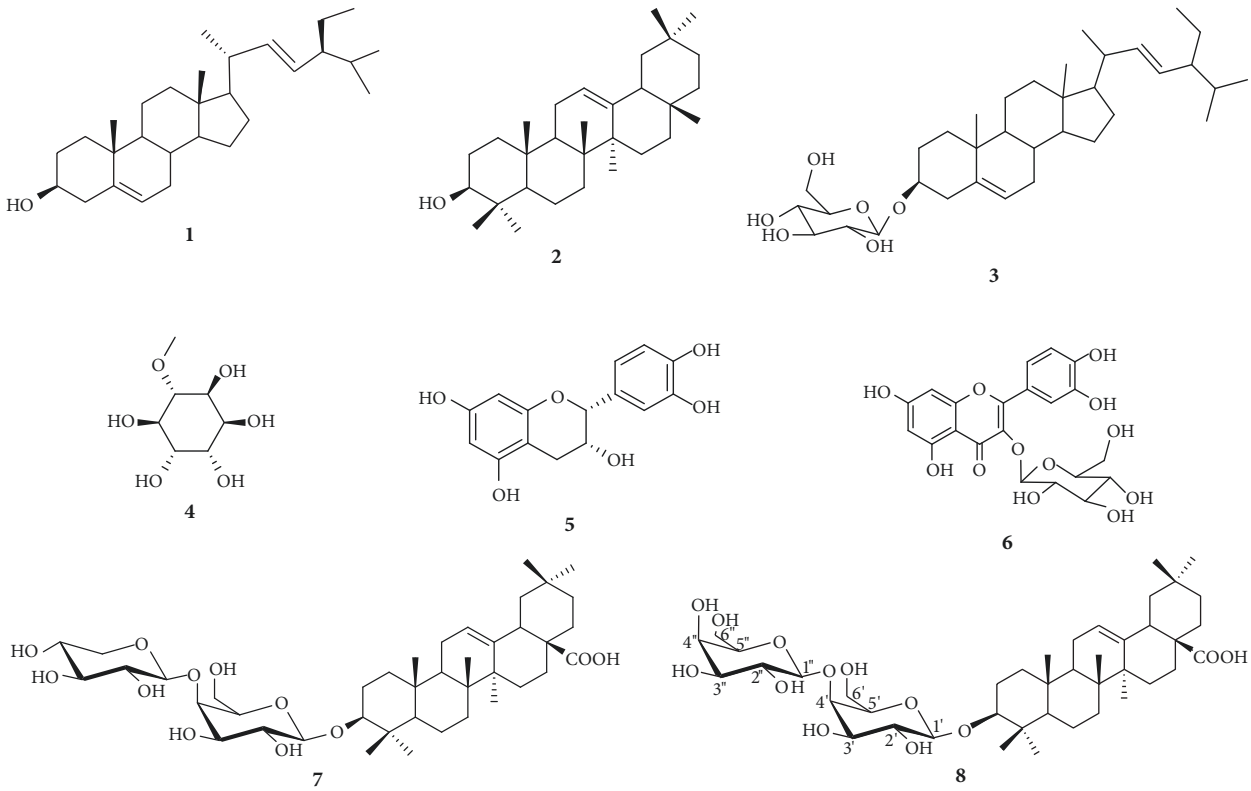


Figure 1: Chemical structures of compounds isolated from the leaf of *Acacia polyacantha*. **1:** stigmasterol, **2:** β-amyrin, **3:** 3-*O*-β-$_D$-glucopyranosylstigmasterol, **4:** 3-O-methyl-D-chiro-inositol, **5:** epicatechin, **6:** quercetin-3-*O*-glucoside, **7:** 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid, and **8:** 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid.

shown in Tables 1 and 2. APL and APB had MIC values ≤ 1024 μg/mL on 11/15 (73.3%) and 7/15 (46.7%) tested bacteria. MIC values of CHL varied between 2-256 μg/mL (Table 1). APLc as well as compounds **1**, **2** and **3** were not active (Table 2). MIC values ≤ 512 μg/mL for fractions and ≤256 μg/mL for compounds were obtained against 8/9 (88.9%) tested bacteria for both APLb and APLd and against 7/9 (77.8%) for compound **8** (Table 2). Analysis of data from Tables 1 and 2 indicated bacteriostatic effects as MBC/MIC ratios were generally above 4 with no MBC value ≤ 1024 μg/mL recorded for the crude extract, fractions, and compounds.

3.3. Role of Efflux Pumps in the Susceptibility of Gram-Negative Bacteria Botanicals and Phytochemicals. The most active extracts, fractions, isolated compound and RA (APL, APLb, APLd, Compound **8**, CHL) were tested in the presence of EPI against 9 bacterial strains including reference strains and MDR phenotypes (Table 3). The results showed that PAβN improves the activity (decrease of MIC values) of APL, APLb, APLd and compound **8** on all tested bacteria with the highest MIC values of 256 μg/mL for crude extract (APL) and compound **8**, and 64 μg/mL for fractions (Table 3). A preliminary study showed that the MIC of PAβN was above 256 μg/mL on the selected bacteria and that the concentration of 30 μg/mL did not affect their growth.

3.4. Antibiotic Resistance Modulating Effects of Botanicals and Phytochemicals. A preliminary study against *P. aeruginosa* PA124 (see supporting information S3) allowed choosing the appropriate subinhibitory concentrations of MIC/2 and MIC/4 as well as APL, compounds **7** and **8** for further studies. These samples were combined with eight antibiotics to evaluate their possible synergistic effects. The results summarized in Tables 4, 5, and 6 showed that the synergistic effects were noted with all the tested samples and many antibiotics. When tested at their MIC/2, the percentages of bacterial strains on which synergism was observed (PBS) were ≥50% when APL was combined with ERY and CIP (Table 4), when compound **7** was combined with ERY, KAN and GEN (Table 5), and when compound **8** was combined with ERY, and DOX (Table 6). At their MIC/4, the PBS ≥50% was obtained when APL was combined with GEN and CIP (Table 4), when compound **7** was combined with ERY and GEN (Table 5), and when compound **8** was combined with ERY and NOR (Table 6).

4. Discussion

4.1. Phytochemistry. The isolated compounds included five terpenoids amongst which were one sterol (stigmasterol; **1**), one triterpene (β-amyrin; **2**), and three saponins

Table 1: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in μg/mL of crude leaf and bark extracts, isolated compounds and chloramphenicol against reference strains and MDR Gram-negative bacteria.

Bacterial strains	Tested samples, MIC and MBC (in bracket) values (μg/mL)		
	Leaf extract (APL)	Bark extract (APB)	Chloramphenicol
Escherichia coli			
ATCC8739	1024 (-)	512 (-)	**2** (64)
AG102	512 (-)	-	32 (256)
AG100Atet	512 (-)	-	32 (256)
ATCC10536	1024 (-)	-	**2** (32)
Enterobacter aerogenes			
ATCC13048	256 (-)	**8** (-)	16 (128)
CM64	-	-	256 (-)
EA27	1024 (-)	256 (-)	32 (256)
EA289	-	-	32 (256)
Klebsiella pneumoniae			
ATCC11296	1024 (-)	-	32 (256)
KP55	-	128 (-)	64 (256)
KP63	-	-	32 (256)
Pseudomonas aeruginosa			
PA01	**64** (-)	256 (-)	64 (-)
PA124	256 (-)	512 (-)	256 (-)
Providencia stuartii			
NEA16	1024 (-)	-	64 (256)
ATCC29916	**8** (-)	256 (-)	64 (256)

(-): MIC or MBC value > 1024 μg/mL for APL and APB or >256 for chloramphenicol; MIC values in bold: significant antibacterial effect [18].

[stigmasterol-3-*O*-β-glucopyranosyl (**3**), **7:** 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid, (**7**) and *O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**8**)], two flavonoids: epicatechin (**5**) and quercetin-3-*O*-glucoside (**6**) and one sugar, 3-O-methyl-D-chiro-inositol (**4**). Previous phytochemical investigations of the leaf of the plant led to the isolation of polyacanthoside A, oleanolic acid, epicatechin, quercetin-3-*O*-glucoside as well as compounds **1**, **4, 3, 5** and **6** and **8** [15]. However, few compounds were reported in this study, probably due to the fact that we focussed only on the bioactive fractions of the leaf extract; meanwhile, less active fractions were not further investigated.

4.2. Antibacterial Activity. It is important to take into consideration the development of resistance by Gram-negative bacteria when searching for new antimicrobial agents. In the present study, several clinical MDR bacteria expressing MDR phenotypes were used. The MIC values of chloramphenicol were above 10 μg/mL on most of the bacterial strains (Table 1), confirming their resistance phenotypes. Established cutoff points for antibacterial activity of botanicals consider that the inhibitory effect is significant when MIC values are below 100 μg/mL, moderate when 100 ≤ MIC ≤ 625 μg/mL, and weak when MIC > 625 μg/mL [18, 29]. On this basis, the antibacterial activity of the crude extracts (APL and APB) could mostly be considered as moderate or poor (Table 1). Nevertheless, MIC values below 100 μg/mL were obtained with APL on the problematic bacterial strain *P. aeruginosa* (PA01) and *P. stuartii* NAE16 (Table 1), as well as with APB against *E. aerogenes* ATCC13048. This data suggested that these extracts could be useful to fight bacterial infections, especially in traditional medicine where they are utilised. It is worth nothing that the MIC values of APL against *P. aeruginosa* (PA01) and *P. stuartii* ATCC29916, or APB against *E. aerogenes* ATCC13048 (Table 1) were lower than those of chloramphenicol, confirming this hypothesis. This was the rational for carrying out bioguided fractionation in order to isolate more active compounds from the leaf extract. Fractions APLa, APLb, and APLd had MIC values below 100 μg/mL against 1, 3, and 7 of the 9 tested bacterial species, respectively (Table 2). This was an indication that fractionation led to more active samples. The activity of phytochemicals was set as significant when MIC<10 μg/mL, moderate when 10<MIC<100 μg/mL, and low when MIC>100 μg/mL [18, 29]. However, less active compounds were obtained from the purified fractions, with none of them displaying MIC value below 10 μg/mL (Table 2). This was an indication that constituents of the extract and fractions synergistically inhibited bacterial growth. All tested samples had MBC/MIC ratios above 4 (Tables 1 and 2), showing that they mostly exerted bacteriostatic effects [30]. To the best of our knowledge, the antibacterial activity of *A. polycantha* as well as that of its most active constituent (compound **8**) was reported for the first time.

Concerning the structure-activity relationship, it appeared that terpenoids **1** and **2** (with no sugar in their chemical structures) and **3** (with only one sugar) as well as

Table 2: MICs and MBCs in μg/mL of fractions and compounds isolated from the leaf (APL) of *Acacia polyacantha* and chloramphenicol against a panel of selected Gram-negative bacteria.

Bacterial strains	Tested samples MIC and MBC (in bracket) values (μg/mL)								
	Fractions from APL			Isolated compounds					Reference antibiotic
	APLa	APLb	APLd	**4**	**5**	**6**	**7**	**8**	chloramphenicol
Escherichia coli									
ATCC8739	-	**16** (-)	**16** (-)	-	-	-	-	32 (-)	**2** (64)
AG102	-	128 (-)	**64** (-)	256 (-)	-	128 (-)	-	32 (-)	32 (256)
Enterobacter aerogenes									
ATCC13048	-	256 (-)	**8** (-)	128 (-)	64 (-)	-	-	32 (-)	16 (128)
EA27	-	-	128 (-)	256 (-)	256 (-)	-	-	-	32 (256)
Klebsiella pneumoniae									
ATCC11229	**16** (-)	**64** (-)	**16** (-)	64 (-)	32 (-)	-	-	32 (-)	32 (256)
KP55	-	**16** (-)	**64** (-)	128 (-)	256 (-)	-	128 (-)	32 (-)	64 (256)
Pseudomonas aeruginosa									
PA01	-	128 (-)	**64** (-)	-	-	-	-	-	64 (-)
PA124	-	256 (-)	**64** (-)	256 (-)	128 (-)	-	-	32 (-)	256 (-)
Providencia stuartii									
ATCC29916	-	128 (-)	-	-	-	-	-	64 (-)	64 (256)

(-): > 512 μg/ml for fractions or > 256 μg/ml for compounds; APL: leaf extract; APLa, APLb, and APLd are fractions from APL; **1:** stigmasterol, **2:** β-amyrin, **3:** 3-*O*-β-$_D$-glucopyranosylstigmasterol, **4:** 3-O-methyl-D-chiro-inositol, **5:** epicatechin, **6:** quercetin-3-*O*-glucoside, **7:** 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid, **8:** 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid; MIC values in bold: significant antibacterial effect [18]. Notes: MICs of fraction APLc > 512 μg/ml and MIC of compounds **1, 2, 3** were > 256 μg/ml in all tested bacteria.

Table 3: MICs (in μg/mL) of crude leaf extract (APL), active fractions (APLa and APLd), 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**8**) and chloramphenicol in the presence of PAßN and fold increase of activity (in parenthesis) against a panel of selected Gram-negative bacteria.

Bacterial strains	Tested samples, MIC in μg/mL in presence of PAßN and fold increase of activity (in parenthesis)				
	Leaf extract	Fractions from APL		Isolated compound	Reference antibiotic
	APL	APLb	APLd	**8**	Chloramphenicol
Escherichia coli					
ATCC8739	256 **(4)**	**4 (4)**	**2 (8)**	**≤2 (≥ 16)**	**≤ 2 (≥ 1)**
AG102	**64 (8)**	**32 (2)**	**8 (16)**	**≤ 2 (≥ 16)**	**4 (8)**
Enterobacter aerogenes					
ATCC13048	**64 (4)**	**4 (2)**	**≤4 (≥ 64)**	**≤ 2 (≥ 16)**	**2 (8)**
EA27	16 **(64)**	**64 (2)**	**4 (128)**	64 **(4)**	**4 (8)**
Klebsiella pneumoniae					
ATCC11229	128 **(8)**	**≤ 4 (≥ 4)**	**≤ 4 (≥ 16)**	**≤ 2 (≥ 16)**	**4 (8)**
KP55	128 **(8)**	**32 (2)**	**4 (4)**	**≤ 2 (≥ 16)**	16 **(4)**
Pseudomonas aeruginosa					
PA01	128 (0.5)	**32 (2)**	**32(4)**	**≤2 (≥ 128)**	**8 (8)**
PA124	128 **(2)**	**32 (2)**	128 **(2)**	16 **(2)**	64 **(4)**
Providencia stuartii					
ATCC29916	**≤ 4 (≥ 2)**	**32 (16)**	**32(4)**	16 **(4)**	16 **(4)**

(-): MIC > 1024 μg/mL for crude extract APL, >512 for fractions APLb and APLd, 256 μg/mL for 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**8**); (): values in parenthesis are fold increase of activity in the presence of PAßN at 30 μg/mL; MIC values in bold: significant antibacterial effects for MIC [18] and significant increase of activity in the presence of PAßN [19, 20]; fold increase of activity determined MIC of sample tested alone (Tables 1 and 2) versus MIC of the sample in the presence of PAßN. The MIC of PAßN was >256 mg/L for *E. coli*, *E. aerogenes*, *K. pneumoniae*, *P. aerogenes,* and *P. stuartii* strains.

Table 4: Antibiotic resistance modulatory activity of leaf extract (APL) of *Acacia polyacantha* against MDR Gram-negative bacteria.

Antibiotics	Extract concentration	Bacteria, MIC (μg/mL) and modulating factors of the leaf extract (in bracket)								
		ATCC8739	AG102	ATCC13048	CM64	KP55	KP63	PA124	NEA16	PBS (%)
ERY	0	128	256	256	256	256	256	256	256	
	MIC/2	256 (0.5)^{a}	**128 (2)^{S}**	**128 (2)^{S}**	256 (1)^{I}	256 (1)^{I}	**32 (8)^{S}**	> 256 (> 1)^{a}	**128 (2)^{S}**	50
	MIC/4	256 (0.5)^{a}	256 (1)^{I}	256 (1)^{I}	**128 (2)^{S}**	256 (1)^{I}	**128 (2)^{S}**	256 (1)^{I}	**128 (2)^{S}**	37.5
CLX	0	256	1024	> 1024	> 1024	1024	512	> 1024	> 1024	
	MIC/2	> 1024 (> 0.25)^{a}	> 1024 (> 1)^{a}	> 1024 (≥ 1)^{I}	> 1024 (≥ 1)^{I}	> 1024 (>1)^{a}	> 1024 (> 0.5)^{a}	> 1024 (≥ 1)^{I}	> 1024 (≥ 1)^{I}	0
	MIC/4	> 1024 (> 0.25)^{a}	> 1024 (> 1)^{a}	> 1024 (≥ 1)^{I}	> 1024 (≥ 1)^{I}	> 1024 (> 1)^{a}	> 1024 (> 0.5)^{a}	> 1024 (≥ 1)^{I}	**1024 (1)^{S}**	12.5
KAN	0	16	16	< 2	16	4	64	8	64	
	MIC/2	128(0.125)^{a}	64 (0.25)^{a}	4 (0.5)^{a}	16 (1)^{I}	**< 2 (2)^{S}**	128 (0.5)^{a}	8(1)^{I}	**< 2(32)^{S}**	25
	MIC/4	128(0.125)^{a}	32 (0.5)^{a}	8 (0.25)^{a}	**8 (2)^{S}**	**< 2 (2)^{S}**	128 (0.5)^{a}	8(1)^{I}	**16 (4)^{S}**	37.5
GEN	0	32	16	16	16	256	32	128	256	
	MIC/2	> 256 (> 0.125)^{a}	> 256 (> 0.062)^{a}	16 (1)^{I}	16 (1)^{I}	256 (1)^{I}	> 256 (> 0.125)^{a}	**64 (2)^{s}**	**16 (16)^{S}**	25
	MIC/4	> 256 (> 0.125)^{a}	**< 2(8)^{S}**	16 (1)^{I}	16 (1)^{I}	**< 2 (128)^{S}**	256 (1)^{I}	**64 (2)^{s}**	**16 (16)^{S}**	50
DOX	0	< 1	4	16	16	4	8	64	32	
	MIC/2	2 (0.5) ^{a}	8 (0.5) ^{a}	32 (0.5)^{a}	16 (1)^{I}	4 (1)	**4 (2)^{S}**	**32 (2)^{S}**	> 128 (> 025)^{a}	25
	MIC/4	< 1 (≤ 1)^{I}	8 (0.5)^{a}	32 (0.5)^{a}	16 (1)^{I}	**< 1 (> 4)^{S}**	8 (1)^{I}	64 (1)^{I}	> 128 (> 025) ^{a}	14.28
CHL	0	2	32	16	256	64	32	256	64	
	MIC/2	2 (1)^{I}	32 (1)^{I}	> 16 (> 1)^{a}	256 (1)^{I}	64 (1)^{I}	32 (1)^{I}	256 (1)^{I}	64 (1)^{I}	0
	MIC/4	2 (1)^{I}	32 (1)^{I}	> 16 (> 1)^{a}	256 (1)^{I}	6 (1)^{I}	32 (1)^{I}	256 (1)^{I}	64 (1)^{I}	0
NOR	0	4	8	32	32	8	8	64	32	
	MIC/2	32 (0.125)^{a}	64 (0.125)^{a}	**16 (2)^{S}**	32 (1)^{I}	64 (0.125)^{a}	64 (0.125)^{a}	**32 (2)^{S}**	32 (1)^{I}	25
	MIC/4	> 128 (> 0.031)^{a}	> 128(> 0.062)^{a}	**16 (2)^{S}**	32 (1)^{I}	> 128 (> 0.062)^{a}	> 128 (> 0.062)^{a}	**32 (2)^{S}**	32 (1)^{I}	25
CIP	0	< 0.5	1	8	8	< 0.5	1	64	8	
	MIC/2	16 (0.031)^{a}	16 (0.0625)^{a}	**4 (2)^{S}**	**4 (2)^{S}**	16 (0.031)^{a}	8 (0.125) ^{a}	**16 (4)**^{S}	**4 (2)^{S}**	50
	MIC/4	16 (0.031)^{a}	16 (0.0625)^{a}	**4 (2)^{S}**	**4 (2)^{S}**	16 (0.031)^{a}	8 (0.125)^{a}	**16 (4)**^{S}	**4 (2)^{S}**	50

ERY: erythromycin; CLX: cloxacillin; KAN: kanamycin; GEN: gentamycin; DOX: doxyciclin; CHL: chloramphenicol; NOR: norfloxacin; CIP: ciprofloxacin; bacterial strains: *Escherichia coli* (AG102, ATCC8739); *Enterobacter aerogenes* (ATCC13048, CM64); *Klebsiella pneumoniae* (KP55, KP63); *P. aeruginosa* PA124; *Providencia stuartii* NEA16; PBS: percentage of bacteria strain on which synergism has been observed. (): ameliorating factor of the antibiotics after association with APF; S: synergy; I: indifference; a: antagonist; values in bold: case of synergy [19, 20].

Table 5: Antibiotic resistance modulatory activity of 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**7**) against MDR Gram-negative bacteria.

Antibiotics	Extract concentration	Bacteria, MIC (μg/mL) and modulating factors of compound **7** (in bracket)								
		ATCC8739	AG102	ATCC13048	CM64	KP55	KP63	PA124	NEA16	PBS (%)
ERY	0	128	256	256	256	256	256	256	256	
	MIC/2	256 (0.5)[a]	**128 (2)[S]**	**128 (2)[S]**	**128 (2)[S]**	**64 (4)[S]**	**128 (2)[S]**	**64 (2)[s]**	256 (1)[I]	**75**
	MIC/4	256 (0.5)[a]	**64 (4)[S]**	**128 (2)[S]**	**128 (2)[S]**	**128 (2)[S]**	**128 (2)[S]**	**64 (2)[s]**	**128 (2)[S]**	**87.5**
CLX	0	256	1024	> 1024	> 1024	1024	512	> 1024	> 1024	
	MIC/2	512 (0.5)[a]	> 1024 (> 1)[a]	**1024 (1)[S]**	> 1024 (≥ 1)[I]	**512 (2)[S]**	**128 (4)[S]**	> 1024 (≥ 1)[I]	> 1024 (≥ 1)[I]	37.5
	MIC/4	512 (0.5)[a]	1024 (1)[I]	**1024 (1)[S]**	> 1024 (≥ 1)[I]	**128 (8)[S]**	> 1024 (> 0.5)[a]	> 1024 (≥ 1)[I]	**1024 (1)[S]**	37.5
KAN	0	16	16	< 2	16	4	64	8	64	
	MIC/2	128 (0.125)[a]	128 (0.125)[a]	< 2 (≤ 1)[I]	**< 2 (> 8)[S]**	**< 2 (> 2)[S]**	**16 (4)[S]**	16 (0.5)[a]	**16 (4)[S]**	50
	MIC/4	128 (0.125)[a]	64 (0.25)[a]	< 2 (≤ 1)[I]	**< 2 (> 8)[S]**	**< 2 (> 2)[S]**	256 (025)[a]	16 (0.5)[a]	**4 (16)[S]**	37.5
GEN	0	32	16	16	16	256	32	128	256	
	MIC/2	64 (0.5)[a]	> 256 (> 0.062)[a]	**4 (4)[S]**	**< 2 (> 8)[S]**	**< 2 (128)[S]**	**16 (2)[S]**	256 (0.5)[a]	**4 (64)[S]**	62.5
	MIC/4	256 (0.125)[a]	> 256 (0.062)[a]	**4 (4)[S]**	**< 2 (> 8)[S]**	**< 2 (128)[S]**	256 (0.125)[a]	256 (0.5)[a]	**4 (64)[S]**	50
DOX	0	< 1	4	16	16	4	8	64	32	
	MIC/2	< 1 (≤ 1)[I]	8 (0.5)[a]	16 (1)[I]	16 (1)[I]	4 (1)[I]	**2 (4)[S]**	**32 (2)[S]**	**16 (2)[S]**	37.5
	MIC/4	4 (0.25)[a]	4 (1)[I]	16 (1)[I]	16 (1)[I]	4 (1)[I]	**2 (4)[S]**	**32 (2)[S]**	**16 (2)[S]**	37.5
CHL	0	2	32	16	256	64	32	256	64	
	MIC/2	2 (1)[I]	32 (1)[I]	16 (1)[I]	256 (1)[I]	64 (1)[I]	32 (1)[I]	256 (1)[I]	64 (1)[I]	0
	MIC/4	2 (1)[I]	**16 (2)[S]**	16 (1)[I]	256 (1)[I]	64 (1)[I]	**16 (2)[S]**	256 (1)[I]	64 (1)[I]	25
NOR	0	4	8	32	32	8	8	64	32	
	MIC/2	64 (0.062)[a]	32 (0.25)[a]	**16 (2)[S]**	32 (1)[I]	64 (0.125)[a]	8 (1)[I]	**32 (2)[S]**	32 (1)[I]	25
	MIC 4	64 (0.062)[a]	32 (0.25)[a]	32 (1)[I]	32 (1)[I]	32 (0.25)[a]	8 (1)[I]	**32 (2)[S]**	32 (1)[I]	12.5
CIP	0	< 0.5	1	8	8	< 0.5	1	64	8	
	MIC/2	2 (0.25)[a]	8 (0.125)[a]	**1 (8)[S]**	8 (1)[I]	8 (0.062)[a]	4 (0.25)[a]	**32 (2)[S]**	**4 (2)[S]**	37.5
	MIC/4	8 (0.062)[a]	4 (0.25)[a]	**2 (4)[S]**	8 (1)[I]	2 (0.25)[a]	4 (0.25)[a]	**32 (2)[S]**	**4 (2)[S]**	37.5

ERY: erythromycin; CLX: cloxacillin; KAN: kanamycin; GEN: gentamycin; DOX: doxyciclin; CHL: chloramphenicol; NOR: norfloxacin; CIP: ciprofloxacin; bacterial strains: *Escherichia coli* (AG102, ATCC8739); *Enterobacter aerogenes* (ATCC13048, CM64); *Klebsiella pneumoniae* (KP55, KP63); *P. aeruginosa* PA124; *Providencia stuartii* NEA16; PBS: percentage of bacteria strain on which synergism has been observed. (): ameliorating factor of the antibiotics after association with compound **7**; S: synergy; I: indifference; a: antagonist; values in bold: case of synergy [19, 20].

Table 6: Antibiotic resistance modulatory activity of 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**8**) against MDR Gram-negative bacteria.

Antibiotics	Extract concentration	Bacteria, MIC (μg/mL) and modulating factors of compound **8** (in bracket)								
		ATCC8739	AG102	ATCC13048	CM64	KP55	KP63	PA124	NEA16	PBS (%)
ERY	0	128	256	256	256	256	256	256	256	
	MIC/2	256 (0.5)[a]	**128 (2)[S]**	256 (1)[I]	**128 (2)[S]**	**128 (2)[S]**	**64 (4)[S]**	**64 (4)[s]**	**128 (2)[S]**	**75**
	MIC/4	128 (1)[I]	**128 (2)[S]**	**128 (2)[S]**	256 (1)[I]	256 (1)[I]	**64 (4)[S]**	**128 (2)[S]**	256 (1)[I]	50
CLX	0	256	1024	> 1024	> 1024	1024	512	> 1024	> 1024	
	MIC/2	1024 (0.25)[a]	> 1024 (> 1)[a]	> 1024 (≥ 1)[I]	> 1024 (≥ 1)[I]	1024 (1)[I]	1024 (0.5)[a]	> 1024 (≥ 1)[I]	> 1024 (≥ 1)[I]	0
	MIC/4	> 1024(>0.25)[a]	> 1024 (> 1)[a]	> 1024 (≥ 1)[I]	> 1024 ≥ 1)[I]	1024 (1)[I]	> 1024 (> 0.5)[a]	> 1024 (≥ 1)[I]	> 1024 (≥ 1)[I]	0
KAN	0	16	16	< 2	16	4	64	8	64	
	MIC/2	**4 (4)[S]**	64 (0.25)[a]	128 (0.015)[a]	128 (0.125)[a]	**< 2 (2)[S]**	256 (0.25)[a]	8 (1)[I]	128 (0.5)[a]	25
	MIC/4	128 (0.125)[a]	32 (0.25)[a]	128 (0.015)[a]	128 (0.125)[a]	**< 2 (2)[S]**	256 (0.25)[a]	8 (1)[I]	128 (0.5)[a]	12.5
GEN	0	32	16	16	16	256	32	128	256	
	MIC/2	256 (0.125)[a]	64 (0.25)[a]	128 (0.125)[a]	**8 (2)[S]**	**64 (4)[S]**	256 (0.125)[a]	**64 (2)[s]**	256 (1)[I]	37.5
	MIC/4	256 (0.125)[a]	> 256 (> 0.062)[a]	128 (0.125)[a]	128 (0.125)[a]	**128(2)[S]**	> 256 (>0.125)[a]	128 (1)[I]	**128 (2)[S]**	25
DOX	0	< 1	4	16	16	4	8	64	32	
	MIC/2	< 1 (≤ 1)[I]	**2 (2)[S]**	16 (1)[I]	32 (0.5)a	**< 1 (> 4)[S]**	**2 (4)[S]**	**32 (2)[s]**	**16 (2)[S]**	62.5
	MIC/4	< 1 (≤ 1) [I]	4 (1)[I]	32 (0.5)a	32 (0.5)a	**< 1 (> 4)[S]**	**2 (4)[S]**	**32 (2)[s]**	32 (1)[I]	37.5
CHL	0	2	32	16	256	64	32	256	64	
	MIC/2	2 (1)[I]	**16 (2)[S]**	16 (1)[I]	256 (1)[I]	**32 (2)[S]**	32 (1)[I]	256 (1)[I]	64 (1)[I]	25
	MIC/4	**1 (2)[S]**	32(1)[I]	16 (1)[I]	256 (1)[I]	**32 (2)[S]**	**16 (2)[S]**	256 (1)[I]	64 (1)[I]	37.5
NOR	0	4	8	32	32	8	8	64	32	
	MIC/2	**2 (2)[S]**	8 (1)[I]	> 128 (> 0.25)[a]	64 (0.5)[a]	**4 (2)[S]**	8 (1)[I]	**32 (2)[S]**	128 (0.125)[a]	37.5
	MIC/4	**< 1 (> 4)[S]**	**2 (4)[S]**	128 (0.25)[a]	64 (0.5)[a]	**4 (2)[S]**	**4 (2)[S]**	**32 (2)[S]**	128 (0.125)[a]	62.5
CIP	0	< 0.5	1	8	8	< 0.5	1	64	8	
	MIC/2	< 0.5 (≤ 1)[I]	**< 0.5 (> 2)[S]**	64 (0.125)[a]	64 (0.125)[a]	< 0.5 (≤ 1)[I]	4 (0.25)[a]	64 (1)[I]	64 (0.125)[a]	12.5
	MIC/4	2 (0.25)[a]	**< 0.5 (> 2)[S]**	64 (0.125)[a]	64 (0.125)[a]	1 (2)[a]	2 (0.5)[a]	64 (1)[I]	64 (0.125)[a]	12.5

ERY: erythromycin; CLX: cloxacillin; KAN: kanamycin; GEN: gentamycin; DOX: doxyciclin; CHL: chloramphenicol; NOR: norfloxacin; CIP: ciprofloxacin; bacterial strains: *Escherichia coli* (AG102, ATCC8739); *Enterobacter aerogenes* (ATCC13048, CM64); *Klebsiella pneumonia* (KP55, KP63); *P. aeruginosa* PA124; *Providencia stuartii* NEA16; PBS: percentage of bacteria strain on which synergism has been observed. (): ameliorating factor of the antibiotics after association with compound **8**; S: synergy; I: indifference; a: antagonist; values in bold: case of synergy [19, 20].

the polyol (**4**) were devoid of antibacterial activity (Table 2). Flavonoids **5** and **6** as well as saponins **7** and **8** had selective and poor antibacterial. Within saponins, it can be noted that the presence of a second galactopyranosyl substituent (compound **8**) instead of xylopyranosyl (compound 7) significantly increased the antibacterial activity, with compound **8** displaying MIC values ≤ 64 μg/mL against 7/9 tested bacteria versus 0/9 for compound **7** (Table 2).

4.3. Role of Bacterial Efflux Pumps. The clinical MDR bacteria tested in this work overexpressed efflux mechanism *via* the efflux pumps of the resistance nodulation cell division (RND) family, namely, AcrAB-TolC for enterobacteria such as *E. coli, E. aerogenes, K. pneumoniae,* and *P. stuartii* and MexAB-OprM for *P. aeruginosa* [31–36]. These efflux pumps expel toxic compounds (including antibiotics) out of the bacterial cytoplasm, preventing them from reaching their intracellular target [37]. Efflux Pump Inhibitors, such as PaβN, could be used to restore the intracellular concentration of antibacterials acting on intracellular target by blocking the bacterial efflux pumps. In the presence of PAβN, it was observed that the activity of the crude extract (except against *P. aeruginosa* PA01), fractions APLb and APLd as well as compound **8** and CHL strongly increased on almost all tested bacteria (Table 3). The fold increase ranged from 2 to ≥ 4 for APLb, from ≥ 1 to 8 for CHL, from 2 to ≥ 128 for compound **8**, from 0.5 to 64 for APL, from 2 to 128 for APLd. This clearly indicated that compound **8** as well as other active constituents of the APL are substrates of bacterial efflux pumps and that they may have an intracellular target [38]. Consequently, the development of an antibacterial drug combination of compound **8**, as well as extracts or fractions with an EPI, could be an interesting strategy to tackle MDR bacterial infections. In effect, modulation factor ≥ 2 define a biologically significant antibiotic resistance modulating substance [19, 20]. Previous study demonstrated that PAβN could also restore the activity of several natural compounds on MDR bacteria expressing active efflux pumps, with MIC values decreasing below 10 μg/mL in most of the tested bacteria for the coumarin, MAB3, the xanthone, laurentixanthone B, the naphthoquinones: diospyrone and plumbagin and the flavonoids: 4-hydroxylonchocarpin and isobavachalcone [22, 23].

4.4. Antibiotic Resistance Modulating Effects. Difficulties in the field of novel antibacterial drug discovery, for combating resistant pathogens, have propelled the search for new alternative medicine to improve or to restore the activity of commonly used antibiotics. Combining antibiotics with botanicals and phytochemicals is an attractive strategy as regards the diversity of secondary metabolites from natural source. If an antibacterial substance improves the activity of at least 70% of the tested antibiotics on more than 70% tested bacterial strains, it might be considered as a potential efflux pump inhibitor [39]. However, this was not the case in the present study, as neither APL nor compounds **7** and **8** were able to exert such degree of synergistic effects with antibiotics (Tables 4–6). However, synergistic effects were observed between APL, compounds **7** and **8** with at least one of the eight tested antibiotics against at least 50% of the MDR bacterial strains (Tables 3–6). This suggests that possible combination of these samples with specific antibiotics could help in antibacterial chemotherapy.

5. Limitations

Our study has limitations. The toxicity of this plant also needs to be performed to evaluate its safety.

6. Conclusion

In the present study, the antibacterial activity of the crude extract, fractions, and compounds from the leaf of *Acacia polyacantha* Willd. (Fabaceae) was investigated. It was found that the leaf extract was more active than the bark extract. The antibacterial constituents of the leaf extract include epicatechin (**5**), quercetin-3-*O*-glucoside (**6**), 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid (**7**), and 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid. Saponin **8** was the major antibacterial constituent of the plant and acted as a substrate of bacterial EPI. Although the crude extract and its constituents were not EPI, they showed synergistic effects with several antibiotics and could be selectively used in bacterial chemotherapy. The overall results demonstrated that *Acacia polyacantha* is a source of antibacterial drug that should be explored further to develop novel substances to combat both sensitive and MDR phenotypes.

Abbreviations

1: Stigmasterol
2: β-amyrin
3: Stigmasterol-3-*O*-β-glucopyranosyl
4: 3-O-methyl-D-chiro-inositol
5: Epicatechin
6: Quercetin-3-*O*-glucoside
7: 3-*O*-[β-$_D$-xylopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid
8: 3-*O*-[β-galactopyranosyl-(1⟶4)-β-$_D$-galactopyranosyl]-oleanolic acid
AcOEt: Ethyl acetate
ATCC: American Type Culture Collection
APB: *Acacia polyacantha* bark extract
APL: *Acacia polyacantha* leaf extract
APLa-d: Fractions of *Acacia polyacantha* leaf extract
CC: Column chromatography
CIP: Ciprofloxacin
$CHCl_3$: Chloroform
CHL: Chloramphenicol
CLX: Cloxacillin
DMSO: Dimethyl sulfoxide
DOX: Doxyciclin

EPI: Efflux pump inhibitor
E. aerogenes: *Enterobacter aerogenes*
E. cloacae: *Enterobacter cloacae*
E. coli: *Escherichia coli*
ERY: Erythromycin
GEN: Gentamycin
INT: *p*-iodonitrotetrazolium chloride
KAN: Kanamycin
K. pneumoniae: *Klebsiella pneumoniae*
MBC: Minimal bactericidal concentration
MDR: Multidrug resistant
MeOH: Methanol
MHB: Mueller Hinton Broth
MIC: Minimal inhibitory concentration
m.p.: Melting point
NMR: Nuclear magnetic resonance
NOR: Norfloxacin
PAßN: Phenylalanine-arginine-β-naphthylamide
P. aeruginosa: *Pseudomonas aeruginosa*
P. stuartii: *Providencia stuartii*
RA: Reference antibiotic.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Flora T. Mambe, Jean Na-Iya, Ghislain W. Fotso, and Fred Ashu carried out the study; Bathélémy Ngameni, Bonaventure T. Ngadjui, Veronique P. Beng, and Victor Kuete designed the experiments. Victor Kuete wrote the manuscript; Victor Kuete supervised the work and provided the bacterial strains; all authors read and approved the final manuscript.

Acknowledgments

Authors are thankful to the Cameroon National Herbarium for identification of plants.

Supplementary Materials

Supplementary file.docx. *S1:* RMN ^{1}H, 13C and major chemical shifts of studied compounds; *S2:* bacterial strains and features; *S3:* preliminary evaluation of antibiotic resistance modulatory activity of selected samples at subinhibitory concentrations against *Pseudomonas aeruginosa* PA124 [1100 KB]. *(Supplementary Materials)*

References

[1] H. Harbottle, S. Thakur, S. Zhao, and D. G. White, "Genetics of antimicrobial resistance," *Animal Biotechnology*, vol. 17, no. 2, pp. 111–124, 2006.

[2] A. A. Amgad, R. P. J. Martin, M. M. Ismail et al., "Antimicrobial activities of seed extracts of mango (Mangifera indica L.)," *Advances in Microbiology*, vol. 2, no. 4, pp. 571–576, 2012.

[3] J. K. Dzotam, I. K. Simo, G. Bitchagno et al., "In vitro antibacterial and antibiotic modifying activity of crude extract, fractions and 3',4',7-trihydroxyflavone from myristica fragrans houtt against MDR Gram-negative enteric bacteria," *BMC Complementary and Alternative Medicine*, vol. 18, no. 1, p. 15, 2018.

[4] V. Kuete, R. Metuno, B. Ngameni et al., "Antimicrobial activity of the methanolic extracts and compounds from treculia obovoidea (Moraceae)," *Journal of Ethnopharmacology*, vol. 112, no. 3, pp. 531–536, 2007.

[5] V. Kuete, J. R. Nguemeving, V. P. Beng et al., "Antimicrobial activity of the methanolic extracts and compounds from Vismia laurentii De Wild (Guttiferae)," *Journal of Ethnopharmacology*, vol. 109, no. 3, pp. 372–379, 2007.

[6] V. Kuete, P. Y. Ango, G. W. Fotso et al., "Antimicrobial activities of the methanol extract and compounds from Artocarpus communis (Moraceae)," *BMC Complementary and Alternative Medicine*, vol. 11, no. 1, 2011.

[7] D. E. Djeussi, J. A. K. Noumedem, J. A. Seukep et al., "Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram-negative bacteria," *BMC Complementary and Alternative Medicine*, vol. 13, article 164, 2013.

[8] J. A. Seukep, A. G. Fankam, D. E. Djeussi et al., "Antibacterial activities of the methanol extracts of seven Cameroonian dietary plants against bacteria expressing MDR phenotypes," *Springerplus*, vol. 2, article 363, 2013.

[9] A. G. Fankam, J. R. Kuiate, and V. Kuete, "Antibacterial activities of beilschmiedia obscura and six other cameroonian medicinal plants against multi-drug resistant gram-negative phenotypes," *BMC Complementary and Alternative Medicine*, vol. 14, article 241, 2014.

[10] I. K. Voukeng, V. Kuete, J. P. Dzoyem et al., "Antibacterial and antibiotic-potentiation activities of the methanol extract of some cameroonian spices against gram-negative multi-drug resistant phenotypes," *BMC Research Notes*, vol. 5, p. 299, 2012.

[11] A. G. Fankam, J. R. Kuiate, and V. Kuete, "Antibacterial and antibiotic resistance modifying activity of the extracts from allanblackia gabonensis, combretum molle and gladiolus quartinianus against gram-negative bacteria including multi-drug resistant phenotypes," *BMC Complementary and Alternative Medicine*, vol. 15, article 206, 2015.

[12] J. K. Dzotam, F. K. Touani, and V. Kuete, "Antibacterial and antibiotic-modifying activities of three food plants (Xanthosoma mafaffa Lam., moringa oleifera (L.) Schott and Passiflora edulis Sims) against multidrug-resistant (MDR) gram-negative bacteria," *BMC Complementary and Alternative Medicine*, vol. 16, no. 1, article 9, 2016.

[13] H. T. Manekeng, A. T. Mbaveng, G. S. Nguenang et al., "Anti-staphylococcal and antibiotic-potentiating activities of seven Cameroonian edible plants against resistant phenotypes," *Investigational Medicinal Chemistry and Pharmacology*, vol. 1, no. 1, article 7, 2018.

[14] C. Orwa, A. Mutua, R. Kindt, R. Jamnadass, and S. Anthony, "Agroforestree Database: a tree reference and selection

guide version 4.0," http://www.worldagroforestry.org/output/agroforestree-database.

[15] G. Fotso W., J. Na-Iya, A. Mbaveng T. et al., "Polyacanthoside A, a new oleanane-type triterpenoid saponin with cytotoxic effects from the leaves of Acacia polyacantha (Fabaceae)," *Natural Product Research (Formerly Natural Product Letters)*, pp. 1–6, 2018.

[16] Y. A. Koudoro, D. C. P. Agbangnan, B. Yèhouénou et al., "Chemical characterization and biological activities of extracts from two plants (Cissus quadrangularis and Acacia polyacantha) used in veterinary medicine in Benin," *Journal of Pharmacognosy and Phytochemistry*, vol. 3, pp. 91–96, 2015.

[17] C. Waterman, R. A. Smith, L. Pontiggia, and A. Der-Marderosian, "Anthelmintic screening of sub-saharan african plants used in traditional medicine," *Journal of Ethnopharmacology*, vol. 127, no. 3, pp. 755–759, 2010.

[18] V. Kuete, "Potential of cameroonian plants and derived products against microbial infections: a review," *Planta Medica*, vol. 76, no. 14, pp. 1479–1491, 2010.

[19] A. G. Fankam, J.-R. Kuiate, and V. Kuete, "Antibacterial and antibiotic resistance modulatory activities of leaves and bark extracts of recinodindron heudelotii (Euphorbiaceae) against multidrug-resistant gram-negative bacteria," *BMC Complementary and Alternative Medicine*, vol. 17, no. 1, article 168, 2017.

[20] J. Kovač, N. Gavarić, F. Bucar, and S. S. Možina, "Antimicrobial and resistance modulatory activity of alpinia katsumadai seed phenolic extract, essential oil and post-distillation extract," *Food Technol Biotechnol*, vol. 52, no. 2, pp. 248–254, 2014.

[21] V. S. Chaturvedula and I. Prakash, "Isolation of stigmasterol and β-sitosterol from the dichloromethane extract of rubus suavissimus," *International Current Pharmaceutical Journal*, vol. 1, no. 9, pp. 239–242, 2012.

[22] V. Kuete, B. Ngameni, J. G. Tangmouo et al., "Efflux pumps are involved in the defense of gram-negative bacteria against the natural products isobavachalcone and diospyrone," *Antimicrobial Agents and Chemotherapy*, vol. 54, no. 5, pp. 1749–1752, 2010.

[23] V. Kuete, S. Alibert-Franco, K. O. Eyong, B. Ngameni et al., "Antibacterial activity of some natural products against bacteria expressing a multidrug-resistant phenotype," *International Journal of Antimicrobial Agents*, vol. 37, no. 2, pp. 156–161, 2011.

[24] N. Sharma, M. K. Verma, D. K. Gupta, N. K. Satti, and R. K. Khajuria, "Isolation and quantification of pinitol in Argyrolobium roseum plant, by 1H-NMR," *Journal of Saudi Chemical Society*, vol. 20, no. 1, pp. 81–87, 2016.

[25] S. B. Mahato and A. P. Kundu, "13C NMR spectra of pentacyclic triterpenoids-a compilation and some salient features," *Phytochemistry*, vol. 37, no. 6, pp. 1517–1575, 1994.

[26] C. S. R. Freire, D. S. C. Coelho, N. M. Santos, A. J. D. Silvestre, and C. P. Neto, "Identification of delta7 phytosterols and phytosteryl glucosides in the wood and bark of several acacia species," *Lipids*, vol. 40, no. 3, pp. 317–322, 2005.

[27] I. K. Adnyana, Y. Tezuka, S. Awale, A. H. Banskota, K. Q. Tran, and S. Kadota, "Quadranosides VI-XI, six new triterpene glucosides from the seeds of Combretum quadrangulare," *Chemical & Pharmaceutical Bulletin*, vol. 48, no. 8, pp. 1114–1120, 2000.

[28] L. W. Xu, J. Chen, H. Y. Qi, and Y. P. Shi, "Phytochemicals and their biological activities of plants in Tagetes L," *Chinese Herbal Medicines*, vol. 4, no. 2, pp. 103–117, 2012.

[29] V. Kuete and T. Efferth, "Cameroonian medicinal plants: Pharmacology and derived natural products," *Frontiers in Pharmacology*, vol. 1, article 123, 2010.

[30] C. Mims, J. Playfair, I. Roitt, D. Wakelin, and R. Williams, "Antimicrobials and chemotherapy," in *Medical Microbiology Review*, CA. Mims, Ed., vol. 35, pp. 1–34, 1993.

[31] J. Chevalier, J.-M. Pages, A. Eyraud, and M. Mallea, "Membrane permeability modifications are involved in antibiotic resistance in klebsiella pneumoniae," *Biochemical and Biophysical Research Communications*, vol. 274, no. 2, pp. 496–499, 2000.

[32] M. Malléa, A. Mahamoud, J. Chevalier et al., "Alkylaminoquinolines inhibit the bacterial antibiotic efflux pump in multidrug-resistant clinical isolates," *Biochemical Journal*, vol. 376, no. 3, pp. 801–805, 2003.

[33] M. Mallea, J. Chevalier, C. Bornet et al., "Porin alteration and active efflux: two in vivo drug resistance strategies used by Enterobacter aerogenes," *Microbiology*, vol. 144, no. 11, pp. 3003–3009, 1998.

[34] M. Viveiros, A. Jesus, M. Brito et al., "Inducement and reversal of tetracycline resistance in Escherichia coli K-12 and expression of proton gradient-dependent multidrug efflux pump genes," *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 8, pp. 3578–3582, 2005.

[35] V. Lorenzi, A. Muselli, A. F. Bernardini et al., "Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 5, pp. 2209–2211, 2009.

[36] Q.-T. Tran, K. R. Mahendran, E. Hajjar et al., "Implication of porins in beta-lactam resistance of providencia stuartii," *The Journal of Biological Chemistry*, vol. 285, no. 42, pp. 32273–32281, 2010.

[37] F. Van Bambeke, J.-M. Pagès, and V. J. Lee, "Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux," *Frontiers in Anti-Infective Drug Discovery*, vol. 1, no. 2, pp. 157–175, 2006.

[38] A. Mahamoud, J. Chevalier, S. Alibert-Franco, W. V. Kern, and J.-M. Pagès, "Antibiotic efflux pumps in gram-negative bacteria: the inhibitor response strategy," *Journal of Antimicrobial Chemotherapy*, vol. 59, no. 6, pp. 1223–1229, 2007.

[39] L. C. Braga, A. A. M. Leite, K. G. S. Xavier et al., "Synergic interaction between pomegranate extract and antibiotics against Staphylococcus aureus," *Canadian Journal of Microbiology*, vol. 51, no. 7, pp. 541–547, 2005.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 2190847, 9 pages
https://doi.org/10.1155/2019/2190847

Research Article

Protective Effects of Evodiamine against LPS-Induced Acute Kidney Injury through Regulation of ROS-NF-κB-Mediated Inflammation

Yan Shi, Qiuju Hua, Na Li, Min Zhao, and Yan Cui

Hospital of Nephrology, The First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan 453100, China

Correspondence should be addressed to Yan Shi; syggdl@126.com

Received 10 October 2018; Accepted 5 February 2019; Published 3 March 2019

Guest Editor: Caio P. Fernandes

Acute kidney injury (AKI) is a critical care syndrome, which is usually associated with sepsis-related endotoxemia. Evodiamine (EVO) is an active ingredient of many traditional medicinal formulations that possess a battery of biological activities. In the study, we aimed to evaluate the potential protective effect of EVO against lipopolysaccharide- (LPS-) induced AKI and cytotoxicity. LPS-resulted pathological injuries were significantly ameliorated by the administration of EVO. EVO reduced the levels of blood urea nitrogen (BUN) and creatinine in LPS-treated rats. EVO also inhibited LPS-induced reduction of cell viability in NRK-52E cells. LPS-resulting increase of TNFα and IL-1β in both serum and kidney of rats and NRK-52E cells was inhibited by EVO. LPS-induced increase of P65 NF-κB expression was markedly inhibited by EVO. EVO-induced reduction of TNFα and IL-1β expression in LPS-treated cells was blocked by overexpression of P65 NF-κB. Moreover, the increase of cell viability in LPS-treated cells induced by EVO was remarkably suppressed by overexpression of P65 NF-κB. LPS-resulting increase of reactive oxygen species (ROS) production was suppressed by EVO. H_2O_2 suppressed EVO-induced decrease of P65 NF-κB expression and increase of cell viability in LPS-treated NRK-52E cells. Moreover, the antioxidant NAC significantly promoted EVO-induced decrease of P65 NF-κB expression and increase of cell viability in LPS-treated NRK-52E cells. In conclusion, EVO had crucial protective effects against LPS-induced AKI and cytotoxicity through the antioxidant activities and thus the inhibition of inflammation. Our data highlight EVO as a potential candidate for the development of new strategies for the treatment of AKI.

1. Introduction

Kidney is an important organ functioning to filter blood and acting as the defense line in the body [1]. It is unfortunate that kidney is one of the most common and direct targets of severe damage [2]. Acute kidney injury (AKI) is a critical care syndrome, which is very common in the elderly with up to 22% mortality of hospitalized patients [3–6]. AKI is usually associated with sepsis-related endotoxemia, which contributes to up to 50% of mortality in ICU patients [7, 8]. The mortality rate in septic AKI patients is higher than nonseptic AKI individuals [8, 9]. Endotoxemia mainly resulted from lipopolysaccharide (LPS), the major component of endotoxin released from the cell wall of Gram-negative bacteria [7]. The LPS-induced endotoxemic AKI in rodent animals is one of the most commonly used animal models to study the pathogenesis and potential treatment of endotoxemic AKI [10]. Endotoxemia-induced AKI could occur under several extremely physiologically stressful conditions, including trauma, burn, and infectious diseases [11]. Renal function is acutely and severely reduced during AKI, characterized by an increase in serum creatinine level and decrease in urine output [12]. AKI also shows the hallmark of renal tubular damage and inflammation [13–15]. LPS activates a renal inflammatory cascade, promotes the release of numerous pro-inflammatory cytokines, and results in kidney end-organ damage [16]. Thus, researchers highlight anti-inflammatory agents that may be developed into drugs for the treatment of endotoxemic AKI [17].

Evodiamine (EVO), ((+)-(S)-8,13,13b,14-tetrahydro-14-methylindolo [2′,3′:3,4] pyrido [2,1-b]quinazolin-5(7H)-one), is an important alkaloidal component extracted from *Evodia rutaecarpa*. EVO is an active ingredient of many traditional medicinal formulations, such as plant extracts

of *E. rutaecarpa (Rutaceae)*, root bark of *Zanthoxylum budrunga* wall, and *Evodiae fructus* [18]. EVO has been shown to possess the properties of analgesia, antiemesis, vascular dilatation, and prevention of tumor growth and metastasis [19, 20]. In particular, EVO exhibits potent anti-inflammatory activities [21–23].

In the study, we designed experiments to evaluate the potential protective effect of EVO against LPS-induced AKI. We found that EVO had critical antioxidant and anti-inflammatory effects through inhibition of ROS/NF-κB signaling and protected against LPS-induced AKI.

2. Materials and Methods

2.1. Chemicals and Reagents. LPS (Escherichia coli 055:B5), N-acetylcysteine (NAC), Evodiamine, and DCFH-DA were obtained from Sigma-Aldrich (St.Louis, MO, USA). The β-actin and P65 NF-κB antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rat TNF-α and IL-1β ELISA kits were obtained from Thermo Fisher Scientific (Rockford, IL, USA).

2.2. Animals and Treatment. Male SD rats (6 -8 weeks) were purchased from the Experimental Animal Center of the First Affiliated Hospital of Xinxiang Medical University. Experiments with animals were conducted in accordance with the guidelines of the National Institutes of Health and the First Affiliated Hospital of Xinxiang Medical University. The mice were housed in plastic cages with 24 ± 2°C and 40-80% humidity with access to food and water at liberty and were kept on a 12 h light/dark cycle.

The rats were randomly allocated into the following groups (n=10):

Control group: rats were injected with equal volume of vehicle.

LPS group: rats were injected intraperitoneally (i.p.) with 15 mg/kg/bw of LPS in 50μL PBS.

LPS + 100 mg/kg Nerolidol group: 1 h after LPS treatment, the rats were injected i.p. with 100 mg/kg EVO.

LPS + 200 mg/kg Nerolidol group: 1 h after LPS treatment, the rats were injected i.p. with 200 mg/kg EVO.

Experiments were terminated 24 h after LPS challenge and the blood samples and kidney tissues were collected.

2.3. Histological Analysis. The kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 5 μm sections. After staining with hematoxylin and eosin (H&E), pathological changes were observed under a light microscope (×200; Olympus, Japan). The score of histological injury was evaluated as previously reported [24].

2.4. Cell Culture and Treatment. The NRK-52E rat proximal tubular cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin in an incubator with 5% CO_2 at 37°C). Cells were cultured in serum-free DMEM with 1 μg/ml LPS in the presence or absence of 10 and 20 μM EVO for 24h. EVO was dissolved in DMSO as stock solution and diluted in serum-free DMEM.

2.5. Transfection of Plasmids. The sequence of P65 was cloned into pCMV vector. Transient transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. 4-6 hours after the transfection, cell growth medium was removed and incubated in media containing 5% FBS. 48 hours after the transfection, cells were incubated with 1 μg/ml LPS with or without 20 μM EVO for 24h. Cell viability and indicated gene expression were determined.

2.6. Cell Viability. The cell viability was determined by the 3-(4,5-di- methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The NRK-52E cells were seeded in 96-well plates at a density of 5×10^4 cells/ml for 24 h and then were cultured in serum-free DMEM with1 μg/ml LPS in the presence or absence of 10 and 20 μM EVO for 24h. Thereafter, 20 μl of MTT was added to each well and incubated for 4 h. After careful removal of medium, 150 μl of DMSO was added. The absorbance at a wavelength of 490 nm was detected on a spectrophotometer (Bio-Rad, CA, USA).

2.7. Biochemical Determination. Serum and kidney homogenates were used for biochemical determination. The levels of BUN were determined using ELISA kits (MyBioSource, CA, USA) according to the manufacturer's instructions. Creatinine level was measured using colorimetric/fluorometric assay kits (BioVision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions. The levels of inflammatory cytokines TNFα and IL-1β in serum and kidney homogenates were measured by ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocols.

2.8. RNA Extraction and Real-Time RT-PCR. Total RNA was isolated from the kidney by using TRIzol reagent as per the manufacturer's instructions (Life Technologies, Carlsbad, CA). Then 1 μg of DNA-free total RNA was reverse-transcribed by use of a one-step RT-PCR kit (TaKaRa, Dalian, China). Reactions were performed in a 50 μL SYBR GREEN PCR volume formatted in CFX96 detection systems (Bio-Rad, Hercules, CA). β-actin was used as an endogenous control for RNA quality and differences among samples. Fold induction was calculated according to the $2^{-\Delta\Delta Ct}$ values.

2.9. Western Blot. Total proteins were extracted using ice-cold RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) and protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Rockford, IL, USA). The extracts were run on an SDS-PAGE gel for Western blot analysis. After electrophoresis, the proteins were electrotransferred to a polyvinylidene

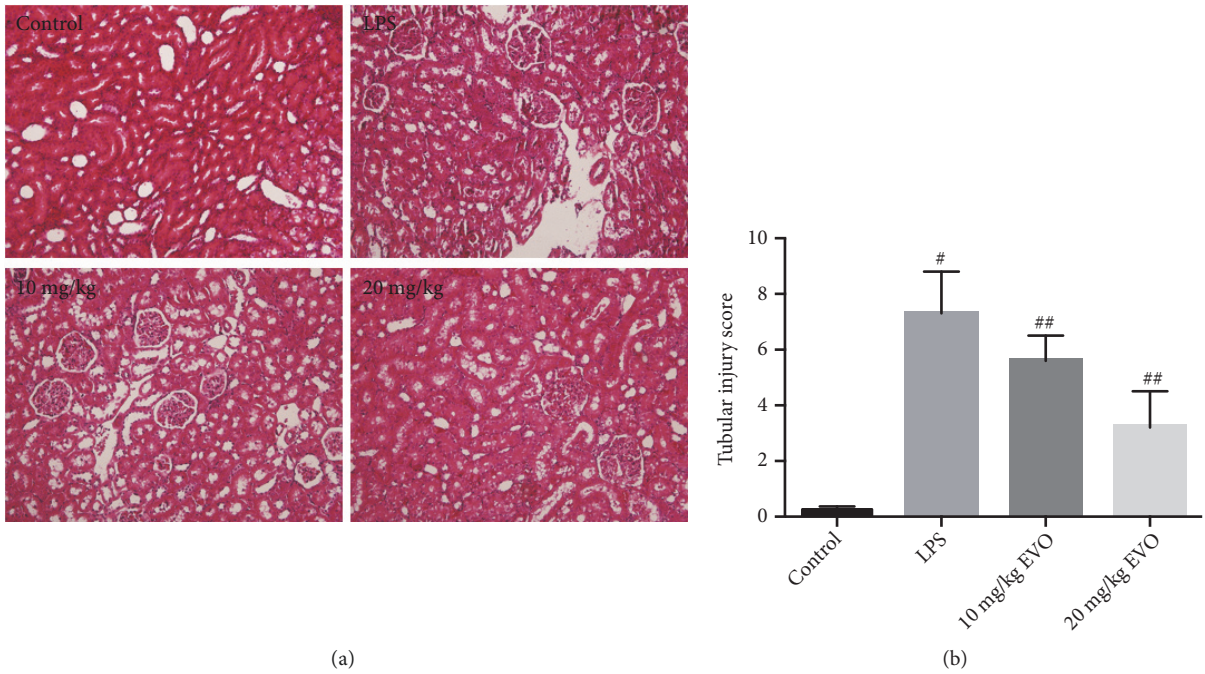


Figure 1: *Effects of EVO on pathological injury of kidney in rats treated by LPS.* Rats were intraperitoneally (i.p.) injected with EVO (10 and 20 mg/kg) 1 h after LPS treatment. Hematoxylin and eosin (HE) staining was conducted to evaluate histological injury of kidney tissues. Representative images of HE staining were shown (a). The score of tubular injury was calculated (b). #p<0.05 vs control group. ##p<0.05 vs LPS group.

difluoride membrane (Millipore Corporation, MA, USA) and non-specific binding of antibodies was blocked with 5% BSA in tris-buffered saline (TBS) for 1 h at room temperature. The membranes were incubated at 4°C overnight with primary antibody in TBST. Then, the membranes were washed four times using TBST with 15 min each time. Membranes were incubated with peroxidase-conjugated IgG secondary antibody for 30 min at 37°C. After washing for four time with TBST, the immune complexes were detected using an ECL kit (Millipore Corporation, MA, USA). Target gene expression levels were normalized to β-actin expression.

2.10. ROS Level. After the treatment, cells were harvested and incubated with 10 μM 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in serum-free DMEM for 30 min at 37°C. Analysis was performed on a flow cytometry (BD, San Jose, CA, USA). ROS level was expressed as folds vs control.

2.11. Statistical Analysis. Data are shown as the means ± standard error of the means (SEM). Statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA). Differences were analyzed by one-way analysis of variance, followed by Dunnett's multiple comparison test. P<0.05 was considered to be statistically significant.

3. Results

3.1. EVO Protects against LPS-Induced Renal Injury In Vivo and In Vitro. LPS-induced rat model of endotoxemic AKI was established in our study. We showed that the injection of LPS induced edema of renal tubular epithelial cells, tubular dilation, and distortion in kidneys of rats. LPS-resulting pathological injuries were significantly ameliorated by the administration of EVO (Figure 1(a)). The tubular injury score in LPS group was significantly reduced by EVO (Figure 1(b)). In addition, LPS induced a marked increase in the levels of blood urea nitrogen (BUN) and creatinine (Figures 2(a) and 2(b)). The treatment of EVO notably reduced the levels of BUN and creatinine (Figures 2(a) and 2(b)). Moreover, NRK-52E cells were exposed to LPS to induce cytotoxicity. In Figure 2(c), we showed that LPS resulted in a significant decrease of cell viability in NRK-52E cells. In the presence of EVO, the reduction of cell viability induced by LPS was notably inhibited (Figure 2(c)). The data demonstrated that EVO protected against LPS-induced AKI in vivo and in vitro.

3.2. EVO Inhibits LPS-Induced Inflammation In Vivo and In Vitro. The Effect of EVO on Inflammation under the Condition of LPS-Induced AKI. In Figures 2(a), 2(b), 2(c), and 2(d), we showed that serum and kidney levels of inflammatory cytokines TNFα and IL-1β were significantly increased. The treatment of EVO induced a marked reduction of the levels of TNFα and IL-1β in LPS-treated rats (Figures 3(a), 3(b), 3(c), and 3(d)). In addition, the mRNA expression of TNFα and IL-1β was notably increased by LPS in NRK-52E cells (Figures 3(e) and 3(f)). LPS-induced increase of TNFα and IL-1β levels was blocked by EVO treatment (Figures 3(e) and 3(f)). The data demonstrated that EVO exhibited anti-inflammatory effects against LPS-induced AKI.

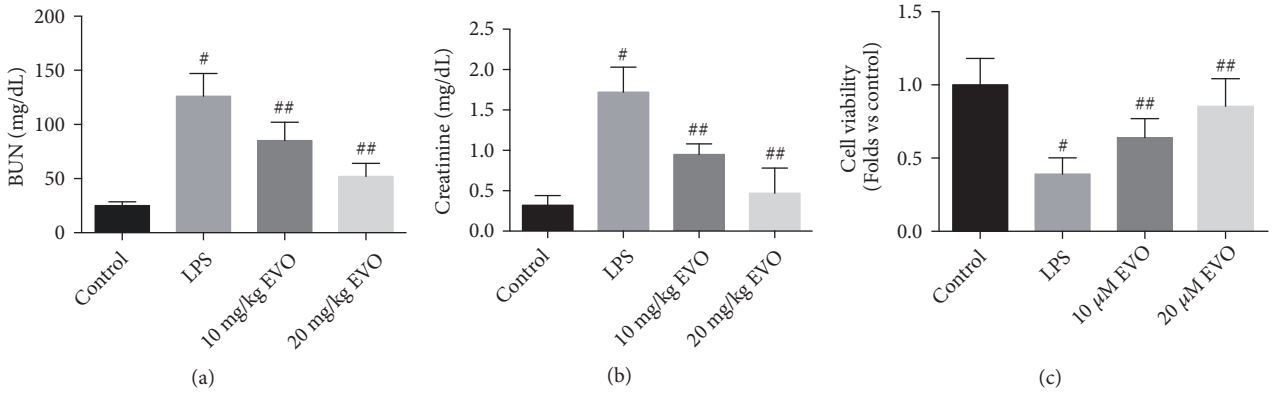


Figure 2: *Effects of EVO on functional injury of kidney in rats and cell viability in NRK-52E cells treated by LPS.* Rats were intraperitoneally (i.p.) injected with EVO (10 and 20 mg/kg) 1 h after LPS treatment. Kidney function was evaluated by the determination of blood urea nitrogen (BUN) (a) and creatinine (b) levels. NRK-52E cells were treated with 1 μg/ml LPS in the presence or absence of 10 and 20 μM EVO for 24h. Cell viability was detected by MTT assay (c). #p<0.05 vs control group. ##p<0.05 vs LPS group.

3.3. Inhibition of NF-κB Expression Is Involved in the Protective Effects of EVO. To explore the mechanism of EVO-induced anti-inflammatory effects, we examined the expression of P65 NF-κB. In Figures 4(a) and 4(b), we showed that LPS resulted in a significant increase of P65 NF-κB mRNA and protein expression in NRK-52E cells. This increase of P65 NF-κB expression was markedly inhibited by EVO (Figures 4(a) and 4(b)). To test whether the reduction of P65 NF-κB expression was involved in the protective effects of EVO against AKI, the expression of P65 NF-κB was upregulated in NRK-52E cells using pCMV-P65 NF-κB. As shown in Figures 4(c) and 4(d), EVO-induced reduction of TNFα and IL-1β expression was blocked by overexpression of P65 NF-κB. Moreover, the increase of cell viability in LPS-treated cells induced by EVO was remarkably suppressed by overexpression of P65 NF-κB (Figure 4(e)). The data demonstrated that downregulation of P65 NF-κB was responsible for the anti-inflammatory effects of EVO and was involved in EVO-induced protective effects against LPS-induced AKI.

3.4. Antioxidant Effect Is Involved in the Protective Effects of EVO. In the next step, we explored the mechanism of EVO-induced inhibition of P65 NF-κB expression. The ROS level was examined and the results showed that LPS resulted in a significant increase in ROS generation in NRK-52E cells (Figure 5(a)). LPS-resulting ROS production was suppressed by EVO in a concentration-dependent manner (Figure 5(a)). This finding indicated that EVO played an antioxidant role under the condition of LPS-induced cytotoxicity in NRK-52E cells. Next, we tested whether the antioxidant role was involved in EVO-induced anti-inflammatory effects and protective effects against LPS-induced cytotoxicity. Hydrogen peroxide (H_2O_2) treatment increased P65 NF-κB expression (Figure 5(b)). H_2O_2 suppressed EVO-induced decrease of P65 NF-κB expression in LPS-treated NRK-52E cells (Figure 5(b)). In addition, EVO-induced increase of cell viability in LPS-treated NRK-52E cells was inhibited by H_2O_2 treatment (Figure 5(c)). Moreover, the antioxidant NAC significantly promoted EVO-induced decrease of P65 NF-κB expression (Figure 5(d)) and increase of cell viability (Figure 5(e)) in LPS-treated NRK-52E cells. The data demonstrated that the antioxidant activity was involved in the anti-inflammatory effects and protective effects of EVO against LPS-induced cytotoxicity.

4. Discussion

LPS is the most common agent that is used to establish endotoxemic AKI animal model and induce cytotoxicity in renal cells. In the current study, we examined the effects of EVO on LPS-induced AKI in rats and LPS-induced in NRK-52E rat proximal tubular cells. We found that EVO ameliorated the histological injury of kidney tissues and improved the function of kidney, as reflected by decrease of BUN and creatinine levels, indicating that EVO protected against LPS-induced AKI. EVO also exhibited cytoprotective effects against LPS in NRK-52E cells.

The kidney is bound with high flow of blood and is sensitive to systemic inflammation. In turn, cytokines and chemokines can be synthesized within the tubular epithelium and released to the circulation [25]. Thereafter, the kidney is easily subject to inflammatory injury [26, 27], resulting in renal dysfunction [28]. EVO has been reported to exhibit anti-inflammatory activities. For example, EVO was found to inhibit nitric oxide and PGE2 synthesis from lipopolysaccharide or IFN-γ-stimulated RAW264.7 cells [22, 23, 29]. EVO may regulate inducible nitric oxide synthase via affecting the expression of inflammatory cytokines, such as TNFα [30]. Fan et al. found that EVO inhibited zymosan-induced production of IL-6, TNFα, and IL-1β at both mRNA and protein levels at 6 h in RAW264.7 cells [31]. However, it was not reported whether EVO had an anti-inflammatory effect in kidney. In the current study, we found that EVO significantly inhibited LPS-induced increase of serum and kidney levels of TNFα and IL-1β. EVO inhibited LPS-induced increase

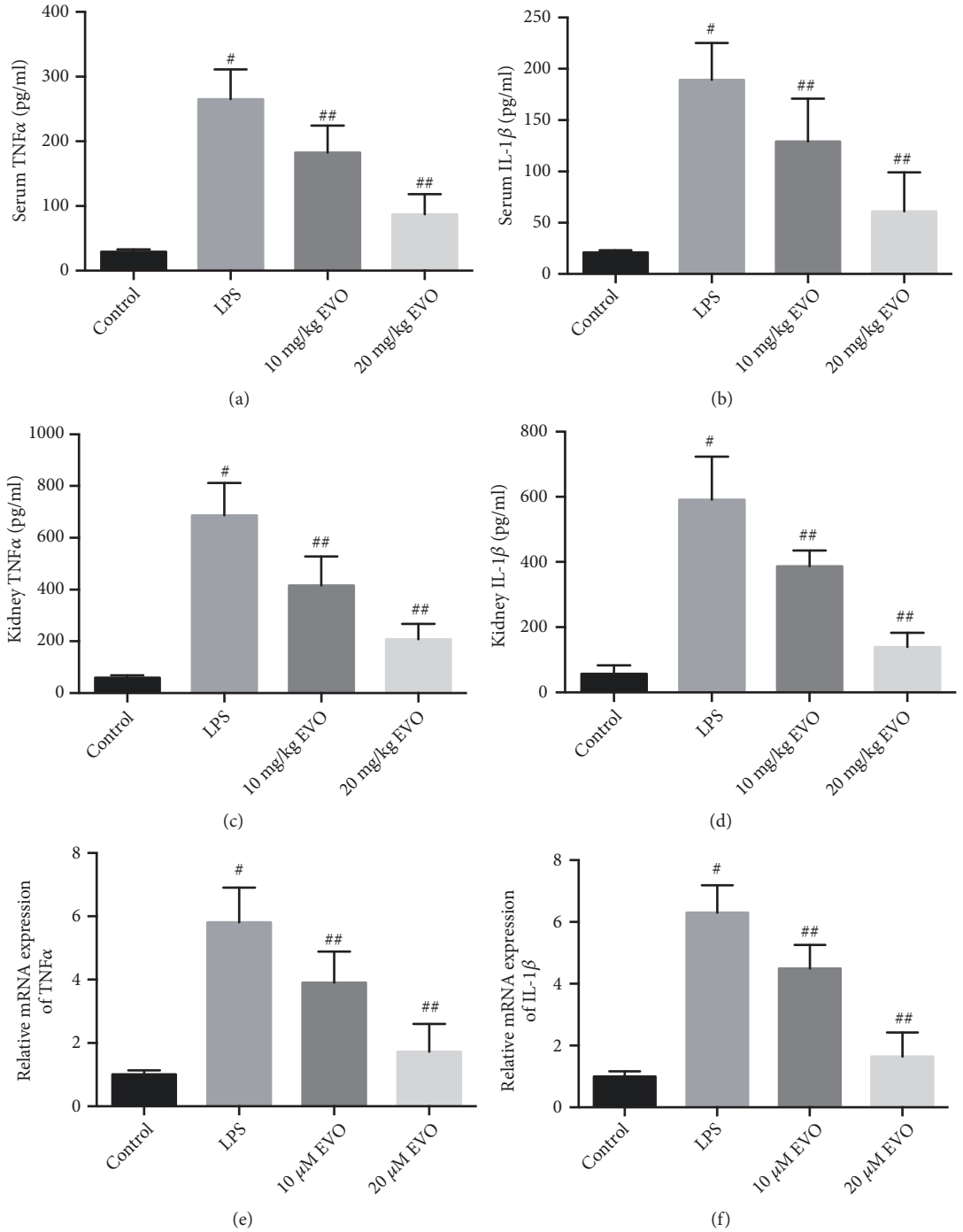


Figure 3: *Effects of EVO on proinflammatory cytokines in rats and in NRK-52E cells treated by LPS.* Rats were intraperitoneally (i.p.) injected with EVO (10 and 20 mg/kg) 1 h after LPS treatment. Inflammation was evaluated by the determination of serum (a and b) and tissue (c and d) levels of proinflammatory cytokines, including TNFα (a and c) and IL-1β (b and d). NRK-52E cells were treated with 1 μg/ml LPS in the presence or absence of 10 and 20 μM EVO for 24h. Relative mRNA expression of TNFα (e) and IL-1β (f) was measured. #p<0.05 vs control group. ##p<0.05 vs LPS group.

of P65 NF-κB expression and overexpression of P65 NF-κB markedly reduced the anti-inflammatory activity and the protective effects of EVO against LPS-induced cytotoxicity in kidney cells. We suggest that the anti-inflammatory activity may participate in the protective effects of EVO against LPS-induced AKI.

The NF-κB signal pathway lies in the center of inflammatory and immune response [32, 33]. ROS has been reported to activate NF-κB through the classical IKK-dependent pathway and induces a positive feedback mechanism associated with inflammation and kidney injury [34–36]. In the current study, we also tested the role of ROS regulation in the

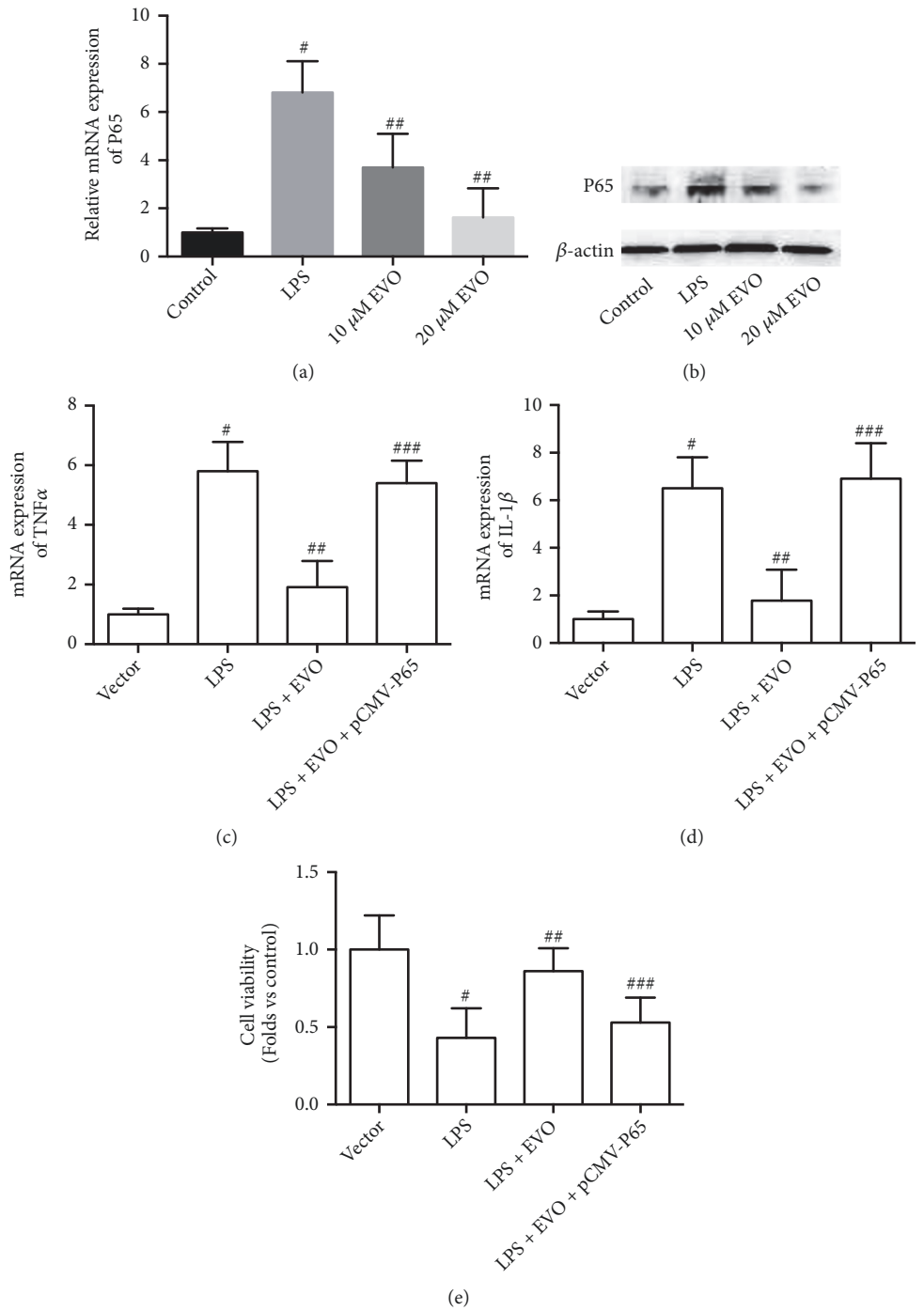


Figure 4: *Role of NF-κB in EVO-induced protective effect against LPS-induced cytotoxicity in NRK-52E cells.* NRK-52E cells were treated with 1 μg/ml LPS in the presence or absence of 10 and 20 μM EVO for 24h. Relative mRNA (a) and protein (b) expression of P65 NF-κB were measured. NRK-52E cells were transfected with pCMV vector or pCMV-P65 and exposed to 1 μg/ml LPS with or without 20 μM EVO for 24h. Relative mRNA expression of TNFα (c) and IL-1β (d) was measured. Cell viability was detected by MTT assay (e). #p<0.05 vs control group. ##p<0.05 vs LPS group. ###p<0.05 vs LPS+EVO group.

protective effect of EVO. We showed that EVO played an antioxidant role in the protection against LPS-induced cytotoxicity. Addition of oxidant H_2O_2 could reverse, but NAC could promote EVO-induced inhibition of P65 NF-κB expression and increase of cell viability in LPS-treated cells. The results suggested that EVO had the anti-inflammatory and renal protective effects via its antioxidant role.

In conclusion, we showed that EVO had crucial renal protective effects against LPS-induced AKI and cytotoxicity through the antioxidant activities and thus the inhibition

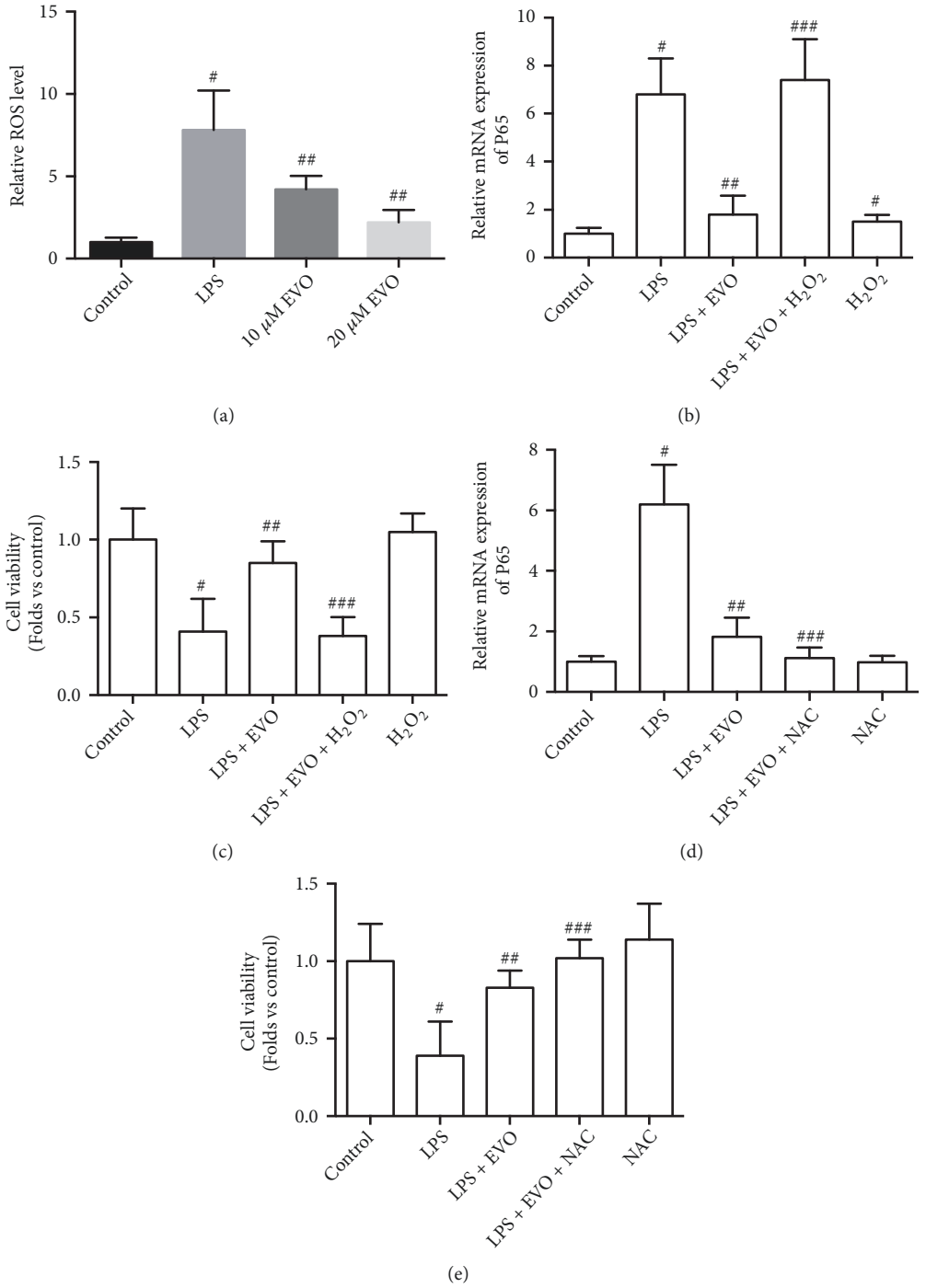


Figure 5: *Role of ROS in EVO-induced protective effect against LPS-induced cytotoxicity in NRK-52E cells.* NRK-52E cells were treated with 1 μg/ml LPS in the presence or absence of 10 and 20 μM EVO for 24h. Relative mRNA (a) and protein (b) expression of P65 NF-κB was measured. NRK-52E cells were transfected with pCMV vector or pCMV-P65 and exposed to 1 μg/ml LPS with or without 20 μM EVO for 24h. Relative mRNA expression of TNFα (c) and IL-1β (d) was measured. Cell viability was detected by MTT assay (e). #p<0.05 vs control group. ##p<0.05 vs LPS group. ###p<0.05 vs LPS+EVO group.

of inflammation. Our data highlight EVO as a potential candidate for the development of new strategies for the treatment of AKI.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] M. Adachi, K. Kitamura, and K. Tomita, "Regulation of sodium and water balance by the kidney," *Nippon Rinsho. Japanese Journal of Clinical Medicine*, vol. 63, no. 1, pp. 45–50, 2005.

[2] S. M. Coldewey, A. I. Khan, A. Kapoor et al., "Erythropoietin attenuates acute kidney dysfunction in murine experimental sepsis by activation of the beta-common receptor," *Kidney International*, vol. 84, no. 3, pp. 482–490, 2013.

[3] S. Dellepiane, M. Marengo, and V. Cantaluppi, "Detrimental cross-talk between sepsis and acute kidney injury: New pathogenic mechanisms, early biomarkers and targeted therapies," *Critical Care*, vol. 20, no. 1, p. 61, 2016.

[4] S. T. Vaara, V. Pettilä, K.-M. Kaukonen et al., "The attributable mortality of acute kidney injury: A sequentially matched analysis," *Critical Care Medicine*, vol. 42, no. 4, pp. 878–885, 2014.

[5] H. Husi, M. D. Sanchez-Niño, C. Delles et al., "A combinatorial approach of Proteomics and Systems Biology in unravelling the mechanisms of acute kidney injury (AKI): Involvement of NMDA receptor GRIN1 in murine AKI," *BMC Systems Biology*, vol. 7, p. 110, 2013.

[6] J. H. Lim, E. N. Kim, M. Y. Kim et al., "Age-associated molecular changes in the kidney in aged mice," *Oxidative Medicine and Cellular Longevity*, p. 171383, 2012.

[7] S. Uchino, J. A. Kellum, R. Bellomo et al., "Acute renal failure in critically ill patients: a multinational, multicenter study," *The Journal of the American Medical Association*, vol. 294, no. 7, pp. 813–818, 2005.

[8] N. C. Riedemann, R.-F. Guo, and P. A. Ward, "The enigma of sepsis," *The Journal of Clinical Investigation*, vol. 112, no. 4, pp. 460–467, 2003.

[9] M. S. Rangel-Frausto, D. Pittet, M. Costigan, T. Hwang, C. S. Davis, and R. P. Wenzel, "The natural history of the systemic inflammatory response syndrome (SIRS): a prospective study," *Journal of the American Medical Association*, vol. 273, no. 2, pp. 117–123, 1995.

[10] W. Wang, E. Zolty, S. Falk et al., "Prostacyclin in endotoxemia-induced acute kidney injury: Cyclooxygenase inhibition and renal prostacyclin synthase transgenic mice," *American Journal of Physiology-Renal Physiology*, vol. 293, no. 4, pp. F1131–F1136, 2007.

[11] S. M. Bagshaw, S. Lapinsky, S. Dial et al., "Acute kidney injury in septic shock: clinical outcomes and impact of duration of hypotension prior to initiation of antimicrobial therapy," *Intensive Care Medicine*, vol. 35, no. 5, pp. 871–881, 2009.

[12] J. Harty, "Prevention and management of acute kidney injury," *The Ulster Medical Journal*, vol. 83, no. 3, pp. 149–157, 2014.

[13] C. M. Elks, S. D. Reed, N. Mariappan et al., "A blueberry-enriched diet attenuates nephropathy in a rat model of hypertension via reduction in oxidative stress," *PLoS ONE*, vol. 6, no. 9, p. e24028, 2011.

[14] A. Zarjou and A. Agarwal, "Sepsis and acute kidney injury," *Journal of the American Society of Nephrology*, vol. 22, no. 6, pp. 999–1006, 2011.

[15] U. Panzer, O. M. Steinmetz, and J. E. Turner, "Resolution of renal inflammation: a new role for NF-kappaB1 (p50) in inflammatory kidney diseases," *American Journal of Physiology-Renal Physiology*, vol. 297, no. 2, pp. F429–439, 2009.

[16] R. W. Schrier and W. Wang, "Acute renal failure and sepsis," *The New England Journal of Medicine*, vol. 351, no. 2, pp. 159–169, 2004.

[17] A. C. Souza, R. A. Volpini, M. H. Shimizu et al., "Erythropoietin prevents sepsis-related acute kidney injury in rats by inhibiting NF-kappaB and upregulating endothelial nitric oxide synthase," *American Journal of Physiology-Renal Physiology*, vol. 302, no. 8, pp. F1045–1054, 2012.

[18] B. S. Joshi, K. M. Moore, S. W. Pelletier, and M. S. Puar, "Alkaloids of zanthoxylum budrunga wall.: NMR assignments of dihydrochelerythrine, (±)-evodiamine and zanthobungeanine," *Phytochemical Analysis*, vol. 2, no. 1, pp. 20–25, 1991.

[19] X. Gao, X.-W. Yang, and P. J. Marriott, "Simultaneous analysis of seven alkaloids in Coptis-Evodia herb couple and Zuojin pill by UPLC with accelerated solvent extraction," *Journal of Separation Science*, vol. 33, no. 17-18, pp. 2714–2722, 2010.

[20] M. Ogasawara, T. Matsunaga, S. Takahashi, I. Saiki, and H. Suzuki, "Anti-invasive and metastatic activities of evodiamine," *Biological & Pharmaceutical Bulletin*, vol. 25, no. 11, pp. 1491–1493, 2002.

[21] Y.-N. Liu, S.-L. Pan, C.-H. Liao et al., "Evodiamine represses hypoxia-induced inflammatory proteins expression and hypoxia-inducible factor 1alpha accumulation in RAW264.7," *Shock*, vol. 32, no. 3, pp. 263–269, 2009.

[22] H.-C. Ko, Y.-H. Wang, K.-T. Liou et al., "Anti-inflammatory effects and mechanisms of the ethanol extract of Evodia rutaecarpa and its bioactive components on neutrophils and microglial cells," *European Journal of Pharmacology*, vol. 555, no. 2-3, pp. 211–217, 2007.

[23] Y. H. Choi, E. M. Shin, Y. S. Kim, X. F. Cai, J. J. Lee, and H. P. Kim, "Anti-inflammatory principles from the fruits of Evodia rutaecarpa and their cellular action mechanisms," *Archives of Pharmacal Research*, vol. 29, no. 4, pp. 293–297, 2006.

[24] A. Tilyek, C. Chai, X. Hou et al., "The protective effects of Ribes diacanthum Pall on cisplatin-induced nephrotoxicity in mice," *Journal of Ethnopharmacology*, vol. 178, pp. 297–306, 2016.

[25] A. I. Lim, L. Y. Chan, S. C. Tang et al., "BMP-7 represses albumin-induced chemokine synthesis in kidney tubular epithelial cells through destabilization of NF-kappaB-inducing kinase," *Immunology and Cell Biology*, vol. 92, no. 5, pp. 427–435, 2014.

[26] G. E. Rodwell, R. Sonu, J. M. Zahn et al., "A Transcriptional profile of aging in the human kidney," *PLoS Biology*, vol. 2, no. 12, p. e427, 2004.

[27] A. Melk, E. S. Mansfield, S.-C. Hsieh et al., "Transcriptional analysis of the molecular basis of human kidney aging using cDNA microarray profiling," *Kidney International*, vol. 68, no. 6, pp. 2667–2679, 2005.

[28] V. Filiopoulos and D. Vlassopoulos, "Inflammatory syndrome in chronic kidney disease: Pathogenesis and influence on

outcomes," *Inflammation & Allergy - Drug Targets*, vol. 8, no. 5, pp. 369–382, 2009.

[29] W.-F. Chiou, Y.-J. Sung, J.-F. Liao, A. Y.-C. Shum, and C.-F. Chen, "Inhibitory effect of dehydroevodiamine and evodiamine on nitric oxide production in cultured murine macrophages," *Journal of Natural Products*, vol. 60, no. 7, pp. 708–711, 1997.

[30] Q.-P. Liu, K. Fruit, J. Ward, and P. H. Correll, "Negative regulation of macrophage activation in response to IFN-gamma and lipopolysaccharide by the STK/RON receptor tyrosine kinase," *The Journal of Immunology*, vol. 163, no. 12, pp. 6606–6613, 1999.

[31] X. Fan, J. Y. Zhu, Y. Sun et al., "Evodiamine inhibits zymosan-induced inflammation in vitro and in vivo: inactivation of nf-kappab by inhibiting ikappabalpha phosphorylation," *Inflammation*, vol. 40, no. 3, pp. 1012–1027, 2017.

[32] M. C. Mulero, A. Bigas, and L. Espinosa, "IkappaBalpha beyond the NF-kB dogma," *Oncotarget*, vol. 4, no. 10, pp. 1550-1551, 2013.

[33] L. Zhang, D. Sun, Y. Bao, Y. Shi, Y. Cui, and M. Guo, "Nerolidol protects against lps-induced acute kidney injury via inhibiting TLR4/NF-κB signaling," *Phytotherapy Research*, vol. 31, no. 3, pp. 459–465, 2017.

[34] G. Gloire, S. Legrand-Poels, and J. Piette, "NF-κB activation by reactive oxygen species: fifteen years later," *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1493–1505, 2006.

[35] S. L. Dodd, B. J. Gagnon, S. M. Senf, B. A. Hain, and A. R. Judge, "Ros-mediated activation of NF-kappaB and Foxo during muscle disuse," *Muscle & Nerve*, vol. 41, no. 1, pp. 110–113, 2010.

[36] K. Jin Jung, D. Hyun Kim, E. Kyeong Lee, C. Woo Song, B. Pal Yu, and H. Young Chung, "Oxidative stress induces inactivation of protein phosphatase 2A, promoting proinflammatory NF-kappaB in aged rat kidney," *Free Radical Biology & Medicine*, vol. 61, pp. 206–217, 2013.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 6191505, 27 pages
https://doi.org/10.1155/2019/6191505

Review Article

An Updated List of Neuromedicinal Plants of Pakistan, Their Uses, and Phytochemistry

Abdul Waheed Khan,[1] **Arif-ullah Khan**,[2] **Syed Muhammad Mukarram Shah**,[3] **Aziz Ullah**,[4] **Muhammad Faheem**,[2] **and Muhammad Saleem**[2]

[1]*Department of Pharmacy, University of Lahore, Islamabad, Pakistan*
[2]*Riphah Institute of Pharmaceutical Sciences, Riphah International University, Islamabad, Pakistan*
[3]*Department of Pharmacy, University of Swabi, Khyber Pakhtunkhwa, Pakistan*
[4]*Department of Pharmacy, Forman Christian College, Lahore, Pakistan*

Correspondence should be addressed to Arif-ullah Khan; arif.ullah@riphah.edu.pk

Received 12 November 2018; Revised 14 January 2019; Accepted 5 February 2019; Published 3 March 2019

Guest Editor: José C. T. Carvalho

Background. Almost every region of Pakistan is stacked with a large number of medicinal plants. Due to high cost and unavailability of allopathic medicines for the neurological diseases, especially in rural areas, traditional healers prescribe phytotherapy for various neurological diseases like epilepsy, depression, anxiety, insomnia, Alzheimer, and migraine. Such treatments are considered to be most effective by the native people. *Methods.* The data was collected from articles published on medicinal plants of various districts of Pakistan, using article search engines like Medline, Pubmed, Web of Science, Science Direct, and Google Scholar. Also, information regarding various neurological uses and mode of applications of medicinal plants was obtained from traditional healers, folk medicine users, and local elderly people having knowledge of medicinal plants. *Results.* A total of 54 families were found to be used in various neurological diseases, of which the highest use was of Solanaceae (22.22%), Asteraceae (12.96%), Lamiaceae, Papaveraceae, and Poaceae, 9% each, and Caprifoliaceae, Cucurbitaceae, Rhamnaceae, and Rosaceae, 5.5% each. According to districts, 15% of plants that were effective in neurological affections were found in Bahawalpur, 11% in Swat, 8% in Muzaffarabad, 7% in Malakand, and 6% in Bahawalnagar, Dir, Gilgat, and Sarghoda each, with 5% in Dera ghazi khan and Jhelum each. According to the plant's habit, out of total of 103 plants, 61.15% were found to be herbs, 22.33% trees, 11.65% shrubs, and 4.85% climbers. According to the part used of plant, whole plant, leaves, fruits, roots, seeds, and flowers were found to be used 32.03%, 24.27%, 20.38%, 16.50%, 13.59%, and 11.65%, respectively. According to disease's types, 45.63% were found to be effective in insomnia, 31.06% in epilepsy 12.62% in depression, 6.80% in anxiety, 7.77% in hysteria, and 5.88% in migraine. *Conclusion.* Taking into consideration this useful knowledge on medicinal properties of the plants for curing neurologic diseases, it is believed that research in areas of ethnomedicine and ethnopharmacology can bring auspicious results that have potential of adding value to the very rich natural resources of Pakistan. This study will help all the researchers from diverse backgrounds working on plants based medicine for neurological diseases.

1. Introduction

Globally, neurological diseases are among the major contributors to mortality and morbidity, particularly in developing nations. The well-known manifestations of neurological diseases include mood swing, restlessness, hopelessness, poor coordination, seizures, impaired cognition, paralysis, distress of sensation, muscle weakness, pain, and confusion [1]. There are more than six hundred neurological diseases, some of which are relatively common and well known while others are rare or poorly recognized [2]. Demographic, socioeconomic, and geographic conditions are the major factors affecting epidemiology of neurological diseases. Globally, the overall burden of neurological diseases is about 6.5%. In lower income countries, neurological diseases range from 4 to 5%, as compared to high income countries where such diseases

Table 1: Global epidemiology of neurological diseases and theircomparative prevalence in Pakistan and neighboring countries.

	Migraine	Stroke	Epilepsy	Depression	Anxiety	Parkinson	Alzheimer
Worldwide	14.9% [49]	5% [50]	0.5-1% [51]	4.4% [52]	3.6% [52]	1% [53]	11.2% [54]
Asia	9.1% [55]	0.94%[56]	0.49% [57]	4.4% [52]	2.8% [58]	0.63% [59]	1.9% [54]
Africa	5.61% [60]	0.4% [61]	1.13% [62]	5.2% [52]	4.4% [52]	0.44% [63]	1.6% [54]
North America	14.4% [64]	2.7% [65]	0.8% [66]	10.6% [67]	7.7% [58]	1.3% [59]	6.4% [54]
South America	11.6% [64]	0.7% [68]	0.98% [57]	13.8% [67]	10.4% [69]	2.3% [59]	4.6% [54]
Europe	15% [70]	6.25%[71]	0.82% [62]	4.2% [52]	3.9% [52]	1.6% [72]	4.4% [54]
Australia	6% [73]	1.8% [74]	0.44% [75]	5.9% [52]	7% [52]	0.46% [76]	6.4% [77]
Pakistan	26.1% [78]	0.25% [79]	2% [4]	4.2% [52]	3.5% [52]	0.23% [51]	1% [4]
India	25.2% [51]	3.69%[71]	0.39% [80]	4.5% [52]	3.0% [52]	0.07% [81]	1.91% [82]
Iran	14% [51]	0.36% [51]	1.8% [80]	4.9% [52]	4.6% [52]	0.29% [83]	2.3% [84]
China	9.3% [78]	4.3% [71]	0.3% [80]	4.2% [52]	3.1% [52]	1.7 % [85]	3.21% [85]
Afghanistan	0.9% [86]	5.2% [87]	8.9% [88]	51.8% [89]	38.5% [89]	35.4% [90]	15.3% [91]

range from 10 to 11%. This high ratio of neurological diseases in advanced countries may be due to their more advanced public health system and health-related facilities that provide and maintain complete data of their patients [1].

About 45 million people of the world, above 18 years of age, suffer from schizophrenia at some stage of their lives, 340 million are affected by depression, and both these diseases are accountable for 60 % of all suicides, while Alzheimer and epilepsy affect about 11 and 45 million people, respectively, around the world accounting for 1% of the total disease burden in the world [3].

In Pakistan, about 10 % people suffer from mental diseases, representing a foggy picture with 2% prevalence of' epilepsy, 5% depression, 1% Alzheimer, and 1.5% schizophrenia [4] as shown in (Table 1). These mental morbidities are responsible for high suicidal rate. Major factors contributing to this alarming increase in mental diseases are unemployment, poverty, political unreliability, violence, and other social horrors and evils beyond the genetic and biological susceptibility [5].

Medicinal plants have been used from the very beginning in health care systems. Studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plant-based medicines. Due to limited access to modern medicine, the local population uses medicinal plants to treat most diseases [6, 7]. Recent focus on plant research has increased worldwide and most evidence has been collected to determine the immense potential of medicinal plants [8]. Medical plants have therapeutic benefits and fewer side effects in comparison with synthetic drugs [9]. Drugs used for neurological diseases along with their side effects are given in (Table 2).

Herbs may provide a source of new compounds including many drugs that are derived from plant sources. For several neurological diseases, modern medicine offers symptomatic treatment that is often expensive and associated with side effects. Indian system of medicine has traditionally been used in several neurological conditions. The accessibility, cost effectiveness, and lower incidence of side effects of plant products offer considerable advantages [10].

Various plant extracts have been screened and investigated for their potential neuropharmacological activities in different experimental models of animals comprising mice and rats. Herbal extracts and natural products including *Bacopa monnieri, Cannabis sativa*, *Solanum nigrum, Withania somnifera, Papaver somniferum*, Zizyphus jujube, *Tribulus terrestris,* and *Verbena officinalis* showed different neuropharmacological activities. These agents can be used alone or as adjuncts to standard drugs, used for various neurological diseases like depression, epilepsy, schizophrenia, Alzheimer, Parkinson, hysteria, melancholia, and dementia, for increasing their efficacy and decreasing side effects.

In developing countries, plant-based medicines are being used by 75-80% of population [11]. The knowledge of indigenous medicinal plants is a part of Pakistani culture and traditionally, majority of Pakistani people use herbal medicines for various diseases [12].

In Pakistan, folk medicines have more use in rural and less developed areas for the treatment of various diseases because of easy access, cost effectiveness, less side effects, and unavailability of allopathic therapeutic agents [13]. This type of treatment, using traditional medicinal flora, is practiced regularly in homes and transferred from generation to generation as a cultural virtue. However, this tradition and associated knowledge are diminishing rapidly due to negligence and less interest of new generation to receive this gift of ethnomedicinal prosperity from their ancestors. Various parameters like industrialization, migration from rural to urban areas for education and jobs, passion towards advanced lifestyles, deforestation, and allopathic medicine might have brought this change in behavior. Therefore, before it is lost forever, this valuable traditional knowledges need to be urgently collected and systematically documented for the interest of humanity [14].

2. Materials and Methods

First the articles published on the medicinal plants of various districts of Pakistan were searched in online research database, i.e., Medline, PubMed, Web of Science, Science

Table 2: Side effects of currently using drugs in treatment of various neurological diseases.

Drug Class	Subclasses	Drugs	Side effects	References
Antidepressants	TCA	Imipramine, Amitriptyline, Desipramine, Nortriptyline, Doxepin	weight gain, sedation, dry mouth, nausea, blurred vision, constipation, tachycardia, dry mouth, constipation, hypotension, increased heart rate	[92]
	MAOI	Isocarboxazid, Phenelzine Tranylcypromine, Selegiline	weight gain, fatigue, sexual dysfunction, nausea, hypotension, dry mouth, diarrhea or constipation, headache, drowsiness, insomnia	
	SSRI	Fluoxetine, Paroxetine, Fluvoxamine, Sertraline, Citalopram	headache, sedation, dizziness, nervousness, somnolence, extrapyramidal effects, nausea, dry mouth, diarrhea, agitation, insomnia, sexual dysfunction, weight gain,	[93]
	SNRI	Venlafaxine, Duloxetine, Desvenlafaxine, Levomilnacipran	nausea, insomnia, dry mouth, headache, increased blood pressure, sexual dysfunction, weight gain, urinary retention, hyponatremia, tremors, vertigo, tachycardia, shock-like sensations, paresthesia, myalgia, tinnitus, neuralgia, ataxia	[92, 94]
	Atypical	Bupropion, Mirtazapine, Trazodone, Vilazodone	headache, agitation, insomnia, sweating, sedation, increased appetite, weight gain, nausea, dizziness	[92]
Anxiolytics	BZDs	Alprazolam, Clonazepam, Lorazepam, Midazolam, Diazepam	sedation, memory disturbances, tolerance, fatigue, dependence, drowsiness, lethargy, At higher dosages, impaired motor coordination, dizziness, vertigo, slurred speech, blurry vision, mood swings, euphoria	[95]
	Azapirones	Buspirone, Binospirone, Gepirone, Tandospirone	dizziness, drowsiness, headaches, restlessness, nausea, diarrhea	[96]
	BAR	Phenobarbital, Amobarbital, Secobarbita, Butabarbital, Pentobarbital	sedation, dizziness, headache, nausea, withdrawal include, tremors, agitation, abnormal breathing, coma, confusion, fainting, hallucinations	[97]
Anti-Alzheimer	AChEIs	Donepezil, Rivastigmine, Galantamine	vomiting, diarrhea, weight loss, bradycardia, insomnia, nausea, agitation, syncope	[98]
	Anti-Aβ	Bapineuzumab, Solanezumab, Gantenerumab	microhemorrhage, vasogenic edema, arrhythmia, skin and subcutaneous tissue disorders	
	NMDAR Antagonists	Memantine	psychosis, nausea, vomiting, memory impairment, and neuronal cell death, drowsiness	[99]
Anti-Parkinson	DA	Bromocriptine, Pergolide, Cabergoline, Pramipexole	nausea, hypotension, confusion, delirium, pulmonary fibrosis, vasospasm, erythromelalgia, sleep attacks	[100]
	COMT Inhibitors	Entacapone, Tolcapone	dyskinesia, nausea, confusion, urine discoloration, diarrhea, abdominal pain	
	MAO-B	Selegiline	confusion, delirium, hallucinations, unusual thoughts or behavior, dizziness, nausea, insomnia, trouble breathing	
Antiepileptic	Sodium Channel Blockers	Phenytoin, Carbamazepine, Lamotrigine, Lacosamide, Oxcarbazepine,	dizziness, drowsiness, diplopia, nausea, vomiting, fatigue, ataxia, neurotoxicity, cardiac arrhythmias, hirsutism, hepatotoxicity, steven-johnson syndrome	[101]
	Calcium Channel Blockers	Ethosuximide, Zonisamide, Trimethadione	nausea, vomiting, headache, mental status changes, neuropathy, change in weight	[102]
	GABA transaminase Inhibitors	Vigabatrin, L-Cycloserine, Ethanolamine-O-Sulfate, Valproate	drowsiness, nystagmus, hyperexcitability, insomnia, fever, memory impairment, depression, confusion, agitation, asthenia, laryngitis, weight gain, vomiting	[103]

TCA: tricyclic antidepressant; MAOI: monoamine oxidase inhibitor; SSRI: selective serotonin reuptake inhibitor; SNRI: serotonin norepinephrine reuptake inhibitor; BZDs: benzodiazepines; BAR: barbiturates; AChEIs: acetylcholinesterase inhibitors; Aβ: amyloid beta; NMDAR: N-methyl-D-aspartate receptor; DA: dopamine agonists; COMT: catechol-O-methyltransferase; MAO-B: monoamine oxidase B; GABA: gamma-aminobutyric acid.

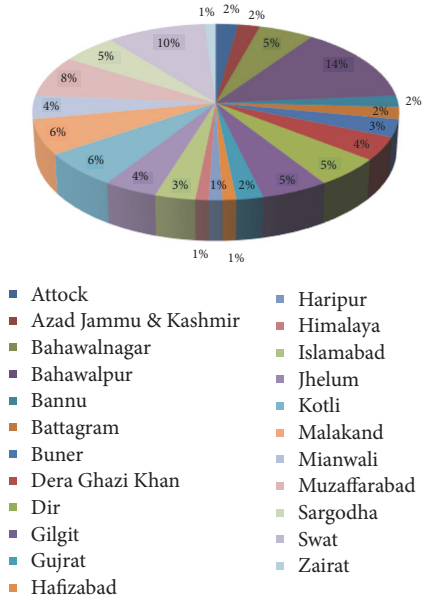


Figure 1: District-wise percentage of plants used for neurological diseases.

Direct, and Google Scholar, by using special key words "medicinal plants", herbal plants, neurological diseases, specific districts names, antialzheimer, antiparkinson, antidepression, sedative, anxiolytic, antiepileptics, epidemiology, and prevalence, from January to March 2018, and downloaded. These entire articles were then viewed and the data of medicinal plants, which have neurological effects, were collected and tabulated in (Table 3). We have personally visited districts Bahawalpur, Bannu, Buner, Dir, Gilgat, Islamabad, Jhelum, Malakand, Mianwali, Rawalpindi, Sargodha, and Swat in April-June 2018 and collected information regarding plants local names, local use, mode of applications, and administration of these plants in neurological diseases from local traditional healers, folk medicine users, and local elderly people of those districts having knowledge of medicinal plants. Information was also collected from distant districts with the help of friends living there via social media (phone calls, text messages, WhatsApp calls and messages, and emails).

3. Results and Discussion

A total of 54 families were found to be useful in various neurological diseases, of which the highest use was of Solanaceae (22.22 %), Asteraceae (12.96 %), Lamiaceae, Papaveraceae, and Poaceae, 9 % each, and Caprifoliaceae, Cucurbitaceae, Rhamnaceae, and Rosaceae, 5.5 % each (Table 3). As per district point of view, 15% plants, effective in neurological affections, were found in Bahawalpur, 11% in Swat, 8 % in Muzaffraabad, 7% in Malakand, and 6% in Bahawalnagar, Dir, Gilgat, and Sarghoda each, with 5% in Dera ghazi khan and Jhelum each (Figure 1).

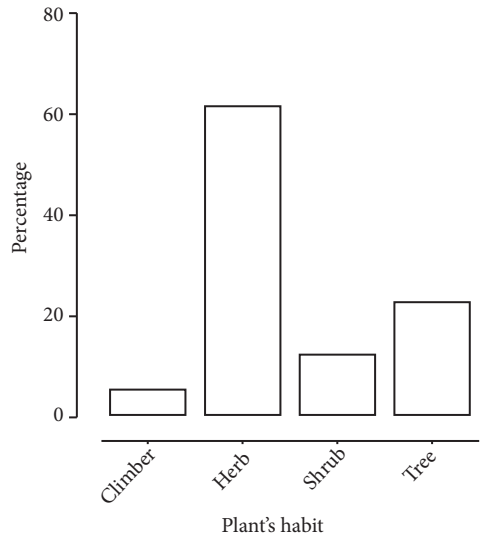


Figure 2: Habit-wise percentage of plants used for neurological diseases.

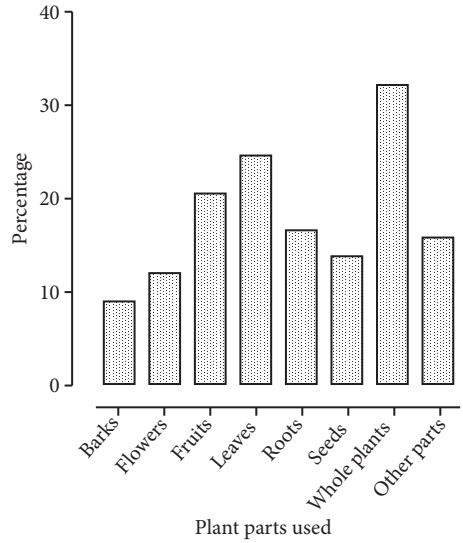


Figure 3: Parts-wise percentage of plants used for neurological diseases.

This district-wise plant distribution will help the researchers, who are willing to research in neuropharmacological area, to easily collect the target plants from the regions to which the plants belong. According to the plant's habit, out of total of 103 plants, 61.15% were found to be herbs, 22.33 % trees, 11.65% shrubs, and 4.85% climbers (Figure 2).

The habit of plants shows that herbs are most important according to neuropharmacological point of view which is another benefit for researchers working in neuropharmacological area to concentrate on herbs more while selecting neurological active plants. According to the part used of plant, whole plant, leaves, fruits, roots, seeds, flowers, and other parts (bulbs, latex, gum, tubers, and rhizome) were found to be used 32.03 %, 24.27 %, 20.38 %, 16.50 %, 13.59 %, 11.65 %, and 15.53 %, respectively (Figure 3). As some plants

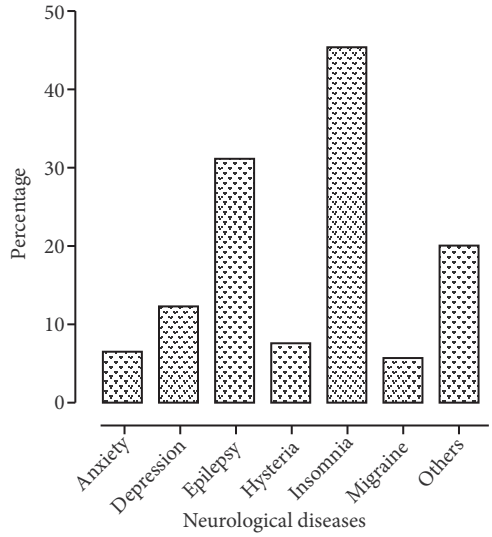


Figure 4: Disease-wise percentage of plants used for neurological diseases.

have more than one part to be used for various neurological diseases, so such plants were counted into percentage of all respective parts. This division of neuropharmacological plants ensures the researchers to select the most appropriate parts of plants having specific neuropharmacological activities, for their research, as used by traditional healers and folk medicine users.

According to disease's types, 45.63 % were found to be of therapeutic value in insomnia, epilepsy (31.06%), depression (12.62%), anxiety (6.80%), hysteria (7.77%), and migraine (5.88%) and 20.38 % in other neurological diseases (neuralgia, mania, Parkinson, schizophrenia, and nerve pain) (Figure 4). As some plants are used for multiple neurological ailments, so such plants were counted into percentage of all respective diseases. This disease-wise plant division will help the local researchers to select their interest areas in the field of neuropharmacology, by selecting the neurological disease, for which most of the plant's percentage was found to be used by traditional healers and folk medicine users in various districts of Pakistan.

The pharmacological activities of plants are due to the presence of various phytochemicals mainly alkaloids, flavonoids, tannins, saponins, resins, glycosides, terpenoids, phenols, sterols, essential oils, vitamins, and nutrients. Some of these are effective in the treatment of neurological diseases; some are useful for cardiovascular, respiratory, and gastrointestinal diseases while others have chemotherapeutic and antibacterial effects. Some of the important phytochemicals of the plants (Table 4) including alkaloids (like nicotine and scopolamine) are reported to have anxiolytic, antidepressant, and anti-Parkinson activities [15–18], saponins (like bacosides) have been reported for anxiolytic, antiepileptic, antiamnesia, and neuroprotective and memory enhancement activities [19–22], terpenoids (like cannabigerol, tetrahydrocannabinol, and cannabidiol) are reported for their neuroprotective effects [23], flavonoids (like kaempferol, luteolin, quercetin, rutin, and hesperidin) have been reported for their anxiolytic, antidepressant, antiepileptic, anti-Alzheimer, and neuroprotective and memory enhancement activities [24–30], glycosides (like hastatoside and verbenalin) are reported for sleep promoting activity [31], steroids (like sitoindosides VII–X and withaferin-A) have been reported for anxiolytic activity [32].

Bacopa monnieri plant is reported for anxiety, depressant, epilepsy, and Parkinsonism and contains alkaloids (Brahmin, nicotine, herpestine, and bacosides A & B), saponins (hersaponin and monnierin), flavonoids (luteolin and apigenin), and sterols like b-sitosterol and stigma-sterol. These constituents are already reported for such neuropharmacological properties and so might be responsible for said activities of this plant [33–36].

Cannabis sativa L. has been reported for the treatment of depression, anxiety, convulsion, Alzheimer, dementia, and insomnia and its constituents responsible for these properties are cannabigerol, tetrahydrocannabinol, and cannabidiol [37–41].

Verbena officinalis Linn. has been reported as anxiolytic, antidepressant, anticonvulsant, and sedative and its constituents responsible for these activities are verbenin, verbenalin, hastatoside, kaempferol, luteolin, verbascoside, aucubin, and apigenin [42–44].

Withania somnifera has been shown to have anxiolytic, antidepressant, anticonvulsant, and anti-Parkinson effects, mainly due to the presence of withanolides, sitoindosides VII–X, and withaferin-A [45–48].

These chemical constituents of plants act on the central nervous system through various mechanisms including regulation of neurotransmitters like adrenergic, cholinergic and serotonergic activity, acting through receptor like GABA and N-methyl-D-aspartate, and ion channels like sodium, potassium, and calcium ion channels. Some of the plant-based drugs and phytochemicals which either are approved or are under clinical trials for the treatment of neurological diseases, mechanism of actions, and their current status in clinical trials are given in (Table 5).

Taking into consideration this useful knowledge on the medicinal properties of plants for curing neurologic diseases, it is believed that the research in the areas of ethnomedicine and ethnopharmacology can bring auspicious results that have potential of adding importance to the very rich natural resources of Pakistan. Various phytochemicals from the above medicinal plants can be further researched under clinical trials and better drugs for treatment of neurological diseases can be obtained with outstanding results and lesser side effects. This study will help all the researchers, especially from Asian countries including Pakistan, China, Iran, India, Sri Lanka, and Bangladesh, working on plants based medicine for neurological diseases.

4. Conclusion

The mental illnesses are one of the major problems of the world mainly in communities presenting with poor socioeconomic conditions. In Pakistan and other countries

Table 3: Traditionally used medicinal plants for the treatment of various neurological diseases.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
1	*Achyranthes aspera*	Ayokanda	Amaranthaceae	Herb	Leaves and Shoot	Nerve tonic	Paste of dried leaves and shoots is applied on head	Sargodha	[104]
2	*Ailanthus altissima*	Backyanra	Simarubacea	Tree	Bark	Hysteria	Decoction of bark to make tea	Malakand	[105]
3	*Albizia lebbeck*	Sirin	Mimosaceae	Tree	Roots	Depression, Migraine and Anxiety	Decoction of root to make tea	Mianwali	[106]
4	*Allium sativum*	Ooga	Amaryllidaceae	Herb	Bulbs and Leaves	Hysteria and Epilepsy	Decoction of bulbs and leaves	Swat	[107]
5	*Alnus nitida*	Geiray	Betulaceae	Tree	Flowers	Insomnia	Powder of dried flowers mixed with water and used orally	Dir	[108]
6	*Alternanthera sessilis*	Waglon	Amaranthaceae	Herb	Leaves	Neuralgia and Sedative	Sniffing of leaves sap	Bahawalpur	[109]
7	*Anagallis arvensis*	Billy booti	Primulaceae	Herb	Whole plant	Nervine, mania and Epilepsy	Extract of whole plant	Bahawalpur	[109]
8	*Artemisia scoparia*	Jaukay	Asteraceae	Herb	Roots	Epilepsy	Powder of roots taken with water	Dir	[108]
9	*Asparagus officinalis*	Phala-moosa	Asparagaceae	Herb	Leaves	Insomnia	Tea of leaves are used on empty stomach	Lahore	[110]
10	*Atropa accuminata*	Bargak	Solanaceae	Herb	Leaves	Insomnia and narcotic	Powder of leaves are taken with water	Dir	[108]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
11	Avena fatua	Jodal	Poaceae	Herb	Seeds	Depression and nervous exhaustion	Either the seeds fluid extract or oatmeal obtained by crushing and grinding seeds	Dera Ghazi Khan	[111]
12	*Avena sativa*	Jai	Poaceae	Herb	Seeds	Nerve tonic and Insomnia	A tincture of juice of immature seeds	Islamabad	[112]
13	*Bacopa monnieri*	Brahmi sak	Scrophulariaceae	Herb	Whole plant	Epilepsy	Extract of whole plant is taken orally	Mianwali	[106]
14	*Buglossoides arvensis*	Kalu	Boraginaceae	Herb	Leaves	Insomnia	Infusion of leaves is used orally	Kotli	[113]
15	*Caltha alba*	Makanpat	Ranunculaceae	Herb	Whole plant	Insomnia	Extract of whole plant	Dir	[108]
16	*Campanula pallida*	Beli Flower	Campanulaceae	Herb	Flowers	Insomnia	An infusion of flowers is used orally	Kotli	[113]
17	*Cannabis Sativa*	Bhang	Cannabaceae	Herb	Flowers	Insomnia	The ground flowers are used by mixing with other fruits	Bannu	[114]
18	*Capparis decidua*	kdler	Capparidaceae	Shrub	Flowers, fruits and shoots	Insomnia	The powder of flowers and shoots while fruits are eaten as such	Gawadar	[115]
19	*Capparis spinosa*	Kawir	Capparidaceae	Shrub	Whole plant	Mental disorders	Fresh extract of whole plant is used	Gilgat	[116]
20	*Carthamus tinctorius*	Tukhmiga-rtum	Asteraceae	Herb	Roots, oil and flowers	Insomnia	Decoction of roots to make tea while oil is applied externally	Rawalpindi	[117]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
21	*Celtis australis*	Karr	Cannabaceae	Tree	Bark	Epilepsy	Decoction of bark is used orally	Sargodha	[118]
22	*Cenchrus pennisetiformis*	Cheetah- gha	Poaceae	Herb	Leaves and fruits	Epilepsy	Extracts and juice of leaves and fruits	Hafizabad	[119]
23	*Citrullus colocynthis*	Tumma	Cucurbitaceae	Climber	Roots and fruits	Epilepsy	The extract of roots is taken with water while fruit's powder is mixed with sugar	Jhelum	[120]
24	*Citrus limon*	Nimboo	Rutaceae	Tree	Whole plant	Anxiety and Depression	whole plant extract	Bahawalpur	[109]
25	*Citrus medica*	Khatti	Rutaceae	Tree	Leaves, seeds, latex	Insomnia	Powder of leaves, seeds and dry latex are taken orally with water	Bahawalpur	[109]
26	*Colebrookia oppositifolia*	Lansa	Lamiaceae	Shrub	Leaves and roots	Epilepsy	Fresh leaves extract and roots decoction tea is taken orally	Malakand	[121]
27	*Commiphora wightii*	Guggul, Mukul	Burseraceae	Herb	Gum	Nervous diseases	The aqueous extract of gum is used	Muzaffarabad	[122]
28	*Convolvulus arvensis*	Baily	Convolvulaceae	Herb	Whole plant	Epilepsy	whole plant extract	Malakand	[121]
29	*Cucurbita maxima*	Walayti kadoo	Cucurbitaceae	Climber	Fruits	Nervous disorders	Juice of both unripe and ripe fruits is used	Azad Jammu & Kashmir	[123]
30	*Cuscuta reflexa*	Bepari, Kasus	Cuscutaceae	Tree	Seeds	Insomnia	An infusion of seed is used	Muzaffarabad	[122]
31	*Cymbopogon citratus*	Lemon- grass	Poaceae	Herb	Oil of whole plant	Nervous system tonic	Oil is externally applied on head	Bahawalpur	[109]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
32	*Cynodon dactylon*	Lawn grass	Poaceae	Herb	Whole plant	Epilepsy and Hysteria	Extracted juice of plant is used	Dera Ghazi Khan	[111]
33	*Cyperus rotundus*	Deela	Cyperaceae	Herb	Tubers	Epilepsy	Oil obtained from tubers are used	Bahawalnagar	[124]
34	*Datura alba*	Datura	Solanaceae	Shrub	Leaves and seeds	Neuralgia, Epilepsy, Hysteria and Insomnia	Lotion of seed's powder is applied locally for neuralgia while tea of leaves is used for Epilepsy	Bahawalpur	[109]
35	*Datura innoxia*	Datura	Solanaceae	Herb	Leaves	Epilepsy and Insomnia	Extract of leaves in water	Dir	[108]
36	*Datura metel*	Dhaturo	Solanaceae	Herb	Leaves and seeds	Epilepsy and Insomnia	Leaves extract and seed's decoction are used	Muzaffarabad	[122]
37	*Datura stramonium*	Datura	Solanaceae	Herb	Whole plant	Insomnia and Parkinson	Extraction of whole plant is used	Dera Ghazi Khan	[111]
38	*Daucus carota*	Gajar	Apiaceae	Herb	Whole plant	Nerve tonic	Eaten as a whole or its juice is used	Sargodha	[104]
39	*Eclipta alba*	Bhringaraj	Asteraceae	Herb	Roots, oil and leaves	Insomnia	Oil is externally applied while roots and leaves extract is used orally	Bahawalpur	[109]
40	*Eruca sativa*	Tara meera	Cruciferaceae	Herb	Whole plant	Epilepsy	Fluid extraction of plant is used	Islamabad	[112]
41	*Evolvulus alsinoides*	Sankha-holi	Convolvulaceae	Herb	Whole plant	Epilepsy	Decoction of whole plant is used	Islamabad	[112]
42	*Ficus lyrata*	Beeri patta	Moraceae	Tree	Leaves	Migraine	Extraction of leaves is used orally	Bahawalpur	[109]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
43	*Flueggea leucopyrus*	Shina	Phyllanthaceae	Shrub	Roots	Epilepsy	Decoction and extraction of roots are used	Dir	[125]
44	*Fumaria indica*	Pitpapra	Fumariaceae	Herb	Leaves and stem	Insomnia	Fresh juice of leaves and stem is used	Rawalpindi	[117]
45	*Gmelina arborea*	Kumbar	Lamiaceae	Tree	Roots	Epilepsy	Extraction and decoction of roots tea is used	Sargodha	[118]
46	*Hyoscyamus niger*	Ajwain-i-Khurasani	Solanaceae	Herb	Leaves and seeds	Insomnia and Nervous affliction	Extraction of fresh leaves and powder of seeds are used orally	Gilgat	[126]
47	*Hypericum perforatum*	Bulhsana	Hypericaceae	Herb	Whole plant	Depression and Insomnia	Fresh extract of whole plant is used orally	Gujrat	[127]
48	*Hyssopus officinalis*	Zufa, Zupa	Lamiaceae	Herb	Whole plant	Nervous affection	Extraction of fresh whole plant	Ziarat	[128]
49	*Indigofera heterantha*	Kainthi	Papilionaceae	Shrub	Whole plant	Epilepsy and neuropathy	Extract of whole plant is used	Gilgat	[116]
50	*Jasminum grandiflorum*	Chanbeli	Oleaceae	Climber	Whole plant	Anxiety, tension and Depression	Oil or tea of leaves and flowers extract are used	Bahawalpur	[109]
51	*Jasminum officinale*	Chanbeli	Oleaceae	Climber	Whole plant	Insomnia	Oil is rubbed on heart as nerve sedative	Swat	[107]
52	*Juglans regia*	Ghuz	Juglandaceae	Tree	Fruits	Depression	Fruits are taken as whole orally	Malakand	[105]
53	*Lactuca serriola*	Berham dandi	Asteraceae	Herb	Whole plant	Memory Enhancing	Fresh plant is ground in water along with black pepper	Jhelum	[120]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
54	*Linum usitatissimum*	Alsi	Linaceae	Herb	Stem	Depression, Schizophrenia and Anxiety	Extraction of fresh stem is used	Kotli	[113]
55	*Lycopersicon esculentum*	Tamator	Solanaceae	Herb	Fruits	Nervous weakness	Eaten as a whole or its juice is used	Sargodha	[104]
56	*Martricaria chamomilla*	Babuna	Asteraceae	Herb	Whole plant	Insomnia	Extraction of whole plant is used orally and oil massage or aromatherapy into skin of head is performed	Rawalpindi	[117]
57	*Martynia annua.*	Bichhu-butti	Martyniaceae	Herb	Leaves and fruits	Epilepsy	Juice of leaves or leaves are cooked to make curry and fruits are taken as dry powder with water	Kotli	[113]
58	*Melia azedarach*	Bakyana	Meliaceae	Tree	Leaves	Hysteria	Decoction of leaves to makes tea	Malakand	[105]
59	*Mimordica dioca*	Jungli karela	Cucurbitaceae	Climber	Fruits and seeds	Insomnia	Fruit's extract and seed oil are used	Mianwali	[129]
60	*Moringa oleifera*	Sohan-jana	Moringaceae	Tree	Seeds and bark	Migraine	Seeds oil used externally while powder of leaves	Gujrat	[127]
61	Ocimum basilicum	Niazbo	Lamiaceae	Herb	Leaves, flowers, seeds and roots	Migraine, Insomnia and Depression	Juice of fresh leaves and flowers while oil of seeds is applied externally on head	Bahawalnagar	[124]
62	*Paeonia emodi*	Mamaikh	Paeoniaceae	Herb	Rhizome	Epilepsy	Rhizome powder is given 1/2 teaspoon twice a day	Malakand	[105]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
63	*Papaver dubium*	Koko-kanga	Papaveraceae	Herb	Flowers	Insomnia	Fluid extract of flowers is used	Kotli	[113]
64	*Papaver hybridum*	Post	Papaveraceae	Herb	Fruits	Insomnia	Fruit and its decoction are used	Jhelum	[120]
65	*Papaver nudicaule*	Zangali kashkash	Papaveraceae	Herb	Flowers	Insomnia	Fluid extract of flowers is used	Buner	[130]
66	*Papaver rhoeas*	Alak jinai	Papaveraceae	Herb	Flowers	Insomnia	Fluid extract of flowers is used	Buner	[130]
67	*Papaver somniferum*	Qash-Qash	Papaveraceae	Herb	Fruit's latex	Insomnia	Latex of unripe fruit is dissolved in water and used orally	Swat	[107]
68	*Parthenium hysterophorus*	Ragweed	Asteraceae	Herb	Leaves	Insomnia	Leaves extraction is used	Buner	[130]
69	*Peganum harmala*	Harmal	Zygophyllaceae	Herb	Seeds	Hysteria	A small amount of seeds added to sufficient grapes juice, boiled to make thick solution and used orally	Dera Ghazi Khan	[111]
70	*Populus caspica*	Nakhtar	Pinaceae	Tree	Fruits	Insomnia	Whole raw fruits are consumed	Malakand	[105]
71	*Primula veris*	Cowslips	Primulaceae	Herb	Flowers	Insomnia	A tasty wine of flowers is made which is used orally	Gilgat	[126]
72	*Prunus persica*	Ardou	Rosaceae	Tree	Leaves, flowers and fruits	Insomnia	Extract of leaves & flowers and fruits are taken as such	Gilgat	[126]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
73	*Punica granatum*	Darrona	Punicaceae	Shrub	Fruits	Memory enhancing	Fruit's juice or fresh seeds are eaten as such	Azad Jammu & Kashmir	[123]
74	*Pyrus communis*	Nashpatai	Rosaceae	Tree	Fruits	Insomnia	Fruits are eaten as such	Dir	[131]
75	*Pyrus pashia*	Tangai	Rosaceae	Herb	Fruits	Insomnia	Fruits are eaten as such	Swat	[107]
76	*Ranunculus muricatus*	Ziar Gulay	Ranunculaceae	Herb	Whole plant	Sciatic and nerve pain	Extraction of dried whole plant is used	Swat	[132]
77	*Raphanus sativus*	Mooli	Brassicaceae	Herb	Seeds	Nervous weakness	Decoction of seeds is used	Sargodha	[104]
78	*Ricinus communis*	Arand	Euphorbiaceae	Shrub	Roots, seeds, leaves	Insomnia and as narcotic	Extract of leaves and roots while oil of seeds are used	Rawalpindi	[117]
79	*Salvadora oleoides*	Peelu	Salvadoraceae	Tree	Whole plant	Epilepsy	Fruit is eaten as raw while tea of leaves and roots are also used	Bahawalpur	[109]
80	*Schinus molle*	False pepper	Anacardiaceae	Tree	Bark and leaves	Depression	Decoction of bark and leaves to make tea	Sargodha	[118]
81	*Scutellaria chamaedrifolia*	Skullcap	Lamiaceae	Herb	Shoots	Insomnia and Depression	Decoction of shoots to make its tea	Swat	[133]
82	*Solanum miniatum*	Peelak	Solanaceae	Herb	Whole plant	Insomnia	whole plant decoction is mixed with sugar	Jhelum	[120]
83	*Solanum nigrum*	Mako	Solanaceae	Herb	Whole plant	Insomnia	Juice of whole plant	Bahawalpur	[109]
84	*Solanum Surratense*	Wara-mara ghinrhye	Solanaceae	Herb	Fruits	Melancholia and Depression	The paste of fruits crushed powders is applied on head externally	Bannu	[114]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
85	*Taxus baccata*	Banhya	Taxaceae	Tree	Leaves and fruits	Epilepsy	Extraction of dried leaves and fruits are consumed as such	Swat	[134]
86	*Taxus wallichiana*	Barmi	Taxaceae	Tree	Bark, leaves and fruits	Epilepsy and Insomnia	Extract of dried bark and leaves while flesh of fruits are consumed	Battagram	[135]
87	*Terminalia arjuna*	Arjun	Combretaceae	Tree	Fruits, bark and leaves	Anxiety	Bark infusion left whole night, then its decoction taken early in the morning and used orally	Bahawalpur	[109]
88	*Tribulus terrestris*	Bakhra	Zygophyllaceae	Herb	Whole plant	Epilepsy and Depression	Powder of dried whole plant	Bahawalnagar	[124]
89	*Valeriana jatamansi*	Mushk-bala	Vahliaceae	Herb	Whole plant	Epilepsy and neurosis	Fresh extract of whole plant	Muzaffarabad	[122]
90	*Verbascum thapsus*	Jungle tambako	Scrophulariceae	Herb	Roots	Migraine	Decoction of root to make tea to use as drink	Mianwali	[106]
91	*Verbena officinalis*	Shamkay	Verbenaceae	Herb	Whole plant	Depression, Migraine and Epilepsy	Extract of dried whole plant is used	Battagram	[135]
92	*Viburnum cotinifolium*	Guch	Caprifoliaceae	Shrub	Stem's bark	Insomnia	Extract of stem's bark	Muzaffarabad	[122]
93	*Viburnum opulus*	Sunaira Phul	Caprifoliaceae	Shrub	Bark	Insomnia and Hysteria	Decoction of bark is used	Muzaffarabad	[122]

Table 3: Continued.

S #	Botanical Name	Local Name	Family	Habitat	Part Used	Used for	Mode of Applications	Location	Reference
94	*Viburnum prunifolium*	Blackhaw	Caprifoliaceae	Tree	Root's bark	Hysteria, Anxiety and Epilepsy	Decoction of root's bark is used	Muzaffarabad	[122]
95	*Vicia sativa*	Muttri	Papilionaceae	Herb	Flowers	Epilepsy and nervous disorders	The juice of flowers petals is used	Kotli	[113]
96	*Viola betonicifolia*	Banafsh	Violaceae	Herb	Whole plant	Epilepsy and nervous disorders	Fresh extract of whole plant orally	Malakand	[105]
97	*Viola canescens*	Banafsha	Violaceae	Herb	Whole plant	Insomnia and Epilepsy	Extract and decoction tea of whole plant	Swat	[133]
98	*Withania coagulans*	Paneer doda	Solanaceae	Herb	Fruits, roots and leaves	Nervous Exhaustion, memory loss and tension	Extract of leaves, roots and fruits are used	Bahawalnagar	[124]
99	*Withania somnifera*	Asgandh	Solanaceae	Shrub	Roots	Insomnia	Powder of roots is taken with water	Bahawalpur	[109]
100	*Xanthium strumarium*	Chota dhatura	Asteraceae	Herbs	Fruits, seeds and roots	Insomnia	Decoction of fruits, roots and seeds to make tea	Attock	[136]
101	*Ziziphus jujuba*	Beri	Rhamnaceae	Tree	Leaves, roots and fruits	Anxiety and Insomnia	Extract of leaves, decoction of roots and dried fruits are consumed	Bahawalnagar	[124]
102	*Ziziphus mauritiana*	Ber	Rhamnaceae	Tree	Roots	Nerve tonic	Decoction of roots is used as tea	Sargodha	[104]
103	Ziziphus nummularia	Jangli beri	Rhamnaceae	Shrub	Leaves and fruits	Insomnia	Extract of leaves while fruits are taken as such	Attock	[136]

Table 4: Phytochemical constituents and pharmacological properties of some well-known medicinal plants.

S.#	Medicinal Plants	Pharmacological Properties	Part used	Phytochemical Constituents	Chemical Compounds Identified	References
1	*Allium sativum*	1. Antidepressant 2. Anticonvulsant 3. Anti-Alzheimer	1. Dried bulbs 2. Oil 3. Whole garlic	Thiosulfinates, sapogenins phenols, saponins, volatile compounds, antioxidants, flavonoids, vitamins, minerals and proteins	Alliin, allixin, 1,2-vinyldithiin, ajoenes, S-allyl-cysteine sulfoxide, calcium, Potassium, vitamin B and vitamin C	[137–140]
2	*Bacopa monnieri*	1. Antidepressant 2. Anxiolytic 3. Anticonvulsant 4. Anti-Parkinson	1. Leaves 2. Stems and leaves 3. Leaves 4. Conc. tincture of plant	Alkaloid,tannin, saponin, phlobatannin, glycoside, terpenoid, flavonoid, sterols, phenol, steroid, anthraquinone and carbohydrate	Brahmin, nicotine, herpestine, bacosides A & B, hersaponin, betulic acid, monnierin, apigenin, b-sitosterol, stigma-sterol and luteolin	[33–36, 141]
3	Cannabis sativa	1. Antidepressant and anxiolytic 2. Anticonvulsant 3. Anti-Alzheimer and antidementia 4. Sedative	1. Leaves 2. Leaves 3. Flowers 4. Whole plant	Alkaloid, flavonoids, tannins, phenols, resins, cardiac glucosides, terpenes, steroids, volatile oils and balsam	Cannabinoids, cannabidiol, dronabinol, cannabigerol, tetrahydrocannabinolic acid, cannabichromenic acid, cannabidiolic acid, anandamide, cannabigerolic acid and cannabichromene	[37–40, 142, 143]
4	*Hyoscyamus niger*	1. Antidepressant 2. Anti-seizure 3. Anti-Parkinson	1. Leaves 2. Seeds 3. Seeds	Alkaloids, withanolide steroids, lignanamides, tyramine derivative, steroidal saponins, glycosides, lignans, coumarinolignan, and flavonoids	Apoatropine, L-DOPA, Cuscohygrine, choline Daturamine, Hyoscine, tropine, hyoscypicrin, phytin, aphoyoscine, alpha and beeta belladonine and Skimmianine	[144–148]
5	*Solanum nigrum*	1. Anti-seizure 2. Sedative	1. Leaves 2. Fruits	Alkaloids, flavonoids, tannins, saponins, glycosides, proteins, carbohydrates, coumarins and phytosterols	Pinoresinol, syringaresinol, medioresinol, scopoletin, tetracosanoic acid and beta-sitosterol	[149–152]

Table 4: Continued.

S.#	Medicinal Plants	Pharmacological Properties	Part used	Phytochemical Constituents	Chemical Compounds Identified	References
6	*Withania somnifera*	1. Anti-Parkinson 2. Anxiolytic and antidepressant 3. Anticonvulsant	1. Whole plant 2. Roots 3. Stems and roots	Alkaloids, steroidal lactones, saponins and iron	Withanolides, withaferins, Withanine, isopellertierine, anferine, Anahygrine, Cuscohygrine, Beta-Sisterol, Chlorogenic acid, Scopoletin, choline, Somniferiene, Somniferinine and Tropanol	[45–47, 153]
7	*Papaver somniferum*	1. Anticonvulsant	1. Seeds	Alkaloids, glycosides, tannins, Phytosterols, Terpenoids, Flavanoids and Carbohydrates	Morphine, Codeine, thebaine, noscapine, papaverine, Salutarifine, meconidine, codmine, neoprene, lanthothine, rophyroxine, narcotisline and papaveramine	[154–159]
8	*Ziziphus jujube*	1. Sedative and hypnotic 2. Anxiolytic 3. Anti-seizure	1. Seeds 2. Leaves 3. Fruits	Triterpenic acids, flavonoids, saponins, cerebrosides, amino acids, phenolic acids, vitamins, total sugars and nucleosides	Zizybeoside I and II, Chryseoriol, Swertisin, Quercetin, Jujubasaponin IV, Lotoside I and II, Zizyphus saponin I and II	[160]
9	*Tribulus terrestris*	1. Anxiolytic 2. Antidepressant 3. Sedative	1. Leaves 2. Whole plant 3. Whole plant	Saponins, flavonoids, glycosides, alkaloids and tannins	Tigogenin, neotigogenin, rutin, chlorogenin, caffeoyl, ruscogenin, kaempferol, tribulosid, terrestribisamide, quercetin, β-sitosterol, stigmasterols, harmane, norharmane and tribulusterine	[161–164]
10	*Verbena officinalis*	1. Antidepressant 2.Anticonvulsant, anxiolytic and sedative	1. Leaves 2. Whole plant	Alkaloids, flavonoids, diterpenes, proteins, amino acids, tannins, saponins, phytosterols and phenolic compounds	Verbenin, oleanolic acid, verbenalin, hastatoside, alpha-sitosterol, ursolic acid, kaempferol, aucubin, luteolin, verbascoside, apigenin, scutellarein, limonene and spathulenol	[42, 43]

Table 4: Continued.

S.#	Medicinal Plants	Pharmacological Properties	Part used	Phytochemical Constituents	Chemical Compounds Identified	References
11	*Albizia lebbeck*	1. Anticonvulsant 2. Nootropic and anxiolytic	1. Leaves 2. Leaves	Alkaloids, flavonoids, phenols, saponins; steroids and terpenoids	Albizia saponins A, B and C, albizinin, melacacidin, catechin lebbecacidin, friedelin, and β-sitosterol	[165–168]
12	*Avena sativa*	1. Antidepressant 2. Anxiolytic	1. Seeds 2. Whole plant	Carbohydrates, alkaloids, flavanoids, steroids, glycosides, saponins, amino acids, gums and mucilage	Gramine, flavone, apigenin and luteolin, flavonolignans, saponins and ferulic acid	[169–171]
13	*Capparis decidua*	1. Sedative and anticonvulsant	1. Flowers and fruits	Alkaloids, glycosides, terpenoids, sterols, flavanoids, phenols and fatty acids	Capparine, cappariline, capparinine, β-sitosterol, capparidisine, capparisine, codonocarpine, Capric acid, cadabacine, quercetin and rutin l-stachydrine	[172, 173]
14	*Citrus limon*	1. Anticonvulsant 2. Sedative, anxiolytic and antidepressant	1. Essential oil of leaves 2. Essential oil of leaves	Phenols, flavonoids, terpenoids, essential oils, carotenoids, citric acid and ascorbic acid	Limonene, α-pinene, β-pinene, linalool, α-terpineol, linalyl acetate, acetate geranyl, nerolidol, acetate neryl, farnesol, sabinene, myrcene, cineol and geranial	[174–176]
15	*Citrullus colocynthis*	1. Anticonvulsant 2. Antidepressant	1. Fruits 2. Fruits	Alkaloids, flavonoids, glycosides, saponosides, Phenolic compounds and ascorbic acid	Colocynthin, colocynthein, colocynthetin, Cucurbitane type triterpen glycoside, quercetin and Flavone	[15, 16, 177]
16	*Datura metel*	1. Antiepileptic 2. Sedative and hypnotics	1. Leaves 2. Seeds	Alkaloids, resins, flavonoids, reducing sugars, tannins, terpenoids and steroid glycosides	Hyoscyamine, scopolamine, atropine, daturabietatriene, daturasterol,, b-sitosterol and Melatonin and serotonin	[17–21]

Table 4: Continued.

S.#	Medicinal Plants	Pharmacological Properties	Part used	Phytochemical Constituents	Chemical Compounds Identified	References
17	Hypericum perforatum	1. Antidepressant 2. Anti-Parkinson 3. Neuroprotective 4. Anticonvulsant 5. Anti-Alzheimer 6. Anxiolytic and sedative	1. Flowers 2. Flowers and leaves 3. Whole plant 4. Flowers and leaves 5. Flowers 6. Flowers	Phenylpropanes, flavonoids, biflavones, phloroglucinols proanthocyanidins, amino acids, essential oil and naphthodianthrones	Hyperoside, adhyperforin Quercitrin, Rutin, Hypericin, Kaempferol, Biapigenin and Hyperforin	[22–28]
18	*Jasminum grandiflorum*	1. Antidepressant 2. Anticonvulsant	1. Essential oil of plant 2. Leaves	Coumarins, steroids, cardiac glycosides, essential oils, flavonoids, phenolics and saponins	Rutin, kaempferol, quercetin, β-primeveroside, kaempferol, hesperidin Methyl jasmonate, methyl anthranilate, linalool β-rutinoside, oleuropein and daucosterol	[29–31]
19	*Lycopersicon esculentum*	1. Antidepressant 2. Anticonvulsant 3. Memory enhancement 4. Anti-Parkinson	1. Fruits 2. Dried fruit extract 3. Dried fruit extract 4. Seeds	flavonoids, tannins, saponin, glycosides, Steroids, fatty acids, carbohydrates and proteins	Chlorogenic acid, rutin, naringenin, noradrenaline lycopene, dopamine, tomatin, tomatoside-A, ascorbic acid, bergapten, serotonin and adrenaline	[32, 178–180]
20	Ocimum basilicum	1. Antidepressant 2. Anticonvulsant 3. Anxiolytic and sedative 4. Enhance memory retention	1. Essential oil 2. Leaves 3. Aerial parts 4. Leaves	Terpenoids, essential oil, polyphenols, tannins and flavonoids	Cineole, geraniol, linalool, cadinol and sabinene, methyl chavicol, β-caryophyllene and neral, quercetin, myricetin, kaempferol, catechin and eugenol	[181–184]
21	*Punica granatum*	1. Antidepressant 2. Anxiolytic and anticonvulsant 3. Anti-Alzheimer 4. Memory enhancement	1. Fruits 2. Leaves 3. Fruits 4. Fruit's peel	Flavonoids, glycosides, amino acids, pectin, indoleamines, tannins, sterols, polyphenols, carbohydrates, ellagitannins, anthocyanins and triterpenoid	Catechin, rutin, quercetin epicatechin, estriol, luteolin kaempferol, anthocyanins, gallagyldilacton, stigmasterol, β-sitosterol, testosterone, tocopherols and isoflavones	[185–188]

Table 5: The different phytochemicals effective in various neurological diseases and their current clinical phase status.

Sr #	Phytochemicals	Source	Family	Disease	Mechanism	Development stage	Trade Name	Reference
1	Cannabidiol	Cannabis sativa	Cannabaceae	Epilepsy	Modulation of intracellular calcium and neuronal inhibition	FDA approved, 2018	Epidiolex as 5- 10 mg/kg/day	[189]
2	Cannabidol	Cannabis sativa L.	Cannabaceae	Chronic Neuropathic pain	CB1 and CB2 receptor activation	FDA approved, 2005	Sativex Spray (CBD 25mg/ml + THC27mg/ml)	[190]
3	Capsaicin	Capsicum annum L.	Solanaceae	Postherpetic neuralgia	TRPV1 activator	FDA approved, 2010	Qutenza as Patch (179mg capsacin)	[190]
4	Curcumin	Curcuma longa	Zingiberaceae	Dementia	Anti-amyloid, AChEI	phase II		[191]
5	Galantamine	*Galanthus nivalis*	Amaryllidaceae	Alzheimer	AChEI, allosteric modulation of nicotinic ACh receptor	FDA approved, 2004	Razadyne as 8-12 mg BD	[192]
6	Huperzine A	*Huperzia serrata*	Huperziaceae	Alzheimer	AChEI, inhibits NMDA and glutamate toxicity	approved in China		[193]
7	Ibogaine	Tabernanthe iboga	Apocynaceae	Parkinson	Dopaminergic agonist, NMDA antagonism	preclinical		[193]
8	Psychollatine	Psychotria umbellate	Rubiaceae	Parkinson	MAO inhibitor	preclinical		[193]
9	Resveratrol	Vitis vinifera L.	Vitaceae	Alzheimer	Reduces Aβ formation and promote Aβ decomposition	phase II		[194]
10	Scyllo-Inositol	Cornus florida L.	Cornaceae	Alzheimer	Breakdown of neurotoxic fibrils, allowing amyloid peptides to clear the body rather than form amyloid plaques	phase II		[195]

FDA: food and drug administration; TRPV1: transient receptor potential vanilloid 1; CB1 and CB2: cannabinoid receptor type 1 & type 2; Ach: acetylcholine; AChEI: acetylcholinesterase inhibitor; CBD: cannabidiol; THC: tetrahydrocannabinol; BD: bis in die; NMDA: N-methyl-D-aspartate; MAO: monoamine oxidase; Aβ: amyloid beta.

of this region, there is no accurate and up to date record of the neurological ailments. In order to find any treatment for these diseases, first realistic survey would be required to find out the exact percentage of various neurological diseases. Being an alarming psychiatric problem, Alzheimer opens a new area of research, affecting an enormous part of world population, but it is still untreatable. A lot of attempts have been conducted but still there is no such drug that can either slow or stop the process of Alzheimer disease. Allopathic medicines are available for psychological diseases including anxiety, depression, epilepsy, Parkison, and Alzheimer, but these are either not so effective or costly or have serious associated adverse effects. The world is full of natural medicinal resources, of which the main source is plant. We should invest money and go for systemic scientific investigations to perceive such drug candidates' form these plants, which are most efficacious, have minor side effects, and are cost friendly. For this purpose, this study is a gift for researchers who have interest to design and perform research based activities in the field of neuropharmacology by evaluating the unexplored medicinal plants mentioned here for their folkloric uses, determining its mechanistic pathways and identifying chemical constituents responsible for therapeutic effects.

Data Availability

No personal data was collected from the interviewees and therefore no such data is kept or shared in any form.

Consent

Prior informed consent was obtained from all participants before conducting interviews. This manuscript does not contain any individual person's data and further consent for publication is not required.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Abdul Waheed Khan, Arif-ullah Khan, and Syed Muhammad Mukarram Shah designed the study, performed field work, and researched various medicinal plants articles on scientific search engines. Aziz Ullah, Muhammad Faheem, and Muhammad Saleem analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors heartily thank all the contributors for taking part and sharing their valuable knowledge with us. They humbly acknowledge Mr. Muhammad Adnan, Mr. Mubashir Shahid, Mr. Rooh Ullah, Mr. Imran ul Haq, Mr. Najeeb Shah, Mr Rizwan Ullah, Ms Qurat ul Ain, Ms. Fizza Bukhari, and Ms Rubia Anwar for their continuous support in the dissemination and collection of the questionnaires in various districts of Pakistan.

References

[1] G. Hussain, H. Anwar, A. Shahzad et al., "Neurological disorder burden in faisalabad, punjab-pakistan: data from the major tertiary carecenters of the city," *Pakistan Journal of Neurological Sciences (PJNS)*, vol. 12, no. 3, pp. 3–10, 2017.

[2] S. Guo, "Using zebrafish to assess the impact of drugs on neural development and function," *Expert Opinion on Drug Discovery*, vol. 4, no. 7, pp. 715–726, 2009.

[3] A. A. Gadit, "State of mental health in Pakistan.," *Journal of the Pakistan Medical Association*, vol. 51, no. 7, pp. 238-239, 2001.

[4] S. Awan, S. Shafqat, A. K. Kamal et al., "Pattern of neurological diseases in adult outpatient neurology clinics in tertiary care hospital," *BMC Research Notes*, vol. 10, no. 1, p. 545, 2017.

[5] W. H. Organization, *Neurological Disorders: Public Health Challenges*, World Health Organization, 2006.

[6] A. Sofowora, E. Ogunbodede, and A. Onayade, "The role and place of medicinal plants in the strategies for disease prevention," *The African Journal of Traditional, Complementary, and Alternative Medicines*, vol. 10, no. 5, pp. 210–229, 2013.

[7] N. H. Rakotoarivelo, F. Rakotoarivony, A. V. Ramarosandratana et al., "Medicinal plants used to treat the most frequent diseases encountered in Ambalabe rural community, Eastern Madagascar," *Journal of Ethnobiology and Ethnomedicine*, vol. 11, no. 1, p. 68, 2015.

[8] J. S. C. Júnior, A. B. Ferraz, T. O. Sousa et al., "Investigation of biological activities of dichloromethane and ethyl acetate fractions of platonia insignis mart. seed," *Basic & Clinical Pharmacology & Toxicology*, vol. 112, no. 1, pp. 34–41, 2013.

[9] M. Govindappa, "A review on role of plant (s) extracts and its phytochemicals for the management of diabetes," *Journal of Diabetes & Metabolism*, vol. 6, no. 7, Article ID 1000565, 2015.

[10] Y. Gupta, "Indian traditional medicine in neurological disorders," *Planta Medica*, vol. 78, no. 05, p. OP19, 2012.

[11] H. Kanwal and B. A. Sherazi, "Herbal medicine: trend of practice, perspective, and limitations in pakistan," *Asian Pacific Journal of Health Sciences*, vol. 4, no. 4, pp. 6–8, 2017.

[12] R. A. Qureshi, M. A. Ghufran, S. Gilani, Z. Yousaf, G. A. Miana, and A. Batool, "Indigenous medicinal plants used by local women in southern himalayan regions of pakistan," *Pakistan Journal of Botany*, vol. 41, no. 1, pp. 19–25, 2009.

[13] A. Mahmood, A. Mahmood, H. Shaheen, R. A. Qureshi, Y. Sangi, and S. A. Gilani, "Ethno medicinal survey of plants from district bhimber azad jammu and kashmir, pakistan," *Journal of Medicinal Plants Research*, vol. 5, no. 11, pp. 2348–2360, 2011.

[14] H. Bhatia, Y. P. Sharma, R. K. Manhas, and K. Kumar, "Ethnomedicinal plants used by the villagers of district Udhampur, J&K, India," *Journal of Ethnopharmacology*, vol. 151, no. 2, pp. 1005–1018, 2014.

[15] B. Pravin, D. Tushar, P. Vijay, and K. Kishanchnad, "Review on citrullus colocynthis," *International Journal of Research in Pharmaceutical Sciences*, vol. 3, no. 1, pp. 46–53, 2013.

[16] S. Mehrzadi, A. Shojaii, S. A. Pur, and M. Motevalian, "Anticonvulsant activity of hydroalcoholic extract of citrullus colocynthis fruit: involvement of benzodiazepine and opioid receptors," *Evidence-Based Complementary and Alternative Medicine*, vol. 21, no. 4, pp. NP31–NP35, 2016.

[17] M. Ali and M. Shuaib, "Characterization of the chemical constituents of datura mete/linn," *Indian Journal of Pharmaceutical Sciences*, vol. 58, no. 6, pp. 243–245, 1996.

[18] S. J. Murch, A. R. Alan, J. Cao, and P. K. Saxena, "Melatonin and serotonin in flowers and fruits of datura metel L," *Journal of Pineal Research*, vol. 47, no. 3, pp. 277–283, 2009.

[19] S. Babalola, M. Suleiman, A. Hassan, and D. Adawa, "Evaluation of datura metel L seed extract as a sedative/hypnotic: a priliminary study," *Journal of Veterinary Advances*, vol. 5, no. 4, p. 857, 2015.

[20] A. Tijani, U. Eyineyi, J. Ibrahim, and S. Okhale, "Neurotoxicological impacts of datura metel linn. (family: solanaceae) leaves extract in mice," *The Journal of Neurobehavioral Sciences*, vol. 2, no. 3, pp. 97–101, 2015.

[21] C. Bhawana, S. Sushil, and S. Amit, "Evaluation of antiepileptic activity of datura metel leaf extract in experimental animal," *International Journal of Research in Pharmacy and Chemistry*, vol. 6, no. 3, 2016.

[22] A. Nahrstedt and V. Butterweck, "Biologically active and other chemical constituents of the herb of hypericum perforation L," *Pharmacopsychiatry*, vol. 30, no. 2, pp. 129–134, 1997.

[23] H. Hosseinzadeh, G.-R. Karimi, and M. Rakhshanizadeh, "Anticonvulsant effect of hypericum perforatum: role of nitric oxide," *Journal of Ethnopharmacology*, vol. 98, no. 1-2, pp. 207-208, 2005.

[24] A. Rezaie, K. R. Dorostkar, M. Pashazadeh, and S. M. Nejad, "Study of sedative and anxiolytic effects of herbal extract hypericum perforatum in comparison with diazepam in rats," *International Journal of Infectious Diseases*, vol. 12, p. e171, 2008.

[25] J. Tian, F. Zhang, J. Cheng, S. Guo, P. Liu, and H. Wang, "Antidepressant-like activity of adhyperforin, a novel constituent of hypericum perforatum L," *Scientific Reports*, vol. 4, p. 5632, 2014.

[26] D. D. Vecchia, M. G. Schamne, M. M. Ferro et al., "Effects of hypericum perforatum on turning behavior in an animal model of parkinson's disease," *Brazilian Journal of Pharmaceutical Sciences*, vol. 51, no. 1, pp. 111–115, 2015.

[27] A. I. Oliveira, C. Pinho, B. Sarmento, and A. C. P. Dias, "Neuroprotective activity of hypericum perforatum and its major components," *Frontiers in Plant Science*, vol. 7, p. 1004, 2016.

[28] K. Zerrouki, N. Djebli, E. E. Ozkan, N. Ozsoy, O. Gul, and A. Mat, "Hypericum perforatum improve memory and learning in alzheimer's model: (experimental study in mice)," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 8, no. 8, pp. 49–57, 2016.

[29] P. Kunhachan, C. Banchonglikitkul, T. Kajsongkram, A. Khayungarnnawee, and W. Leelamanit, "Chemical composition, toxicity and vasodilatation effect of the flowers extract of jasminum sambac (L.) ait. "g. duke of tuscany"," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 471312, 7 pages, 2012.

[30] R. K. Gupta and P. S. Reddy, "Antinociceptive and anticonvulsant activities of hydroalcoholic extract of Jasminum grandiflorum (jasmine) leaves in experimental animals," *Pharmacognosy Research*, vol. 5, no. 4, pp. 286–290, 2013.

[31] S. Umukoro, A. Adebesin, G. Agu, O. Omorogbe, and S. B. Asehinde, "Antidepressant-like activity of methyl jasmonate involves modulation of monoaminergic pathways in mice," *Advances in Medical Sciences*, vol. 63, no. 1, pp. 36–42, 2018.

[32] P. Milind and M. Suman, "Eat tomato a day to keep depression at bay," *Asian Journal of Biological Sciences*, vol. 4, no. 2, pp. 258–262, 2009.

[33] S. K. Bhattacharya and S. Ghosal, "Anxiolytic activity of a standardized extract of *Bacopa monniera*: an experimental study," *Phytomedicine*, vol. 5, no. 2, pp. 77–82, 1998.

[34] D. Kaushik, A. Tripathi, R. Tripathi, M. Ganachari, and S. A. Khan, "Anticonvulsant activity of bacopa monniera in rodents," *Brazilian Journal of Pharmaceutical Sciences*, vol. 45, no. 4, pp. 643–649, 2009.

[35] P. Jadiya, A. Khan, S. R. Sammi, S. Kaur, S. S. Mir, and A. Nazir, "Anti-parkinsonian effects of bacopa monnieri: insights from transgenic and pharmacological caenorhabditis elegans models of parkinson's disease," *Biochemical and Biophysical Research Communications*, vol. 413, no. 4, pp. 605–610, 2011.

[36] M. A. Mannan, A. B. Abir, and M. R. Rahman, "Antidepressant-like effects of methanolic extract of bacopa monniera in mice," *BMC Complementary and Alternative Medicine*, vol. 15, no. 1, p. 337, 2015.

[37] E. A. Carlini and J. M. Cunha, "Hypnotic and antiepileptic effects of cannabidiol," *The Journal of Clinical Pharmacology*, vol. 21, 1, no. 8-9, pp. 417S–427S, 1981.

[38] J. T. Pickens, "Sedative activity of cannabis in relation to its Δ'-trans-tetrahydrocannabinol and cannabidiol content," *British Journal of Pharmacology*, vol. 72, no. 4, pp. 649–656, 1981.

[39] L. Volicer, M. Stelly, J. Morris, J. McLaughlin, and B. J. Volicer, "Effects of dronabinol on anorexia and disturbed behavior in patients with alzheimer's disease," *International Journal of Geriatric Psychiatry*, vol. 12, no. 9, pp. 913–919, 1997.

[40] A. R. M. de Schier, N. P. O. de Ribeiro, D. S. Coutinho et al., "Antidepressant-like and anxiolytic-like effects of cannabidiol: a chemical compound of Cannabis sativa," *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)*, vol. 13, no. 6, pp. 953–960, 2014.

[41] S. Valdeolivas, C. Navarrete, I. Cantarero, M. L. Bellido, E. Muñoz, and O. Sagredo, "Neuroprotective properties of cannabigerol in huntington's disease: studies in R6/2 mice and 3-nitropropionate-lesioned mice," *Neurotherapeutics*, vol. 12, no. 1, pp. 185–199, 2015.

[42] T. Jawaid, S. A. Imam, and M. Kamal, "Antidepressant activity of methanolic extract of Verbena Officinalis Linn. plant in mice," *Asian Journal of Pharmaceutical and Clinical Research*, vol. 8, no. 4, pp. 308–310, 2015.

[43] A. W. Khan, A.-U. Khan, and T. Ahmed, "Anticonvulsant, anxiolytic, and sedative activities of Verbena officinalis," *Frontiers in Pharmacology*, vol. 7, 2016.

[44] Y. Makino, S. Kondo, Y. Nishimura, Y. Tsukamoto, Z.-L. Huang, and Y. Urade, "Hastatoside and verbenalin are sleep-promoting components in verbena officinalis," *Sleep and Biological Rhythms*, vol. 7, no. 3, pp. 211–217, 2009.

[45] S. K. Bhattacharya, A. Bhattacharya, K. Sairam, and S. Ghosal, "Anxiolytic-antidepressant activity of Withania somnifera glycowithanolides: an experimental study," *Phytomedicine*, vol. 7, no. 6, pp. 463–469, 2000.

[46] M. Ahmad, S. Saleem, A. S. Ahmad et al., "Neuroprotective effects of Withania somnifera on 6-hydroxydopamine induced Parkinsonism in rats," *Human & Experimental Toxicology*, vol. 24, no. 3, pp. 137–147, 2005.

[47] S. K. Raju, P. L. Basavanna, H. N. Nagesh, and A. D. Shanbhag, "A study on the anticonvulsant activity of Withania somnifera (Dunal) in albino rats," *National Journal of Physiology, Pharmacy and Pharmacology*, vol. 7, no. 1, pp. 17–21, 2017.

[48] S. K. Bhattacharya, R. K. Goel, R. Kaur, and S. Ghosal, "Anti-stress activity of sitoindosides VII and VIII, new acylsterylglucosides from withania somnifera," *Phytotherapy Research*, vol. 1, no. 1, pp. 32–37, 1987.

[49] R. C. Burch, S. Loder, E. Loder, and T. A. Smitherman, "The prevalence and burden of migraine and severe headache in the united states: updated statistics from government health surveillance studies," *Headache: The Journal of Head and Face Pain*, vol. 55, no. 1, pp. 21–34, 2015.

[50] D. Kadojić, M. Dikanović, M. Bitunjac, V. Vuletić, L. Čengić, and B. Bijelić, "Epidemiology of Stroke," *Periodicum Biologorum*, vol. 114, no. 3, pp. 253–257, 2012.

[51] G. Hussain, A. Rasul, H. Anwar et al., "Epidemiological data of neurological disorders in pakistan and neighboring countries: a review," *Pakistan Journal of Neurological Sciences (PJNS)*, vol. 12, no. 4, pp. 52–70, 2017.

[52] W. H. Organization, *Depression and Other Common Mental Disorders: Global Health Estimates*, 2017.

[53] D. M. Radhakrishnan and V. Goyal, "Parkinson's disease: a review," *Neurology India*, vol. 66, no. 7, p. 26, 2018.

[54] C. P. Ferri, M. Prince, C. Brayne et al., "Global prevalence of dementia: a Delphi consensus study," *The Lancet*, vol. 366, no. 9503, pp. 2112–2117, 2005.

[55] K. P. Peng and S. J. Wang, "Epidemiology of headache disorders in the a sia-p acific region," *Headache: The Journal of Head and Face Pain*, vol. 54, no. 4, pp. 610–618, 2014.

[56] N. Venketasubramanian, B. W. Yoon, J. Pandian, and J. C. Navarro, "Stroke epidemiology in south, east, and south-east asia: A review," *Journal of Stroke*, vol. 19, no. 3, pp. 286–294, 2017.

[57] S. Churl, "The worldwide prevalence of epilepsy: a systematic review and meta-analysis," *Epilepsy Currents*, vol. 13, p. 322, 2013.

[58] O. Remes, C. Brayne, R. van der Linde, and L. Lafortune, "A systematic review of reviews on the prevalence of anxiety disorders in adult populations," *Brain and Behavior*, vol. 6, no. 7, p. e00497, 2016.

[59] T. Pringsheim, N. Jette, A. Frolkis, and T. D. L. Steeves, "The prevalence of parkinson's disease: a systematic review and meta-analysis," *Movement Disorders*, vol. 29, no. 13, pp. 1583–1590, 2014.

[60] Y. W. Woldeamanuel, A. P. Andreou, and R. P. Cowan, "Prevalence of migraine headache and its weight on neurological burden in Africa: A 43-year systematic review and meta-analysis of community-based studies," *Journal of the Neurological Sciences*, vol. 342, no. 1-2, pp. 1–15, 2014.

[61] D. Adeloye, "An estimate of the incidence and prevalence of stroke in Africa: A systematic review and meta-analysis," *PLoS ONE*, vol. 9, no. 6, p. e100724, 2014.

[62] Diseases, O.M.d.l.S.P.f.N et al., *Atlas: Epilepsy Care in The World*, World Health Organization, 2005.

[63] D. G. Massi, "Epidemiology of Parkinsons disease in Africa: Challenges and opportunities. Parkinsonism Related Disorders," in *Epidemiology of Parkinson's disease in Africa: Challenges and opportunities. Parkinsonism Related Disorders*, pp. 46–e10, p. e10, 46, 2018.

[64] R. B. Lipton and M. E. Bigal, "Migraine: epidemiology, impact, and risk factors for progression," *Headache: The Journal of Head and Face Pain*, vol. 45, pp. S3–S13, 2005.

[65] D. Mozaffarian, E. J. Benjamin, A. S. Go et al., "Heart disease and stroke statistics—2016 update: a report from the american heart association," *Circulation*, p. CIR. 0000000000000350, 2015.

[66] W. H. Theodore, S. S. Spencer, S. Wiebe et al., "Epilepsy in north america: a report prepared under the auspices of the global campaign against epilepsy, the international bureau for epilepsy, the international league against epilepsy, and the world health organization," *Epilepsia*, vol. 47, no. 10, pp. 1700–1722, 2006.

[67] G. Y. Lim, W. W. Tam, Y. Lu, C. S. Ho, M. W. Zhang, and R. C. Ho, "Prevalence of depression in the community from 30 countries between 1994 and 2014," *Scientific Reports*, vol. 8, no. 1, p. 2861, 2018.

[68] G. Saposnik and O. H. D. Brutto, "Stroke in South America: a systematic review of incidence, prevalence, and stroke subtypes," *Stroke*, vol. 34, no. 9, pp. 2103–2107, 2003.

[69] A. J. Baxter, K. M. Scott, T. Vos, and H. A. Whiteford, "Global prevalence of anxiety disorders: a systematic review and meta-regression," *Psychological Medicine*, vol. 43, no. 5, pp. 897–910, 2013.

[70] L. J. Stovner and C. Andree, "Prevalence of headache in europe: a review for the eurolight project," *The Journal of Headache and Pain*, vol. 11, no. 4, pp. 289–299, 2010.

[71] N. P. Şensöz, Ü. T. Börü, C. Bölük et al., "Stroke epidemiology in Karabük city Turkey: Community based study," *eNeurologicalSci*, vol. 10, pp. 12–15, 2018.

[72] M. d. de Rijk, C. Tzourio, M. M. Breteler et al., "Prevalence of parkinsonism and parkinson's disease in europe: the europarkinson collaborative study. european community concerted action on the epidemiology of parkinson's disease," *Journal of Neurology, Neurosurgery & Psychiatry*, vol. 62, no. 1, pp. 10–15, 1997.

[73] E. Shao, J. Hughes, and R. Eley, "The presenting and prescribing patterns of migraine in an australian emergency department: a descriptive exploratory study," *World Journal of Emergency Medicine*, vol. 8, no. 3, p. 170, 2017.

[74] W. A. D. o. Health, *Epidemiology Profile of Neurological Conditions in Western Australia*, Health Strategy and Networks Branch, Australia, 2015.

[75] M. Bellon, R. J. Panelli, and F. Rillotta, "Epilepsy-related deaths: An Australian survey of the experiences and needs of people bereaved by epilepsy," *Seizure*, vol. 29, pp. 162–168, 2015.

[76] P. Mehta, A. Kifley, J. J. Wang, E. Rochtchina, P. Mitchell, and C. M. Sue, "Population prevalence and incidence of Parkinson's disease in an Australian community," *Internal Medicine Journal*, vol. 37, no. 12, pp. 812–814, 2007.

[77] M. Prince, R. Bryce, E. Albanese, A. Wimo, W. Ribeiro, and C. P. Ferri, "The global prevalence of dementia: a systematic review and metaanalysis," *Alzheimer's & Dementia*, vol. 9, no. 1, Article ID e2, pp. 63–75, 2013.

[78] B. Ray, N. Paul, A. Hazra et al., "Prevalence, burden, and risk factors of migraine: a community-based study from eastern india," *Neurology India*, vol. 65, no. 6, pp. 1280–1288, 2017.

[79] M. I. khan, J. I. Khan, S. I. Ahmed, and U. U. Haq, "The epidemiology of stroke in a developing country (Pakistan)," *Journal of Neurology & Stroke*, vol. 8, no. 1, 2018.

[80] H. Ebrahimi, M. Shafa, and S. H. Asl, "Prevalence of active epilepsy in kerman, iran: a house based survey," *Acta Neurologica Taiwanica*, vol. 21, no. 3, pp. 115–124, 2012.

[81] A. K. Verma, J. Raj, V. Sharma, T. B. Singh, S. Srivastava, and R. Srivastava, "Epidemiology and associated risk factors of parkinson's disease among the north indian population," *Clinical Epidemiology and Global Health*, vol. 5, no. 1, pp. 8–13, 2017.

[82] P. S. Mathuranath, A. George, N. Ranjith et al., "Incidence of alzheimer's disease in india: a 10 yearsfollow-up study," *Neurology India*, vol. 60, no. 6, p. 625, 2012.

[83] S.-M. Fereshtehnejad, M. Shafieesabet, A. Rahmani, A. Delbari, and J. Lökk, "Medium-to-high prevalence of screening-detected parkinsonism in the urban area of Tehran, Iran: Data from a community-based door-to-door study," *Neuropsychiatric Disease and Treatment*, vol. 11, pp. 321–332, 2015.

[84] S. Gholamzadeh, B. Heshmati, A. Manni, P. Petramfar, and Z. Baghery, "The prevalence of Alzheimer's disease; its risk and protective factors among the elderly population in Iran," *Shiraz E Medical Journal*, vol. 18, no. 9, 2017.

[85] J. Ni, F. Han, J. Yuan et al., "The discrepancy of neurological diseases between china and western countries in recent two decades," *Chinese Medical Journal*, vol. 131, no. 8, pp. 886–891, 2018.

[86] L. J. Stovner, E. Nichols, T. J. Steiner et al., "Global, regional, and national burden of migraine and tension-type headache, 1990–2016: a systematic analysis for the global burden of disease study 2016," *The Lancet Neurology*, vol. 17, no. 11, pp. 954–976, 2018.

[87] V. L. Feigin, M. H. Forouzanfar, R. Krishnamurthi et al., "Global and regional burden of stroke during 1990–2010: findings from the global burden of disease study 2010," *The Lancet*, vol. 383, no. 9913, pp. 245–255, 2014.

[88] P. Ventevogel, W. van de Put, H. Faiz, B. van Mierlo, M. Siddiqi, and I. H. Komproe, "Improving access to mental health care and psychosocial support within a fragile context: a case study from afghanistan," *PLoS Medicine*, vol. 9, no. 5, p. e1001225, 2012.

[89] S.-M. Shin, H. J. Kim, L. Liw, and S. Kim, "Depression and PTSD in pashtun women in kandahar, afghanistan," *Asian Nursing Research*, vol. 3, no. 2, pp. 90–98, 2009.

[90] E. R. Dorsey, A. Elbaz, E. Nichols et al., "Global, regional, and national burden of parkinson's disease, 1990–2016: a systematic analysis for the global burden of disease study 2016," *The Lancet Neurology*, vol. 17, no. 11, pp. 939–953, 2018.

[91] G. Miller, "A battle no soldier wants to fight," *American Association for the Advancement of Science*, vol. 333, no. 6042, pp. 517-518, 2011.

[92] D. Santarsieri and T. L. Schwartz, "Antidepressant efficacy and side-effect burden: a quick guide for clinicians," *Drugs in Context*, vol. 4, 2015.

[93] J. M. Ferguson, "SSRI antidepressant medications: adverse effects and tolerability," *Primary Care Companion to the Journal of Clinical Psychiatry*, vol. 3, no. 1, pp. 22–27, 2001.

[94] A. F. Carvalho, M. S. Sharma, A. R. Brunoni, E. Vieta, and G. A. Fava, "The safety, tolerability and risks associated with the use of newer generation antidepressant drugs: a critical review of the literature," *Psychotherapy and Psychosomatics*, vol. 85, no. 5, pp. 270–288, 2016.

[95] C. E. Griffin III, A. M. Kaye, F. R. Bueno, and A. D. Kaye, "Benzodiazepine pharmacology and central nervous system-mediated effects," *Ochsner Journal*, vol. 13, no. 2, pp. 214–223, 2013.

[96] F. Batool, "Buspirone and anxiety disorders: a review with pharmacological and clinical perspectives," *The Internet Journal of Pharmacology*, vol. 5, no. 2, 2007.

[97] H. M. Kwon, J. W. Baek, S. P. Lee, and J. I. Cho, "A fatal adverse effect of barbiturate coma therapy: dyskalemia," *Korean Journal of Neurotrauma*, vol. 12, no. 2, pp. 156–158, 2016.

[98] F. Stella, M. Radanovic, P. R. Canineu, V. J. R. de Paula, and O. V. Forlenza, "Anti-dementia medications: current prescriptions in clinical practice and new agents in progress," *Therapeutic Advances in Drug Safety*, vol. 6, no. 4, pp. 151–165, 2015.

[99] Y.-J. Huang, C.-H. Lin, H.-Y. Lane, and G. E. Tsai, "NMDA neurotransmission dysfunction in behavioral and psychological symptoms of Alzheimer's disease," *Current Neuropharmacology*, vol. 10, no. 3, pp. 272–285, 2012.

[100] V. S. C. Fung, M. A. Hely, G. De Moore, and J. G. L. Morris, "Drugs for parkinson's disease," *Australian Prescriber*, vol. 24, no. 4, pp. 92–95, 2001.

[101] M. J. Brodie, "Sodium channel blockers in the treatment of epilepsy," *CNS Drugs*, vol. 31, no. 7, pp. 527–534, 2017.

[102] B. J. Kopecky, R. Liang, and J. Bao, "T-type calcium channel blockers as neuroprotective agents," *Pflügers Archiv - European Journal of Physiology*, vol. 466, no. 4, pp. 757–765, 2014.

[103] J. T. Lerner, N. Salamon, and R. Sankar, "Clinical profile of vigabatrin as monotherapy for treatment of infantile spasms," *Neuropsychiatric Disease and Treatment*, vol. 6, p. 731, 2010.

[104] R. Qureshi, M. Ilyas, G. Rahim, W. Ahmad, H. Shaheen, and K. Ullah, "Ethnobotanical study of bhera, district sargodha, pakistan," *Archives Des Sciences*, vol. 65, no. 11, pp. 690–707, 2012.

[105] T. A. Alamgeer, M. Rashid, M. N. H. Malik, and M. N. Mushtaq, "Ethnomedicinal survey of plants of valley alladand dehri, tehsil batkhela, district malakand, pakistan," *International Journal of Basic Medical Sciences and Pharmacy (IJBMSP)*, vol. 3, no. 1, 2013.

[106] A. Shah, S. K. Marwat, and F. Gohar, "Ethnobotanical study of medicinal plants of semi-tribal area of Makerwal & Gulla Khel (lying between Khyber Pakhtunkhwa and Punjab Provinces), Pakistan," *American Journal of Plant Sciences*, vol. 4, no. 01, p. 98, 2013.

[107] I. Iqbal and M. Hamayun, "Studies on the traditional uses of plants of malam jabba valley, district swat, pakistan," *Ethnobotanical Leaflets*, vol. 2004, no. 1, p. 15, 2004.

[108] A. Hazrat, M. Nisar, J. Shah, and S. Ahmad, "Ethnobotanical study of some elite plants belonging to dir, kohistan valley, khyber pukhtunkhwa, pakistan," *Pakistan Journal of Botany*, vol. 43, no. 2, pp. 787–795, 2011.

[109] M. Haider and L. Zhong, "Ethno-medicinal uses of plants from district bahawalpur, pakistan," *Current Research Journal of Biological Sciences*, vol. 6, pp. 183–190, 2014.

[110] A. A. Shah, M. Ramzan, and R. Saba, "Ethnoecological studies of herbs and shrubs of miani sahib graveyard, lahore city, punjab, pakistan," *Journal of Bioresource Management*, vol. 3, no. 2, p. 5, 2016.

[111] A. B. Gulshan, A. A. Dasti, S. Hussain, M. I. Atta, and M. Amin-ud-Din, "Indigenous uses of medicinal plants in rural areas of dera ghazi khan, punjab, pakistan," *Journal of Agricultural & Biological Science*, vol. 7, no. 9, pp. 750–762, 2012.

[112] S. S. Ahmad, "Medicinal wild plants from lahore-islamabad motorway (M-2)," *Pakistan Journal of Botany*, vol. 39, no. 2, pp. 355–375, 2007.

[113] M. Ajaib, Z. Khan, and A. Zikrea, "Ethnobotanical survey of some important herbaceous plants of district kotli, azad jammu & kashmir," *Biologia (Pakistan)*, vol. 60, no. 1, pp. 11–22, 2014.

[114] S. U. Khan, R. U. Khan, I. Ullah, S. Mehmood, A. Muhammad, and M. Ullah, "Morpho-anatomical study of selected plants of district bannu, khyber pakhtunkhwa, pakistan," *Pakistan Journal of Weed Science Research*, vol. 19, no. 4, pp. 447–464, 2013.

[115] A. Noman, I. Hussain, Q. Ali, M. A. Ashraf, and M. Z. Haider, "Ethnobotanical studies of potential wild medicinal plants of ormara, gawadar, pakistan," *Emirates Journal of Food and Agriculture*, vol. 25, no. 10, pp. 751–759, 2013.

[116] M. R. Awan, Z. Jamal, and A. Khan, "Ethno-botanical studies of economically important plants from mountainous region of Gilgit-Baltistan," *Science, Technology and Development*, vol. 32, pp. 308–318, 2013.

[117] S. Z. Husain, R. N. Malik, M. Javaid, and S. Bibi, "Ethonobotanical properties and uses of medicinal plants of morgah biodiversity park, rawalpindi," *Pakistan Journal of Botany*, vol. 40, no. 5, pp. 1897–1911, 2008.

[118] A. Shah, S. Rahim, K. H. Bhatti et al., "Ethnobotanical study and conservation status of trees in the district sargodha, punjab, pakistan," *Phyton, International Journal of Experimental Botany*, vol. 84, no. 1, pp. 34–44, 2016.

[119] M. Umair, M. Altaf, and A. M. Abbasi, "An ethnobotanical survey of indigenous medicinal plants in hafizabad district, Punjab-Pakistan," *PLoS ONE*, vol. 12, no. 6, Article ID e0177912, 2017.

[120] H. Iqbal, Z. Sher, and Z. U. Khan, "Medicinal plants from salt range pind dadan khan, district jhelum, punjab, pakistan," *Journal of Medicinal Plants Research*, vol. 5, no. 11, pp. 2157–2168, 2011.

[121] W. Murad, A. Ahmad, G. Ishaq, M. S. Khan, M. A. H. Khan, and I. Ullah, "Ethnobotanical studies on plant resources of hazar nao forest, district malakand, pakistan," *Pakistan Journal of Weed Science Research*, vol. 18, no. 4, 2012.

[122] Z. I. Awan, H. Rehman, A. A. Awan, F. A. Minhas, and M. N. Khan, "Ethnobotanical importance of some highly medicinal plants of district muzaffarabad, pakistan with special reference to the species of the genus viburnum," *IOSR Journal of Pharmacy and Biological Sciences*, vol. 6, no. 2, pp. 53–66, 2013.

[123] A. M. Hussain, M. S. A. Abbasi, N. Hussain, and S. A. Majid, "A survey of important indigenous medicinal plants of district bhimber azad jammu & kashmir, pakistan," *International Journal of Advanced Research*, vol. 1, pp. 635–644, 2013.

[124] M. F. Nisar, F. Jaleel, and S. M. Haider, "Exploration of ethno-medicinal plants and their ritual uses in bahawalnagar, pakistan," *Middle-East Journal of Scientific Research*, vol. 21, no. 9, pp. 1466–1471, 2014.

[125] A. T. Khalil, Z. K. Shinwari, M. Qaiser, and K. B. Marwat, "Phyto-therapeutic claims about euphorbeaceous plants belonging to pakistan; an ethnomedicinal review," *Pakistan Journal of Botany*, vol. 46, no. 3, pp. 1137–1144, 2014.

[126] S. M. Wazir, A. A. Dasti, and J. Shah, "Common medicinal plants of chapursan valley, gojal ii, gilgit-pakistan," *Journal of Research (Science) Bahauddin Zakariya University, Multan, Pakistan*, vol. 15, pp. 41–43, 2004.

[127] A. M. M. Kanwal, S. Shaukat, R. Javed, and R. Ilyas, "Exploration of ethnomedicinal values of imperative plants of district gujrat, pakistan," *Middle-East Journal of Scientific Research*, vol. 7, pp. 397–400, 2011.

[128] A. M. Sarangzai, A. Ahmed, and S. K. Laghari, "Traditional uses of some useful medicinal plants of ziarat district balochistan, pakistan," *FUUAST Journal of Biology*, vol. 3, no. 1, p. 101, 2013.

[129] A. Ghani, Z. Ali, and S. Perveen, "Folk recipes and ethno botanical survey of medicinal plants mianwali district (pakistan)," *International Journal of Current Pharmaceutical Research*, vol. 4, no. 2, pp. 61–63, 2012.

[130] Z. Sher, Z. U. D. Khan, and F. Hussain, "Ethnobotanical studies of some plants of chagharzai valley, district buner, pakistan," *Pakistan Journal of Botany*, vol. 43, no. 3, pp. 1445–1452, 2011.

[131] M. Shuaib and I. Khan, "Study of Medicinal Plants of Lower Dir, Timergara, Tehsil Balambat, Khyber Paktunkhaw-Pakistan," *American-Eurasian Journal of Agricultural & Environmental Sciences*, vol. 2015., 15, p. 2088, 2088.

[132] I. Ahmad, M. Ibrar, Barkatullah, and N. Ali, "Ethnobotanical study of tehsil kabal, swat district, KPK, pakistan," *Journal of Botany*, vol. 2011, Article ID 368572, 9 pages, 2011.

[133] M. Ilyas, R. Qureshi, Z. K. Shinwari, M. Arshad, S. N. Mirza, and Zia-Ul-Haq, "Some ethnoecological aspects of the plants of qalagai hills, kabal valley, swat, pakistan," *International Journal of Agriculture and Biology*, vol. 15, no. 5, pp. 801–810, 2013.

[134] N. Ahmad, S. Anwar, H. Fazal, and B. H. Abbasi, "Medicinal plants used in indigenous herapy by people of madyan valley in district swat, pakistan," *International Journal of Medicinal and Aromatic Plants*, vol. 3, no. 1, pp. 47–54, 2013.

[135] F. Haq, H. Ahmad, and M. Alam, "Traditional uses of medicinal plants of nandiar khuwarr catchment (district battagram), pakistan," *Journal of Medicinal Plants Research*, vol. 5, no. 1, pp. 39–48, 2011.

[136] R. A. Qureshi and M. A. Ghufran, "Indigenous knowledge of selected medicinal wild plants of district attock, punjab, pakistan," *Pakistan Journal of Botany*, vol. 39, pp. 2291–2299., 2007.

[137] N. B. Chauhan and J. Sandoval, "Amelioration of early cognitive deficits by aged garlic extract in alzheimer's transgenic mice," *Phytotherapy Research*, vol. 21, no. 7, pp. 629–640, 2007.

[138] D. Dhingra and V. Kumar, "Evidences for the involvement of monoaminergic and GABAergic systems in antidepressant-like activity of garlic extract in mice," *Indian Journal of Pharmacology*, vol. 40, no. 4, pp. 175–179, 2008.

[139] U. Advani, A. Ansari, and E. Menghani, "Anticonvulsant potentials of sesamum indicum and allium sativum oil alone and in combination in animal models," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 3, 4, pp. 154–158, 2011.

[140] N. Martins, S. Petropoulos, and I. C. F. R. Ferreira, "Chemical composition and bioactive compounds of garlic (Allium sativum L.) as affected by pre- and post-harvest conditions: A review," *Food Chemistry*, vol. 211, pp. 41–50, 2016.

[141] P. Jain, H. P. Sharma, F. Basri, K. Priya, and P. Singh, "Phytochemical analysis of bacopa monnieri (L.) wettst. and their antifungal activities," *Indian Journal of Traditional Knowledge*, vol. 16, no. 2, pp. 310–318, 2017.

[142] E. B. Russo, "Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects," *British Journal of Pharmacology*, vol. 163, no. 7, pp. 1344–1364, 2011.

[143] B. S. Audu, P. C. Ofojekwu, A. Ujah, and M. N. O. Ajima, "Phytochemical, proximate composition, amino acid profile and characterization of marijuana (cannabis sativa L.)," *The Journal of Phytopharmacology*, vol. 3, no. 1, pp. 35–43, 2014.

[144] H. M. Reza, H. Mohammad, E. Golnaz, and S. Gholamreza, "Effect of methanolic extract of hyoscymus niger L. on the seizure induced by picritoxin in mice," *Pakistan Journal of Pharmaceutical Sciences*, vol. 22, no. 3, pp. 308–312, 2009.

[145] J. Li, J. Shi, X.-W. Yu et al., "Chemical and pharmacological researches on Hyoscyamus niger," *Chinese Herbal Medicine*, vol. 3, pp. 117–126, 2011.

[146] T. Sengupta, J. Vinayagam, N. Nagashayana, B. Gowda, P. Jaisankar, and K. P. Mohanakumar, "Antiparkinsonian effects of aqueous methanolic extract of *Hyoscyamus niger* seeds result from its monoamine oxidase inhibitory and hydroxyl radical scavenging potency," *Neurochemical Research*, vol. 36, no. 1, pp. 177–186, 2011.

[147] J. Talairach and P. Thournoux, *Co-Planar Stereotaxic Atlas of the Human Brain*, Thieme Medical Publishers, Stuttgart, Germany, 1988.

[148] K. Aparna, A. Joshi, and M. Vyas, "Adverse reaction of Parasika Yavani (Hyoscyamus niger Linn): Two case study reports," *AYU (An International Quarterly Journal of Research in Ayurveda)*, vol. 36, no. 2, p. 174, 2015.

[149] R. M. Perez G., J. A. Perez L., L. M. Garcia D., and H. Sossa M., "Neuropharmacological activity of Solanum nigrum fruit," *Journal of Ethnopharmacology*, vol. 62, no. 1, pp. 43–48, 1998.

[150] N. N. Wannang, J. A. Anuka, H. O. Kwanashie, S. S. Gyang, and A. Auta, "Anti-seizure activity of the aqueous leaf extract of *Solanum nigrum* Linn. (solanaceae) in experimental animals," *African Health Sciences*, vol. 8, no. 2, pp. 74–79, 2008.

[151] Y. Zhao, F. Liu, and H.-X. Lou, "[Studies on the chemical constituents of Solanum nigrum].," *Zhong yao cai = Zhongyaocai = Journal of Chinese medicinal materials*, vol. 33, no. 4, pp. 555-556, 2010.

[152] M. A. B. Nyeem, "Solanum nigrum (Maku): A review of pharmacological activities and clinical effects," *IJAR*, vol. 3, no. 1, pp. 12–17, 2017.

[153] S. C. Kaul and R. Wadhwa, *Science of Ashwagandha: Preventive and Therapeutic Potentials*, Springer International Publishing, Cham, 2017.

[154] W.-H. Peng, M.-T. Hsieh, Y.-S. Lee, Y.-C. Lin, and J. Liao, "Anxiolytic effect of seed of *Ziziphus jujuba* in mouse models of anxiety," *Journal of Ethnopharmacology*, vol. 72, no. 3, pp. 435–441, 2000.

[155] J.-G. Jiang, X.-J. Huang, and J. Chen, "Comparison of the sedative and hypnotic effects of flavonoids, saponins, and polysaccharides extracted from Semen Ziziphus Jujube," *Natural Product Research (Formerly Natural Product Letters)*, vol. 21, no. 4, pp. 310–320, 2007.

[156] M. Pahuja, J. Mehla, K. H. Reeta, S. Joshi, and Y. K. Gupta, "Hydroalcoholic extract of Zizyphus jujuba ameliorates seizures, oxidative stress, and cognitive impairment in experimental models of epilepsy in rats," *Epilepsy & Behavior*, vol. 21, no. 4, pp. 356–363, 2011.

[157] S. Kumaravel and K. Alagusundaram, "Antimicrobial activity and Phytochemical analysis of selected Indian spices," *Journal of Pure and Applied Microbiology*, vol. 8, no. 5, pp. 4131–4136, 2014.

[158] R. Sabbaghzadeh and M. Asadbegi, "Effects of Methanolic extracts of Papaver Somniferum on Picrotoxin induced seizure in mice," *Advances in Environmental Biology*, vol. 8, no. 10, pp. 740–743, 2014.

[159] I. Rayment, H. M. Holden, M. Whittaker et al., "Structure of the actin-myosin complex and its implications for muscle contraction," *Science*, vol. 261, no. 5117, pp. 58–65, 1993.

[160] S. Wang, J. Zhang, Z. Zhang et al., "Identification of chemical constituents in the extract and rat serum from Ziziphus Jujuba mill. By HPLC-PDA-ESI-MSn," *Iranian Journal of Pharmaceutical Research*, vol. 13, no. 3, pp. 1055–1064, 2014.

[161] Z. Wang, D. Zhang, S. Hui, Y. Zhang, and S. Hu, "Effect of tribulus terrestris saponins on behavior and neuroendocrine in chronic mild stress depression rats," *Journal of Traditional Chinese Medicine*, vol. 33, no. 2, pp. 228–232, 2013.

[162] S. Ahmed, S. Lutfullah, I. Ahmed, R. Farooq, and J. Iqbal, "Anxiolytic activity of Tribulus terrestris on elevated plus maze," *Journal of Applied Pharmaceutical Science*, vol. 4, no. 2, pp. 126–128, 2014.

[163] S. Chhatre, T. Nesari, G. Somani, D. Kanchan, and S. Sathaye, "Phytopharmacological overview of *Tribulus terrestris*," *Pharmacognosy Reviews*, vol. 8, no. 15, pp. 45–51, 2014.

[164] O. A. Phillips, K. T. Mathew, and M. A. Oriowo, "Antihypertensive and vasodilator effects of methanolic and aqueous extracts of *Tribulus terrestris* in rats," *Journal of Ethnopharmacology*, vol. 104, no. 3, pp. 351–355, 2006.

[165] V. S. Kasture, C. T. Chopde, and V. K. Deshmukh, "Anticonvulsive activity of Albizzia lebbeck, Hibiscus rosa sinesis and Butea monosperma in experimental animals," *Journal of Ethnopharmacology*, vol. 71, no. 1-2, pp. 65–75, 2000.

[166] H. D. Une, V. P. Sarveiya, S. C. Pal, V. S. Kasture, and S. B. Kasture, "Nootropic and anxiolytic activity of saponins of Albizzia lebbeck leaves," *Pharmacology Biochemistry & Behavior*, vol. 69, no. 3-4, pp. 439–444, 2001.

[167] V. Padamanabhan, M. Ganapathy, and V. K. Evanjelene, "Preliminary phytochemical and anti-bacterial studies on flowers and pods of Albizia lebbeck (Benth)," *International Journal of Emerging Technology and Advanced Engineering*, vol. 3, no. 9, pp. 541–544, 2013.

[168] S. Desai, P. Tatke, and S. Gabhe, "Isolation of catechin from stem bark of Albizia lebbeck," *International Journal of Analytical, Pharmaceutical and Biomedical Sciences*, vol. 3, no. 2, pp. 31–35, 2014.

[169] R. Singh, S. De, and A. Belkheir, "Avena sativa (oat), a potential neutraceutical and therapeutic agent: an overview," *Critical Reviews in Food Science and Nutrition*, vol. 53, no. 2, pp. 126–144, 2013.

[170] K. Usha Rani, M. Ramaiah, K. Nagaphani, V. Preethi, and M. Srinadh, "Screening for antidepressant-like effect of methanolic seed extract of Avena sativa using animal models," *Pharmacognosy Journal*, vol. 6, no. 3, pp. 86–92, 2014.

[171] D. Kaur, A. Kamboj, and R. Shri, "Comparative evaluation of anxiolytic effects of various extracts of oats (Avena sativa), rice bran (Oryza sativa) and spinach (Spinacia oleracea) in experimental animals," *International Journal of Pharmaceutical Sciences and Research*, vol. 7, no. 10, p. 4110, 2016.

[172] M. Goyal, B. P. Nagori, and D. Sasmal, "Sedative and anticonvulsant effects of an alcoholic extract of Capparis decidua," *Journal of Natural Medicines*, vol. 63, no. 4, pp. 375–379, 2009.

[173] P. D. Verma, R. D. Dangar, K. N. Shah, D. M. Gandhi, and B. N. Suhagia, "Pharmacognostical potential of Capparis decidua Edgew," *Journal of Applied Pharmaceutical Science*, vol. 1, no. 10, pp. 6–11, 2011.

[174] L. M. Campêlo, S. G. Lima, C. M. Feitosa, and R. M. Freitas, "Evaluation of central nervous system effects of Citrus limon essential oil in mice," *Revista Brasileira de Farmacognosia*, vol. 21, no. 4, pp. 668–673, 2011.

[175] L. M. Lopes Campêlo, C. Gonçalves e Sá, A. A. C. de Almeida et al., "Sedative, anxiolytic and antidepressant activities of Citrus limon (Burn) essential oil in mice," *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, vol. 66, no. 8, pp. 623–627, 2011.

[176] A. Ben Hsouna, N. Ben Halima, S. Smaoui, and N. Hamdi, "Citrus lemon essential oil: Chemical composition, antioxidant

and antimicrobial activities with its preservative effect against Listeria monocytogenes inoculated in minced beef meat," *Lipids in Health and Disease*, vol. 16, no. 1, 2017.

[177] S. Najafi, N. Sanadgol, B. S. Nejad, M. A. Beiragi, and E. Sanadgol, "Phytochemical screening and antibacterial activity of Citrullus colocynthis (Linn.) schrad against Staphylococcus aureus," *Journal of Medicinal Plants Research*, vol. 4, no. 22, pp. 2321–2325, 2010.

[178] V. Kumar, S. K. Sharma, K. Nagarajan, and P. K. Dixit, "Effects of lycopene and sodium valproate on pentylenetetrazol-induced kindling in mice," *Iranian Journal of Medical Sciences*, vol. 41, no. 5, pp. 430–436, 2016.

[179] K. Gokul, "Oral supplements of aqueous extract of tomato seeds alleviate motor abnormality, oxidative impairments and neurotoxicity induced by rotenone in mice: relevance to Parkinson's disease," *Neurochemical Research*, vol. 39, no. 7, pp. 1382–1394, 2014.

[180] J. Bae, M. Han, H. Shin et al., "Lycopersicon esculentum extract enhances cognitive function and hippocampal neurogenesis in aged mice," *Nutrients*, vol. 8, no. 11, p. 679, 2016.

[181] J. S. Oliveira, L. A. Porto, and C. S. Estevam, "Phytochemical screening and anticonvulsant property of Ocimum basilicum leaf essential oil," *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, vol. 8, no. 3, 2009.

[182] S. Sarahroodi, S. Esmaeili, Z. Hemmati, P. Mikaili, and Y. Saberi, "The effects of green Ocimum basilicum hydroalcoholic extract on retention and retrieval of memory in mice," *Ancient Science of Life*, vol. 31, no. 4, p. 185, 2012.

[183] M. Rabbani, S. E. Sajjadi, and A. Vaezi, "Evaluation of anxiolytic and sedative effect of essential oil and hydroalcoholic extract of Ocimum basilicum L. and chemical composition of its essential oil," *Research in Pharmaceutical Sciences*, vol. 10, no. 6, pp. 535–543, 2015.

[184] S. S. Ali, M. G. Abd El Wahab, N. N. Ayuob, and M. Suliaman, "The antidepressant-like effect of Ocimum basilicum in an animal model of depression," *Biotechnic & Histochemistry*, vol. 92, no. 6, pp. 390–401, 2017.

[185] S. Adiga, P. Trivedi, V. Ravichandra, D. Deb, and F. Mehta, "Effect of Punica granatum peel extract on learning and memory in rats," *Asian Pacific Journal of Tropical Medicine*, vol. 3, no. 9, pp. 687–690, 2010.

[186] S. Das and P. Sarma, "A study on the anticonvulsant and antianxiety activity of ethanolic extract of punica granatum linn," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 6, no. 2, pp. 389–392, 2014.

[187] T. Yuan, H. Ma, W. Liu et al., "Pomegranate's neuroprotective effects against alzheimer's disease are mediated by urolithins, its ellagitannin-gut microbial derived metabolites," *ACS Chemical Neuroscience*, vol. 7, no. 1, pp. 26–33, 2015.

[188] R. Shastry, A. Sharma, V. Sayeli, and U. S. Dinkar, "Screening of antidepressant activity of punica granatum in mice," *Pharmacognosy Journal*, vol. 9, no. 1, pp. 27–29, 2017.

[189] J. Wise, "FDA approves its first cannabis based medicine," *British Medical Journal Publishing Group*, 2018.

[190] A. G. Atanasov, B. Waltenberger, E. M. Pferschy-Wenzig et al., "Discovery and resupply of pharmacologically active plant-derived natural products: a review," *Biotechnology Advances*, vol. 33, no. 8, pp. 1582–1614, 2015.

[191] N. Brondino, S. Re, and A. Boldrini, "Curcumin as a therapeutic agent in dementia: a mini systematic review of human studies," *The Scientific World Journal*, vol. 2014, Article ID 174282, 6 pages, 2014.

[192] N. Gurnani, D. Mehta, M. Gupta, and B. k. Mehta, "Natural Products: source of potential drugs," *African Journal of Basic & Applied Sciences*, vol. 6, pp. 171–186, 2014.

[193] S. Girdhar, A. Girdhar, S. K. Verma, V. Lather, and D. Pandita, "Plant derived alkaloids in major neurodegenerative diseases: from animal models to clinical trials," *Journal of Ayurvedic and Herbal Medicine*, vol. 1, no. 3, pp. 91–100, 2015.

[194] C. Sawda, C. Moussa, and R. S. Turner, "Resveratrol for alzheimer's disease," *Annals of the New York Academy of Sciences*, vol. 1403, no. 1, pp. 142–149, 2017.

[195] M. S. Rafii, B. G. Skotko, M. E. McDonough et al., "A randomized, double-blind, placebo-controlled, phase II study of oral ELND005 (scyllo -inositol) in young adults with down syndrome without dementia," *Journal of Alzheimer's Disease*, vol. 58, no. 2, pp. 401–411, 2017.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 1237301, 19 pages
https://doi.org/10.1155/2019/1237301

Research Article

Acmella oleracea (L) R. K. Jansen Reproductive Toxicity in Zebrafish: An *In Vivo* and *In Silico* Assessment

Gisele Custodio de Souza,[1,2] Arlindo César Matias Pereira,[2] Muller Duarte Viana,[2] Adriana Maciel Ferreira,[2] Ianna Dias Ribeiro da Silva,[3] Monaliza Maia Rebelo de Oliveira,[3] Wagner Luiz Ramos Barbosa,[3] Luciane Barros Silva,[4] Irlon Maciel Ferreira,[1,2] Cleydson Breno Rodrigues dos Santos,[1,4] and José Carlos Tavares Carvalho[1,2]

[1]*Programa de Pós-Graduação em Inovação Farmacêutica, Departamento de Ciências Biológicas e da Saúde, Colegiado de Farmácia, Universidade Federal do Amapá, Rodovia Juscelino Kubitschek, Km 02, 68902-280 Macapá, AP, Brazil*
[2]*Laboratório de Pesquisa em Fármacos, Departamento de Ciências Biológicas e da Saúde, Colegiado de Farmácia, Universidade Federal do Amapá, Macapá, AP, Brazil*
[3]*Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Pará, Belém, PA, Brazil*
[4]*Laboratório de Modelagem e Química Computacional, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Macapá, AP, Brazil*

Correspondence should be addressed to José Carlos Tavares Carvalho; farmacos@unifap.br

Received 2 January 2019; Revised 5 February 2019; Accepted 19 February 2019; Published 3 March 2019

Academic Editor: Wen-yi Kang

The plant species *Acmella oleracea* L. is used in the north of Brazil for the treatment of a range of illnesses, such as tuberculosis, flu, cough, and rheumatism and as an anti-inflammatory agent; besides, hydroethanolic formulations with this species are popularly used as a female aphrodisiac agent. However, currently, there are no studies performed evaluating its effect on embryonic development. Hence, this research aimed to evaluate the effects of the hydroethanolic extract of *A. oleracea* (EHFAo) on the reproductive performance (parental) and embryonic development (F1 generation) of zebrafish, at concentrations of 50, 100, and 200 μg/L. Histopathology of parental gonads after 21 days of exposure to EHFAo reveals few alterations in the ovaries and testes, not impairing the reproduction; an increase of eggs deposition was observed in animals treated with EHFAo at the highest concentrations. Nevertheless, concerning the embryonic development of F1, teratogenic effects were observed including tail deformation, cardiac and yolk edema, scoliosis, and growth retardation; these alterations were more prominent in the groups born from progenitors exposed to the highest concentrations (100 and 200 μg/L.); but only the occurrence of yolk and cardiac edema had a statistically significant difference when compared to the control group. The chromatographic analysis shows that spilanthol (affinin) was the primary compound found in the EHFAo. Hence, *in silico* assessment was performed to evaluate the pharmacokinetic and toxicological properties of this molecule and 37 metabolites derived from it. Overall, our data show that the treatment caused no detrimental changes in progenitors regarding their gonads or fertility but caused some potentially teratogenic activity in embryos, which may be due to the action of spilanthol's metabolites M3, M6, M7, M8, M16, M28, and M31.

1. Introduction

The plant species *Acmella oleracea* (L) R. K. Jansen is popularly known as "jambú", albeit other vernacular names are used, such as "agrião-do-Pará", "agrião-bravo", "botão-de-ouro", "jambuaçu", "abecedária", "agrião-do-Brasil", "mastruço", "agrião-do-norte" [1], "jaguaçú", "erva-maluca", and "jagurama" [2]. This species, from the family Asteraceae, is native from oriental Amazon and is highly cultivated in the states of Pará and Amapá due to its relevance in local cuisine and folk medicine [3, 4].

The most representative compounds of this species are alkylamides, specially spilanthol ((2E,6Z,8E)-N-Isobutyl-2,6,8-decatrienamide), also called Affinin; this molecule is known for its pharmacological properties [5–7]. In folk medicine, inflorescences and leaves from *A. oleracea* are

used to treat mouth and throat ailments, tuberculosis [2, 7, 8], as a diuretic agent [4, 9, 10], to treat flu and cough, as an antibacterial, antifungal, antimalarial [11–13], and insecticide [11, 14], and to treat rheumatism, as an anti-inflammatory, analgesic, and local anesthetic agent. The inflorescences are also used as a local anesthetic for toothaches [3].

Worldwide, researches are performed continuously to improve our understanding of how diseases work and how we can treat them. In this context, zebrafish (*Danio rerio*) is a remarkable model organism, particularly for assessment of acute [15] and reproductive [16–18] toxicity of compounds, either natural [19, 20] or synthetic [21].

Among the methods used to assess a compound's reproductive toxicity with zebrafish, the immersion method is highlighted due to its simplicity and reproducibility. The fish is exposed to a tested compound added to water, allowing the researcher to evaluate if this compound interferes in reproductive parameters, if it causes any toxicity-induced histological changes in the gonads, and finally if it causes any teratogenic or even lethal effects in the fish.

The metabolism of an initial drug or xenobiotic can produce metabolites with considerably different pharmacological and physical-chemical properties; this consequently has relevant implications regarding the safety and effectiveness of the compound [22, 23]. To reduce potential risks which caused the metabolism of a given drug, it is necessary to employ reliable methods to predict it. However, due to restrictions to study human metabolism of xenobiotic compounds, a computational approach is currently the method of choice [22].

Considering these information, this study aimed to evaluate the reproductive performance of zebrafish treated with the hydroethanolic extract from the flowers of *Acmella oleracea* (L) R. K. Jansen; evaluate the effects of the treatment on embryonic development of the offspring (F1 generation) from exposed progenitors; and perform *in silico* assessment of the principal molecule of EHFAo—spilanthol—and its metabolites, to appraise their potential toxicity to the human body.

2. Materials and Methods

2.1. Plant Material. *Acmella oleracea* (L.) R. K. Jansen flower samples were collected in September 2016, District of Fazendinha (S 0° 02′30.40 ″/W 5106′37.5 ″), in the City of Macapá, State of Amapá, Brazil. A dried plant specimen was stored in IAN Herbarium (Embrapa Amazônia Oriental, Bélem, Pará, Brazil), under the identification n° 196011.

2.2. A. Oleracea Flowers Hydroethanolic Extract (EHFAo) Preparing. Selected fresh flowers were ground to powder and then cold macerated for seven days in 70% hydroethanolic solution. The resulting solution was filtered and concentrated in a rotary evaporator (Quimis Model Q 218.2) at 40°C for complete evaporation of the solvent. Subsequently, this concentrate was freeze-dried, yielding 2.5%.

2.3. EHFAo Analysis: Ultra High-Performance Liquid Chromatography (UHPLC-ESI-MS). EHFAo analysis was performed using an Agilent 1290 (Agilent®) Liquid Chromatograph with DAD detector, coupled to an Agilent G400 Triple Quadrupole Electrospray Mass Spectrometer in positive ionization mode.

Samples containing 5 mg/mL of the extract were prepared with methanol, filtered in microfilters, and then analyzed on a reverse phase column (ZORBAX XDB C8; 2.1 x 50 mm 3.5 micron), eluted with water and (A) 0.1% acetic acid and (B) acetonitrile (40:60) in isocratic mode, with 2 μL of injection volume, flow rate of 0.05 mL/min, and 1,200 bars of pressure limit, in 13 min. of analysis time. The column temperature was kept at 40°C, the thermostat at 20°C and the samples were kept at room temperature. The compounds were detected at 230 nm. Mass spectrometry was performed through electrospray ionization in full scan mode, operating between 50 and 700 m/z, with 50 V of collision energy. Nitrogen gas was used as nebulizer (45 psi), with a flow rate of 5 L/min in positive mode. The mass found was registered in positive ionization mode, and the spectra of the fragments were identified according to the literature.

2.4. Animals. The project of this research was approved by the Animals Use Ethics Committee (CEUA) from the Federal University of Amapá (UNIFAP), under registration n° 002/2018.

Both sexes of *Danio rerio* (n = 48 fishes) were used (AB wild-type). The animals were about six months old, 3.5-4.0 cm in length and 650 mg in weighing. All animals were bought from the company Acqua New Aquarium and Fish Ltda. (Igarassu, PE, Brazil), kept in quarantine, and then acclimated over a month before the experiments, performed in the Zebrafish Room from the Drugs Research Laboratory of the Federal University of Amapá (UNIFAP).

The fish were kept in water tanks (25 L) equipped with running water system at temperatures between 25-29°C; pH between 8,4 and 8.6; hardness between 140 and 145 mg/l $CaCO_3$; and 90% of dissolved O_2. The fish were stored in the proportion of 1 fish per water liter, in a light/dark cycle of 12/12 hours. All animals were fed twice a day with ration flakes (Alcon Colours) [15].

2.5. Experimental Design. After the acclimation period, the animals (n = 48) were randomly divided into four groups (n = 12/group), with a proportion of two male fishes for each female fish.

The groups were divided as follows:

A: parental generation treated with regular water from the system (control group);

B: parental generation treated with EHFAo at 50 μg/l diluted in 1 L of regular water;

C: parental generation treated with EHFAo at 100 μg/l diluted in 1 L of regular water;

D: parental generation treated with EHFAo at 200 μg/l diluted in 1 L of regular water.

Table 1: Teratogenic and lethal effects observed in zebrafish embryos throughout the development period.

Developmental toxicity		24 hpf	48 hpf	72 hpf	96 hpf
Lethal effects	Coagulated eggs[a]	+	+	+	+
	No heartbeat[b]		+	+	+
Teratogenic effects	Tail malformation[c]		+	+	+
	Scoliosis			+	+
	Yolk edema		+	+	+
	Growth retardation[d]		+	+	+
	Cardiac edema		+	+	+

[a] Coagulated eggs are milky white and look dark when seen under optical microscopy.
[b] No record of heart beats for one minute.
[c] Tail malformation occurred when an embryo had a curved, twisted, or hook-like tail.
[d] Growth retardation was evaluated by comparing treated embryos with control ones (size, development stage). At 72 and 96 hpf, growth retardation was considered when embryo's size was less than 2.9 and 3.3 mm, respectively.

One week before the experiments, a pretreatment was performed with all groups using only regular water from the system. During this period, the eggs were collected, quantified, and evaluated until 96 hpf. This procedure was carried out to establish base values for each group.

After this pretreatment, the groups were treated according described throughout 21 days. Every day, 50% of water from the tanks was renewed, containing the same original concentration of EHFAo designed for the respective group, prepared from a mother solution tank, changed weekly [16]. The concentrations used over the experiment were established after a preliminary acute toxicity test (48 h), in which eggs, mortality, and teratogenesis were evaluated until 96 hpf.

2.6. Egg Collection and Maintenance. After mating and spawning, fertilization occurred about 30 minutes after the lights were turned on. The eggs were collected, quantified, and washed using regular water from the system and then stored on inert Petri dishes with regular water (changed 70% daily); these Petri dishes were stored in a stove (SOLAB SL-102/630) at 28°C ± 2 until 96 hpf [24].

2.7. Teratogenesis and Lethality Evaluation. A daily observation was performed to evaluate the embryos in 24, 48, 72, and 96 hpf. The embryos were classified according to the severity of morphological defects and signs of toxicity (Table 1).

2.8. Gonads Histopathological Analysis. After the 21 days of exposure to EHFAo, the animals were euthanized according to the method described by Castro [25]. After this procedure, the specimens were fixed in Bouin solution, decalcified using EDTA at 7%, processed and stained with Hematoxylin & Eosin, as described by Souza et al. [15]. Histological changes were qualitatively and quantitatively appraised as described by Souza et al. [15].

2.9. Statistical Analysis. The values of eggs deposition were expressed as a mean ± standard error of the mean (SEM). To evaluate morphologic deviations among embryos compared to the control group was performed the bidirectional analysis of variance Kruskal-Wallis, followed by the post hoc test of Dunn. Results with $p < 0.05$ were considered statistically significant. All statistical analysis was performed using the software GraphPad Prism v. 5.0.

2.10. In Silico Evaluation of Spilanthol Metabolism. Spilanthol and its metabolites were appraised using the online server PreADMET (https://preadmet.bmdrc.kr/). This server calculated pharmacokinetic properties, such as solubility in pure water and skin permeability (P_{Skin}). PreADMET predicts *in vitro* values of P_{Skin}, and the result is given as log Kp.

Kp (cm/hour) is defined, according to Singh [26], as

$$\mathrm{Kp} = \mathrm{Km} * \frac{\mathrm{D}}{\mathrm{h}} \quad (1)$$

Km represents the coefficient of distribution between the stratum corneum and vehicle; **D** represents the average diffusion coefficient (cm^2/h); and **h** represents the thickness of the skin (cm).

PreaADMET also predicted values of plasmatic protein binding (PPB), blood-brain barrier penetration (BBB), permeability in Caco-2 cells, toxicological properties—including environmental (using the species Minnow as a parameter) and developmental (using Medaka fish as a parameter) [27]—and the risk of cardiac toxicity due to inhibition of the human gene ether-a-go-go-related (hERG). The structures assessed were further analyzed using the software Derek [28], to compare its results with those of PreADMET.

3. Results

When analyzed through UHPLC-ESI-MS, the extract had a peak at 2.61 minutes corresponding to spilanthol in the chromatogram of total ions (TIC), with 22.56% of relative abundance, determined by integration of the peaks (Figure 1).

3.1. Gonads Histopathology. In females from the control group, all follicles—such as the perinucleolar, alveolar cortical, vitellogenic, and mature follicles—were regular (Figures 2(a) and 2(e)).

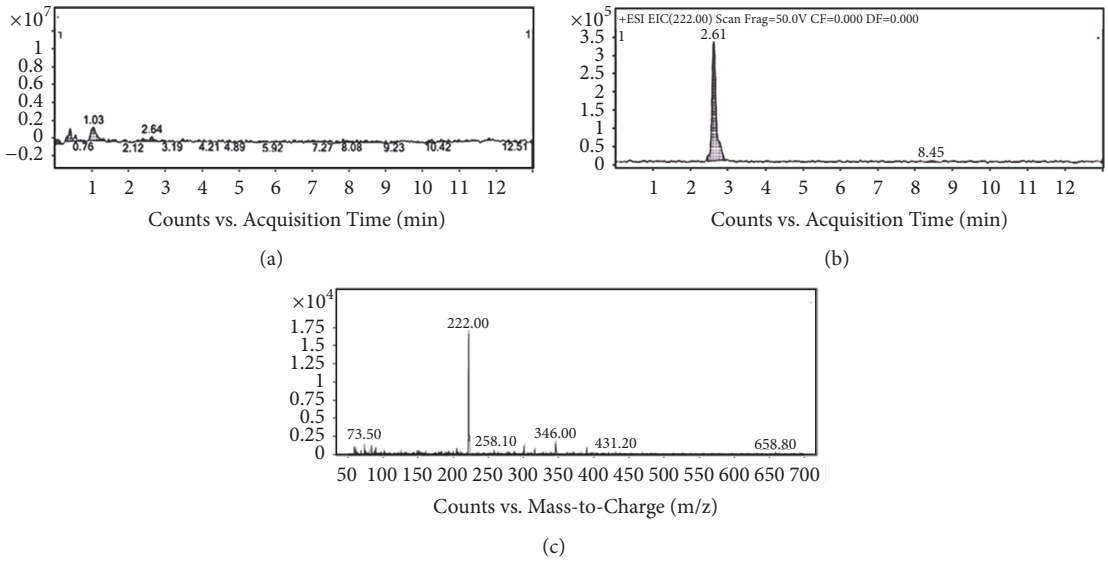


Figure 1: UHPLC-DAD-ESI-MS Analysis of the EHFAo: total ions chromatogram (a); extracted ions chromatogram (b); and mass spectrum in scan mode (c).

After 21 days of treatment with EHFAo (at 50, 100, and 200 μg/L), mild alterations were observed in females' ovaries from the treated groups, including perifollicular cells hyperplasia (a level II severity change found in < 20% of the tissue), as shown in Figure 2(d). Moderate alterations were also observed, like interstitial fibrosis (level III severity change in ≤ 30% of this tissue), as shown in Figures 2(b) and 2(h). Finally, oocytes atresia was observed, but only in females treated with the highest concentration (200 μg/L), as shown in Figure 2(c).

In males' testis from the control group, it is observed that the parenchyma is organized with numerous highly developed spermatocytes, all cell components discernible, and abundant normally distributed spermatozoa. In the interstice several conjunctive cells and blood vessels are seen. Leydig cells are regularly arranged, with round nuclei, sometimes forming agglomerates in the interstitial space. Lastly, the spermatozoa had round-shaped heads, although some were oval-shaped (Figure 3(b)).

In males exposed to EHFAo at 50 μg/L no sign of severe histopathological changes was observed; only interstitial cells had barely discernible hyperplasia (< 20%; a level I severity alteration), as shown in Figure 3(d).

Among male animals treated with EHFAo at 100 and 200 μg/L, few cellular features were altered, for instance, clear signals of testis parenchyma degeneration were detected, a level III severity alteration (Figure 3(h)). In animals exposed to EHFAo at 100 μg/L, this alteration was present in 50% of the tissue, while those exposed to EHFAo at 200 μg/L had 60% of the tissue altered. In these groups the highest percentage of mature spermatozoa (Figures 3(c), 3(e), and 3(f)), hyperplasia of interstitial cells, and development of asynchronous gonads was observed, a level II severity alteration, in ≥ 20% of the tissue (Figures 3(b) and 3(g)).

3.2. Fertility. Figure 4 shows the average number of collected eggs over 21 days of treatment with EHFAo. In the period before treatment, the spawning occurred in regular intervals among all groups, with 100% of fertilization and hatching.

The highest numbers of deposited eggs were observed in the groups exposed to EHFAo at 100 and 200 μg/L (126.5 ± 20.2 and 136.4 ± 24.7, respectively); a total of 18 spawns were registered in the group treated with the concentration 100 μg/L and 15 spawns in the group treated with the concentration 200 μg/L. The treatment resulted in an apparent concentration-dependent increase of spawning.

3.3. Embryos Lethality and Teratogenesis Assessment. Lethal effects observed in the embryos, whose progenitors were exposed to EHFAo, included coagulation and absence of heartbeats, registered up to 96 hpf. These lethalities were more prominent in the groups whose progenitors were exposed to the highest concentrations (Figure 5). Mortalities caused by the occurrence of several alterations in one single embryo were also registered up to 96 hpf.

The number of embryos with at least one malformation was higher between 48 and 72 hpf. In this period, all larvae had already hatched (Figure 6), allowing a better view of embryos' structure. After 96 hpf, occurrences of malformations decreased in all groups.

The most severe and evident malformations recorded were yolk and cardiac edema (Figures 7(d), 7(h), 7(i), and 7(k)). The total number of cardiac edema occurrences was 38, 147, and 196, in groups B, C, and D, respectively, while the number of yolk edema occurrences was 42, 153, and 175. However, only group D—whose progenitors were exposed to EHFAo at 200 μg/L—had a statistically significant difference compared to the control group (A). Larvae with such malformations did not survive after 96 hpf.

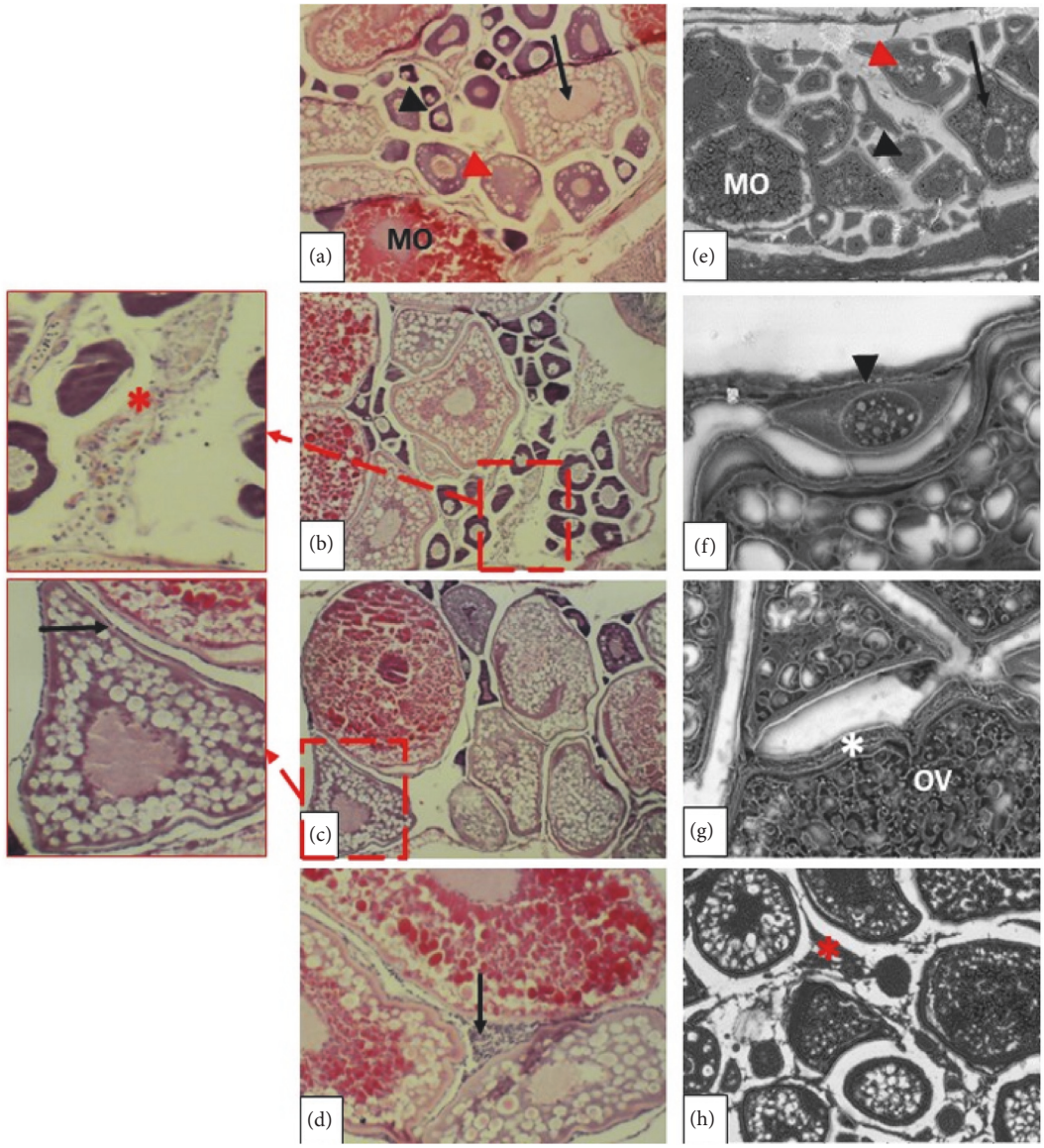


Figure 2: Longitudinal sections of zebrafish ovaries. In figures (a) (H&E), (e), (f), and (g) (SEM) normal aspects of zebrafish ovaries are observed, with perinucleolar oocyte (black arrowhead), primary vitellogenic oocyte (red arrowhead), vitellogenic oocyte (black arrow), mature oocyte (MO), chorion (asterisk), and vitellogenic oocyte (OV). In (b) (H&E) and (h) (SEM) interstitial fibrosis is observed (red asterisk), present in all groups treated with EHFAo. In (c) oocyte atresia is observed (black arrow), observed only in the group treated with EHFAo at 200 μg/L. In figure (d) hyperplasia of perifollicular cells is observed (black arrow), observed in all treated groups.

Tail deformity (Figures 7(e), 7(h), 7(j), 7(l), 7(m), and 7(n)) and retarded growth (Figure 7(n)) were frequently observed alterations, but without a statistically significant difference when compared to the control group. These alterations resulted in impaired larval movement, which was evident in groups C and D. The least frequent alteration was scoliosis, with two occurrences in group A, 5 in B, 18 in C, and 22 in D.

3.4. Spilanthol Metabolism In Silico Assessment. Figure 8 shows spilanthol metabolites predicted by the software MetaTox and the reactions to form them. The software DL_{50} value for spilanthol was 20.80 mg/kg in rats. Among the chemical reactions predicted by the enzyme CYP450 (Figure 8) were aliphatic hydroxylation, C-oxidation, N-glucuronidation, N-acetylation, epoxidation, and glutathionylation.

As shown in Table 2, the metabolite M8 is formed through epoxidation and catalyzed by epoxide hydrolase. According to the software, M8 has a potential risk to cause cardiac insufficiency and is potentially carcinogenic for male rats and female mice. M33 is a phase I reaction (oxidation) metabolite and was predicted to be potentially carcinogenic in the liver of male mice, in the lungs of female mice, and for tumor bearer male rats [29]. The metabolite M18 was predicted to

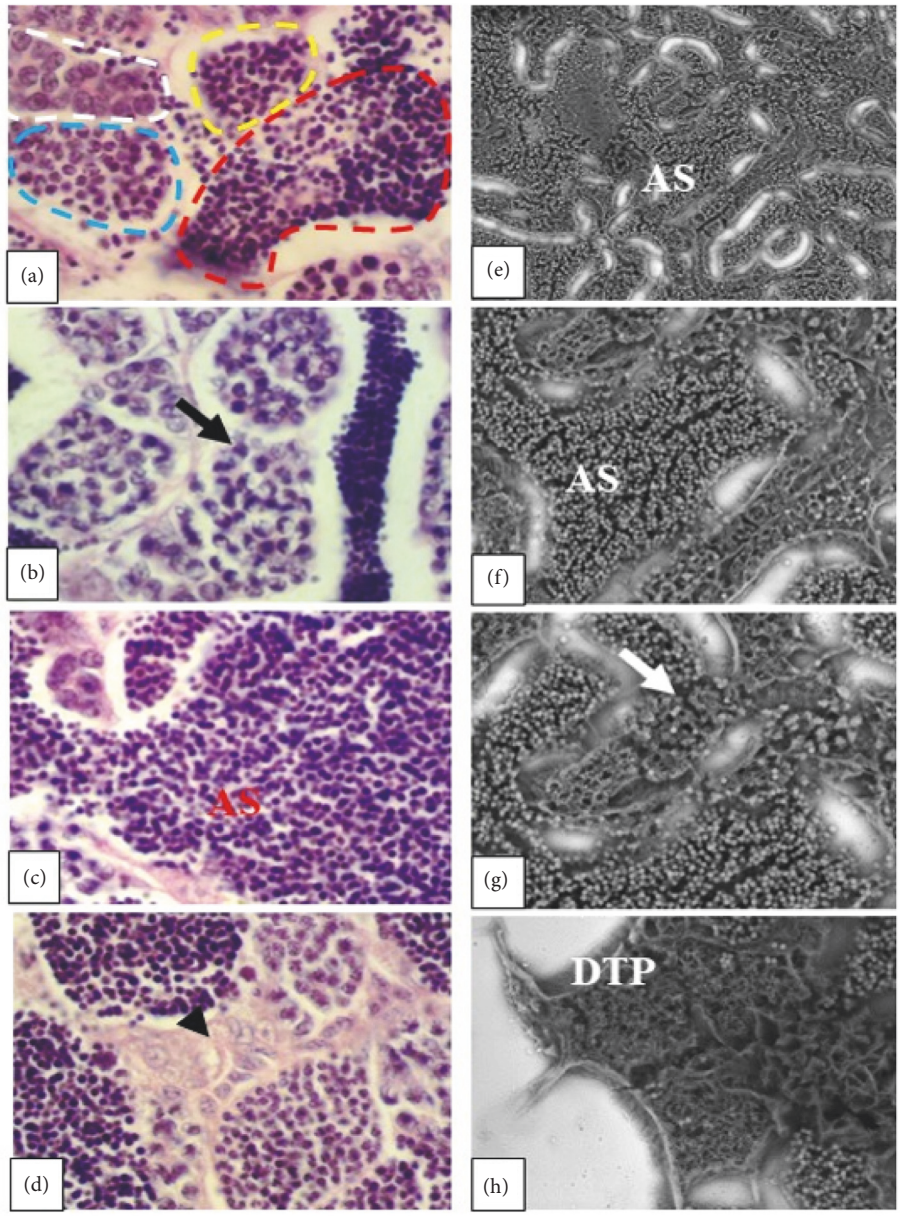


Figure 3: Longitudinal sections of zebrafish testes. In figures (a) (H&E), (e), and (f) (SEM) normal aspects of zebrafish testes are observed, with mature spermatozoa (red dashed lines), spermatocytes (blue dashed lines), type-II spermatocytes (white dashed lines), and plenty of spermatozoa (AS). In figure (b) (H&E) and (g) (SEM) the development of asynchronous gonads is observed (black and white arrow) in groups treated with EHFAo at 100 and 200 μg/L. In figure (c) (H&E) plenty of mature spermatozoa is observed (groups treated with EHFAo at 100 and 200 μg/L). In (d) (H&E) hyperplasia of interstitial cells is observed (arrowhead), in the group treated with EHFAo at 50 μg/L. In (h) (SEM), testicular parenchyma degeneration is observed (DTP), observed in groups treated with EHFAo at 100 and 200 μg/L.

be formed through N-acetylation, one of the major routes of biotransformation from xenobiotics with an aromatic amine ($R\text{-}NH_2$), which are converted into aromatic amides (R-NH-COCH3) [30].

The metabolite M26 is formed through aliphatic hydroxylation, as shown in Figure 9. In simple hydrocarbons with linear chain, aliphatic hydroxylation occurs in terminal methyl groups and internal methylene groups. The oxidation of some aliphatic alkenes produces metabolites reactive enough to bind covalently to the heme portion of cytochrome P450. M26 showed no side effect.

As for M28 and M35, both are phase II metabolites. M28 is formed through a conjugation reaction with the tripeptide glutathione (Gly-Cys-Glu). The products of conjugation between glutathione and xenobiotics are fundamentally different from those formed by conjugation with other amino acids and dipeptides [31]; M28 showed no side effect but was predicted to be potentially carcinogenic for the kidney

Table 2: Most probable metabolites for spilanthol, with their respective chemical reactions, probability values, and predicted DL_{50}.

Metabolites	Phase type	Chemical Reaction	Probability (PR)	DL_{50}
M8	Phase I reaction (Oxidation)	Epoxidation	0,9991	46.49 mg/kg
M18	Phase II biotransformation reaction	N- Acetylation	0,8985	26.28 mg/kg
M26	Phase I reaction (Oxidation)	aliphatic hydroxylation	0,8072	33.14 mg/kg
M28	Phase II biotransformation reaction	Glutathionation	0,7797	327.74 mg/kg
M33	Phase I reaction (Oxidation)	C-oxidation	0,6322	28.69 mg/kg
M35	Phase II biotransformation reaction	N-glucuronidation	0,5135	81.34 mg/kg

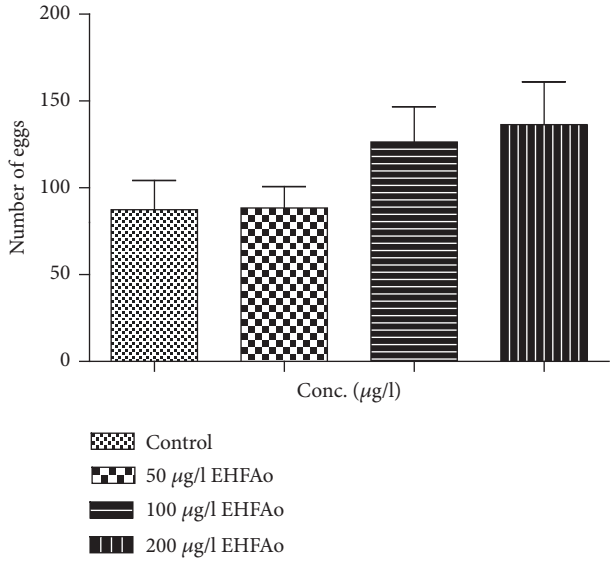


Figure 4: Average values of eggs production in zebrafish over the treatment period (21 days) with EHFAo. The eggs deposition was higher in groups treated with the two highest doses of EHFAo. The numbers of eggs from each group were compared among each other using the software GraphPad Prism 5.0, but the difference was not statistically significant ($p > 0.05$).

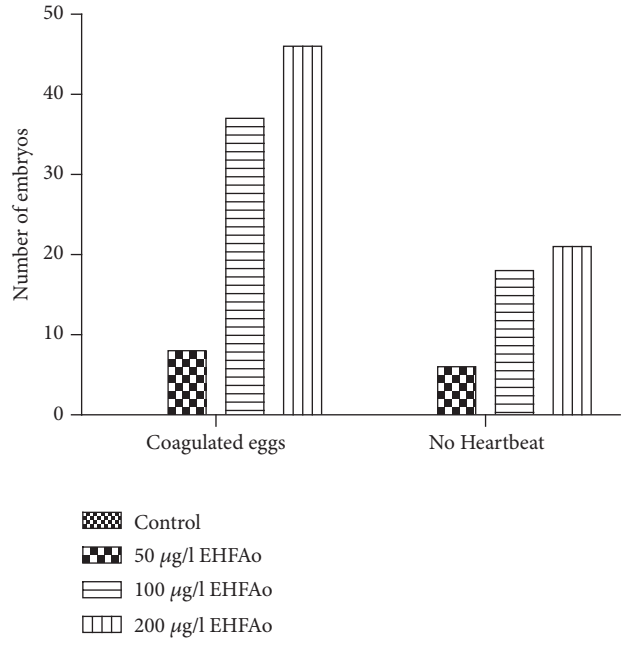


Figure 5: The graph shows the numbers of embryos with lethal abnormalities. The groups treated with EHFAo at 100 and 200 μg/L had the highest incidence of lethalities, and coagulation was the most frequent egg abnormality.

of male mice and skin of male rats. The metabolite M35 is formed through N-glucuronidation; this reaction requires the cofactor uridine diphosphate glucuronic acid (UDPGA) and is catalyzed by the enzyme UDP-glucuronosyltransferase (UGTs), which is located in the endoplasmic reticulum of the liver, and other tissues, such as the kidney [32]. The site of glucuronidation is often an electron-rich nucleophilic heteroatom, which in the case of spilanthol is the nitrogen. As side effects, M35 showed to be toxic to nephrons and carcinogenic to the kidney of male mice.

3.5. In Silico Assessment of Spilanthol Pharmacokinetics. The prediction of Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) for spilanthol and 37 metabolites formed from it is shown in Table 3; this table also shows values of solubility in pure water, absorption (P_{Skin} and Caco-2 cells), among others.

The solubility in pure water is a property of singular interest in drug development since it is directly related to the compound's pharmacokinetics. Spilanthol had a value of 384.737 cm/h, as shown in Table 3. In this study, the

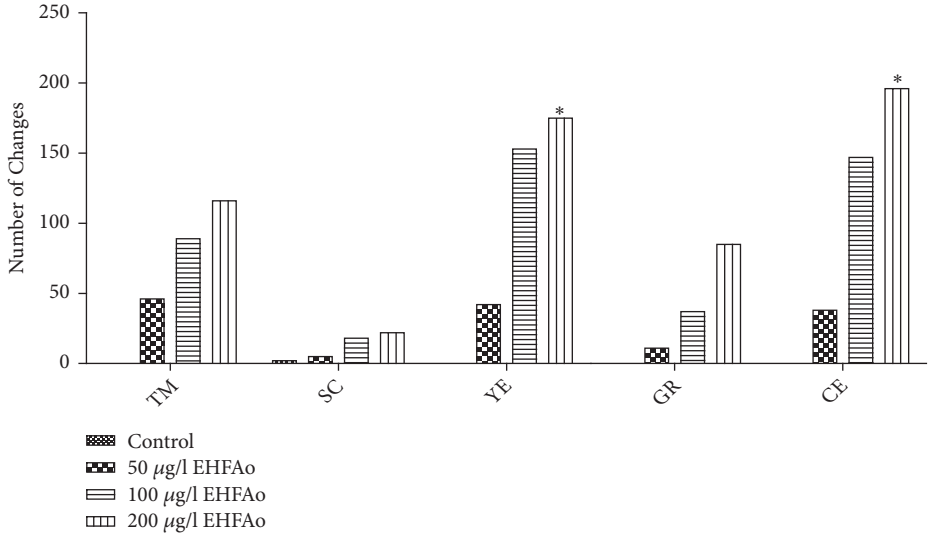


Figure 6: Embryonic and larvae changes in the offspring from the parents exposed to treatment with EHFAo for 21 days. $* p < 0.05$, statistically significant difference compared to the control group; TM: tail malformation, SC: scoliosis, YE: yolk edema, GR: growth retardation, and CE: cardiac edema.

highest P_{Skin} values were those of spilanthol (-0.860042), M9 (-0.992692), M21 (-0.996746), and M22 (-0.994307). Most values of permeability in Caco-2 cells (P_{CaCo2}), shown in Table 3, are comprised between 4 and 70; the minimum value of this property was for M34 (10.5575 nm/s), and the maximum values were for M8 (56.2978 nm/s) and spilanthol (53.0118 nm/s). None of the molecules had a high permeability value (≥ 70). The metabolites M13, 15, 16, 21, 25, 30, 36, and 37 had values inferior to 4.

Moreover, distribution properties (PPB % and $C_{Brain}C_{Blood}$) were evaluated for spilanthol and its 37 metabolites. Spilanthol and the molecules M1, M3, M4, M6, M9, and M21 had a plasma protein binding index (PPB) equal to 100%.

From 38 molecules assessed (Table 3), 20 of them were classified as active in the central nervous system, with $C_{Brain}C_{Blood}$ values > 1. From these, the highest values were from spilanthol (6.711), M1 (2.065), M2 (2.491), M3 (2.662), M4 (2.771), M5 (2.572), M6 (2.669), M18 (2.245), and M21 (2.245). Molecules with $C_{Brain}C_{Blood}$ values < 1, on the other hand, are classified as inactive in SNC; among these molecules are M12, M13, M23, M24, M27, M29, M35, M36, and M37, whose values ranged between 0.030 and 0.038.

As shown in Table 3, M10 and M21 have a high probability of being hERG-type K^+ channels blocker; 13 other metabolites were classified as ambiguous, and 23 had a medium probability of causing this effect. Also, these metabolites showed no probability to be toxic environmentally (Minnow) or in the development (Medaka).

Additional toxicological data for spilanthol and its 37 metabolites tested using the software Derek are shown in Table 4. Overall, this data indicates that spilanthol and 18 of its metabolites show toxicity to skin sensibility. According to the software Derek, this property is due to the pharmacophore groups: alpha or beta-unsaturated amide or precursor, alpha or beta-unsaturated ketone or precursor, epoxide, aldehyde, and alpha or beta-unsaturated aldehyde or precursor. The molecules M3, M6, M7, M8, M16, M28, and M31 had a probability of causing chromosome damage by inducing molecular structural changes. According to the software Derek, this potential to cause chromosome damage is due to the presence of alpha and beta-unsaturated ketones (in M3, M6, M28, and M31) and epoxide (in M7, M8, and M16).

As shown in Table 4, nine molecules were attributed of being potentially carcinogenic to humans due to the pharmacophore groups: alpha or beta-unsaturated aldehyde, ketone or imine; epoxide; and alpha or beta-unsaturated amide, nitrile, or nitro compound. Other molecules were attributed to being potentially toxic in the development (M7, M8, and M16), cause eye and skin irritation in humans (M7, M8, M16, and M33) or irritation in the respiratory tract (M33). From the 38 molecules screened, 23 had none toxicologic alert for humans.

4. Discussion

In mass spectra analysis of the EHFAo (Figure 1), there is a peak with retention time equal to 2.64 minutes and 22.76% of relative area; this peak corresponds to the fragment m/z = 222 (base peak), which according to the literature is spilanthol ((2E, 6Z, 8E)-N-isobutyldeca-2,6,8-trienamide). Spilanthol is an alkylamide, which are molecules with a medium to long fatty acid chain (8 to 18 carbons), often aliphatic and with one amide group [33]. The extracted ions chromatogram was analyzed to find out the exact retention time of peak from spilanthol, and it was observed that the peak base m/z = 222 was eluted in 2.61 minutes.

Some other fragments were detected in product ions analysis, the fragment m/z 69 can be attributed to the group isobutylnitrile ($C_4H_7N^+$), while the fragment m/z 53 can be

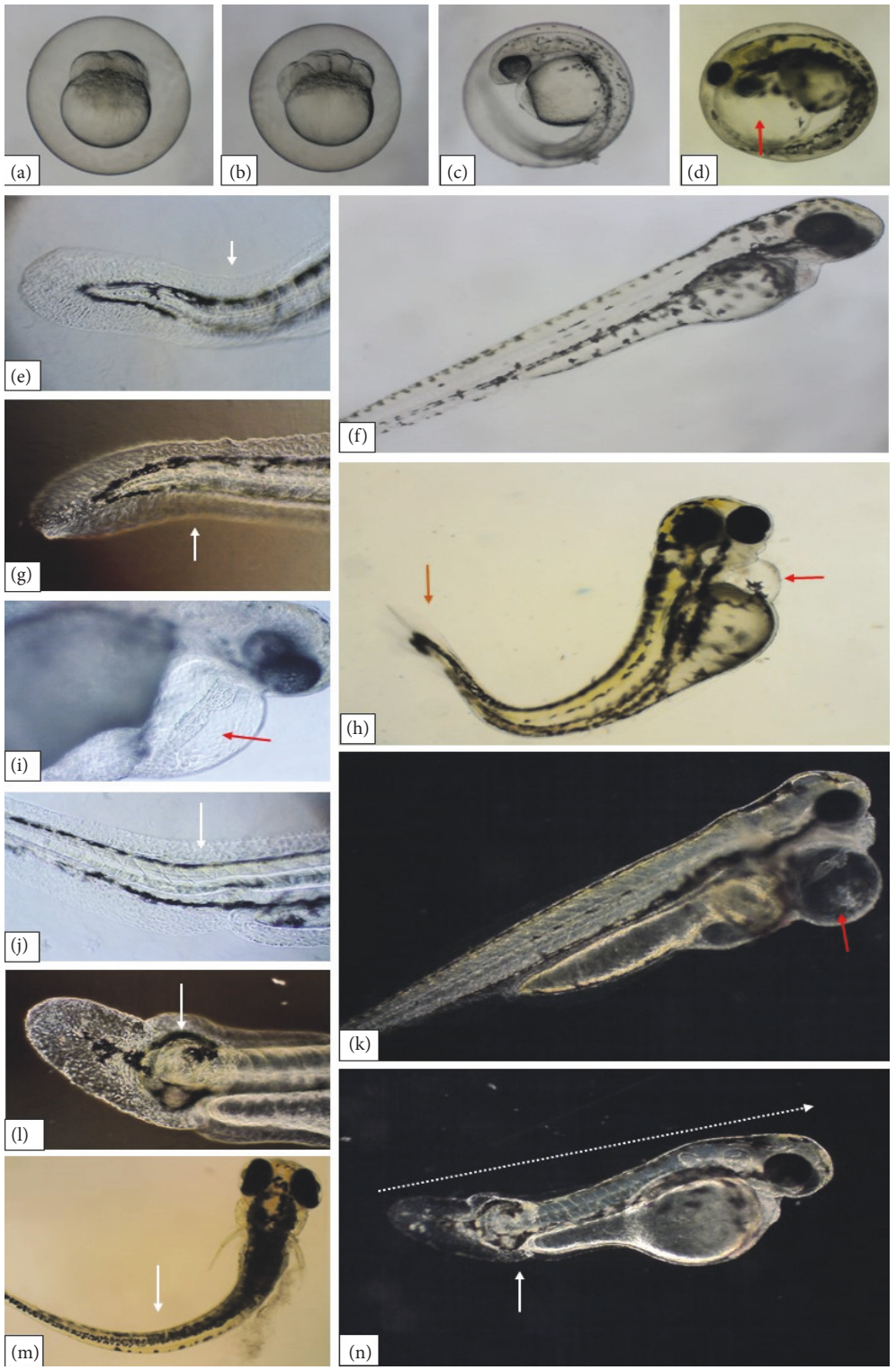


Figure 7: Representative pictures of zebrafish embryos and larvae from groups (a), (b), (c), and (d), whose parental generation was exposed to EHFAo at 50, 100, and 200 μg/L. (a) Normal embryo with two cells (0.75 hpf); (b) normal embryo with 16 cells (1.5 hpf); normal embryo (c); and larva (f) at 24 and 48 hpf. White arrows indicate tail malformation in (e), (g), (h), (l), (m), and (n). Cardiac edema in 24 hpf embryo is shown in (d), and 96 hpf larvae in (i), (h), and (k) (red arrows). In (n) a fish with retarded growth is observed (white dashed arrow).

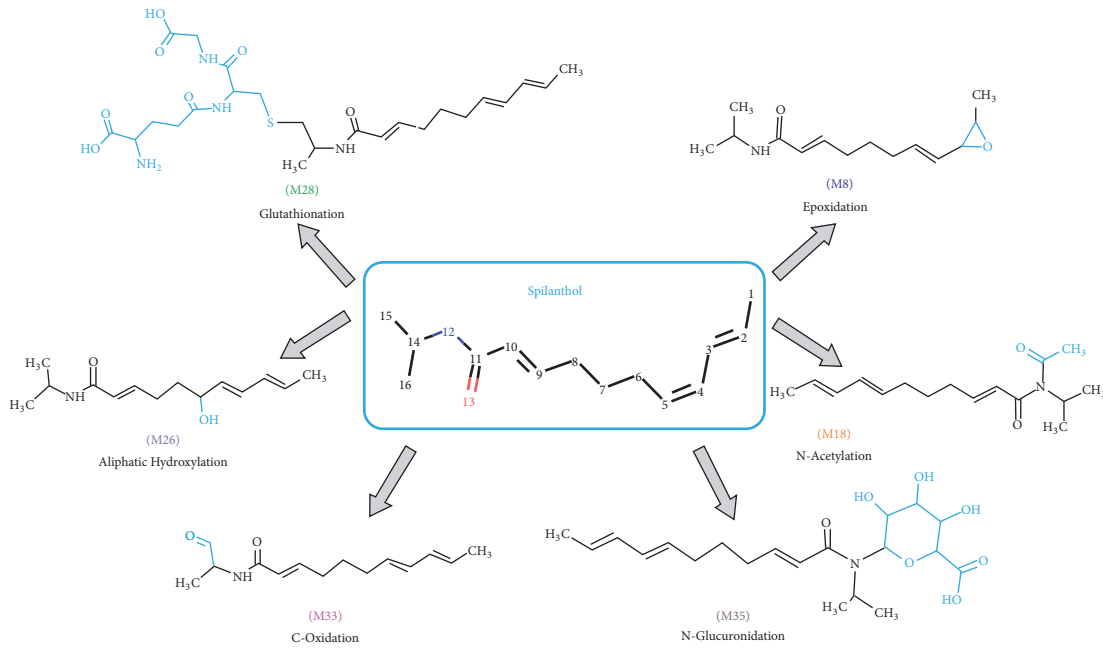


Figure 8: MetaTox results from spilanthol and its metabolites with their respective chemical reactions.

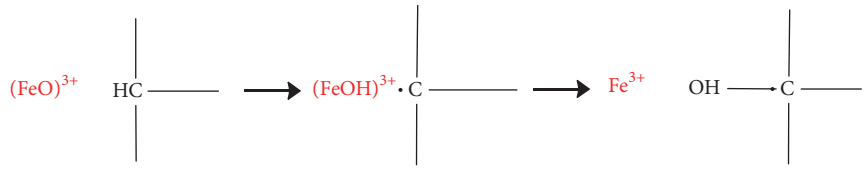


Figure 9: P450 enzymes catalyze hydroxylation preferably. Adapted from [23].

$C_3H_3N^+$, and m/z 41 can be $C_2H_3N^+$. The fragment m/z 29 suggests a loss of carbonyl group from spilanthol, and the fragment m/z 149 indicates rupture of a C-N bond, with loss of the amine group. Singh and Chattuverdi [34] also detected spilanthol in the leaves of *Spilanthes acmella* through electrospray ionization, evidenced by a base peak m/z = 222. These authors also reported a fragment m/z = 99, which could be isobutyl isocyanate (C_5H_9NO).

Spilanthol has several pharmacological activities reported [35], among them, its local anesthetic activity is the most reported [7, 35–42]; other activities are analgesic [43], antioxidant [39], anti-inflammatory [38, 44, 45], antiwrinkle [46], antifungal [35], aphrodisiac [35], antimalarial [47], among others.

Until now, no research had been performed testing the hydroethanolic extract of *Acmella oleracea*, or its primary compound—spilanthol—over reproductive parameters of zebrafish or any teleost, neither its effect in the embryonic development of any model. In rats [48], EHFAo was reported to cause no maternal toxicity and significantly increased females' proestrus phase (88.91 and 444.57 mg/kg) when compared to a control group.

In this study, histopathology of female zebrafish ovaries showed oocyte atresia in the group treated with EHFAo at the highest concentration. This tissue alteration had also been reported to be caused by treatment with plant sterols, for instance, in studies with *Oryzias latipes* exposed to the phytoestrogens genistein, equol, and the bioflavonoid quercetin [49, 50]. Oocyte atresia is a natural process induced through apoptosis for ovarian maintenance; it is involved in growth and postovulatory regression of teleost fishes [51]. However, the increased occurrence of oocyte atresia can be a general response to endocrine disturbance and chemical-induced toxicity [52].

Hyperplasia and hypertrophy of perifollicular cells, observed in the groups exposed to EHFAo, are the increase of total number (hyperplasia) or size (hypertrophy) found in epithelial cells from granulosa or theca folliculi of a developing follicle [53]; this can be perceived as an increase of height and in the total number of granulosa cells, giving a pseudostratified layer appearance. Since perifollicular cells are involved in the production of aromatase in animals [54, 55], it is plausible that the increased size and number of these cells are a compensatory mechanism to maintain aromatase in appropriate levels for vitellogenesis [53].

Interstitial fibrosis occurs with an increased amount of fibrous connective tissue inside testicular or ovarian interstice [53]; this alteration indicates chemical-induced chronic stress

Table 3: Predictions of pharmacokinetic parameters of spilanthol and its metabolites.

Metabolites	Pharmacokinetic						Acute Toxicity (LC_{50} – mg/l)	
	$S_{Pure\ water\ (cm/h)}$	P_{skin}	$PPB_{(\%)}$	$BBB_{(C.brain/C.blood)}$	hERG	CaCo2	Medaka	Minnow
Spilathol	384.737	-0.860	100.00	6.711	m_risk	53.0118	0.0181	0.01775
M1	901.681	-1.163	100.00	2.065	m_risk	25.6981	0.0450	0.04509
M2	631.747	-1.167	93.224	2.491	m_risk	25.698	0.0386	0.0409
M3	310.115	-1.125	100.00	2.662	m_risk	25.6981	0.0353	0.03059
M4	310.115	-1.113	100.00	2.771	m_risk	25.6949	0.0359	0.03348
M5	631.747	-1.150	92.756	2.572	m_risk	41.9066	0.0553	0.03904
M6	310.115	-1.139	100.00	2.669	m_risk	25.6981	0.0359	0.03040
M7	1206.68	-1.728	96.916	1.336	m_risk	47.8535	0.0828	0.0801
M8	845.441	-1.683	83.732	1.576	m_risk	56.2978	0.0958	0.06987
M9	776.796	-0.992	100.00	1.106	m_risk	19.7788	0.0731	0.06476
M10	68578	-3.067	66.967	0.875	l_risk	20.9729	31.35	16.810
M11	3973.59	-1.185	87.469	1.954	m_risk	23.6658	0.0819	0.09516
M12	142.092	-2.934	31.644	0.037	ambiguous	0.611474	0.2308	0.3764
M13	252.203	-2.635	34.217	0.038	ambiguous	14.5677	0.2754	0.3786
M14	48.8697	-2.936	35.301	0.040	ambiguous	0.624655	0.1404	0.2630
M15	48.8697	-2.931	35.216	0.063	ambiguous	0.626143	0.1305	0.2620
M16	728.976	-1.608	83.202	1.444	m_risk	47.7725	0.0902	0.08946
M17	428.204	-1.099	90.967	0.429	m_risk	49.8748	0.0133	0.01459
M18	1366.64	-1.114	93.160	2.245	m_risk	23.6658	0.0643	0.07021
M19	3102.58	-1.190	89.623	1.804	m_risk	25.6981	0.0705	0.09478
M20	48.869	-2.910	28.384	0.043	ambiguous	0.645312	0.2711	0.2598
M21	1007.38	-0.996	100.00	2.958	l_risk	37.5329	0.0275	0.02863
M22	863.164	-0.994	88.489	1.786	m_risk	25.8079	0.0923	0.1140
M23	99.5542	-2.918	32.768	0.034	ambiguous	1.14185	0.2015	0.3296
M24	99.5542	-2.914	31.183	0.030	ambiguous	0.686328	0.291	0.3299
M25	3102.58	-1.195	61.667	1.811	m_risk	25.6981	0.1203	0.09362
M26	5553.90	-1.294	68.862	0.402	m_risk	26.3048	0.1695	0.1878
M27	86.740	-2.565	36.681	0.036	ambiguous	14.7041	0.2742	0.2633
M28	2344.76	-1.252	90.150	0.726	m_risk	34.1402	0.0555	0.08731
M29	118.492	-2.979	31.622	0.033	ambiguous	0.782091	0.5623	0.4349

Table 3: Continued.

Metabolites	Pharmacokinetic							
	$S_{Pure\ water\ (cm/h)}$	P_{skin}	$PPB_{(\%)}$	$BBB_{(C.brain/C.blood)}$	hERG	CaCo2	Acute Toxicity (LC_{50} – mg/l)	
							Medaka	Minnow
M30	6320.38	-1.230	76.605	1.610	m_risk	18.0322	0.1370	0.1216
M31	2344.76	-1.258	62.141	0.727	m_risk	34.1402	0.135	0.08580
M32	699.357	-1.051	95.684	1.263	m_risk	27.5265	0.0472	0.0458
M33	2358.30	-1.161	90.422	1.133	m_risk	27.5255	0.0546	0.04960
M34	1569.72	-3.016	77.128	0.052	ambiguous	10.5575	0.1132	0.1838
M35	257.656	-2.846	33.003	0.033	ambiguous	0.625941	0.2805	0.3762
M36	257.656	-2.845	29.616	0.030	ambiguous	0.654714	0.1285	0.3777
M37	524.879	-2.830	31.206	0.038	ambiguous	0.561316	0.1639	0.4769

Table 4: Toxicity results according to the software Derek.

Metabolites	Toxicity Prediction Alert (Lhasa prediction)	Toxicophoric Group	Toxicity Alert
Spilanthol	Skin sensitization in human	alpha, beta-Unsaturated amide or precursor	Plausible
M1, M5, M9, M11, M18, M25, M26 e M30	Skin sensitization in human	alpha, beta-Unsaturated amide or precursor	Plausible
M3	Chromosome damage in vitro in human	alpha, beta-Unsaturated ketone	Equivocal
	Skin sensitization in human	alpha, beta-Unsaturated ketone or precursor alpha, beta-Unsaturated amide or precursor	Plausible
M6	Carcinogenicity in human	alpha, beta-Unsaturated aldehyde, ketone or imine	Plausible
	Chromosome damage in vitro in human	alpha, beta-Unsaturated ketone	Equivocal
	Skin sensitization in human	alpha, beta-Unsaturated amide or precursor	Plausible
	Skin sensitization in human	alpha, beta-Unsaturated ketone or precursor alpha, beta-Unsaturated amide or precursor	Plausible
M7, M8, and M16	Carcinogenicity in human Chromosome damage in vitro in human Chromosome damage in vivo in human Developmental toxicity in human Irritation (of the eye) in human Irritation (of the skin) in human	Epoxide	Plausible
	Skin sensitization in human	Epoxide alpha, beta-Unsaturated amide or precursor	
M19	Carcinogenicity in human	alpha, beta-Unsaturated amide, nitrile or nitro compound	Equivocal
	Skin sensitization in human	alpha, beta-Unsaturated amide or precursor	Plausible
M28, M31	Carcinogenicity in human	alpha, beta-Unsaturated aldehyde, ketone or imine	Plausible
	Chromosome damage in vitro in human	alpha, beta-Unsaturated ketone	Equivocal
	Skin sensitization in human	alpha, beta-Unsaturated ketone or precursor alpha, beta-Unsaturated amide or precursor	Plausible
M32	Skin sensitization in human	Aldehyde alpha, beta-Unsaturated amide or precursor	Plausible
M33	Carcinogenicity in human Irritation (of the eye) in human Irritation (of the respiratory tract) in human Irritation (of the skin) in human	alpha, beta-Unsaturated aldehyde, ketone or imine alpha, beta-Unsaturated aldehyde	Plausible
	Skin sensitization in human	alpha, beta-Unsaturated aldehyde or precursor alpha, beta-Unsaturated amide or precursor	
M36	Carcinogenicity in human	alpha, beta-Unsaturated amide, nitrile or nitro compound	Equivocal

[53, 56]. In this study, all females exposed to EHFAo had this tissue alteration, sometimes accompanied by inflammation and accumulation of protein liquid.

The occurrence of protein liquid has been linked to treatments with estrogenic compounds, and the fluid is presumably vitellogenin or a derivative [53]. The increase of fibrous connective tissue and other interstitial tissue alterations are in accordance with others reports of exposure to endocrine disruptors (e.g., E2, EE2, nonylphenol, isoflavones, octylphenol, and PCBs) using either zebrafish [50] or other species (e.g., *Oryzias latipes*, *Dicentrarchus labrax*, *Chalcalburnus tarichi*, *Cyprinodon variegatus*, and *Oreochromis niloticus*), both in testes and in ovaries [49, 57–63]. This data indicates that connective tissue proliferation occurs to replace damaged structures.

In male fishes, histopathology showed a high percentage of spermatocytes and mature spermatozoa in the groups treated with the two highest concentrations of EHFAo, indicating that exposure to the extract at these concentrations induced spermatogenesis; this was also reported in studies with fishes exposed to androgens and pulp mill effluent [64].

In the groups exposed to the two highest concentrations of EHFAo testicular parenchymal degeneration was observed; in this situation apoptotic germ cells are observed, characterized by cell shrinkage, nuclear condensation, and fragmentation. No inflammation was observed along with these cells. When testicular degeneration is too extensive, this may lead to local or general loss of germinal epithelium [53].

Development of asynchronous gonads was registered only in the groups exposed to the extract at 100 and 200 μg/L. This term is used to denote spermatocytes containing a mix of spermatocytes and spermatids, or spermatocytes containing primary spermatocytes in more than one meiotic phase [53].

The effects of EHFAo in eggs production and fertilization seem to be due to its influence over gonads' steroids. In this study an increase of eggs production in the treated groups was observed when compared to the control group. The results suggest that exposure of fishes to EHFAo stimulated the production of sexual hormones, which could be through modification of steroid enzymes or an indirect feedback effect [65], resulting in increased production of egg cells and spermatozoa.

Other studies were performed to assess the effect of *Acmella oleracea* extract over the sexual behavior of male rats [66] and maternal toxicity in female Wistar rats [48]. These studies indicate a potential aphrodisiac activity in *A. oleracea*, attributed to its N-alkylamides. However, until the present study, no assessment had been performed over its effects in the embryonic development.

Even though the treatment upregulated reproductive parameters in parents, it negatively affected the embryonic development of the offspring, evidenced by an increased rate of mortality and developmental defects, such as cardiac and yolk edema, tail malformation, and scoliosis. Some other authors had reported these developmental defects caused by treatment with other compounds [67–69].

Lethal and detrimental effects observed were more pronounced with the two highest concentrations of EHFAo, showing that these concentrations can be harmful to the offspring, and this toxicity is passed somehow from the treated parents to them. It is important to notice that the control group had no increase of lethality rates, and only two larvae had scoliosis (72 hpf). Other compounds were reported to induce developmental toxicity in the offspring after parental treatment, such as bisphenol S [70], fusaric acid [71], selenomethionine [72], ZnO nanoparticles [73], and azoxystrobin [74].

Based on previous studies, some mechanisms proposed to explain a compounds' toxicity induced in the offspring after treatment of parental generation are as follows:

(1) The treatment could induce alterations on egg cells and spermatozoa, resulting in affected development of the offspring [75, 76].

(2) Bioaccumulation of the compound and its metabolites in parental fishes could further induce its deposition from the eggs to the larvae, even if these compounds are absent in water [77–79].

(3) The embryos could absorb aqueous chemical products from parental fish tanks after spawning [76].

However, since the increased rates of lethalities and detrimental effects were accompanied by increased eggs deposition, the former could be a consequence of the latter, since the parental resources to produce a regular number of eggs (e.g., yolk) would be distributed among a higher number, resulting in less resource per egg, and hence favoring detrimental effects. Still, it is not possible to fully elucidate the mechanism of which the EHFAo induces toxicity to the offspring; this is due to an impossibility to measure the transference of EHFAo's compounds or derived metabolites from the parent to the embryo.

Some of the pharmacokinetic properties of isolated spilanthol or extracts containing it were already assessed in vivo or in vitro; in both cases the *in silico* values reported here can be helpful. In the total absence of any data, these values can serve as a parameter for new studies; if there is data, the results can be compared. It is important to notice also that some studies were performed with solutions containing spilanthol, and the in silico values here are deducted from spilanthol alone.

The water solubility of a compound is useful due to its influence in pharmacokinetic properties. The *in silico* prediction of spilanthol solubility in pure water was 384.737 cm/h. With 221.34 g/mol, this molecule is lipophilic (LogP = 3.39) [80], and despite the low solubility in water of spilanthol alone, the whole extract (EHFAo) could be fully dispersed in the water tanks at the concentrations used. The probable physical form of spilanthol is micelles, like other N-alkylamides [81].

Skin permeability is a crucial parameter mainly for transdermal administration of drugs and to evaluate the risks of a chemical that could accidentally touch the skin [82]. In this study, spilanthol and its 37 compounds had negative values of skin permeability, indicating high permeability of these molecules. This is in accordance with Boonen et al. [5] who tested the permeability in human skin of spilanthol-containing solutions with different solvents, such as 65% ethanol and 10% propylene glycol using a Franz diffusion

cell system; overall, these authors showed that the spilanthol-containing solutions were able to permeate the skin. Also, Spiegeleer et al. [81] showed that spilanthol could be used to enhance skin permeability through increased partitioning of the molecules.

Caco-2 cells are currently being employed as the primary cell model to assess a compound's permeability and absorption. Table 3 shows the values of permeability in Caco-2 cells from spilanthol and its metabolites, and most values are average ($4 \leq x \leq 70$; spilanthol: 53); this is in accordance with Veryser et al. [80], who showed in vitro that spilanthol could permeate Caco-2 cells from the apical to the basolateral side and vice versa, which this was further confirmed *in vivo* in rats intestinal lumen. According to these authors, the lipophilic nature of this molecule favors transcellular transport. Boonen et al. [83] also showed that the ethanol extract of *Spilanthes acmella* (= *Acmella oleracea*) could permeate buccal mucosa in a Franz diffusion cell system.

The binding between plasma protein-drug can affect this drug's half-life; also, the bound moiety can act as a chemical reservoir for the drug, since the bound drug will be released to maintain the balance, while the unbound moiety will be metabolized and excreted from the body [84, 85]. As shown in Table 3, spilanthol and the molecules M1, M3, M4, M6, M9, and M21 had the highest PPB values. No *in vitro* or *in vivo* studies with this property were found.

The blood-brain barrier penetration index (BBB), shown in Table 3, is another crucial aspect of a drug. As shown in Table 3, spilanthol BBB was > 1, indicating that this molecule can pass through BBB; this is in accordance with Veryser et al. [80], who showed that spilanthol could rapidly cross the BBB in mice due to its lipophilic nature.

The human ether-a-go-go-related gene (hERG) is codified for a protein that forms a voltage-dependent potassium ion channel found in heart and nervous system; this channel is essential for the repolarization during the cardiac action potential. Conductance alterations of this channel, especially channel block, can lead to an impaired action potential [86]. Due to its importance in the regulation of the cardiac action potential, drugs that can interact with hERG are currently being taken out of the market, since this can result in cardiac arrhythmia and sudden death [86, 87]. In this study, only the molecules M10 and M21 had a considerable risk to interact with hERG.

Minnow and Medaka indexes indicate environmental and developmental toxicity, respectively. Their values were not significative, which is in accordance with Farias et al. [87], who evidenced that natural molecules induce none or few of these toxicity values. Note that this lack of developmental toxicity *in silico* corroborates the hypothesis that the detrimental and lethal effects observed in zebrafish embryos are more likely to be due to the increased egg deposition than a direct toxic effect by the extract.

The occurrence of skin sensitization alert indicates that a molecule can potentially induce skin sensitization, but it also depends if this molecule is absorbed through the skin. Usually, small lipophilic molecules pass more easily through skin and hence are more likely to induce sensitization. As shown in Table 4, 18 molecules were predicted to induce sensitization; this alert is due to the presence of amides, ketones, and alpha or beta-unsaturated precursors that can interact with skin proteins through a Michael addition reaction [88].

In this study, seven molecules were predicted of being potentially injurious for chromosomes. This alert is due to the presence of alpha or beta-unsaturated ketones (vinylketones), which can induce chromosomal aberration *in vitro* and in the L5178Y TK +/- assay. These compounds manage to induce weak positive results in relatively cytotoxic concentrations, such as alpha-ionone [89] and phorone [90]. On the other hand, negative or ambiguous results were attributed to some alpha and beta-unsaturated ketones in the micronucleus test *in vivo*, like curcumin [91], 2-cyclohexene-1-one [92, 93], and methyl vinyl ketone [94, 95].

Alpha and beta-unsaturated ketones possess an electrophilic center (Michael's acceptor) and hence, were attributed to react with DNA bases [96]; for instance, 2-cyclohexene-1-one and methyl vinyl ketone were shown to form guanosine adducts *in vitro* [97]. It is possible that the formation of such adducts can contribute to the potential of these molecules to induce chromosomal damage; also, there is some evidence that reactive epoxide species can be at least partially involved in the mutagenic activity of this kind of compound through metabolic activation [98].

Table 4 shows alerts of the potential genotoxic carcinogenicity found in 9 molecules, among them aldehydes, ketones, alpha, and beta-unsaturated imines. In laboratory conditions, alpha and beta-unsaturated compounds undergo a Michael-type conjugated addition with nucleophile species [99]; the carcinogenicity found in aldehydes and alpha or beta-unsaturated ketones is presumably due to nucleophilic attacks in their double bonds by DNA bases.

Epoxides are strong alkylating agents; their pharmacophore was identified as active in human developmental toxicity predicted in the molecules M6, M7, and M15; this occurs due to the opening of the molecule's ring, forming a reactive ion that can alkylate the DNA, culminating in adverse effects for the developing fetus. Diepoxides are usually more reactive than monoepoxides; however, carbonyl and thiocarbonyl groups can mitigate this reactivity. Currently, there are no studies reporting teratogenesis in animals or humans caused by treatment with these chemicals [100].

Eye, skin, and respiratory tract irritation alerts were registered in four molecules. This irritation is attributed to epoxy groups and due to their lower molecular mass (MM < 200), tends to be more irritating. The occurrence of two epoxide groups instead of one results in higher irritancy; however, low-mass (< 300) epoxides with low water solubility are usually only slightly irritating for eyes and skin [101].

Except for corrosive and highly reactive compounds, the potential to induce eye and skin irritation of a chemical relies on its physicochemical properties. Skin penetration is higher in low-mass (< 500) and relatively lipophilic molecules (Log P (octanol/water) = 1-4), while ocular irritation is higher in water-soluble compounds that dissolve quickly in the eye's tear film, cornea, and conjunctiva [102].

The software MetaTox is the first web application in which the generation of a metabolic pathway is coordinated with

acute toxicity prediction. This website is useful at the beginning of drug development and offers some clinical advantages and cost reduction [25]. Some reactions of spilanthol metabolism are linked to its toxicity, such as hydroxylation, C-oxidation, N-glucuronidation, N-acetylation, epoxidation, and glutathionation. Spilanthol and the metabolites M18, M26, M28, and M33 had no collateral effects predicted.

5. Conclusion

Currently, this is the first study evaluating the reproductive and developmental toxicity of the hydroethanolic extract of *Acmella oleracea* (EHFAo). In parent zebrafish, this treatment resulted in few gonads tissue alterations, without interfering in reproduction and significantly increased eggs deposition; this has important implications for studies of nonintentional pharmaceutical treatments. Moreover, the results show that parental treatment can only induce lethal and teratogenic effects in the highest concentrations of EHFAo (100 and 200 μ/L). However, the exact mechanism of which the extract could induce toxicity at these concentrations in the embryos could not be fully elucidated since it is not possible to measure its transference from mother fish to embryos. It is possible that detrimental effects could be due to the increased deposition of eggs since it would result in few resources per egg.

Considering prediction errors, the results of pharmacokinetics parameters are, in general, within the limits of clinical relevance given in the literature for water solubility, skin permeability, PPB, BBB, and hERG.

In silico, spilanthol and 18 of its metabolites had skin sensitization potential attributed to more than one pharmacophore groups, and the molecules M3, M6, M7, M8, M16, M28, and M31 were predicted to cause chromosomal damage. For spilanthol, metabolism reactions included aliphatic hydroxylation, C-oxidation, N-glucuronidation, N-acetylation, epoxidation, and CYP450 enzyme glutathionation.

Data Availability

The statistical, figures, and tables data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank CAPES (no. 3292/2013 AUXPE) and CNPq Proc. 402332/2013-0.

References

[1] L. C. Di Stasi, *Plantas Medicinais na Amazônia e na Mata Atlântica*, Editora UNESP, São Paulo, 2002.

[2] H. Lorenzi and F. J. A. Matos, *Plantas Medicinais do Brasil: Nativas e Exóticas Cultivadas*, Instituto Plantarum, Nova Odessa, SP, USA, 2002.

[3] H. Villachica, J. E. U. Carvalho, C. H. Muller, S. C. Diaz, and M. Almanza, *Frutales y Hortalizas Promissórios de la Amazônia*, Lima: TCA; Secretaria Protempore, 1996.

[4] W. D. Ratnasooriya, K. P. P. Pieris, U. Samaratunga, and J. R. A. C. Jayakody, "Diuretic activity of Spilanthes acmella flowers in rats," *Journal of Ethnopharmacology*, vol. 91, no. 2-3, pp. 317–320, 2004.

[5] J. Boonen, B. Baert, N. Roche, C. Burvenich, and B. De Spiegeleer, "Transdermal behaviour of the N-alkylamide spilanthol (affinin) from Spilanthes acmella (Compositae) extracts," *Journal of Ethnopharmacology*, vol. 127, no. 1, pp. 77–84, 2010.

[6] M. Jacobson, "The structure of espilanthol," *Chemistry & Industry*, vol. 12, pp. 50-51, 1957.

[7] V. Prachayasittikul, S. Prachayasittikul, S. Ruchirawat, and V. Prachayasittikul, "High therapeutic potential of Spilanthes acmella: a review," *EXCLI Journal*, vol. 12, pp. 291–312, 2013.

[8] C. Storey and J. I. Salem, "Lay use of amazonian plants for the treatment of tuberculosis," *Acta Amazonica*, vol. 27, no. 3, pp. 175–182, 1997.

[9] A. Gerbino, G. Schena, S. Milano et al., "Spilanthol from Acmella oleracea lowers the intracellular levels of cAMP impairing NKCC2 phosphorylation and water channel AQP2 membrane expression in mouse kidney," *PLoS ONE*, vol. 11, no. 5, pp. 1–22, 2016.

[10] R. Yadav, N. Yadav, M. D. Kharya, and R. Savadi, "Preliminary studies on diuretic effect of Spilanthes acmella leaves extracts in rats," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 3, no. 3, pp. 245–247, 2011.

[11] R. S. Ramsewak, A. J. Erickson, and M. G. Nair, "Bioactive N-isobutylamides from the flower buds of Spilanthes acmella," *Phytochemistry*, vol. 51, no. 6, pp. 729–732, 1999.

[12] R. Favoreto and B. Gilbert, "Estado da Arte/State of the Art - *Acmella oleracea* (L.) R. K. Jansen (Asteraceae) – Jambu," *Revista Fitos*, vol. 5, no. 1, pp. 83–91, 2010.

[13] N. Vijendra and K. P. Kumar, "Traditional knowledge on ethnomedicinal uses prevailing in tribal pockets of Chhindwara and Betul Districts, Madhya Pradesh, India," *African Journal of Pharmacy and Pharmacology*, vol. 4, no. 9, pp. 662–670, 2010.

[14] S. C. Moreno, G. A. Carvalho, M. C. Picanço, E. G. F. Morais, and R. M. Pereira, "Bioactivity of compounds from Acmella oleracea against Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) and selectivity to two non-target species," *Pest Management Science*, vol. 68, no. 3, pp. 386–393, 2012.

[15] J. C. Tavares Carvalho, "Obtainment and Study of the Toxicity of Perillyl Alcohol Nanoemulsion on Zebrafish (Danio rerio)," *Journal of Nanomedicine Research*, vol. 4, no. 4, pp. 18–20, 2016.

[16] OECD: Gideline 229, "Fish Short Term Reproduction Assay, 2012".

[17] J. Han, Q. Wang, X. Wang et al., "The synthetic progestin megestrol acetate adversely affects zebrafish reproduction," *Aquatic Toxicology*, vol. 150, pp. 66–72, 2014.

[18] J. Y. Hoo, Y. Kumari, M. F. Shaikh, S. M. Hue, and B. H. Goh, "Zebrafish: a versatile animal model for fertility research," *BioMed Research International*, vol. 2016, Article ID 9732780, 20 pages, 2016.

[19] I. V. F. Santos, G. C. Souza, G. R. Santana et al., "Histopathology in Zebrafish (Danio rerio) to Evaluate the Toxicity of Medicine: An Anti-Inflammatory Phytomedicine with Janaguba Milk (Himatanthus drasticus Plumel), Cap 3, 2018".

[20] M. N. Forsatkar, M. Hedayatirad, and A. C. Luchiari, ""Not tonight zebrafish": the effects of Ruta graveolens on reproduction," *Pharmaceutical Biology*, vol. 56, no. 1, pp. 60–66, 2018.

[21] Q. Xu, D. Wu, Y. Dang, L. Yu, C. Liu, and J. Wang, "Reproduction impairment and endocrine disruption in adult zebrafish (Danio rerio) after waterborne exposure to TBOEP," *Aquatic Toxicology*, vol. 182, pp. 163–171, 2017.

[22] J. Kirchmair, A. H. Göller, D. Lang et al., "Predicting drug metabolism: experiment and/or computation?" *Nature Reviews Drug Discovery*, vol. 14, no. 6, pp. 387–404, 2015.

[23] A. Parkinson and B. Ogilvie, "Biotransformation of Xenobiotics, 2007".

[24] OECD: Guideline 236, "Fish Embryo Acute Toxicity (FET) Test, 2013".

[25] V. L. S. S. Castro, "Uso de Animais de experimentação e legislação correlata: orientações sobre estudos com peixes e roedores," in *Jaguariúna, SP: Embrapa Meio Ambiente*, p. 27, SP, 2013.

[26] S. Singh and J. Singh, "Transdermal drug delivery by passive diffusion and iontophoresis: a review," *Medicinal Research Reviews*, vol. 13, no. 5, pp. 569–621, 1993.

[27] V. S. Freitas-Blanco, M. Franz-Montan, F. C. Groppo et al., "Development and evaluation of a novel mucoadhesive film containing acmella oleracea extract for oral mucosa topical anesthesia," *PLoS ONE*, vol. 11, no. 9, pp. 1–18, 2016.

[28] D. M. Sanderson and C. G. Earnshaw, "Computer prediction of possible toxic action from chemical structure; the DEREK system," *Human & Experimental Toxicology*, vol. 10, no. 4, pp. 261–273, 1991.

[29] V. A. Ramchandani, "Genetics of alcohol metabolism," in *Alcohol, Nutrition, and Health Consequences*, pp. 15–25, Humana Press, 2013.

[30] E. Sim, A. Abuhammad, and A. Ryan, "Arylamine N-acetyltransferases: from drug metabolism and pharmacogenetics to drug discovery," *British Journal of Pharmacology*, vol. 171, no. 11, pp. 2705–2725, 2014.

[31] H. Sies, *Glutathione Conjugation*, Academic Press, London, UK, 1988.

[32] S. Taina, K. Mika, K. Tiia, L. Antti, and F. Moshe, "UDP-glucuronosyltransferases in conjugation of 5alpha- and 5beta-androstane steroids," *Drug Metabolism & Disposition*, vol. 37, 2009.

[33] N. Nakatani and M. Nagashima, "Pungent Alkamides from Spilanthes acmella L. var. oleracea Clarke," *Bioscience, Biotechnology, and Biochemistry*, vol. 56, no. 5, pp. 759–762, 1992.

[34] M. Singh and R. Chaturvedi, "Evaluation of nutrient uptake and physical parameters on cell biomass growth and production of spilanthol in suspension cultures of Spilanthes acmella Murr," *Bioprocess and Biosystems Engineering*, vol. 35, no. 6, pp. 943–951, 2012.

[35] S. Dubey, S. Maity, M. Singh, S. A. Saraf, and S. Saha, "Phytochemistry, pharmacology and toxicology of spilanthes acmella: a review," *Advances in Pharmacological Sciences*, vol. 2013, Article ID 423750, 9 pages, 2013.

[36] V. G. Cilia-López, B. I. Juárez-Flores, J. R. Aguirre-Rivera, and J. A. Reyes-Agüero, "Analgesic activity of Heliopsis longipes and its effect on the nervous system," *Pharmaceutical Biology*, vol. 48, no. 2, pp. 195–200, 2010.

[37] K. L. Tiwari, S. K. Jadhav, and V. Joshi, "An updated review on medicinal herb genus spilanthes," *Journal of Chinese Integrative Medicine*, vol. 9, no. 11, pp. 1170–1178, 2011.

[38] A. M. A. Dias, P. Santos, I. J. Seabra, R. N. C. Júnior, M. E. M. Braga, and H. C. De Sousa, "Spilanthol from Spilanthes acmella flowers, leaves and stems obtained by selective supercritical carbon dioxide extraction," *The Journal of Supercritical Fluids*, vol. 61, pp. 62–70, 2012.

[39] G. R. P. I. Abeysiri, R. M. Dharmadasa, D. C. Abeysinghe, and K. Samarasinghe, "Screening of phytochemical, physico-chemical and bioactivity of different parts of Acmella oleraceae Murr. (Asteraceae), a natural remedy for toothache," *Industrial Crops and Products*, vol. 50, pp. 852–856, 2013.

[40] J. Paulraj, R. Govindarajan, and P. Palpu, "The genus *Spilanthes* ethnopharmacology, phytochemistry, and pharmacological properties: a review," *Advances in Pharmacological Sciences*, vol. 2013, Article ID 510298, 22 pages, 2013.

[41] M. R. Rios and H. F. Olivo, "Natural and synthetic alkylamides: applications in pain therapy," in *Studies in Natural Products Chemistry*, Atta-Ur-Rahman, Ed., pp. 79–118, Elsevier, New York, NY, USA, 2014.

[42] A. Hajdu, *An Ethnopharmacological Survey Conducted in the Bolivian Amazon, and Identification of N-alkylamides and Lignans from Lepidium meyenii and Heliopsis helianthoides var. scabra with Effects on the Central Nervous System*, University of Szeged, Szeged, Hungary, 2014.

[43] M. Déciga-Campos, M. Arriaga-Alba, R. Ventura-Martínez, B. Aguilar-Guadarrama, and M. Y. Rios, "Pharmacological and toxicological profile of extract from Heliopsis longipes and affinin," *Drug Development Research*, vol. 73, no. 3, pp. 130–137, 2012.

[44] L.-C. Wu, N.-C. Fan, M.-H. Lin et al., "Anti-inflammatory effect of spilanthol from Spilanthes acmella on murine macrophage by down-regulating LPS-induced inflammatory mediators," *Journal of Agricultural and Food Chemistry*, vol. 56, no. 7, pp. 2341–2349, 2008.

[45] I. Hernández, L. Márquez, I. Martínez et al., "Anti-inflammatory effects of ethanolic extract and alkamides-derived from Heliopsis longipes roots," *Journal of Ethnopharmacology*, vol. 124, no. 3, pp. 649–652, 2009.

[46] F. Demarne and G. Passaro, "Use of an Acmella oleracea extract for the botulinum toxin-like effect thereof in an anti-wrinkle cosmetic composition," US Patent, no. 7, pp. 531-193, 2009.

[47] A. Sharma, V. Kumar, R. S. Rattan, N. Kumar, and B. Singh, "Insecticidal toxicity of spilanthol from Spilanthes acmella Murr. Against Plutella xylostella L," *American Journal of Plant Sciences*, vol. 03, no. 11, pp. 1568–1572, 2012.

[48] C. F. da Rocha, Y. D. Medeiros Souza Lima, H. O. Carvalho et al., "Action of the hydroethanolic extract of the flowers of Acmella oleracea (L.) R.K. Jansen on the reproductive performance of Wistar females rats: a popular female aphrodisiac from the Amazon," *Journal of Ethnopharmacology*, vol. 214, pp. 301–308, 2018.

[49] Y. Kiparissis, G. C. Balch, T. L. Metcalfe, and C. D. Metcalfe, "Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka (Oryzias latipes)," *Environmental Health Perspectives*, vol. 111, no. 9, pp. 1158–1163, 2003.

[50] L. P. Weber, R. L. Hill Jr., and D. M. Janz, "Developmental estrogenic exposure in zebrafish (Danio rerio): II. Histological evaluation of gametogenesis and organ toxicity," *Aquatic Toxicology*, vol. 63, no. 4, pp. 431–446, 2003.

[51] A. W. Wood and G. J. Van Der Kraak, "Apoptosis and ovarian function: novel perspectives from the teleosts," *Biology of Reproduction*, vol. 64, no. 1, pp. 264–271, 2001.

[52] I. Christianson-Heiska, P. Smeds, N. Granholm, E. Bergelin, and B. Isomaa, "Endocrine modulating actions of a phytosterol mixture and its oxidation products in zebrafish (Danio rerio)," *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, vol. 145, no. 4, pp. 518–527, 2007.

[53] OECD, "OECD Guidance document for the diagnosis of endocrine-related histopathology of fish gonads," *OECD Environmental Health and Safety Publications, Series on Testing and Assessment*, vol. 123, pp. 1–42, 2010.

[54] R. H. Devlin and Y. Nagahama, "Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences," *Aquaculture*, vol. 208, no. 3-4, pp. 191–364, 2002.

[55] Y. Nagahama, "The functional morphology of teleost gonads," in *Fish Physiology*, W. S. Hoar, D. J. Randall, and E. M. Donaldson, Eds., vol. IX, pp. 223–275, Academic Press, New York, NY, USA, 1983.

[56] V. S. Blazer, "Histopathological assessment of gonadal tissue in wild fishes," *Fish Physiology and Biochemistry*, vol. 26, no. 1, pp. 85–101, 2002.

[57] M. Blázquez, S. Zanuy, M. Carrillo, and F. Piferrer, "Structural and functional effects of early exposure to estradiol-17β and 17α-ethynylestradiol on the gonads of the gonochoristic teleost Dicentrarchus labrax," *Fish Physiology and Biochemistry*, vol. 18, no. 1, pp. 37–47, 1998.

[58] A. M. Coimbra and M. A. Reis-Henriques, "Nile tilapia, oreochromis niloticus L., reproduction inhibition by dietary exposure to aroclor 1254," *Bulletin of Environmental Contamination and Toxicology*, vol. 75, no. 2, pp. 407–412, 2005.

[59] A. M. Coimbra and M. A. Reis-Henriques, "Tilapia larvae aroclor 1254 exposure: Effects on gonads and circulating thyroid hormones during adulthood," *Bulletin of Environmental Contamination and Toxicology*, vol. 79, no. 5, pp. 488–493, 2007.

[60] M. A. Gray, A. J. Niimi, and C. D. Metcalfe, "Factors affecting the development of testis-ova in medaka, Oryzias latipes, exposed to octylphenol," *Environmental Toxicology and Chemistry*, vol. 18, no. 8, pp. 1835–1842, 1999.

[61] B. Kaptaner and G. Ünal, "Effects of 17α-ethynylestradiol and nonylphenol on liver and gonadal apoptosis and histopathology in Chalcalburnus tarichi," *Environmental Toxicology*, vol. 26, no. 6, pp. 610–622, 2011.

[62] C. D. Metcalfe, T. L. Metcalfe, Y. Kiparissis et al., "Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (Oryzias latipes)," *Environmental Toxicology and Chemistry*, vol. 20, no. 2, pp. 297–308, 2001.

[63] E. J. Zillioux, I. C. Johnson, Y. Kiparissis et al., "The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17α-ethynylestradiol," *Environmental Toxicology and Chemistry*, vol. 20, no. 9, pp. 1968–1978, 2001.

[64] S. Örn, S. Yamani, and L. Norrgren, "Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and japanese medaka after exposure to 17α-ethinylestradiol and 17β-trenbolone," *Archives of Environmental Contamination and Toxicology*, vol. 51, no. 2, pp. 237–243, 2006.

[65] L. J. Mills and C. Chichester, "Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations?" *Science of the Total Environment*, vol. 343, no. 1-3, pp. 1–34, 2005.

[66] V. Sharma, J. Boonen, N. S. Chauhan, M. Thakur, B. de Spiegeleer, and V. K. Dixit, "Spilanthes acmella ethanolic flower extract: LC-MS alkylamide profiling and its effects on sexual behavior in male rats," *Phytomedicine*, vol. 18, no. 13, pp. 1161–1169, 2011.

[67] L. Yang, N. Y. Ho, R. Alshut et al., "Zebrafish embryos as models for embryotoxic and teratological effects of chemicals," *Reproductive Toxicology*, vol. 28, no. 2, pp. 245–253, 2009.

[68] Z.-G. Lu, M.-H. Li, J.-S. Wang, D.-D. Wei, Q.-W. Liu, and L.-Y. Kong, "Developmental toxicity and neurotoxicity of two matrine-type alkaloids, matrine and sophocarpine, in zebrafish (Danio rerio) embryos/larvae," *Reproductive Toxicology*, vol. 47, pp. 33–41, 2014.

[69] A. Saley, M. Hess, K. Miller, D. Howard, and T. C. King-Heiden, "Cardiac toxicity of triclosan in developing zebrafish," *Zebrafish*, vol. 13, no. 5, pp. 399–404, 2016.

[70] K. Ji, S. Hong, Y. Kho, and K. Choi, "Effects of bisphenol S exposure on endocrine functions and reproduction of zebrafish," *Environmental Science & Technology*, vol. 47, no. 15, pp. 8793–8800, 2013.

[71] E. S. Yin, M. Rakhmankulova, K. Kucera et al., "Fusaric acid induces a notochord malformation in zebrafish via copper chelation," *BioMetals*, vol. 28, no. 4, pp. 783–789, 2015.

[72] J. C. Raine, L. Lallemand, C. M. Pettem, and D. M. Janz, "Effects of chronic dietary selenomethionine exposure on the visual system of adult and F1 generation zebrafish (Danio rerio)," *Bulletin of Environmental Contamination and Toxicology*, vol. 97, no. 3, pp. 331–336, 2016.

[73] J. Du, J. Tang, S. Xu et al., "Parental transfer of perfluorooctane sulfonate and ZnO nanoparticles chronic co-exposure and inhibition of growth in F1 offspring," *Regulatory Toxicology and Pharmacology*, vol. 98, pp. 41–49, 2018.

[74] F. Cao, H. Li, F. Zhao et al., "Parental exposure to azoxystrobin causes developmental effects and disrupts gene expression in F1 embryonic zebrafish (Danio rerio)," *Science of the Total Environment*, vol. 646, pp. 595–605, 2019.

[75] N. Aluru, J. F. Leatherland, and M. M. Vijayan, "Bisphenol a in oocytes leads to growth suppression and altered stress performance in juvenile rainbow trout," *PLoS ONE*, vol. 5, no. 5, Article ID 10741, 2010.

[76] M. Galus, S. Rangarajan, A. Lai, L. Shaya, S. Balshine, and J. Y. Wilson, "Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring," *Aquatic Toxicology*, vol. 151, pp. 124–134, 2014.

[77] J. K. Thomas and D. M. Janz, "In ovo exposure to selenomethionine via maternal transfer increases developmental toxicities and impairs swim performance in F1 generation zebrafish (Danio rerio)," *Aquatic Toxicology*, vol. 152, pp. 20–29, 2014.

[78] Q. Wang, N. L.-S. Lai, X. Wang et al., "Bioconcentration and transfer of the organophorous flame retardant 1,3-dichloro-2-propyl phosphate causes thyroid endocrine disruption and developmental neurotoxicity in zebrafish larvae," *Environmental Science & Technology*, vol. 49, no. 8, pp. 5123–5132, 2015.

[79] L. Yu, Y. Jia, G. Su et al., "Parental transfer of tris(1,3-dichloro-2-propyl) phosphate and transgenerational inhibition of growth of zebrafish exposed to environmentally relevant concentrations," *Environmental Pollution*, vol. 220, pp. 196–203, 2017.

[80] L. Veryser, L. Taevernier, T. Joshi et al., "Mucosal and blood-brain barrier transport kinetics of the plant N-alkylamide spilanthol using in vitro and in vivo models," *BMC Complementary and Alternative Medicine*, vol. 16, pp. 1–12, 2016.

[81] B. De Spiegeleer, J. Boonen, S. V. Malysheva et al., "Skin penetration enhancing properties of the plant N-alkylamide

spilanthol," *Journal of Ethnopharmacology*, vol. 148, no. 1, pp. 117–125, 2013.

[82] A. Sadym, A. Lagunin, D. Filimonov, and V. Poroikov, "Prediction of biological activity spectra via the Internet," *SAR and QSAR in Environmental Research*, vol. 14, no. 5-6, pp. 339–347, 2003.

[83] J. Boonen, B. Baert, C. Burvenich, P. Blondeel, S. De Saeger, and B. De Spiegeleer, "LC-MS profiling of N-alkylamides in Spilanthes acmella extract and the transmucosal behaviour of its main bio-active spilanthol," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 53, no. 3, pp. 243–249, 2010.

[84] W. B. Pratt and P. Taylor, Eds., *Principles of Drug Action: The Basis of Pharmacology*, Churchill Livingstone, New York, NY, USA, 3rd edition, 1990.

[85] Ajay, G. W. Bemis, and M. A. Murcko, "Designing libraries with CNS activity," *Journal of Medicinal Chemistry*, vol. 42, no. 24, pp. 4942–4951, 1999.

[86] G. Sliwoski, S. Kothiwale, J. Meiler, and E. W. Lowe Jr., "Computational methods in drug discovery," *Pharmacological Reviews*, vol. 66, no. 1, pp. 334–395, 2014.

[87] I. V. Farias, L. G. Faqueti, V. F. Noldin et al., "Cytotoxic phloroglucinol meroterpenoid from Eugenia umbelliflora fruits," *Phytochemistry Letters*, vol. 27, pp. 187–192, 2018.

[88] J. Ashby, D. A. Basketter, D. Paton, and I. Kimber, "Structure activity relationships in skin sensitization using the murine local lymph node assay," *Toxicology*, vol. 103, no. 3, pp. 177–194, 1995.

[89] A. Kasamaki, H. Takahashi, N. Tsumura, J. Niwa, T. Fujita, and S. Urasawa, "Genotoxicity of flavoring agents," *Mutation Research Letters*, vol. 105, no. 6, pp. 387–392, 1982.

[90] K. L. Dearfield, K. Harrington-Brock, C. L. Doerr, J. R. Rabinowitz, and M. M. Moore, "Genotoxicity in mouse lymphoma cells of chemicals capable of Michael addition," *Mutagenesis*, vol. 6, no. 6, pp. 519–525, 1991.

[91] National Toxicology Program (NTP), *Bone Marrow Micronucleus: Study Summary of Curcumin (CAS No. 458-37-7)*, National Toxicology Program Web Server, 1999.

[92] National Toxicology Program (NTP), *Bone Marrow Micronucleus: Study Summary of 2-cyclohexen-1-one (CAS No. 930-68-7)*, National Toxicology Program Web Server, 1994.

[93] National Toxicology Program (NTP), *Peripheral Blood Micronucleus: Study Summary of 2-cyclohexen-1-one (CAS No. 930-68-7)*, National Toxicology Program Web Server, 1998.

[94] National Toxicology Program (NTP), *Bone Marrow Micronucleus: Study Summary of Methyl Vinyl Ketone (CAS No. 78-94-4)*, National Toxicology Program Web Server, 1999.

[95] National Toxicology Program (NTP), *Peripheral Blood Micronucleus: Study Summary of Methyl Vinyl Ketone (CAS No. 78-94-4)*, National Toxicology Program Web Server, 1998.

[96] J. Ashby, R. W. Tennant, E. Zeiger, and S. Stasiewicz, "Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 42 chemicals tested for carcinogenicity by the U.S. National Toxicology Program," *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, vol. 223, no. 2, pp. 73–103, 1989.

[97] F. L. Chung, K. Roy, and S. S. Hecht, "Study of reactions of .alpha.,.beta.-unsaturated carbonyl compounds with deoxyguanosine," *The Journal of Organic Chemistry*, vol. 53, no. 1, pp. 14–17, 1988.

[98] T. Neudecker, E. Eder, C. Deininger, C. Hoffman, and D. Henschler, "Mutagenicity of methylvinyl ketone in Salmonella typhimurium TA100 - indication for epoxidation as an activation mechanism," *Mutation Research Letters*, vol. 227, no. 2, pp. 131–134, 1989.

[99] S. Patai and Z. Rappoport, "Nucleophilic attacks on carbon-carbon double bonds," *The Chemistry of the Alkenes*, vol. 1, pp. 469–584, 2010.

[100] J. L. Schardein, "Pesticides," in *Chemically Induced Birth Defects*, pp. 819–873, 3rd edition, 2000.

[101] L. Kanerva, B. Bjorkner, T. Estlander, R. Jolanki, and K. Tarvainen, "Plastic materials: occupational exposure, skin irritancy and its prevention," in *The Irritant Contact Dermatitis Syndrome*, P. G. M. van der Valk and H. I. Maibach, Eds., pp. 127–155, CRC Press, Boca Raton, Fla, USA, 1996.

[102] T. H. Gardiner, J. M. Waechter, and D. E. Stevenson, "Epoxy compounds," in *Patty's Industrial Hygiene and Toxicology*, G. D. Clayton and F. E. Clayton, Eds., vol. 2a, pp. 329–444, John Wiley, New York, NY, USA, 4th edition, 1993.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 2807926, 8 pages
https://doi.org/10.1155/2019/2807926

Research Article

Jian-Pi-Yi-Shen Decoction Relieves Renal Anemia in 5/6 Nephrectomized Rats: Production of Erythropoietin via Hypoxia Inducible Factor Signaling

Jianping Chen[1], **Fochang Wang**[1], **Shiying Huang**[1], **Xiaoyan Liu**[2], **Zhonggui Li**[1], **Airong Qi**[2], **Xinhui Liu**[2], **Tiegang Yi**[1] and **Shunmin Li**[1,2]

[1]*Shenzhen Key Laboratory of Hospital Chinese Medicine Preparation, Shenzhen Traditional Chinese Medicine Hospital, The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, Shenzhen, China*
[2]*Department of Nephrology, Shenzhen Traditional Chinese Medicine Hospital, The Fourth Clinical Medical College of Guangzhou University of Chinese Medicine, Shenzhen, China*

Correspondence should be addressed to Jianping Chen; lycjp@126.com and Shunmin Li; zyylishunmin@163.com

Received 7 January 2019; Accepted 12 February 2019; Published 3 March 2019

Guest Editor: José Carlos Tavares Carvalho

Jian-Pi-Yi-Shen (JPYS) is one of the herbal medicines for treatment of anemic patients with chronic kidney disease (CKD). However, less of scientific evidence to support JPYS involved in treating anemia has been revealed. Here, an animal study was performed to investigate its hematopoietic activities and the underlying mechanism. The 5/6 nephrectomized inductions of CKD anemic rats were randomly divided into two groups: CKD group and JPYS group. Sham-operated rats served as sham group. JPYS (1.36 g/kg/d) was administered orally to CKD rats daily for six consecutive weeks. Results showed that JPYS treatment notably improved renal function and pathological injury in CKD rats. JPYS also restored the hematological parameters, including red blood cells, hemoglobin, and hematocrit. In parallel, the reduction level of EPO was reversed by JPYS. Furthermore, JPYS induced the accumulation of hypoxia inducible factor (HIF)-α protein expression. Collectively, these results provide convincing evidence for JPYS decoction in ameliorating CKD-associated anemia, and its mechanism might be related to regulate EPO production via HIF signaling pathway.

1. Introduction

Renal anemia is a common complication of chronic kidney disease (CKD) [1]. A relative deficiency of erythropoietin (EPO) production is the central cause that renal anemia develops [2]. Recombinant human EPO (rHuEPO) and erythropoiesis-stimulating agents (ESAs) are being applied to correct anemia in patients with CKD [3]. However, in the last decade, the ESA treatment-related harms, including increased mortality, cardiovascular events, and cancer progression, have raised our concerns and stimulated researchers' interest in finding alternative therapeutic approaches [4, 5]. Traditional Chinese medicine (TCM) has been widely used in China and other areas for centuries, which has been considered as an alternative medicinal purpose for a wide range of diseases, including the prevention and treatment of CKD and its associated complications, i.e., anemia [6–8]. Therefore, TCM is of great interest for being developed as a potential drug for treatment of CKD anemia.

Jian-Pi-Yi-Shen (JPYS), a Chinese herbal decoction, consists of Astragali Radix, Salviae Miltiorrhizae Radix et Rhizoma, Dioscoreae Rhizoma, Cistanches Herba, and other four ingredients. JPYS has been clinically prescribed to patients with CKD associated anemia for decades, as it is believed to possess the efficacies of fortifying the spleen, tonifying the kidney, activating blood, and resolving stasis. Previous pharmacological studies have supported that JPYS can improve renal function and kidney injury in CKD rats [9–11] and can stimulate the transcriptional expression of EPO in cultured kidney HEK293T cells [12]. Besides, the extract of Astragali Radix and Salviae Miltiorrhizae Radix et Rhizoma deriving from JPYS also has been found to

ameliorate adenine-induced CKD rats [13]. These findings confirm the beneficial role of JPYS for treatment of CKD anemia. However, the molecular mechanism of JPYS in treating renal anemia still needs to be further studied.

The discovery of hypoxia-inducible factor (HIF) pathway in controlling EPO gene transcription has been regarded as a novel foundation that stimulates endogenous EPO production to promote physiologic erythropoietic response [14, 15]. Thus, HIF activation and increased production of endogenous EPO can be useful for therapeutic indications and manipulated for the treatment of renal anemia in CKD. Taking together, we speculate that JPYS could ameliorate renal anemia in CKD rats by targeting HIF-mediated EPO expression pathway. In this study, the improvement of anemia in CKD rats by JPYS treatment and the involvement of HIF signaling in JPYS-treated rats, including renal functions, hematological parameters, and EPO concentrations, as well as HIF activation, were investigated.

2. Materials and Methods

2.1. Preparation of JPYS Extract. JPYS extract was prepared as previously described [12]. In brief, eight herbs of JPYS were weighed and extracted in boiling water twice for 1 hour. After centrifugation, the supernatant was dried under reduced pressure to powder, and it was stored at -80°C. Before the treatment, the powder was redissolved with Milli-Q water and vortexed at room temperature.

2.2. Animals. Male Sprague-Dawley (SD) rats, eight weeks old, were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China) and maintained in a specific pathogen-free (SPF) animal facility under a 12-hour light/12-hour dark cycle. Rodent food and drinking water were provided freely. All experiments were performed with protocols approved by the Institutional Animal Care Use Committee of Guangzhou University of Chinese Medicine and in accordance with National Institutes of Health Guideline for the care and use of laboratory animals (NIH Publications No. 80–23, revised 1996).

2.3. Induction of CKD Anemia Rats. The 5/6 nephrectomy was performed in two steps as previously described [9]. The sham group underwent the same operation consisting of laparotomy and manipulation of the renal pedicles, except for the destruction of renal tissue. All the surgical operation was performed under anesthesia with sodium pentobarbital (50 mg/kg body weight, intraperitoneal injection). The 5/6 Nx rats were randomly divided into two groups: rats without drug treatment (CKD group) and rats receiving JPYS treatments at dose of 1.36 g/kg/d (CKD + JPYS group). The JPYS extract was administered orally (by gavage). The same amount of distilled water was given to sham group (n = 6). After six weeks of treatment, all rats were euthanized. Blood samples were obtained from cardiac puncture. Kidneys were removed and preserved; one part of kidney was fixed in neutral formalin and embedded in paraffin for further histological analysis; another part was dissected in ice-cold PBS to remove the medulla and snap frozen in liquid nitrogen and stored at -80°C for further western blotting analysis.

2.4. Biochemical Analysis. Red blood cell (RBC), hemoglobin (Hb), and hematocrit (HCT) were executed using the Hematology Systems (Siemens 2021i, Erlangen, Germany), according to the manufacturer's instruction manual. Blood urea nitrogen (BUN), serum creatinine (Scr), and EPO levels were measured using ELISA kits according to the manufacturer's instructions (Thermo, Waltham, Massachusetts).

2.5. Histological Analysis. The extent of renal pathological injury was examined using periodic acid-Schiff (PAS) and Masson staining. The quantitative analysis approach was conducted as described previously [10, 16]. Briefly, tubular atrophy score in PAS staining was classified as follows: 0, normal tubules; 1, rare single atrophic tubules; 2, several clusters of atrophic tubules; 3, massive atrophy. Approximately 40-50 of glomerular tuft area in each rat and six rats per group were measured using Nikon NIS-Elements BR software (version 4.10.00, Nikon, Japan) as to quantify glomerular changes. The fibrotic area in Masson staining was determined by using Image J (NIH, Bethesda, MD). A minimum of 10 microscopic fields (200x) of each rat and six rats per group were captured. Atrophy score, glomerular change, and fibrotic area were measured randomly.

2.6. Western Blot Analysis. Proteins were extracted from snap-frozen kidney cortexes and were quantified with a Bio-Rad protein assay. Equal amounts of protein lysates were loaded and separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes or polyvinylidene difluoride membranes (Millipore, USA). Nonspecific proteins were blocked by incubating the membranes in 5% non-fat milk for 1 hour at room temperature. The membranes were then incubated with primary antibodies at 4°C overnight for specific proteins, followed by incubation with HRP-conjugated secondary antibodies for 1 hour at room temperature. HRP activity was visualized using Clarity Western ECL Substrate and a ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). Image Lab software version 5.1 was applied for densitometric analysis (Bio-Rad Laboratories, USA). The following primary antibodies were used in this study: polyclonal anti-HIF-2α from rabbit (Abcam; ab199; 1:200 dilution), polyclonal anti-HIF-3α from rabbit (Abcam; ab176464; 1:1000 dilution), and monoclonal anti-β-actin from mouse (Sigma; A5441; 1:5000 dilution).

2.7. Statistical Analysis. Data are expressed as Mean ± SEM. Statistical significance among groups was evaluated by one-way ANOVA and post hoc analysis with the Least Significant Difference (LSD) test or the Games-Howell test. $P < 0.05$ was considered statistically significant. All data were performed using SPSS statistics software (version 16.0, SPSS Inc., Chicago, IL, USA).

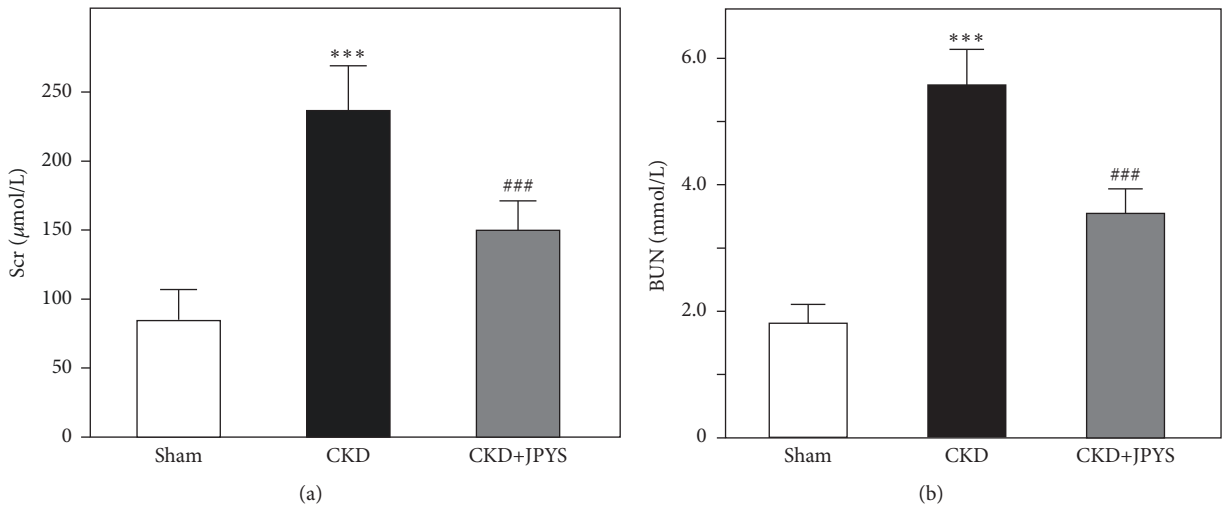


Figure 1: *JPYS improves renal function of CKD rats*. The levels of Scr (a) and BUN (b) were measured by ELISA. Values are expressed as the Mean ± SEM, where n = 6 rats per group ($***p < 0.001$ compared with the sham group; $^{###}p < 0.001$ compared with the CKD group).

3. Results

3.1. JPYS Improves Renal Function of CKD Rats. Before the treatment of extract onto the animals, JPYS was chemically standardized [11]. The minimum requirement of identified chemical amounts for 1 g of dried extract powder of JPYS should be no less than the established parameters, i.e., sodium danshensu (0.45 mg/g); salvianolic acid B (1.80 mg/g); echinacoside (0.50 mg/g); calycosin 7-O-β-glucoside (0.68 mg/g); acteoside (0.10 mg/g); liquiritin (0.60 mg/g); astragaloside IV (0.05 mg/g); formononetin (0.60 mg/g). The extraction yield was ~32.59 ± 1.1% (w/w, Mean ± SD, n = 3). The JPYS extract being used in this study met the aforesaid requirements, which could guarantee the repeatability of biological results.

To reveal the improvement of renal function by JPYS, the levels of BUN and Scr, two well-known indicators of renal function, were analyzed. Compared with sham group, rats in the CKD group showed significant higher BUN and Scr levels, indicating the decline of renal function. Compared with CKD group, treatment with JPYS extract robustly downregulated the BUN and Scr levels (Figure 1). In addition, the levels of plasma ALT and AST did not show significant difference among three groups (Supplementary Figure 1), indicating JPYS treatment would not cause hepatic toxicity in rats.

3.2. JPYS Attenuates Renal Pathological Injury in CKD Rats. PAS staining showed that renal tubules appeared normal in the sham group with tubular atrophy score at ~0.5, yet the massive tubular atrophy was observed in CKD rats with increased tubular atrophy score at ~2.5. Compared with CKD group, the renal injury and tubular atrophy were alleviated in JPYS-treated group and that of score dropped to ~1.2 (Figures 2(a) and 2(b)). In addition, glomerular injury was also observed in CKD rats. Glomerular tuft area was robustly enlarged in CKD group compared with sham group, the area of which was sufficiently decreased after JPYS treatment (Figure 2(c)). Furthermore, Masson staining demonstrated that severe interstitial fibrosis from CKD group occurred, which was elevated more markedly than that of sham group. In JPYS treatment group of rats, the fibrotic area was significantly reduced as compared to that of CKD rats (Figures 3(a) and 3(b)).

3.3. JPYS Restores the Hematological Parameters of CKD-Induced Anemic Rats. In Figure 4, the blood hematological parameters, including RBC, Hb, and HCT, were measured. Data showed that the levels of RBC, Hb, and HCT were statistically significant decreased in CKD-induced rats, confirming the 5/6 nephrectomy conduction successfully induced anemia in rats. For the CKD rats treated with JPYS extract, the decreased levels of RBC, Hb, and HCT were obviously restored, from 7.4 to 8.5×10^{12}/L (P=0.03) in RBC, 12.4 to 13.9 g/dL (P=0.02) in Hb, and 38.9 to 45.6% (P=0.04) in HCT.

3.4. JPYS Induces the Production of EPO. Renal anemia is mainly considered to be less of EPO production from the kidney. To investigate the inductive effect of JPYS on EPO, the levels of endogenous serum EPO were determined by ELISA. EPO levels were dramatically dropped in CKD group, the levels of which could be significantly raised near to normal in JPYS-treated rats (Figure 5). We further performed western blot analysis to test the activation of HIF-α protein. In CKD group, the expressions of HIF-2α and HIF-3α were increased slightly as compared to sham group. The accumulations of HIF-2α and HIF-3α by JPYS administration were much stronger than that of CKD rats (Figure 6).

4. Discussion

Anemia, an almost irreducible complication of CKD, occurs more frequently in patients with advanced kidney dysfunction and relates to quality of life and mortality in CKD patients [17, 18]. Anemia in CKD is mainly due to inadequate amount of EPO production of injured kidneys, and the EPO

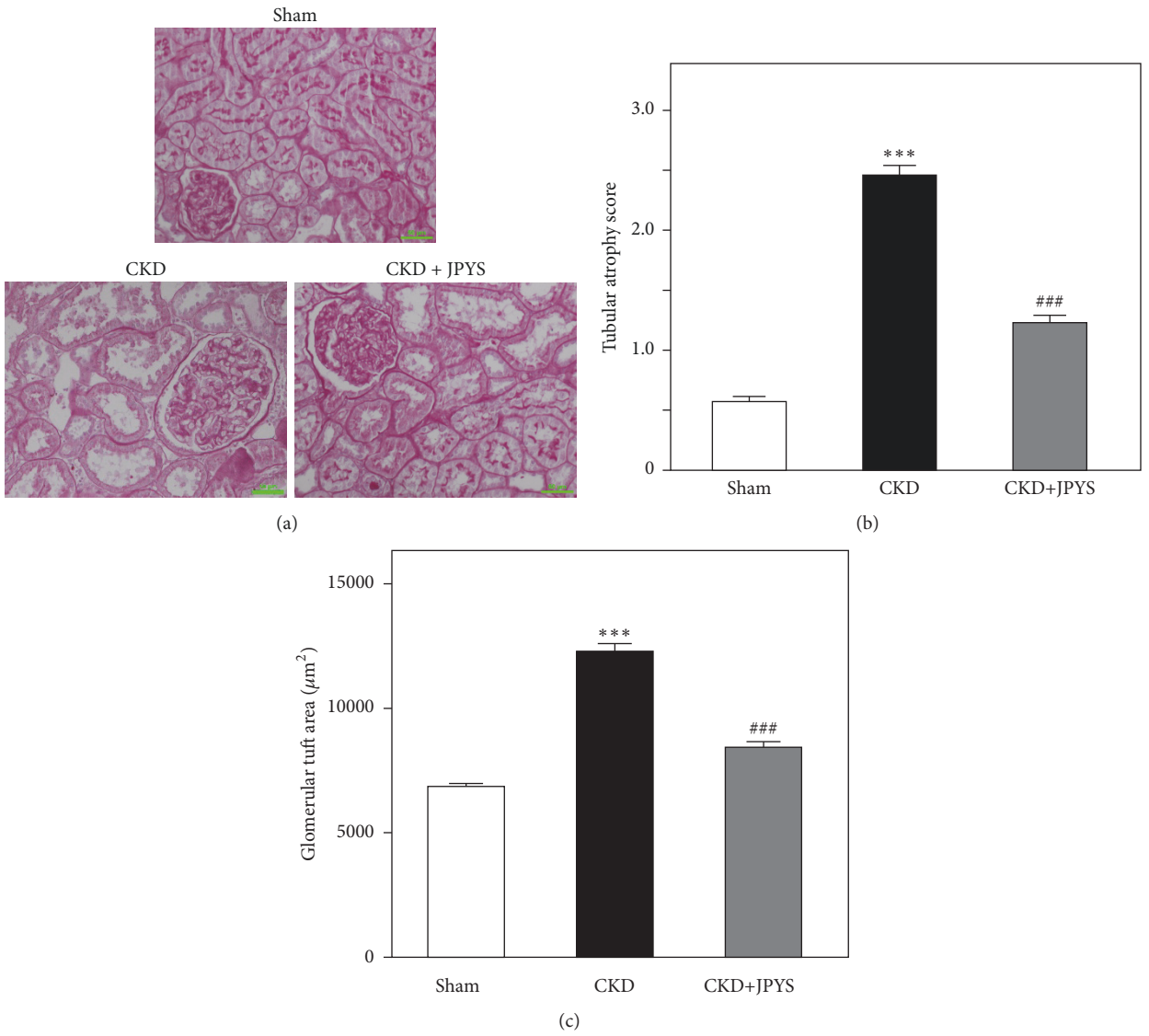


Figure 2: *JPYS attenuates renal tubular atrophy in CKD rats.* (a) PAS staining was employed to depict the kidney characteristics in each group. (b) Tubular atrophy score was quantified in each group. (c) Glomerular tuft area was quantified in each group. Representative images are shown at identical magnification, ×200, scale bar = 50 μm. Data are presented as the Mean ± SEM, n = 6 rats per group ($^{***}P < 0.001$ compared with the sham group; $^{###}P < 0.001$ compared with the CKD group).

deficiency is proposed to be the central feature of CKD associated anemia [1, 19]. HIF is a critical intermediate in the defense mechanisms against hypoxia and EPO is one of its target genes. Herein, targeting HIF stabilization in increasing more physiologic EPO levels offers a novel approach to improve the management of anemia [14].

In the present study, we investigated the effect of JPYS on improving renal anemia in CKD rats. JPYS significantly improves renal function and hematological parameters of CKD-induced anemic rats. These effects could be involved with the production of EPO via HIF signaling pathway. In line with these findings, our previous studies revealed that JPYS remarkably retards development and progression of CKD in animal model and induced expression of EPO in cultured kidney cells [9, 10, 12]. The results of this study demonstrated that the serum EPO levels were decreased in CKD rats. Consistent with our finding, Rahman et al. reported that the plasma EPO levels were remarkably reduced in animal models of renal anemia [20]. Yu et al. found that the mRNA expression of EPO was upregulated in rat remnant kidney [21]. We speculate that the variation of EPO levels in between plasma and kidney may be due to the progression stage of CKD. Indeed, the plasma EPO levels were changed during the development of CKD rats [20]. Similarly, acute renal injury increases EPO production at the beginning of the disease with a notable tendency to reduce just after the progress of injury [22].

HIF is a heterodimer comprising of an α and β subunit. The α and β subunits of HIF bind together in the cell nucleus to form a functional dimer, which results in activating

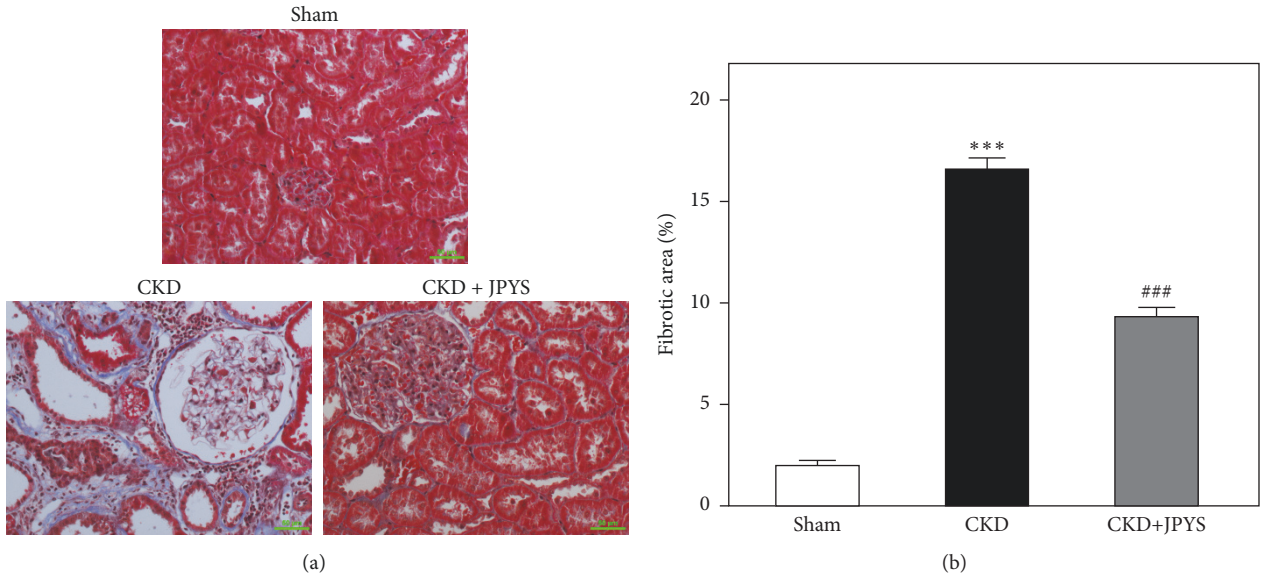


FIGURE 3: *JPYS reduces renal interstitial fibrosis in CKD rats*. (a) Masson staining was used to observe the renal tissue in each group. (b) Fibrotic area was quantified in each group. Representative images are shown at identical magnification, ×200, scale bar = 50 μm. Data are presented as the Mean ± SEM, n = 6 rats per group ($^{***}p < 0.001$ compared with the sham group; $^{\#\#\#}p < 0.001$ compared with the CKD group).

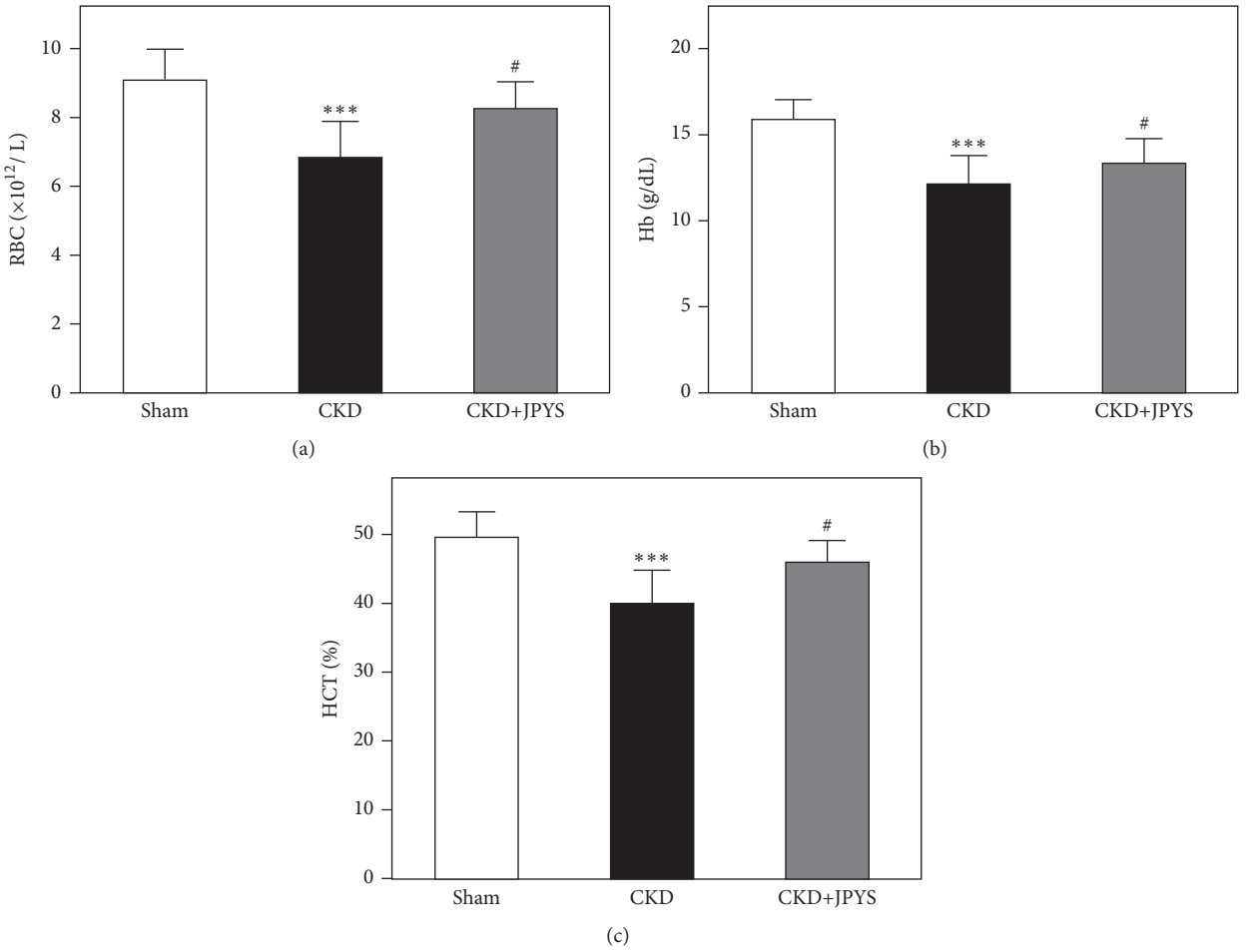


FIGURE 4: *JPYS restores the hematological parameters in CKD rats*. The levels of RBC (a), Hb (b), and HCT (c) were detected by ELISA kit. Values are expressed as the Mean ± SEM, where n = 6 rats per group ($^{***}p < 0.001$ compared with the sham group; $^{\#}p < 0.05$ compared with the CKD group).

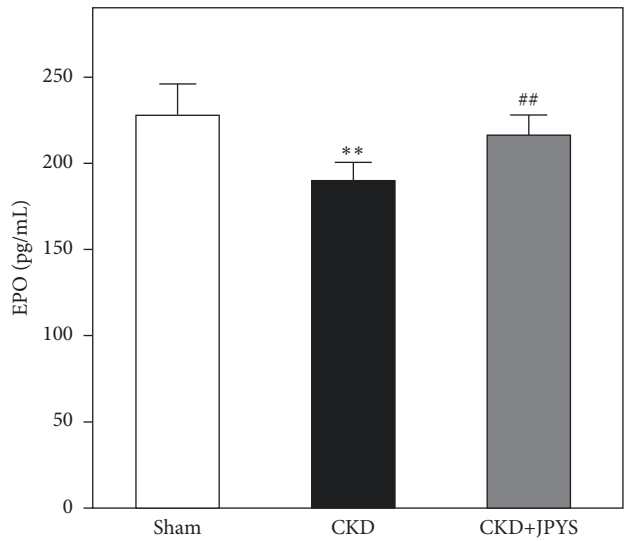


Figure 5: *JPYS stimulates EPO production in CKD rats*. EPO levels in each group were measured by ELISA. Values are expressed as the Mean ± SEM, where n = 6 rats per group ($^{**}p < 0.01$ compared with the sham group; $^{\#\#}p < 0.01$ compared with the CKD group).

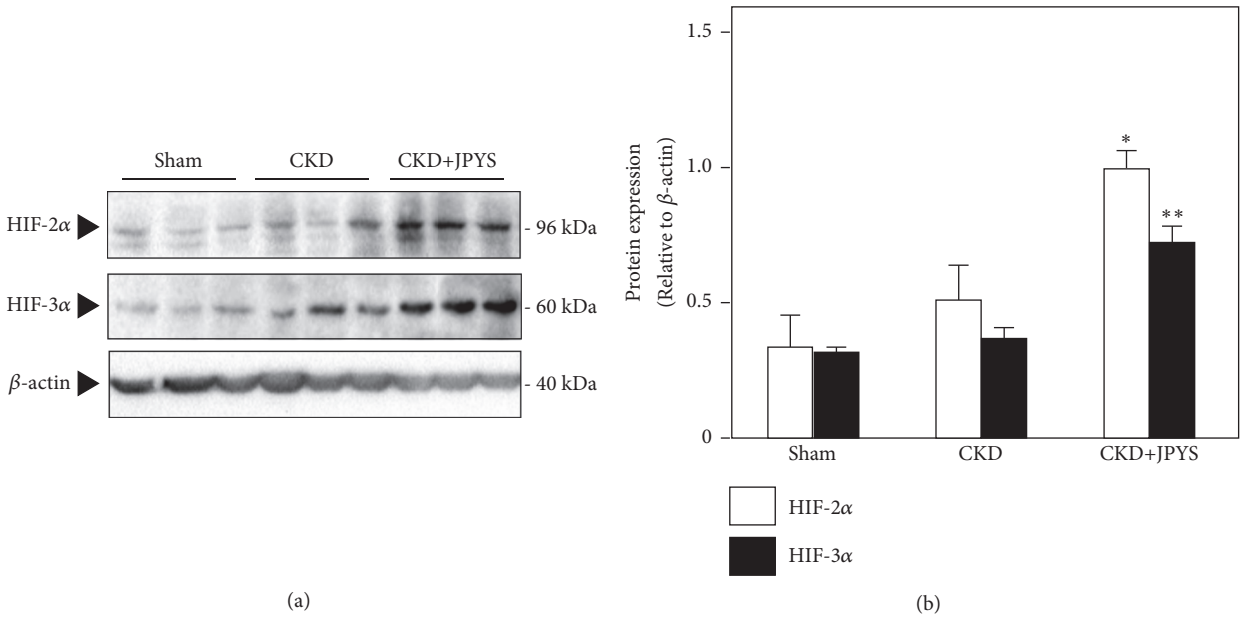


Figure 6: *Effects of JPYS on protein expressions of HIF-2α and HIF-3α in renal tissue of CKD rats*. (a) The protein lysates of kidney tissue were collected to determine the expressions of HIF-2α and HIF-3α by western blot. (b) Quantification of blot intensity for HIF-2α and HIF-3α was conducted. Representative western blot images are shown. Data are expressed as the Mean ± SEM, where n = 6 rats per group ($^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the CKD group).

transcriptional expression of erythropoietin [14]. In general, HIF-β is constitutively expressed and present excess, while HIF-α is subjected to ubiquitination and proteasomal degradation under normoxic conditions [19]. However, hypoxia causes HIF-α stabilization by inhibition of prolyl hydroxylase domain proteins. These findings represent a novel therapeutic application against anemia in chronic kidney disease [23]. HIF-α exists in three isoforms, HIF-1α, HIF-2α, and HIF-3α. In recent findings, HIF-1α plays a vital role in the response to local ischemia and has effects on angiogenesis and anaerobic metabolism [14]. HIF-2α is reported to be the key regulator of endogenous EPO gene transcription [24]. In agreement with this, our current study revealed that the protein level of HIF-2α and EPO was robustly upregulated by JPYS treatment, which supported JPYS would have benefits in anemia of CKD via HIF activation. However, the HIF-1α protein level was not detected in all experimental groups. We speculated that HIF-1α may play a role in the early period of CKD. In support of this, it was found that HIF-1 governed the response to hypoxia at the beginning and was degraded over

time, whereas HIF-2 became main regulator and mediated chronic hypoxia [25, 26]. The roles of HIF-3α are less known, although it is generally considered as a negative regulator of HIF-1α and HIF-2α [27]. Recent findings suggest that there is a switch in HIF signaling, passing the signaling from HIF-1 to HIF-2 and finally to HIF-3 during prolonged hypoxia, where HIF-3 is considered to promote angiogenesis and long-term survival [25]. In present study, JPYS treatment induced the expression of HIF-3α protein level. Angiopoietin-1 is one of the important angiogenic factors for angiogenesis. Decreased angiopoietin-1 level has been identified and the angiopoietin-1/VEGF-A ratio was decreased in patients with CKD [28]. Future prospective studies are needed to examine whether HIF-3 can stimulate angiogenesis as to play a role in progression of CKD.

The anemia of CKD has been also reported to be associated with inflammatory cytokine levels [29]. The suppressive effects of proinflammatory cytokines on erythropoiesis are one of the key factors that cause the anemia of inflammation [30]. In our previous findings, JPYS regulated the cytokine expressions in cultured macrophages and 5/6 nephrectomized CKD rat [10, 12]. We speculate that the trophic role of JPYS in renal anemia may be also associated with inhibition of inflammation. In line with this, it has been found that the increased erythropoiesis via HIF signaling inhibits hepcidin expression, whereas the upregulated hepcidin levels in patients with CKD are associated with increased inflammatory activity [17, 31].

5. Conclusion

In conclusion, JPYS decoction could relieve renal anemia in 5/6 nephrectomized rats, which might be associated with upregulation of erythropoietin via hypoxia inducible factor signaling.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Jianping Chen and Fochang Wang are co-first authors.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by Natural Science Foundation of China (81804052 and 81603437), Natural Science Foundation of Guangdong Province (2015A030310247 and 2018A030313305), and the Shenzhen Science and Technology Plan Project (JSGG20141017103353178, ZDSYS201606081515458, JCYJ20160428182041577, and JCYJ20170307154652899).

Supplementary Materials

Supplementary Figure 1: Effect of JPYS on liver function in CKD rats. The levels of ALT (A) and AST (B) were measured by ELISA. Data are expressed as the Mean ± SEM, where n = 6 rats per group. *(Supplementary Materials)*

References

[1] S. Fishbane and B. Spinowitz, "Update on anemia in ESRD and earlier stages of CKD: core curriculum 2018," *American Journal of Kidney Diseases*, vol. 71, no. 3, pp. 423–435, 2018.

[2] J. L. Babitt and H. Y. Lin, "Mechanisms of anemia in CKD," *Journal of the American Society of Nephrology*, vol. 23, no. 10, pp. 1631–1634, 2012.

[3] S. Fishbane, B. Singh, S. Kumbhat, W. A. Wisemandle, and N. E. Martin, "Intravenous epoetin Alfa-epbx versus epoetin alfa for treatment of anemia in end-stage kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 13, no. 8, pp. 1204–1214, 2018.

[4] S. C. Palmer, V. Saglimbene, D. Mavridis et al., "Erythropoiesis-stimulating agents for anaemia in adults with chronic kidney disease: a network meta-analysis," *The Cochrane Database of Systematic Reviews*, no. 12, p. D10590, 2014.

[5] G. M. Chertow, J. Liu, K. L. Monda et al., "Epoetin alfa and outcomes in dialysis amid regulatory and payment reform," *Journal of the American Society of Nephrology*, vol. 27, no. 10, pp. 3129–3138, 2016.

[6] F. Dou, H. Miao, J.-W. Wang et al., "An integrated lipidomics and phenotype study reveals protective effect and biochemical mechanism of traditionally used Alisma orientale Juzepzuk in chronic kidney disease," *Frontiers in Pharmacology*, vol. 9, p. 53, 2018.

[7] X. Wang, S. Yu, Q. Jia et al., "NiaoDuQing granules relieve chronic kidney disease symptoms by decreasing renal fibrosis and anemia," *Oncotarget* , vol. 8, no. 34, pp. 55920–55937, 2017.

[8] K. Wang, J. Wu, J. Xu et al., "Correction of anemia in chronic kidney disease with angelica sinensis polysaccharide via restoring EPO production and improving iron availability," *Frontiers in Pharmacology*, vol. 9, p. 803, 2018.

[9] D. Wang, J. Chen, X. Liu et al., "A Chinese herbal formula, Jian-Pi-Yi-Shen decoction, improves muscle atrophy via regulating mitochondrial quality control process in 5/6 nephrectomised rats," *Scientific Reports*, vol. 7, no. 1, p. 9253, 2017.

[10] J. Lu, X. Liu, Y. Liao, D. Wang, J. Chen, and S. Li, "Jian-Pi-Yi-Shen formula regulates inflammatory cytokines production in 5/6 nephrectomized rats via suppression of NF-kappaB activation," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 7203547, 7 pages, 2018.

[11] X. Liu, J. Chen, X. Liu et al., "Jian-Pi-Yi-Shen Formula ameliorates chronic kidney disease: involvement of mitochondrial quality control network," *BMC Complementary and Alternative Medicine*, vol. 18, no. 1, p. 340, 2018.

[12] J. Chen, A. Gong, X. Liu et al., "A Chinese herbal decoction, Jian-Pi-Yi-Shen, regulates the expressions of erythropoietin and pro-inflammatory cytokines in cultured cells," *BMC Complementary and Alternative Medicine*, vol. 18, no. 1, p. 119, 2018.

[13] X. Liu, S. Huang, F. Wang et al., "Huangqi-danshen decoction ameliorates adenine-induced chronic kidney disease by modulating mitochondrial dynamics," *Evidence-Based Complementary and Alternative Medicine*, vol. 2019, Article ID 9574045, 8 pages, 2019.

[14] J. M. Kaplan, N. Sharma, and S. Dikdan, "Hypoxia-inducible factor and its role in the management of anemia in chronic kidney disease," *International Journal of Molecular Sciences*, vol. 19, no. 2, p. E389, 2018.

[15] C. M. Wyatt and T. B. Drüeke, "HIF stabilization by prolyl hydroxylase inhibitors for the treatment of anemia in chronic kidney disease," *Kidney International*, vol. 90, no. 5, pp. 923–925, 2016.

[16] X. Liu, J. Wu, J. Wang et al., "Possible role of mitochondrial injury in Caulis Aristolochia manshuriensis-induced chronic aristolochic acid nephropathy," *Drug and Chemical Toxicology*, vol. 40, no. 1, pp. 115–124, 2017.

[17] F. Locatelli, S. Fishbane, G. A. Block, and I. C. MacDougall, "Targeting hypoxia-inducible factors for the treatment of anemia in chronic kidney disease patients," *American Journal of Nephrology*, vol. 45, no. 3, pp. 187–199, 2017.

[18] S. Li, R. N. Foley, and A. J. Collins, "Anemia and cardiovascular disease, hospitalization, end stage renal disease, and death in older patients with chronic kidney disease," *International Urology and Nephrology*, vol. 37, no. 2, pp. 395–402, 2005.

[19] M. Sugahara, T. Tanaka, and M. Nangaku, "Prolyl hydroxylase domain inhibitors as a novel therapeutic approach against anemia in chronic kidney disease," *Kidney International*, vol. 92, no. 2, pp. 306–312, 2017.

[20] A. Rahman, D. Yamazaki, A. Sufiun et al., "A novel approach to adenine-induced chronic kidney disease associated anemia in rodents," *PLoS ONE*, vol. 13, no. 2, Article ID e192531, 2018.

[21] X. Yu, Y. Fang, H. Liu et al., "The balance of beneficial and deleterious effects of hypoxia-inducible factor activation by prolyl hydroxylase inhibitor in rat remnant kidney depends on the timing of administration," *Nephrology Dialysis Transplantation*, vol. 27, no. 8, pp. 3110–3119, 2012.

[22] S. Morgera, P. Heering, T. Szentandrasi, C. Niederau, and B. Grabensee, "Erythropoietin in patients with acute renal failure and continuous veno- venous haemofiltration," *International Urology and Nephrology*, vol. 29, no. 2, pp. 245–250, 1997.

[23] A. A. Joharapurkar, V. B. Pandya, V. J. Patel, R. C. Desai, and M. R. Jain, "Prolyl hydroxylase inhibitors: a breakthrough in the therapy of anemia associated with chronic diseases," *Journal of Medicinal Chemistry*, vol. 61, no. 16, pp. 6964–6982, 2018.

[24] M. Xu, J. S. Nagati, J. Xie et al., "An acetate switch regulates stress erythropoiesis," *Nature Medicine*, vol. 20, no. 9, pp. 1018–1026, 2014.

[25] M. Serocki, S. Bartoszewska, A. Janaszak-Jasiecka, R. J. Ochocka, J. F. Collawn, and R. Bartoszewski, "miRNAs regulate the HIF switch during hypoxia: a novel therapeutic target," *Angiogenesis*, vol. 21, no. 2, pp. 183–202, 2018.

[26] M. Flisinski, E. Wisniewska-Chudy, A. Brymora, A. Stefanska, P. Strozecki, and J. Manitius, "Chronic kidney disease leads to hypoxia inducible factor-1alpha to hypoxia inducible factor-2alpha switch in the gastrocnemius muscle," *Journal of Physiology and Pharmacology*, vol. 68, no. 3, pp. 419–425, 2017.

[27] M. Heikkilä, A. Pasanen, K. I. Kivirikko, and J. Myllyharju, "Roles of the human hypoxia-inducible factor (HIF)-3α variants in the hypoxia response," *Cellular and Molecular Life Sciences*, vol. 68, no. 23, pp. 3885–3901, 2011.

[28] C. E. Anderson, L. L. Hamm, G. Batuman et al., "The association of angiogenic factors and chronic kidney disease," *BMC Nephrology*, vol. 19, no. 1, 2018.

[29] S. R. Keithi-Reddy, F. Addabbo, T. V. Patel, B. V. Mittal, M. S. Goligorsky, and A. K. Singh, "Association of anemia and erythropoiesis stimulating agents with inflammatory biomarkers in chronic kidney disease," *Kidney International*, vol. 74, no. 6, pp. 782–790, 2008.

[30] P. G. Fraenkel, "Anemia of Inflammation: A Review," *Medical Clinics of North America*, vol. 101, no. 2, pp. 285–296, 2017.

[31] J. Zaritsky, B. Young, H.-J. Wang et al., "Hepcidin—a potential novel biomarker for iron status in chronic kidney disease," *Clinical Journal of the American Society of Nephrology*, vol. 4, no. 6, pp. 1051–1056, 2009.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 8986038, 10 pages
https://doi.org/10.1155/2019/8986038

Research Article

BuShenKangShuai Tablet Alleviates Hepatic Steatosis via Improving Liver Adiponectin Resistance in ApoE$^{-/-}$ Mice

Shu-chao Pang,[1] **Shuo Wang**,[2] **Mei-ling Chen**,[2] **Jun-ping Zhang**,[1] **Yuan-yuan Wang**,[2] **Hui-yun Jia**,[2] **Li-yuan Bi**,[2] **and Hui Wang**[2]

[1]*First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*
[2]*Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*

Correspondence should be addressed to Mei-ling Chen; custard.chen@tjutcm.edu.cn and Jun-ping Zhang; tjzhtcm@163.com

Received 23 December 2018; Revised 27 January 2019; Accepted 3 February 2019; Published 13 February 2019

Guest Editor: José C. T. Carvalho

BuShenKangShuai tablet (BSKS) is a Chinese herbal compound, which has been used to treat nonalcoholic fatty liver disease and cardiovascular diseases in clinic for over four decades. This study intends to explore whether BSKS administration can alleviates hepatic steatosis via improving liver adiponectin resistance in ApoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were fed with western-type diet for 6 weeks and then were administrated with BSKS or atorvastatin for 6 weeks by gavage, and then blood and liver were collected for analysis. The results showed that BSKS attenuated hepatic steatosis, decreased blood lipids, and increased the serum level of adiponectin. We also found that adiponectin resistance in the liver was improved by BSKS, while the expression of TLR4 and NF-κB p65 was inhibited, followed by the suppression of proinflammatory mediators of TNF-α. Our data provided evidence that BSKS was able to alleviate hepatic steatosis in vivo. The underlying mechanism of BSKS was focused on improving liver adiponectin resistance, thereby regulating dyslipidemia and inhibiting inflammatory signaling pathway.

1. Introduction

Data from clinical, experimental, and epidemiological studies indicate that nonalcoholic fatty liver disease (NAFLD), characterized by predominantly macrovesicular hepatic steatosis, is usually considered to be the hepatic manifestation of the metabolic syndrome [1].The overall prevalence of NAFLD has been significantly growing over the past few decades, mainly as a result of its close relationship with two major worldwide epidemics, obesity and diabetes mellitus [2]. In many cases, mortality in patients with NAFLD has dramatically risen, compared with the age- and gender-matched general population [3]. The reason is that NAFLD refers to a cluster of pathological spectrum risk factors, ranging from indolent hepatic steatosis, associated with an asymptomatic benign clinical process, to advanced liver diseases (nonalcoholic steatohepatitis (NASH) to fibrosis, cirrhosis, and hepatocellular carcinoma) and progressive cardiovascular diseases and/or metabolic diseases with higher cancer risks [4]. The exact pathogenesis of NAFLD and peculiarly the mechanisms leading to disease progression have not been thoroughly elucidated. Understanding NAFLD and its management is a crucial issue in current clinical practice.

Increasing evidence indicates that the pathogenesis of NAFLD is hastened by a disturbance in adipocytokines production [5]. Among them, adiponectin, as the most abundant and adipose-specific adipokine with an important anti-inflammatory, insulin-sensitizing, and antiatherogenic role, generally predicts steatosis grade and the severity of NAFLD. Furthermore, to some extent, adiponectin can attenuate liver inflammation and fibrosis [6]. Several studies have demonstrated that hypoadiponectinemia plays an important pathophysiological role in the progression of NAFLD [7]. Data from a large, multiethnic population-based cohort have also revealed that adiponectin levels are negatively related to hepatic steatosis even after correction for ethnicity, extrahepatic abdominal adiposity, and insulin sensitivity [8]. Taking these properties into account, it may be a potential way to prevent NAFLD through elevating serum level of adiponectin [9]. Current therapeutic strategies have focused

on the indirect upregulation of adiponectin on the basis of pharmacotherapy or lifestyle modifications [6].

However, there are some contradictory results from studies where adiponectin was not associated with disease progression between patients with NASH and cirrhosis and those with hepatocellular carcinoma [10]. Indeed, studies on human or animal models have demonstrated that adiponectin increases in cirrhosis and hepatocellular carcinoma. The above-mentioned discrepancies among studies could be potentially attributed to the reduced liver function or compensative increased production of proinflammatory cytokines that may affect the increased adiponectin, independently of body composition and the presence of metabolic diseases [11, 12]. Overall evidence suggests the presence of a correlation between adiponectin levels and progressive hepatocellular damage [13]. Moreover, defective adiponectin activity has already been demonstrated in chronic liver diseases, but in this case the mechanism seems associated with adiponectin resistance, leading to hyperadiponectinemia, particularly in patients with severe fibrosis [14]. Thus, the expression of adiponectin in NAFLD, remains controversial. And the change of adiponectin activity in steatotic liver needs to be studied.

BuShenKangShuai tablet (BSKS) is a Chinese herbal compound, which has been used to treat NAFLD and cardiovascular diseases in clinic for over four decades. Our previous study has verified that BSKS could not only improve blood lipids metabolism, but also reduce the ratio of liver weight and body weight, and the ratio of white adipose tissue and body weight in high fat (HF) fed mice, fed with western-type diet ($P < 0.05$) [15]. What is more, it has also been found that BSKS could alleviate the clinical symptoms of patients with coronary heart disease angina pectoris and NAFLD in clinical research [16]. However, the mechanism of BSKS treating on NAFLD is still unclear. In this study, we try to answer above questions and intend to investigate the exact expression of adiponectin in HF-fed ApoE$^{-/-}$ mice and whether BSKS can target on liver adiponectin resistance to attenuate the degree of hepatic steatosis in ApoE$^{-/-}$ mice.

2. Materials and Methods

2.1. Animals. In this study, all of the animal experiments were carried out in accordance with institutional guidelines and under protocols approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (Tianjin, China). Eight-week-old male C57BL/6J mice and ApoE$^{-/-}$ mice on C57BL/6J genetic background (20–22g) were purchased from Beijing Huafukang Bioscience Co., Ltd. (Beijing, China) (Certificate no. SCXK (Jing) 2014-0004). Mice were housed in cages (4/cage) at a constant environment (room temperature 21–24°C, room humidity 41–62%) under a 12 h light-dark cycle and received standard diet and water ad libitum.

2.2. Drugs. BuShenKangShuai tablet (Cat. No. TJZB-Z2008110052, Specification: 0.5g/tablet) was supplied by Pharmacy Department of First Teaching Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China), whose quality control was on the basis of the medical institutions standards of Tianjin Food and Drug Administration. Atorvastatin tablet (Cat. No. H20051407, Specification: 10 mg/tablet) was produced by Pfizer Pharmaceutical Co., Ltd. (Dalian, China).

2.3. Experimental Design. After a 7-day acclimation period, C57BL/6J mice were fed with a normal diet and designated as control group (n=10), whereas ApoE$^{-/-}$ mice were fed with western-type diet (21% fat and 0.15% cholesterol, Cat. No. H10141, Beijing Huafukang Bioscience Co., Ltd.) for 6 weeks and then were randomly divided into model group (n=10), BSKS group (n=10), and atorvastatin group (n=10). Mice in model group were administered 0.3 ml isopycnic sterile distilled water by gavage, and mice in BSKS group were administered 1365 mg/kg BSKS tablets by gavage, while mice in atorvastatin group were administered 3 mg/kg atorvastatin by gavage (BSKS and atorvastatin solution preparation: BSKS tablets and atorvastatin tablets were squashed by pestle and then were moved to a tube and dissolved in 0.3 ml isopycnic sterile distilled water for each mouse). All groups underwent intervention at a fixed time once a day.

Following 6 weeks of treatment, animals were anesthetized by injecting with 10% chloral hydrate intraperitoneally. Blood was sampled from mouse eyes (orbital canthus venous plexus) and then centrifuged at 3000 r/min for 10 min, and the serum was collected for subsequent detection. Liver tissues were dissected carefully from mice and placed in a physiological saline. Part of liver tissue was fixed in 10% neutral formaldehyde buffer solution, and the remainder was placed in Eppendorf tubes and immersed in liquid nitrogen to snap-freeze them.

2.4. Hematoxylin and Eosin Staining. Fixed liver tissues were dehydrated and embedded in paraffin. Five micrometer cross sections were prepared and stained with Hematoxylin and Eosin. The area of liver tissue was measured by two independent observers using image analysis software (Image-Pro Plus 6.0, Media Cybernetics, Inc., Rockville, MD, USA).

2.5. Blood Lipids Detection and Enzyme-Linked Immunosorbent Assay (ELISA). Serum levels of low-density lipoproteins cholesterol (LDL-C) and high-density lipoproteins cholesterol (HDL-C) (Cat. No. A113-1 and A112-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were detected by double reagent direct method. Total cholesterol (TC) and triglyceride (TG) (Cat. No. A111-1 and A110-1 Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were tested by single reagent COD-PAP method. The colorimetric analysis was performed by using a microplate reader (TECAN, Männedorf, Switzerland). All measurements were performed in accordance with the manufacturer's instructions, and each sample was measured in duplicate.

Level of adiponectin in blood was measured by using mouse-specific ELISA kits according to the manufacturer's instructions (Cat. No. SEA605Mu, Wuhan USCN Business Co., Ltd., Wuhan, China). Briefly, samples were incubated for

30 min at 37°C and then were exposed to biotin-conjugated detection antibody and streptavidin-HRP, respectively, for 60 min at 37°C. Stabilized chromogen and stop solution were added to terminate the reaction, then plates were read at 450 nm (OD values) within 2 hours using a spectrophotometer.

2.6. Western Blot Analysis. Liver tissue (~50 mg Sol) was homogenized (5,000 μl/g tissue, 1:5 dilution) in ice-cold lysis buffer suitable for protein extraction and preserving phosphorylation states of proteins. Homogenates was centrifuged at 12,000 rpm for 20 min at 4°C in a microcentrifuge, then the supernatant was aspirated in a fresh tube, and protein content was performed using BSA as standards. Equal amounts of protein (30 μg protein/lane) were solubilized in 4 ×Laemmli's buffer, boiled (95°C, 5 min), electrophoresed using 8-12% SDS-polyacrylamide gels in a Tris/HCl buffer system, and followed by electrophoretic transfer to a PVDF microporous membrane. The membranes were blocked for 1 h and then incubated overnight at 4°C with the following specific primary antibodies: anti-adiponectin antibody (1:1000, Cat. No. 2789s, Cell Signaling Technology), anti-adiponectin receptor 1 (AdipoR1) antibody (1:1000, Cat. No. ab126611, Abcam), anti-adiponectin receptor 2 (AdipoR2) antibody (1:1000, Cat. No. sc-514045, Santa Cruz), anti- Toll-like receptor 4 (TLR4) antibody (1:1000, Cat. No. 14358s, Cell Signaling Technology), anti- nuclear factor-kappa B p65 (NF-κB p65) antibody (1:1000, Cat. No. 8242s, Cell Signaling Technology), and antitumor necrosis factor-α (TNF-α) antibody (1:1000, Cat. No. ab6671, Abcam). After incubation with appropriate secondary antibodies goat anti-rabbit IgG H&L (HRP) (1:1000, Cat. No. ab6721, Abcam) for 1 h, membranes were washed with phosphate-buffered saline three times. Specific bands of target proteins were detected using the enhanced chemiluminescence method (Syngene Chemigenius2; PerkinElmer, Waltham, MA) and quantified with densitometry (Gene Tools software; PerkinElmer). All bands were analyzed semiquantitatively with Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.7. Immunohistochemical Staining. Paraffin-embedded liver tissues fixed in 10% formalin were sectioned at 5-μm thick and placed onto poly-L-lysine-coated slides. Coverslips were incubated for 1 h at 60°C and then processed by conventional dewaxing. After incubation with 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity, the sections were washed 3 times with sterile distilled water, treated with normal goat serum for 30 min, and then incubated with primary antibodies at 4°C overnight. Immunohistochemical staining was conducted using antibodies against TLR4 (1:50, Cat. No. ab47093, Abcam), NF-κB p65 (1:1000, Cat. No. ab7970, Abcam), and TNF-α (1:200, Cat. No. ab6671, Abcam) as primary antibodies. After being rinsed with PBS 3 times (10 min each), the slides were incubated with the secondary antibody goat anti-rabbit IgG H&L (HRP) (1:1000, Cat. No. ab6721, Abcam) for 45 min at 37°C and then washed with PBS 3 times (10 min each). The sections were incubated in the horseradish peroxidase streptavidin for 15 min at 37°C. After being washed with PBS 3 times (10 min each), the sections were stained with DAB- H_2O_2. The sections were counterstained lightly with hematoxylin, dehydrated in ethanol, cleared in xylene, mounted with neutral rubber sealant, and then observed under the bright field microscope (Olympus, CX21). Pictures were counted in 5 microscopic fields chosen randomly at ×400 magnification under the microscope. Image analysis software (Image-Pro Plus 6.0) was used to calculate the ratio of positive to all areas, and the average value was taken.

2.8. Statistical Analysis. All parameters were expressed as mean ± S.D. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test for multiple comparisons. All analyses were performed using the SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. BSKS Alleviates Hepatic Steatosis in ApoE$^{-/-}$ Mice. To explore whether BSKS can improve the histopathological changes in hepatic steatosis caused by western-type diet, we examined liver tissues of mice with pathological section staining (Figure 1). The results of H&E staining have shown that the basic structure of the hepatic lobule was still complete, and no significant hepatic steatosis could be found in the control group. Compared with the control group, hepatocyte nuclear ballooning, hepatocyte apoptosis, Mallory's hyaline, and inflammation foci were the main types of hepatic histopathological changes in the model group. After the treatment (BuShenKangShuai tablet or atorvastatin), the histopathological changes were significantly improved, especially in the atorvastatin group, in which the hepatic cells were arranged more closely.

3.2. BSKS Decreased Blood Lipids Level. The imbalance in lipids metabolism is tightly associated with NAFLD, representing an emerging health concern. Lipid deposition within hepatocytes accumulation, resulting from a disparity between lipids availability and lipid disposal in the liver, is described as hepatic steatosis [17]. To further prove the antihepatic steatosis effect of BSKS, we next evaluated circulating lipids level (Figure 2). The result demonstrated that serum TG, TC, and LDL-C in the model group were significantly higher ($P < 0.05$), but the level of HDL-C in the model group was much lower ($P < 0.05$), in contrast with the control group. After drug administration treatment for 6 weeks, we found that BSKS showed similar lipid-lowering effect with atorvastatin, which significantly reduced serum TG, TC, and LDL-C and increased HDL-C ($P < 0.05$). These results provided evidence that the anti-hepatic steatosis effect of BSKS might be due to improving blood lipids metabolism.

3.3. BSKS Promoted the Binding Sensitivity of Adiponectin to Its Receptor. To clarify whether the expression of adiponectin

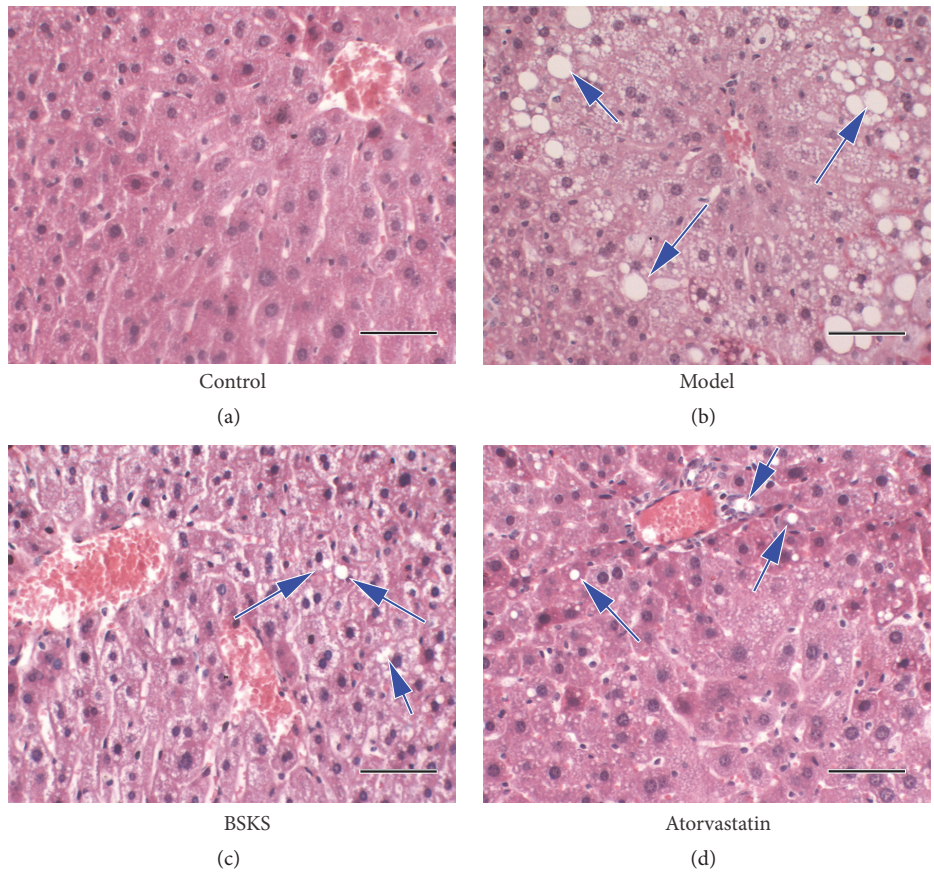


Figure 1: Effect of BuShenKangShuai tablet (BSKS) on the hepatic steatosis in ApoE−/− mice. Representative photomicrographs of Hematoxylin and Eosin staining of liver. The area of liver tissue was measured by Image-Pro Plus 6.0 (magnification, ×400). (a) No significant hepatic steatosis could be found in control group. (b) Hepatocyte nuclear ballooning, hepatocyte apoptosis, Mallory's hyaline, and inflammation foci were the main types of hepatic histopathological changes in model group. (c) The above histopathological changes were significantly improved in BSKS group. (d) Atorvastatin could also significantly alleviate the above histopathological changes. Except for this, the hepatic cells were arranged more closely in atorvastatin group. Scale bars 50 μm.

in blood and liver tissues of ApoE$^{-/-}$ mice, fed with western-type diet, was different and how it happened, we firstly performed ELISA to examine the serum level of adiponectin. Furthermore, given that adiponectin sensitivity, mediated by adiponectin/adiponectin receptors signaling, plays a significant role in the pathogenesis of NAFLD [18], we performed western blot analysis of adiponectin, AdipoR1, and AdipoR2 in adiponectin target liver tissue. In comparison to the control group, the model group expressed the lower serum level of adiponectin ($P < 0.05$) (Figure 3(a)), but higher liver tissue levels of adiponectin, AdipoR1, and AdipoR2 ($P < 0.05$) (Figure 3(b)). After treatment with BSKS and atorvastatin for 6 weeks, the serum level of adiponectin increased ($P < 0.05$) (Figure 3(a)), while the expression of adiponectin, AdipoR1, and AdipoR2 in the liver significantly decreased ($P < 0.05$), compared with the model group (Figure 3(b)).

3.4. BSKS Decreased Inflammatory Mediators Pathway. Inflammation is thought to be the driving force behind NAFLD and the progression to NASH, hepatic fibrosis, and subsequent cirrhosis [19]. Based on this, we tried to find whether BSKS could affect inflammatory mediator pathway. Primarily, we performed immunohistochemical staining of TLR4/NF-κB p65 pathways in the liver. As demonstrated in Figure 4, the immunohistochemical staining results of TLR4, NF-κB p65, and TNF-α in the liver showed that, in the control group, there were a small number of tiny tan particles scattered throughout the cytoplasm and/or the nucleus, while, in model group, the brown area was expanded, and the level of TLR4, NF-κB p65, and TNF-α had significantly increased ($P < 0.05$). As expected, after the treatment with BSKS or atorvastatin for 6 weeks, the positive staining area of TLR4, NF-κB p65, and TNF-α in the liver was decreased, in contrast with the model group ($P < 0.05$).

Furthermore, western blotting was performed to quantify the protein level of TLR4, NF-κB p65, and TNF-α in the liver. Coincidentally, the results were consistent with the immunohistochemical staining results. We found that protein levels of TLR4, NF-κB p65, and TNF-α were significantly increased in the model group, as compared to the control group ($P < 0.05$). Similarly, BSKS or atorvastatin treatment remarkably

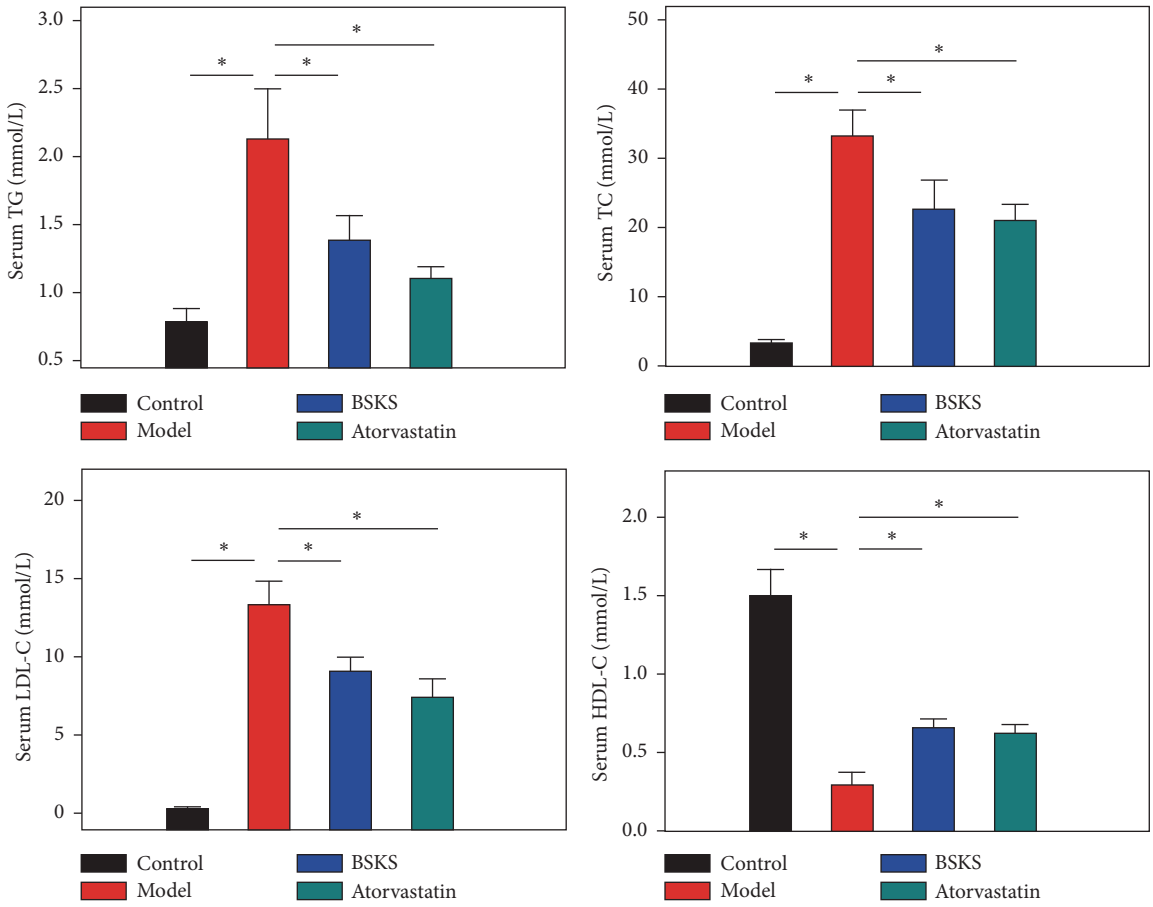


Figure 2: Effect of BSKS on blood lipids. The serum TG, TC, LDL-C, and HDL-C of mice were detected and compared between the control group and model group, model group and drug treatment groups ($n = 10$). Data are shown as mean ± S.D and compared by one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test for individual comparisons. $^{n.s.}P > 0.05$; $^{*}P < 0.05$.

inhibited the protein expression of TLR4, NF-κB p65, and TNF-α, compared with the model group ($P < 0.05$) (Figure 5).

4. Discussion

Obesity and especially visceral fat accumulation cause dyslipidemias and insulin resistance (IR), which are common risk factors for hepatic steatosis. Currently, the theory of systemic lipotoxicity (deleterious effects of lipid accumulation in nonadipose tissues are known as lipotoxicity [20]) has been applied to NAFLD, where excessive or dysfunctional regulation of free fatty acids (FFAs) and/or their metabolites induces hepatocytes injury and lipoapoptosis [19, 21]. The primary factor in the mechanism of the induction of dyslipidemias and IR by obesity is the abnormal expression of adipocytokines, physiologically active substances which are released by adipose tissue.

Adiponectin, an adipocytokine in inverse proportion to the body mass index that enhances the burning of fatty acids and the anti-inflammatory effect and improves IR and the pathological condition of NAFLD, can be used as an effective therapy against dyslipidemias and IR that accompanies obesity or NAFLD [18]. Adiponectin works, depending on binding to adiponectin receptors. Two seven-transmembrane domains proteins, AdipoR1 and AdipoR2, which are ubiquitously expressed, have been identified to function as its receptors and mediate increased fatty acid oxidation and glucose uptake by adiponectin. AdipoR1 is abundantly expressed in heart and skeletal muscle, whereas AdipoR2 is supposed to be the main receptor in the liver, suggesting an association with the pathology of liver diseases [22]. Some studies [18, 23] have shown that hepatic adiponectin sensitivity and resistance mediated by adiponectin/AdipoR signaling play a vital role in the pathogenesis of NAFLD. Evidence of adiponectin resistance has also been shown in obesity and following chronic HF-fed conditions. Customarily, people believe that the decreased expression of AdipoR1 and/or AdipoR2 leads to a decrease in adiponectin binding, and this in turn leads to decrease effects of adiponectin, termed adiponectin resistance, the so-called vicious cycle [24]. Based on this, elevating the expression of AdipoR1 and/or AdipoR2 should be a useful treatment to improve adiponectin resistance.

However, this study showed that the model group, fed with western-type diet for 12 weeks, expressed lower serum level of adiponectin ($P < 0.05$), but higher liver tissue protein levels of adiponectin, AdipoR1, and AdipoR2 ($P < 0.05$), in

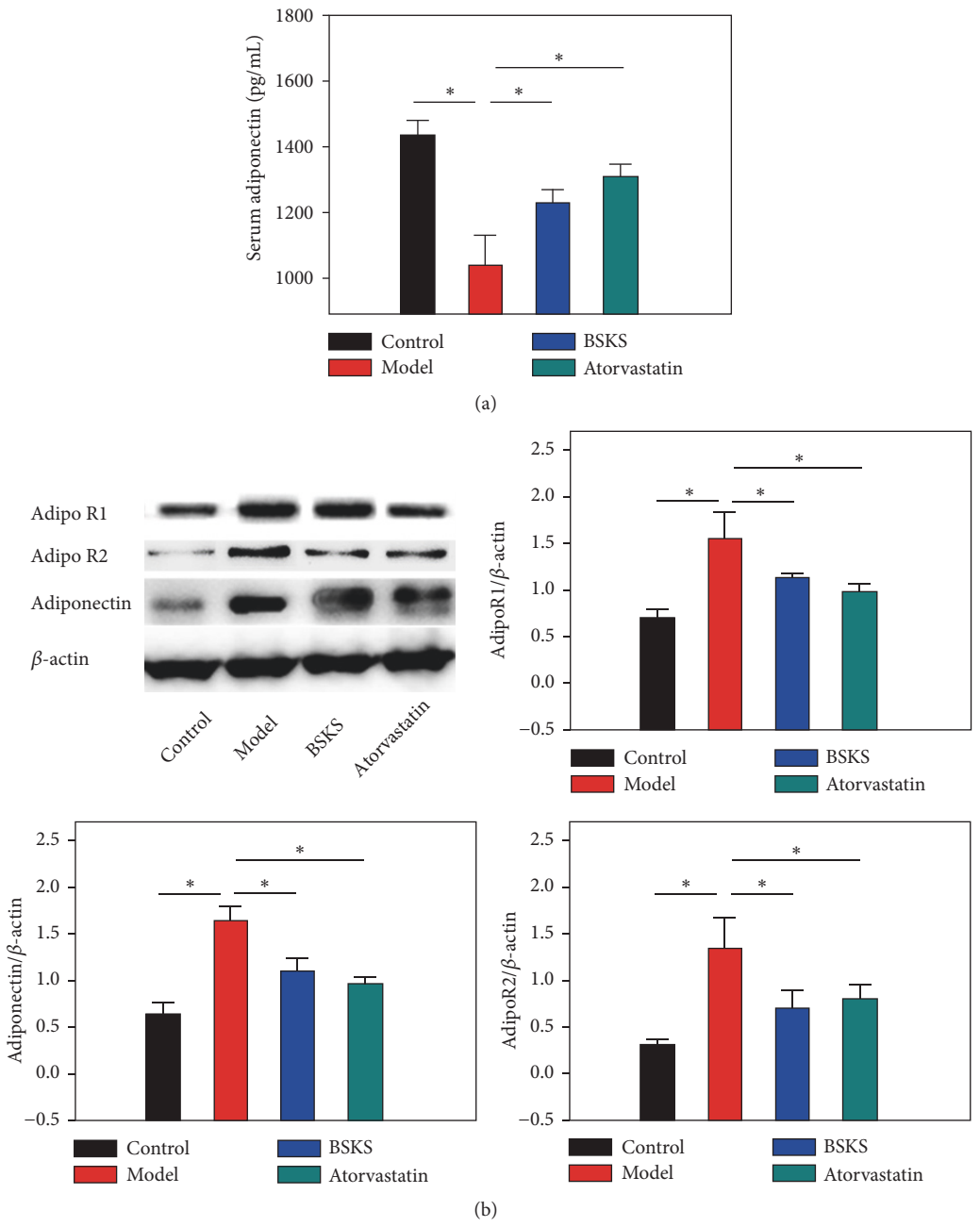


FIGURE 3: Effect of BSKS on the serum level of adiponectin and the expression of adiponectin and its receptors in the liver tissue. (a) The serum level of adiponectin was detected by ELISA ($n = 10$). (b) The expressions of adiponectin, AdipoR1, and AdipoR2 in the liver were detected by western blot ($n = 3$). The results were quantitatively compared between the control group and model group, model group and drug treatment groups. Data are shown as mean ± S.D and compared by ANOVA followed by LSD test for individual comparisons. $^{n.s.}P > 0.05$; $^{*}P < 0.05$.

contrast with the control group. While the serum level of adiponectin was upregulated, the expression of adiponectin, AdipoR1, and AdipoR2 in the liver was decreased by BSKS treatment. Changes in adiponectin and its receptors protein content do not appear to be a likely cause of adiponectin resistance. The elevated adiponectin level with adiponectin resistance is a compensatory response under the condition of an unusual discordance between IR and adiponectin unresponsiveness. Therefore, we cannot rule out the possibility that adiponectin, AdipoR1, and AdipoR2 conformation or association with the plasma membrane or other required molecules could have been altered by the HF-diet. These indicate that the low ability of adiponectin and/or low sensitivity of adiponectin binding to adiponectin receptors may provide a novel possible molecular mechanism to induce adiponectin resistance, rather than the previously well-known recognized downregulation of adiponectin receptors to induce adiponectin resistance. The decreased adiponectin

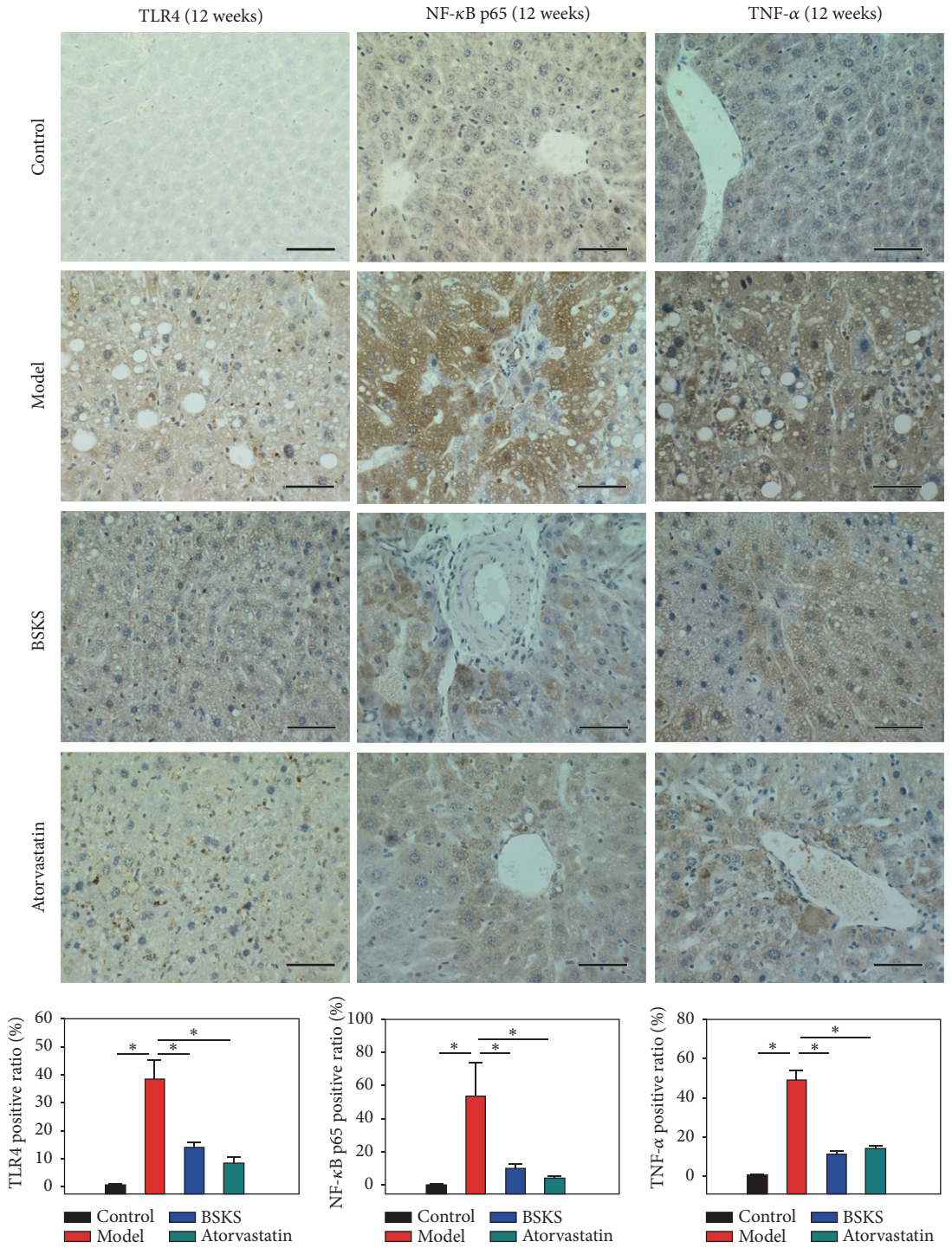


Figure 4: Effect of BSKS on TLR4 mediated signaling pathway, including TLR4, NF-κB p65, and TNF-α, which were detected by immunohistochemical staining. The percentage of immunohistochemical staining area was quantitatively analyzed by using Image-Pro Plus 6.0 (magnification, ×400). The results were quantitatively compared between the control group and model group, model group and drug treatment groups ($n = 10$). TLR4 mediated signaling pathway was activated in model group. BSKS and atorvastatin could inhibit TLR4 mediated signaling pathway. Data are shown as mean ± S.D and compared by ANOVA followed by LSD test for individual comparisons. $^{n.s.}P > 0.05$; $^{*}P < 0.05$. Scale bars 50 μm.

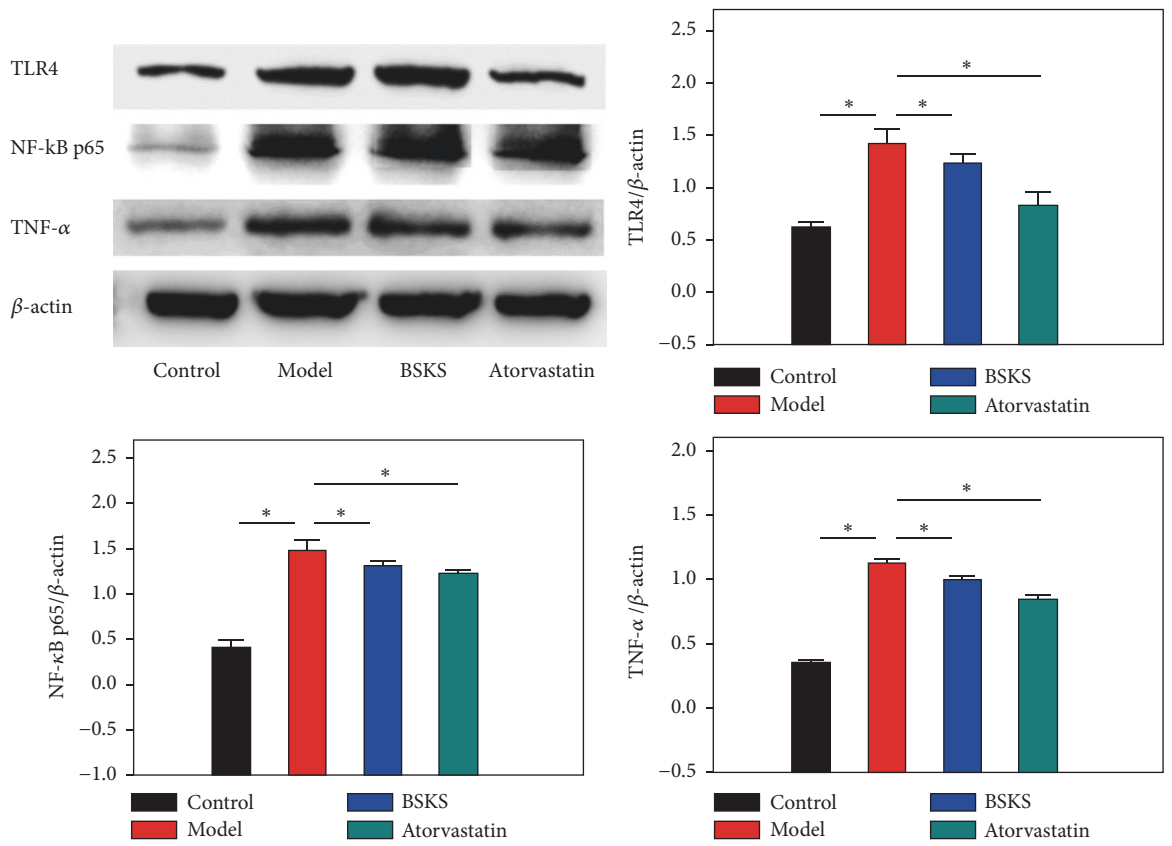


Figure 5: Effect of BSKS on TLR4 mediated signaling pathway, including TLR4, NF-κB p65, and TNF-α. The results were quantitatively compared between the control group and model group, model group and drug treatment groups ($n = 3$). The western blot results were coincidentally consistent with the immunohistochemical staining results. Data are shown as mean ± S.D and compared by ANOVA followed by LSD test for individual comparisons. $^{n.s.}P > 0.05$; $^{*}P < 0.05$.

response in HF-fed animals was not always attributable to a decrease in receptor content. Similar results have already been reported in muscle of HF-fed rats [24] and adipose tissues of HF-fed mice [25].

Bobbert et al. [26] believe that diet-induced hyperlipidemia was the main cause of adiponectin resistance, and vice versa. In this study, we found that hepatic adiponectin resistance was linearly associated with hyperlipidemia. BSKS could improve liver adiponectin resistance, as well as declining blood lipids in the HF-fed mice model. Otherwise, the result of this study indicated that decreased adiponectin signaling or disruption of adiponectin receptors activity served as an upstream pathway of increased inflammation in the liver. Previous study [19] has already suggested that the presence of inflammation on liver biopsy is associated with the development of advanced fibrosis in NAFLD. The presence of acinar and portal inflammation is increasingly recognized as an important histological feature of NASH. Thus, the therapy of anti-inflammation is a useful way to improve hepatic steatosis. There is an evidence that adiponectin attenuates liver inflammation by reducing the release of proinflammatory cytokines, the activation of hepatic stellate cells, and cell death of hepatocytes [21]. The underlying mechanism of adiponectin against hepatic inflammation is due to inhibition of TLR4 mediated signaling in the liver [27]. Here, in our study, adiponectin in the blood was upregulated, and adiponectin resistance in the liver was improved by BSKS treatment, while the expression of TLR4 and NF-κB p65 in the liver was inhibited by BSKS, followed by the suppression of proinflammatory mediators of TNF-α.

These results suggested that BSKS could alleviate hepatic steatosis in HF-fed mice, through improving the degree of liver adiponectin resistance, which might be involved in regulating dyslipidemia and inhibiting the inflammatory response via TLR4 and NF-κB p65 signaling pathway.

5. Conclusions

This study indicated that the development of hepatic steatosis was able to be alleviated by BSKS in vivo. The underlying mechanism of BSKS was focused on improving liver adiponectin resistance, thereby improving lipid metabolism and inhibiting inflammatory signaling pathway. However, further studies are needed to illustrate which active ingredient of BSKS plays the major role in improving adiponectin resistance.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Shu-chao Pang performed data analyses and wrote the manuscript; Shuo Wang should be regarded as first joint author. Yuan-yuan Wang, Hui-yun Jia, Li-yuan Bi, and Hui Wang performed the experiments. Mei-ling Chen and Jun-ping Zhang contributed to the conception of the study.

Acknowledgments

This work is financially supported by the National Natural Science Foundation of China (No. 81703702, 81403334) and Natural Science Foundation of Tianjin Municipal Education Commission (No. 2018KJ012).

References

[1] G. Marchesini, E. Bugianesi, G. Forlani et al., "Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome," *Hepatology*, vol. 37, no. 4, pp. 917–923, 2003.

[2] M. Hamaguchi, N. Takeda, T. Kojima et al., "Identification of individuals with non-alcoholic fatty liver disease by the diagnostic criteria for the metabolic syndrome," *World Journal of Gastroenterology*, vol. 18, no. 13, pp. 1508–1516, 2012.

[3] M. Lazo, R. Hernaez, S. Bonekamp et al., "Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study," *BMJ*, vol. 343, no. 7836, Article ID d6891, p. 1245, 2011.

[4] S. Petta, A. Gastaldelli, E. Rebelos et al., "Pathophysiology of non alcoholic fatty liver disease," *International Journal of Molecular Sciences*, vol. 17, no. 12, p. E2082, 2016.

[5] N. K. Gatselis, G. Ntaios, K. Makaritsis, and G. N. Dalekos, "Adiponectin: a key playmaker adipocytokine in non-alcoholic fatty liver disease," *Clinical and Experimental Medicine*, vol. 14, no. 2, pp. 121–131, 2014.

[6] C. Finelli and G. Tarantino, "What is the role of adiponectin in obesity related non-alcoholic fatty liver disease?" *World Journal of Gastroenterology*, vol. 19, no. 6, pp. 802–812, 2013.

[7] K. Krawczyk, P. Szczesniak, A. Kumor et al., "Adipohormones as prognostric markers in patients with nonalcoholic steatohepatitis (NASH)," *Journal of Physiology and Pharmacology*, vol. 60, Suppl. 3, pp. 71–75, 2009.

[8] A. T. Turer, J. D. Browning, C. R. Ayers et al., "Adiponectin as an independent predictor of the presence and degree of hepatic steatosis in the dallas heart study," *The Journal of Clinical Endocrinology & Metabolism*, vol. 97, no. 6, pp. E982–E986, 2012.

[9] S. A. Polyzos, K. A. Toulis, D. G. Goulis, C. Zavos, and J. Kountouras, "Serum total adiponectin in nonalcoholic fatty liver disease: A systematic review and meta-analysis," *Metabolism - Clinical and Experimental*, vol. 60, no. 3, pp. 313–326, 2011.

[10] V. W.-S. Wong, G. L.-H. Wong, P. C.-L. Choi et al., "Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years," *Gut*, vol. 59, no. 7, pp. 969–974, 2010.

[11] U. J. F. Tietge, K. H. W. Böker, M. P. Manns, and M. J. Bahr, "Elevated circulating adiponectin levels in liver cirrhosis are associated with reduced liver function and altered hepatic hemodynamics," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 287, no. 1, pp. E82–E89, 2004.

[12] T. A. Salman, N. Allam, G. I. Azab, A. A. Shaarawy, M. M. Hassouna, and O. M. El-Haddad, "Study of adiponectin in chronic liver disease and cholestasis," *Hepatology International*, vol. 4, no. 4, pp. 767–774, 2010.

[13] S. Corbetta, A. Redaelli, M. Pozzi et al., "Fibrosis is associated with adiponectin resistance in chronic hepatitis C virus infection," *European Journal of Clinical Investigation*, vol. 41, no. 8, pp. 898–905, 2011.

[14] L. Valenti, R. Rametta, M. Ruscica et al., "The I148M PNPLA3 polymorphism influences serum adiponectin in patients with fatty liver and healthy controls," *BMC Gastroenterology*, vol. 412, p. 111, 2012.

[15] S.-c. Pang, L. Peng, J.-p. Zhang et al., "Bushenkangshuai tablet reduces atherosclerotic lesion by improving blood lipids metabolism and inhibiting inflammatory response via TLR4 and NF-κB signaling pathway," *Evidence-Based Complementary and Alternative Medicine*, vol. 2018, Article ID 1758383, 9 pages, 2018.

[16] S. C. Pang, M. L. Chen, S. C. Lv et al., "The clinical research of patients with coronary heart disease angina pectoris and nonalcoholic fatty liver disease intervened by bushen kangshuai tablet," *Chinese Journal of Gerontology*, vol. 37, no. 9, pp. 2139–2141, 2017.

[17] N. Duarte, I. C. Coelho, R. S. Patarrão et al., "How inflammation impinges on NAFLD: a role for kupffer cells," *BioMed Research International*, vol. 2015, Article ID 984578, 11 pages, 2015.

[18] K. Tomita, Y. Oike, T. Teratani et al., "Hepatic AdipoR2 signaling plays a protective role against progression of nonalcoholic steatohepatitis in mice," *Hepatology*, vol. 48, no. 2, pp. 458–473, 2008.

[19] O. Sîrbu, M. Floria, P. Dăscălița et al., "Non-alcoholic fatty liver diseaseFrom the cardiologist perspective," *Anatolian Journal of Cardiology*, vol. 16, no. 7, pp. 534–541, 2016.

[20] M. A. Abdul-Ghani, F. L. Muller, Y. Liu et al., "Deleterious action of FA metabolites on ATP synthesis: possible link between lipotoxicity, mitochondrial dysfunction, and insulin resistance," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 295, no. 3, pp. E678–E685, 2008.

[21] M. Gil-Campos, R. Cañete, and A. Gil, "Adiponectin, the missing link in insulin resistance and obesity," *Clinical Nutrition*, vol. 23, no. 5, pp. 963–974, 2004.

[22] T. Yamauchi, J. Kamon, Y. Ito et al., "Cloning of adiponectin receptors that mediate antidiabetic metabolic effects," *Nature*, vol. 423, no. 6941, pp. 762–769, 2007.

[23] A. Tsuchida, T. Yamauchi, Y. Ito et al., "Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity," *The Journal of Biological Chemistry*, vol. 279, no. 29, pp. 30817–30822, 2004.

[24] K. L. Mullen, J. Pritchard, I. Ritchie et al., "Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 296, no. 2, pp. R243–R251, 2009.

[25] D. Gu, Z. Wang, X. Dou et al., "Inhibition of ERK1/2 pathway suppresses adiponectin secretion via accelerating protein degradation by Ubiquitin-proteasome system: relevance to obesity-related adiponectin decline," *Metabolism - Clinical and Experimental*, vol. 62, no. 8, pp. 1137–1148, 2013.

[26] T. Bobbert, J. Weicht, K. Mai, M. Möhlig, A. F. H. Pfeiffer, and J. Spranger, "Acute hyperinsulinaemia and hyperlipidaemia modify circulating adiponectin and its oligomers," *Clinical Endocrinology*, vol. 71, no. 4, pp. 507–511, 2009.

[27] S. A. Polyzos, J. Kountouras, C. Zavos, and E. Tsiaousi, "The role of adiponectin in the pathogenesis and treatment of non-alcoholic fatty liver disease," *Diabetes, Obesity and Metabolism*, vol. 12, no. 5, pp. 365–383, 2010.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 1832025, 10 pages
https://doi.org/10.1155/2019/1832025

Research Article

Adenosine Attenuates LPS-Induced Cardiac Dysfunction by Inhibition of Mitochondrial Function via the ER Pathway

Mengnan Zeng,[1,2] Beibei Zhang,[1,2] Benke Li,[1,2] Yuxuan Kan,[1,2] Shengchao Wang,[1,2] Weisheng Feng ,[1,2] and Xiaoke Zheng [1,2]

[1]*Henan University of Chinese Medicine, Zhengzhou 450046, China*
[2]*Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment & Chinese Medicine Development of Henan Province, Zhengzhou 450046, China*

Correspondence should be addressed to Weisheng Feng; fwsh@hactcm.edu.cn and Xiaoke Zheng; zhengxk.2006@163.com

Received 27 September 2018; Revised 14 November 2018; Accepted 28 November 2018; Published 10 January 2019

Academic Editor: Lucindo Quintans-Júnior

Sepsis is a life-threatening organ dysfunction syndrome with a high rate of mortality. It is caused by an abnormal immune response to infection, and the occurrence of sepsis-induced cardiomyopathy is the primary cause of death. The present study was designed to examine the effects of adenosine on lipopolysaccharide- (LPS-) induced cardiac anomalies and the underlying mechanisms involved. Adenosine (25, 50, and 100 mg/kg, i.g., 2 times/day) was administered for three days, followed by the induction of sepsis by intraperitoneal injection of LPS (10 mg/kg/2h). The effects of adenosine on inflammatory factors, LVEF, LVFS, and MAPK in septic rats (half male and half female) were observed. Subsequently, the effect of adenosine (10 μM) on the mitochondrial function of H9c2 cells stimulated with LPS (20 μg/mL, 24 h) was observed in the presence and absence of the estrogen receptor-specific antagonist ICI182,780. The results show that medium to high doses of adenosine can significantly promote cardiac function (LVEF and LVFS) and reduce the levels of inflammatory factors (TNF-α, IL-6, PCT, and cTnI) and p-JNK in septic rats, with a significant difference seen between male and female rats. The results of flow cytometry show that adenosine significantly inhibited increases in ROS levels, mitochondrial membrane potential, and the swelling degree of mitochondria in H9c2 cells stimulated with LPS, but this effect could be blocked by ICI182,780, indicating that adenosine attenuated LPS-induced cardiac dysfunction by inhibiting mitochondrial function via the ER pathway.

1. Introduction

Sepsis is a life-threatening organ dysfunction syndrome caused by an abnormal immune response to infection [1] and is one of the major causes of death in critically ill patients. According to the latest statistical data [2], the mortality rate of patients with sepsis is as high as 24.3%, and the occurrence of sepsis-induced cardiomyopathy is the primary cause of death. In a study by Pulido et al. [3], the recorded incidence of sepsis-induced cardiomyopathy was reported to be as high as 64%. In recent years, with the development of technology monitoring cardiac function, a greater understanding of sepsis-induced cardiomyopathy has been reached. The heart is an organ rich in mitochondria, and the mitochondrial dysfunction in sepsis is therefore receiving increasing attention [4]. Studies have shown that many factors are associated with the development of myocardial damage in sepsis, such as excessive production of ROS and destruction of mitochondrial membrane potential [5, 6]; however, the specific underlying mechanism of myocardial damage in patients with sepsis remains unclear. Gram-negative bacteria which could secrete lipopolysaccharide are the main pathogens that induce sepsis. In this study, LPS was used to induce sepsis.

Adenosine, a compound produced by linking the N-9 of adenine to the C-1 of D-ribose with a β-glycosidic bond, is an endogenous nucleoside distributed throughout human cells and can be directly phosphorylated in the myocardium, forming adenylate [7]. Adenosine has a physiological effect in many systems (e.g., cardiovascular system) and tissues. For instance, adenosine slows atrioventricular node conduction, blocks atrioventricular node reentry, and restores the

normal sinus rhythm of patients with paroxysmal supraventricular tachycardia (PSVT) (with or without preexcitation syndrome) [8, 9]. Whether adenosine can contribute to protection of cardiomyopathy induced by LPS has not been reported. In our previous studies, it has been reported that adenosine has estrogen-like effects mediated by the estrogen receptor (ER) [10]. Other studies have shown that drugs with estrogen-like activity can protect the immune system of mice with traumatic blood loss and reduce the occurrence of immunosuppression [11, 12]. Furthermore, estradiol (E2) has been demonstrated to modulate the function of LPS-treated macrophages and H9c2 cells [13, 14], suggesting a possible link between estrogen-like activity and sepsis. Nevertheless, it remains to be clarified whether the estrogen-like effects of adenosine can contribute to protection of cardiac dysfunction associated with LPS-induced sepsis. This study was designed to examine the effects of adenosine on LPS-induced cardiac anomalies and the underlying mechanisms involved.

Astragali Radix (named Huang Qi in China, called HQ in this study) is a traditional Chinese medicine that has the effect of supplementing qi, mainly containing astragalus saponins, sucrose, astragalus polysaccharides, amino acids, selenium, zinc, copper, and so on [15]. Researches have reported that HQ have many kinds of pharmacological activities, such as strong heart, diuresis, and antithrombotic, fight free radical damage, protect myocardium, and enhance the immune function [16]. HQ is used for treatment of myocardial injury, such as sepsis, diabetes, and hypertension [17]. In addition, HQ has estrogen-like activity. In our study, HQ is a positive control drug.

2. Materials and Methods

2.1. Plant Material and Reagents. Adenosine and LPS were purchased from Sigma (St Louis, MO, USA); Huangqi Zhusheye (referred to HQ, positive control group in this study) was purchased from Zbd Pharmaceutical Co., Ltd. (HeiLongJiang, China). Dulbecco's modified Eagle medium (DMEM, Gibco, Pittsburgh, PA, USA), heat-inactivated fetal calf serum (HyClone, Logan, UT, USA), methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) (Amresco, Seattle, WA, USA), ICI182,780 (Tocris, Bristol, UK), and Salirasib (FST, APExBIO, Amazon, USA). A microplate reader was also used (Bio-Rad, Hercules, CA, USA) were used.

2.2. Cell Culture and Treatment. H9c2 cells for *in vitro* experiments were purchased from ATCC (Rockville, MD, USA) and cultured in DMEM supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin and 10% heat-inactivated fetal calf serum at 37°C in a humidified incubator with 5% CO_2 (Thermo Scientific, Waltham, MA, USA). There were four experimental groups in the present study: control, model (LPS 20 μg/mL), HQ (0.1 mg/mL, LPS 20 μg/mL, positive control), and adenosine (10μM, LPS 20 μg/mL) groups. H9c2 cells were seeded on a 100 mm × 20 mm cell culture flask at a density of 2×10^5 cells/mL. Following treatment with HQ and adenosine for 24 h, the cells were collected. For antagonism, the estrogen antagonist Faslodex (ICI182,780, 1 μM; ER nonspecific) was added 30 min prior to treatment with HQ and adenosine to evaluate whether the observed effects were mediated by the ER. Cells from each group were used for mitochondrial swelling, membrane potential ($\Delta\Psi$m), and ROS detection assays.

2.3. Evaluation of the Degree of Mitochondrial Swelling [18]. To determine the large amplitude swelling of H9c2 cells, the isolation of the mitochondria and the cytosol was performed using a Cell Mitochondria Isolation kit (SM0020; Beijing Solarbio Science & Technology Co., Ltd., China). Briefly, H9c2 cells in each group were incubated in ice-cold mitochondrial lysis buffer for 20 min. The cell suspension was transferred to a glass homogenizer and homogenized for 20 strokes. The homogenate was subjected to centrifugation at 800 x g for 15 min at 4°C to remove the nuclei and unbroken cells. The supernatant was collected and centrifuged again at 12,000 x g for 15 min at 4°C to obtain the mitochondrial fraction. Samples of mitochondria were dissolved in buffer and subjected to flow cytometry (FCM; BD FACS Aria III; BD Biosciences, USA).

2.4. Evaluation of Mitochondrial Membrane Potential ($\Delta\Psi m$) [18]. $\Delta\Psi$m was assessed using FCM with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole-carbocyanide iodine staining (JC-1; GK3610; Genview; Beijing, China). H9c2 cells in each group were stained with JC-1 for 20 min at 37°C and subjected to FCM. JC-1 (red fluorescence) represented normal cells, and monomeric JC-1 (green fluorescence) represented cells in which $\Delta\Psi$m was increased.

2.5. Determination of Intracellular ROS Levels. The production of ROS in H9C2 cells was fluorometrically monitored using the nonfluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (CA1410; Beijing Solarbio Science & Technology Co., Ltd, China). DCFH-DA passively diffuses into cells and is deacetylated, becoming a fluorescent compound, 2′,7′-dichlorofluorescein (DCFH). DCFH reacts with ROS to form the fluorescent product, DCF, which is trapped inside the cells. Cells in each group cultured on 6-well dishes were trypsinized and collected by centrifugation. DCFH-DA, diluted in DMEM to a final concentration of 1 μM, was added to the H9c2 cells and incubated at 37°C for 20 min. Subsequently, H9c2 cells were washed three times with PBS, and DCF fluorescence was measured by FCM. Mean fluorescence intensity values represented the levels of intracellular ROS.

2.6. Animals. The present study was conducted in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. Male (6 weeks old; 200 ± 20 g) and female (6 weeks old; 180 ± 20 g) Wistar rats were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Ethical approval reference number: SCXK2016-0011). All rats were housed in cages on a 12 h/12 h light-dark schedule at a controlled temperature (22°C)

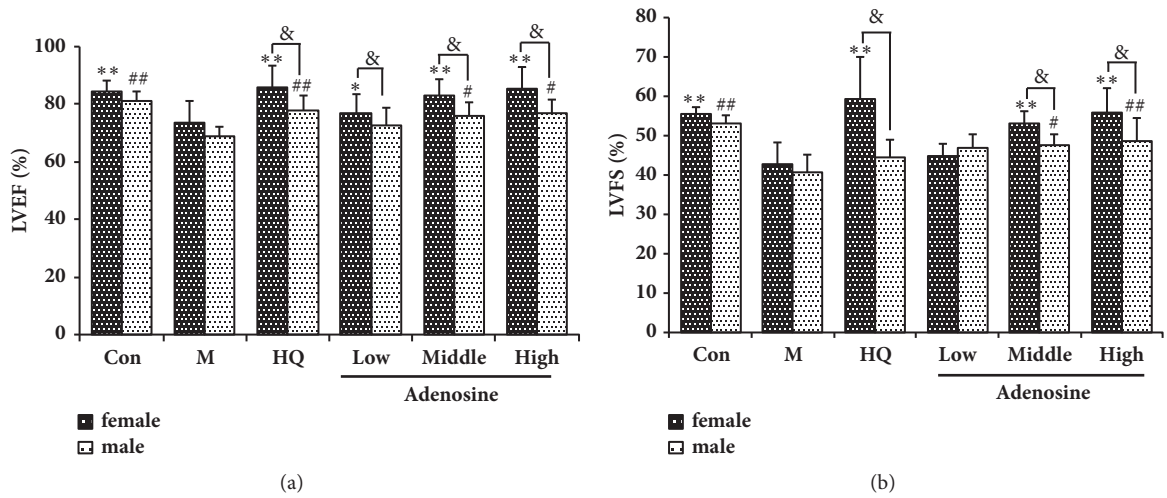


Figure 1: Effects of adenosine on heart function in LPS-induced septic rats as assessed by ultrasound. Female and male rats received LPS (10 mg/kg, i.p., 2 h) prior to gavage with different doses of adenosine. (a) Effects of different doses of adenosine on LVEF. (b) Effects of different doses of adenosine on LVFS. X ± SD, n = 8 rats per group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus female model group; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ versus male model group; $^{\&}P < 0.05$ versus male rats in each group.

with free access to food and water at the Laboratory Animal Research Center of Henan University of Chinese Medicine. All the procedures for the care of the rats were in accordance with the institutional guidelines for animal use in research. Following adaptive feeding for one week, 75 male and 75 female rats received LPS (10 mg/kg, i.p.) induced the sepsis disease, and were divided into five groups, with 15 female and 15 male rats in each: model (LPS 10 mg/kg, i.p), HQ (2000 mg/kg + LPS 10 mg/kg, i.p.), low-dose adenosine (Ad, 25 mg/kg + LPS 10 mg/kg, i.p), mid-dose adenosine (Ad, 50 mg/kg+ LPS 10 mg/kg, i.p), and high-dose adenosine (Ad, 100 mg/kg+ LPS 10 mg/kg, i.p) groups. In addition, 10 female and 10 male rats received the same volume of saline (i.p.) as a control group. Two hours later, treatment rats were gavaged with different doses of adenosine, while control rats were gavaged with water. The doses were administered as described above twice a day for 3 consecutive days. Eighteen hours after the final administration, the animals were anesthetized with ketamine hydrochloride to measure heart function, and blood was subsequently collected using the abdominal aortic method. The carefully dissected hearts were weighed on a sensitive torsion balance and immediately frozen in liquid nitrogen at -80°C.

2.7. Cytometric Bead Array (CBA). CBA was performed according to the manufacturer's instructions. Heart tissue was incubated with lysate treatment reagent (6299995; BD Biosciences; New York, USA) on ice for 15 minutes to release the intracellular phosphorylated extracellular-regulated protein kinases 1/2 (p-ERK1/2), c-Jun N-terminal kinase (p-JNK), and p-p38 proteins into the supernatant. A total of 50 μL heart tissue lysate from each sample was incubated with 50 μL anti-p-ERK1/2 (7205548; BD), anti-p-JNK (7170701; BD), or anti-p-p38 (7202572; BD) antibody conjugated to beads and 50 μL PE-labeled anti-p-ERK1/2, anti-p-JNK, and anti-p-p38 in the dark at room temperature for 2 hours with sufficient shaking. Following washing with CBA wash buffer, the samples were resuspended in 500 μL CBA wash buffer and analyzed immediately by FCM using the Cell Quest software (BD). The Tracking Beads system was used according to the manufacturer's guidelines.

2.8. ELISA. Plasma was collected and used to detect the levels of interleukin 6 (IL-6; R180109-003a; Neobioscience Co., Ltd., Shenzhen, China), tumor necrosis factor α (TNF-α; R180109-102a; Neobioscience Co., Ltd., Shenzhen, China), procalcitonin (PCT; CSB-E13419r; CUSABIO BIOTECH CO., Ltd., Wuhan, China), and cardiac troponin I (cTnI; 2HSPA1018; Life Diagnostics, lnc., West Chester, PA) according to the respective manufacturer's instructions.

2.9. Statistical Analysis. Analyses were performed using the SPSS 20.0 software (IBM; New York, NY, USA). Statistical significance was assessed in comparison with the respective control for each experiment using one-way ANOVA. A p value less than 0.05 was accepted as significant.

3. Results

3.1. Effect of Adenosine on Heart Function in LPS-Induced Septic Rats. Ultrasound revealed that LPS challenge significantly decreased left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS), the effect of which was abrogated by adenosine. As a positive control, HQ also increased LVEF and LVFS in LPS-induced septic rats. Interestingly, the effects of HQ and adenosine were different between male and female rats; HQ and adenosine displayed a better effect on heart function in female rats, as shown in Figure 1.

3.2. Effects of Adenosine on Proinflammatory Cytokines in LPS-Induced Septic Rats. To assess the effect of adenosine on

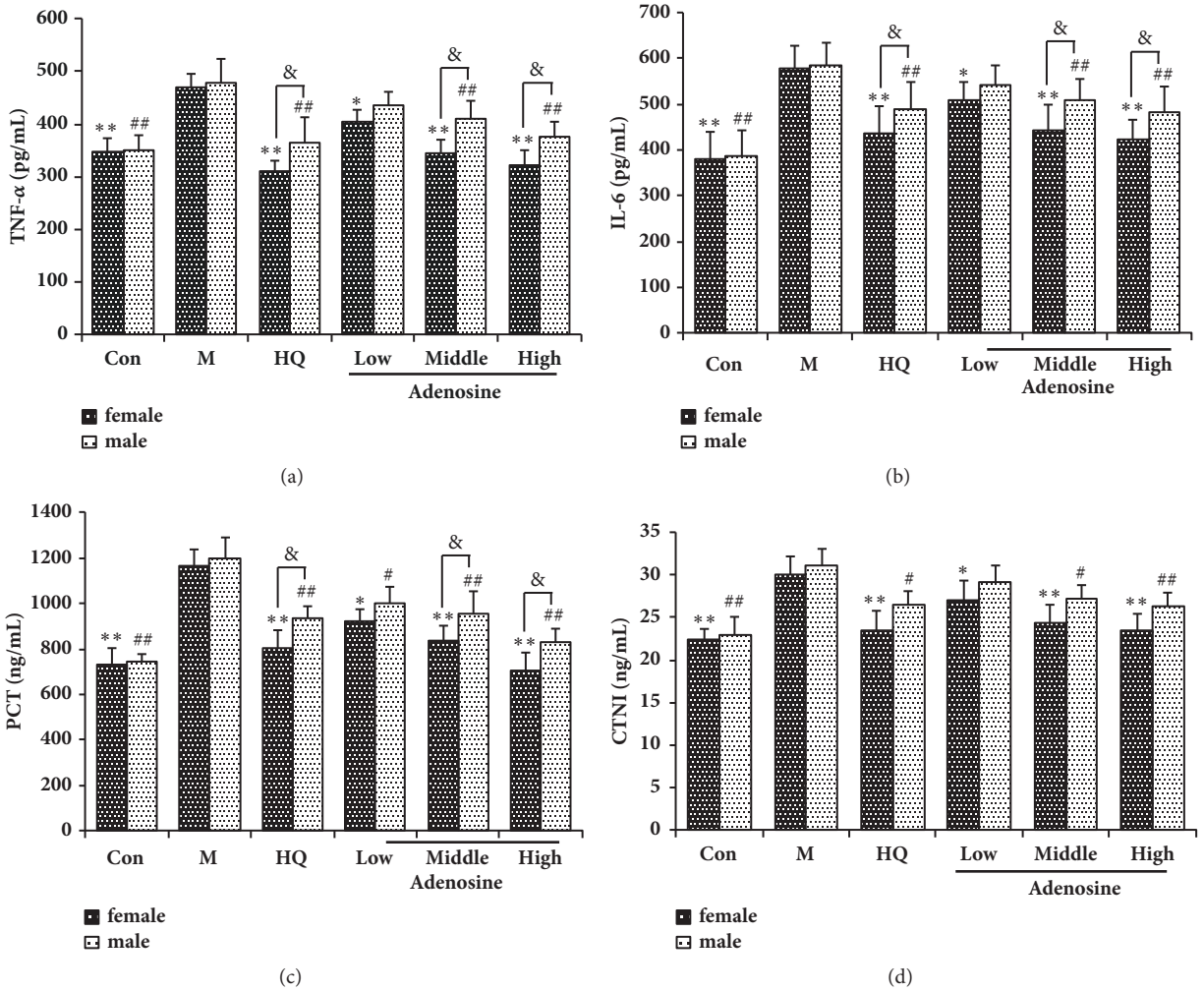


Figure 2: Effects of adenosine on proinflammatory cytokine levels in LPS-induced septic rats as assessed by ELISA. Female and male rats received LPS (10 mg/kg, i.p., 2 h) prior to gavage with different doses of adenosine. (a) Effects of different doses of adenosine on TNF-α. (b) Effects of different doses of adenosine on IL-6. (c) Effects of different doses of adenosine on PCT. (d) Effects of different doses of adenosine on cTnI. X ± SD, n = 8 rats per group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus female model group; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ versus male model group; $^{\&}P < 0.05$ versus male rats in each group.

proinflammatory cytokine production, the levels of TNFα, IL-6, PCT, and cTnI were evaluated by ELISA. As shown in Figure 2, LPS significantly elevated the levels of all the measured proinflammatory cytokines, while HQ and adenosine attenuated this increase, thereby conferring cardioprotection. Moreover, we also observed that the effect of HQ and adenosine showed better effects of mitigated cytokines in female rats.

3.3. Effects of Adenosine on the MAPK Signaling Pathway in LPS-Induced Septic Rats. To investigate whether the MAPK signaling pathway contributes to the cardioprotection offered by adenosine against LPS treatment, the levels of phosphorylated stress signaling molecules p38, ERK, and JNK were evaluated by CBA using flow cytometry. The results demonstrated that HQ and adenosine decreased the phosphorylation of JNK and p38 and increased the phosphorylation of ERK. In addition, the effects of HQ and adenosine on the phosphorylation levels of all three kinases were different between male and female rats, the latter of which had a better prognosis. The results are shown in Figure 3.

3.4. The Antagonistic Activity of ICI182,780 against Adenosine on the Mitochondrial Swelling in LPS-Stimulated H9c2 Cells. With the aim of understanding mitochondrial function more objectively, mitochondrial swelling was evaluated by FCM the following determination of the FSC-SSC parameters. FSC correlates highly with cell size or volume, and SSC is related to granularity and the refractive index. The quantitative degree of mitochondrial swelling was represented by the FSC/SSC ratio. LPS significantly elevated the levels of mitochondrial swelling in H9c2 cells, while HQ and adenosine attenuated this increase. Moreover, ICI182,780 could block the effect of HQ and adenosine on mitochondrial swelling but had no effect alone. The results are shown in Figure 4.

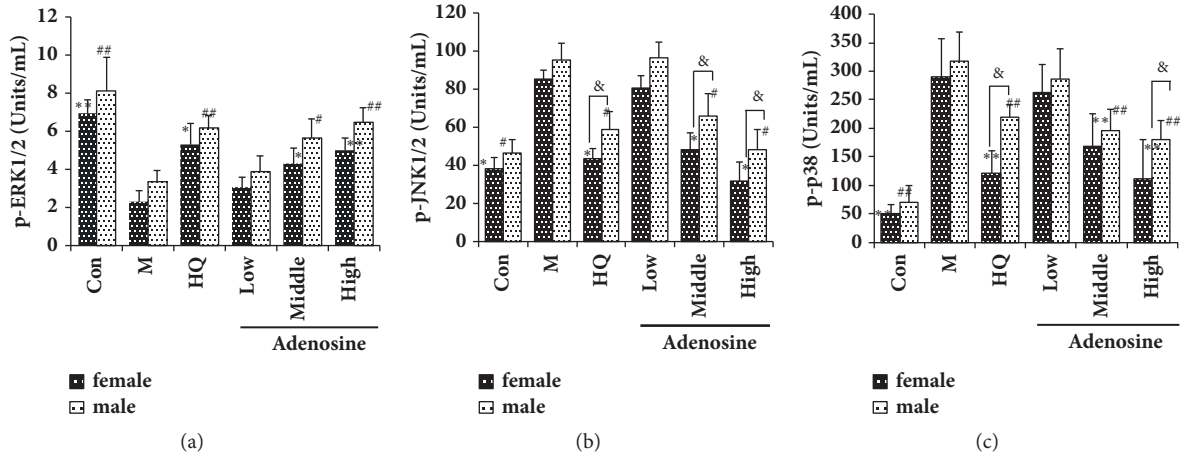


Figure 3: Effects of adenosine on the MAPK signaling pathway in LPS-induced septic rats as assessed by CBA. Female and male rats received LPS (10 mg/kg, i.p., 2 h) prior to gavage with different doses of adenosine. (a) Effects of different doses of adenosine on the phosphorylation of ERK. (b) Effects of different doses of adenosine on the phosphorylation of JNK. (c) Effects of different doses of adenosine on the phosphorylation of p38. X ± SD, n = 8 rats per group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus female model group; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ versus male model group; $^{\&}P < 0.05$ versus male rats in each group.

3.5. The Antagonistic Activity of ICI182,780 against Adenosine on the MMP (ΔΨm) in LPS-Stimulated H9c2 Cells. For a more objective understanding of MMP, FCM was used to detect ΔΨm; JC-1 aggregates in the mitochondria and emits red fluorescence, which can be easily monitored. Treatment of H9c2 cells with LPS for 24 h resulted in the dissipation of MMP, as shown by reduced JC-1 staining. The red fluorescence intensity in the HQ and adenosine groups was stronger than that in the LPS group, indicating that LPS significantly elevated the levels of ΔΨm in H9c2 cells, while HQ and adenosine attenuated this increase. In addition, ICI182,780 could block the effect of HQ and adenosine on ΔΨm, but had no effect alone. The results are shown in Figure 5.

3.6. The Antagonistic Activity of ICI182,780 against Adenosine on ROS Production in LPS-Stimulated H9c2 Cells. To explore the molecular mechanism of adenosine, the levels of ROS were detected by FCM. As shown in Figure 6, LPS significantly elevated the levels of ROS in H9c2 cells, while HQ and adenosine attenuated this increase. Moreover, ICI182,780 could block the effects of HQ and adenosine on ROS production to differing degrees, but had no effect alone.

4. Discussion

Sepsis-induced myocardial dysfunction (SIMD) is an important predictive factor for the prognosis of sepsis [19]. SIMD is a common complication of severe sepsis, with clinical manifestations including myocardial damage and cardiac dysfunction. The mechanism of SIMD is very complicated; excessive release of cytokines [20], excessive activation of complement [21], apoptosis of myocardial cells [22], and an imbalance in energy metabolism are all considered related factors [23]. Through clinical research, Edo Y et al. observed a reduction in LVEF and left ventricular dilatation in patients in the early stage of sepsis [24]. Due to the portability and noninvasiveness of the echocardiograph, it has become the most commonly used tool for the assessment of cardiac function. Therefore, in the present study, the effect of adenosine on cardiac function in septic rats was first assessed by echocardiography. The results show that HQ (2000 mg/kg) and adenosine (50 and 100 mg/kg) can significantly improve LVEF and LVFS in septic rats, thus promoting their cardiac function. Interestingly, the therapeutic effect of HQ and adenosine in female septic rats was significantly superior to that in male septic rats ($P < 0.05$).

Gram-negative bacteria [25] are the main pathogens that induce sepsis. The lipopolysaccharides (LPS) located in the cell wall of these bacteria are lipid and polysaccharide complexes that can activate mononuclear cells and macrophages, causing many pathophysiological changes, including the release of a large number of proinflammatory factors such as TNF-α and IL-6. Procalcitonin (PCT) is a nonhormone glycoprotein that is released into the circulatory system of patients with severe systemic infections, particularly those caused by bacteria, and is used as the most accurate serological diagnostic marker for sepsis to judge the type and severity of infection [26]. cTnI is a marker of myocardial injury, with high sensitivity and specificity, and is of great significance for the diagnosis and risk stratification of myocardial infarction. In recent years, studies have found that cTnI is elevated in patients with sepsis-related cardiomyopathy, and its increase often reflects the severity of myocardial cell injury, which is closely related to disease severity and mortality [27]. The results show that HQ (2000 mg/kg) and adenosine (50 and 100 mg/kg) significantly reduced serum TNF-α, IL-6, PCT, and cTnI levels in septic rats. Similarly, HQ and adenosine had a significant male–female difference in the regulation of serum inflammatory factors in septic rats ($P < 0.05$).

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific and are widely distributed in eukaryotic cells. Typical MAPKs include c-Jun-N-terminal kinase (JNK), p38 kinases, and extracellular signal transduction

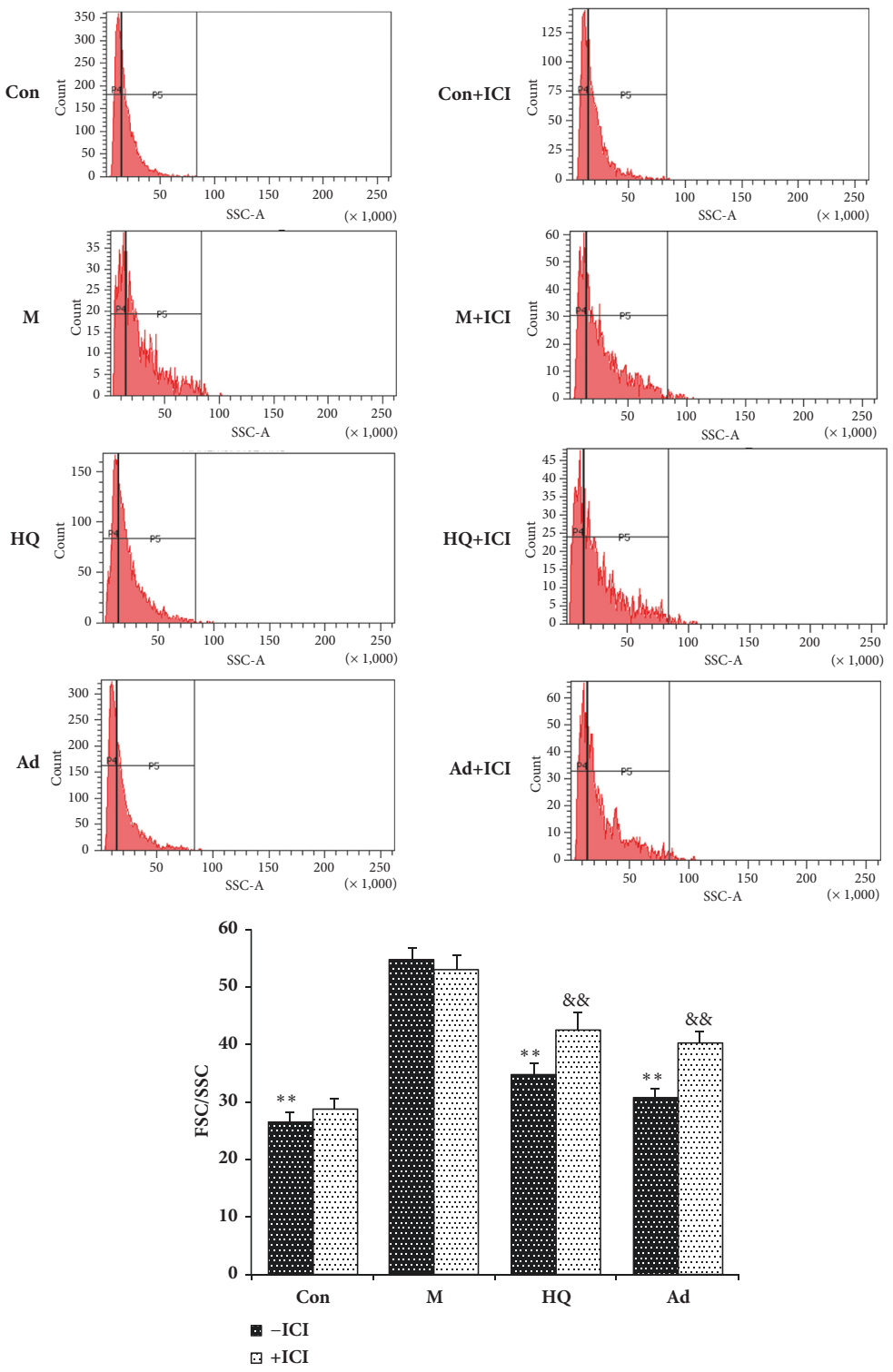


Figure 4: The antagonistic activity of ICI182,780 against adenosine on the mitochondrial swelling in LPS-stimulated H9c2 cells as assessed by FCM. H9c2 cells seeded on a 100 mm × 20 mm cell culture flask at a density of 2×10^5 cells/mL were treated with HQ (0.1 mg/mL) and adenosine (10 μM) for 24 h. In another experiment, the ER nonspecific antagonist Faslodex (ICI182,780, 1 μM) was added 30 min prior to HQ (0.1 mg/mL) and adenosine (10 μM) to evaluate whether the observed effects elicited by HQ and adenosine were mediated by the ER. Cells in each group were collected 24 h later and mitochondrial swelling was assessed. X ± SD, n = 3, $^{**}P < 0.01$ versus model group (-ICI); $^{\&\&}P < 0.05$ versus each group.

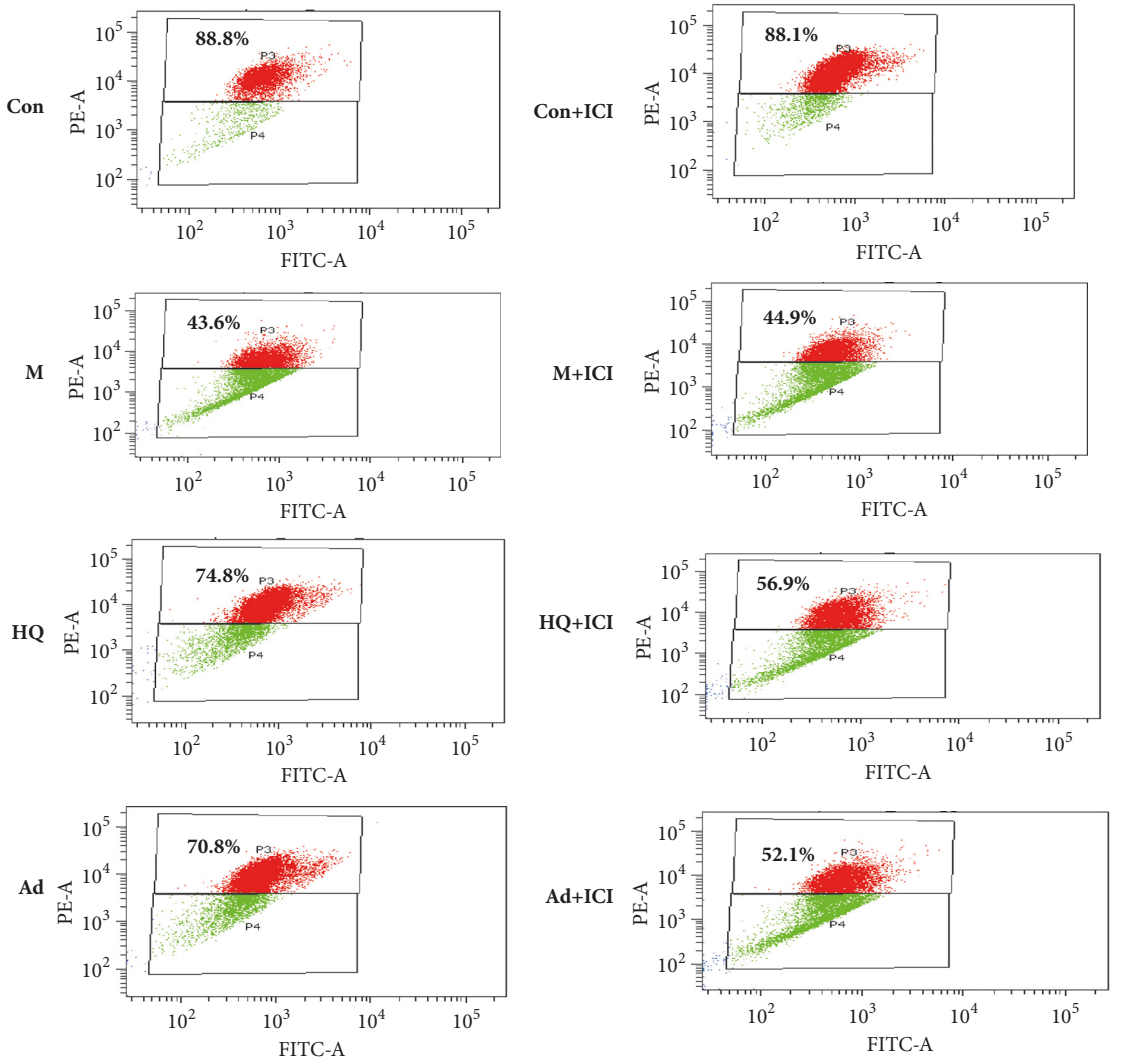


Figure 5: The antagonistic activity of ICI182,780 against adenosine on MMP (or ΔΨm) in LPS-stimulated H9c2 cells as assessed by FCM. H9c2 cells seeded on a 100 mm × 20 mm cell culture flask at a density of 2×10^5 cells/mL were treated with HQ (0.1 mg/mL) and adenosine (10 μM) for 24 h. In another experiment, the ER nonspecific antagonist Faslodex (ICI182,780, 1 μM) was added 30 min prior to HQ (0.1 mg/mL) and adenosine (10 μM) to evaluate whether the observed effects elicited by HQ and adenosine were mediated by the ER. Cells in each group were collected 24 h later and ΔΨm was assessed.

kinase (ERK). Serine/threonine-specific protein kinases can be activated by extracellular signals or stimuli such as physical stress, proinflammatory cytokines, growth factors, and bacterial complexes, and this signal is subsequently amplified to the nucleus by a phosphorylation cascade. Therefore, MAPKs indirectly regulate the activity of transcription factors, resulting in altered expression of corresponding genes and regulation of various important cell physiological/pathological processes such as cell growth, cell differentiation, environmental stress adaptation, and inflammatory response [28, 29]. The results of the present study show that HQ (2000 mg/kg) and adenosine (50 and 100 mg/kg) could significantly reduce the levels of p-JNK and p-P38 and increase the level of p-ERK in septic rats. Moreover, the effect of adenosine on p-JNK levels also showed a significant male–female difference ($P < 0.05$). It can be speculated that adenosine may play a protective role in septic rats through the JNK pathway.

Gender differences can cause changes and differences in the pharmacokinetics and pharmacodynamics of endogenous and exogenous compounds. Female animals contain higher levels of estrogen, which affects the bioavailability, distribution, absorption, metabolism, and excretion of drugs. In order to rule out this interference, we observed the effects of adenosine on male and female rats induced by LPS, respectively. Interestingly, results showed that the therapeutic effect of adenosine in female septic rats was significantly superior to that in male septic rats. This might be associated with higher levels of estrogen receptors in female mice; adenosine also has estrogen-like activity [10]. Subsequently, the underlying mechanisms of adenosine on the mitochondrial function of H9c2 cells stimulated with LPS were observed in the presence

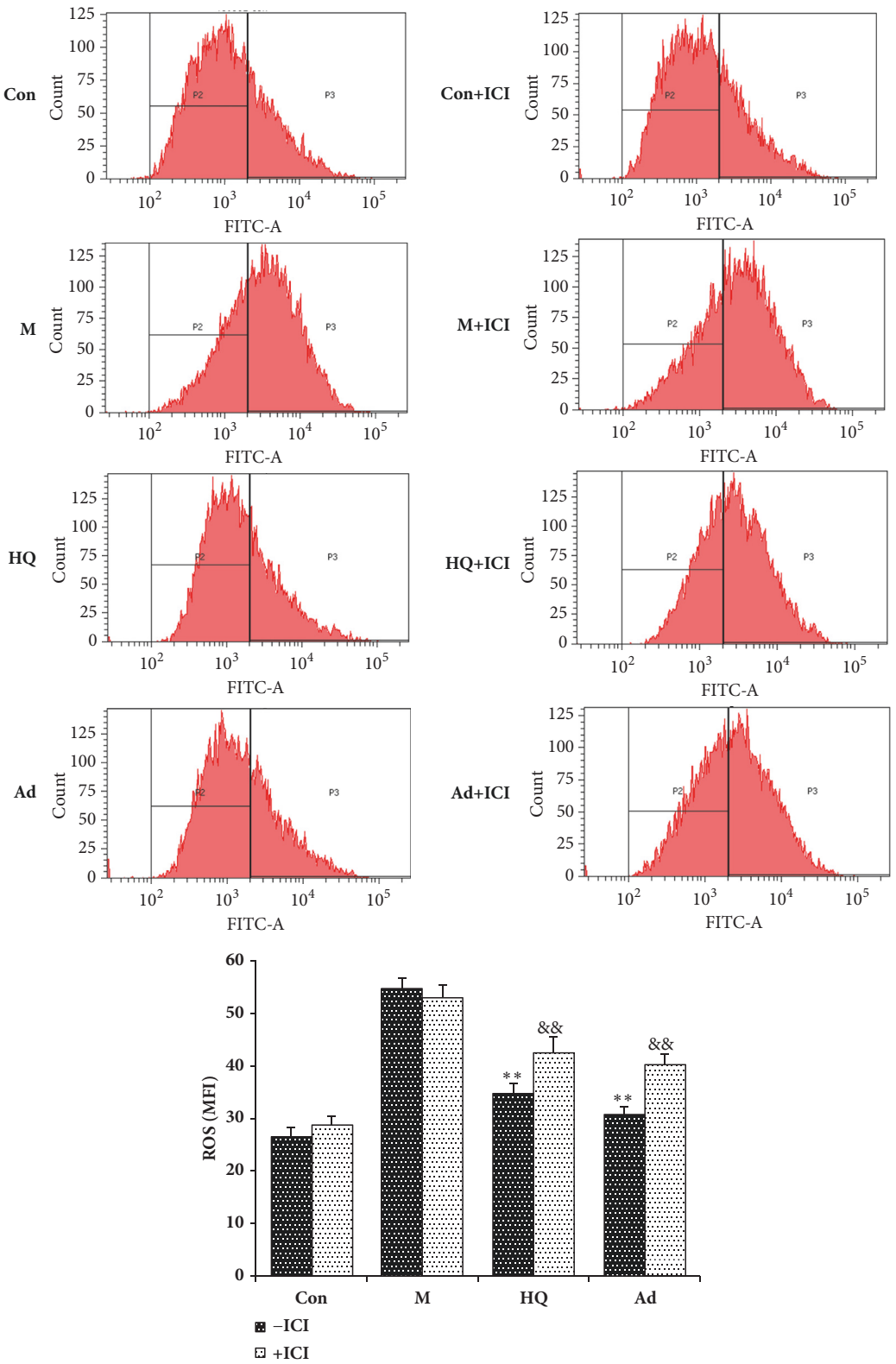


Figure 6: The antagonistic activity of ICI182,780 against adenosine on ROS production in LPS-stimulated H9c2 cells as assessed by FCM. H9c2 cells seeded on a 100 mm × 20 mm cell culture flask at a density of 2×10^5 cells/mL were treated with HQ (0.1 mg/mL) and adenosine (10 μM) for 24 h. In another experiment, the ER nonspecific antagonist Faslodex (ICI182,780, 1 μM) was added 30 min prior to HQ (0.1 mg/mL) and adenosine (10 μM) to evaluate whether the observed effects elicited by HQ and adenosine were mediated by the ER. Cells in each group were collected 24 h later and ROS levels were assessed. X ± SD, n = 3, $^{**}P < 0.01$ versus model group (-ICI); $^{\&\&}P < 0.05$ versus each group.

and absence of the estrogen receptor-specific antagonist ICI182,780.

In recent years, mitochondrial dysfunction has been recognized as one of the major factors in the pathogenesis of sepsis. The reported mechanism includes excessive ROS production during sepsis, which causes oxidative stress injury and results in oxidative damage to the mitochondrial respiratory chain and insufficient ATP production, thereby impairing mitochondrial membrane potential and ultimately leading to mitochondrial permeability transition, mitochondrial energetic dysfunction, and organelle swelling. Our FCM results show that HQ (0.1mg/mL) and adenosine (10 μM) could significantly reduce ROS levels and mitochondrial membrane potential and inhibit the increase in mitochondrial swelling in H9c2 cells induced by LPS (20 μg/mL, 24 h). This effect could be blocked by the specific estrogen receptor antagonist ICI182,780, suggesting that the protective effect of adenosine in H9c2 cells is mediated by the ER.

Previous studies have shown that the condition of sepsis is closely related to gender. In comparison with male septic animals, the prognosis is better for females, which may be explained by the fact that female animals have more anti-inflammatory factors, such as interleukin 10 (IL-10), and drugs with estrogen-like activity can protect the immune system of mice with blood loss, reducing the occurrence of immunosuppression. This indicates that there may be a connection between estrogen-like activity and sepsis symptoms. Accordingly, previous studies in our laboratory have shown that adenosine has estrogen-like activity and that its function is mediated by estrogen receptors. The present study demonstrates that there was a male–female difference in the therapeutic effect of HQ and adenosine in septic rats and that the improvement in mitochondrial function in H9c2 cells elicited by HQ and adenosine could be antagonized by ICI182,780. It is reasonable to suggest, therefore, that HQ and adenosine executes its protective function via the ER pathway.

In our study, HQ is a positive control drug. HQ has estrogen-like activity which was used for treatment of myocardial injury, such as sepsis, diabetes, and hypertension. Results of animal experiments and cell experiments also showed that HQ attenuated LPS-induced cardiac dysfunction by inhibiting mitochondrial function via the ER pathway. Moreover adenosine has the same molecular mechanism as HQ.

5. Conclusion

Medium and high doses of adenosine could significantly promote cardiac function (LVEF and LVFS) and reduce inflammatory factors (TNF-α, IL-6, PCT, and cTnI) and p-JNK levels in septic rats, with an obvious male–female difference. The results of experiments using ICI182,780 to antagonize the effect of adenosine on LPS-stimulated H9c2 cells show that adenosine could significantly inhibit the increases in ROS levels, mitochondrial membrane potential, and mitochondrial swelling and that this effect could be blocked by the estrogen receptor-specific antagonist ICI182,780. In conclusion, adenosine attenuated LPS-induced cardiac dysfunction by inhibiting mitochondrial function via the ER pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Mengnan Zeng and Xiaoke Zheng designed and performed the experiments and analyzed the raw data. Beibei Zhang, Benke Li, Yuxuan Kan, and Shengchao Wang assisted with the experiments. Weisheng Feng supervised the project.

Acknowledgments

Our work was supported by the Central Government Guide Local Science and Technology Development Funds (14104349) and the study on the key technology for quality control and the key characteristics of *Rehmannia glutinosa*, *Dioscorea opposita* Thunb., and *Achyranthes bidentata* Blume from Henan Province (171100310500). Thanks for Meng Li supervised the project.

References

[1] R. P. Dellinger, M. M. Levy, and A. Rhodes, "Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012," *Critical Care Medicine*, vol. 42, no. 1, pp. 580–637, 2014.

[2] C. Fleischmann, D. O. Thomas, M. Hartmann et al., "Hospital incidence and mortality rates of sepsis," *Deutsches Ärzteblatt International*, vol. 113, no. 10, pp. 159–166, 2016.

[3] J. N. Pulido, B. Afessa, M. Masaki et al., "Clinical spectrum, frequency, and significance of myocardial dysfunction in severe sepsis and septic shock," *Mayo Clinic Proceedings*, vol. 87, no. 7, pp. 620–628, 2012.

[4] N. Varga, J. C. Ruiz-Rodríguez, and R. Ferrer, "Melatonin and mitochondrial dysfunction are key players in the pathophysiology of sepsis," *Enfermedades Infecciosas y Microbiología Clínica*, vol. 36, no. 9, pp. 535–538, 2018.

[5] S. Li, H. Wu, D. Han et al., "A Novel Mechanism of Mesenchymal Stromal Cell-Mediated Protection against Sepsis: Restricting Inflammasome Activation in Macrophages by Increasing Mitophagy and Decreasing Mitochondrial ROS," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 3537609, 15 pages, 2018.

[6] J. Lyu, G. Zheng, Z. Chen et al., "Sepsis-induced brain mitochondrial dysfunction is associated with altered mitochondrial Src and PTP1B levels," *Brain Research*, vol. 1620, pp. 130–138, 2015.

[7] B.-G. Han, J.-W. Cho, Y. D. Cho et al., "Crystal structure of human transglutaminase 2 in complex with adenosine triphosphate," *International Journal of Biological Macromolecules*, vol. 47, no. 2, pp. 108–115, 2010.

[8] W. Geldenhuys, A. Hanif, J. Yun, and M. Nayeem, "Exploring Adenosine Receptor Ligands: Potential Role in the Treatment of Cardiovascular Diseases," *Molecules*, vol. 22, no. 6, p. 917, 2017.

[9] M. Peleli and M. Carlstrom, "Adenosine signaling in diabetes mellitus and associated cardiovascular and renal complications," *Molecular Aspects of Medicine*, vol. 55, pp. 62–74, 2017.

[10] M. Zeng, L. Zhang, M. Li et al., "Estrogenic Effects of the Extracts from the Chinese Yam (Dioscorea opposite Thunb.) and Its Effective Compounds in Vitro and in Vivo," *Molecules*, vol. 23, no. 11, pp. 330–343, 2018.

[11] E. J. Filardo, "A role for G-protein coupled estrogen receptor (GPER) in estrogen-induced carcinogenesis: Dysregulated glandular homeostasis, survival and metastasis," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 176, pp. 38–48, 2018.

[12] P. Habib, D. Dreymueller, B. Rösing et al., "Estrogen serum concentration affects blood immune cell composition and polarization in human females under controlled ovarian stimulation," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 178, pp. 340–347, 2018.

[13] T.-C. Chao, H.-H. Chao, M.-F. Chen, J. A. Greager, and R. J. Walter, "Female sex hormones modulate the function of LPS-treated macrophages," *American Journal of Reproductive Immunology*, vol. 44, no. 5, pp. 310–318, 2000.

[14] Y. Tien, J. Lin, C. Lai et al., "Carthamus tinctorius L. prevents LPS-induced TNFα signaling activation and cell apoptosis through JNK1/2–NFκB pathway inhibition in H9c2 cardiomyoblast cells," *Journal of Ethnopharmacology*, vol. 130, no. 3, pp. 505–513, 2010.

[15] Z. Liang, B. Du, L. Xie et al., "Pulverization Using Liquid Nitrogen Significantly Improves Physical Properties of Powder and Extraction Yield of Polysaccharides of Astragalus mongholicus," *International Journal of Food Engineering*, vol. 13, no. 2, pp. 164–175, 2017.

[16] L. Zhou and X. Mei, "Review of study on peripheral nerve regeneration promoted by traditional chinese medicines," *Zhongguo Zhongyao Zazhi*, vol. 36, no. 1, pp. 77–80, 2011.

[17] H. Tian, J. Lu, H. He et al., "The effect of Astragalus as an adjuvant treatment in type 2 diabetes mellitus: A (preliminary) meta-analysis," *Journal of Ethnopharmacology*, vol. 191, pp. 206–215, 2016.

[18] G. Zheng, J. Lyu, S. Liu et al., "Silencing of uncoupling protein 2 by small interfering RNA aggravates mitochondrial dysfunction in cardiomyocytes under septic conditions," *International Journal of Molecular Medicine*, vol. 35, no. 6, pp. 1525–1536, 2015.

[19] Y. Liu and Z. Lin, "Pathogenesis of sepsis-induced myocardial dysfunction," *Zhonghua Wei Zhong Bing Ji Jiu Yi Xue*, vol. 30, no. 4, pp. 374–376, 2018.

[20] G. Singh, G. Singh, R. Bhatti et al., "Indolyl-isoxazolidines attenuate LPS-stimulated pro-inflammatory cytokines and increase survival in a mouse model of sepsis: Identification of potent lead," *European Journal of Medicinal Chemistry*, vol. 153, pp. 56–64, 2018.

[21] M. M. Markiewski, R. A. DeAngelis, and J. D. Lambris, "Complexity of complement activation in sepsis," *Journal of Cellular and Molecular Medicine*, vol. 12, no. 6a, pp. 2245–2254, 2008.

[22] X. Li, R. Luo, R. Jiang et al., "The role of the Hsp90/Akt pathway in myocardial calpain-induced caspase-3 activation and apoptosis during sepsis," *BMC Cardiovascular Disorders*, vol. 13, no. 1, pp. 8–13, 2013.

[23] H. Yorulmaz, E. Ozkok, G. Ates, A. Aksu, N. Balkıs, and S. Tamer, "Ghrelin: Impact on Muscle Energy Metabolism in Sepsis," *International Journal of Peptide Research and Therapeutics*, vol. 24, no. 2, pp. 259–264, 2018.

[24] E. Y. Birati, H. Mathelier, and M. Molina, "Comparison of Causes of Death After Heart Transplantation in Patients With Left Ventricular Ejection Fractions =35% Versus >35%," *American Journal of Cardiology*, vol. 117, no. 8, pp. 1322–1326, 2018.

[25] S. K. Vanaja, A. J. Russo, B. Behl et al., "Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation," *Cell*, vol. 165, no. 5, pp. 1106–1119, 2016.

[26] J. Szederjesi, E. Almasy, A. Lazar, A. Huţanu, I. Badea, and A. Georgescu, "An Evaluation of Serum Procalcitonin and C-Reactive Protein Levels as Diagnostic and Prognostic Biomarkers of Severe Sepsis," *The Journal of Critical Care Medicine*, vol. 1, no. 4, pp. 147–153, 2015.

[27] R. Haines, S. Crichton, J. Wilson, D. Treacher, and M. Ostermann, "Cardiac biomarkers are associated with maximum stage of acute kidney injury in critically ill patients: A prospective analysis," *Critical Care*, vol. 21, no. 1, 2017.

[28] J. N. Sanchez, T. Wang, and M. S. Cohen, "BRAF and MEK Inhibitors: Use and Resistance in BRAF-Mutated Cancers," *Drugs*, vol. 78, no. 5, pp. 549–566, 2018.

[29] L. Xing, H. Ni, and Y. Wang, "Quercitrin attenuates osteoporosis in ovariectomized rats by regulating mitogen-activated protein kinase (MAPK) signaling pathways," *Biomedicine & Pharmacotherapy*, vol. 89, pp. 1136–1141, 2017.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 7517431, 11 pages
https://doi.org/10.1155/2019/7517431

Research Article

Tetrandrine-Induced Autophagy in MDA-MB-231 Triple-Negative Breast Cancer Cell through the Inhibition of PI3K/AKT/mTOR Signaling

Yubo Guo [1] **and Xiaohua Pei** [2]

[1] *Beijing University of Chinese Medicine, Beijing 100029, China*
[2] *The Fangshan Hospital, Beijing University of Chinese Medicine, Beijing 102488, China*

Correspondence should be addressed to Xiaohua Pei; pxh_127@163.com

Received 21 October 2018; Revised 24 November 2018; Accepted 3 December 2018; Published 1 January 2019

Academic Editor: Lucindo Quintans-Júnior

The present study examined the effects of tetrandrine suppressing proliferation, targeting LC3, p62, and Beclin-1 autophagy genes by inhibiting PI3K/AKT/mTOR signaling in Triple-negative breast cancer (TNBC) MDA-MB-231 cell. Cell viability and apoptosis were evaluated by MTT and Annexin-V/PI double staining. Cytotoxicity was determined with LDH assay. Western Blot and Immunofluorescence were used to measure the protein levels of p62/SQSTM1, Beclin1, LC3-II/LC3-I, and PTEN/PI3K/AKT/mTOR signaling. Results showed that tetrandrine inhibited the MDA-MB-231 cell proliferation and induced the apoptosis. Tetrandrine at doses of 12.8, 16.1, and 25.7μmol/L showed significant cytotoxicity on MDA-MB-231 cells ($p<0.01$). Tetrandrine induced MDA-MB-231 cell autophagy by decreasing p62/SQSTM1 expression, improving the expression of Beclin1 and LC3-II/LC3-I ($p<0.01$), inhibiting the PI3K/AKT /mTOR pathway by downregulating the expression of p-AKT ^{ser473}/AKT, p-PI3K/PI3K p110α, and p-mTOR ^{ser2448}/mTOR and upregulating PTEN expression. These findings revealed that tetrandrine could suppress proliferation and induce autophagy in MDA-MB-231 cell by inhibiting the PI3K/AKT/mTOR pathway and might be a promising anti-triple-negative breast cancer drug.

1. Introduction

Breast cancer is the most common women cancer and one of the leading causes of mortality in females [1]. Triple-negative breast cancer (TNBC), which accounts for approximately 10-20% of all breast cancers, has high proliferation, high histological grade, and poor prognosis [2]. Due to the lack of expression of ER, PR, and Her2/Neu, the current triple-negative breast cancer targeted therapy is ineffective and there are no established targeted agents for TNBC and basal-like BC. Although TNBC is sensitive to chemotherapy, compared with other subtypes, TNBC has a high recurrence rate with distant metastases, high metastasis rate, high recurrence rate, and poor overall survival [3].

Autophagy is a self-adaption and has a double-edged sword function in tumor metastasis. Autophagy has three types: macroautophagy, microautophagy, and chaperone-mediated autophagy. Autophagy in present study refers to macroautophagy [4]. Malignant tumor cells are usually apoptotic defects and autophagy is a form of cell death that is parallel to apoptosis. Autophagy may serve as an alternative mechanism for cell death when tumor cells have a defect in apoptosis or when apoptosis is inhibited [5]. The use of autophagy inducers can induce excessive autophagy of tumor cells and play synergistic effects on chemotherapeutic drugs beneficial to treatment [6]. Beclin1, LC3, and p62, the three major participants in autophagy, facilitate evaluation of autophagy levels of cells [7]. Studies have shown that LC3 and Beclin-1 are essential for cell proliferation, survival, migration, and invasion and may contribute to tumor growth and the development of highly invasive and metastatic TNBC cells. Targeted therapy of autophagy genes may be a potential therapeutic strategy in TNBC breast cancer [8].

Tetrandrine is a bisbenzylisoquinoline alkaloid extracted from the root of *Stephania tetrandra* S. Moore [9]. Although it has a wide range of pharmacological effects on lung cancer, nasopharyngeal carcinoma, laryngeal cancer, oral cancer, liver cancer [10–14], and so on, it has been demonstrated that tetrandrine has distinctive effect on breast cancer. For example, tetrandrine exhibited antitumor effects by inhibiting the growth of transplanted tumor and inducing the formation of apoptotic bodies of MCF-7 tumor-bearing nude mice [15]. Tetrandrine can reverse multidrug resistance of breast cancer cell MCF-7/Dox [16], enhance the cytotoxicity of Dox to MCF-7/Dox tumor cells, and significantly improve the reversal fold (RF) value [17, 18]. Besides, tetrandrine can enhance the sensitivity of tumor cells to high-energy radiation and DNA damage of anticancer drugs and exert a synergistic antitumor effect [19]. Tetrandrine also can play antitumor angiogenesis, antioxidative, and regulate the immune system function [20]. Although tetrandrine has a significant role in antibreast cancer, its mechanism on anti-triple-negative breast cancer (TNBC) remains unclear.

The PI3K/AKT/mTOR pathway has been shown to play significant roles in the development, progression, and metastatic of TNBC breast cancer [21, 22]. Mutations of the PTEN-PI3K-AKT axis occur in approximately 30% of breast cancers [23]. Besides, autophagy differs depending on the upstream regulatory signal pathway and is mainly dependent on mammalian rapamycin target protein mTOR signaling pathway. The typical AKT/protein kinase B mammalian rapamycin target (mTOR) signaling pathway is known to initiate vesicle bilayer formation in autophagy. AKT can activate autophagy-dependent classical mTOR pathway and inhibition of AKT/mTOR pathway can induce autophagy and help block breast cancer progression. The PI3K, which is particularly relevant to cancer, converts PIP2 to PIP3. PIP3 binds to AKT and PDK1, causing PDK1 to phosphorylate Ser308 and Ser473 of the AKT protein, resulting in activation of AKT. AKT phosphorylates TSC1/2 and activates the mTOR complex (mTORC1) to activate the translation of proteins and enhances the growth of cancer cells. PTEN is a PIP3-phosphatase that functions in contrast to PI3K, which converts PIP3 to PIP2 by dephosphorylating. Around 40% of BC shows loss of expression of PTEN, especially in hormone-receptor- (HR-) negative breast cancer [24]. It is reported that tetrandrine might lead to autophagic induction through PKC-α inactivation [25]. However, it is unclear whether tetrandrine can induce autophagy in triple-negative breast cancer cells and whether its mechanism inhibits PI3K/AKT/mTOR pathway.

In light of these findings, we investigate whether tetrandrine could suppress proliferation in human triple-negative breast cancer MDA-MB-231 cell targeting autophagy and its potential association with the PTEN/PI3K/AKT/mTOR signaling pathway.

2. Materials and Methods

2.1. Chemicals and Reagents. Tetrandrine was purchased from National Institutes for Food and Drug Control. RPIM was purchased from Gibco Life Technologies, Grand Island, NY, USA (lot. No: 1894129). Fetal bovine serum (FBS) was purchased from ExCell Bio Inc., Australia (lot. No: 11G047). Penicillin Streptomycin (100 Units/mL Penicillin and 100μg/mL Streptomycin) was purchased from Gibco Life Technologies, Grand Island, NY, USA (lot. No: 1857814). ly294002 (a specific inhibitor of PI3K) was purchased from Selleck.cn, Houston, Texas, USA (lot. No: S110503), which served as a positive control for this experiment. LDH assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Cat. No: A020-2). BCA protein quantitation assay and whole cell lysis assay were purchased from Nanjing Keygen Biotech. Co., Ltd. (Cat. No: KGPBCA, Cat. No: KGP103). Rabbit anti-LC3 Polyclonal Antibody (Cat. No: 12135-1-AP), Rabbit anti-p62/SQSTM1 Polyclonal Antibody (Cat. No: 18420-1-AP), Rabbit anti-Beclin1 Polyclonal Antibody (Cat. No: 11306-1-AP), Mouse anti-PTEN Monoclonal Antibody (Cat. No: 60300-1-Ig), Mouse anti-PI3K p85 (alpha) Monoclonal Antibody (Cat. No: 60225-1-Ig), Rabbit anti-PI3K p110 (alpha) Polyclonal Antibody (Cat. No: 21890-1-AP), Mouse anti-AKT Monoclonal Antibody (60203-2-Ig), and Mouse anti-beta Actin Monoclonal antibody (Cat. No: 66009-1-Ig) were purchased from Proteintech Group, Inc. (Chicago, USA). Rabbit anti-Phospho-PI3 Kinase p85 (Tyr458)/p55(Tyr199) Polyclonal Antibody and Rabbit anti-Phospho-Akt(Ser473) (D9E)XP® Monoclonal Antibody were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA), and Mouse Anti-mTOR Monoclonal Antibody[53E11] ab87540 and Rabbit Anti-mTOR(phosphor S2448) Monoclonal antibody [EPR426(2)](ab109268) were purchased from Abcam Biocompany (Cambridge, MA, USA). FITC conjugated Annexin-V apoptosis detection kit instructions (Becton, Dickinson and Company, Franklin Lake, New Jersey, USA, lot. No: 7040932). All other reagents were obtained from Sigma-Adrich Co. (St. Louis, MO, USA). The standard of tetrandrine was dissolved in DMSO (concentration <0.1%) before adding to the RPMI-1640 culture medium making the final concentration 64.2 μmol/L, protected from light and stored in a refrigerator at -20°C.

2.2. Cell Cultures. Human breast cancer cell MDA-MB-231 was purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and cultured with PRIM 1640 (Gibco Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS, ExCell Bio Inc., Australia) and 1% Penicillin Streptomycin at 37°C with 5% CO^2 in a humidified cell incubator under 95%/5% (v/v) mixture of air and CO^2.

2.3. Assessment of Cell Viability by MTT Method. The cell viability of human breast cancer cell MDA-MB-231 treated with tetrandrine was investigated using MTT assay. MDA-MB-231 cells (5×10^4 cells/ml) were seeded in 96-well plates and cultured with normal medium to adherent. Cells were treated with tetrandrine at doses of 0, 0.8, 1.6, 3.2, 6.4, 12.8, 16.1, 25.7, 32.1, 51.4, and 64.2μmol/L for 24h, 48h, and 72h. A volume of 20μl of MTT 5mg/ml was added to each well and incubated for another 4h at 37°C. The medium

was then aspirated carefully without disturbing the blue formazan crystals. DMSO was added (150μl/well) shaking for 15min. The optical density (OD) value was measured at 490 nm on a multifunctional microplate reader (FLUO star Omega, BMG Labtech, Germany). The results were expressed as a percentage of the absorbance present in treated cells compared with control cells. Cell proliferation inhibition rate formula: [1-(drug group OD value/Control group OD value)] × 100%. The IC50 of 72h was calculated using GraphPad Prism 5.01 software.

2.4. Apoptosis Assessment by Flow Cytometry. Cells were seeded in 6-well plates at a density of 1×10^6/well and treated with different concentrations of tetrandrine for 72h. Cells were harvested without EDTA-trypsin digestion solutions (Solarbio science & technology Co., Ltd., Beijing, lot. No: 20171024), washed twice with PBS, and resuspended in 200μl Binding Buffer, Pipette 100 μl of the cell suspension into Eppendorf tube 1.5ml). The cell suspension was mixed with 5μl of Annexin-V-fluorescein isothiocyanate (FITC) and 5μl Propidium Iodide (PI) according to the FITC conjugated Annexin-V apoptosis detection kit instructions (Becton, Dickinson and Company, Franklin Lake, New Jersey, USA, lot. No: 7040932), incubated for 15 min in the dark at room temperature. Then 150μl binding buffer was added to each tube and they were analyzed with a flow cytometer within 1 hour.

2.5. The Cytotoxicity Assessment by LDH Assay. LDH assay was used to test the cell toxicity of tetrandrine on human breast cancer MDA-MB-231 cell. Cells were seeded in 6-well plates at a density of 1×10^6/well and treated with different concentrations of tetrandrine for 48h. The cell supernatant was collected and 0.2μmol/ml standard solutions of pyruvate were both added 20μl to a 96-well plate. Set up blank group and Control group. According to LDH kit instructions, mix with matrix buffer 25μl and the cell supernatant sample group was added in coenzyme 1 application solution 5μl. Mix well and incubate at 37°C for 15min. Add 2,4-dinitrophenylhydrazine 25μl to the 96-well plate. Mix well and incubate at 37°C for 15min. Then it was the 0.4 mol/l NaOH solution 250μl. Mix well and stand at room temperature 5min. The optical density (OD) value was measured at 450 nm on a multifunctional microplate reader (FLUOstar Omega, BMG Labtech, Germany).

2.6. Immunofluorescence Analysis. The logarithmic growth phase of MDA-MB-231 cells was made into cell suspension, dropping 500μl (1×10^6/well) into a glass bottom cell culture dish with 20mm diameter and incubating it 4-5h for adherent cells. It was treated with different concentrations of tetrandrine for 48h and fixed with 4% precooled paraformaldehyde for 20 minutes, Permeate with 0.2% Triton X-100 for 10 minutes. 10% Goat serum blocked them for 30 minutes. Incubate primary antibody and put into the wet box overnight at 4°C. Wash three times with PBS. Fluorescent secondary antibody was incubated at room temperature for 30min (away from light). Wash three times with PBS. DAPI (Solarbio, lot. No: 20170815) stained nuclei. Use the Laser confocal scanning microscope to take pictures and observe the protein expression and their analysis was with Image Pro Plus software 6.0.

2.7. Western Blot Analysis. The total protein of cells was extracted by protein extraction kit and quantified by BCA method conducted according to the procedure as previously described [26] with some modifications. Briefly, 20 μg of protein from the MDA-MB-231 cell was on 10% polyacrylamide gel by SDS-PAGE electrophoresis (concentration gel for 30min, separation gel for 1.5h) followed by transferring onto PVDF membrane (Immobilon®-P Transfer Membranes, 0.45μm, lot. No: K5PA9282A). Then the membrane was subsequently incubated with appropriate primary antibody (1:500) for overnight at 4°C, and the corresponding HRP labeled secondary antibodies at room temperature for 1h. Add ECL chemiluminescence solution (Proteintech, lot. No: B500023) on the membrane, with a gel imager (Azure c500, Azure Bio systems, USA) exposure. Grayscale values were analyzed with ImageJ and internal controls were used with β-actin.

2.8. Statistical Analysis. All results were expressed as mean ± standard deviation (SD). One-way ANOVA was performed between multiple groups using SPSS software (Version 20.0) when homogeneity of variance and normality were met. Otherwise, Dunnett's T3 and nonparametric tests were conducted between multiple groups, respectively. $p<0.05$ was considered statistical difference and $p<0.01$ was considered statistical significant difference.

3. Results

3.1. Effects of Tetrandrine on MDA-MB-231 Cell Viability. MTT method was used to detect the inhibition rate of tetrandrine in human breast cancer MDA-MB-231 cell proliferation. MDA-MB-231 cell was respectively treated with tetrandrine and LY294002. Results showed that tetrandrine at doses of 6.4, 12.8, 16.1, 25.7, 32.1, 51.4, 64.2μmol/L inhibited the MDA-MB-231 cell proliferation, as shown in Figure 1(a). The IC50 at 72 hours of tetrandrine on MDA-MB-231 breast cancer cells proliferation was 28.06μmol/L, with 95% confidence interval (17.81-44.20) (Figure 1(c)). LY294002 at doses of 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600μmol/L inhibited the MDA-MB-231 cell proliferation, as shown in Figure 1(b). The IC50 at 72 hours of LY294002 functioned on MDA-MB-231 cell was 38.32, with 95% confidence interval (27.17-54.06) (Figure 1(d)).

Figures 1(a)–1(d) showed inhibition rate of cell proliferation and IC50 of tetrandrine and LY294002. MTT method was used to measure the cell proliferation inhibition rate. Different doses of tetrandrine (0, 0.8, 1.6, 3.2, 6.4, 12.8, 16.1, 25.7, 32.1, 51.4, 64.2μmol/L) and LY294002 (0, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600μmol/L) were given to MDA-MB-231 cell for 24, 48, and 72 hours.

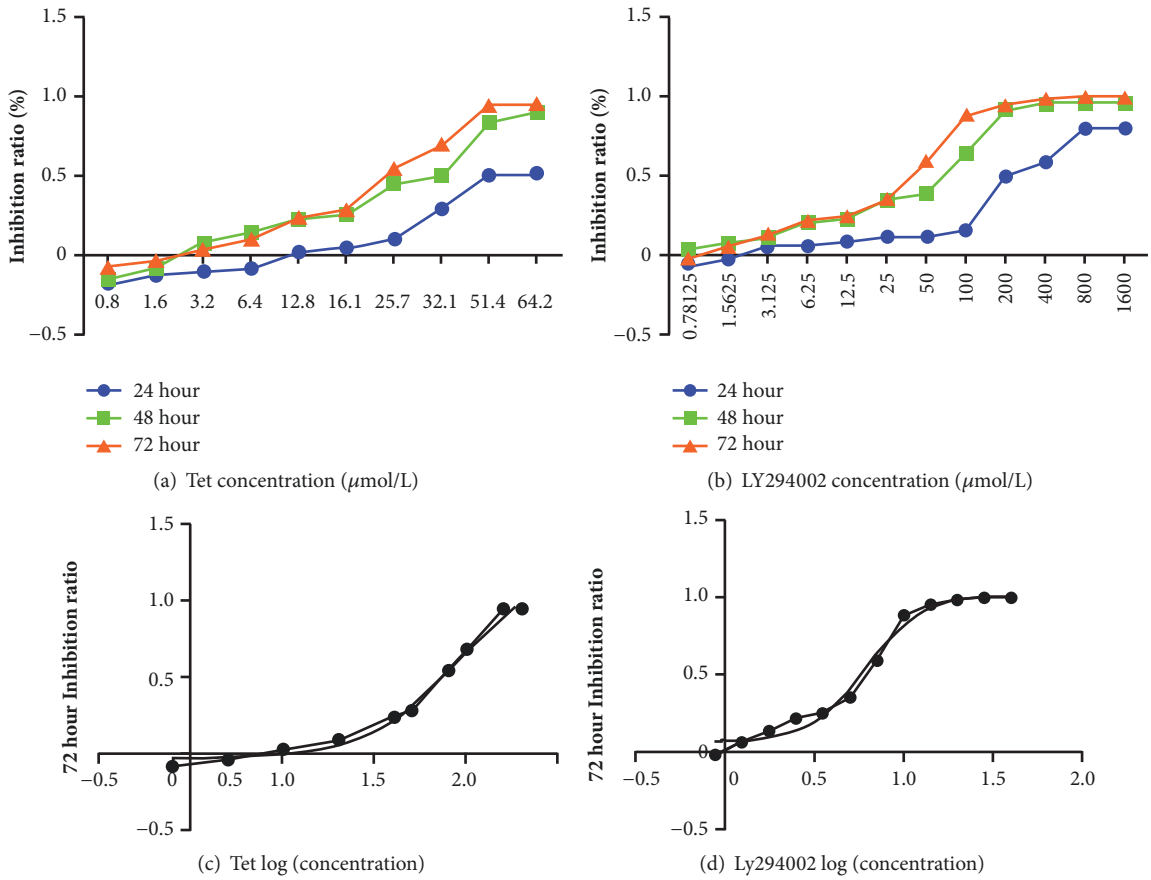


Figure 1: The inhibition rate and IC50 of tetrandrine and LY294002 on MDA-MB-231 cells.

3.2. Effects of Tetrandrine on MDA-MB-231 Cell Apoptosis. Cells (2×10^5 cells/well) were incubated with or without tetrandrine at doses of 0, 6.4, 12.8, 16.1, and 25.7μmol/L for 72h and then were harvested for staining with Annexin-V and PI double staining to measure the percentage of apoptotic cells by flow cytometry. MDA-MB-231 cell was treated with or without tetrandrine for 72h. Tetrandrine at doses of 0, 6.4, 12.8, 16.1, and 25.7μmol/L induced MDA-MB-231 cell apoptosis rate of 10.4%, 17.5%, 27.4%, 34.1%, and 38.1%, respectively. Compared with the Control group, the tetrandrine group of the normal quadrant were significantly decreased and the early apoptotic quadrant were significantly increased ($p<0.01$), as shown in Figure 2 and Table 1.

Figures 2(a)–2(e) and Table 1 showed the cell apoptosis rate in different concentrations of tetrandrine groups. Annexin-V and PI double staining was used to measure the cell apoptosis. Different doses of tetrandrine (0, 6.4, 12.8, 16.1, 25.7μmol/L) were given to MDA-MB-231 cell for 72 hours (Table 1 ∗compared with the Control group; $p<0.05$ was considered statistical difference and $p<0.01$ was considered significant difference).

3.3. Effects of Tetrandrine on MDA-MB-231 Cytotoxicity. LDH (lactate dehydrogenase) is a stable protein that exists in the cytoplasm of normal cells. Once the cell membrane is damaged, LDH is released to the outside of the cell to increase the LDH activity in the extracellular fluid. Compared with Control group, tetrandrine at doses of 12.8, 16.1, and 25.7μmol/L showed obvious cytotoxicity to human breast cancer MDA-MB-231 cell ($p<0.01$, Figure 3).

Figure 3 showed the cytotoxicity in different concentrations of tetrandrine groups. LDH assay was used to measure the cytotoxicity. Different doses of tetrandrine (0, 3.2, 6.4, 12.8, 16.1, and 25.7μmol/L) were given to MDA-MB-231 cell for 48 hours (Figure 3 ∗compared with the Control group; $p<0.05$ was considered statistical difference and $p<0.01$ was considered significant difference).

3.4. Effects of Tetrandrine on Beclin1, LC3, and p62/SQSTM1 Expression. Previous studies [25] have shown that tetrandrine can induce autophagy in tumor cells. In order to observe whether tetrandrine could induce autophagy in triple-negative breast cancer cells and select appropriate concentration of tetrandrine, Beclin1, LC3, and p62/SQSTM1, the three major proteins involved in the autophagy process, were examined by Western Blot analysis and also confirmed by immunofluorescence staining assay. Results of Western Blot showed that tetrandrine at dose of 25.7μmol/L decreased p62/SQSTM1 ($p<0.01$) and at doses of 12.8, 16.1,

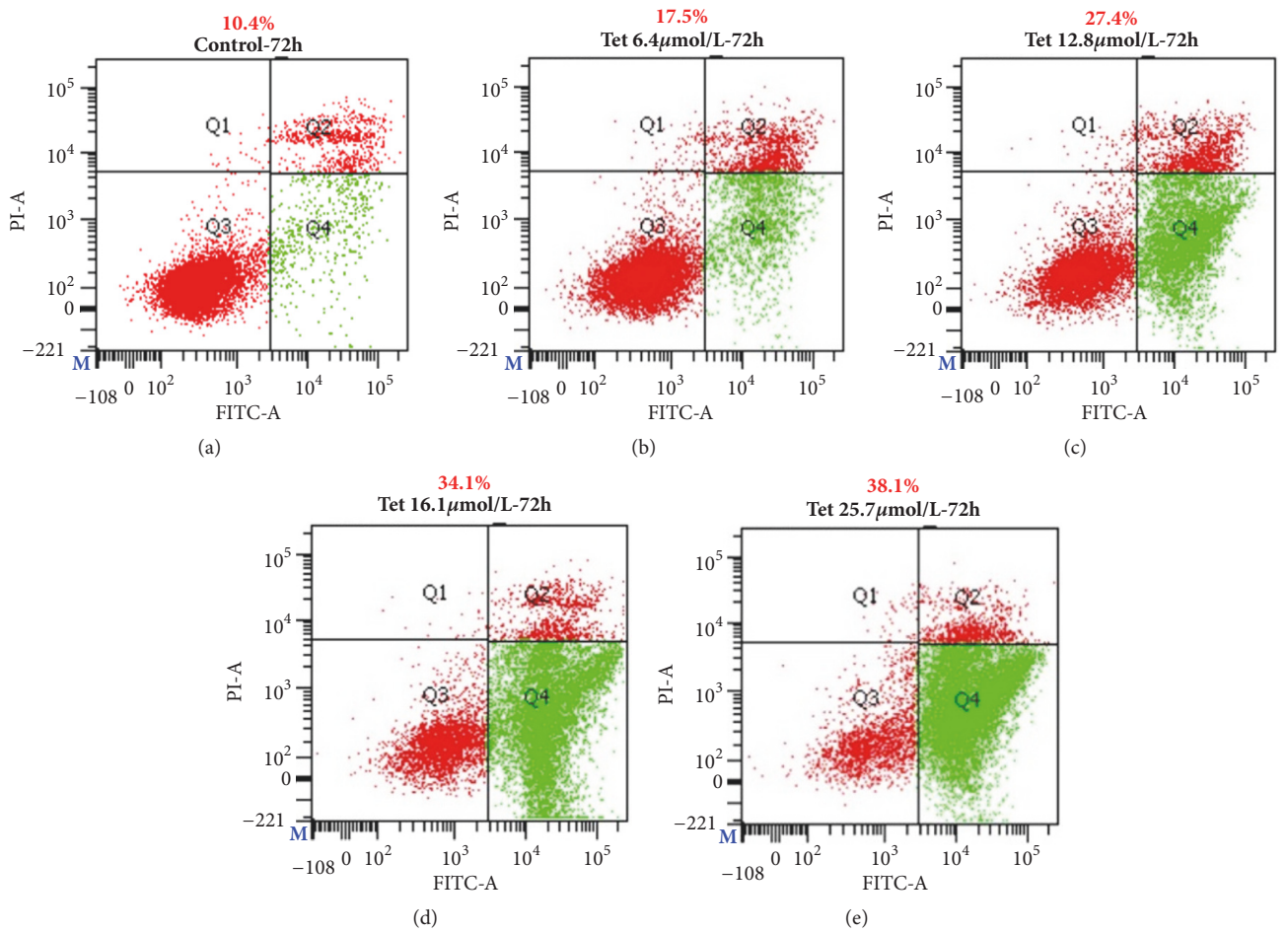


Figure 2: Comparison of apoptosis rate in different concentrations of tetrandrine group in human breast cancer MDA-MB-231 cells with Annexin-V and PI double staining.

Table 1: Comparison of apoptosis rate in different concentrations of tetrandrine group in MDA-MB-231 cells (Mean±SD) (n=3).

Group	Normal cell(%)	Early apoptosis(%)	Late apoptosis(%)	Necrosis(%)
Control	70.65±1.63	5.00±0.71	5.35±0.78	0.25±0.07
Tet (6.4μmol/L)	49.85±1.77**	10.45±0.21**	6.80±0.57	0.55±0.07*
Tet (12.8μmol/L)	25.05±1.91**	20.75±0.92**	5.70±0.42	0.25±0.07
Tet (16.1μmol/L)	13.10±1.56**	29.95±0.49**	3.45±0.49	0.35±0.07
Tet (25.7μmol/L)	8.70±1.13**	33.90±0.42**	3.40±0.71	0.25±0.07

and 25.7μmol/L improved LC3-II/LC3-I expression ($p<0.01$) as shown in Figures 4(a1)–4(c1). Immunofluorescence staining had shown that p62/SQSTM1 and the autophagy flow of LC3 were distributed in the cytoplasm, while Beclin1 was expressed in the cytoplasm and nucleus. Tetrandrine (12.8μmol/L) improved the activation of autophagy flow of LC3 and Beclin1 expression and inhibited the expression of p62/SQSTM1 in MDA-MB-231 cell. It was found that tetrandrine could induce autophagy in triple-negative breast cancer cells. With the increase of concentration, Tet induced the autophagy of human triple-negative breast cancer cells MDA-MB-231.

Figures 4(a)–4(c) showed Beclin1, LC3, and p62/SQSTM1 protein expression of MDA-MB-231 with different concentrations of tetrandrine intervened. Immunofluorescence staining was used to measure the protein expression of Beclin1, LC3, and p62/SQSTM1, which were observed with the laser confocal scanning microscope (original magnification, ×600) (Figures 4(a2)–4(c2)) and their analysis was with Image Pro Plus software 6.0. White arrow in Figure 4(c2) represents autophagy flow. Western Blot was used to measure the protein levels of Beclin1, LC3, and p62/SQSTM1 expression and their analysis was with ImageJ. Different concentrations of tetrandrine (0, 3.2, 6.4, 12.8, 16.1, and 25.7μmol/L) were

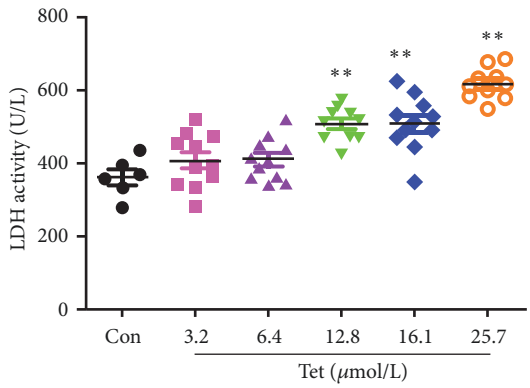


FIGURE 3: Comparison of the cytotoxicity of different concentrations of tetrandrine in MDA-MB-231 cell.

given to MDA-MB-231 cell for 48 hours (Figures 4(a1)–4(c1) ∗compared with the Control group; $p<0.05$ was considered statistical difference and $p<0.01$ was considered significant difference).

3.5. Effects of Different Concentrations of Tetrandrine on PI3K/AKT/mTOR Pathway in MDA-MB-231 Cells. Increased activity of the PI3K/AKT pathway is often associated with multiple cancers. Autophagy relies mainly on the mammalian target of rapamycin (mTOR) pathway. We investigated the effect of tetrandrine on the PI3K/AKT/mTOR signaling pathway in vitro by incubating MDA-MB-231 cells with different concentrations of tetrandrine (0, 3.2, 6.4, 12.8, 16.1, and 25.7μmol/L) for 48h. Western Blot analysis, as shown in Figures 5(a)–5(e), indicated that tetrandrine could reduce the expression of p-akt^{ser473}/akt, p-PI3K/PI3K p110α, p-PI3K/PI3K p85α, p-mTOR^{ser2448}/mTOR and improved the expression of PTEN ($p<0.05,0.01$) compared with Control group, inhibiting the PI3K-AKT-mTOR pathway.

Figure 5 showed the protein levels of p-akt^{ser473}, akt, p-PI3K, PI3K p110α, p-PI3K, PI3K p85α, p-mTOR^{ser2448}, mTOR in different concentrations of tetrandrine groups. The Western Blot method was used to measure the protein expression levels of the PTEN/PI3K/AKT/mTOR pathway and their analysis was with ImageJ. Different concentrations of tetrandrine (0, 3.2, 6.4, 12.8, 16.1, and 25.7μmol/L) were given to MDA-MB-231 cell for 48 hours (Figures 5(a)–5(e) ∗compared with the Control group; $p<0.05$ was considered statistical difference and $p<0.01$ was considered significant difference).

3.6. Effects of Tetrandrine (12.8μmol/L) and ly294002 (50μmol/L) Inhibitor of PI3K/AKT Pathway on MDA-MB-231 Cells. To further examine whether tetrandrine-induced autophagy involves the PI3K/AKT/mTOR signaling pathway, we pretreated MDA-MB-231 cells with tetrandrine (12.8μmol/L) and ly294002 (50μmol/L) inhibitor of PI3K/AKT signaling pathway and then the levels of AKT, p-AKT ^{ser473}, p110α, p-PI3K, mTOR, and p-mTOR^{ser2448} were examined. As shown in Figures 6(a)–6(e), results indicated that tetrandrine (12.8μmol/L) could inhibit the PI3K/AKT/mTOR signaling pathway by improving expression of PTEN and reducing expression of p-AKT ^{ser473}/AKT, p-PI3K/p110α, p-mTOR^{ser2448}/mTOR ($p<0.05,0.01$). Furthermore, the immunofluorescence staining results, as shown in Figure 6(f), indicated that, in the blank group, PTEN was only expressed in the cytoplasm of MDA-MB-231 cell. After intervention with tetrandrine (12.8μmol/L), PTEN was expressed both in the nucleus and cytoplasm. PTEN protein level in tetrandrine group was significantly higher than the Control group ($p<0.01$), while lower than the LY294002 group ($p<0.01$).

Figures 6(a)–6(f) showed the protein levels of p-akt^{ser473}, akt, p-PI3K, PI3K p110α, p-PI3K, PI3K p85α, p-mTOR^{ser2448}, mTOR in different concentrations of tetrandrine groups. The Western Blot method was used to measure the protein expression levels of the PTEN/PI3K/AKT/mTOR signaling and their analysis was with ImageJ. Immunofluorescent staining, observed with the laser confocal scanning microscope (original magnification, ×600) (f), was used to measure the PTEN expression and the analysis was with Image Pro Plus software 6.0. The PTEN expression in MDA-MB-231 cell nucleus was indicated with the white arrows. Tetrandrine (12.8μmol/L) and ly294002 (50μmol/L) were given (Figure 6, ∗compared with the Control group; $p<0.05$ was considered statistical difference and $p<0.01$ was considered significant difference).

4. Discussion

This study revealed that tetrandrine could inhibit the proliferation of triple-negative breast cancer MDA-MB-231 cells, induce early apoptosis, and have certain toxicity to MDA-MB-231 cell in a concentration and time dependent manner. The mechanism is probably due to the fact that tetrandrine could induce MDA-MB-231 cell autophagy by reducing the expression of p62 and increasing the expression of Beclin1, LC3-II/LC3-I. The triple-negative breast cancer MDA-MB-231 cells autophagy induced by tetrandrine was by inhibiting the PI3K/AKT/mTOR signaling via upregulating PTEN expression and downregulating p-akt^{ser473}/akt, p-PI3K/PI3K p110α, p-mTOR^{ser2448}/mTOR. LY294002 inhibited PI3K/AKT signaling pathway by reducing p-PI3K/p85α and p-PI3K/p110α, to inhibit breast cancer cell proliferation and induce apoptosis.

Our previous study found that tetrandrine had a significant inhibitory effect on MCF-7 xenograft tumor in nude mice. The nude mice were subcutaneously inoculated with MCF-7 breast cancer cells in the right axilla of the nude mice, and the nude mice model of breast cancer transplanted tumor was inoculated for 7 days. Tetrandrine treatment was given for 15 days. It was found that tetrandrine had obvious antitumor growth effect and induced breast cancer cell apoptotic bodies in vivo [15]. Furthermore, in vitro experiments, it is reported that tetrandrine combined with cisplatin was applied to breast cancer cell MDA-MB-231 for 24 hours, the surface structure of the cell membrane was destroyed, and pores were formed. After 48 hours, the

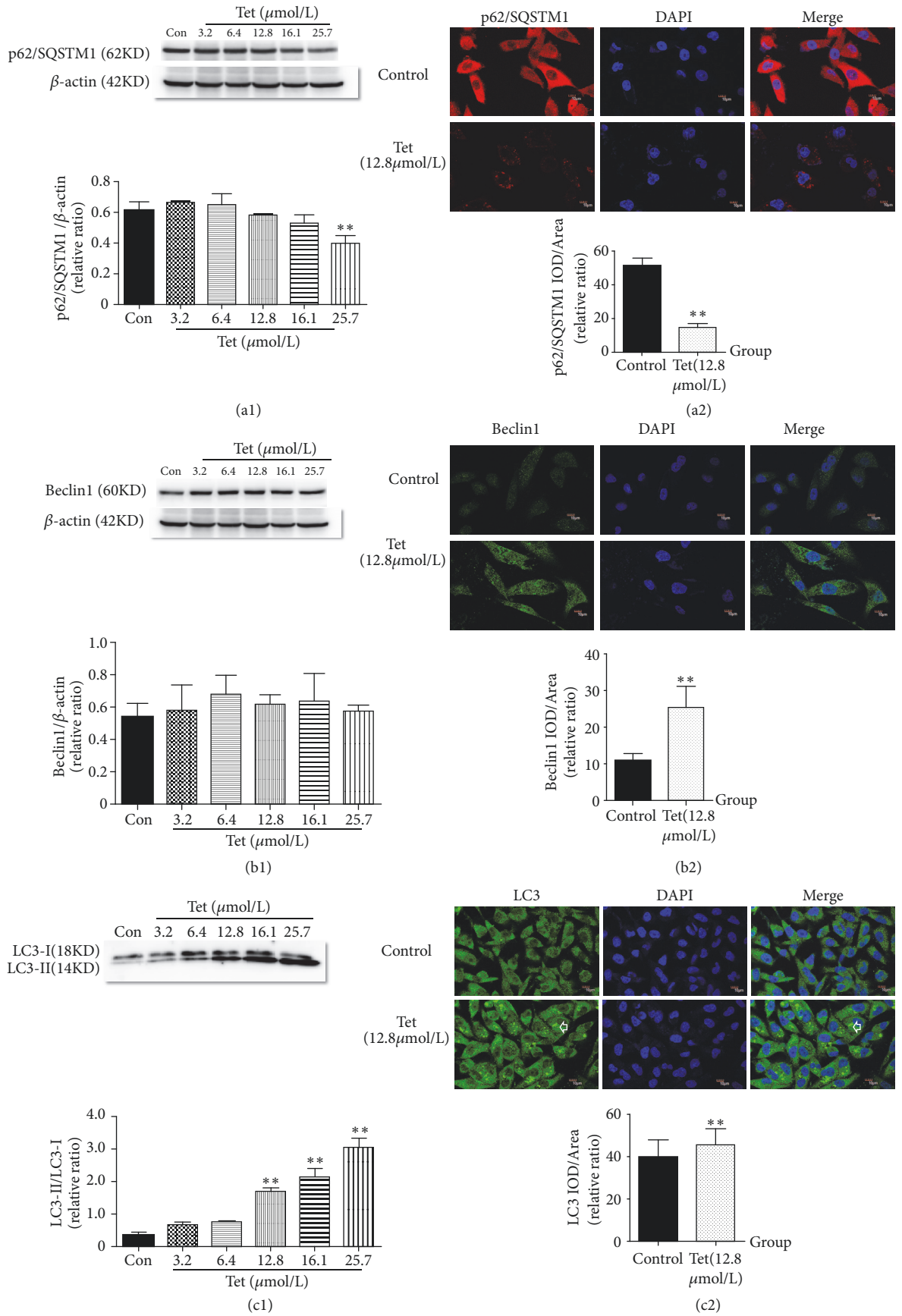


Figure 4: Comparison of p62/SQSTM1, Beclin1, and LC3 in MDA-MB-231 cells intervened with different concentrations of tetrandrine.

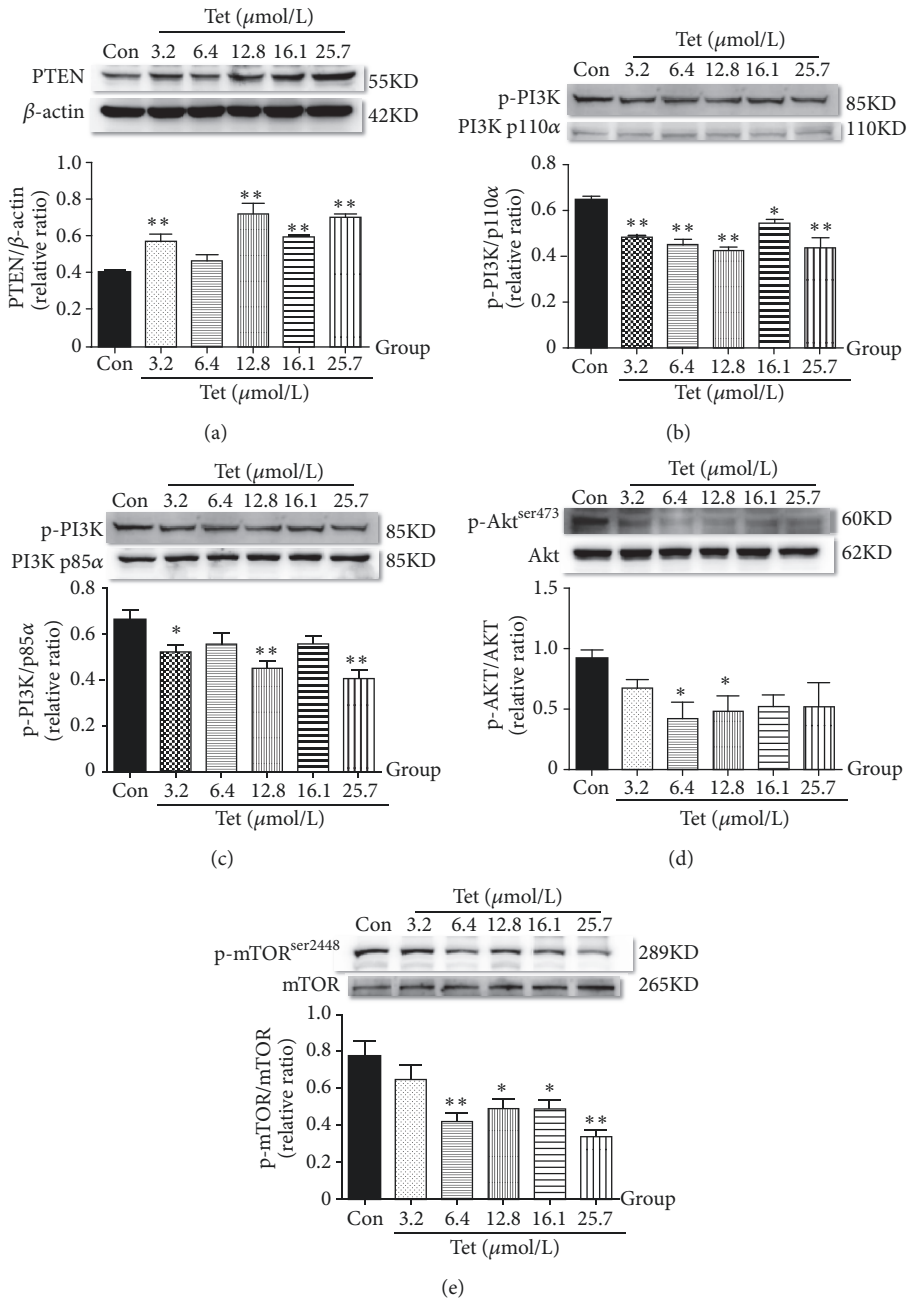


Figure 5: Comparison of the protein levels of PI3K/AKT/mTOR pathway in MDA-MB-231 cells intervened with different concentrations of tetrandrine.

cell membrane was severely damaged. The cell cycle ratio increased to 51.7% in S phase, which improved the anticancer drugs of cancer cells sensitivity [27]. Consistent with the results of the previous study, this study found that tetrandrine could inhibit the proliferation of triple-negative breast cancer cells MDA-MB-231, inducing early apoptosis after 72h ($p < 0.01$).

Autophagy is a double-edged sword for tumor therapy. One is that autophagy protects tumor cells and reduces the adverse effects of the surrounding environment. The other is to upregulate the autophagy activity of tumor cells and initiate programmed cell death. Studies have shown that knocking down the autophagy genes LC3 and Beclin1 could lead to a significant decrease in autophagy and inhibit the proliferation, migration, and invasion of triple-negative breast cancer MDA-MB-231 cells [8]. Tetrandrine, which can reverse the multidrug resistance of tumor cells, inhibiting tumor angiogenesis and playing an antitumor effect, is a potent cell autophagy agonist [28]. In this study, we found that tetrandrine could inhibit the proliferation of MDA-MB-231 cells and induce autophagy and apoptosis by decreasing p62/SQSTM1 expression and

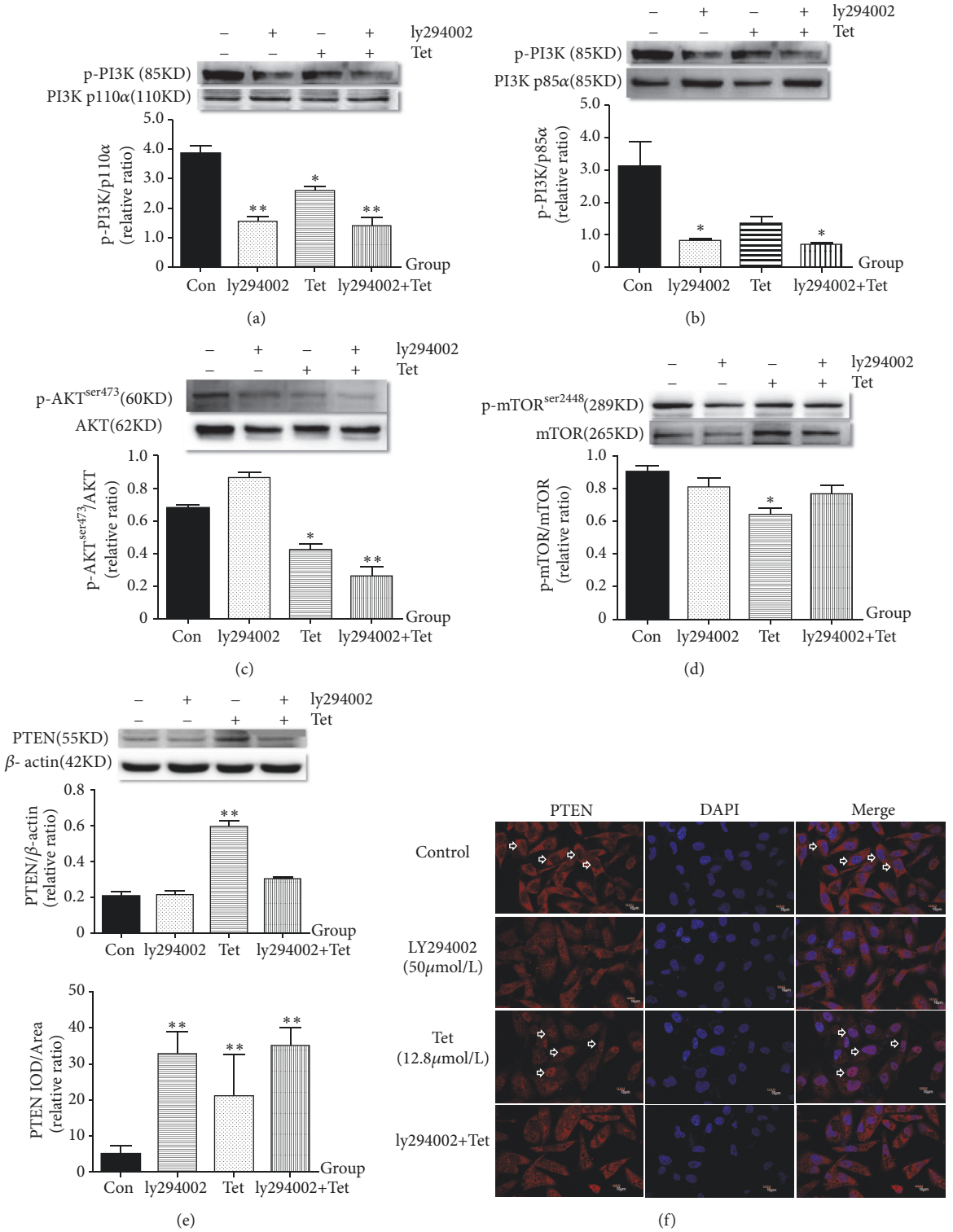


Figure 6: Comparison of the protein levels of PTEN/PI3K/AKT/mTOR pathway in different groups.

increasing Beclin1 and LC3 expression, thereby reversing the protective effect of autophagy on TNBC cells. The results of our study showed that tetrandrine could activate autophagy and induce autophagic death in TNBC cells.

To further reveal the activating autophagy mechanism of tetrandrine, the PI3K/AKT/mTOR pathway was considered. It was reported that TNBC was subclassified into at least six tumor molecular subtypes including a mesenchymal-like subset that was highly sensitive to

PI3K/mTOR inhibitors in vitro and in vivo [24]. Our study showed that LY294002, as the PI3K inhibitor which served as a positive control for this experiment, could reduce p-PI3K/p85α and p-PI3K/p110α, increase the expression of PTEN, and block the PI3K/AKT pathway to inhibit the triple-negative breast cancer MDA-MB-231 cell proliferation, which suggested that the inhibitor of PI3K/AKT pathway played an important role in the development of breast cancer. Moreover, our research found that tetrandrine was also an inhibitor of PI3K/AKT/mTOR signaling pathway and could significantly inhibit the proliferation and induce apoptosis in triple-negative breast cancer MDA-MB-231 cells.

The phosphoinositol-3-kinase family is divided into four different classes: Class I, Class II, Class III, and Class IV. Among them, the most widely studied is Class I PI3K. Class IA PI3K is composed of a heterodimer between a p110 catalytic subunit and a p85 regulatory subunit. The ways in which p85 subunits contribute to cancer and the effective means to pharmacologically inhibit these mechanisms are still unclear [29]. The gene encoding for PI3K catalytic subunit p110α is mutated in 20-40% of breast cancer [30]. In our study, it was found that tetrandrine could inhibit p-PI3K/PI3K p110α, which provided a novel drug for the study of PIC3A gene mutation of TNBC. Therefore, tetrandrine inhibited the triple-negative breast cancer MDA-MB-231 cell proliferation and induced autophagy likely by the inhibition of PI3K/AKT/mTOR signaling pathway.

PTEN is a tumor suppressor gene that inhibits cell proliferation by inhibiting the phosphoinositide 3-kinase (PI3K) signaling pathway [31]. PTEN deletion was significantly associated with estrogen receptor negative (ER-), especially in triple-negative breast cancer [32]. Studies had shown that degrading PTEN through lysosome-mediated activation of PI3K/AKT/GSK3β/SNAI1 signaling pathway could promote the metastasis and progression of EMT and breast cancer tumors, which showed that the loss of PTEN contributed to the development of breast cancer [33]. In our study, tetrandrine could significantly increase PTEN content compared with the Control group ($p<0.01$) by expressing both nucleus and cytoplasm, inhibiting the progression of TNBC. These data suggested that tetrandrine might be a PTEN enhancer, which provide clinical targeted drugs for Triple-negative breast cancer.

However, some limitations should be noted in the current study. It is reported that hyperactivation of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is implicated in the tumor genesis of ER+ breast cancer and in resistance to endocrine therapy [34]. Our future endeavors still need to further explore the relationship between tetrandrine with ER+ breast cancer and endocrine therapy resistance. Besides, tetrandrine as the inhibitor of PI3K/AKT/mTOR also needs to be further exploited through rational combinations with immunotherapies and targeted therapies to improve Triple-negative breast cancer clinical outcomes.

5. Conclusions

In conclusion, we have proposed a novel mechanism of tetrandrine inducing autophagy on the triple-negative breast cancer MDA-MB-231 cells. High expression of p62 and low expression of Beclin1 and LC3-II/LC3-I in the human breast cancer MDA-MB-231 cells lead to autophagy and apoptosis defects which accelerate breast cancer progression. Intervention on MDA-MB-231 cell with tetrandrine inhibits the proliferation and induces autophagy through inhibiting the PI3K/AKT/mTOR signaling via upregulating PTEN expression and downregulating p-akt^{ser473} /akt, p-PI3K/PI3K p110α, p-mTOR^{ser2448} /mTOR, suggesting tetrandrine may serve as a promising active antitumor drug, by a direct regulation of the PI3K/AKT/mTOR pathway in the triple-negative breast cancer MDA-MB-231 cell. The present paper also warrants further study of tetrandrine in the treatment of triple-negative breast cancer with autophagocytosis and targeted therapy of chemotherapeutic drugs.

Data Availability

Authors make data supporting the conclusions of the study available to all interested researchers upon request through the authors themselves. Xiaohua Pei should be contacted to request the data and the email address is pxh_127@163.com.

Disclosure

The funding agencies have no roles in the study design; in data collection, analysis, and interpretation; in the writing of the report; and in the decision to submit the article for publication.

Conflicts of Interest

All authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Xiaohua Pei contributed to the design of the study and analytic strategy; Yubo Guo completed the experiment, analyzed the data, and wrote the paper.

Acknowledgments

This work was financially supported by grants from National Natural Science Foundation of China (NSFC81774319), Beijing Natural Science Foundation Project (7182098), and the independent subject graduate student projects of Beijing University of Traditional Chinese Medicine (2018-JYBZZ-XS235).

References

[1] E. Schwich and V. Rebmann, "The inner and outer qualities of extracellular vesicles for translational purposes in breast cancer," *Frontiers in Immunology*, vol. 9, 2018.

[2] P. Sharma, "Update on the Treatment of Early-Stage Triple-Negative Breast Cancer," *Current Treatment Options in Oncology*, vol. 19, no. 5, 2018.

[3] C. Neophytou, P. Boutsikos, and P. Papageorgis, "Molecular mechanisms and emerging therapeutic targets of triple-negative breast cancer metastasis," *Frontiers in Oncology*, vol. 8, 2018.

[4] J. Zhang, Z. Yang, and L. Xie, "Statins, autophagy and cancer metastasis," vol. 45, pp. 745–752, 2013.

[5] J. Chen, Y. Zhu, W. Zhang et al., "Delphinidin induced protective autophagy via mTOR pathway suppression and AMPK pathway activation in HER-2 positive breast cancer cells," *BMC Cancer*, vol. 18, no. 1, 2018.

[6] C. Y. Wang, Y. Jiao, and B. Zhang, "Research advance on autophagy and its role in cancer therapy," *E-journal of Translational Medicine*, vol. 4, no. 5, pp. 65–69, 2017.

[7] Q. Zhu and F. Lin, "Molecular markers of autophagy," *Acta Pharmaceutica Sciencia*, vol. 51, pp. 33–38, 2016.

[8] Z. Hamurcu, N. Delibaşı, S. Geçene et al., "Targeting LC3 and Beclin-1 autophagy genes suppresses proliferation, survival, migration and invasion by inhibition of Cyclin-D1 and uPAR/Integrin β1/ Src signaling in triple negative breast cancer cells," *Journal of Cancer Research and Clinical Oncology*, vol. 144, no. 3, pp. 415–430, 2018.

[9] B. Han, G. J. Wang, and H. L. Luo, "Research progress of pharmacological effects of Tetrandrine," *Chinese Journal of Clinical Rational Drug Use*, vol. 7, no. 3A, pp. 198-199, 2014.

[10] M. Xu and B. Zhou, "Reversal of resistance to chemotherapy and apoptosis in lung cancer by tetrandrine," *New Journal of Traditional Chinese Medicine*, vol. 38, no. 6, pp. 90-91, 2006.

[11] H. P. Wang, L. Ye, C. Wang et al., "Study of the inhibitive effects of tea polyphenols and tetrandrine combined with antineoplastic drugs on the HNE-1 (200) cell line," *Journal of Emergency in Traditional Chinese Medicine*, vol. 19, no. 1, pp. 102–103+108, 2010.

[12] L. Ye and H. Y. Chen, "Reversal effect of tetrandrine on multidrug resistance of the KBV200 cell line," *Journal of Chongqing Medical University*, vol. 33, no. 8, pp. 967–969, 2008.

[13] J.-C. Lien, M.-W. Lin, S.-J. Chang et al., "Tetrandrine induces programmed cell death in human oral cancer CAL 27 cells through the reactive oxygen species production and caspase-dependent pathways and associated with beclin-1-induced cell autophagy," *Environmental Toxicology*, vol. 32, no. 1, pp. 329–343, 2017.

[14] W. Y. Deng, X. C. Sun, and S. Y. Li, "The effect of tetrandrine on proliferation and apoptosis of hum an liver cancer cell line 7402," *Journal of Medical Postgraduates*, vol. 20, no. 2, pp. 138–141, 2007.

[15] X. H. Pei, "Antitumor Effect of Tetrandrine on MCF-7 in Tumor Bearing Nude Mice," in *Proceedings of the The 12th National Conference on Chinese Medicine and Integrated Traditional Chinese and Western Medicine Breast Diseases Conference*, vol. 6, pp. 325–330, 2011.

[16] T. X. Wang, M. Li, and Y. Zhang, "Study on isotetrandrine enhancing the effect of doxorubicin-induced apoptosis on breast cancer cell lines MCF," in *7/DOX*, Maternal and Child Health Care of China, vol. 23, no. 35, pp. 5052-5053, 2008.

[17] T.-X. Wang and X.-H. Yang, "Reversal effect of isotetrandrine, an isoquinoline alkaloid extracted from Caulis Mahoniae, on P-glycoprotein-mediated doxorubicin-resistance in human breast cancer (MCF-7/DOX) cells," *Acta Pharmaceutica Sinica*, vol. 43, no. 5, pp. 461–466, 2008.

[18] J. H. Wang, Z. G. Ye, A. X. Sun et al., "Reversal of anti-apoptotic action by tetrandrine in the human breast carcinoma doxorubicin-resistant MCF-7 cells," *Chinese Journal of Histochemistry and Cytochemistry*, vol. 9, no. 4, pp. 436–439, 2000.

[19] X. C. Sun, J. J. Wang, Y. S. Zhen et al., "Potentiation of radiosensitivity by tetrandrine in human breast cancer cells and its mechanism," *Chinese Journal of Radiological Medicine and Protection*, vol. 23, no. 3, pp. 160–162, 2003.

[20] X. P. Qian, B. R. Liu, J. Hu et al., "Inhibitory effect of tetrandrine on angiogenesis," *Chinese Journal of Cancer*, vol. 27, no. 10, pp. 1050–1055, 2008.

[21] T. Li, Q. M. Zhou, and W. H. Zhang, "Advances in Research of PI3K/Akt/mTOR Signaling Pathway for Treatment of Triple Negative Breast Cancer," *Chinese Journal of Cancer*, vol. 27, no. 1, pp. 40–45, 2018.

[22] Y. T. Zhou, E. B. Rucker, B. P. Zhou et al., "Autophagy regulation in the development and treatment of breast cancer," *Acta Biochimica et Biophysica Sinica*, vol. 48, no. 1, pp. 60–74, 2016.

[23] J. S. O'Donnell, D. Massi, M. W. L. Teng, and M. Mandala, "PI3K-AKT-mTOR inhibition in cancer immunotherapy, redux," *Seminars in Cancer Biology*, vol. 48, pp. 91–103, 2018.

[24] N. Dey, P. De, and B. Leyland-Jones, "PI3K-AKT-mTOR inhibitors in breast cancers: From tumor cell signaling to clinical trials," *Pharmacology & Therapeutics*, vol. 175, pp. 91–106, 2017.

[25] V. K. W. Wong, W. Zeng, J. Chen et al., "Tetrandrine, an Activator of Autophagy, Induces Autophagic Cell Death via PKC-α Inhibition and mTOR-Dependent Mechanisms," *Frontiers in Pharmacology*, vol. 8, 2017.

[26] L. L. Wang, R. F. Ma, and Y. B. Guo, "Antioxidant Effect of Fructus Ligustri Lucidi Aqueous Extract in Ovariectomized Rats Is Mediated through Nox4-ROS-NF-kB Pathway," *Frontiers in Pharmacology*, vol. 8, p. 266, 2017.

[27] S. M. Luo, C. H. Ke, J. Y. Cai et al., "Effects of tetrandrine combined with cisplatin on breast cancer cells in vitro," *Biotechnology*, vol. 21, no. 3, pp. 64–69, 2011.

[28] H. Wang, T. Liu, L. Li et al., "Tetrandrine is a potent cell autophagy agonist via activated intracellular reactive oxygen species," *Cell Bioscience*, vol. 5, p. 4, 2015.

[29] L. M. Thorpe, H. Yuzugullu, and J. J. Zhao, "PI3K in cancer: Divergent roles of isoforms, modes of activation and therapeutic targeting," *Nature Reviews Cancer*, vol. 15, no. 1, pp. 7–24, 2015.

[30] N. Sobhani, G. Roviello, S. P. Corona et al., "The prognostic value of PI3K mutational status in breast cancer: A meta-analysis," *Journal of Cellular Biochemistry*, vol. 119, no. 6, pp. 4287–4292, 2018.

[31] F. Khan, A. Esnakula, L. J. Ricks-Santi et al., "Loss of PTEN in high grade advanced stage triple negative breast ductal cancers in African American women," *Pathology - Research and Practice*, vol. 214, no. 5, pp. 673–678, 2018.

[32] N. Jones, F. Bonnet, S. Sfar et al., "Comprehensive analysis of PTEN status in breast carcinomas," *International Journal of Cancer*, vol. 133, no. 2, pp. 323–334, 2013.

[33] M. Wang, H. Wu, S. Li et al., "SYNJ2BP promotes the degradation of PTEN through the lysosome-pathway and enhances breast tumor metastasis via PI3K/AKT/SNAI1 signaling," *Oncotarget* , vol. 8, no. 52, pp. 89692–89706, 2017.

[34] E. M. Ciruelos Gil, "Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer," *Cancer Treatment Reviews*, vol. 40, no. 7, pp. 862–871, 2014.

Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2019, Article ID 8976937, 8 pages
https://doi.org/10.1155/2019/8976937

Research Article

Ethnomedical Survey of the Plants Used by Traditional Healers in Narok County, Kenya

Gabriel Kigen,[1] **Zipporah Kamuren**,[1] **Evangeline Njiru**,[2] **Bernard Wanjohi**,[3] **and Wilson Kipkore**[4]

[1]*Department of Pharmacology and Toxicology, Moi University School of Medicine, P.O. Box 4606-30100, Eldoret, Kenya*
[2]*Department of Internal Medicine, Moi University School of Medicine, P.O. Box 4606-30100, Eldoret, Kenya*
[3]*Department of Wildlife Management, University of Eldoret, P.O. Box 1125-30100, Eldoret, Kenya*
[4]*Department of Forestry, University of Eldoret, P.O. Box 1125-30100, Eldoret, Kenya*

Correspondence should be addressed to Gabriel Kigen; kigengfk@gmail.com

Received 6 November 2018; Accepted 13 November 2018; Published 1 January 2019

Guest Editor: José C. T. Carvalho

Most of the plants used by herbalists amongst the various Kenyan communities have not been documented despite their widespread use. The purpose of this research was to document the medicinal plants used by the herbalists from the Maasai, a community that still relies on herbal medicine to a large extent for the provision of medical services. Semistructured interviews, direct observations, group discussions, and in-depth interviews were used to collect information from the traditional healers. A total of 47 plant species belonging to 31 families were identified. They were used in the treatment of 33 medical and 4 veterinary conditions.

1. Introduction

Medicinal plants still play an important role in primary healthcare in many sub-Saharan African countries due to variety of reasons including lack of health services, cultural norms, and traditional beliefs [1–3]. Many patients in these countries combine traditional medicine (TM) with conventional medicine, especially those with chronic diseases [4]. The use of TM is in most cases widespread and not only limited to the rural areas or low-income settings, but also in urban and well to do settings [3, 5, 6]. In addition, there is a growing global demand for traditional and complementary medicine [2]. The knowledge of these medicinal preparations is therefore important in order to enable health practitioners to be aware of the kind of TM their patients are on, so as to minimize potential adverse effects resulting from herb-drug interactions [7]. The documentation of the type of medicinal plants used by the traditional medical practitioners (TMPs) and the conditions treated is crucial to this endeavor [4]. In addition, databases containing this information would also be important for research and potential development of new drugs, as many of the drugs in current use have been developed from medicinal plants [8, 9]. Examples of these include paclitaxel, an antitumour drug developed from the bark of *T. brevifolia* [10], the antimalarial drug artemisinin from *Artemisia annua* [11], digoxin from *Digitalis lanata*, atropine from *Atropa belladona,* aspirin from *Filipendula ulmaria,* and several other drugs. However, despite the widespread use of medicinal plants in Kenya, several have not been documented [4, 12]. The main aim of this research was to document the medicinal plants used by the Maasai community in Narok County, one of the regions in Kenya whereby the practice of herbal medicine is still widespread for future research.

2. Materials and Methods

2.1. Study Area. The study was conducted in two locations within Narok County, Olpusimoru (2°1′0″S, 36°54′0″E) a highland area located in the Northern part; and Sekenani (1°30′58.33″S, 35°20′19.63″E) a lowland area located in the South-Western region [Siana Ward] [Figure 1]. Olpusimoru is a mountainous forested terrain with an average altitude of 2478 metres and high rainfall, while Sekenani has an average

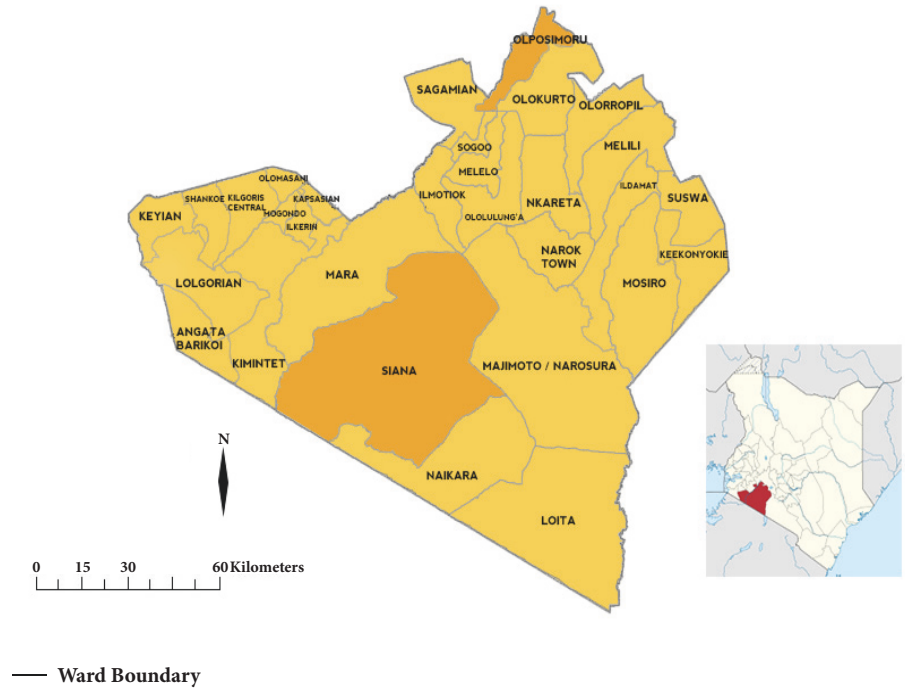


Figure 1: Map of Narok County showing Olpusimoru and Siana wards and its position within Kenyan map.

altitude of 1820 m with comparatively low rainfall. It borders the globally famous Maasai Mara National Reserve on the East [13, 14].

2.2. Data Collection. Ethnobotanical data was collected from TMPs between March and December 2016. The research team is comprised of professionals from the medical field and botany, including a physician (EN), two clinical pharmacologists (GK and ZK), a taxonomist (BW), and a plant specialist (WK). There were also two local lead persons, one from each site who accompanied the team at each visit in order to direct and introduce them to the TMPs. All TMPs that we met were willing to participate in the research. A total of 37 TMPs comprising 20 men and 17 women aged between 42 and 85 were interviewed. Semistructured interviews, direct observations, group discussions, and in-depth interviews were used to collect ethnopharmacological information [15–17]. The participant's biodata, conditions they treated, methods of treatment, medicinal plants used, methods of preparation and administration, and dosing forms were recorded. They were also asked to explain the manner in which they arrived at a diagnosis. At the end of each interview, the informants were requested to accompany the research team to sites where they collected the plants and assist in identification. Preliminary identification of the plants was then done by BW and WK, and the plants and their surrounding habitats photographed. The voucher specimens were then collected using standard botanical procedures, and further identification and confirmation were performed using the relevant taxonomic keys at University of Eldoret Herbarium where the specimens were subsequently deposited [18, 19]. The data was then compared to related research that has been carried out in the region.

2.3. Data Analysis. The medicinal importance of each plant species used was calculated as per the use-value index for each plant species *(UVs)* using the formula:

$$UVs = \frac{U}{N} \quad (1)$$

where U is the number of different uses mentioned by each TMP (informant) whereas N is the total number of TMPs interviewed during the survey [20, 21]. The *UV* index theoretically varies from 0, which implies that none of the informants mention any use of the plant, to 1 whereby the plant is most frequently mentioned as useful in treatment of the highest number of conditions.

3. Results and Discussion

A total of 47 plant species belonging to 31 families were identified, out of which 36 (77%) were from the lowland area (Sekenani), while 11 (23%) species were from the highlands (Olpusimoru) [Table 1]. The plant details including the voucher numbers are outlined in Table 2.

Medicinal Plant Uses. The plants were used in the treatment of 33 medical and 4 veterinary disorders. The detailed list of the plants and their respective medicinal uses are outlined in Table 2 and Supplementary Material section (available here). The most frequently used plant was *Solanum incanum* which had seven medical uses ($UV = 0.19$) followed by *Olea europaea subsp. cuspidata* which had five applications ($UV = 0.14$). *Asparagus africanus*, *Carissa edulis, Commiphora africana, Elaeodendron buchananii,* and *Kigelia africana* had four medicinal uses each ($UV = 0.11$).

Table 1: Classification of medicinal plants.

	Family	Species	
		No	*Members*
1.	FABACEAE	5	*Albizia gummifera, Dichrostachys cinerea, Erythrina senegalensis, Senegalia senegal, Vachellia nilotica*
2.	SOLANACEAE	4	*Physalis peruviana, Solanum arundo, Solanum incanum, Solanum mauense*
3.	AMARANTHACEAE	2	*Achyranthes aspera, Aerva javanica*
4.	APOCYNACEAE	2	*Acokanthera schimperi, Carissa edulis*
5.	BURSERACEAE	2	*Commiphora africana, Ficus sycomorus*
6.	CELASTRACEAE	2	*Elaeodendron buchananii, Mystroxylon aethiopicum*
7.	EUPHORBIACEAE	2	*Clutia abyssinica, Croton dichogamous*
8.	MALVACEAE	2	*Grewia bicolor, Sida cuneifolia*
9.	RHAMNACEAE	2	*Rhamnus prinoides, Ziziphus mucronata*
10.	RUBIACEAE	2	*Galium aparinoides, Pavetta subcana*
11.	RUTACEAE	2	*Teclea nobilis, Toddalia asiatica*
12.	APIACEAE	1	*Anthriscus sylvestris*
13.	ASPARAGACEAE	1	*Asparagus africanus*
14.	ASTERACEAE	1	*Acmella calirhiza*
15.	BIGNONIACEAE	1	*Kigelia africana*
16.	BORAGINACEAE	1	*Cordia monoica*
17.	CANELACEAE	1	*Warburgia ugandensis*
18.	CAPPARIDACEAE	1	*Boscia angustifolia*
19.	COLCHICACEAE	1	*Gloriosa superba*
20.	COMMELINACEAE	1	*Aneilema equinoctiale*
21.	CRASSULACEAE	1	*Kalanchoe crenata*
22.	CUCURBITACEAE	1	*Momordica friesiorum*
23.	EBENACEAE	1	*Diospyros abyssinica*
24.	FLACOURTIACEAE	1	*Dovyalis abyssinica*
25.	LABIATAE	1	*Leonotis mollissima*
26.	OLEACEAE	1	*Olea europaea subsp. cuspidata*
27.	PRIMULACEAE	1	*MyrsineaAfricana*
28.	ROSACEAE	1	*Prunus africana*
29.	SANTALACEAE	1	*Osyris lanceolata*
30.	SAPINDACEAE	1	*Pappea capensis*
31.	VITACEAE	1	*Cissus fischeri*
	Total	**47**	

Most of the plants used by traditional healers in Kenya have not documented despite the imminent risk of disappearance of this plants due to several factors including deforestation and overexploitation [4]. In addition, the practice is usually a guarded family secret, and some of the siblings may not be willing to inherit the art due to changing lifestyles [22]. The lack of adequate regulation of the practice in Kenya has also led to infiltration by several quacks. However, the Maasai is one community in Kenya which still practices TM to a large extent owing to several reasons including lack of adequate health facilities and traditional values [23]. Some of the reported plants have been evaluated *in vitro* and found to exhibit pharmacological activities related to the uses described by the TMPs [24]. These include *Aerva javanica, Asparagus africanus, Carissa edulis, Sida cuneifolia, and Solanum incanum* which have demonstrated to possess antibiotic/antifungal activities [25–28], while *Gloriosa superba* that is used as an abortifacient has oxytocic activity [29]. The plants used by the TMPs are largely similar to those used by their Kalenjin counterparts that we have reported before, although for different medicinal uses [3, 12, 30, 31]. Additionally, the methods of preparations are slightly different as the Maasai TMPs tend to use a lot of cold herbal infusions prepared by soaking the plant parts in water and hardly use the burnt leaves/barks as their counterparts

4. Conclusions

It is important to document traditional medicinal plants used by the various communities in Kenya in order to develop a database for future research. The risk of the rapid disappearance of the knowledge on traditional medicine calls for an urgent multidisciplinary approach towards conserving the information before it is lost forever. Some of these plants

Table 2: Medicinal plant uses.

No	Botanical Name	Family	Voucher No.	Maa name	Habitat	Parts used	Method of preparation	Medicinal uses
1.	*Achyranthes aspera* L.	AMARANTHACEAE	OLP/08/15/007	*Olerubat*	Highland	*Roots*	*Boiled*	*Arthritis*
2.	*Acmella calirhiza* Del	ASTERACEAE	OLP/08/15/009	*Ekum*	Highland	*Flowers*	*Crushed and mixed with water*	*Oral thrush in children*
3.	*Acokanthera schimperi* (A.DC.) Schweinf	APOCYNACEAE	MAU/08/15/027	*Olmorijioi*	Lowland	*Roots* *Bark*	*Boiled* *Soaked in water*	*Syphilis* *Arrow poison*
4.	*Aerva javanica* (Burm.f.) Shult.	AMARANTHACEAE	MAU/08/15/032	*Eleleshwa-ekop*	Lowland	*Flowers*	*Ground into paste & mixed with water*	*East Coast Fever in cattle*
5.	*Albizia gummifera* (J.F. Gmel.) C.A.Sm.	FABACEAE	MAU/08/15/018	*Osupakupe*	Lowland	*Pods* *Roots*	*Crushed* *Pounded*	*Stomachache* *Skin disorders*
6.	*Aneilema aequinoctiale* P. Beauv	COMMELINACEAE	MAU/08/15/020	*Enkaiteteyiai*	Lowland	*Leaves* *Flowers*	*Soaked in water* *Pressed to produce juice*	*Malnutrition, colds* *Ocular disorders*
7.	*Anthriscus sylvestris* (L.) Hoffm	APIACEAE	MAU/08/15/028	*Oldule*	Lowland	*Seeds*	*Mixed with honey and chewed*	*Chesty colds*
8.	*Asparagus africanus* Lam	ASPARAGACEAE	MAU/08/15/006	*Empereempapa*	Lowland	*Leaves, stem & roots* *Leaves* *Roots*	*Soaked in water* *Soaked in water* *Soaked in water* *Chewed*	*Mental illness* *Wounds* *Venereal diseases* *Cough & sore throat*
9.	*Boscia angustifolia* Harvey	CAPPARIDACEAE	MAU/08/15/001	*Oloireroi*	Lowland	*Leaves* *Bark*	*Pounded* *Crushed & mixed with water*	*Cattle fever* *Gynaecological disorders*
10.	*Carissa edulis* Harv	APOCYNACEAE	MAU/08/15/003	*Olamuriaki*	Lowland	*Roots*	*Boiled*	*Lower abdominal pains in pregnancy, gonorrhea, chest pains, polio symptoms*
11.	*Cissus fischeri* Gilg	VITACEAE	MAU/08/15/011	*Osurkurtuti*	Lowland	*Leaves*	*Soaked in water*	*Respiratory disorders in cattle*
12.	*Clutia abyssinica* Jaub. & Spach.	EUPHORBIACEAE	OLP/08/15/011	*Enkiparnyeny*	Highland	*Roots*	*Boiled*	*Appetizer*
13.	*Commiphora africana* (A. Rich.) Endl	BURSERACEAE	MAU/08/15/034	*Osilalei*	Lowland	*Roots* *Bark* *Fruits*	*Boiled* *Chewed* *Boiled*	*Swollen testicles, abdominal pains* *Snake bite* *Typhoid*
14.	*Cordia monoica* Roxb	BORAGINACEAE	MAU/08/15/031	*Oseki*	Lowland	*Leaves, bark* *Roots* *Leaves*	*Leaves- boiled, bark - pounded* *Boiled* *Pounded*	*Leprosy* *Mental illness* *Ocular disorders*
15.	*Croton dichogamous Pax*	EUPHORBIACEAE	MAU/08/15/008	*Ollokirdangai*	Lowland	*Roots*	*Boiled*	*Polio-like symptoms, gonorrhea, chest pain*

Table 2: Continued.

No	Botanical Name	Family	Voucher No.	Maa name	Habitat	Parts used	Method of preparation	Medicinal uses
16.	*Dichrostachys cinerea* Wight et Arn	FABACEAE	MAU/08/15/009	*Emerrumori*	Lowland	*Leaves*	*Pounded*	*Local anaesthesia, ulcers, gonorrhea*
17.	*Diospyros abyssinica* Hiern	EBENACEAE	MAU/08/15/025	*Olchartuyian*	Lowland	*Bark*	*Pounded & soaked in water*	*Malaria, ocular d/orders in livestock*
18.	*Dovyalis abyssinica* (A. Rich.) Warb	FLACOURTIACEAE	OLP/08/15/006	*Olmorogi*	Highland	*Roots* *Leaves*	*Boiled* *Chewed*	*Gonorrhea* *Toothache*
19.	*Elaeodendron buchananii* (Loes) Loes.	CELASTRACEAE	MAU/08/15/004	*Osoket*	Lowland	*Roots* *Roots* *Leaves*	*Dried and ground to powder* *Boiled or dried and ground to powder* *Chewed*	*Wounds, syphilis* *Respiratory d/orders* *Diarrhoea*
20.	*Erythrina senegalensis DC.*	FABACEAE	MAU/08/15/036	*Ol-oboni*	Lowland	*Roots*	*Boiled*	*Polio-like symptoms, gonorrhea, chest pain*
21.	*Ficus sycomorus* L.	BURSERACEAE	MAU/08/15/024	*Olngaboli*	Lowland	*Roots*	*Boiled, chewed*	*Abortifacient*
22.	*Galium aparinoides* Forssk	RUBIACEAE	MAU/08/15/026	*Olngeriantus*	Lowland	*Whole plant*	*Pounded & soaked in water or boiled*	*Throat cancer in cattle*
23.	*Gloriosa superba* L.	COLCHICACEAE	MAU/08/15/022	*Sakutayei*	Lowland	*Roots*	*Chewed or soaked in water*	*Abortifacient*
24.	*Grewia bicolor*	MALVACEAE	MAU/08/15/014	*Ositeti*	Lowland	*Roots*	*Soaked in water*	*Respiratory d/orders, snake bite*
25.	*Kalanchoe crenata* (Andrews) Haw	CRASSULACEAE	OLP/08/15/008	*Ormasilig*	Highland	*Leaves*	*Warmed*	*Poultice*
26.	*Kigelia africana* (Lam.) Benth.	BIGNONIACEAE	MAU/08/15/021	*Oldarpoi*	Lowland	*Fruits* *Roots* *Bark* *Leaves*	*Brewed* *Boiled* *Boiled* *Boiled*	*Measles in children* *Abortifacient* *Headache* *Malaria*
27.	*Leonotis mollissima* Guerke	LABIATAE	OLP/08/15/003	*Olbibi*	Highland	*Leaves*	*Soaked in water or boiled*	*Antiseptic, skin rashes, blood purifier*
28.	*Momordica friesiorum* (Harms) C. Jeffrey	CUCURBITACEAE	OLP/08/15/001	*Esumeito*	Highland	*Roots*	*Pounded & mixed with water*	*Induce vomiting*
29.	*Myrsine Africana* L.	PRIMULACEAE	OLP/08/15/004	*Seketet*	Highland	Seeds	*Ground*	*Antihelminthic, heartburn*
30.	*Mystroxylon aethiopicum* (Thunb.) Loes.	CELASTRACEAE	MAU/08/15/035	*Olodonganayioi*	Lowland	*Bark*	*Boiled*	*Colic pain, especially in children*
31.	*Olea europaea subsp. cuspidata* (Wall. ex G. Don) Cif.	OLEACEAE	MAU/08/15/023	*Oloirien*	Lowland	*Bark* *Leaves* *Roots*	*Pounded and soaked in water* *Boiled* *Boiled*	*Antihelminthic* *Liver disease* *Polio-like symptoms, gonorrhea, chest pain*
32.	*Osyris lanceolata* Hochst. & Steud. ex A. DC.	SANTALACEAE	MAU/08/15/016	*Olosesiai*	Lowland	*Bark* *Leaves* *Roots*	*Boiled* *Pounded* *Boiled*	*Abdominal pains in children* *Diarrhoea* *Gonorrhea*
33.	*Pappea capensis* Eckl. & Zeyh	SAPINDACEAE	MAU/08/15/029	*Olkisik-ongo*	Lowland	*Bark*	*Boiled*	*Abdominal disorders*
34.	*Pavetta subcana* Hiern.	RUBIACEAE	MAU/08/15/002	*Olabai*	Lowland	*Roots* *Whole plant*	*Boiled* *Soaked in water*	*Gonorrhea* *Cough in calves, fleas*
35.	*Physalis peruviana* L.	SOLANACEAE	OLP/08/15/010	*Ormumai*	Highland	*Roots*	*Squeezed/chewed*	*Tonsillitis*

Table 2: Continued.

No	Botanical Name	Family	Voucher No.	Maa name	Habitat	Parts used	Method of preparation	Medicinal uses
36.	*Prunus africana* (Hook.f.) Kalkman	ROSACEAE	MAU/08/15/012	*Olkujuk*	Lowland	*Leaves* *Bark*	*Pounded & soaked in water* *Pounded & mixed with water*	*Appetizer* *Stomachache*
37.	*Rhamnus prinoides* L'Hér.	RHAMNACEAE	OLP/08/15/002	*Olkonyel*	Highland	*Roots* *Stem*	*Boiled* *Pounded and mixed with water*	*Gonorrhea, arthritis* *Preservative*
38.	*Senegalia senegal* (L.) Britton & P. Wilson	FABACEAE	MAU/08/15/010	*Oitioibor*	Lowland	*Roots* *Bark*	*Boiled* *Boiled*	*Purgative, constipation & gonorrhea* *Diarrhoea & abdominal disorders*
39.	*Sida cuneifolia* Roxb	MALVACEAE	MAU/08/15/005	*Olonini*	Lowland	*Roots*	*Chewed* *Boiled*	*Sore throat* *Reduce foetal movements in pregnancy*
40.	*Solanum arundo*	SOLANACEAE	MAU/08/15/013	*Esokawai*	Lowland	Roots	*Chewed, pounded & soaked in water*	*Fever*
41.	*Solanum incanum* L.	SOLANACEAE	MAU/08/15/015	*Entulelei*	Lowland	*Roots* *Leaves* *Fruits*	*Boiled* *Raw roots used* *Chewed and applied* *Juice*	*Abdominal pains, fever* *Toothache* *Snake bite* *Chest pain, wounds & skin disorders, Respiratory disorders in sheep*
42.	*Solanum mauense* Bitter.	SOLANACEAE	MAU/08/15/007	*Olesayiet*	Lowland	*Berries* *Roots*	*Cooked* *Boiled*	*Pneumonia* *Anthrax in both humans and animals*
43.	*Teclea nobilis* Del.	RUTACEAE	MAU/08/15/019	*Olgilai*	Lowland	*Leaves, roots*	*Boiled*	*Pneumonia, arthritis*
44.	*Toddalia asiatica* (L.) Lam	RUTACEAE	MAU/08/15/030	*Oleparmunyio*	Lowland	*Bark* *Roots*	*Boiled or soaked in water* *Boiled*	*Respiratory disorders* *Malaria*
45.	*Vachellia nilotica* (L.) P.J.H.Hurter & Mabb	FABACEAE	MAU/08/15/033	*Olkiloriti*	Lowland	*Bark*	*Pounded and mixed with water*	*Stomachache, indigestion*
46.	*Warburgia ugandensis* Sprague.	CANELACEAE	OLP/08/15/005	*Osokonoi*	Highland	*Bark*	*Pound and mixed with water* *Boiled, ground to powder*	*Malaria, abdominal disorders* *Arthritis*
47.	*Zizyphus mucronata* Willd.	RHAMNACEAE	MAU/08/15/017	*Oloilalei*	Lowland	*Roots* *Bark*	*Soaked in water* *Boiled*	*Snake bite* *Arthritis, stomachache*

may contain undiscovered pharmacological properties which can serve as ingredients for the development of new drugs as has happened in Asia with the discovery of artemisinin. Additionally, medical personnel would also have an idea of the kind of herbal medicine that their patients may be taking and therefore minimize toxic effects through herb-drug interactions.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors are very grateful to John Ole Tira and Richard Ole Kuyo for their assistance in introduction to TMPs and identification of medicinal plants in Sekenani and Olpusimoru sites, respectively.

Supplementary Materials

Details of the medicinal plant uses. *(Supplementary Materials)*

References

[1] A. A. Abdullahi, "Trends and challenges of traditional medicine in Africa," *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 8, pp. 115–123, 2011.

[2] WHO, *WHO Traditional Medicine Strategy 2014-2023*, 2017, http://www.who.int/medicines/publications/traditional/trm_strategy14_23/en/.

[3] W. Kipkore, B. Wanjohi, H. Rono, and G. Kigen, "A study of the medicinal plants used by the Marakwet Community in Kenya," *Journal of Ethnobiology and Ethnomedicine*, vol. 10, article 24, 2014.

[4] G. K. Kigen, H. K. Ronoh, W. K. Kipkore, and R. JK, "Current trends of traditional herbal medicine practice in Kenya: a review," *The African Journal of Pharmacology and Therapeutics*, vol. 2, no. 1, pp. 32–37, 2013.

[5] J. W. Stanifer, U. D. Patel, F. Karia et al., "The determinants of traditional medicine use in northern tanzania: a mixed-methods study," *PLoS ONE*, vol. 10, no. 4, p. e0122638, 2015.

[6] P. P. Mhame, K. Busia, and O. M. Kasilo, *Clinical Practices of African Traditional Medicine*, Africa Health Monitor, 2010.

[7] P. S. Fasinu, P. J. Bouic, and B. Rosenkranz, "An overview of the evidence and mechanisms of herb–drug interactions," *Frontiers in Pharmacology*, vol. 3, article 69, 2012.

[8] D. S. Fabricant and N. R. Farnsworth, "The value of plants used in traditional medicine for drug discovery," *Environmental Health Perspectives*, vol. 109, no. 1, pp. 69–75, 2001.

[9] W. Sneader, *Drug Discovery: A History*, John Wiley and Sons, Ltd, Hoboken, New Jersey, NJ, USA, 2005.

[10] M. E. Wall and M. C. Wani, "Camptothecin and taxol: discovery to clinic–thirteenth Bruce F. Cain Memorial Award Lecture," *Cancer Research*, vol. 55, pp. 753–760, 1995.

[11] L. H. Miller and X. Su, "Artemisinin: discovery from the Chinese herbal garden," *Cell*, vol. 146, no. 6, pp. 855–858, 2011.

[12] G. Kigen, W. Kipkore, B. Wanjohi, B. Haruki, and J. Kemboi, "Medicinal plants used by traditional healers in Sangurur, Elgeyo Marakwet County, Kenya," *Pharmacognosy Research*, vol. 9, no. 4, pp. 333–347, 2017.

[13] Olpusimoru, "Olpusimoru Forest; geoview.info," 2017, http://ke.geoview.info/olpusimoru_forest,181965.

[14] Sekenani, "Sekenani; geoview.info," 2017, http://ke.geoview.info/sekenani,1611716061n.

[15] J. R. Stepp, "Advances in Ethnobiological Field Methods," *Field Methods*, vol. 17, no. 3, pp. 211–218, 2016.

[16] M. N. Alexiades and J. W. Sheldon, *Selected Guidelines for Ethnobotanical Research: A Field Manual*, vol. 10 of *Advances in Economic Botany*, New York Botanical Garden Bronx, New York, NY, USA, 1996.

[17] G. Martin, *Ethnobotany—A Manual of Methods*, Earthsacn Publishers Limited, London, UK, 2004.

[18] H. Beentje, J. Adamson, and D. Bhanderi, "Kenya trees, shrubs, and lianas," in *National Museums of Kenya*, 1994.

[19] A. D. Q. Agnew, *Upland Kenya Wild Flowers and Fern*, Nature Kenya–The East Africa Natural History Society, Nairobi, Kenya, 3rd edition, 2013.

[20] U. P. Albuquerque, R. F. Lucena, J. M. Monteiro, A. T. Florentino et al., "Evaluating two quantitative ethnobotanical techniques," *Ethnobotany Research and Applications*, vol. 4, pp. 51–60, 2006.

[21] J. Tardío and M. Pardo-De-Santayana, "Cultural importance indices: a comparative analysis based on the useful wild plants of Southern Cantabria (Northern Spain)," *Economic Botany*, vol. 62, no. 1, pp. 24–39, 2008.

[22] R. W. Bussmann, G. G. Gilbreath, J. Solio et al., "Plant use of the Maasai of Sekenani Valley, Maasai Mara, Kenya," *Journal of Ethnobiology and Ethnomedicine*, vol. 2, article 22, 2006.

[23] J. W. Kiringe, "A survey of traditional health remedies used by the maasai of southern kaijiado district, Kenya," *Ethnobotany Research and Applications*, pp. 61–74, 2006.

[24] M. E. Parker, S. Chabot, B. J. Ward, and T. Johns, "Traditional dietary additives of the Maasai are antiviral against the measles virus," *Journal of Ethnopharmacology*, vol. 114, no. 2, pp. 146–152, 2007.

[25] A. O. Maima, S. N. Ndwigah, G. N. Thoithi et al., "Antimicrobial properties of some medicinal plants of the luo community of kenya," *African Journal of Pharmacology and Therapeutics*, vol. 3, 2014.

[26] A. Teka, J. Rondevaldova, Z. Asfaw et al., "In vitro antimicrobial activity of plants used in traditional medicine in Gurage and Silti Zones, south central Ethiopia," *BMC Complementary and Alternative Medicine*, vol. 15, article 286, 2015.

[27] B. Taye, M. Giday, A. Animut, and J. Seid, "Antibacterial activities of selected medicinal plants in traditional treatment of human wounds in Ethiopia," *Asian Pacific Journal of Tropical Biomedicine*, vol. 1, no. 5, pp. 370–375, 2011.

[28] F. U. Mufti, H. Ullah, A. Bangash et al., "Antimicrobial activities of Aerva javanica and Paeonia emodi plants," *Pakistan Journal of Pharmaceutical Sciences*, pp. 565–569, 2012.

[29] A. A. Malpani, "Effect of the aqueous extract of Gloriosa superba Linn (Langli) roots on reproductive system and cardiovascular parameters in female rats," *Tropical Journal of Pharmaceutical Research*, vol. 10, 2011.

[30] G. Kigen, F. Some, J. Kibosia et al., "Ethnomedicinal plants traditionally used by the keiyo community in Elgeyo Marakwet County, Kenya," *Journal of Biodiversity, Bioprospecting and Development*, vol. 1, pp. 132–143, 2014.

[31] G. Kigen, A. Maritim, F. Some et al., "Ethnopharmacological survey of the medicinal plants used in Tindiret, Nandi County, Kenya," *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 13, pp. 156–168, 2016.