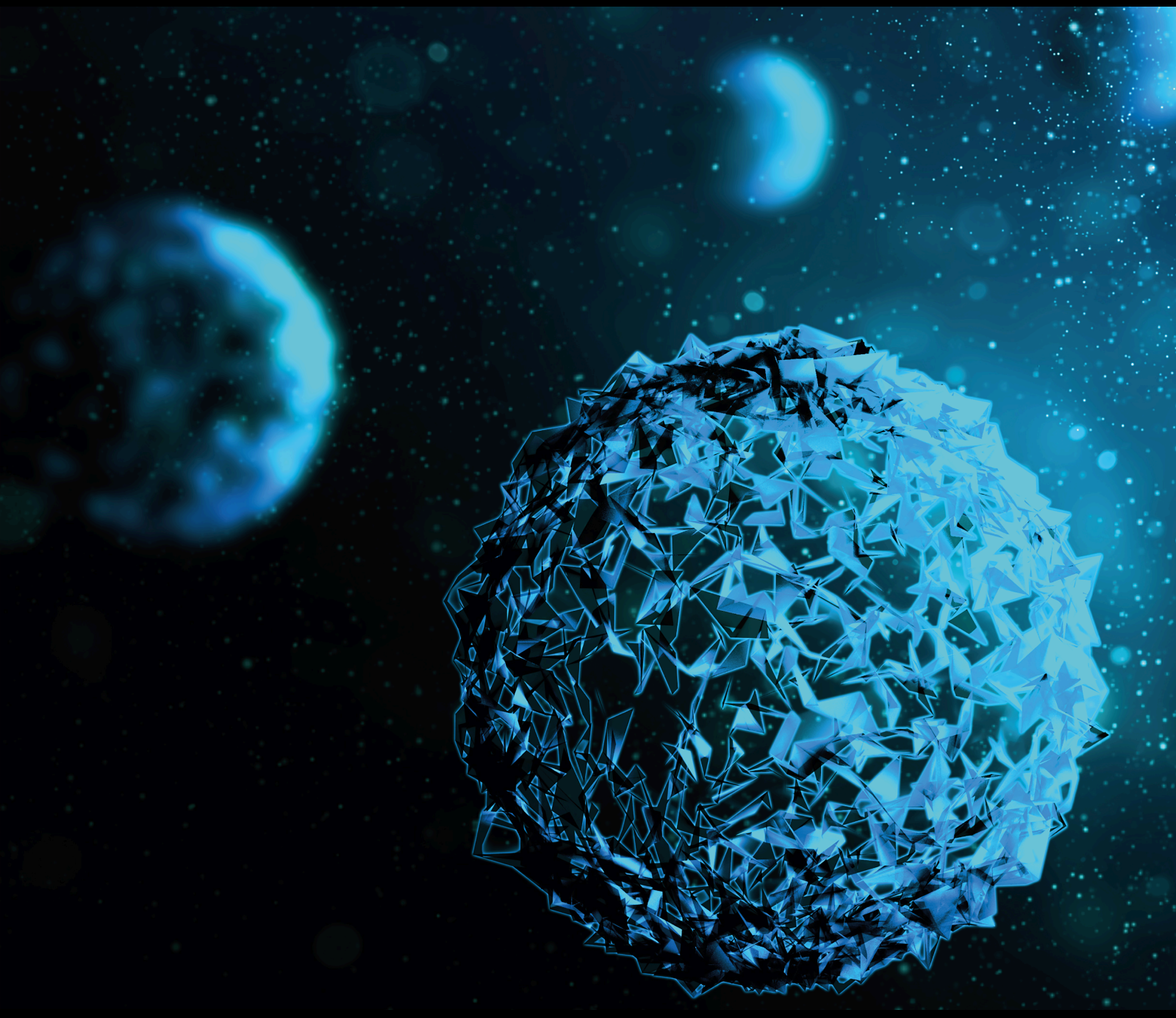
BioMed Research International

Beneficial and Harmful Effects of Aromatic Medicinal Plants

Lead Guest Editor: Riaz Ullah
Guest Editors: Najeeb Rehman, Ahmed Bari, and Abdelaaty Shahat



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Toxicology

Contents

Contents

Hindawi
BioMed Research International
Volume 2024, Article ID 9817451, 1 page
https://doi.org/10.1155/2024/9817451

Retraction

Retracted: Targeting Streptomyces-Derived Streptenol Derivatives against Gynecological Cancer Target PIK3CA: An In Silico Approach

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Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] H. Jemmy Christy, S. Vasudevan, S. Sudha et al., "Targeting Streptomyces-Derived Streptenol Derivatives against Gynecological Cancer Target PIK3CA: An In Silico Approach," *BioMed Research International*, vol. 2022, Article ID 6600403, 15 pages, 2022.

Hindawi
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Retracted: Unani Formulation Habb-e-Suranjan: A Treasure of Biological Activities

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Hindawi
BioMed Research International
Volume 2024, Article ID 9840153, 1 page
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Retraction

Retracted: Dioscorea nipponica Makino Relieves Ovalbumin-Induced Asthma in Mice through Regulating RKIP-Mediated Raf-1/MEK/MAPK/ERK Signaling Pathway

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Hindawi
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Retraction

Retracted: Toxicological and Teratogenic Effect of Various Food Additives: An Updated Review

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[1] S. Sambu, U. Hemaram, R. Murugan, and A. A. Alsofi, "Toxicological and Teratogenic Effect of Various Food Additives: An Updated Review," *BioMed Research International*, vol. 2022, Article ID 6829409, 11 pages, 2022.

Hindawi
BioMed Research International
Volume 2024, Article ID 9754274, 1 page
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Retraction

Retracted: A Network Pharmacology Method Combined with Molecular Docking Verification to Explore the Therapeutic Mechanisms Underlying Simiao Pill Herbal Medicine against Hyperuricemia

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Research Article

A Network Pharmacology Method Combined with Molecular Docking Verification to Explore the Therapeutic Mechanisms Underlying Simiao Pill Herbal Medicine against Hyperuricemia

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Objective. Hyperuricemia (HUA) is a common metabolic disease caused by disordered purine metabolism. We aim to reveal the mechanisms underlying the anti-HUA function of Simiao pill and provide therapeutic targets. *Methods*. Simiao pill-related targets were obtained using Herbal Ingredients' Targets (HIT), Traditional Chinese Medicine Systems Pharmacology (TCMSP), and Traditional Chinese Medicine Integrated Database (TCMID). HUA-associated targets were retrieved from GeneCards, DisGeNET, and Therapeutic Targets Database (TTD). Protein-protein interaction (PPI) network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, ggraph and igraph R packages. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using ClusterProfiler. The top 10 core targets were identified through cytoHubba. Molecular docking was conducted using PyMOL and AutoDock high-performance liquid chromatograph (HPLC) analysis was performed to identify effective compounds of Simiao pill. *Results*. Simiao pill-HUA target network contained 80 targets. The key targets were mainly involved in inflammatory responses. Insulin (INS), tumor necrosis factor (TNF), interleukin-6 (IL6), interleukin 1 beta (IL1B), vascular endothelial growth factor A (VEGFA), leptin (LEP), signal transducer and activator of transcription 3 (STAT3), C-C motif chemokine ligand 2 (CCL2), interleukin-10 (IL10), and toll like receptor 4 (TLR4) were the top 10 targets in the PPI network. GO analysis demonstrated the main implication of the targets in molecular responses, production, and metabolism. KEGG analysis revealed that Simiao pill might mitigate HUA through advanced glycation end-product- (AGE-) receptor for AGE- (RAGE-) and hypoxia-inducible factor-1- (HIF-1-) associated pathways. IL1B, IL6, IL10, TLR4, and TNF were finally determined as the promising targets of Simiao pill treating HUA. Through molecular docking and HPLC analysis, luteolin, quercetin, rutaecarpine, baicalin, and atractylenolide I were the main active compounds. *Conclusions*. Simiao pill can mitigate HUA by restraining inflammation, mediating AGE-RAGE- and HIF-1-related pathways, and targeting IL1B, IL6, IL10, TLR4, and TNF.

1. Introduction

Hyperuricemia (HUA) is a common metabolic disorder triggered by abnormal purine metabolism, with a rising incidence worldwide [1, 2]. It has been reported that the prevalence of HUA was 11.9–25.0% in Europe, 11.3–47% in the United States, 26.8% in Japan, and 13.1–13.3% in China [3]. The onset of HUA is dominantly due to the boosted formation or decreased excretion of uric acid [4]. Uric acid is the product of purine breakdown, which circulates in the ionized form of urate at the normal physiological pH of 7.4. Purine metabolism primarily occurs in the liver, also produced in any other tissue with xanthine oxidase (intestines) [5]. HUA can lead to gout and kidney stones [6, 7], and it is closely linked with the development of cardiovascular disease [8]. Although the exact cause and pathogenesis remain unclear, inflammation and oxidative stress have been demonstrated to be closely associated with

the pathological mechanisms of HUA [9]. Noteworthily, HUA tends to be asymptomatic, bringing a challenge to the management and treatment of HUA.

In the last decades, the therapeutic strategies of HUA have counted on the decline in uric acid. Xanthine oxidase inhibitors such as allopurinol [10] and febuxostat [11] have been regarded as promising uric acid-lowering drugs to treat HUA [12]. Despite significant improvements in anti-HUA agents, current drug therapy is insufficient to cure HUA and prevent the onset of HUA-associated disorders. Therefore, safer and more effective drugs for treating HUA are desperately needed.

Traditional Chinese medicine (TCM) has gained global attention owing to its favorable efficacy and safety in the treatment of various diseases, and the application of herbal remedies is increasing worldwide. Plants have been the main source of tradition medicines since ancient times [13]. Some Chinese herbs or their active ingredients, such as plantain [14] and berberine [15], have exhibited curative potential for HUA. Simiao pill, a TCM formula, consists of Rhizoma Atractylodis, Cortex Phellodendri, Radix Achyranthis Bidentatae, and Semen Coicis at the ratio of 1 : 2 : 1 : 2 [16, 17]. Pharmacological research has indicated that Simiao pill contains a variety of natural active compounds, such as flavonoids and phenols [18]. Interestingly, recent studies have demonstrated that some phytochemicals including flavonoids and phenols possess antioxidative activity, which benefit to the treatment of various diseases and health care [13, 19, 20]. Simiao pill is used as a damp-removing agent with the function of eliminating heat and dispelling dampness, which can treat damp-heat betting-induced arthralgia [21]. Modern pharmacological study has indicated that Simiao pill has multiple pharmacological properties, including anti-inflammation [22], and it has been utilized to treat rheumatoid arthritis [23]. Interestingly, recent research has indicated the anti-HUA effect of Simiao pill [24]. Nevertheless, research into the potential anti-HUA function and its mechanisms is still lacking.

Network pharmacology is an emerging tool for data collection and analysis of TCM, which combines bioinformatics with system medicine [25, 26]. In recent years, network pharmacology has been broadly used for investigation into TCM due to its capability of clarifying the features of multiple ingredients and targets of TCM [26]. Herein, we probed into the active components of Simiao pill and relevant therapeutic targets based on these facts. Through network pharmacology and molecular docking, we aim to uncover the mechanisms underlying Simiao pill treating HUA, thereby providing therapeutic targets against HUA. Besides, we conducted high-performance liquid chromatograph (HPLC) analysis for quality control and verification of the potential active ingredients of Simiao pill against HUA. The workflow of this study is shown in Figure 1.

2. Materials and Methods

2.1. Databases and Analysis Tools. Platforms or tools utilized in this study are shown as follows: Herbal Ingredients' Targets (HIT; v2.0; http://hit2.badd-cao.net), Traditional Chinese Medicine Systems Pharmacology (TCMSP; http://tcmspw.com/tcmsp.php), Traditional Chinese Medicine Integrated Database (TCMID; v2.0; http://www.megabionet.org/tcmid/), GeneCards (v3.0; https://www.genecards.org/), DisGeNET (v7.0; http://www.disgenet.org/), Therapeutic Targets Database (TTD; https://idrblab.org/ttd/), PubChem (https://pubchem.ncbi.nlm.nih.gov), UniProt (https://www.uniprot.org/), VennDiagram (v1.7.3; http://bioinfogp.cnb.csic.es/tools/venny/index.html), Cytoscape (v3.7.2, http://www.cytoscape.org/), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/cgi/input.pl), ggraph (v2.0.5), igraph (v1.3.1), cytoHubba (v0.1), Metascape (http://metascape.org/), ClusterProfiler (v4.0, https://bioconductor.org/packages/release/clusterProfiler.html), circlize R package (v0.4.15), Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, https://www.rcsb.org/), PyMOL (v2.1), AutoDock (v1.5.6), ZINC (https://zinc.docking.org/), and Open Babel graphical user interface (GUI; v2.3.1).

2.2. Screening of Active Components of Simiao Pill and Relevant Targets. Active components of Simiao pill were retrieved from HIT [27], TCMSP [28], and TCMID [29] databases. After duplication deletion, the structures of active components were obtained from the PubChem database [30].

Absorption, distribution, metabolism, and excretion (ADME) filtration is of great significance to the development of drugs [31, 32]. Based on ADME properties, the criteria of "quantitative estimate of drug – likeness (QED) ≥ 0.2" [33] was applied to sort out active components of Simiao pill. Then, targets related to active components were obtained from the HIT database. Some target genes repeated were excluded. The targets were standardized using the UniProt database [34] with the species set to "*Homo sapiens*."

2.3. Collection of HUA-Related Targets. Potential HUA-related targets were obtained from GeneCards [35], DisGeNET [36], and TTD [37] databases with the search term "hyperuricemia". Similarly, HUA-associated targets repeated were eliminated. The obtained targets were standardized using the UniProt database with the species limited to "*Homo sapiens*."

2.4. Identification of Simiao Pill-HUA Intersection Targets and Protein-Protein Interaction (PPI) Network Analysis. Common targets of Simiao pill and HUA were acquired using VennDiagram. Then, overlapping targets of active components of Simiao pill and HUA were entered into the STRING database [38] to establish the PPI network. The ggraph and igraph R packages were utilized to visualize the protein interactions. The nodes of the PPI network represent proteins, and the edges represent the relations between two proteins. In the current study, we set the species to "*Homo sapiens*" and the confidence score of ">0.4" to screen out candidate targets. The other parameters for PPI network were default: network type = full STRING network; size cutoff = no more than 10 interactors. Besides, the cytoHubba plugin was used for screening the core

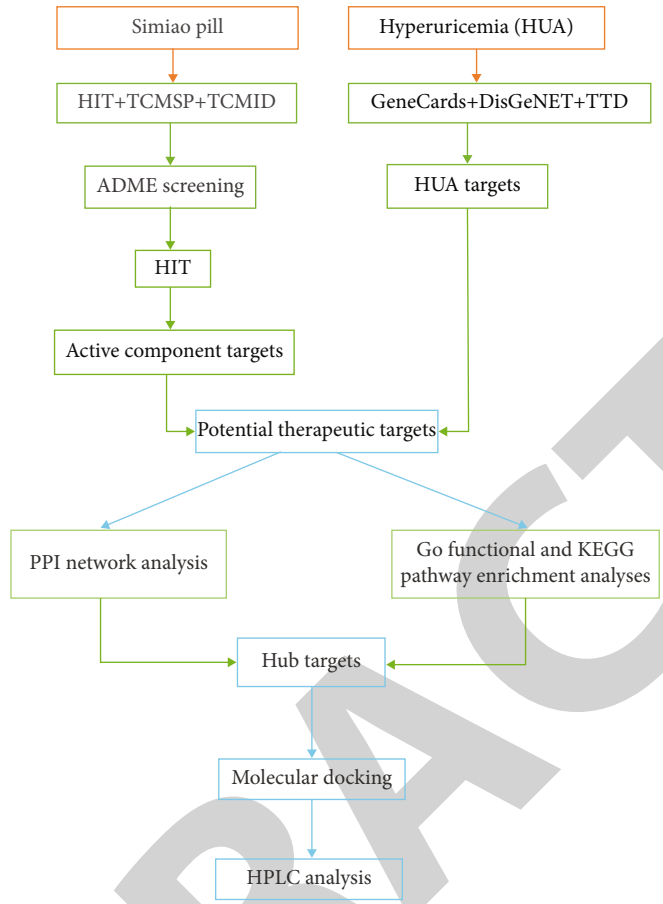

Figure 1: Workflow of the network pharmacology and molecular docking-based study.

targets (top 10) with the parameters set to "ranking method = maximal clique centrality (MCC); Hubba nodes = top 10 nodes ranked by MCC (default)".

2.5. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyses. In this study, Metascape was applied for GO and KEGG pathway enrichment analyses with the species of "*Homo sapiens*." Metascape is a web-based portal, which provides gene annotation and analysis resource [39]. GO analysis comprises biological process (BP), cellular component (CC), and molecular function (MF). We performed KEGG analysis to find out the pathway mechanisms of HUA associated with the overlapping targets. ClusterProfiler in R package [40] was utilized to visualize the results of GO and KEGG pathway enrichment. To determine whether the target gene set is significantly associated with specific gene ontology and pathways, the hypergeometric distribution model was used. The following hypergeometric distribution formula was applied:

$$P = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}, \quad (1)$$

where N is the total number of genes; M is the number of annotated genes in GO or KEGG pathways; n is the number of imported target genes; and k is the number of common genes. P value < 0.01 was considered statistically significant [41].

2.6. Screening of GO-KEGG-Based Hub Targets. Top 10 hub genes originating from GO functional annotation and KEGG pathway analyses were obtained using the cytoHubba plugin. Similarly, the following parameters of cytoHubba were applied: ranking method = maximal clique centrality (MCC); Hubba nodes = top 10 nodes ranked by MCC (default). The circlize R package was applied to visualize the hub target network.

2.7. Molecular Docking. Molecular docking was conducted to verify the interactions between active components and core target proteins of Simiao pill treating HUA. Crystal structures of candidate proteins were downloaded from RCSB PDB [42]. PyMOL and AutoDock tools were used to remove water molecules and ligands, add hydrogen, optimize amino acids, and calculate charges for the structures. The structures were saved in PDB format. Subsequently, 3D structures of the active components in mol2 format were downloaded from ZINC [43]. All structure files were converted into PDBQT format by Open Babel GUI software. Docking pairs simultaneously that met the criteria of the binding free

energy < −5.0 kcal/mol and the formation of hydrogen bonds were considered effective [44] and used for subsequent analysis. Finally, AutoDock Vina was used for molecular docking, and PyMOL was for visualization of the results.

2.8. Preparation of Simiao Pill Sample Solutions and Standard Solutions. Simiao pill used in this study was provided by the Department of Pharmacy of Hangzhou Wuyunshan Hospital (Hangzhou, China). All the preparation procedures conformed to the standards of the Chinese Pharmacopoeia (2020 edition). Briefly, 0.2 g of Simiao pill powder was accurately weighed out and added into 40 mL solution of hydrochloric acid-methanol (1 : 100) for 45 min of ultrasound (power: 250 W; frequency: 33 kHz). After cooling, the hydrochloric acid-methanol solution (1 : 100) was added to 50 mL, followed by shaking and filtering. The subsequent filtrate was obtained and injected through a 0.22 μm filter membrane for subsequent high-performance liquid chromatograph (HPLC) analysis.

Ingredients used to prepare the standard solutions were purchased from Shanghai Yuanye Bio-Technology Co., Ltd, and Shanghai Chunxiao Bio-Technology Co., Ltd (Shanghai, China). The ingredients used were as follows: chlorogenic acid (#B20782, HPLC ≥ 98%, Yuanye), magnoflorine (#B20882, HPLC ≥ 98%, Yuanye), berberine hydrochloride (#B21449, HPLC ≥ 98%, Yuanye), luteolin (#B20888, HPLC ≥ 98%, Yuanye), palmitic acid (#1957-10-3, HPLC ≥ 98%, Chunxiao), wogonin (#B20489, HPLC ≥ 98%, Yuanye), atractylenolide I (#B20054, HPLC ≥ 98%, Yuanye), osthole (#B21152, HPLC ≥ 98%, Yuanye), quercetin (#B20527, Yuanye), rutaecarpine (#B21314, HPLC ≥ 98%, Yuanye), paeonol (#B20266, HPLC ≥ 98%, Yuanye), kaempferol (#B21126, HPLC ≥ 98%, Yuanye), baicalin (#B20570, HPLC ≥ 98%, Yuanye), vitamin E (#B21296, HPLC ≥ 97%, Yuanye), and phytosterol (#B20272, HPLC ≥ 98%, Yuanye). For the preparation of standard solutions, 10 mg of these reference sample components was accurately weighed and dissolved in 10 mL methanol, respectively. Then, equal volumes (10 mL) of these stock solutions were blended to obtain the mixed standard solution.

2.9. HPLC Analysis. HPLC analysis was performed on the 1260 Infinity II liquid chromatograph system (Agilent, Palo Alto, California, USA) with 10 μL mixed standard solution and sample solution, respectively, injected. The mobile phase consisted of acetonitrile (A) and 0.1% phosphoric acid (B). The procedure of gradient elution was set as follows: 0~30 min, 7%~18%A; 30~45 min, 18%~22.5%A; 45~50 min, 22.5%~24%A; 50~65 min, 24%~48%A; 65~75 min, 48%~51%A; 75~90 min, 51%~70%A; 90~95 min, 70%~7%A; and 95~100 min, 7%~7%A. The operation time was set to 100 min, the flow rate to 1 mL/min, the column temperature to 30°C, and the detection wavelength to 280 nm.

3. Results

3.1. Identification of Simiao Pill-HUA Candidate Targets. After retrieval from HIT, TCMSP, and TCMID databases, 282 active compounds of Simiao pill were obtained, excluding those repeated or without PubChem ID (Table S1). A total of 264 active compounds met the criterion of "QED ≥0.2" and were used for the following selection of candidate target genes (Table S2). Also, we acquired 606 target genes related to the active compounds of Simiao pill after searching from the HIT database (Table S3).

Table 1: Overlapping targets of active components of Simiao pill and hyperuricemia in this study.

ID	Gene name	UniProtKB	ID	Gene name	UniProtKB
1	PPARA	Q07869	41	GAA	P10253
2	HTR2A	P28223	42	G6PC1	P35575
3	P2RX7	Q99572	43	TLR2	O60603
4	FASN	P49327	44	CASP8	Q14790
5	VEGFA	P15692	45	EDN1	P05305
6	ERCC1	P07992	46	SLC22A2	O15244
7	APP	P05067	47	STAT3	P40763
8	TNF	P01375	48	TP53	P04637
9	IL6	P05231	49	EPO	P01588
10	MAPK1	P28482	50	TLR4	O00206
11	NQO1	P15559	51	LPL	P06858
12	MAOA	P21397	52	NOS3	P29474
13	IL1B	P01584	53	IFNG	P01579
14	CYP2E1	P05181	54	CYP11A1	P05108
15	PPARG	P37231	55	INS	P01308
16	ESR1	P03372	56	SLC2A2	P11168
17	SELE	P16581	57	CXCL8	P10145
18	CAT	P04040	58	CRP	P02741
19	MYC	P01106	59	GCG	P01275
20	TGFB1	P01137	60	UCP2	P55851
21	GPT	P24298	61	CYP2C8	P10632
22	IGF1R	P08069	62	PARP1	P09874
23	SOD1	P00441	63	JAK2	O60674
24	SIRT1	Q96EB6	64	CYP2C19	P33261
25	LEP	P41159	65	CD40LG	P29965
26	SERPINE1	P05121	66	ABCG2	Q9UNQ0
27	ADIPOQ	Q15848	67	CCL2	P13500
28	AGT	P01019	68	IL10	P22301
29	POMC	P01189	69	XDH	P47989
30	COL1A1	P02452	70	NLRP3	Q96P20
31	RAPGEF4	Q8WZA2	71	APOE	P02649
32	KHK	P50053	72	ACAT1	P24752
33	GCK	P35557	73	APOB	P04114
34	NGF	P01138	74	COG2	Q14746
35	LCN2	P80188	75	TNFRSF11B	O00300
36	NPPA	P01160	76	ALDH2	P05091
37	NPPB	P16860	77	G6PD	P11413
38	HNF1A	P20823	78	IL6R	P08887
39	HNF4A	P41235	79	IL18	Q14116
40	NFATC1	O95644	80	SLC6A3	Q01959

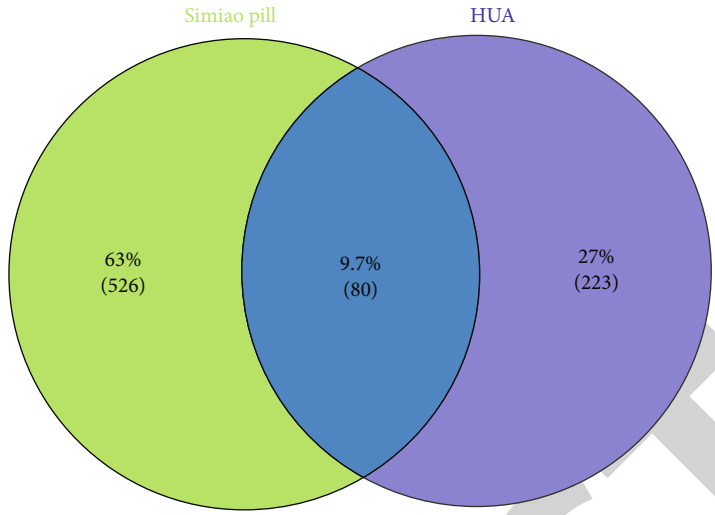

Figure 2: Acquisition of intersection targets related to active components of Simiao pill and HUA using VennDiagram. HUA: hyperuricemia.

Moreover, 303 HUA-associated target genes were obtained from GeneCards, DisGeNET, and TTD databases, excluding those repeated or without UniProt ID (Table S4). Significantly, we acquired the intersections between the above 606 active compound-related and 303 disease-related genes, resulting in 80 Simiao pill-HUA targets (Table 1 and Figure 2).

3.2. PPI Network of Simiao Pill-HUA Common Targets. To clarify the potential links among the identified 80 targets, the PPI network analysis was performed. In the current research, the PPI network included 79 nodes (proteins; degree ≥ 2) and 1,083 edges (interaction pairs; score > 0.4). The top 10 hub proteins (red nodes) were presented as follows: insulin (INS; degree = 66), tumor necrosis factor (TNF; degree = 61), interleukin-6 (IL6; degree = 60), interleukin 1 beta (IL1B; degree = 55), vascular endothelial growth factor A (VEGFA; degree = 51), leptin (LEP; degree = 50), signal transducer and activator of transcription 3 (STAT3; degree = 48), C-C motif chemokine ligand 2 (CCL2; degree = 46), interleukin-10 (IL10; degree = 46), and toll-like receptor 4 (TLR4; degree = 43) (Table S5 and Figure 3). These core genes might be significantly implicated in the pathogenesis of Simiao pill treating HUA.

3.3. GO and KEGG Pathway Enrichment Analyses. To explore the biological functions of 80 potential targets, GO enrichment analysis was conducted. In this study, 1,612 BP, 19 CC, and 50 MF terms correlated with the 80 targets were obtained (P value < 0.01) (Table S6). Then, the top 5 enriched GO items were, respectively, screened out of BP, CC, and MF terms. The results of GO analysis showed that the candidate targets were mainly concentrated in BPs, including response to peptide and regulation of small molecule metabolic process; CCs, such as membrane raft and membrane microdomain; MFs, such as signaling receptor activator activity and receptor ligand activity (Table 2 and Figure 4(a)). Also, we applied KEGG pathway analysis to probe into the pathway mechanisms underlying these targets. The top 15 enriched KEGG pathways were identified, including the AGE-RAGE signaling pathway in diabetic complications and HIF-1 signaling pathway (P value < 0.01) (Table 3 and Figure 4(b)).

3.4. Identification of Hub Genes Based on GO and KEGG Analyses. To find out which target acts as a vital switch in the regulation of the above 15 enriched biological functions and 15 pathways, we further screened out the hub targets using the cytoHubba plugin. The interaction network of the candidate targets; 15 GO terms of top 5 BP, CC, and MF; and top 15 KEGG pathways is shown in Figure 5(a). According to the maximal clique centrality (MCC) scores, the 10 hub GO-KEGG genes were acquired, namely, TNF (MCC score = 23), IL1B (MCC score = 20), IL6 (MCC score = 18), transforming growth factor beta 1 (TGFB1; MCC score = 17), interferon gamma (IFNG; MCC score = 16), toll like receptor (TLR2; MCC score = 15), TLR4 (MCC score = 14), IL10 (MCC score = 14), C-X-C motif chemokine ligand 8 (CXCL8; MCC score = 14), and Janus kinase 2 (JAK2; MCC score = 13) (Figure 5(b)).

3.5. Molecular Docking of Active Compounds and Pivotal Targets. Molecular docking is a favorable structure-based method to clarify the binding ability of small molecules and interactions between active components and protein targets [45]. Therefore, we utilized molecular docking to further validate the reliability of key targets for Simiao pill treating HUA. In this study, we found 5 overlapping targets (IL1B, IL6, IL10, TLR4, and TNF) from the PPI and GO-KEGG hub gene networks (Figure 6) and 22 active compounds associated with these genes (QED ≥ 0.3) (Table 4).

IL1B, IL6, IL10, TLR4, and TNF were docked with the 22 active compounds, generating 29 decently bound pairs with binding free energy < −5.0 kcal/mol and the formation of

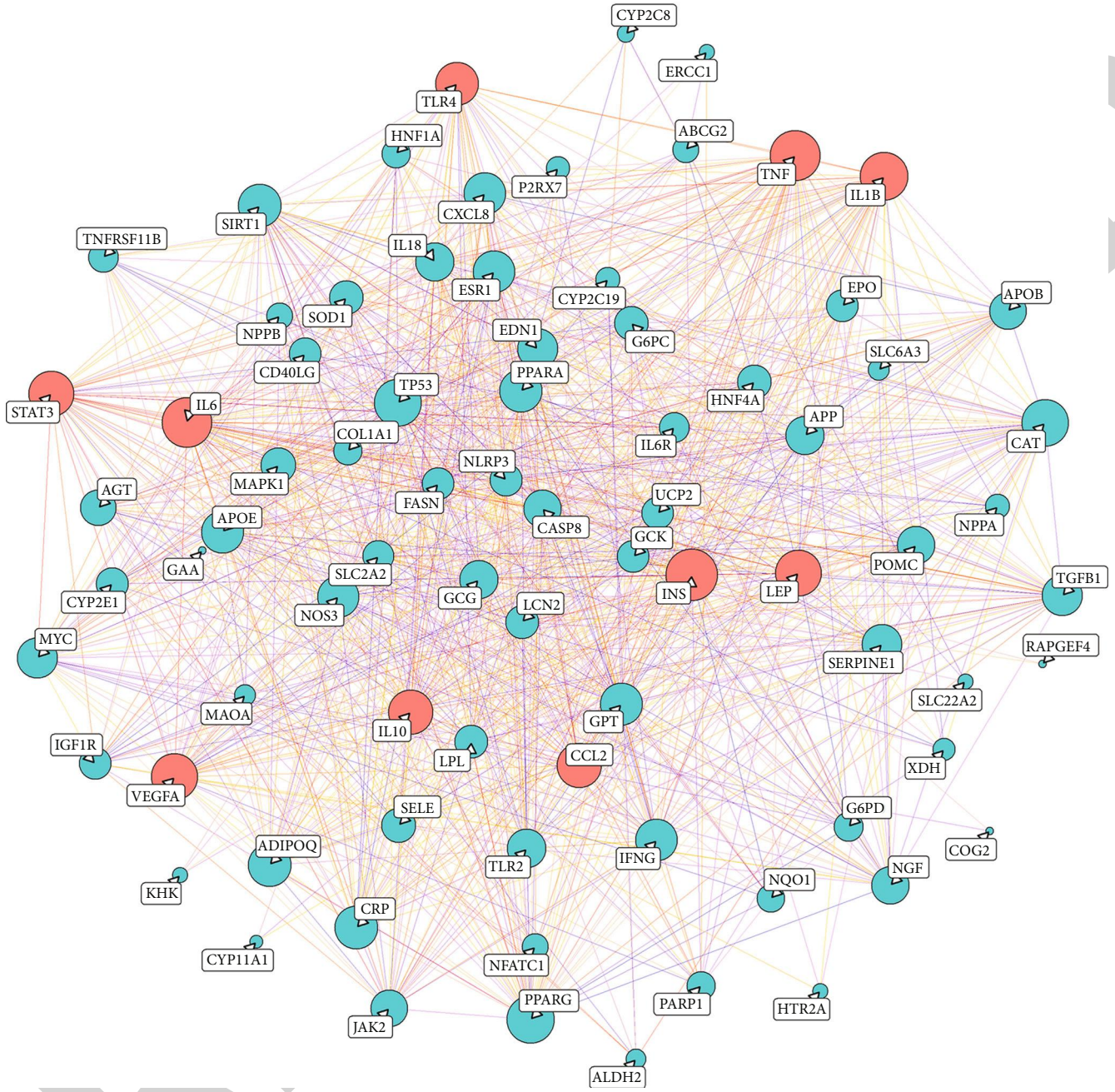

Figure 3: Construction of PPI network diagram using the STRING database, visualized by ggraph and igraph R packages. Red nodes represent the top 10 hub genes. PPI: protein-protein interaction.

hydrogen bonds (Table 5). The top 10 binding pairs (binding free energy ≤ −8.0 kcal/mol) are presented in Figure 7, and other pairs are shown in Figure S1.

3.6. HPLC Analysis of the Potential Effective Compounds of Simiao Pill. HPLC analysis was conducted for quality control and identification of the main components of Simiao pill. Herein, chlorogenic acid, magnoflorine, berberine hydrochloride, luteolin, palmitic acid, wogonin, atractylenolide I, osthole, quercetin, rutaecarpine, paeonol, kaempferol, baicalin, vitamin E, and phytosterol were selected for HPLC analysis. Simiao pill is composed of Rhizoma Atractylodis, Cortex Phellodendri, Radix Achyranthis Bidentatae, and Semen Coicis. According to the HIT, TCMSP, and TCMID databases, luteolin, palmitic acid, wogonin, atractylenolide I, and osthole were the active components of Rhizoma Atractylodis; quercetin, rutaecarpine, and paeonol were produced by Cortex Phellodendri; kaempferol, baicalin, and vitamin E were derived from Radix Achyranthis Bidentatae; phytosterol was from Semen Coicis. Chlorogenic acid, magnoflorine, and berberine hydrochloride serve as the signature components for the quality control of Simiao pill.

Table 2: Top 5 enriched BP, CC, and MF terms of GO enrichment analysis in this study.

Ontology	ID	Description	*P* value
BP	GO:1901652	Response to peptide	8.27533*E*-26
BP	GO:0062012	Regulation of small molecule metabolic process	1.916*E*-24
BP	GO:0043434	Response to peptide hormone	1.18917*E*-22
BP	GO:0001819	Positive regulation of cytokine production	6.22927*E*-21
BP	GO:0006109	Regulation of carbohydrate metabolic process	5.53126*E*-20
CC	GO:0045121	Membrane raft	8.05101*E*-09
CC	GO:0098857	Membrane microdomain	8.3307*E*-09
CC	GO:0005901	Caveola	4.30163*E*-08
CC	GO:0031983	Vesicle lumen	8.88621*E*-08
CC	GO:0031904	Endosome lumen	2.94786*E*-07
MF	GO:0030546	Signaling receptor activator activity	4.94658*E*-19
MF	GO:0048018	Receptor ligand activity	5.77182*E*-18
MF	GO:0005126	Cytokine receptor binding	3.83607*E*-14
MF	GO:0005125	Cytokine activity	7.90798*E*-14
MF	GO:0005179	Hormone activity	1.25158*E*-10

HPLC results showed that luteolin and atractylenolide I were the effective components of Simiao pill compared with those of the mixed standard solution. Furthermore, the proportion of luteolin and atractylenolide I in the sample solution of Simiao pill was 56.2% and 1.39%, respectively (Figure 8).

4. Discussion

HUA is a metabolic disease resulting from disordered uric acid metabolism [46]. At present, TCM remains an appealing choice for the treatment of HUA. Some TCM formulas, such as Ermiao Wan [47] and CoTOL [48], have been applied to treat HUA. As a TCM formula, Simiao pill is well-known for its antirheumatic function, which comprises Rhizoma Atractylodis, Cortex Phellodendri, Radix Achyranthis Bidentatae, and Semen Coicis [16]. According to TCM theory, Rhizoma Atractylodis possesses dampness-eliminating and spleen-tonifying functions; Cortex Phellodendri has heat and dampness-eliminating effect; Radix Achyranthis Bidentatae possess liver and kidney-strengthening function; Semen Coicis has spleen-tonifying and diuretic effects [21]. Modern pharmacological research has demonstrated that Simiao pill exerts multiple pharmacological effects, such as anti-inflammatory [22] and uric acid-lowing functions [49]. Otherwise, an *in vivo* study has declared that Simiao pill can alleviate urate underexcretion in HUA mice [50]. These facts prompted us to further explore the curative effects of Simiao pill on HUA. Herein, we employed network pharmacology analysis to find out the mechanisms underlying Simiao pill mitigating HUA. In the current study, we preliminarily identified 264 active compounds of Simiao pill based on ADME screening and 606 relevant target genes. Meanwhile, 303 HUA-related target genes were obtained. Noteworthily, 80 intersection targets related to active compounds of Simiao pill and HUA were acquired for further analysis. Among them, 5 hub genes (IL1B, IL6, IL10, TLR4, and TNF) and 22 relevant active ingredients were identified as underlying molecular interaction mechanisms of Simiao pill treating HUA.

In the PPI network of 80 targets, INS, TNF, IL6, IL1B, VEGFA, LEP, STAT3, CCL2, IL10, and TLR4 were regarded as the top 10 vital targets of Simiao pill treating HUA. Interestingly, some of these core targets were proinflammatory factors, such as TNF, IL6, and IL1B. Evidence has demonstrated that inflammation is significantly implicated in the pathogenesis of HUA [51–53]. Specifically, Nod-like receptor protein 3 (NLRP3) inflammasome is frequently activated during HUA, leading to the secretion of IL1B and other proinflammatory cytokines [52]. Furthermore, an *in vivo* study has indicated that TLR4 inactivation-induced downregulation of TNF, IL6, and IL1B can mitigate the pathological injury of HUA [54]. Additionally, inhibition of the STAT3 signaling pathway can be beneficial to HUA amelioration, as evidenced by relevant studies [55, 56]. These results indicate that Simiao pill may be used to treat HUA owing to its anti-inflammatory function.

At the GO function level, we found that the 80 candidate genes were mainly enriched in BPs, such as response to peptide, regulation of small molecule metabolic process, and positive regulation of cytokine production. Evidence has demonstrated that multiple peptides and proteins are implicated in the onset and progression of HUA [57–60]. A metabolomics analysis-based study has indicated that the pathological mechanisms are associated with the metabolism of various small molecules, such as glycerophospholipid and arachidonic acid [61]. Cytokines have significant implications for the occurrence and development of HUA [62]. For example, an *in vivo* study found downregulation of IL10 (an anti-inflammatory cytokine)

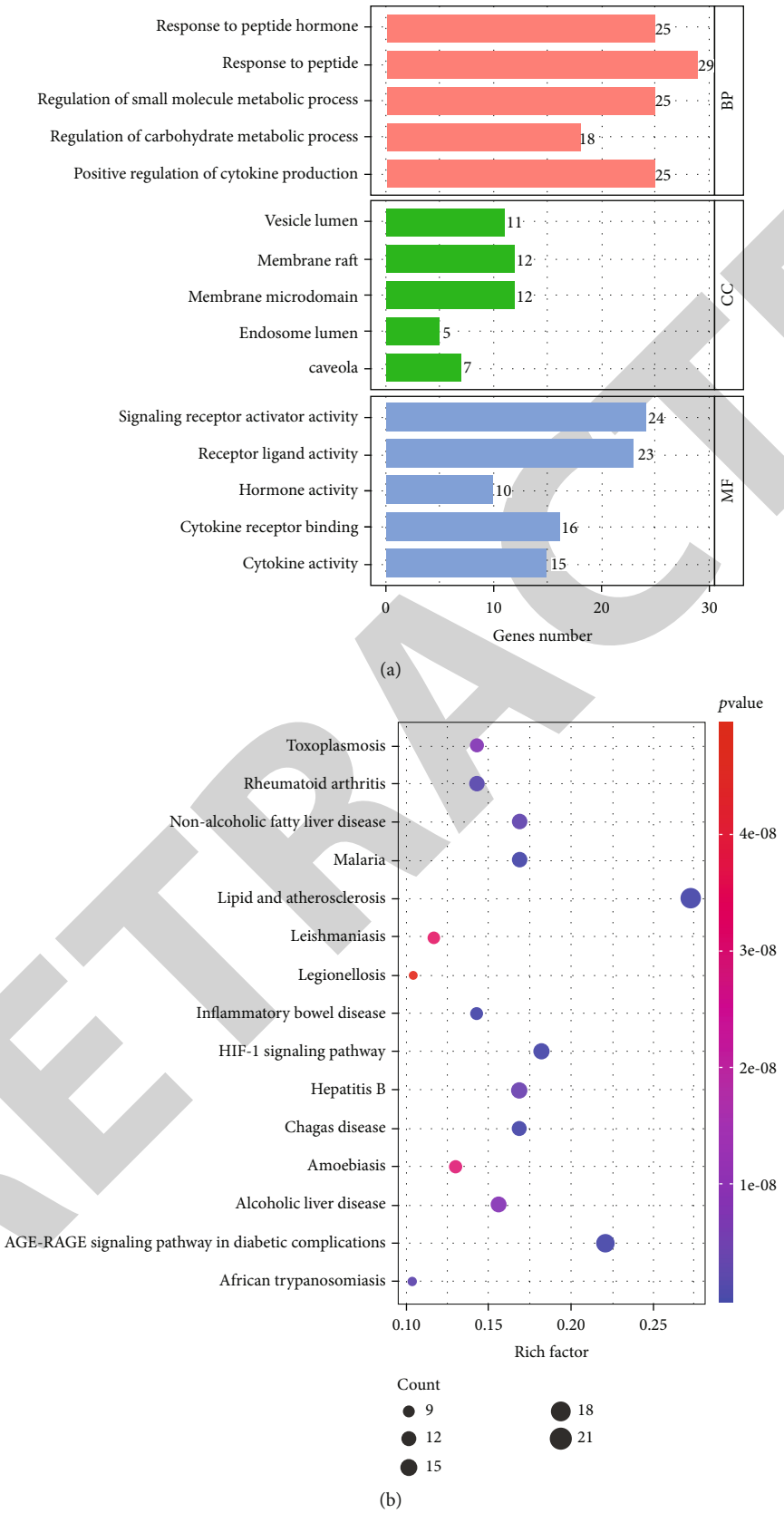

Figure 4: GO functional and KEGG pathway enrichment analyses. (a) Fifteen vitally enriched GO functions, including top 5 BP, CC, and MF terms. (b) Top 15 enriched KEGG pathways. P value < 0.01. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BP: biological process; CC: cellular component; MF: molecular function.

in HUA mice, and it showed that upregulation of IL10 inhibited HUA progression [63]. Besides, these targets were closely associated with some MFs, including signaling receptor activator activity and receptor ligand activity. These findings indicate that a wide range of molecules are involved in the pathological mechanisms of HUA, and the regulation of molecular response, production, and metabolism is crucial to HUA progression.

At the KEGG pathway level, our data showed that the mechanisms by which Simiao pill treats UC were primarily related to the AGE-RAGE signaling pathway in diabetic complications and HIF-1 signaling pathway. Accumulating evidence has demonstrated that HUA interacts with type 2 diabetes mellitus [60, 64, 65]. High serum uric acid level can contribute to the development of type 2 diabetes mellitus [64], and glucose metabolism disorder, in turn, can lead to increased serum uric acid [66]. Significantly, the coordination of the advanced glycation end-product (AGE) and its receptor (RAGE) can trigger inflammation [67, 68]. Hypoxia-inducible factor-1 (HIF-1) is one of the core regulators of cellular responses under low-oxygen circumstances [69]. Otherwise, HIF-1α is implicated in the regulation of inflammatory factors, such as TNF-α, IL1B, and IL-6 [70]. Therefore, the HIF-1 signaling pathway plays a vital part in the inflammatory response. These results indicate that Simiao pill may participate in the treatment of HUA by targeting AGE-RAGE- and HIF-1-related cascade signaling pathways.

Noteworthily, we finally determined 5 core targets according to the PPI and GO-KEGG networks, namely, IL1B, IL6, IL10, TLR4, and TNF. Besides, we found 22 active ingredients of Simiao pill associated with these 5 targets, such as paeonol and wogonin. Crucially, the 5 target proteins exhibited a close affinity with the active compounds, as 29 binding pairs had favorable molecular docking scores of "binding free energy < −5.0 kcal/mol". Molecular docking results showed that the 12 components kaempferol, luteolin, rutaecarpine, baicalin, quercetin, vitamin E, paeonol, atractylenolide I, osthole, phytosterol, palmitic acid, and wogonin were the pivotal active compounds of Simiao pill in the treatment of HUA. Among these, the active compounds luteolin, quercetin, rutaecarpine, and baicalin displayed strong docking activities with most of the core targets (binding free energy < −7.0 kcal/mol). Also, HPLC analysis was performed to further verify the potential active components of Simiao pill. The results of HPLC analysis showed that luteolin and atractylenolide I were the effective components of Simiao pill. Significantly, luteolin accounted for 56.2%, higher than any other detected compounds in Simiao pill. Combining molecular docking and HPLC analysis, we determined luteolin, rutaecarpine, baicalin, quercetin, and atractylenolide I as the main components of Simiao pill against HUA.

Luteolin belongs to the flavone family, initially found in some fruits and vegetables with potent anti-inflammatory pharmacological function [71]. A previous investigation indicated that luteolin might act on TNF and medicate the HIF-1 pathway implicated in the treatment of HUA [14]. Recent research has indicated that luteolin can effectively reduce the expression of proinflammatory factors IL1B and IL-6 to mitigate TNF-α-induced cellular inflammatory injury [72]. Rutaecarpine, an alkaloid extracted from the unripe fruit of E. rutaecarpa, possesses an anti-inflammatory function [73]. Rutaecarpine has been reported to inhibit the expression of IL1B, IL6, and TNF and promote IL10 expression to ameliorate the histopathology damage caused by inflammation [74–76]. Baicalin, a flavonoid compound isolated from radix scutellariae, is characterized by its potent anti-inflammatory effect [77]. Similarly, baicalin can mitigate inflammation by suppressing the expression of IL1B, IL6, and TNF and enhancing IL10 expression [78]. Quercetin is a flavonoid abounding in fruits and vegetables with multiple pharmacological effects, such as antioxidation and anti-inflammation [79]. A recent study has indicated that quercetin may ameliorate HUA by inhibiting TLR4 cascade signaling and downregulating the expression of IL1B, IL6, and TNF [3]. As a eudesmane-type sesquiterpenoid lactone derivative of Rhizoma Atractylodis macrocephalae, atractylenolide I possesses various biological activities, such an-inflammatory and anticancer activities [80]. Evidence has demonstrated the crosstalk between atractylenolide I-induced downregulation of IL1B, IL6, and TNF and TLR4 signaling [81]. Noteworthily, atractylenolide I has been determined as one of the main active components of Rhizoma Atractylodis to treat gouty arthritis via downregulating IL1B, IL6, TNF, and uric acid [82]. Consistent with the previous studies, our data imply that Simiao pill may treat HUA by targeting the release of inflammatory factors and inflammation-related signaling pathways.

However, there are still some limitations in our research. First, we only investigated the potential therapeutic mechanisms of Simiao pill on HUA, without further analyzing

Table 3: Top 15 enriched KEGG pathways of KEGG enrichment analysis in this study.

ID	Description	*P* value
hsa04933	AGE-RAGE signaling pathway in diabetic complications	2.14*E*-17
hsa05417	Lipid and atherosclerosis	2.89*E*-16
hsa05144	Malaria	4.47*E*-16
hsa04066	HIF-1 signaling pathway	1.04*E*-12
hsa05142	Chagas disease	8.03*E*-12
hsa05321	Inflammatory bowel disease	1.53*E*-11
hsa05323	Rheumatoid arthritis	8.42*E*-10
hsa05143	African trypanosomiasis	1.36*E*-09
hsa04932	Nonalcoholic fatty liver disease	1.66*E*-09
hsa05161	Hepatitis B	2.86*E*-09
hsa05145	Toxoplasmosis	6.29*E*-09
hsa04936	Alcoholic liver disease	6.85*E*-09
hsa05146	Amoebiasis	3.30*E*-08
hsa05140	Leishmaniasis	3.58*E*-08
hsa05134	Legionellosis	4.99*E*-08

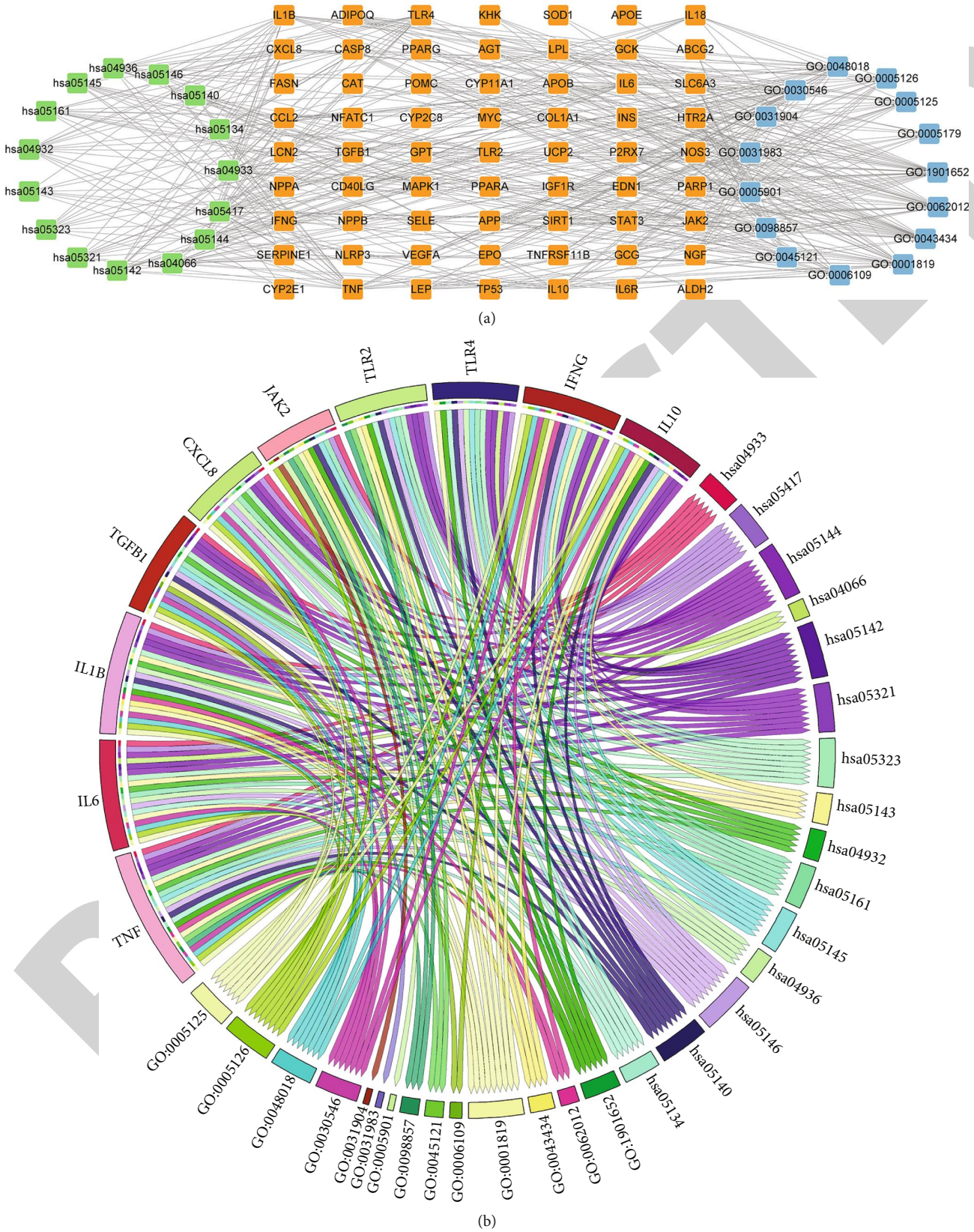

FIGURE 5: Identification of 10 hub targets based on GO functional and KEGG pathway enrichment analysis. (a) Construction of the interaction network of candidate targets; top 5 BP, CC, and MF terms of GO enrichment analysis; and top 15 KEGG items. (b) Identification of 10 hub targets corresponding to the GO-KEGG network using the cytoHubba plugin. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

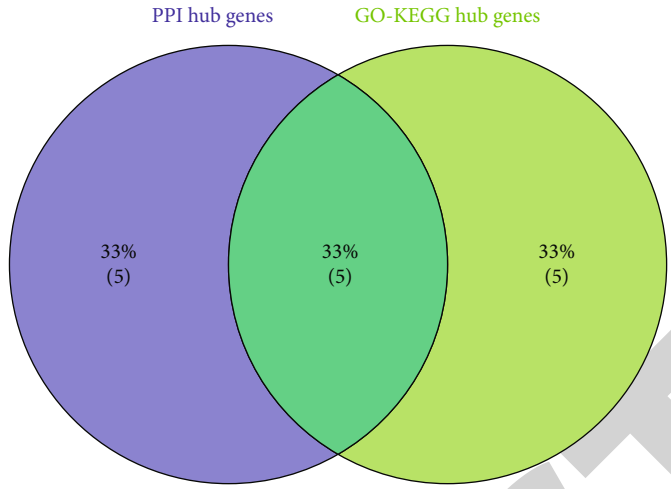

Figure 6: Acquisition of 5 intersection targets from the PPI and GO-KEGG networks of hub targets using VennDiagram. PPI: protein-protein interaction; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table 4: Active compounds of Simiao pill related to hub targets.

Pubchem_ID	cpd_name	QED
CID:10228	Osthole	0.61
CID:11092	Paeonol	0.68
CID:12389	Tetradecane	0.37
CID:14985	Vitamin E	0.36
CID:177	Acetaldehyde	0.36
CID:248	Betaine	0.51
CID:440917	Limonene	0.49
CID:5280343	Quercetin	0.43
CID:5280445	Luteolin	0.51
CID:5280863	Kaempferol	0.55
CID:5281515	Beta-caryophyllene	0.5
CID:5281520	Alpha-humulone	0.49
CID:5281703	Wogonin	0.76
CID:5283349	2,4-Decadienal	0.25
CID:5321018	Atractylenolide I	0.47
CID:5997	Phytosterol	0.49
CID:6184	Hexanal	0.39
CID:64982	Baicalin	0.3
CID:65752	Rutaecarpine	0.54
CID:702	Alcohol	0.41
CID:8181	Methyl palmitate	0.3
CID:985	Palmitic acid	0.41

the interactions between its active components. Second, the core target genes and pathways were determined at the network pharmacology level and have not been experimentally validated. Third, additional *in vivo* and *in vitro* studies are needed to further verify the efficacy of Simiao pill in treating HUA and its molecular mechanisms. These limitations will be perfected in our subsequent investigations.

Table 5: Results of molecular docking in this study.

Gene	Compound	PubChem ID	Free binding energy (kcal/mol)
IL1B	Kaempferol	CID:5280863	-7.8
	Luteolin	CID:5280445	-7.7
	Quercetin	CID:5280343	-8
	Rutaecarpine	CID:65752	-9.9
	Baicalin	CID:64982	-9.3
	Vitamin E	CID:14985	-6.5
	Paeonol	CID:11092	-5.5
IL6	Atractylenolide I	CID:5321018	-7.3
	Kaempferol	CID:5280863	-7
	Luteolin	CID:5280445	-7.1
	Baicalin	CID:64982	-8.1
	Vitamin E	CID:14985	-6.1
	Osthole	CID:10228	-6
	Phytosterol	CID:5997	-6
	Palmitic acid	CID:985	-5.2
IL10	Quercetin	CID:5280343	-7.3
	Baicalin	CID:64982	-9
TLR4	Wogonin	CID:5281703	-7.8
	Luteolin	CID:5280445	-7.7
	Quercetin	CID:5280343	-8.1
	Rutaecarpine	CID:65752	-8.3
TNF	Atractylenolide I	CID:5321018	-7.7
	Wogonin	CID:5281703	-7.8
	Kaempferol	CID:5280863	-7.8
	Luteolin	CID:5280445	-7.9
	Quercetin	CID:5280343	-8.1
	Rutaecarpine	CID:65752	-8.3
	Baicalin	CID:64982	-8.4
	Vitamin E	CID:14985	-7.1

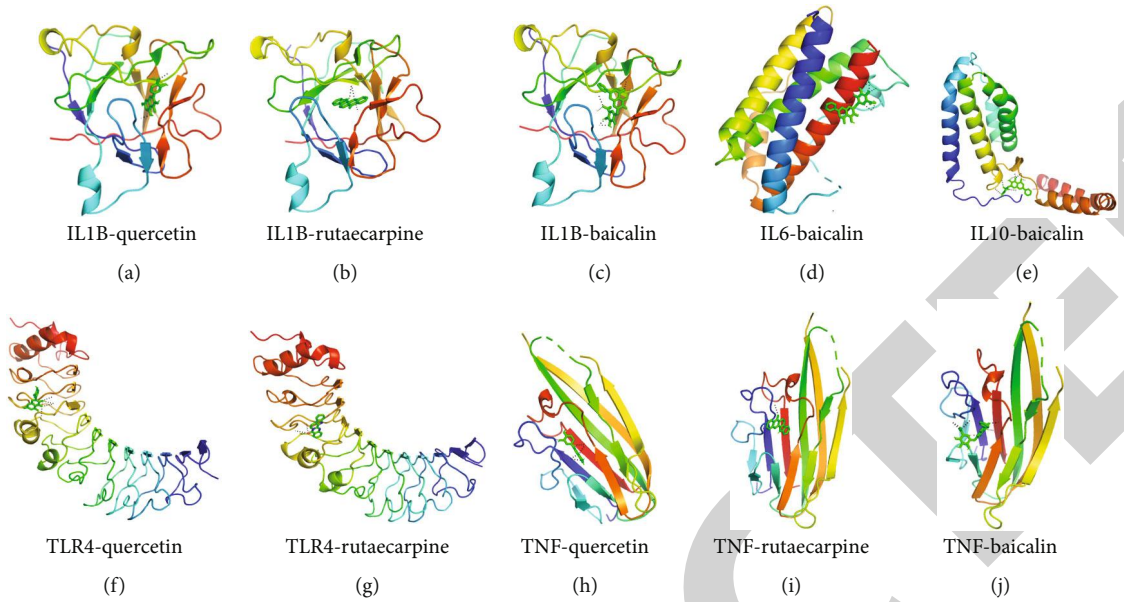

Figure 7: Top 10 well-binding pairs of target protein-active compound through molecular docking.

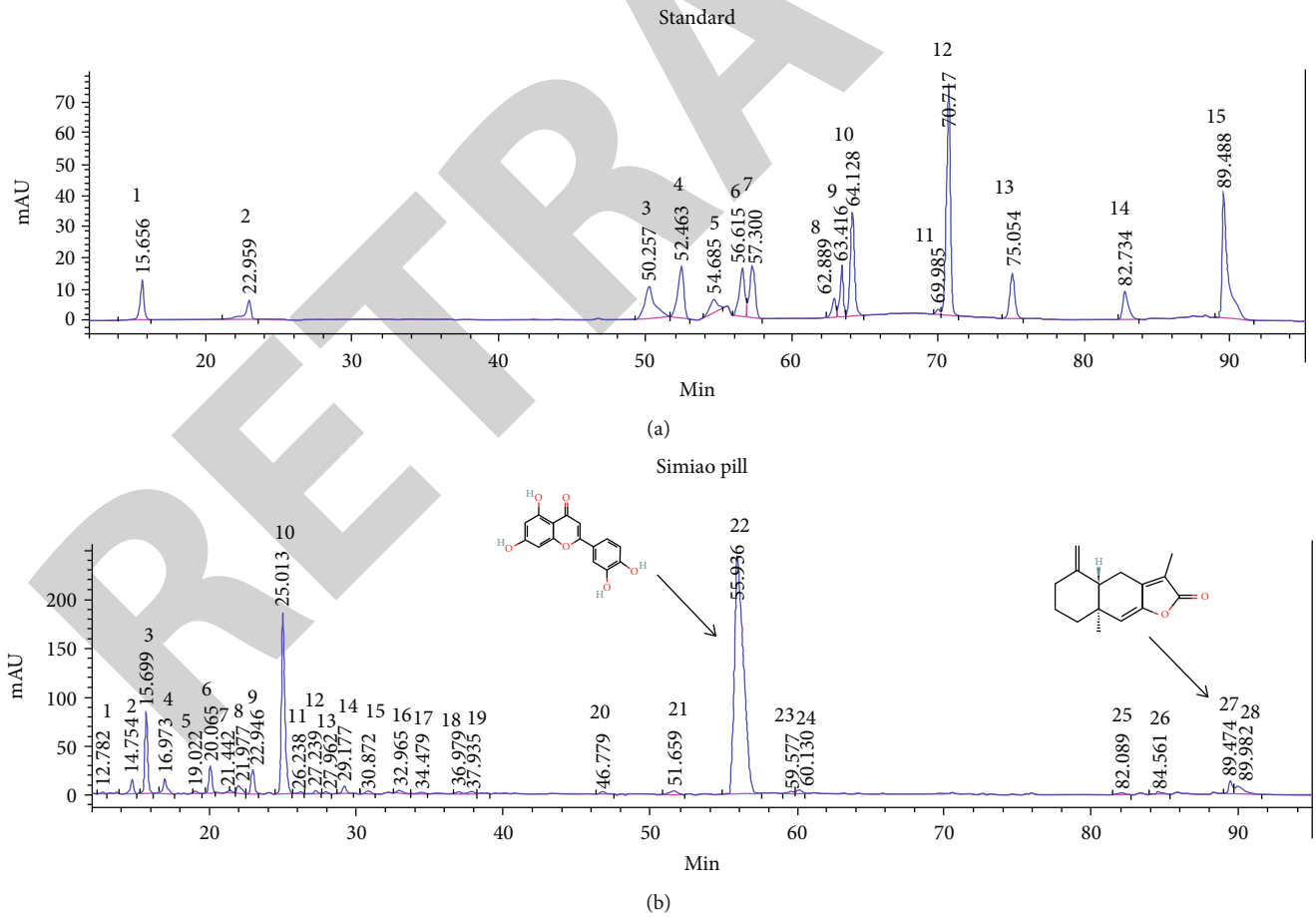

Figure 8: Quality control and main compound validation of Simiao pill through HPLC analysis. (a) Chromatogram of the mixed standard solution (1: chlorogenic acid; 2: magnoflorine; 3: baicalin; 4: berberine hydrochloride; 5: palmitic acid; 6: luteolin; 7: quercetin; 8: kaempferol; 9: paeonol; 10: wogonin; 12: rutaecarpine; 13: osthole; 14: phytosterol; 15: atractylenolide I). (b) Chromatogram of the Simiao pill sample solution (22: luteolin; 27: atractylenolide I). HPLC: high-performance liquid chromatograph.

5. Conclusions

Based on the network pharmacology-molecular docking method, we found that luteolin, quercetin, rutaecarpine, baicalin, and atractylenolide I were the pivotal active ingredients of Simiao pill against HUA, which acted on the key targets, IL1B, IL6, IL10, TLR4, and TNF. This study revealed that Simiao pill could ameliorate HUA mainly by inhibiting inflammation and targeting AGE-RAGE- and HIF-1- associated signaling pathways. There is an essential need for both *in vivo* and *in vitro* experiments to further validate the action mechanisms of the core components of Simiao pill alleviating HUA. Our study provides a direction for the follow-up research into the pharmacological mechanisms of Simiao pill against HUA and offers a reference for the development of novel anti-HUA drugs.

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 is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Figure S1: nineteen well-binding pairs of target protein-active compound through molecular docking, apart from the top 10 pairs. Table S1: active components of Simiao pill. Table S2: active components of Simiao pill after ADME screening. ADME: absorption, distribution, metabolism, and excretion. Table S3: targets of active components of Simiao pill. Table S4: targets of hyperuricemia. Table S5: nodes of PPI network. PPI: protein-protein interaction. Table S6: GO enrichment results. GO: Gene Ontology. *(Supplementary Materials)*

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Research Article

Dracocephalum kotschyi Boiss. *In Vitro* Efficacy on Growth and Apoptosis Induction in *Leishmania major* Promastigotes

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Dracocephalum kotschyi Boiss. is a plant generally used in modern medicine to treat many human illnesses. It is also used to prevent tumor cell proliferation throughout the world. This study's objective was to evaluate this plant's in vitro efficacy on growth and apoptosis induction in *Leishmania major* promastigotes. To do this, the essential oil is extracted for the test following the collection and identification of *D. kotschyi*. The essential oil was analyzed using a GC-MS analyzer. Promastigotes of *L. major* were cultured in RPMI-1640 media, and the MTT assay and a flow cytometry analysis were carried out on promastigotes that had entered the log phase. To differentiate between viable, necrotic, and apoptotic treated or untreated promastigotes, the flow cytometry method of double staining with annexin V-FLUOS and propidium iodide (PI) was used. Given the results obtained, 11 phytochemicals were identified in the essential oil of this plant. Copaene (22.15%), methyl geranate (16.31%), geranial (13.78%), and carvone (11.34%) were the main substances. The essential oil of *D. kotschyi* inhibits the proliferation of *L. major* promastigotes at 921 μg/mL, 252 μg/mL, and 416 μg/mL, respectively, after 24 h, 48 h, and 78 h. The cells were divided into four quadrates based on cell phases using the flow cytometry approach by double staining with annexin V-FLUOS and propidium iodide (PI): necrosis (Q1), late apoptosis (Q2), early apoptosis (Q3), and viable (Q4) quadrates. Overall, it is apparent that the different concentrations induced cell apoptosis in promastigotes. Observation under the light microscope at ×100 magnification showed that the different doses of *D. kotschyi* essential oil caused apparent alterations in the treated promastigotes. In this work, *D. kotschyi* essential oils induce programmed death on *L. major* promastigotes. This study opens many research perspectives, such as investigating the mechanisms of action and the production of a phytomedicine based on this plant.

1. Background

Biologically active natural substances, including medicinal plants, have been used for decades to prevent diseases and treat cellular disorders. According to Kinghorn et al., many pharmacologically effective medications have been produced from natural resources, such as medicinal herbs [1]. Numerous plants' therapeutic potential for treating diseases is still being studied. The prevalence of medicinal plants among people worldwide is rising due to their low risk of adverse effects, accessibility, and affordability [2].

Dracocephalum kotschyi Boiss. is a medicinal plant of the Labiatae family used in many countries. It is an aromatic plant that grows in Iran's high, mountainous regions [3–6]. In traditional medicine, several medicinal properties have been attributed to it. So this plant is used to treat congestion, stomach aches, headaches, and liver disease [7–9]. *Dracocephalum kotschyi* is a plant usually used in modern medicine to treat many human illnesses and prevent tumor cell proliferation worldwide [10–12]. *D. kotschyi* has some of biological and pharmacological activities that have been described, counting antibacterial [13], anti-inflammatory, and antifungal effects [14]. This plant also has antispasmodic, analgesic, antihyperlipidemic, and immunomodulatory activity [15]. The different biological properties of this plant are due to the bioactive chemical compounds of this plant. The essential oil of *D. kotschyi* contains flavonoids, which have antimicrobial characteristics, whereas α-terpineol and limonene

have antinociceptive qualities [15]. The anticancer effects are brought on by methoxylated flavones such as luteolin, isokaempferid, apigenin, crisimaritin, penduletin, and xanthomicrol [16]. For example, some *in vitro* and *in vivo* studies have shown that apigenin induces cell apoptosis [17, 18]. Indeed, apoptosis is a programmed cell death process that involves cascades of events depending on energy and various morphological features [17]. Extrinsic (death receptor) and intrinsic (mitochondrial) pathways play a significant role in the induction of apoptosis. Apigenin is an effective agent for triggering apoptosis through the intrinsic or extrinsic pathway in human cancer cells. Apigenin treatment of the prostate cancer cell lines PC-3 and DU145 resulted in apoptosis via decreasing Bcl-2 and Bcl-xL proteins and increasing the active form of the Bax protein [19]. Through altering the amount of mitochondrial protein expression, apigenin can also cause cell apoptosis via upregulating the expression of the Bim protein and decreasing the expression of the Mcl-1 protein. To cause mitochondria-dependent cell apoptosis, this synergizes with the Bcl-2 inhibitor ABT-263 [20]. The antioxidant activity of the plant is probably caused by polyphenolic compounds such as flavonoids, chlorogenic acid, phenylpropanoids, caffeic acid, and caffeic acid [16].

Additionally, it was established that *D. kotschyi* had cytotoxic, antiproliferative, and apoptotic effects on cancer cells *in vitro* [15]. According to one research, the most beneficial fractions were CH2Cl2, luteolin, and essential oil (EO), which caused morphological changes in the cells. The biological properties of luteolin include antioxidant, anti-inflammatory, and anticancer activities. For *D. kotschyi*, the effect of flavonoids on tumor cell inhibition has been reported. This plant's flavonoids are considered the most effective chemicals [15].

Of all the studies on the biological properties of *D. kotschyi*, very few have evaluated the *in vitro* efficacy of this plant on the growth and apoptosis induction in *Leishmania major* promastigotes. A variety of protozoa from the genus *Leishmania* cause the vector-borne disease leishmaniasis. It is an emerging disease with high morbidity and mortality rates [21]. It has three primary forms: cutaneous, mucocutaneous, and visceral. Several drugs are used to treat this disease, but high toxicity levels have been established for these drugs [22, 23]. Pentavalent antimonials, amphotericin B, paromomycin, and pentamidine are the most frequently prescribed drugs for leishmaniasis. These medications have serious side effects, must be taken in high dosages for long periods, and must be administered parenterally [24]. It has also been established that drugs capable of initiating apoptosis of *Leishmania* promastigotes would be very effective in treating this parasitic disease [25]. The simplest model for screening is the promastigote since the parasites grow in cell-free media [21]. The inhibition of promastigote multiplication is assessed within three days, during which the control organism multiplies three to six times. Since promastigotes are not the desired parasites, some researchers like this screening method due to its simplicity [21]. The multiple therapeutic uses of *D. kotschyi* are of particular interest. In this regard, the current study has evaluated the *in vitro* efficacy of this plant on the growth and apoptosis induction in *L. major* promastigotes.

2. Materials and Methods

2.1. Plant Material and Preparation of Essential Oil. In October 2019, the plant utilized in this study was collected in Isfahan, Iran. Prof. Ghanadian verified it at the pharmacognosy department. The pharmacy school herbarium had a reference specimen (No. 1519). We had permission to collect *Dracocephalum kotschyi* from the Research and Ethics Committee of Islamic Azad University, Shahrekord Branch (date 2021/12/08, IR.IAU.SHK.REC.1400.061).

The plant parts were mechanically pulverized using an electric mixer after the aerial parts of the plants were dried in the lab at 16°C in the shade. The essential oils were obtained after three hours of hydrodistillation with a Clevenger apparatus. The oil was put into sealed vials, dissolved in hexane (Merck, Darmstadt, Germany), and dried on anhydrous sodium sulfate before being stored in the dark at 4-6°C.

2.2. Analysis Using Gas Chromatography-Mass Spectrometry (GC/MS). The essential oil was analyzed using a GC with an Agilent 7890A coupled to a 5975C mass detector with a triple quadrupole electron ionization (EI) mass analyzer. An HP-5 GC capillary column (30 m × 0.25 mm; film thickness 0.25 μm) was used to prepare the GC. The oven was preheated to 50°C and left there for two minutes. It was increased by 8°C/min to 250°C and then 250-330°C by 3°C/min for 58 min. At a 2 mL/min flow rate, helium served as the carrier gas. The injector and detector were both operated at a temperature of 280°C. Ion source temperature (230°C), mass range (50-700), and ionization voltage were the MS's parameters (70 Ev). MSD ChemStation was the operating system that was used. The chemicals were identified by comparing the mass spectra and retention times to data from the literature [21].

2.3. Culture of L. major Promastigotes (MRHO/IR/75/ER). Promastigotes of the Iranian standard strain of *L. major* (MRHO/IR/75/ER) were purchased from the Pasteur Institute (Tehran, Iran). According to previous research, *L. major* promastigotes (MRHO/IR/75/ER) were grown in RPMI-1640 medium (without phenol red) at 25 ± 1°C in 25 mM HEPES (pH 7.2) and enhanced with 10% heat-inactivated FBS, antibiotics (100 μg/mL streptomycin and 100 IU/mL penicillin), and L-glutamine (2 mM). Each vial's culture was checked every day for bacterial and fungal contamination. At 25 ± 1°C, the flasks were incubated. The following assays were then run-on promastigotes that had reached the log phase. Three times were done for each test [23].

2.4. MTT Assay. A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) kit was employed to obtain the 50% inhibitory concentration (IC_{50}). In order to do it, 5 mg of MTT powder in 1 mL of PBS sterile solution (5 mg/mL) was dissolved to prepare the MTT reagent. A 96-well microplate was used for the MTT assay (5 mg/mL, 20 μL/well). After adding 2×10^5 promastigotes/100 μL/well, *D. kotschyi* essential oil in different increasing concentrations ranging from 125, 250, 500, 1000, and 2,000 μL/mL was added to treat the parasites. The plates are incubated

at 25 ± 1°C for 24, 48, and 72 h. Promastigote viability using the MTT assay was examined. Then, absorbance at a wavelength of 540 nm was measured using an ELISA reader device. The following formula was used to determine the percentage of cells that were exposed and unexposed in the samples [25, 26]:

$$\text{Viable promastigotes}\,(\%) = \frac{(A_T - A_B)}{(A_C - A_B)} \times 100, \qquad (1)$$

where A_T is the absorbance of the exposed promastigotes, A_C is the absorbance of the unexposed promastigotes 2,000,000, and A_B is the absorbance of the blank.

Final results were compared to positive controls (promastigotes treated with 20 μL of amphotericin B 20 μg/mL) and negative controls (promastigotes treated with 100 μL of DMSO 1% without essential oil) to determine the IC50 (that inhibited half of the promastigotes growth). Observation of morphological changes in treated and untreated promastigotes with D. kotschyi essential oil.

2.5. Flow Cytometry Analysis. Using the flow cytometry technique, double staining with annexin V-FLUOS and propidium iodide (PI) allowed us to differentiate between promastigotes that had been treated with essential oil and were viable, necrotic, or apoptotic, as well as between those that had not. Annexin-V can differentiate between necrotic cells (PI-positive/upper left) and normal cells (both annexin-V and PI negative/lower left), apoptotic cells (only annexin-V positive as early apoptosis/lower right), and late apoptosis cells (both annexin-V and PI-positive/upper right) [27]. Promastigotes of essential oil that had been treated (2×10^6 parasites/mL) and those that had not were centrifuged for ten minutes at 1400*g* after being twice washed with cold PBS solution following the manufacturer's instructions. Then, they were incubated in 100 μL of annexin-V FLUOS with PI for 15 minutes at room temperature and in a dark area. A FACS Calibur flow cytometer (Becton Dickinson, USA) was used to analyze the samples after they had been examined. The percentage of positive cells for each sample was calculated [27].

2.6. Relevance of the Methodology. The methodology used in this study is consistent with current regulations and institutional guidelines and is replicable. All references used have been provided for ease of understanding.

2.7. Statistical Analysis. The *in vitro* antileishmanial activity's IC50 was calculated using a linear regression test and SPSS software version 19 (SPSS Inc., Chicago, IL, USA). The means and standard deviation were calculated using ANOVA. Tukey's multiple comparisons test was used to compare each experimental batch to the control batch at varied concentrations (ANOVA two-way). The tests were conducted at a 5% significance level.

3. Results

3.1. Extraction Yield of Essential Oils and GC/MS Analysis. The yield of the extraction was 1.1%. There were found to be eleven substances. They represent 91.5 percent of the oil. Copaene (22.15%), geranial (13.78%), methyl geranate (16.31%), and carvone (11.34%) were the main substances [28].

Table 1: The number of live promastigotes (1×10^6) at different concentrations of essential oil and as a function time.

Concentration (μg/mL)	24 h	48 h	72 h
125	0.85 ± 0.01	0.68 ± 0.01	0.69 ± 0.097
250	0.84 ± 0.011	0.61 ± 0.04	0.68 ± 0.03
500	0.82 ± 0.007	0.60 ± 0.07	0.660 ± 0.03
1,000	0.78 ± 0.014	0.58 ± 0.02	0.63 ± 0.08
2,000	0.72 ± 0.068	0.57 ± 0.05	0.58 ± 0.03
Positive control	0.74 ± 0.007	0.63 ± 0.04	0.68 ± 0.04
Negative control	0.86 ± 0.01	0.88 ± 0.04	0.99 ± 0.04

3.2. The Number of Live Promastigotes at Different Concentrations of Essential Oil. Table 1 shows the number of live promastigotes at different concentrations of *D. kotschyi* essential oil after 24 hours, 48 hours, and 72 hours of incubation. This data shows that the number of live promastigotes after 72 hours of incubation is higher than after 48 hours. It is inferred from this finding that the essential oil of *D. kotschyi* affected the promastigotes of *L. major*.

Figure 1 shows the number of live promastigotes (1×10^6) as a function of different doses (dose-response) of essential oil. This figure shows that as the dose or concentration of essential oil increases, the number of living promastigotes in the reaction medium decreases. We deduce from this that there is a dose-response reaction to the number of living promastigotes.

3.3. Effect of D. kotschyi Essential Oil on the Cell Proliferation of Leishmania major Promastigotes. Cell viability was used to determine the percentage of growth inhibition (GI%) and the half-maximal inhibitory concentration (IC_{50}) of essential oil of *D. kotschyi* on *L. major* promastigotes. Essential oils were tested at the following concentrations: 125, 250, 500, 1000, and 2000 μg/mL. Figure 2 shows the comparative effects the essential oil concentrations on *L. major* promastigotes after 24-h, 48-h, and 72-h exposure. After 24 to 72 hours of incubation, the number of viable parasites increased considerably in the negative control, indicating the absence of inhibition. The positive control showed parasite inhibition after 48 and 72 hours of incubation. For the essential oil of *D. kotschyi*, it was noted that the inhibitory activity was concentration-dependent. The highest inhibitory activity of the essential oil is observed at the concentration of 2000 μg/mL, so the higher the concentration, the higher the activity.

The half inhibitory concentration (IC50) of the different concentrations (250, 500, 1000, and 2000 μg/mL) of essential oil on *L. major* promastigotes is determined and reported in Table 2. The concentrations of *D. kotschyi* essential oil vary with time.

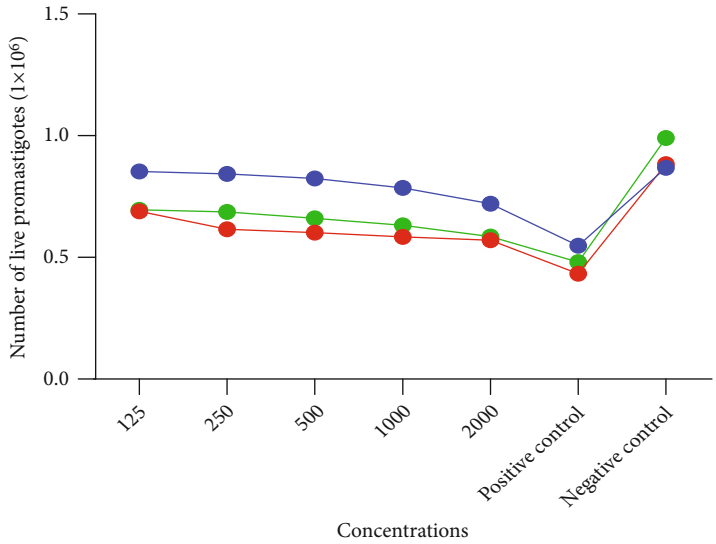

Figure 1: Number of live promastigotes (1×10^6) as a function of different doses (dose-response) of essential oil.

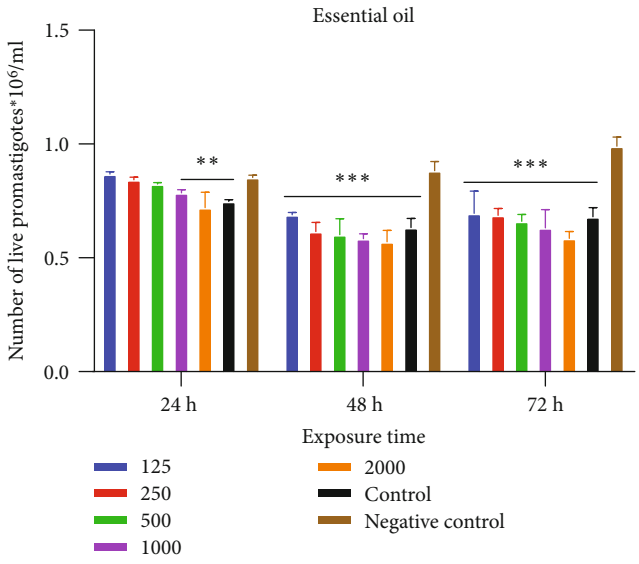

Figure 2: Comparison effects of the concentrations of the essential oil on *Leishmania major* promastigotes. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 2: IC_{50} at 24 hours, 48 hours, and 72 hours and R^2.

Time	IC_{50} (μg/mL)	R^2
24 hours	921	0.89
48 hours	252	0.84
72 hours	416	0.94
Positive control	255	0.87

3.4. Flow Cytometry Was Used to Identify Phosphatidylserine at the Outer Membrane of Apoptotic Cells. The difference between necrosis and apoptosis was made using an annexin-V FLUOS staining kit, and the results were evaluated using the flow cytometry method. Figure 3 provides further information. The flow cytometric tests with 2000 μg/mL and 1000 μg/mL of essential oil at 24 and 48 hours resulted in a two-dimensional plot of Annexin V-FITC against PI. The FSC/SSC plot to the untreated cells was used to establish the analysis border (control). By placing the most significant number of dots in the Q4 region of the control sample, the borders of the four quadrants (Q1-Q4) were established. Based on the cell phases, the cells were divided into four quadrates: necrosis (Q1), late apoptosis (Q2), early apoptosis (Q3), and viable (Q4) quadrates. Overall, it was found that the different concentrations induced cell apoptosis of promastigotes. Additionally, after 42 hours of incubation, the experimental group's percentage of promastigotes in the early and late stages of apoptosis changed with time.

Light microscopic observation under ×100 magnification showed that different doses of *D. kotschyi* essential oil cause apparent alterations in treated promastigotes, including cytoplasmic condensation, cell shrinkage, and immobility after 24 hours. In contrast, no visible changes were observed in the control group in cell morphology and promastigote multiplication. Figure 4 shows the morphological alterations in exposed and unexposed promastigotes throughout time.

4. Discussion

The main objective of this study is to evaluate the *in vitro* efficacy of *Dracocephalum kotschyi* on growth and apoptosis induction in *Leishmania major* promastigotes.

The biological activity, including anticancer, antidiabetic, analgesic, and cardioprotective hepatoprotective, and food potential of medicinal plants are due to the strength of secondary metabolites contained within the plant and the diversity of these secondary metabolites [29–31]. This study investigated for the first time the phytochemical constituents of the essential oil of *D. kotschyi* by the gas chromatography-mass spectrometry (GC/MS) analysis technique. This work identified eleven (11) different chemical groups in the essential oil of *D. kotschyi*. Copaene (22.15 percent), methyl geranate (16.31 percent), geranial (13.78 percent), and carvone (13.78 percent) were the most

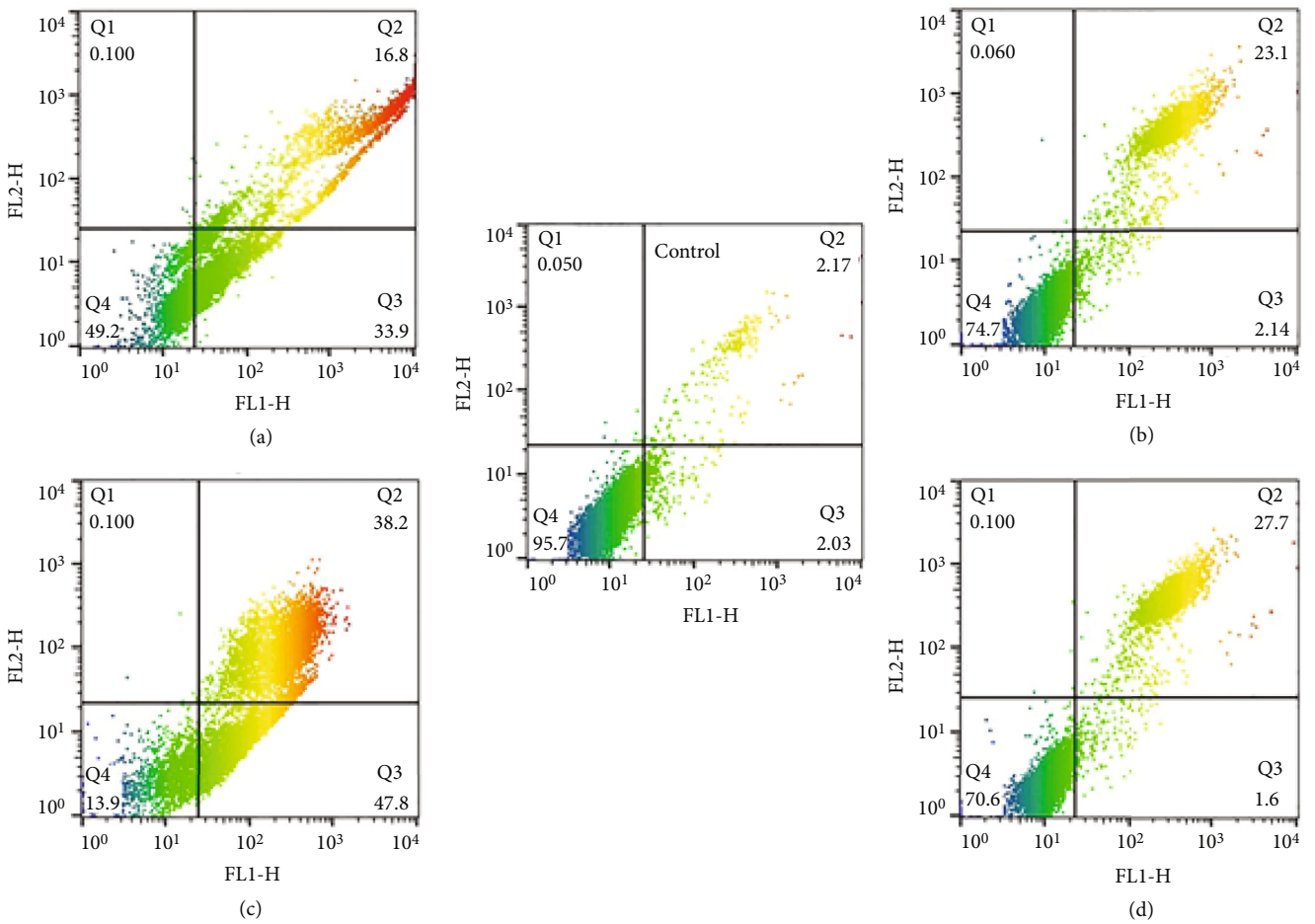

Figure 3: Differentiate between viable, necrotic, and apoptotic treated or untreated promastigotes with essential oil by flow cytometry analysis. Legends: (a) 2000 μg/mL 24 h =50.7% ($Q2 + Q3$) induced cell apoptosis; (b) 1000 μg/mL 24 h =25.24% ($Q2 + Q3$) induced cell apoptosis; (c) 2000 μg/mL 48 h =86% ($Q2 + Q3$) induced cell apoptosis; and (d) 1000 μg/mL 48 h =29.3% ($Q2 + Q3$) induced cell apoptosis.

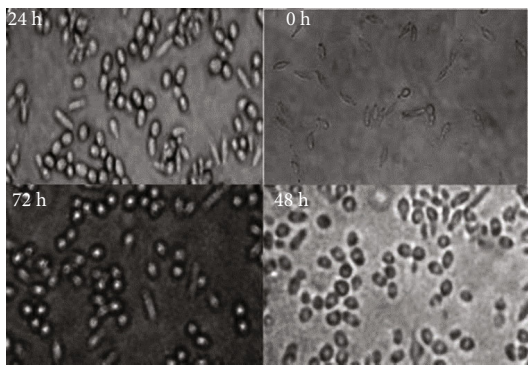

Figure 4: Analysis of the morphology of *L. major* treated with *D. kotschyi* essential at 0, 24, 48, and 72 hours after treatment using light microscopy (magnification, × 100).

common compounds (11.34 percent). Khamesipour et al. [25] made the same findings. Shakib et al. [28] discovered oxygenated sesquiterpenes, monoterpene hydrocarbons, sesquiterpene hydrocarbons, and oxygenated monoterpenes in the essential oils of *D. kotschyi*. Flavonoids such as calycopterin, xanthomicrol, apigenin 4'-O-β-d-glucopyranoside, isokaempferide, luteolin, luteolin 7-O-β-d-glucopyranoside, acetin 7-O-β-d-glucopyranoside, luteolin 3'-O-β-d-glucuronide, and apigenin were also identified according to the works of Zeng et al. [32]. These variations can be related to several parameters: the season of harvest, the method of collection, the method of extraction, the maturity of the plant, and the conditions of drying and grinding. The constituents of this plant are thought to be responsible for its biological characteristics. Flavonoids do, in fact, possess antibacterial properties [33]. The anticancer effects are carried on by methoxylated flavones such as apigenin, luteolin, isokaempferid, crisimaritin, penduletin, and xanthomicrol [16, 34]. The antioxidant activity of this plant's essential oil is usually related to phenolic components such as caffeic acid, chlorogenic acid, and phenylpropanoids [35].

The majority of drugs used to treat leishmaniasis now have several limitations, including high toxicity, challenging treatment schedules, and the development of resistance. New, safer, more potent, and economically feasible drugs are urgently required to treat leishmaniasis. Medicinal herbal products have been used to treat multiple human diseases for thousands of years. The *in vitro* efficacy of *D. kotschyi* on growth and apoptosis induction in *L. major* promastigotes was evaluated in this study.

The impact of *D. kotschyi* on the proliferation of *L. major* promastigotes analyzed using the MTT cell proliferation assay showed that promastigotes in the untreated control group continued to proliferate in a time-dependent manner (from 24 hours to 72 hours). Contrarily, within 24 hours after treatment with *D. kotschyi*, the number of promastigotes decreases sharply, and the decreased continued again for 72 hours. There was a significant difference in promastigote proliferation after 72 hours when comparing *D. kotschyi*-treated promastigotes with untreated promastigotes from the control group. These data suggest that the essential oil of *D. kotschyi* affects cell proliferation and induces apoptosis of target cells. Similar findings regarding the antiproliferative and apoptosis-inducing activities of curcumin on *L. major* were made in the study of Elamin et al. [21]. The latter investigated curcumin's effect on the cell cycle and showed a curcumin-dependent increase of S-phase cells that reached about 33% versus 14% in the control group after 16 hours of incubation.

This phenomenon was investigated to determine whether apoptosis is the cause of the cell death process brought on by *D. kotschyi*. Using flow cytometry after Annexin V/propidium iodide (PI) staining, different *D. kotschyi* were used to treat *L. major* promastigotes for 48 hours before they were stained and sorted. Four cell groupings could be seen in the data. Viable cells (Annexin V-/PI-) in bottom left; early apoptotic cells (Annexin V+/PI-) in bottom right; late apoptotic cells (Annexin V+/P+) in top right; and necrotic cells (Annexin V-/PI+) in top left (Figure 2). The proportion of early and late apoptosis corresponded to the proportion of apoptosis after determining the percentage of spontaneous apoptosis. Significantly, the essential oil of *D. kotschyi* increased the rate of apoptosis-induced mortality, and this effect was dose-dependent. The number of promastigotes that underwent apoptosis changed with a dose-response. These data could be explained by the fact that the essential oil of *D. kotschyi* acted on the target cells through cell signaling pathways related to cell apoptosis. In medulloblastoma cells, apoptosis caused by plant extracts would be accomplished through the mitochondrial pathway by downregulating Bcl-2, an antiapoptotic effector downstream of Shh signaling, according to Elamin et al. [21]. The programmed cell death (apoptosis) induced by *D. kotschyi* on *L. major* promastigotes could effectively eliminate the parasite, thus providing an alternative treatment to modern drugs. The same observations were made in Shaabani et al. [36]. They worked on human cancer cells. It was demonstrated through their study that the essential oil of *D. kotschyi* induced apoptosis in human glioblastoma U87 cells in a dose-response manner. Additionally, compared to the control group, there were significantly more cells in the sub-G1 phase. The findings show that *D. kotschyi* extracts cause cell apoptosis in U87 cells, in addition to measuring the proliferation of U87 cells and investigating the apoptotic effects of *D. kotschyi* on glioma cells. Based on cellular function, *D. kotschyi* extracts would have acted on ROS. Several studies have shown that ROS is essential in controlling a number of cellular functions, such as cell proliferation, inflammatory responses, and cell death [37, 38]. The fact that *D. kotschyi* extracts have been shown to increase ROS levels in cells over time significantly supports the theory that *D. kotschyi* causes cell death by causing oxidative stress and an accumulation of reactive oxygen species. Furthermore, it is known that many alterations occur during apoptosis in three essential steps, namely, cell membrane modifications, the cytoplasmic or mitochondrial pathway, and cell nucleus modifications [37, 38]. Different doses of *D. kotschyi* essential oil cause apparent alterations in treated promastigotes, such as cell shrinkage, cytoplasmic condensation, and immobility, beginning within 24 hours, according to light microscopic examination at a magnification of ×100.

This activity would be caused by the presence of luteolin, naringenin, penduletin, xanthomicrol, apigenin, isokaempferide, cirsimaritin, and calycopterin, according to Moghaddam et al. [39]. Cirsimaritin, penduletin, xanthomicrol, and calycopterin are methoxylated hydroxyflavones that were specifically effective against tumor cells. Luteolin caused tumor cells to undergo apoptosis, showing that it has apoptosis-inducing effects on the target organ [34]. Therefore, the main components of *D. kotschyi* and its isolated compounds exhibit a variety of activities affecting several targets and participate in the induction of apoptotic cell death in cancer, providing information on how to improve the effectiveness of cancer medications in people [34].

5. Conclusion

There are now new possibilities for treating leishmaniasis due to natural substances previously used in traditional medicine. On *L. major* promastigotes, the essential oil of *D. kotschyi* exerts lethal effects that cause the parasite to induce apoptosis. More research is required to understand further this natural substance's method of action and its toxic effects on other cells, particularly *in vitro*.

Data Availability

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are also available from the corresponding author.

Ethical Approval

This study does not involve any human or animal testing. The Research and Ethics Committee of Islamic Azad University, Shahrekord Branch, approved the experiments (Date 2021/12/08, IR.IAU.SHK.REC.1400.061).

Consent

Not applicable.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

All authors whose names appear on the submission made substantial contributions to the conception and design of the work, acquisition, analysis, and interpretation of data. All authors read and approved the last version to be published. Neda Kosari conducted the experiments. Faham Khamesipour designed the experiments, contributed to analyzing, developed the theory, designed the experiments, and contributed to analyzing and interpreting the data and writing the manuscript.

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Research Article

Antiobesity Potential of Bioactive Constituents from Dichloromethane Extract of *Psoralea corylifolia* L. Seeds

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Purpose. Effectively controlling the accumulation of adipose tissue can be a therapeutic strategy for treating obesity, which is a global problem. The present study was designed for comparative assessment of *in vitro* antiobesity activities of the *Psoralea corylifolia*-dichloromethane seed extract (DCME) and the isolated phytochemicals, bakuchiol, isopsoralen, and psoralen, through antiadipogenesis and pancreatic lipase (PL) inhibition assays. *Material and Methods. In vitro* pancreatic lipase activity was determined spectrophotometrically by measuring the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) to *p*-nitrophenol at 405 nm, and adipogenesis was assayed in 3 T3-L1 adipocytes (by using Oil Red O staining) using *P. corylifolia*-dichloromethane seed extract (DCME) and individual compounds, isolated from the extract. *Result.* Antilipase as well as antiadipogenesis activity was displayed by both the DCME and the compounds. Maximum antilipase property was recorded in DCME (26.02 ± .041%) at 100 μg/ml, while, among the isolated compounds, bakuchiol exhibited a higher activity (24.2 ± 0.037 %) at 100 μg/ml concentration, compared to other isolates. DCME was found to exhibit antiadipogenesis property, 75 ± 0.003% lipid accumulation, compared to the control at 100 μg/ml dose. Bakuchiol, isopsoralen, and psoralen inhibited the lipid accumulation in 3T3-L1 preadipocytes, 78.06 ± 0.002%, 80.91 ± 0.004%, and 80.91 ± 0.001%, respectively, lipid accumulation in comparison to control at 25 μM dose. *Conclusion.* The present study highlights the antiobesity potential of *P. corylifolia* and its active constituents. Thus, it can be concluded that *P. corylifolia* has the potential to treat obesity and related diseases; however, further research on dose standardization and clinical trials are required.

1. Introduction

Obesity can be defined as the abnormal enhancement in the mass of adipose tissue in the body [1]. It is a complex multifactorial disease and a global health issue that leads to metabolic syndrome, diabetes, hypertension, heart failure, renal failure, and other comorbidities [2, 3]. When an adult's body mass index (BMI), a measurement of body fat based on height and weight, surpasses 25 or 30 kg/m^2, they are called overweight or obese [4, 5]. Undoubtedly, obesity is caused by a sedentary lifestyle and the ingestion of extra calories on a regular basis. Overweight and obesity prevalence rates have

roughly doubled globally since 1980, with over one-third of the world's population being categorized as overweight or obese [6].

Energy intake starts from fat absorption after digestion of fat into monoglycerides and fatty acids. Lipase is a crucial enzyme in the absorption of lipids [7]. The pancreatic lipase, which is responsible for the hydrolysis of 50-70% of total dietary fats, is known to facilitate fat digestion [8]. However, its suppression can lower the fat level in the blood and can be a useful strategy for the management of obesity [9]. Adipogenesis is a complex multistep process through which preadipocytes are converted into mature, lipid-containing adipocytes [10]. In obesity, adipocytes experience unusual augmentation characterized by enhanced numbers of fat cells storing their lipids through excessive adipocyte differentiation. So, disruption of adipocyte differentiation may decrease fat storage.

Although traditional therapies such as lifestyle modification (diet and exercise) and medication are useful, their weight loss results are limited. Despite the advances in our understanding of obesity, clinical therapy of the condition faces several challenges [11]. Patients and practitioners may be disappointed by the less favorable results of weight loss after consuming most of the pharmaceuticals, as well as weight recovery once they are discontinued. Hence, weight-loss medications are not a popularly used practice and may create some lingering doubts regarding obesity pharmacotherapy's long-term safety [12]. Currently, the US Food and Drug Administration has approved four weight-loss medicines: orlistat, marketed as Xenical® by Roche, Switzerland; Alli® by GlaxoSmithKline, UK; Contrave® by Nalpropion Pharmaceuticals, USA; Belviq® by Eisai, Japan; and Qsymia® by Vivus, USA. But existing synthetic medications have been linked to heart attacks, strokes, and liver damage [13].

Medicinal plants from any region serve as a natural reservoir for medications, and they are a boon as they can treat several ailments quickly and effectively [14]. Natural products have the advantage of providing complex phytochemical combinations with high structural complexity and biological potency [15]. Herbal treatments are independent of any age group or sex and help to stay healthy and active in the long run. In fact, they are the best remedy to decrease body fat and weight [16]. Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use.

Psoralea corylifolia L. (family: Fabaceae) is one such widely used medicinal plant whose extracts/compounds are reported to exhibit estrogenic, anticancer, antioxidant, antimicrobial, antidepressant, anti-inflammatory, osteoblastic, and hepatoprotective effects, among others [17]. The effect of *P. corylifolia* seed extract on high fat diet-induced nonalcoholic fatty liver disease (NAFLD) in C57BL/6 mice was studied, and it could decrease body weight as well as glucose level in blood. It also improved the glucose tolerance and insulin sensitivity [18]. Prenylated flavonoid-standardized extract (PFE) from the seeds of *P. corylifolia* was found to drastically reduce bodyweight, fat mass, and white adipose cells in the high fat diet-induced obese mice [19]. *P. corylifolia* is less explored plants on account of its antihyperlipidemic activity. Although there are several medicines in the market to treat obesity, prolonged intake can result in serious negative effects. As a result, scientists are on the lookout for novel natural sources with potential antiobesity activity. In order to find new bioactive natural antiobesity agents, compounds isolated from the *P. corylifolia* seed DCME were evaluated for their antilipase as well as antiadipogenesis activity. This is the first report wherein antiadipogenesis activity of the *P. corylifolia* DCME seed extract as well as of the isolated compounds has been reported.

2. Material and Methods

2.1. Plant Material. Seeds of *P. corylifolia* were procured from CSIR-IIIM Jammu and submitted to the Crude Drug Repository (CDR) of Janaki Ammal Herbarium at IIIM Jammu. CDR accession number allotted to the voucher specimen was CDR 4242. Dried seeds were grinded using a mechanical grinder, and the seed powder was sealed in a plastic bag and stored at room temperature (in a vacuum desiccator) until further use.

2.2. Chemicals and Reagents. Mouse 3T3-L1 preadipocytes were procured from the National Centre for Cell Science, Pune, India. Petroleum ether, dichloromethane, methanol, ethyl acetate, dimethyl-thiazol-diphenyl tetrazolium bromide, Dulbecco's modified eagle medium, dexamethasone, Bovine calf serum, penicillin, streptomycin, 3-isobutyl-1-methylxanthine, dimethyl sulfoxide (DMSO), Oil Red O, isopropyl alcohol, and formaldehyde were procured from Sigma Chemical Co. USA. All the chemicals utilized were of analytical grade.

2.3. Preparation of Plant Extracts. 100 g of dried seeds were grinded, and then extracts were prepared by the maceration method. Three different solvents (petroleum ether, dichloromethane, and methanol) were used for sequential extraction starting from low polarity to high polarity [20, 21]. The same fresh solvent was used for two more extractions before switching to the next polar solvent. Plant extracts were evaporated to obtain the dry residue. The percentage yield of the dried extract was computed.

2.4. Ultrafast Liquid Chromatography (UFLC). The analysis of DCME and the compounds isolated from the same extract was performed using Shimadzu UFLC system which was equipped with a gradient solvent quarternary pump (LC-20 AD), autosampler SIL-20A HT, column oven CTO-10ASvp, and detector PDA SPD-M20A. The analytes were separated by using column Merck RP-18e LiChrospher, having particle size 5 μm. With gradient elution using acetonitrile (A) and 0.1 percent v/v formic acid in water (B) at a flow rate of 0.75 ml/min, the total run duration was 50 minutes. The gradient was as follows (in terms of v/v percent of B): 0–0.01 min, 90; 0.01-15 min, 90 to 70; 15-25 min, 70 to 40; 25-35 min, 40 to 0; 35-40 min, 0; 40-45, 0 to 90; and 45-50 min, 90.

2.5. Isolation and Characterization. For isolating the secondary metabolites from *P. corylifolia* seeds, silica gel column chromatography technique was applied to the DCME. A vertical glass column (40 mm wide by 60 mm long) made of borosilicate material was used for the fractionation. The column was rinsed well with acetone and was completely dried before packing. A piece of glass wool was placed at the bottom of the column with the help of a glass rod. Silica slurry was prepared with hexane and was poured from the top of the column approximately 2/3rd of the column. The extract (9.46 g) was loaded onto column packed with 60-120 mesh size silica gel and gradually eluted with 1 to 15% (vol/vol) ethyl acetate (EtOAc) in n-hexane. Each chromatographic fraction which was collected was 15-20 ml and was examined by TLC using TLC silica gel $60\,F_{254}$ sheets (Merck). The separated compound on TLC was detected under the UV Fluorescence Analyzing Cabinet (Sunstar). After the evaluation of the TLC, similar R_f value fractions were pooled and allowed to evaporate. Analysis of ^{1}H NMR (400 MHz) and ^{13}C NMR (125 MHz) spectra was done with Bruker FT-NMR spectrometer and compared with the data published [22] which allowed to predict the structure of the isolated compounds.

2.6. Pancreatic Lipase Inhibition Assay. The pancreatic lipase inhibition assay was performed according to the procedure by Sridhar et al. [23] with slight modification. The enzyme solution was prepared immediately before use, and for this, 25 mg of porcine pancreatic lipase was suspended in 5 ml of the TrisHCl buffer (pH 7.4). The solution was then subjected to centrifugation (4000 rpm, 18°C for 10 min). The supernatant was collected and used. For all, i.e., DCME, the isolated compounds and the positive control (orlistat), different concentrations (100 μg/ml, 50 μg/ml, and 25 μg/ml) were prepared in TrisHCl buffer containing 1% dimethyl sulfoxide. The final 1000 μl reaction mixture is comprised of a preincubated mixture (5 min at 37°C) of 875 μl of buffer, 100 μl of enzyme, and 20 μl of DCME/isolated compounds/orlistat of different concentrations, followed by the addition of 5 μl of the substrate (4-nitrophenyl butyrate, 10 mM in acetonitrile).

The absorbance of the final mixture was determined using the microplate spectrophotometric reader (Spectra Max ABS Plus, USA) after 5 min at 405 nm. The assay was performed in triplicate, and the percentage inhibition was calculated using the formula

$$\%\text{Inhibition} = \left[\frac{(\text{AE} - \text{AT})}{\text{AE}}\right] \times 100, \quad (1)$$

where AE is the absorbance of enzyme control (without inhibitor) and AT is the difference between the absorbance of test sample, with and without substrate.

2.7. Cell Viability Assay. An MTT (dimethyl-thiazol-diphenyl tetrazolium bromide) assay was done in order to find out the cytotoxic effect of DCME/isolated compounds on the 3T3L1 cell line [24]. Undifferentiated 3T3L1 cells were seeded in a 96-well culture plate, and the density was kept at 1×10^4 cells/ml. After 24 h, the medium was replaced with a medium having DCME or isolates at different concentrations. Incubation was done in a 5% CO_2 atmosphere at 37°C for 24 h. The test solutions in the wells were removed after incubation, and 10 μl of MTT (5 mg/ml MTT in PBS (pH = 7.4)) was added to each well. Thereafter, the plates were incubated at 37°C for 4 h. The supernatant was withdrawn, and 100 μl of DMSO was added to the plates and agitated to dissolve the formazan that had formed. The absorbance was read at 570 nm using a microplate reader (SpectraMax i3 Molecular Devices, USA). Undifferentiated preadipocytes treated with 1% DMSO were used as control. The growth inhibition percentage was computed.

$$\%\text{Inhibition} = \left(\frac{\text{Mean test OD}}{\text{Mean non-treated OD}}\right) \times 100. \quad (2)$$

2.8. Cell Culture and Antiadipogenesis Assay. Mouse 3T3-L1 preadipocytes were cultured in a 250-ml culture flask with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Bovine Calf Serum (BCS) and 1% penicillin-streptomycin. Whole cell culture was maintained at 37° C in a humidified incubator along with a continuous supply of 95% O_2 and 5% CO_2 [25]. Postconfluent cells (80% confluency) were then stimulated to differentiate by incubating for 48 h in the medium containing basal medium (DMEM) with 10% BCS, 1% penicillin-streptomycin solution, and IDM cocktail (1 μg/ml insulin, 0.1 μM dexamethasone, and 0.1 mM 3-isobutyl-1-methylxanthine (IBMX)). The medium was replaced after two days with maintenance medium containing DMEM supplemented with 10% BCS, 1% penicillin-streptomycin solution, and 1 μg/ml insulin for the next ten days, with medium replacement on every alternate day. Treatment of various compounds was given in differentiation medium as well as in maintenance medium for 12 days total. The control group was the cell lines treated with differentiated medium plus maintenance medium.

2.9. Oil Red "O" Staining. Lipid accumulation was measured using a method previously described [26]. During adipogenesis experiment, 3T3-L1 cells were treated with plant extracts/compounds. Cells were rinsed in PBS and then fixed with 3% formaldehyde in PBS for 30 minutes at room temperature before being incubated with oil red "O" solution (0.5 percent oil red "O" dye in isopropanol: water; 60 : 40). The excess dye solution was withdrawn, and the cells were rinsed with water to eliminate any remaining dye. An inverted microscope was used to capture images of the treated cells (Leica DMI 6000 B, Germany). After air drying the stained cells overnight, bounded dye separation in isopropanol was performed, and absorbance was read at 520 nm using an ELISA plate reader (SpectraMax i3 Molecular Devices, USA).

2.10. Statistical Analysis. The data was reported as mean ± SEM. GraphPad Prism software was used to analyze the significant differences between the means of different groups through one-way analysis of variance (ANOVA). This was followed by Tukey's post hoc test for comparison of means.

For all results, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Determination of Extractive Value of P. corylifolia. The dry powdered plant material of *P. corylifolia* (seeds) was extracted with solvents of different polarity, viz. petroleum ether, dichloromethane, and methanol using a maceration process. In a weighing bottle, 100 g of coarsely powdered plant material (seeds) was weighed and put to a dry 1-liter conical flask. Thereafter, petroleum ether was poured into the flask (500 ml). The flask was corked and incubated at room temperature for 24 h, with intermittent shaking. The liquid was filtered into a 500-ml beaker using a Whatman No. 1 filter paper. The residue was collected, dried, and transferred to a dry 1-liter flask, which was then filled with 500 ml dichloromethane and stirred for 24 h at 120 rpm at 37° C. The liquid was filtered into a 500-ml beaker using Whatman No. 1 filter paper. The residue was collected, dried, and transferred to a dry 1-liter flask, which was then filled with 500 ml of methanol, corked, and stirred for 24 h. The liquid was filtered into a 500-ml beaker using Whatman No. 1 filter paper. The same fresh solvent was used for two extractions before switching to the next polar solvent. To obtain crude extracts, all the solvents were evaporated under vacuum. When compared to other extracts, studies found that DCME had the highest extractive value.

3.2. Isolation and Biochemical Characterization of Phytochemicals. The DCME of *P. corylifolia* seeds yielded three major constituents. Using the slurry pack method with 100% hexane, 9.6 g of crude extract was put into the column. The sample was then gradually eluted in n-hexane with 1 to 15% (vol/vol) ethyl acetate (EtOAc). Each chromatographic fraction was then examined by TLC. Compound 1 having R_f value of 0.65 was obtained from fractions 7 to 19 [up to 5% (vol/vol) ethyl acetate in n-hexane]. TLC examination of the fraction was performed in a solvent ratio of 20% ethyl acetate in hexane. The combined organic solvent from different fractions was evaporated under vacuum to obtain compound 1 (0.6 g) as brown oily. The UFLC analysis of compound obtained above (compound 1) was carried out, and the chromatogram (Figure 1(d)) showed one single peak at the retention time of 40.3 min. This suggests that compound 1 is a pure molecule, and the structural elucidation using NMR spectra indicates that compound 1 is "bakuchiol."

Compound 2 was obtained from the later obtained fractions 22-34 [solvent system up to 13% (vol/vol) ethyl acetate in n-hexane) of DCME which were then combined. The TLC analysis of the combined fractions was performed in a solvent system containing 20% ethyl acetate in hexane, after which the result was observed under UV light, and the R_f value of compound 2 was found to be 0.45. The combined organic solvent from different fractions was evaporated under vacuum to obtain compound 2 (0.09 g). The UFLC analysis of compound obtained above was carried out, and the chromatogram showed one single peak at the retention time of 28.5 min. Compound 2 was obtained as a white crystalline solid having melting point of 137-138°C.

Characteristic absorption as well as splitting pattern in 1H-NMR of compound 2 are in consonance with the 1H-NMR absorption and splitting pattern already reported by Jiangning et al. [22]. NMR spectra indicate that compound 2 is "isopsoralen."

Compound 3 (40 mg) was isolated from subfraction obtained by combining fractions 35-45, which was subjected to repeated CC using gradient elution with 0-15% ethyl acetate in n-hexane as analyzed by TLC. The R_f value of compound 3 was found to be 0.31. The combined organic solvent was evaporated under vacuum to obtain compound 3 as white solid (0.04 g) having melting point 163-164°C. The UFLC analysis of compound 3 was carried out, and the chromatogram (Figure 1(b)) showed one single peak at the retention time of 28.0 min. NMR spectra studies indicate that compound 3 is "psoralen." After isolation of three major chemical constituents from DCME, our next aim was to study the antiobesity potential of these components.

3.3. Antilipase Activity of DCME and Isolated Compounds of P. corylifolia. Lipids are an essential component of human nutrition. Inhibiting the digestion of dietary fats can be a rational target for pharmaceutical intervention [27]. The antilipase activity of the DCME of *P. corylifolia* seeds, bakuchiol (PC1), isopsoralen (PC2), and psoralen (PC3) was performed at 100 μg/ml, 50 μg/ml, and 25 μg/ml concentration. The percentage inhibition of PL enzyme recorded in each experiment is shown in Figure 2.

3.4. MTT Assay. Before testing the extracts/compounds with the 3 T3-L1 cell line, a toxicity test was required to determine the concentration at which the extract becomes toxic, in order to rule out the possibility that the extract does not influence cell viability. The required level of viability should be greater than 90% [28].

For toxicity analysis, 3 T3-L1 cells were treated with DCME at different concentrations 100, 125, 150, 175, 200, and 400 μg/ml. At concentrations up to 100 μg/ml, DCME of *P. corylifolia* showed no substantial toxicity to 3 T3 -L1 cells, as shown in Figure 3(a). The viability of 3T3-L1 cells was severely reduced at concentrations more than 100 μg/ml. For further tests, safe doses of DCME were kept 100 μg/ml.

The MTT assay was also used to determine the effect of bakuchiol, isopsoralen, and psoralen from *P. corylifolia* seed on 3T3-L1 cell viability and the cells were treated with different concentrations of pure compounds, i.e., 12.5, 25, and 50 μM. At concentrations up to 25 μM, none of the isolates were found to be toxic to 3T3 -L1 cells. The viability of 3T3-L1 cells was reduced at concentrations greater than 25 μM (Figure 3(b)). Thus, safe doses of PC-1, PC-2, and PC-3 were determined as up to 25 μM for further experiments.

3.5. Antiadipogenesis Assay. The differentiation of preadipocytes to mature adipocytes is connected with the increase in the number of cells stained with Oil Red O and lipid

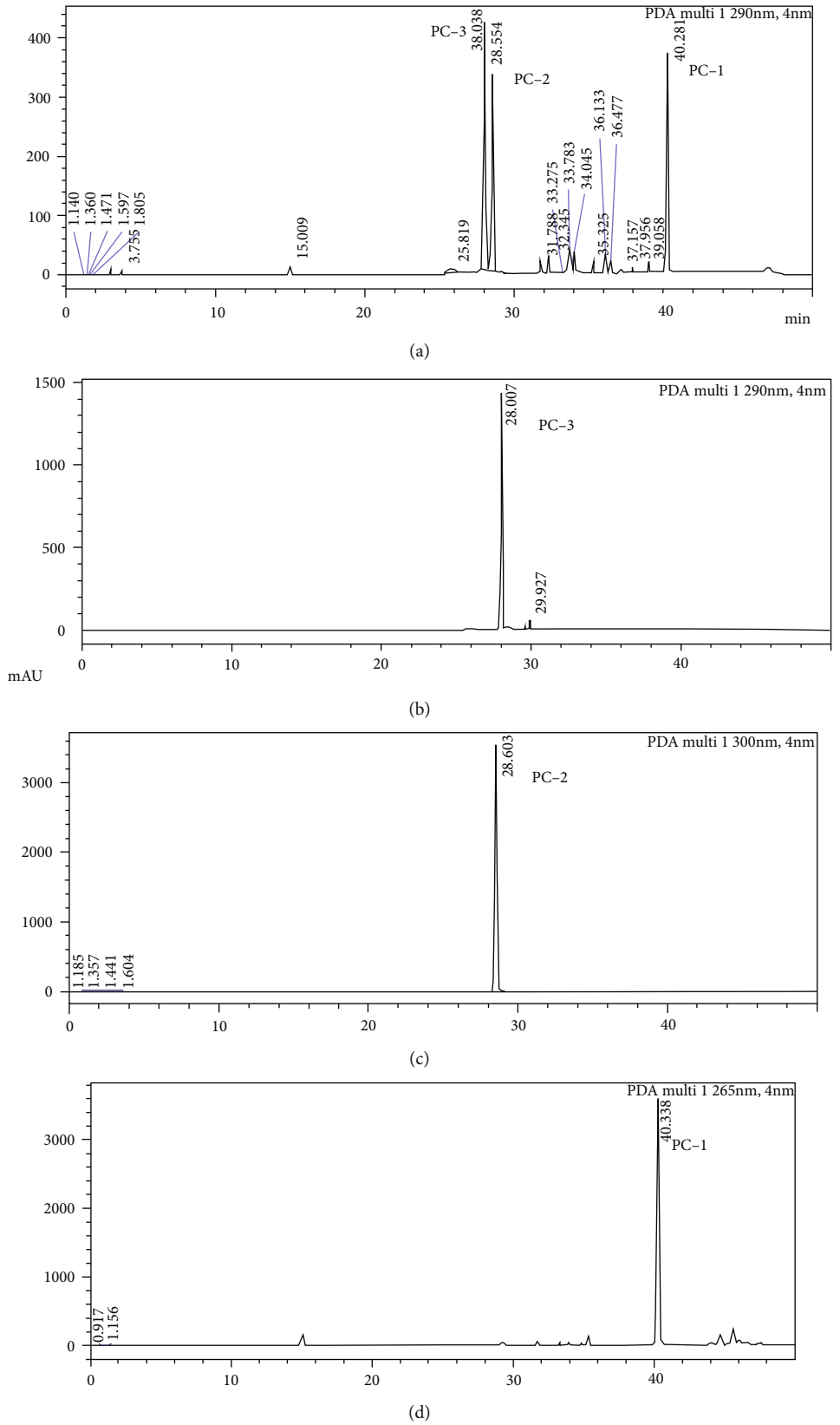

Figure 1: Ultrafast liquid chromatography (UFLC) chromatograms of *Psoralea corylifolia* seed extract and isolates. (a) Chromatogram of dichloromethane extract DCME, (b) chromatogram of PC-3 isolated by column chromatography from DCME, (c) chromatogram of PC-2 isolated by column chromatography from DCME, and (d) chromatogram of PC-1 isolated by column chromatography from DCME.

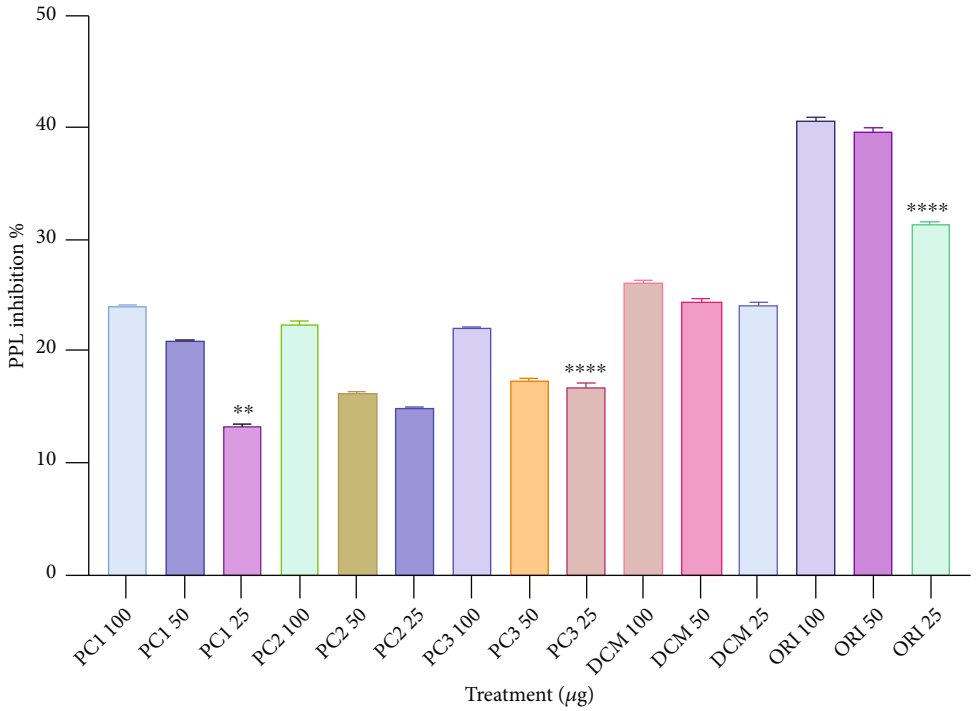

FIGURE 2: Percentage inhibition of pancreatic lipase enzyme by DCM extracts and isolated compounds. Each value is expressed as a mean ± SEM. Each experiment was performed in triplicate ($^{*}p < 0.05$, $^{**}p < 0.01^{***}$ <0.001). Results revealed that all the isolates as well as extract exhibited a dose-dependent inhibitory activity against pancreatic lipase.

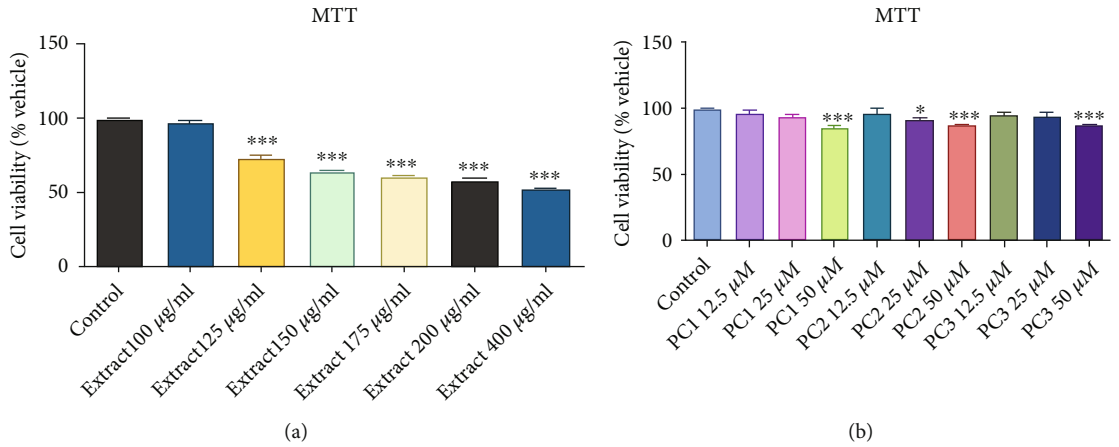

FIGURE 3: Viability of 3 T3-L1 adipocytes under various concentrations of (a) DCM extract of *P. corylifolia* seeds and (b) isolated compounds PC1 (bakuchiol), PC2 (isopsoralen), and PC3 (psoralen) as determined by MTT assay. Data are expressed as mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01^{***}$ <0.001 as compared to control).

accumulation. 3T3-L1 cells were treated with differentiation media plus 100 μg/ml of DCM extract or 25 μM of bakuchiol/isopsoralen/psoralen for 48 hours to see if *P. corylifolia* influences adipogenesis. Further, the cells were shifted to maintenance medium (basal medium with 1 μg/ml insulin) for 10 days with medium replacement on every alternate day. For the antiadipogenesis experiment, extracts or isolates were introduced to differentiation and maintenance medium in 0.1% DMSO carrier for up to 12 days. Control is cells treated with differentiation media plus maintenance media.

After staining with Oil Red O stain on day 12th, cells were imaged using an inverted microscope. The Oil Red O stain demonstrates a reduction in the quantity and size of lipid droplets accumulated in the cells treated with extract or isolates, when viewed under microscope. DCM extract as well as bioactive compounds treatment led to inhibition of adipogenesis (Figures 3(a)–3(e)). After capturing images, OD detection was performed. The results indicated that treatment with the extract or compounds led to a significant decrease of OD (Figure 4). Specifically, treatment with DCM extract,

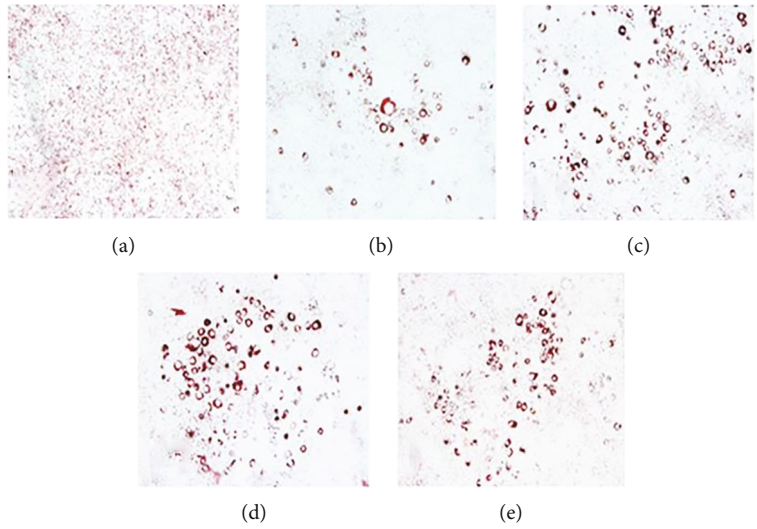

Figure 4: Images of treated cells obtained using an inverted microscope. (a) Control, (b) PC1 treated, (c) PC2 treated, (d) PC3 treated, and (e) DCM extract treated. Red spots in images represent area stained by Oil Red O Dye.

bakuchiol, isopsoralen, and psoralen led lipid accumulation to $75 \pm 0.003\%$, $78.06 \pm 0.002\%$, $80.91 \pm 0.004\%$, and $80.91 \pm 0.001\%$ in comparison to control, respectively (Figure 5).

4. Discussions

Obesity is currently one of the main public health concerns since it is a major contributor to the global burden of chronic diseases, including cardiovascular disease, nonalcoholic fatty liver disease, type 2 diabetes mellitus, and certain types of cancer. Obesity-associated comorbidities can impact function and quality of life as well as mental health also [29]. Phytochemicals are plant-based chemicals that have been shown to be useful in the treatment of chronic diseases like cancer, diabetes, and obesity. Polyphenols and flavonoids have been shown to be particularly abundant in extracts that may be useful against diseases [30].

Phytochemical studies indicated that coumarins, flavonoids, and meroterpenes are the main components of *P. corylifolia*. Some of its isolated compounds are bakuchiol, psoralidin, psoralen, isopsoralen, bavadin, isobavachalcone, corylin, daidzin, and genistein. The extracts and active components of *P. corylifolia* demonstrated multiple biological activities, including estrogenic, antitumor, antioxidant, antimicrobial, antidepressant, anti-inflammatory, osteoblastic, hepatoprotective [17], and antiobesity activities [31]. Due to the various biological activities of this plant, much interest has been generated, suggesting further study on its effect on antiobesity. Polyphenols, flavonoids, terpenoids, alkaloids, saponins, carboxylic acids, glycosides, and tannins are some of the natural secondary metabolites found in plants that have been reported to have antiobesity properties through various modes of action [32]. In the present study, we found that the isolated phytochemicals from *P. corylifolia* possess antiobesity activity, and bakuchiol is a type of meroterpenoid, and psoralen and isopsoralen are coumarins (phenolic substance). Our data supports the potential of terpenoids and phenols as antiobesity agents. Moreover, in a study,

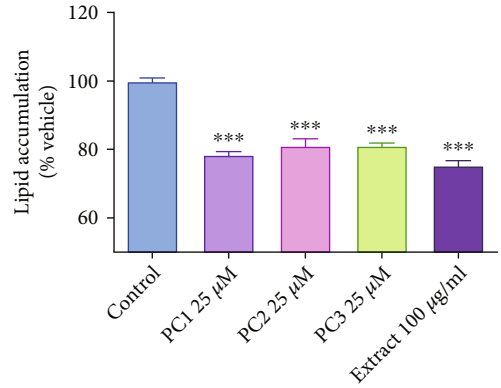

Figure 5: Inhibition of fat accumulation in 3T3-L1 via suppression of adipogenesis by DCM extract/isolated compounds from *Psoralea corylifolia* as determined by Oil O Red solvent extraction. Data are expressed as mean ± SEM ($n = 5$). (* <0.05, ** <0.01, and *** <0.001 versus the control group). The control group was the cell lines treated with differentiated medium plus maintenance and was considered as 100% lipid accumulation.

PCS extract treatment significantly attenuated lipid accumulation in liver and adipose tissue and reduced serum lipid and hepatic triglyceride levels [18]. So, this study favors our work to choose *P. corylifolia* for antiobesity activity.

One of the most extensively utilized models for examining the potential efficacy of natural products as antiobesity medicines is pancreatic lipase inhibition [33]. Since inhibitors of digestive lipase should be useful antiobesity agents, recent research has focused on identifying novel and safe lipase inhibitors from natural sources like phytic acid, tannin, saponins, and Oolong tea [34]. In our study, by assessing the hydrolysis of p-NPB to p-nitrophenol, an in vitro pancreatic lipase model was utilized to assess the inhibitory effect on pancreatic lipase of all isolates as well as of DCME of *P. corylifolia* at concentrations of 25, 50, and 100 μg/ml. The results from our study indicated that DCM extract of

P. corylifolia seeds, bakuchiol, isopsoralen, and psoralen exhibited inhibitory activity against pancreatic lipase which is consistent with the activity reported by other groups [35]. For comparison the antilipase activity of orlistat (positive control) was also studied which was found to be more active than our isolated compounds. Noteworthy, weaker inhibitory effect of plant extracts and their isolated compounds on pancreatic lipase than orlistat has been observed by others [36]. Weaker antilipase effect than orlistat may be ascribed to the complexity of composition and multiple interactions between different components. Although orlistat is an available market drug, but some of its most common side effects (gastrointestinal toxicity, liver damage, pancreatic damage, kidney damage, metabolic system abnormalities, and a high cancer risk) severely limit its therapeutic applicability, particularly for long-term use [37].

When energy intake exceeds energy expenditure over time, the surplus energy is stored in the form of triglycerides in adipose tissue. An increase in cell size, cell number, or both can result in increased adipose tissue mass [38]. Preadipocyte differentiation has become an area of intense research in recent years with in vitro models of adipogenesis, such as the 3T3-L1 cell line, being used to investigate it [39]. Lipid droplet accumulation and preadipocytes differentiation into mature adipocytes are regarded as the hallmark events in obesity [40]. We examined the effects of the compounds on the inhibition of lipid accumulation after conducting the MTT assay. In the present study, as detected by the Oil Red O staining, adipogenesis and lipid accumulation were markedly inhibited by treatment with DCME of *P. corylifolia* seeds, bakuchiol, isopsoralen, and psoralen in 3T3-L1 adipocytes. It is interesting to note that DCME displayed a higher activity compared to the isolated compounds. This could be due the cumulative effect of other known compounds such as genistein [41, 42], bavachin [43], isobavachalcone [44], and corylin [45] in the *P. corylifolia* seed extract which are known to possess antiobesity potential. There are several reports which revealed the inhibition of adipogenesis via regulation of PPARγ and C/EBPα expression in 3T3-L1 cells [46]. *P. corylifolia* may inhibit adipocyte differentiation by suppressing PPARγ and C/EBPα transcriptional activity in 3T3-L1 adipocytes.

5. Conclusions

Through our studies on PCSE, we claim that *P. corylifolia* possesses antiobesity potential, but there are only few scientific evidences to support this claim [31]. The antiobesity activity of *P. corylifolia* seed extract might be due to the presence of coumarins [47], flavonoids [48], etc. Present work disclosed that bakuchiol, isopsoralen, and psoralen isolated from PCSE inhibited pancreatic lipase 24.2 ± 0.037, 22.69 ± 0.026, and 22.24 ± 0.057 percent, respectively, at 100 μg/ml concentration. Bakuchiol, isopsoralen, and psoralen decreased lipid accumulation in 3T3-L1 preadipocytes with $78.06 \pm 0.002\%$, $80.91 \pm 0.004\%$, and $80.91 \pm 0.001\%$ lipid accumulation, respectively, in comparison to control at 25 μM dose. These findings indicate the involvement of phytochemicals derived from *P. corylifolia* in regulating adipocyte differentiation, making it a promising candidate for the treatment of obesity-related diseases. This work highlights the therapeutic role of PCSE in metabolic disorders, which can help researchers across globe to analyze the various constituents responsible for antiobesity activity on specific receptors in adipose tissue. Thus, extensive research could be planned to determine the precise mode of action of the aforementioned bioactive compounds.

Data Availability

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

Additional Points

Highlights. (i) Bioactive constituents (bakuchiol, isopsoralen, and psoralen) were isolated from *Psoralea corylifolia*-dichloromethane seed extract. (ii) *Psoralea corylifolia*-dichloromethane seed extract exhibited antiadipogenesis and pancreatic lipase activities. (iii) Seed extract and isolated compounds showed therapeutic potential against adipogenesis

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

N.M., B.K., and J.S. wrote the first draft of the manuscript. The final draft was read and edited by J.K., M.B., P.G., A.K., I.M., A.K.R., R.P., and B.A.S. All authors have made a substantial, direct and intellectual contribution to the work and approved it for publication. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials

Supplementary Table 1 The extractive value of different extracts. Supplementary Table 2: spectral and melting point data of the compounds isolated from *Psoralea corylifolia* L. Supplementary Figure 1: 1H NMR and 13C NMR of different isolates, isolated by column chromatography, from DCM extract of *Psoralea corylifolia*. (a) 1H NMR of PC-1, (b) 13C NMR of PC-1, (c) 1H NMR of PC-II, (d) 13C NMR of PC-II, (e) 1H NMR of PC-III, and (f) 13C NMR of PC-III. *(Supplementary Materials)*

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Research Article

Ethnopharmacological Survey, Mineral and Chemical Content, *In Vitro* Antioxidant, and Antibacterial Activities of Aqueous and Organic Extracts of *Chamaerops humilis* L. var. argentea Andre Leaves

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Introduction. The present study is carried out for the first time on *Chamaerops humilis* L. var. argentea Andre from the region of Taza using an ethnopharmacological survey, an experimental study of the mineralogical and chemical compositions, and evaluations of the antioxidant and antibacterial activities. *Methods.* After conducting the ethnopharmacological survey, a mineralogical and phytochemical study involving the preparation of aqueous and organic extracts was done. Essential oils were also extracted by hydrodistillation. Subsequently, qualitative and quantitative chemical analyses were performed. *In vitro* evaluation of antioxidant activities was performed by five tests (H_2O_2, DPPH, ABTS, FRAP, and RP) and antibacterial activities by the disc method and determination of MIC and MBC. A principal component analysis (PCA) was performed to visualize the different correlations. *Results.* The different parts of the plant are used for the treatment of digestive disorders, cardiovascular diseases, and diabetes. In addition, the leaves are rich in mineral compounds, catechic tannins, flavonoids, and sterols. However, they have some traces of essential oils. The quantitative analysis revealed that the ethanolic macerated had a higher content of total polyphenols (100.27 ± 1.95 mg EAG/g E) and catechic tannins (52.11 ± 1.02 mg EC/g E). This extract had a strong antioxidant capacity (H_2O_2 (37.34 ± 0.55%), DPPH (IC_{50} = 31.18 ± 0.66 μg/ml), ABTS (108.28 ± 1.29 mg E AA/g E), FRAP (148.85 ± 0.43 mg E T/g E), and RP (10.86 ± 0.01 mg E AA/g E). The same extract had a bactericidal effect against *Staphylococcus aureus*. Principal component analysis (PCA) showed that antioxidant activity was highly correlated with the chemical composition of *C. humilis* leaves; a high correlation was recorded between the total polyphenol content and ABTS (r = 0.9779), FRAP (r = 0.9644), DPPH (r = 0.9418), and PR (r = 0.9271) tests. In addition, cathectic tannins were highly correlated with the tests of DPPH (r = 0.9753) and ABTS (r = 0.8843). Flavonoids were similarly correlated with DPPH (r = 0.8897) and ABTS (r = 0.7599) tests. *Conclusion.* These results could justify the traditional use of the leaves of *Chamaerops humilis* in the region of Taza for the treatment of some diseases.

1. Introduction

For several years, the use of herbal preparations has been of great importance in the therapeutic field. We also notice that this sector concerns the developed countries as well as the developing countries. For the first countries, the mistrust towards synthetic products and the desire to consume organic extracts are the main factors that encourage them to use medicinal and aromatic plants in their daily life. In the United States of America, for example, 25% of prescribed

medicines are plant based [1]. In Europe, the number of food supplements produced from plants has grown exponentially in 2002 [2]. Developing countries consider traditional medicine as part of the cultural heritage; the population of these countries has a know-how related to the uses of plants in traditional pharmacopoeia. Similarly, they use medicinal plants and herbal remedies for economic reasons related to purchasing power which is low, difficulties in accessing health care due to the high cost of synthetic drugs.

In Morocco, ensuring access to primary health care and universal health coverage for the population remains a major challenge. Despite the efforts made in recent years, the problem of access to health care is far from being solved, which pushes the population to seek other accessible and less expensive means for the treatment of diseases, including medicinal plants. Indeed, Morocco has a very important floristic wealth characterized by a great diversity of plants with 4200 species and a large number of species and subspecies [3]. This natural wealth places Morocco in an important position among other Mediterranean countries. Morocco also has a long history in phytotherapy especially in rural areas [4].

Similarly, the region of Taza (north-east Morocco) has an important natural richness and a local traditional know-how related to the uses of medicinal plants in phytotherapy [5, 6]. *Chamaerops humilis* L. var. argentea Andre is among the very abundant plants in the region of Taza and is the only palm species naturally distributed in Europe and Africa [7]. In Europe, *Chamaerops humilis* is present on the coasts of southern Portugal, southern and eastern Spain, south-eastern France, and western Italy [8, 9]. The species is also found on most of the large islands in the western Mediterranean, namely, the Balearic Islands, Sardinia, Sicily, and Malta. In Corsica, it does not seem to be indigenous [10]. In Africa, it is mainly located in Morocco but also in northern Algeria and northern Tunisia. The species has the widest distribution in Morocco, where 2 varieties have been distinguished: a green-leaf form (var. *humilis*) and a glaucous-leaf form (var. argentea André) [9, 11, 12]. First, *C. humilis* or dwarf palm belongs to the Arecaceae family (Palmaceae), it is small in size and can grow up to 1.5 m, it is a dioecious plant, its leaves are palmate and sclerophyllous [13], and the fruits are "polydrupes" that ripen in autumn (September–November). They are attached to inflorescences up to 30 cm long (37–91 fruits per branch), and the female inflorescences are solid, brownish, and bear fruits or only the calyx; in contrast, the males have smaller and very fragile inflorescences [14].

In addition to the abundance of the variety *Chamaerops humilis* L. var. argentea Andre in the region of Taza. To our knowledge, no ethnopharmacological survey nor mineralogical analysis or studies of the chemical composition and antibacterial activity have been carried out on *Chamaerops humilis* L. var. argentea Andre in the region of Taza. In Morocco, we identified from the literature only one preliminary study made on the species *Chamaerops humilis* on a single extract and only one test of antioxidant activity [15]. In Algeria, an ethnopharmacological study was conducted on the species *Chamaerops humilis* [16] and another on the chemical composition of the species *Chamaerops humilis* [17], and finally, another ethnobotanical study was conducted still in Algeria on *Chamaerops humilis* variety argentea André but without going to pharmacological tests to assess the traditional uses mentioned by the respondents [18]. Studies have declared the use of the plant in phytotherapy; the aerial part is against pyelonephritis and prostatitis [19]; the fruits and roots are also used to treat type 2 diabetes [20], the plant is also used for the treatment of digestive system disorders and in veterinary medicine [21]; the fruits are used as antidiarrheal medicine [22] and to treat digestive system disorders in general [23]. Digestive disorders are typically associated with a long line of acute and chronic human diseases [24]. The digestive system contains intestinal microbiota that includes all the bacteria that live in symbiosis and have a crucial role in the digestion of food. Any imbalance can lead to an increase in the proliferation of pathogenic bacteria, and consequently, a change in the composition of the microbiota of the gastrointestinal tract can lead to hypochlorhydria [25], to abdominal discomfort, bloating, and diarrhea, which can cause hypophosphatemia and hypomagnesemia that affect the normal functioning of our organs [26–28]. In addition, researches have reported the role of minerals for the proper functioning of the digestive system; the increase of dietary calcium reduces the risk of developing colon adenomas, a reduction in the risk of colorectal cancer [29]. In addition, the various fermentation processes in the rumen require an adequate supply of minerals [30].

Faced with this observation and these data, we were interested in conducting a global study on *Chamaerops humilis* L. var. argentea Andre to highlight its therapeutic interest by adopting the ethnopharmacological approach. Thus, we were interested on the one hand in the study of the interest of *C. humilis* in traditional medicine via an ethnopharmacological survey conducted in the province of Taza and, on the other hand, to carry out an analysis of its mineralogical and chemical composition and a study of biological activities in order to confirm or deny the use of this plant in traditional medicine. The chemical study included aqueous extraction (decoction, infusion, and maceration) and organic extraction with Soxhlet and cold by three solvents (ethanol, chloroform, and hexane), the screening of secondary metabolites, and the quantification of phenolic compounds.

The biological study consisted of an *in vitro* evaluation of the antioxidant activity by five tests: hydrogen peroxide scavenging test (H_2O_2), DPPH (2,2-diphenyl-1-picrylhydrazil) free radical scavenging test, ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate) or TEAC (Trolox equivalent antioxidant capacity), ferric-reducing antioxidant power assay (FRAP), and the reducing power (RP) test. And then, a study on antibacterial activity by the disc method and the determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) was done. Finally, a principal component analysis (PCA) was performed to investigate the correlations between the chemical composition of the different extracts and their biological activities and also between

the five tests used for the evaluation of the antioxidant activity.

2. Material and Methods

2.1. Ethnopharmacological Survey

2.1.1. Study Site. The province of Taza is located in north-eastern Morocco east of the Fez-Meknes region, situated between the Pre-rif in the north and the Middle Atlas in the south, characterized by a semicontinental climate with a Mediterranean influence [31].

The province is characterized by a remarkable diversity and natural wealth because it has one of the oldest national parks in Morocco: the Tazekka National Park [32].

2.1.2. Data Collection. The ethnopharmacological survey was conducted in four stations in the province of Taza: Taza city, Bab El Mrouj, Gueldaman, and Bab Boudir. The choice of survey stations was based on their abundance of *Chamaerops humilis* L. var. argentea Andre that makes its traditional use easy and therefore allows the accumulation of traditional knowledge by the local population (Figures 1 and 2).

The method used was a questionnaire developed and adopted by the Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health and Quality of Life (SNAMOPEQ), Polydisciplinary Faculty of Taza, Sidi Mohamed Ben Abdellah University (USMBA) of Fez, Morocco [5, 6, 33]. The survey was conducted using a questionnaire with a simple choice, multiple choice, and open-ended questions about the informant and *C. humilis* and its medicinal aspects. The questions were written in French language, and due to the fact that the province of Taza is with multiple tribes (Branès, Ghiata, Tsoul, and the Amazighs) and each tribe is individualized by a separate dialect and to avoid difficulties in understanding the questions, we helped the informants to understand the questionnaire with their own dialects (Arabic/Amazigh) [33–38]. The data obtained were later translated into French and English [39]. The survey was conducted among traditional practitioners, herbalists, farmers, nomads, and a total of 239 people.

The information collected concerned the profile of the respondent (age, gender, and level of education) and ethnopharmacological data such as the vernacular name of the plant, part used, methods of preparation, and uses of the plant.

2.2. Mineralogical and Chemical Compositions

2.2.1. Plant Material. The plant material consists of the leaves of the plant *Chamaerops humilis* L. var. argentea Andre collected in February 2017 from the mountains of Bab Boudir at 42.3 km from the city of Taza, geographical coordinates N 34°405.635′, W 004°05.900′, altitude: 1358 meters (Figure 1).

The botanical identification of the plant *Chamaerops humilis* L. var. argentea Andre was made by Dr. Abdelmajid Khabbach, botanist at the Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health and Quality of Life (SNAMOPEQ), at the Polydisciplinary Faculty of Taza, based on the works of Valdés and his collaborators and Richard [40, 41] (Figure 3).

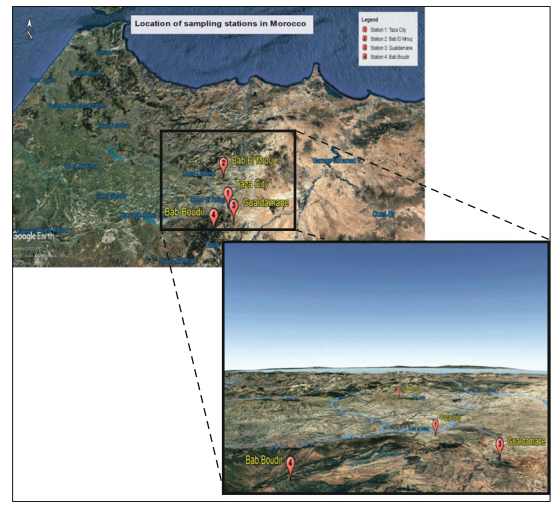


FIGURE 1: 3D geographical map representing the location of the stations of the ethnopharmacological survey *on Chamaerops humilis* L. var. argentea Andre in the study area: province of Taza (north-east Morocco).

A reference sample SB2017 of the leaves of *Chamaerops humilis* L. var. argentea Andre was deposited in the herbarium of the SNAMOPEQ of the Polydisciplinary Faculty of Taza, Morocco.

2.2.2. Mineralogical Study. The quantitative analysis of mineral elements was done according to the protocol of Arora and collaborators [42]. The test procedure has been described in detail in our previous work [43–45].

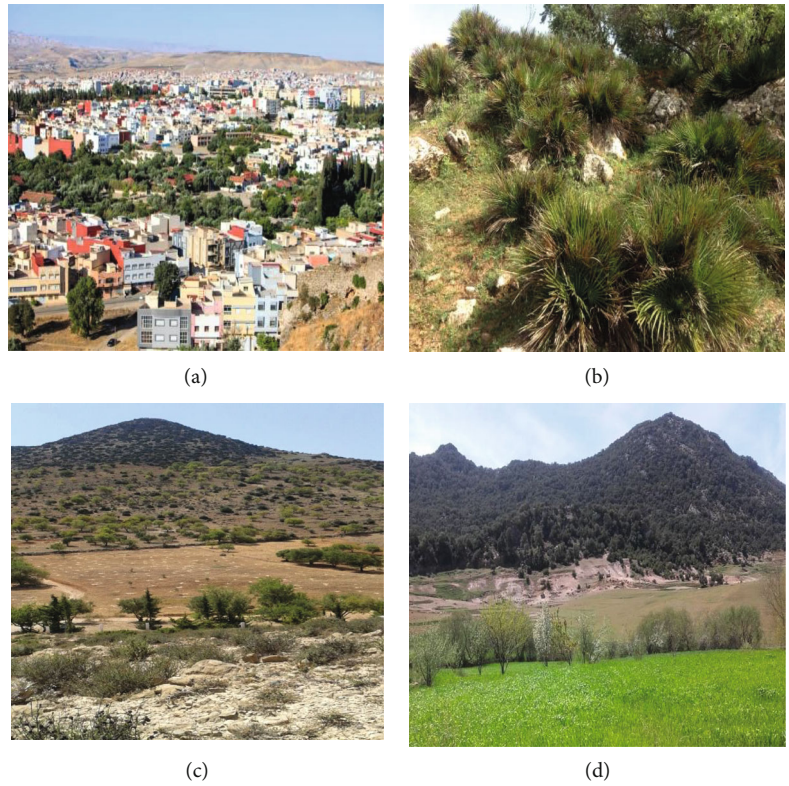
2.3. Phytochemical Study

2.3.1. Preparation of Extracts. The extraction of bioactive molecules contained in the leaves of *C. humilis* was carried out by two methods: an aqueous extraction with distilled water in three ways: decoction, infusion, and maceration, and another organic one by hot Soxhlet using organic solvents of different polarities (ethanol, chloroform, and hexane) and cold by maceration in ethanol. The extraction procedure employed is detailed in our previous work [43].

After removing the solvents under reduced pressure at 40°C in a Buchi R-210 Rotavapor, all extracts obtained were weighed for yield calculation and stored at 4°C until use.

2.3.2. Extraction of Essential Oils by Hydrodistillation. The extraction of essential oils was performed by hydrodistillation using the Clevenger apparatus. The leaves of *Chamaerops humilis* var. argentea André are immersed in a flask containing distilled water, and then, the whole is brought to boil using a flask heater.

2.3.3. Phytochemical Screening. Phytochemicals were characterized on both the powdered plant material and the aqueous and organic extracts of the plant using staining and precipitation reactions as described by a previous work in



(a) (b) (c) (d)

Figure 2: Survey stations: (a) Taza city, (b) Bab El Mrouj, (c) Gueldamane, and (d) Bab Boudir.

Figure 3: *Chamaerops humilis* L. var. argentea Andre (pictures taken on 16 April 2017 in Bab Boudir, located 60 km from the city of Taza; geographical coordinates: N 34°405.635′, W 004°05.900′).

our laboratory [43, 44, 46–49]. The characterization reagents were used to investigate the following chemical groups: catechic tannins and gall tannins using the ferric chloride $FeCl_3$ reaction [50], flavonoids using the cyanidine reaction [51], saponins using the foaming technique [52], alkaloids by two reagents, Dragendorff (potassium iodobismuthate) and Valser-Mayer (potassium tetraiodomercurate) [53], sterols with the Liebermann reaction [51], anthracenosides by the Borntraeger reaction [54], free quinones by sodium hydroxide [55], and anthraquinones with potassium hydroxide for plant material and with ammonia solution for aqueous and organic plant extracts [56].

2.3.4. Quantification of Phenolic Compounds. The results of phytochemical screening of *C. humilis* leaves directed us in the quantification of total polyphenols, flavonoids, and catechic tannins according to the protocols described in previous works of our laboratory [43, 44, 46–49].

(1) Determination of Total Polyphenols. The determination of total polyphenols in the extracts of *C. humilis* leaves was performed according to the Folin-Ciocalteu reagent method [57]; the test protocol is described in previous publications [43, 47, 49, 58]. The total polyphenol content of the extracts was expressed as microgram (μg) equivalent of gallic acid per milligram (mg) of extract (μg GAE/mg E).

(2) Determination of Flavonoids. Total flavonoids were quantified using the technique adapted by Mihai and collaborators with aluminum trichloride and soda [59]; the methodology for the quantification of flavonoids is detailed in previous works [49, 57]. The flavonoid content was expressed as μg quercetin equivalent/mg extract (μg QE/mg E).

(3) Determination of Catechic Tannins. Catechin tannins are determined by the vanillin method using the procedure reported by Joslyn [60]. This method is based on the ability of vanillin to react with condensed tannin units in the presence of acid to produce a colored complex measurable at 500 nm. The detailed protocol is described in our previous work [35, 46–48]. Tannin concentration was expressed as

microgram equivalents of catechin per milligram of extract (μg CE/mg) from the calibration curve.

2.3.5. Antioxidant Activity. The determination of the antioxidant capacity of aqueous and organic extracts of the leaves of *C. humilis* was carried out *in vitro* by five tests: hydrogen peroxide scavenging test H_2O_2, free radical scavenging test DPPH (2,2-diphenyl-1-picrylhydrazil), ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate) method or TEAC (Trolox Equivalent Antioxidant Capacity), iron reduction antioxidant capacity (FRAP), and the reducing power test (PR).

(1) Hydrogen Peroxide Scavenging Assay (H_2O_2). The study of H_2O_2 scavenging activity was performed by the method described by Ruch and collaborators [61]; the experimental protocol was described by later works [43, 44, 46–49]. The results are expressed as percent inhibition according to the following formula:

$$\%H_2O_2 \text{ scavenging} = \left[\frac{(AC - AT)}{AC}\right] \times 100[6], \quad (1)$$

where *AC* is the absorbance of the control and AT is the absorbance of the test.

(2) Determination of the Antifree Radical Activity by the DPPH Test. The activity of the DPPH radical scavenging (2,2-diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$) from the nine aqueous and organic extracts of *C. humilis* is determined according to the method of [62], described by previous studies from our laboratory [43, 44, 46–49]. The results obtained were compared to the DPPH scavenging activity for Trolox, BHT, and ascorbic acid which were used as reference standards. The results are expressed as percent inhibition using the following formula:

$$I(\%) = \left[\frac{(\text{Abs} - \text{Abse})}{\text{Abs}}\right] \times 100, \quad (2)$$

where *I* (%) is the percent inhibition, Abs is the absorbance of negative control, and Abse is the absorbance of the sample.

The IC_{50} (50% inhibitory concentration) is the concentration of the tested sample capable of reducing 50% of the $DPPH^{\cdot}$ radical which was determined graphically from the percentage of scavenging effect versus the corresponding sample concentration.

(3) Trolox Equivalent Antioxidant Capacity Using ABTS (TEAC). The evaluation of the $ABTS^{-}$ radical scavenging capacity was determined by the method of [63]. The technique of this assay has been detailed in our subsequent work [43, 44, 46–49]. The total antioxidant capacity of the 9 aqueous and organic extracts of the plant was expressed as mg of Trolox equivalent per gram of extract (μg of TE/mg E).

(4) Ferric-Reducing Antioxidant Power Assay (FRAP). Determination of the iron reduction antioxidant capacity (FRAP) of our 9 aqueous and organic extracts was determined according to the method described by Benzie and Strain [64]. The detailed protocol refers to several references from our previous studies [43, 44, 46–49]. Results are expressed as micrograms of Trolox equivalent per milligram of extract (μg TE/mg E).

(5) Reducing Power Assay (RP). The reducing power of iron was determined according to the method of Oyaizu [65]. The experimental protocol was detailed in our previous work [43, 44, 46–49]. Results are expressed as μg ascorbic acid equivalent per milligram of extract (μg EAA/mg E).

2.3.6. Antibacterial Activity. The antibacterial activity evaluation tests were performed on *Staphylococcus aureus* CECT976, *Bacillus subtilis* DSM6633, and *Listeria innocua* CECT 4030 for $Gram^{+}$ bacteria and *Escherichia coli* K12, *Proteus mirabilis*, and *Pseudomonas aeruginosa* CECT118 for $Gram^{-}$ bacteria. The evaluation of the antibacterial activity of the different organic extracts was carried out by two methods, the disc diffusion method in an agar medium, and then the determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

(1) Agar Diffusion Method. Organic extracts at concentrations of 40, 80, and 100 mg/ml were prepared and dissolved in 10% dimethyl sulfoxide (DMSO).

Microbial suspensions in the exponential phase of growth (approximately 10^8 CFU/m according to the McFarland scale) were plated on sterile Mueller Hinton agar contained in the petri dishes. Sterile Whatman paper discs with a diameter of 6 mm impregnated with 40 mg/ml, 80 mg/ml, and 100 mg/ml of organic extracts were aseptically deposited on the seeded agar, and then, the plates were incubated at 37°C for 24 h. Antibacterial activity is determined by measuring the diameter of the inhibition zone around each disc. The experiment was performed in triplicate for each microbial species. 10% DMSO was used as a negative control, and tetracycline and Amikacin were used as positive controls [66].

(2) Macrodilution Method in Liquid Medium. (2)1. Determination of the Minimum Inhibitory Concentration (MIC). The MIC is the lowest concentration of a substance that can inhibit the growth of bacteria for 18 to 24 hours at 37°C. The MIC was performed only for extracts that have shown sensitivity during the agar diffusion method. It was evaluated by the microdilution method in agar medium. The initial concentration was 80 mg/ml.

100 μl of liquid culture medium (Mueller-Hinton) was introduced in each well, then, 100 μl of the extracts to be tested was added, and successive dilutions were carried out. Finally, the wells were inoculated with 10 μl of the suspension of microorganisms at final inoculum concentrations of 10^8 CFU/ml.

The microplate was covered and incubated at 37°C for 24 hours. 10 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was added to each well; after a 15 minute reincubation at 37°C, the reading was taken. The appearance of a purple coloration shows bacterial growth. The MIC is the lowest concentration of the test substance at which no visual disturbance is observed [67].

(2)2. Determination of the Minimum Bactericidal Concentration (MBC). In order to determine the MBC values, we used well solutions with an extract concentration equal to or higher than the obtained MIC values. 10 μl of each well was plated on Mueller-Hinton medium in petri dishes. After incubation for 24 to 37°C, the MBC, defined as the lowest concentration showing no bacterial growth, was determined.

2.3.7. Data Analysis. For the ethnopharmacological survey, the data recorded on the forms used for the survey were then processed and entered into the Excel software. The analysis of the data used descriptive statistics methods. Thus, the qualitative variables are described using the numbers and percentages by the following formula:

$$\text{Frequency in\%} = \left(\frac{\text{xi}}{N}\right) * 100, \quad (3)$$

where xi is the number of employees of a given value and N is the total number of employees.

The statistical analysis of the data was done using the one-way analysis of variance (ANOVA) followed by the Tukey post test. All the results obtained were expressed as mean ± standard deviation. All results were analyzed using GraphPad Prism 5 statistical analysis software, and differences were considered statistically significant for $p < 0.05$ in all statistical analyses.

Principal component analysis (PCA), developed by Pearson, was also used to analyze the correlations between the chemical composition of the aqueous and organic extracts prepared from the leaves of *C. humilis* and their biological activity and also between the different tests used for the evaluation of the antioxidant activity.

3. Results

3.1. Ethnopharmacological Study

3.1.1. Information about the Respondents. The analysis of the information collected on the respondents made it possible to obtain data on age, gender, and level of education.

(1) Age Factor. According to the data analysis, the average age of the respondents is 50 years. The frequency of use of *Chamaerops humilis* L. in traditional medicine changes with the age group, and people over 70 years old has the highest frequency of use with a percentage of 100%. Followed by the age groups [50-69 years] and [30-49 years] with frequencies of 90.74 and 81.19% respectively (Figure 4).

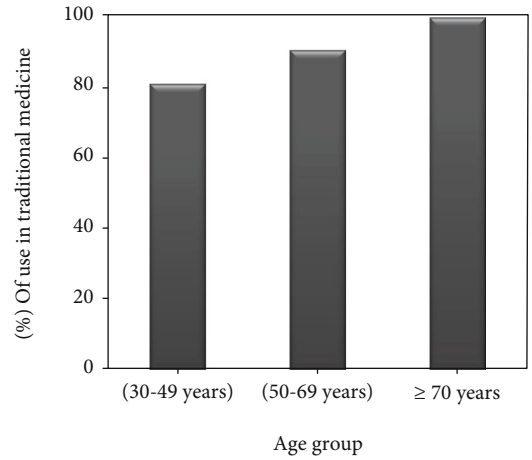


Figure 4: Distribution of users of *C. humilis* in traditional medicine according to age group in the region of Taza, Morocco.

(2) Gender Factor. The survey was conducted among 239 people who are divided into 144 men and 95 women. The results obtained show that they have a frequency of users of *C. humilis* very close, and it is 84.21% for women and 90.27% for men (Figure 5).

(3) Level of Study. The data obtained show that there is a significant effect on the level of education of the populations surveyed on the frequency of medicinal use of *C. humilis*; the vast majority of users of *C. humilis* are illiterate with an average of 65.27% (Figure 3). This category presents a percentage of 89.10% regarding the traditional therapeutic use of the studied species. However, people with primary and secondary education have a significant percentage of use which is 88.57% and 55.55%, respectively, while those with university education use this medicinal plant very seldom with a percentage of 25% (Figure 6).

3.1.2. Vernacular Names of C. humilis. According to the population surveyed, in the Arabic dialect, the entire plant is known by the name Doum or "Douma" to designate a single plant or "Tigztamet" in Amazigh. Each part of the plant also has a distinct vernacular name; the leaves are called "Dûm" or "Laâzaf" in Arabic dialect, and the fruits are called "Lghaz" and "Ghaz dûm" in Arabic dialect or "Aghaz" in Amazigh. The palm heart is called "Jammar" or "Jammara" to refer to a single unit of the palm heart in dialectal Arabic; in Amazigh, it is called "Agnit" and the spadix is called "Al baâouche" in dialectal Arabic or "Timjjat wgnit" or "Timjjin wgnit" as a single spadix in Amazigh.

3.1.3. Medicinal Interest of C. humilis. The medicinal interest of *C. humilis* concerned several aspects such as the part used, the disease treated, the mode of preparation, and other uses of this plant in the Moroccan traditional medicine.

(1) Parts Used of C. humilis. The results obtained (Figure 4) show that heart of palm and spadice are the most used parts in the four surveyed stations with a similar percentage of 79.49%, followed by fruits, leaves, and roots with percentages

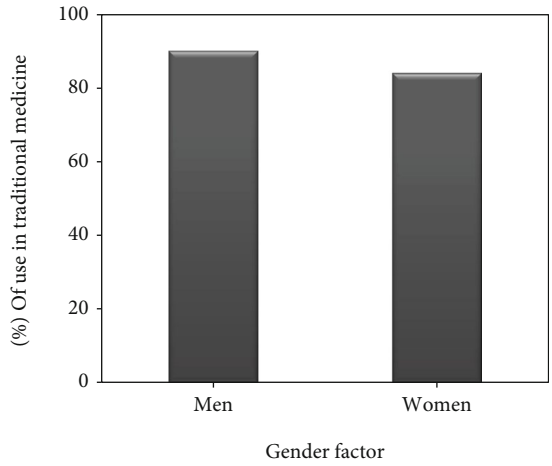


Figure 5: Medicinal uses of *C. humilis* according to gender in the region of Taza, Morocco.

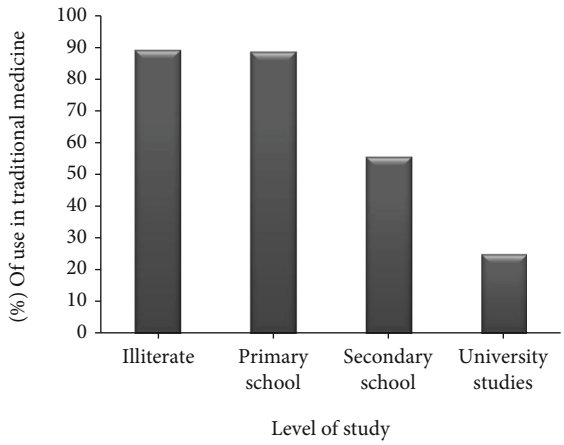


Figure 6: Level of study of *C. humilis* users in the Taza region, Morocco.

of 24.26%, 8.78%, and 4.60%, respectively. In addition, during the survey, we noticed that most of the respondents do not differentiate between the two parts, heart of palm and spadice; they usually mention of the heart of palm, except in the last station of «Bab Boudir» (Figure 7).

(2) Diseases Treated by C. humilis. The analysis of the information collected by the surveys carried out in the four survey stations shows that the three parts, palm heart, spadice, and fruits, are used for the treatment of digestive disorders (bloating, ulcer, intestinal worms, and inflammations), diabetes, cardiovascular system disorders, respiratory system disorders, and hepatitis. In addition, the fruits are also used to solve fertility problems, especially in women, and to strengthen the immune system. The roots are used for the treatment of digestive disorders and diabetes and in association with *Lawsonia inermis* or "natural henna" (belonging to the family Lythraceae, genus *Lawsonia*, species *inermis*)

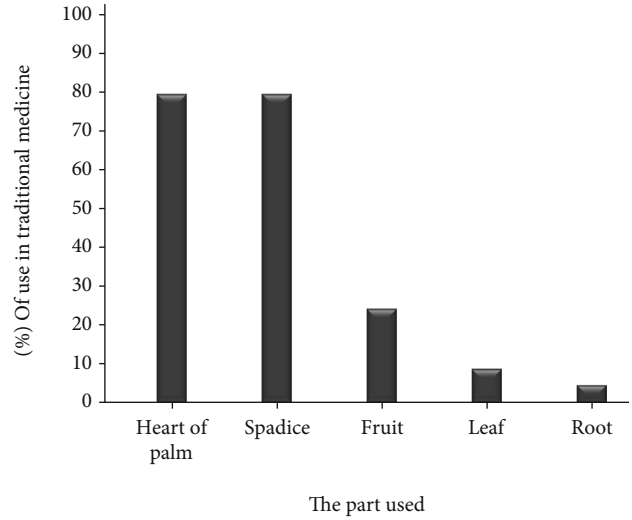


Figure 7: Parts used of *C. humilis* in the region of Taza, Morocco.

against hair loss. Our data indicate that the leaves are also used to treat digestive system disorders in humans and livestock (Table 1).

(3) Preparation Method of C. humilis. During the survey, we found that palm heart and spadice are generally used raw without preparation with a percentage of 95.38% and can be used roasted, in decoction or in combination, with a percentage of 2.56%, 1.02%, and 1.02% (Figure 8).

The fruits are also used raw with a percentage of 59.32% or in association with pure honey or olive oil (38.98%); they are weakly used in decoction (1.69%). On the other hand, we found that the decoction dominates as a method of preparation of the roots (90.90%) and the infusion of the roots is less used with a percentage of 9.09%.

The leaves are crushed and used with a percentage of 47.82%, in decoction with 34.78% and in aqueous maceration with 17.39%.

(4) Other Uses of the Plant C. humilis. The data obtained from the surveys carried out in the four stations show that saw palmetto has several uses other than medicinal. This species can serve as food for humans (palm heart, spadice, and fruit) and food for animals (leaf and fruit); the leaves are used extensively for the manufacture of handicrafts and can be used as a tool for washing dishes (a type of sponge). Also, they are used in the past for the production of vegetable hair, the leaves and fruits are also used to practice magic, and the whole plant is used as firewood (Table 2).

3.2. Mineralogical Analysis of Chamaerops humilis L. var. argentea *Leaves.* Mineralogical analysis by inductively coupled plasma atomic emission spectrometry (ICP-AES) revealed the presence of iron, potassium, phosphorus, magnesium, sodium, copper, calcium, zinc, selenium, and strontium (Table 3).

Table 1: Diseases treated according to the part used of *C. humilis* in the region of Taza, Morocco.

Parts of the plant *C. humilis*	Diseases treated
Palm heart	Digestive disorders, diarrhea, inflammation, diabetes, toning, cardiovascular disease, respiratory system disorders, hepatopathy
Spadice	Digestive disorders, diarrhea, inflammation, diabetes, toning, cardiovascular disease, respiratory system disorders, hepatopathy
Fruit	Digestive disorders, diarrhea, inflammation, diabetes, toning, cardiovascular disease, respiratory system disorders, hepatopathy, fertility, immune system disorders
Root	Digestive disorders, diabetes, hair loss
Leaves	Digestive disorders for humans and livestock

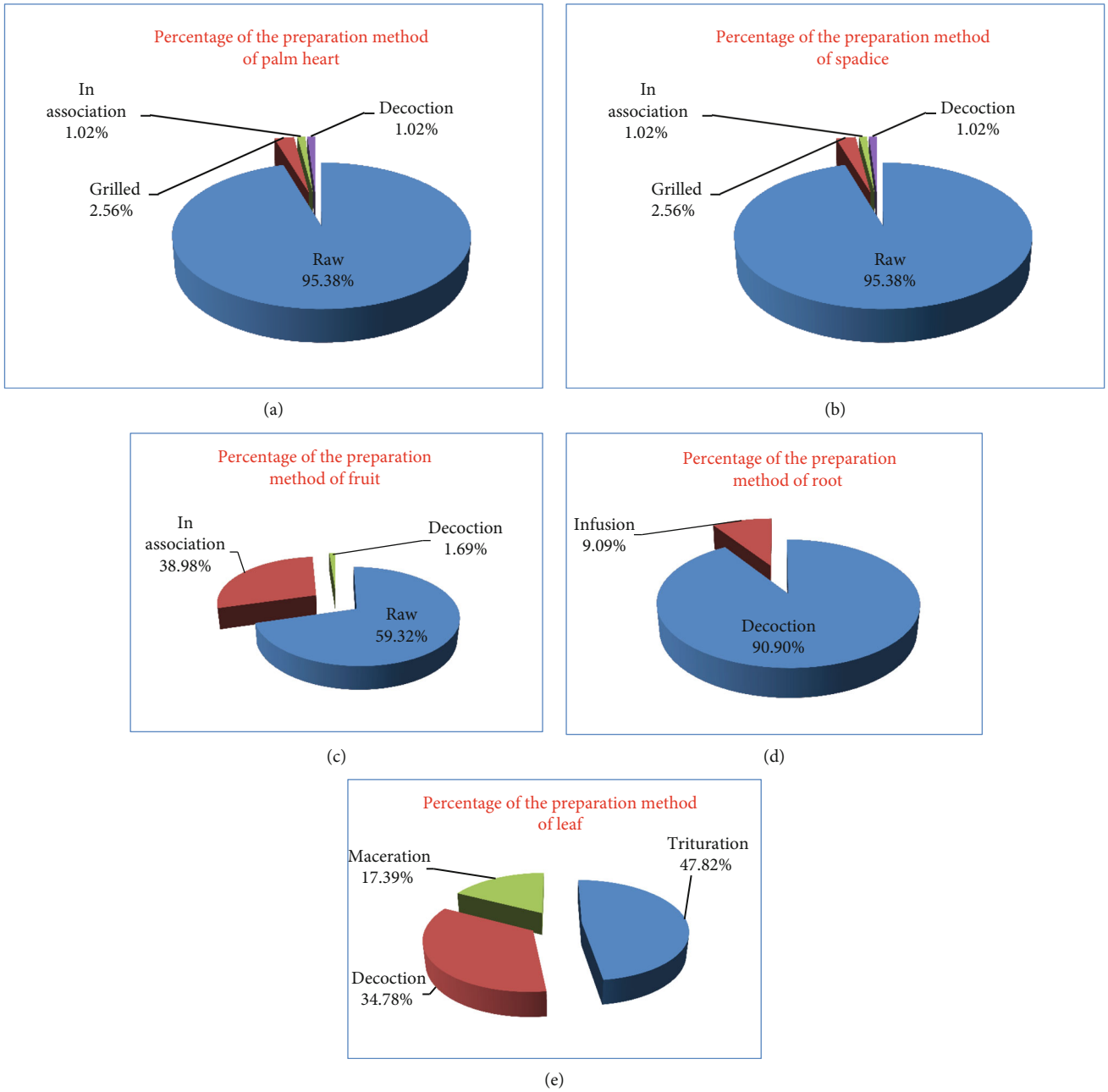


Figure 8: Percentage of preparation methods of different parts of *C. humilis* in the region of Taza, Morocco. (a) Heart of palm; (b) spadice; (c) fruits; (d) root; (e) leaf.

Table 2: The different uses of *C. humilis* in the region of Taza, Morocco.

Part of the plant	Type of application
Palm heart, spadice	Therapeutic
	Human alimentation
Fruit	Therapeutic
	Human alimentation
	Animal feed
	Magic
Leaves	Therapeutic
	Human alimentation
	Basketry dishwashing tool
	Magic
The entire plant	Firewood

Table 3: Mineral composition of *Chamaerops humilis* L. var. argentea leaves in mg/kg of plant material.

Minerals	Content in $mg \cdot kg^{-1}$ of dry matter
Iron (Fe)	82395.00
Potassium (K)	9354.90
Phosphorus (P)	1828.62
Magnesium (Mg)	1312.47
Sodium (Na)	627.03
Copper (Cu)	542.64
Calcium (Ca)	92.19
Zinc (Zn)	66.15
Selenium (Se)	3.00
Strontium (Sr)	3.00

Table 3 illustrates the results of the study of mineral contents of the leaves of *C. humilis*. The analysis of minerals showed that this plant contains significant contents of iron, potassium, phosphorus, and magnesium with values of 82395.00, 9354.9, 1828.62, and 1312.47 $mg \cdot kg^{-1}$, respectively. Sodium, copper, calcium, and zinc are present in average amounts with values of 627.03, 542.64, 92.19, and 66.15 $mg \cdot kg^{-1}$, respectively. Selenium and strontium are present with low levels of about 3.00 $mg \cdot kg^{-1}$ for each.

3.3. Phytochemical Study

3.3.1. Yields of Aqueous and Organic Extractions. Aqueous and organic extraction of *C. humilis* leaves resulted in the yields presented in Table 4.

From the results in Table 4, we can observe that organic extraction gives the slightly better yields compared to aqueous extraction and that hot extraction gives the best yields compared to cold extraction by maceration for both aqueous and organic extraction modalities.

For aqueous extracts, the decocted (10%) is richer in extractable matter followed by the infused (2.2%) and the macerated (1.2%). For organic extracts, the best yield was obtained by the most polar solvent and ethanol hot by Soxhlet (10.84%), followed by ethanolic macerated (3.18%), then chloroformic extract (1.245%), followed by chloroformic macerate (1.040%) and hexanic extract (0.820%), and lastly hexanic macerate (0.433%).

Table 4: Yields of aqueous and organic extractions obtained from the leaves of *C. humilis*.

	Extracts	Yield in %
Aqueous extracts	**Decocted**	**10.00**
	Infused	2.20
	Aqueous macerated	1.20
Organic extracts	**Ethanolic extract**	**10.84**
	Ethanolic macerated	3.18
	Chloroformic extract	1.24
	Chloroformic macerated	1.04
	Hexanic extract	0.82
	Hexanic macerated	0.43

3.3.2. Extraction by Hydrodistillation. The extraction by hydrodistillation of essential oils from the leaves of *Chamaerops humilis* var. argentea André gave only a few traces that are stuck to the internal surface of Clevenger. The extraction yield was not determined due to the small amount extracted.

3.3.3. Phytochemical Screening. Phytochemical screening was carried out on the powder and on the aqueous and organic extracts of the leaves of *C. humilis*. The tests carried out are aimed at highlighting the presence of chemical families: catechic tannins and gall tannins, flavonoids, saponins, alkaloids, sterols, anthracenosides, free quinones, and anthraquinones, and the results obtained are represented in Table 5.

The results of phytochemical screening carried out on the powder of plant material and different aqueous and organic extracts of the leaves of *C. humilis* are mentioned in Table 5. The powder of the leaves of *C. humilis* contains the catechic tannins, flavonoids, saponins, sterols, and free quinones. Concerning the aqueous extracts, the decocted and infused contain catechic tannins, flavonoids, and saponins and the decocted contains in addition the free quinones, whereas the macerated, cold-prepared extract contains only the catechic tannins and saponins.

For the organic extracts, ethanolic extract and ethanolic macerated contain catechic tannins, flavonoids, saponins, and free quinones; chloroformic extract and chloroformic macerate contain catechic tannins; hexanic extract and hexanic macerate contain catechic tannins and sterols. For the other families of secondary metabolites, gall tannins, alkaloids, anthracenosides, and anthraquinones are absent in both the leaf powder and in all the aqueous and organic extracts prepared from them (Table 5).

3.3.4. Phytochemical Dosage. The secondary metabolites to be assayed (total polyphenols, flavonoids, and catechic tannins)

Table 5: Chemical composition of the powder and aqueous and organic extracts of the leaves of *C. humilis*.

Leaf powder/aqueous and organic extracts	Catechic tannins	Gallic tannins	Flavonoids	Saponins	Alkaloids	Sterols	Anthracenosides	Free quinones	Anthraquinones
Powder of leaves	+++	−	+	+++	−	+++	−	+	−
Decocted	+++	−	++	+++	−	−	−	+	−
Infused	+++	−	+	+	−	−	−	−	−
Aqueous macerated	+++	−	—	+++	−	−	−	−	−
Ethanolic extract	+++	−	+	++	−	−	−	++	−
Ethanolic macerated	+++	−	++	+	−		−	++	−
Chloroformic extract	+	−	−	−	−	−	−	−	−
Chloroformic macerated	++	−	−	−	−	−	−	−	−
Hexanic extract	+	−	−	−	−	++	−	−	−
Hexanic macerated	+	−	−	−	−	+++	−	−	−

(+++): strong presence; (++): medium presence; (+): weak presence; (−): absence.

were selected based on the results of phytochemical screening of leaves of *C. humilis*. The results of the assay are presented in Table 6.

The results of quantitative analyses of phenolic compounds in aqueous and organic extracts of *C. humilis* leaves are reported in Table 6. The organic extracts contain high contents of total polyphenols, flavonoids, and catechic tannins compared to the aqueous extracts. For the aqueous extracts, the decocted has the highest content of total polyphenols of 13.23 ± 0.19 mg EAG/g E in comparison with the infused (2.11 ± 0.03) and macerated (2.02 ± 0.04) with a statistically significant difference.

For the organic extracts, we found that the total polyphenol contents of the different organic extracts varied between 20.8 ± 1.33 and 100.27 ± 0.66 mg EAG/g E. The highest concentration of phenols was measured in the ethanolic macerated, with a level of 100.27 ± 1.95 mg EAG/g E and the hot Soxhlet-prepared ethanolic extract (96.99 ± 0.82 mg EAG/g E) with a statistically insignificant difference, followed by the chloroformic and hexanic macerates, respectively, with a statistically insignificant difference and lastly the chloroformic and hot prepared hexanic extracts, with values of: 37.51 ± 1.66, 34.77 ± 1.19, 28.04 ± 0.43, and 20.8 ± 1.33 mg EAG/g E with a statistically significant difference.

3.3.5. Determination of Flavonoids. Concerning the aqueous extracts, we found that the decocted has the highest flavonoid content (56.36 ± 0.39 mg EQ/g E), followed by the infused (41.94 ± 0.09 mg EQ/g E) and lastly the aqueous macerate (4.48 ± 0.26 mg EQ/g E) with a statistically nonsignificant difference between the three extracts. For the organic extracts, the chloroformic macerate has high flavonoid contents with 542.7 ± 24.03 mg EQ/g E, followed by the ethanolic macerated (468.25 ± 9.07 mg EQ/g E) and the ethanolic extract (457.98 ± 5.18 mg EQ/g E) prepared by hot Soxhlet with a statistically significant difference. The chloroformic extract and the hexanic macerate present flavonoid contents of 356.87 ± 10.90 and 335.48 ± 10.67 mg EQ/g E, respectively, with a statistically nonsignificant difference and lastly the hexanic extract with a content of 273.25 ± 8.92 mg EQ/g E (Table 6).

3.3.6. Determination of Tannins. The results presented in Table 6 reveal that the decocted has a significant amount of catechic tannins (10.08 ± 0.07), followed by the infused (1.28 ± 0.03) and lastly the macerated (1.26 ± 0.04) with a statistically nonsignificant difference between the three aqueous extracts. For organic extracts, ethanolic macerated is highly rich in tannins with a value of 52.11 ± 0.24 mg EC/g E, with a statistically nonsignificant difference with ethanolic extract (50.27 ± 0.99 mg EC/g E), followed by chloroformic macerate, chloroformic extract with a nonsignificant difference, and lastly hexanic macerate and hexanic extract with a nonsignificant difference, with values of 39.05 ± 0.78, 35.72 ± 2.35, 30.16 ± 1.02, and 25.5 ± 0.51 mg EC/g E (Table 6), respectively.

3.3.7. Antioxidant Activity. Antioxidant activity was evaluated for aqueous and organic extracts of *C. humilis* leaves using H_2O_2, DPPH, ABTS, FRAP, and RP assays. The results obtained are presented in Table 7.

(1) Hydrogen Peroxide Scavenging Assay (H_2O_2). The results obtained revealed that all the extracts have a strong capacity to neutralize hydrogen peroxide clearly superior to the reference drug ascorbic acid (14.35 ± 0.002%) at a concentration of 100 μg/ml and the decocted (aqueous extract) proved to be the most active (45.77 ± 0.15%) compared to all the extracts tested and its activity is three times higher than that of ascorbic acid.

For aqueous extracts, in comparison with decocted (45.77 ± 0.15%), the infused came second (29.65 ± 0.31%) with statistically significant difference, followed by aqueous macerate (29.24 ± 0.62%) with statistically insignificant difference with the infused and significant with the decocted. The decocted presented a high hydrogen peroxide inhibition value compared to those of the organic extracts with a statistically significant difference. Regarding the organic extracts, ethanolic macerated showed a high hydrogen peroxide inhibition rate followed by hot methanolic extract with statistically insignificant difference, followed by chloroformic macerate and chloroformic extract with statistically insignificant difference;

Table 6: Total polyphenol, flavonoid, and catechic tannin contents of aqueous and organic extracts of *C. humilis* leaves.

	Extracts	Total polyphenols (μg GAE/mg E)	Flavonoids (μg QE/mg E)	Catechic tannins (μg CE/mg E)
Aqueous extracts	Decocted	**13.23 ± 0.19**^{a}	**56.36 ± 0.39**^{a}	**10.08 ± 0.07**^{a}
	Infused	2.11 ± 0.03^{b}	41.94 ± 0.09^{a}	1.28 ± 0.03^{a}
	Aqueous macerated	2.02 ± 0.04^{b}	4.48 ± 0.26^{a}	1.26 ± 0.04^{a}
Organic extracts	Ethanolic extract	**96.99 ± 0.82**^{c}	**457.98 ± 5.18**^{b}	**50.27 ± 0.99**^{b}
	Ethanolic macerated	**100.27 ± 0.66**^{c}	**468.25 ± 9.07**^{b}	**52.11 ± 0.24**^{b}
	Chloroformic extract	28.04 ± 0.43^{d}	**356.87 ± 10.90**^{c}	35.72 ± 2.35^{c,d}
	Chloroformic macerated	37.51 ± 1.66^{e}	542.70 ± 24.03^{d}	39.05 ± 0.78^{d}
	Hexane extract	20.80 ± 1.33^{f}	273.25 ± 8.92^{e}	25.50 ± 0.51^{e}
	Hexanic macerated	34.77 ± 1.19^{e}	335.48 ± 10.67^{c,e}	30.16 ± 1.02^{c,e}

All results presented are the mean of three individual replicates (= 3 ± SEM). Values with the same superscript letters in the same row are not significantly different ($p < 0.05$).

Table 7: Antioxidant activity of aqueous and organic extracts of *C. humilis* leaves.

Extracts/tests		H_2O_2 (%)	DPPH (IC_{50}) (μg/ml)	ABTS (mg TE/g E)	FRAP (mg TE/g E)	RP (mg AAE/g E)
Aqueous extracts	Decocted	**45.77 ± 0.15**^{a}	**6814.00 ± 0.08**^{a}	**14.44 ± 0.003**^{a}	**23.44 ± 0.07**^{a}	**2.46 ± 0.04**^{a}
	Infused	29.65 ± 0.31^{b}	10280.00 ± 0.04^{a}	2.49 ± 0.19^{a}	6.67 ± 0.017^{b}	**2.49 ± 0.01**^{a}
	Aqueous macerated	29.24 ± 0.62^{b}	9260.00 ± 0.39^{a}	2.46 ± 0.05^{a}	5.34 ± 0.14^{c}	0.31 ± 0.007^{a}
Organic extracts	Ethanolic extract	**36.80 ± 0.33**^{c}	**44.25 ± 2.31**^{a}	**78.21 ± 0.98**^{b}	**144.71 ± 0.35**^{d}	**10.14 ± 0.04**^{b}
	Ethanolic macerated	**37.34 ± 0.55**^{d,c}	**31.18 ± 0.66**^{b}	**108.28 ± 1.29**^{c}	**148.85 ± 0.43**^{e}	**10.86 ± 0.01**^{c}
	Chloroformic extract	34.83 ± 0.26^{e,c}	490.06 ± 0.54^{c}	30.10 ± 0.75^{d}	16.77 ± 0.69^{f}	1.14 ± 0.05^{d}
	Chloroformic macerated	35.79 ± 0.29^{f,c,d,e}	491.53 ± 2, 08^{d,b,c}	41.93 ± 0.62^{e}	17.21 ± 0.10^{g,f}	2.12 ± 0.05^{e}
	Hexanic extract	33.96 ± 0.17^{g,e,f}	975.83 ± 11.83^{e,b,c,d}	14.87 ± 2.31^{f}	9.53 ± 0.23^{h}	0.92 ± 0.07^{f,d}
	Hexanic macerated	21.59 ± 1.14^{h}	335.10 ± 0.5^{f,d,e}	30.15 ± 3.76^{g,d}	39.62 ± 0.08^{i}	1.41 ± 0.02^{g}
Reference standards	Ascorbic acid	14.35 ± 0.002^{i}	0.17 ± 0.02^{g,b,c,d,e,f}	—	—	—
	BHT	—	1.59 ± 0.13^{h}	—	—	—
	Trolox	—	1.75 ± 0.09^{i}	—	—	—

All results expressed are the mean of three individual replicates (= 3 ± SEM). Values with the same superscript letters in the same row are not significantly different ($p < 0.05$).

hexanic extract shows statistically insignificant difference with chloroformic macerate with inhibition percentages of 37.34 ± 0.55, 36.80 ± 0.33, 35.79 ± 0.29, 34.83 ± 0.26, and 33.96 ± 0.17%; the hexanic macerate comes last with 21.59 ± 1.14% and with a statistically significant difference with all the tested extracts (Table7).

(2) 2,2-Diphenyl-1-Picrylhydrazil Free Radical Scavenging Activity (DPPH). The lowest IC_{50} value corresponds to the highest extract efficiency. The DPPH free radical scavenging capacity is proportional to the increase in concentration of the tested samples. Both aqueous and organic extracts of *C. humilis* leaves show significant capacity to scavenge the DPPH free radical (Table7). For the aqueous extracts, the decocted shows the highest capacity to scavenge the free radical with an IC_{50} value of 6814 ± 0.08 μg/ml, followed by the macerate (9260 ± 0.39 μg/ml) and then the infused (10280 ± 0.04 μg/ml) with a statistically significant difference between the three extracts. The organic extracts have a significantly higher free radical capacity than the aqueous extracts with a statistically significant difference. Ethanolic macerated exhibited the best activity with an IC_{50} of 31.18 μg/ml ± 0.66 followed by hot ethanolic extract, hexanic macerate, hot chloroformic extract, chloroformic macerate with a statistically insignificant difference, and lastly hot hexanic extract which exhibits a statistically significant difference with the other organic extracts and the IC_{50} are 44.25 ± 2.31, 335.1 ± 0.5, 490.06 ± 0.54, 491.53 ± 2.08, and 975.83 ± 11.83 μg/ml).

From these results, it seems that via the DPPH test, all the extracts studied have an antioxidant activity but lower than that of the reference standards (BHT, Trolox, and ascorbic acid) with a statistically significant difference (Table 7).

(3) $ABTS^+$ Radical Scavenging Test. Aqueous extracts recorded a low antiradical potency towards the ABTS radical; decocted comes first with a value of 14.44 ± 0.003 mg TE/g E, followed by infused (2.49 ± 0.19 mg TE/g E) and aqueous macerated (2.46 ± 0.05 mg TE/g E) with a statistically nonsignificant difference between the three extracts. For the organic extracts studied, the best antiradical activity is recorded for ethanolic macerated (108.28 ± 1.29 mg TE/g E) and ethanolic extract (78.21 ± 0.98 mg TE/g E) with a statistically nonsignificant difference; chloroformic extract and chloroformic macerate present a scavenging activity of the $ABTS^+$ radical of 30.1 ± 0.75 and 41.93 ± 0.62 mg TE/g E, respectively, with a statistically significant difference. Hexanic extract and hexanic macerate showed considerable antiradical capacities of 14.87 ± 2.31 and 30.15 ± 3.76 mg TE/g E, respectively, with a statistically significant difference (Table 7).

(4) Ferric-Reducing Antioxidant Power Assay (FRAP). The antioxidant activity of an extract by the FRAP method corresponds to the capacity of this extract to reduce Fe^{3+} to ferrous ion (Fe^{2+}), which is reflected by the increase in absorbance at 593 nm. The results obtained show that the iron reduction capacity varies according to the extracts; for the aqueous extracts, the decocted has a reducing activity of 23.44 ± 0.07 mg TE/g E, followed by the infused (6.67 ± 0.017 mg TE/g E) and lastly the macerated (5.34 ± 0.14 mg TE/g E); the aqueous extracts present a statistically significant difference. In addition, the decocted showed a high capacity of reduction of ferric ions (Fe^{3+}) in comparison with three organic extracts: chloroformic extract, chloroformic macerate, and hexanic extract with a statistically significant difference.

As for the organic extracts, this reduction is much higher for ethanolic macerated (148.85 ± 0.43 mg TE/g E) and ethanolic extract (144.71 ± 0.35 mg TE/g E) with a statistically significant difference, followed by hexanic macerate, chloroformic macerate, chloroformic extract, and at the end hexanic extract, with values of 39.62 ± 0.08, 17.21 ± 0.10, 16.77 ± 0.69, and 9.53 ± 0.23 mg TE/g E, respectively; the organic extracts show a nonsignificant difference between them, except that the statistical difference is nonsignificant between chloroformic extract and chloroformic macerate (Table 7).

(5) Reducing Power Assay (RP). The reducing power of organic extracts of *C. humilis* leaves is much higher than that of aqueous extracts. From the results obtained, we found that for aqueous extracts, the infused has the highest reducing power (2.495 ± 0.01 mg EAA/g E) followed by the decocted (2.46 ± 0.04 mg EAA/g E) and aqueous macerated (0.31 ± 0.007 mg EAA/g E) with a statistically insignificant difference between the three extracts.

Regarding the organic extracts, the ethanolic macerated presents the highest iron reduction capacity (10.86 ± 0.01 mg EAA/g E), followed by the ethanolic extract (10.14 ± 0.04 mg EAA/g E) with a statistically significant difference; nevertheless, the chloroformic and hexanic macerates and the chloroformic extract and the hexanic extract present low reducing powers with values of (2.12 ± 0.05, 1.41 ± 0.02, 1.14 ± 0.05, and 0.92 ± 0.07 mg EAA/g E) with a statistically significant difference between chloroformic and hexanic macerates and a statistically nonsignificant difference between chloroformic and hexanic extracts (Table 7).

The decocted and infused showed higher reducing power than the extracts: chloroformic and hexanic prepared hot by Soxhlet and chloroformic and hexanic macerated with a statistically significant difference.

3.3.8. Antibacterial Activity

(1) Agar Diffusion Method. The disc diffusion method allowed highlighting the antibacterial power of the organic extracts of *C. humilis* on the selected strains which is translated by the appearance of inhibition zones. Extracts with inhibition zones greater than 15 mm are considered to possess strong antibacterial power; 8 to 15 mm are moderate, and inhibition zones of 1 to 8 mm represent weak antimicrobial activities [68]. The results are shown in Table 8.

The antibacterial activity of the organic extracts of the leaves of *C. humilis* towards 6 bacterial strains was tested by the diffusion method in agar medium. After 24 hours of incubation, the diameters of the inhibition zones around the disc were read and the results obtained are presented in Table 8.

Ethanolic extract showed moderate inhibitory activity against the following strains: *Proteus mirabilis* and *Listeria innocua* CECT 4030 with an inhibition diameters of 12 and 8.5 mm, respectively, and 8 mm was recorded for *Escherichia coli*, *Pseudomonas aeruginosa* CECT 118, *Staphylococcus aureus* CECT 976, and *Bacillus subtilis* DSM 6633 strains. The ethanolic macerated exhibited strong antimicrobial activity against *Proteus mirabilis* with an inhibition diameter of 16 mm at a concentration of 80 mg/ml and moderate inhibitory activity against the strains: *Listeria innocua* CECT 4030 and *Bacillus subtilis* DSM 6633 with an inhibition diameter of 14 mm at a concentration of 100 mg/ml; ethanolic macerated showed moderate activity against *Escherichia coli*, *Pseudomonas aeruginosa* CECT 118, and *Staphylococcus aureus* CECT 976 strains reflected by an inhibition diameter of 8 mm at concentration 100 mg/ml. Chloroformic extract showed moderate antimicrobial activity on *Proteus mirabilis* (10 mm), *Escherichia coli* (8 mm), *Pseudomonas aeruginosa* CECT 118 (8 mm), *Staphylococcus aureus* CECT 976 (8 mm), *Listeria innocua* CECT 4030 (8 mm), and *Bacillus subtilis* DSM 6633 (8 mm) at 100 mg/ml. Hexanic macerate showed moderate inhibitory power on the six strains tested: *Proteus mirabilis* (9 mm), *Listeria innocua* CECT 4030 (9 mm), *Pseudomonas aeruginosa* CECT 118 (8 mm), *Staphylococcus aureus* CECT 976 (8 mm), *Bacillus subtilis* DSM 6633 (8 mm), and *Escherichia coli* (7 mm) at 100 mg/ml. Chloroformic macerate and hexane extract showed no inhibition against the tested strains.

Table 8: Antibacterial activity of organic extracts from the leaves of *C. humilis*.

Extracts/bacterial strains	Ethanolic extract (mg/ml)			Ethanolic macerated (mg/ml)			Chloroformic extract (mg/ml)			Chloroformic macerated (mg/ml)			Hexanic extract (mg/ml)			Hexanic macerated (mg/ml)			Standard (−) (μg/ml)	Standard (+)
	40	**80**	**100**	**40**	**80**	**100**	**40**	**80**	**100**	**40**	**80**	**100**	**40**	**80**	**100**	**40**	**80**	**100**	**T/AK 20/30**	**DMSO (10%)**
	Inhibition zone (mm)																			
E. coli	—	7	8	—	7	8	—	7	8	—	—	—	—	—	—	—	7	7	12/T	—
P. m	7	**12**	**12**	11.5	**16**	**16**	7	9	10	—	—	—	—	—	—	8	9	9	22/T	—
P. a	—	7	8	—	7	8	—	7	8	—	—	—	—	—	—	—	7	8	27/AK	—
S. a	—	7	8	—	7	8	—	7	8	—	—	—	—	—	—	—	7	8	12/T	—
Lis	—	7	8.5	9	**14**	**14**	—	7	8	—	—	—	—	—	—	—	7	9	11/T	—
B. s	—	7	8	10.5	10	**14**	—	7	7.5	—	—	—	—	—	—	7	7	8	10/T	—

T: tetracycline; AK: amikacin; *E. coli*: *Escherichia coli*; *P. m*: *Proteus mirabilis*; *P. a*: *Pseudomonas aeruginosa* CECT 118; *S. a*: *Staphylococcus aureus* CECT 976; *B. s*: *Bacillus subtilis* DSM 6633; *Lis*: *Listeria innocua* CECT 4030; (−): absence of inhibition.

Table 9: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of organic extracts from the leaves of *C. humilis*.

Extracts/bacterial strains	Ethanolic extract			Ethanolic macerated			Chloroformic extract			Hexanic macerated		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
	mg/ml			mg/ml			mg/ml			mg/ml		
E. coli	*5*	ND	—	*2.5*	80	32	20	80	*4*	10	ND	—
P. m	20	40	**2**	10	40	*4*	10	80	8	10	40	*4*
P. a	10	40	*4*	*2.5*	40	16	10	80	8	5	ND	—
S. a	20	80	*4*	10	40	*4*	40	80	**2**	20	ND	—
Lis	*5*	80	16	*2.5*	40	16	*1.25*	40	32	*2.5*	80	32
B. s	40	ND	—	5	ND	—	5	ND	—	20	ND	—

E. coli: *Escherichia coli*; *P. m*: *Proteus mirabilis*; *P. a*: *Pseudomonas aeruginosa* CECT 118; *S. a*: *Staphylococcus aureus* CECT 976; *B. s*: *Bacillus subtilis* DSM 6633; *Lis*: *Listeria innocua* CECT 4030; MBC/MIC ≤ 4: bactericidal power; MBC/MIC > 4: bacteriostatic power; ND: not determined.

(2) Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Organic Extracts of C. humilis Leaves. The MIC and MBC were determined only for the most active extracts found during the evaluation of antibacterial activity by the disc diffusion method; these concentrations will allow to determine the nature of the antibacterial activity: bacteriostatic or bactericidal.

The results and ratios of MIC and MBC are mentioned in Table 9. The ethanolic extract showed a low MIC value in the range of 5 mg/ml for *Escherichia coli* and *Listeria innocua* CECT 4030; *Pseudomonas aeruginosa* CECT 118 had a MIC value of 10 mg/ml; *Proteus mirabilis* and *Staphylococcus aureus* CECT 976 showed a MIC value in the range of 20 mg/ml; the highest MIC was obtained by *Bacillus subtilis* of 40 mg/ml. For the ethanolic macerated, the lowest MIC was 2.5 mg/ml recorded for the strains: *Escherichia coli*, *Pseudomonas aeruginosa* CECT 118, and *Listeria innocua* CECT 4030. *Bacillus subtilis* strain DSM 6633 had a MIC value of 5 mg/ml; *Proteus mirabilis* and *Escherichia coli* presented a MIC value of 10 mg/ml. The chloroform extract had the lowest MIC value of 1.25 mg/ml for *Listeria innocua* CECT 4030, followed by an MIC value of 5 mg/ml for *Bacillus subtilis* DSM 6633 and 10 mg/ml for *Proteus mirabilis* and for *Pseudomonas aeruginosa* CECT 118. *Escherichia coli* and *Staphylococcus aureus CECT 976* had MIC values of 20 and 40 mg/ml, respectively. Regarding hexane macerate, the lowest MIC value was obtained with *Listeria innocua* CECT 4030 (2.5 mg/ml), followed by *Pseudomonas aeruginosa* CECT 118, *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus* CECT 976, and *Bacillus subtilis* DSM 6633 with MIC values of 5, 10, 10, 20, and 20 mg/ml, respectively.

According to Table 9, the ratios of MBC/MIC indicated are between 2 and 32 mg/ml; we can deduce that our extracts have both bactericidal and bacteriostatic power on the six tested strains.

The MBC/MIC ratio indicates that the ethanolic extract presents a bactericidal activity against *Proteus mirabilis*, *Pseudomonas aeruginosa* CECT 11, and *Staphylococcus aureus* CECT 976 with values of 2, 4, and 4 mg/ml, respectively; on the other hand, this extract presents a bacteriostatic activity against *Listeria innocua* CECT 4030 (16 mg/ml). The ethanolic macerated has a bactericidal power on two tested strains: *Proteus mirabilis* and *Staphylococcus aureus* CECT 976 with a value of 4 mg/ml and a bacteriostatic effect against *Pseudomonas aeruginosa* CECT 11 and *Listeria innocua* CECT 4030 with a value of 16 mg/ml, while the *Escherichia coli* strain presents a resistance towards this same extract.

The chloroformic extract also has a bactericidal effect against *Staphylococcus aureus* CECT 976 and *E. coli* with 2 and 4 mg/ml, respectively, and a bacteriostatic power against *Proteus mirabilis* and *Pseudomonas aeruginosa* CECT 118 of 8 mg/ml. Hexanic macerate has a bactericidal effect against *Proteus mirabilis* (4 mg/ml), but *Listeria innocua* CECT 4030 seems to be resistant to this extract (32 mg/ml).

The results of the study of the antibacterial activity of the extracts of the leaves of *C. humilis* showed that the extracts have antimicrobial activities of varying degrees against the tested strains.

3.4. Principal Component Analysis (PCA). The principal component analysis (PCA) allowed us to visualize the correlation between aqueous and organic extracts and their antioxidant capacity by the different tests used and the correlation between the activity of the extracts and their content in chemical compounds.

The PCA was carried out on nine individuals which are divided into three aqueous extracts (decocted, infused, and macerated) and six organic extracts (ethanolic extract, ethanolic macerated, chloroformic extract, chloroformic macerated, hexanic extract, and hexanic macerated) of the leaves of *C. humilis*, and this is in relation to eight variables represented, on the one hand, by the content of chemical compounds (total polyphenols, flavonoids, and catechic tannins) and, on the other hand, by the five tests used for the evaluation of the antioxidant activity: H_2O_2, DPPH, ABTS, FRAP, and PR.

3.4.1. Correlation Matrix. According to the principal component analysis (PCA), the two main axes (F1 and F2) describe 90.48% of the total variance of the observations. We can therefore conclude that the interpretations made from this PCA will be highly significant.

Table 10: Correlation matrix between phytochemical data (total polyphenols, flavonoids, and catechic tannin contents) and antioxidant activity via the five tests (H_2O_2, DPPH, ABTS, FRAP, and RP) of *C. humilis* leaves.

Variables	H_2O_2	DPPH	ABTS	FRAP	RP	Total polyphenols	Flavonoids	Catechic tannins
H_2O_2	**1**							
DPPH	0.0543	**1**						
ABTS	0.2503	**0.9214**	**1**					
FRAP	0.2240	0.8377	**0.9341**	**1**				
RP	0.3280	0.7581	**0.9104**	**0.9743**	**1**			
Total polyphenols	0.2142	**0.9418**	**0.9779**	**0.9644**	**0.9271**	**1**		
Flavonoids	0.0676	**0.8897**	0.7599	0.5584	0.4982	0.7559	**1**	
Catechic tannins	0.1619	**0.9753**	**0.8843**	0.7472	0.6812	**0.8924**	**0.9532**	**1**

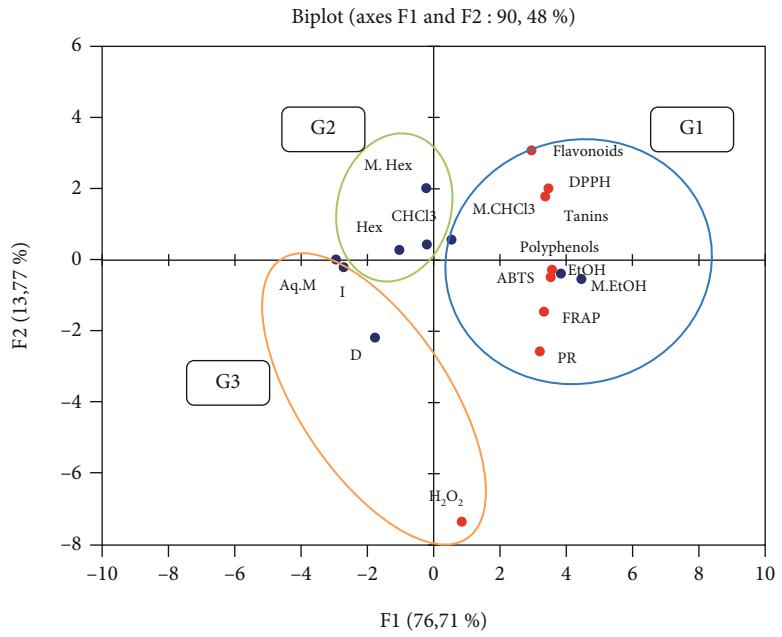


Figure 9: Projection of individuals on the factorial plane (F1 × F2). G1: group 1; G2: group 2; G3: group 3; D: decocted; I: infused; Aq. M: aqueous macerated; EtOH: ethanolic extract; M. EtOH: ethanolic macerate; $CHCL_3$: chloroformic extract; M. $CHCl_3$: chloroformic macerated; Hex: hexane extract; M. Hex: hexanic macerated.

PCA showed that the DPPH test is highly positively correlated with the ABTS test ($r^2 = 0.9214$) and the latter is highly positively correlated with the FRAP ($r^2 = 0.9341$) and RP ($r^2 = 0.9104$) tests. The FRAP test shows a strong positive correlation with the RP test ($r^2 = 0.9743$). Regarding chemical composition, the total polyphenol content is highly positively correlated with all tests except H_2O_2 (DPPH $r^2 = 0.9418$, ABTS $r^2 = 0.9779$, FRAP $r^2 = 0.9644$, and RP $r^2 = 0.9271$). The flavonoid content was also strongly positively correlated with the test (DPPH $r^2 = 0.8897$). In addition, catechic tannins are highly positively correlated with DPPH ($r^2 = 0.9753$) and ABTS ($r^2 = 0.8843$) tests. In addition, total polyphenols are highly positively correlated with the catechic tannin content ($r^2 = 0.8924$) (Table 10).

3.5. Graphical Representation of the Principal Component Analysis (PCA). Figure 9 shows the distribution of the 9 individuals (extracts) in three groups G1, G2, and G3:

Group 1 gathers the ethanolic extract and the ethanolic macerated and the chloroformic macerate; the first two extracts present the highest contents of total polyphenols and tannins; they also express a better antiradical activity by the DPPH and ABTS tests and a high reducing power by the FRAP and RP tests. Total polyphenols and tannins seem to play a crucial role in the antioxidant effect. Chloroformic macerate contains a high content of flavonoids and shows a relatively high antiradical activity with the DPPH test.

Group 2 contains chloroformic extract, hexanic extract, and hexanic macerate; the chloroformic extract has a medium content of tannins; moreover, the hexanic extract and the hexanic macerate are moderately rich in flavonoids. These extracts present low antioxidant activity compared to the other organic extracts.

Group 3 includes the aqueous extracts: decocted, infused, and macerated which express low contents of phenolic compounds and a reduced antioxidant activity with

all the tests except for the decocted which presents a notable activity via the test of H_2O_2 (Figure 9).

4. Discussion

4.1. Ethnopharmacological Survey. According to the results of the ethnopharmacological survey conducted on the species *C. humilis*, the average age of the respondents is 50.47 years; the evolution of the use of the plant in traditional medicine with age can be explained by the fact of the accumulation of experience and traditional phytotherapeutic knowledge with age. These results are similar to those advanced by Medjati in Algeria who found that the frequency of use of *C. humilis* in the medicinal field increases with the age of the respondents; people over 60 years old have the highest percentage of use (100%) [16].

For the percentage of use of the species in traditional medicine, it is very close between women (84.21%) and men (90.27%); our results corroborate with the ethnobotanical works carried out in Algeria on the same species with a very close percentage; it is 80% for women and 84.71% for men [16]. This is not the case for studies that have shown that ethnobotanical knowledge related to other species varies mostly with gender; the work conducted by Boulfia and his collaborators in the same area of the present study showed that men use medicinal plants more frequently than women [5, 6]. Another ethnobotanical study conducted in Morocco by Mehdioui and Kahouadji whose aim is at describing the different uses of medicinal plants in one of the communes of the province of Essaouira showed that women have more knowledge about medicinal species compared to men (53% versus 47%) [69]. In other ethnobotanical studies conducted in northern Morocco, the first was done to identify medicinal plants used in the treatment of diabetes and hypertension, among the population living in the forest of Izarène in northern Morocco, which revealed that it is women who use medicinal plants much more than men (74.36% against 25.64%) [70]; the second conducted in Mechra Bel Ksiri on medicinal plants used in traditional medicine in the treatment of urinary tract infections showed that women use medicinal plants much more (87%) than men (61.90) [71]. An ethnobotanical survey was conducted in the region of Rabat-Sale-Kenitra on the plants used for the treatment of chronic diseases and showed that women have a good knowledge of traditional medicine than men, with a frequency of 59.05% against 40.94% for men [72].

According to the data obtained, illiterate people use medicinal plants much more to treat their illnesses (65.27%), which could be explained by the high cost of treatments offered by modern medicine, which are the limits that can justify this recourse to traditional pharmacopoeia, also because illiterate people are the oldest and have much more information about medicinal plants, since this segment of the population of respondents holds much of the ancestral knowledge that is part of the oral tradition. Leonti assumes that illiterate societies generally transmit cultural knowledge orally in a vertical fashion [73]. Ribeiro and collaborators also found that 75% of respondents acquired their knowledge of plant use from family tradition in an ethnobotanical survey conducted in Brazil [74]. Similarly, the respondents stated that the therapeutic recommendations on the plant in question are made by their grandfathers and grandmothers, which means vertically.

The results obtained with people with a higher level of education can be explained by the fact that they are aware of the toxicity caused by the misuse of medicinal plants. Our results corroborate with other ethnobotanical surveys done at the national level such as an ethnobotanical study conducted in the High Moulouya which proved that the dominant users of traditional medicine are illiterate people with a percentage of 41% [75]. Our results are also in agreement with other studies and ethnobotanical surveys conducted in Algeria such as Medjati who found that there is a correlation between the level of study and the medicinal use of *Chamaerops humilis* between illiterate people, primary-level people, secondary-level people, and university-level people with percentages of 96.7%, 89.2%, 83%, and 58.9%, respectively, [16]. Other studies were conducted nationwide, such as that of Mehdioui and Kahouadji who showed that the vast majority of users of medicinal plants are illiterate, with a percentage of 66% followed by people with a primary level with 26%, while those with secondary and university levels of education use very little medicinal plants (secondary 7%, university 1%) [69]. Hmamouchi and his collaborators conducted a study on the traditional practices of using Moroccan medicinal plants in rheumatology; in this study, they found that there is a positive correlation between the level of study and the percentage of use of medicinal plants (illiterate 53%, secondary 19%, and university 4%) [76].

According to the results of the survey, we found that the names attributed to *C. humilis* and its different parts vary according to the dialect of the respondent (Arabic/Amazigh). In this sense, studies carried out in Morocco have reported that *C. humilis* is called "Dûm" in the region of Taounat [77]. In the region of Sidi Bennour, the whole plant is also called "Eddoum" and the fruit "Lghaz" [78]. In the Middle Atlas of Morocco, the palm heart has a different name; it is called "Jmakh" [79]. In Algeria, the whole plant takes a similar name to that in Morocco; it is called "Doum" [16, 80].

The heart of palm and spadices are the most used parts by the population of the region of Taza (79.49%). These results are consistent with those obtained by Medjati and collaborators Okkacha and collaborators who showed that the most used parts in Algeria are the heart of palm and spadices [16, 18].

The survey allowed us to list a number of chronic diseases treated by the heart of palm: digestive disorders, cardiovascular system disorders, respiratory system disorders, diabetes, hepatitis, hair loss, and weakening of the immune system. Our results are consistent with those of a survey conducted in the province of Taounate which showed that the fruits in decoction are used as a treatment for liver disorders, gallstones, and against diabetes [77]. Another study conducted in the Moroccan central plateau with the objective of describing the medicinal plants used in the treatment of dermatological conditions indicated that the fruits of *Chamaerops humilis* are used against digestive disorders [81].

In Algeria, according to a survey conducted by Medjati et al., the heart of palm and spadices are used against gastrointestinal diseases and the fruits as antiseptic, the roots are used in decoction against intestinal worms and as a treatment for anemia, they are also used in women for cleaning the uterus after childbirth, and the leaves are used against gastrointestinal attacks and hepatitis [16].

Okkacha and his collaborators also demonstrated from a survey conducted in Algeria that the heart of palm is used against bloating, gastric pain, and constipation, while spadices are used as a tonic; on the other hand, the therapeutic use of the fruits of *Chamaerops humilis* is not reported by the respondents; on the other hand, the leaves of *Chamaerops humilis* are used for the treatment of diabetes [18].

The heart of palm and spadices are generally consumed raw without preparation (95.38%); this could be explained by the abundance of this plant in the region; the heart of palm is considered the fruit of the season, and it is sold everywhere in the province of Taza, which is in agreement with the results obtained by two studies conducted in Algeria by Medjati et al. and by Okkacha and his collaborators who found that the heart palm is consumed raw in salad. The fruits are also used raw with a percentage of 59.32% or in association with pure honey or olive oil (38.98%) and weakly used in decoction (1.69%). This use can be explained by the synergic effect of this part with the honey or the olive oil (Figure 8). On the other hand, the decoction dominates as a mode of preparation for the roots (90.90%), the infusion mode is less used among the respondents with 9.09% (Figure 8), and the surveyed population thinks more about the decoction because it allows to warm the body and to disinfect the roots which are in direct contact with the soil to eliminate the toxic effect of some recipes. During our survey, we clearly found that only the leaves in the fresh state are used after grinding (47.82%), decoction (34.78%), and maceration (17.39%). For Okkacha and his collaborators, the leaves are used much more in maceration; in addition, Medjati et al. showed that the leaves are used in maceration or else in decoction [16, 18]. These same surveys did not indicate the use of one or more parts in combination with other substances.

C. humilis has several uses other than being medicinal: food for humans (heart of palm, spadice, and fruit), food for animals (leaves and fruit), handicraft use (leaves), and tool for dishes (leaves), and the whole plant is used as firewood. These results confirm the results of other work done on the same species; Guarrera and Savo conducted a study on the traditional use of wild food plants in Italy; they found that the bud of *Chamaerops humilis* was consumed as a salad in Sicily [82]. Other ethnobotanical studies conducted on *Chamaerops humilis* in Algeria by Okkacha and collaborators in 2011 found that heart of palm, spadice, and leaves are used for therapeutic purposes [18], while Medjati et al. showed that all parts of the plant are used therapeutically [16].

4.2. Mineralogical Composition of C. humilis Leaves. Mineralogical analysis shows that leaves of *C. humilis* constitute a resource of minerals, with a high content of iron, potassium, phosphorus, and magnesium, in addition to significant quantities of sodium, copper, and zinc. Calcium and strontium are present at low levels. Iron is a fundamental constituent for plants and a vital element of hemoglobin; it also plays an important role in DNA synthesis and electron transport. However, if the concentration of iron exceeds this limit, the ingestion of these plants can lead to the accumulation of iron in the human body [37]. In addition, the analyzed part has significant contents of elements K, Ca, Cu, and Zn which are responsible for insulin secretion by islet beta cells and are involved in the potentiation of insulin action [83]; this could be due to their antioxidant effect. The results also show that the studied part is rich in magnesium that is an essential mineral for the human body; it is necessary for the synthesis of ATP, proteins, bone structure, stabilization of membranes, and metabolism of blood sugar and also intervenes in the functioning of many enzymatic systems at the level of the human body. Sodium in turn is an important component in the regulation of osmotic balance of all intra/intercellular fluids [84]. Selenium in turn at low doses is capable of blocking free radical synthesis [85]. In addition, according to Kolachi and colleagues, selenium can reduce the incidence of cancer and associated mortality in humans [86]. According to Pilmane and colleagues, strontium has a dual action on bones: it is able to improve bone formation and slow bone resorption [87].

The leaves of *C. humilis* provide a source of minerals that is likely responsible for its biological activities; according to Grela and colleagues, the antioxidant activity was attributed to mineral components such as copper, manganese, and iron [88]. A study by Pandya found that low doses of trace minerals such as Cu and Zn inhibit the production of oxidative free radicals [85]. In addition, mineral imbalance would change the content of flavonoids considered as the proven antioxidant compound [89]. Our recent study conducted on *Haloxylon scoparium* had shown that the high antioxidant capacity of aqueous and organic extracts of this plant is correlated and explained by its richness in Fe (60909.00), K (27452.10), Mg (10059.90), P (1125.39), Na (1054.65), and Cu (438.93 mg/kg) [43].

Studies conducted on plants collected in the region of Taza, Morocco, have also revealed the richness of the said plants in mineral elements that are involved in their biological properties: *Juglans regia* has a high level of Fe (19849.8), K (3487.8), Mg (2631.03), and P (691.02 mg/kg), *Leopoldia comosa* is rich in Fe (33552), K (1843.14), P (756.36), Na (439.65), Cu (303.9), Mg (272.37), and Ca (20.55 mg/kg), and *Ajuga iva* has contents of 112.00 mg/l Fe, 44.071 mg/l K, and 16.572 mg/l Na [44, 90, 91].

4.3. Phytochemical Study of the Leaves of C. humilis

4.3.1. The Yield of the Extractions. The phytochemical study carried out on the leaves of *C. humilis* revealed that the best yield was obtained with the most polar solvents and that the yields of hot extractions are higher than those obtained cold. The yield of the extraction varies according to the plant species, the part used in the extraction, the period of harvest of the plant, the type of soil, the climate, the geographical position, the conditions of drying in particular the duration, the

form of the plant material (powder or fragments), the nature of the solvent used in the extraction and its polarity, and the conditions of extraction (temperature, duration of extraction, and ratio solvent/plant material).

According to Mylonaki and collaborators, the concentration of ethanol and the duration of extraction influence the extraction yield of *Olea europaea* [92]. Similarly, Wu and colleagues found that temperature, extraction time, and solvent to plant material ratio affect the yield of *Zizyphus jujuba* [93].

Studies conducted in our laboratory under the same experimental conditions as the present study show that the part of the plant, the choice of solvent, the extraction modality (aqueous or organic), and the temperature (hot or cold extraction by maceration) influence the extraction yields, the contents of secondary metabolites of the extracts, and consequently their biological activities. Indeed, Bouabid and her collaborators reported that the best yields are obtained with polar solvents and hot extraction gives the best yields compared to cold extractions of the underground part of *Atractylis gummifera* [46, 58]. Senhaji and her collaborators found that the aqueous macerate and the methanolic extract prepared hot from the aerial part of *Ajuga iva* have the highest yield of 13.31 and 9%, respectively. Similarly, the aqueous macerate and methanolic extract of the aerial part of *Anabasis aretioides* show the best yield of 3.41 and 3.39%, respectively, [47, 48]. Boulfia and his collaborators found that ethanolic extract and decocted of *Juglans regia* have the highest yields of 22.04 and 14%, respectively [49]. In addition, the highest yield was obtained by the decocted prepared from *Leopoldia comosa* [44]. Another study recently conducted on *Haloxylon scoparium* showed that the two extracts were prepared under heat and with the most polar solvents (water and methanol): the decocted and the methanolic extract show the highest yields of 16, 8, and 14.35% [43].

4.3.2. Extraction by Hydrodistillation. The extraction by hydrodistillation of essential oils from the leaves of *Chamaerops humilis* var. argentea André has given only a few traces whose calculation of the yield has been infeasible. Works were carried out on the same species with the technique of hydrodistillation but in different geographical locations whose results obtained are also variable. Indeed, Okkacha and his collaborators in Algeria reported that extraction of essential oils from *Chamaerops humilis* leaves yielded only trace amounts of essential oil, while in Morocco, Khoudali and his collaborators found that extraction of essential oils from *Chamaerops humilis* leaves produced 0.19% [15, 94].

Several factors can affect the extraction of essential oils in the same species including the season and area of harvest, the stage of growth, the method of extraction, and the condition of the plant material spawned or dried. According to Ranjitha and Vijiyalakshmi, among all the extraction methods, the supercritical carbon dioxide method is the most effective method for extraction of essential oils [95].

4.3.3. Phytochemical Screening. Phytochemical screening carried out on both the leaf powder and the aqueous and organic extracts prepared from them showed that the plant material of the studied part contains sterols, saponins, flavonoids, free quinones, and catechic tannins. The ethanolic extract, ethanolic macerated, decocted, and infused possess flavonoids, saponins, quinones, and catechic tannins. Sterols are present only in the hexanic extract and the hexanic macerate. Alkaloids are absent in all extracts and in the powder of the studied plant part.

The complete study of the phytochemical screening highlights the presence of chemical compounds with interesting biological activities. The presence of tannins, endowed with tissue renewal properties, is powerful in the healing process of wounds due to diabetes, flavonoids possess antioxidant properties, and they could be used to prevent atherosclerosis [96]. The therapeutic effects of the leaves of *C. humilis* against digestive disorders in cattle are produced by different chemical compounds which are sterols, total polyphenols, flavonoids, catechic tannins, and quinones. The presence of these compounds in this part would justify its use in the traditional pharmacopoeia.

The phytochemical study reported by Benmehdi and collaborators showed that the aqueous, methanolic, and diethyl ether extracts of saw palmetto leaves contained the gall tannins, while the catechic tannins were absent in all the extracts studied [17]; another phytochemical study done on the methanolic extract of the leaves showed the presence of tannins, flavonoids, quinones, and saponins [97]. Khoudali and his collaborators found that the methanolic extract of *Chamaerops humilis* leaves contains both gallic and catechic tannins [15].

Under the same operating conditions, a work conducted in our SNAMOPEQ on plants from the same region as *C. humilis* states that phytochemical screening revealed the presence of catechic tannins, saponins, and sterols in the aerial part of *Anabasis aretioides* [47]; the underground part of *Atractylis gummifera* is rich in catechic tannins and flavonoids [46]. *Juglans regia* is rich in catechic tannins, flavonoids, anthraquinones, and free quinones; *Leopoldia comosa* has catechic tannins, flavonoids, and free quinones [44, 49]. The aerial part of *Ajuga iva* contains catechic tannins, flavonoids, saponins, and sterols [48].

4.3.4. Determination of Total Polyphenols, Flavonoids, and Catechic Tannins Contents. Phenolic compounds are more abundant in the organic extracts compared to the aqueous extracts. For the latter, the decocted presents the highest content of total polyphenols (13.23 ± 0.19 mg EAG/g E), flavonoids (56.36 ± 0.39 mg EQ/g E), and tannins (10.08 ± 0.07 mg EC/g E). These results show that high temperature allows a better extraction of total polyphenols, flavonoids, and catechic tannins. For organic extracts, ethanolic macerated and ethanolic extracts were richer in total polyphenols (100.27 ± 0.66; 96.99 ± 0.82 mg EAG/g E) and tannins (52.11 ± 0.24; 50.27 ± 0.99 mg EC/g E) with a nonsignificant difference. For flavonoids, chloroformic macerate contains the highest flavonoid content (542.7 ± 24.03 mg EQ/g E), followed by ethanolic macerated which also has high flavonoid content with a significant difference with chloroformic macerate. From these results, it is deduced that the phenolic content in the extracts examined

depends on the polarity of the solvent used and the method of extraction; Dary and collaborators conducted an extraction optimization study of three bioactive alkaloids: palmatine, roemerine, and tetrahydropalmatine from *Stephania cambodica* tuber; they found that microwave-assisted extraction (MAE) was more efficient than ultrasonic-assisted extraction (UAE) for the extraction of tetrahydropalmatine but UAE increased the yield of palmatine and roemerine. In addition, the liquid-to-solid ratio, percentage of solvent, and extraction time also influenced the extraction of these three alkaloids [98].

A study made in Algeria by Benahmed and collaborators revealed that the cold-prepared methanolic macerated of *Chamaerops humilis* leaves presents a value of 26.8 ± 0.41 mg/g in polyphenols and 40.7 mg EC/g E in flavonoids; these values are still low compared to the content of total polyphenols (100.27 ± 0.66 mg EAG/g E) and flavonoids (468.25 ± 9.07 mg EQ/g E) obtained in our study. Similarly, for a study conducted in Morocco, Khoudali and colleagues quantified polyphenols (99.8 mg EAG/g E) and flavonoids (3.70 mg EQ/g E) at the level of methanolic extract of leaves of the same species [15]. These values remain low compared to those of the ethanolic and ethanolic macerated extract determined in our present study. This quantitative difference is due to several factors related to the variety; in our study, we worked on the variety argentea, part of the plant, place, and stage of harvest of the plant and also to the experimental conditions that we adopted for our study: choice of extraction solvents, modalities of lime and cold, and aqueous and organic extractions. Studies carried out on plants from the region of Taza, Morocco, under the same experimental conditions by Senhaji and his collaborators confirm these findings. Indeed, the decocted and aqueous macerate of the aerial part of *Anabasis aretioides* present high contents of total polyphenols (1.78 ± 0.003; 0.92 ± 0.03 mg EAG/g E); moreover, ethyl acetate is the recommended solvent for the extraction of total polyphenols [47]. Another study conducted on the underground part of *Atractylis gummifera* revealed that the methanolic macerated is richer in total polyphenols (102.88 ± 1.38 mg EAG/g E), flavonoids (17.25 ± 0.06 mg ER/g E), and tannins (144.09 ± 3.96 mg EC/g E) [58]. According to Senhaji and collaborators, decoction is the mode that allows high extraction of total polyphenols (3.75 ± 0.02 EAG/g E), flavonoids (22.40 ± 0.36 mg ER/g E), and tannins (15.49 ± 0.17 mg EC/g E) from the aerial part of *Ajuga iva* [48]. Boulfia and collaborators found that the acetone macerate of *Juglans regia* contained the highest content of phenolic compounds (327.972 ± 0.06 μg EAG/mg E), flavonoids (1267.981 ± 2.911 μg EQ/mg E), and catechic tannins (38.056 ± 1.886 μg EC/g E). In addition, the diethyl ether extract prepared from *Leopoldia comosa* bulb contains high content of total polyphenols (129.75 ± 0.29 μg EAG/mg E), flavonoids (988.26 ± 0.18 μg EQ/mg E), and tannins (30.22 ± 0.15 μg EC/g E) [44, 49]. Our recent study done on *Haloxylon scoparium* revealed that the methanolic extract is rich in total polyphenols (161.65 ± 1.52 μg EAG/mg E) and catechic tannins are abundant in the ethyl acetate extract (23.69 ± 0.6 μg EC/mg E); the decocted also has a high flavonoid content (306.59 ± 4.35 μg EQ/mg E) [43].

The catechic tannins contained at the level of medicinal plants also present therapeutic virtues. According to Zahoui et al., the catechic tannins present at the level of the leaves of *Combretum micranthum* give it a powerful diuretic power [99] and anti-inflammatory properties, thanks to its astringent character; the tannins can contribute to the treatment of acute diarrhea [100], which confirms the use of the leaves of *C. humilis* to treat problems of the digestive system. The existence of sterols in the studied plant part confirms its use in traditional medicine as an antimicrobial agent. According to a study done by Geethalakshmi and Sarada, sterols isolated from *Trianthema decandra* L. possess antimicrobial activity *in vitro* against Gram-positive and Gram-negative bacteria [101].

4.3.5. Antioxidant Activity. In this study, we noticed that the decocted has the highest antioxidant capacity for DPPH, ABTS, H_2O_2, and FRAP tests in comparison with the infused and aqueous macerated. This same extract has a higher scavenging capacity than the reference standard: ascorbic acid (14.35 ± 0.002) in the H_2O_2 test. For the reducing power (RP) test, we notice that the infused and decocted extracts have a better reducing power with a statistically nonsignificant difference between these two aqueous extracts. The evaluation of the antiradical and antioxidant activities of the organic extracts reveals that the ethanolic macerated presents the highest activity for the five tests used: DPPH (CI 50 = 31.18 ± 0.66 μg/ml), ABTS (108.28 ± 1.29 mg ET/g E), H_2O_2 (37.34 ± 0.55%), FRAP (148.85 ± 0.4323 mg ET/g E), and RP (10.86 ± 0.01 mg EAA/g E). In addition, the ethanolic macerated shows a significantly higher percentage of inhibition than the reference standard for the H_2O_2 test. This extract also shows a statistically insignificant difference with the ethanolic extract prepared at hot Soxhlet for both H_2O_2 (36.51 ± 0.33%) and DPPH (IC 50 = 44.25 ± 2.31 μg/ml) tests and with the chloroformic macerate for the H_2O_2 test (35.79 ± 0.29%).

These results show that the leaves of *C. humilis* are endowed with a remarkable antioxidant power, which could explain the use of this part in phytotherapy to solve digestive problems. Indeed, numerous scientific researches have pointed out the link between reactive oxygen species (ROS) and digestive pathologies; free radicals are involved in many human gastrointestinal diseases such as gastritis, hepatitis (e.g., infectious and toxic etiologies), inflammatory bowel diseases, and digestive cancers [102, 103].

In comparison within the same species and the same studied part, our results of the DPPH antiradical activity are not very close to those obtained in Morocco by Khoudali and his collaborators, on the methanolic extract with an IC_{50} = 24.5 μg/ml. On the other hand, our ethanolic extracts show a higher capacity to trap the radical than those obtained by Benahmed and collaborators for the methanolic extract (IC_{50} = 180.71 μg/ml) prepared from Algerian *Chamaerops humilis* leaves and by Gonçalves and collaborators on the methanolic macerated of *Chamaerops humilis* leaves harvested in Portugal (IC_{50} = 346 μg/ml) [15, 97, 104]. This variation is due to the geographical area, harvesting season, preparation form, and temperature and extraction time adopted for each study.

The reducing power of *C. humilis* is probably due to the existence of the hydroxyl group in phenolic compounds that can be like an electron donor. Because of this, antioxidants are considered to be reducers and inactivators of oxidants [105]. Gonçalves and collaborators tested the reducing power of methanolic extract of *Chamaerops humilis* leaves harvested from Algare, a region in the south of Portugal; they found that they present a value of 434.34 ± 13.71 μmol/g E [104]. Under the same operative conditions as in our study, a phytochemical study conducted by Bouabid and collaborators showed that the methanolic macerate of the underground part of *Atractylis gummifera* L. presents the most expressed antioxidant activity for the H_2O_2 test (19.24 ± 1.102%), ABTS (122.6 ± 0.63 mg TE/g E), FRAP (102.5 ± 1.66 mg TE/g E), and RP (96.15 ± 1.12 mg EAA/g E) [58]. In addition, Senhaji and her collaborators conducted a phytochemical study on the aerial part of *Ajuga iva* subsp.; they found that the decocted had a strong reducing power compared to the infused and aqueous macerate for FRAP and RP tests [91]. Another phytochemical study conducted on the aerial part of *Anabasis aretioides* showed that the methanolic macerated exhibited the highest antioxidant activity for all four assays: DPPH (IC_{50} = 52.91 ± 0.24 μg/ml), ABTS (48.99 ± 1.316 μg TE/mg E), FRAP (99.73 ± 3.570 μg TE/mg E), and RP (72.176 ± 0.540 μg AAE/mg E) [47]. Boulfia and his collaborators found that the best antioxidant activity of *Juglans regia* bark was obtained with the acetone macerate which was found to be the most active according to the five different tests: H_2O_2 (24.13 ± 1.81%), DPPH (IC_{50} = 5.573 μg/ml), ABTS (602.29 ± 0.34 μg ET/mg E), FRAP (759.11 ± 0.27 μg TE/mg E), and RP (685.68 ± 0.82 μg AAE/mg E); the diethyl ether extract of *Leopoldia comosa* also showed antioxidant power via the same tests: H_2O_2 (62.67 ± 0.06%), DPPH (IC_{50} = 10.08 ± 0.01 μg/ml), ABTS (381.63 ± 0.63 μg ET/mg E), FRAP (394.77 ± 0.74 μg TE/mg E), and RP (356.7 ± 0.92 μg AAE/mg E) [44, 49]. Evaluation of the antioxidant activity of the methanolic extract of the aerial part of *Haloxylon scoparium* showed a high antioxidant capacity compared to the other extracts tested: H_2O_2 (20.91 ± 0.27%), DPPH (IC_{50} = 39.63 ± 2.03 μg/ml), ABTS (50.75 ± 0.72 μg ET/mg E), FRAP (163.37 ± 1.52 μg TE/mg E), and RP (116.18 ± 8.19 ± 0.82 μg AAE/mg E) [43].

The results obtained in the present study confirm the existence of a certain correlation between the content of phenolic compounds and the antiradical and antioxidant activities. In fact, the extract and ethanolic macerated prepared hot by Soxhlet and cold by maceration showed high contents of total polyphenols and were found to be the extracts with the highest antifree radical and antioxidant activity in the five tests used. Indeed, the antioxidant activity may be due to the inhibition of radical formation or the scavenging of the formed radicals [106]. Furthermore, mineral elements could also be responsible for the antioxidant capacity of *Chamaerops humilis*; a study conducted on *Phoenix dactylifera* fruits showed a strong correlation between antioxidant activity and mineral composition; the K content was strongly correlated with the FRAP test (r^2 = 0.800) and with the H_2O_2 test (r^2 = 0.889) [107]. From our mineralogical analysis, we found that *C. humilis* leaves are rich in potassium (9354.90 mg·kg^{-1} dry matter) which might be responsible for its antioxidant power. In addition, several works have announced the contribution of minerals as antioxidant elements. For Grela and collaborators, the antioxidant activity is due to mineral compounds such as copper, manganese, and iron [88].

4.3.6. Antibacterial Activity. The results of the study of the antibacterial activity of the extracts of the leaves of *C. humilis* show that the extracts have antimicrobial activities of varying degrees against the tested strains with inhibition diameters from 7 to 16 mm. The ethanolic macerated shows the highest inhibition against *Proteus mirabilis* with an inhibition diameter of 16 mm at 80 mg/ml and medium inhibition against *Bacillus subtilis* DSM 6633 and *Listeria innocua* CECT 4030 with a zone of 14 mm at 100 mg/ml. This is in agreement with the high amount of polyphenols contained in this extract (100.27 ± 1.95 mg EAG/g E) and tannins (52.11 ± 1.02 mg EC/g E).

Ethanolic and chloroformic extracts showed moderate inhibition against *Proteus mirabilis* of 11 mm and 10 mm, respectively, at 100 mg/ml. The ethanolic extract contains the saponins and coumarins, compounds with antimicrobial action; a work by Abd El-Fattah and collaborators confirmed the antibacterial effect of synthetic coumarin derivatives against *B. Subtilis* (28 mm), *P. Aeruginosa* (22 mm), and *E. Coli* (24 mm) [108]. In addition, Dong and collaborators reported that saponin "-O-β-D-glucopyranosyl-(1→3)-α-larabinopyranosyl-phytolaccagenic acid-27-oxo-28-O-β-D-glucopyranosyl" extracted from *Chenopodium quinoa* Willd had an inhibitory effect on the growth of *S. aureus* (11.70 mm) [109].

Hexanic macerate had a moderate inhibitory effect against *Proteus mirabilis*, *Listeria innocua* CECT 4030, and *Bacillus subtilis* DSM 6633 with a zone of 9, 9, and 8 mm, respectively, at 100 mg/ml. From phytochemical screening, we found that this extract contains sterols which are probably responsible for this antimicrobial action. A study conducted by Geethalakshmi and Sarada shows that sterols isolated from the leaves of *T. decandra* had a growth inhibitory action against *Staphylococcus aureus* MTCC 29213 (20 mm), *Escherichia coli* MTCC 443 (23 mm), *Pseudomonas aeruginosa* MTCC 1035 (23 mm), and *Bacillus subtilis* MTCC 121 (22 mm) [101]. In addition, Nazarparvar and collaborators showed that n-butanol extract of *Nigella sativa* is rich in terpenoids and exhibited antimicrobial effect against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Yersinia enterocolitica* [110].

Based on the results reported in Table 9, the MBC/MIC ratio ranged from 2 to 32 mg/ml. The MBC/MIC ratio indicates that the ethanolic macerate had bactericidal power on two strains tested: *Proteus mirabilis* and *Staphylococcus aureus* CECT 976, and a bacteriostatic effect against *Pseudomonas aeruginosa* CECT 11 and *Listeria innocua* CECT 4030. The ethanolic extract showed bactericidal activity against *Proteus mirabilis*, *Pseudomonas aeruginosa* CECT 11, and *Staphylococcus aureus* CECT 976. According to Armbruster and collaborators, *Proteus mirabilis* is capable of causing various human infections, especially those of the gastrointestinal tract [111]; this explains the traditional use

of *Chamaerops humilis* leaves by the population for the treatment of digestive system problems in livestock. This extract also exhibits bacteriostatic activity against *Listeria innocua* CECT 4030. The chloroformic extract prepared by hot Soxhlet also shows bactericidal effect against *Staphylococcus aureus* strain CECT 976 and *E. Coli* and a bacteriostatic power against *Pseudomonas aeruginosa* CECT 118 and *Proteus mirabilis*.

Under the same operating conditions as in the present study, the chloroform extract of the aerial part of *Anabasis aretioides* tested in our laboratory gave identical MIC value for *Pseudomonas aeruginosa*, *Staphylococcus aureus* CECT976, and *Bacillus subtilis* DSM 6633 strains in the range of 100 mg/ml and 50 mg/ml for *Escherichia coli* K12 and *Proteus mirabilis* [47]. Lamchouri and her collaborators reported that ethyl acetate extract of the aerial part of *Haloxylon scoparium* exhibits moderate antibacterial activity (7–12 mm) against *Staphylococcus aureus* strain [112]. In the present study, hexane macerate of *Chamaerops humilis* shows bactericidal effect against *Proteus mirabilis*; on the other hand, *Listeria innocua* CECT 4030 seems to be resistant towards this extract. This activity could be explained by the nature of the antimicrobial molecules extracted and soluble in the hexanic macerate. Indeed, the phytochemical screening mentioned the presence of sterols which may be responsible for the inhibition of *Proteus mirabilis*. In this sense, Singh and collaborators found that the highest antibacterial capacity was observed for sterols in *E. hirta* fruits (21 mm) [113]. In addition, studies conducted on plants from Taza region reported that *Ajuga iva* had the highest inhibition diameter for petroleum ether extract against *Proteus mirabilis* strain (14 mm) [91]; *Juglans regia* acetone macerate was found to be the most active with an inhibition diameter of 17 and 18 mm against *Proteus mirabilis* and *Pseudomonas aeruginosa* [90].

4.4. Principal Component Analysis (PCA). According to the principal component analysis, the DPPH, ABTS, FRAP, and RP tests used for the evaluation of antioxidant activity are highly correlated with each other: DPPH with ABTS ($r^2 = 0.92$), ABTS with FRAP ($r^2 = 0.93$), ABTS with RP ($r^2 = 0.91$), FRAP with RP ($r^2 = 0.97$), and RP with ABTS ($r^2 = 0.91$). Indeed, these correlations show that our extracts possess both a better scavenging power towards free radicals demonstrated by the tests (DPPH and ABTS) and also show a better Fe(III)-reducing capacity via the tests (FRAP, RP). This probably shows the presence in our extracts of antioxidant molecules that can intervene by two types of reaction mechanisms. The reactions involved may act according to the test used; for the FRAP and RP tests, it is a reduction of Fe (III), based on an electron transfer. Concerning the DPPH and ABTS tests, these two radicals can be neutralized either by direct reduction via electron transfer or by trapping the radical via a hydrogen atom transfer [114]. Regarding the H_2O_2 assay, it shows the lowest correlation to the other assays which could be explained by the nature of each oxidizing radical and its mechanism of action on antioxidant compounds; according to Winterbourn, hydrogen peroxide is a strong oxidant with two electrons but much of its two-electron oxidation reactions are too slow to be biologically interesting [115].

The content of polyphenols presents a high correlation with the results of the antioxidant activity obtained by the four tests: (ABTS $r^2 = 0.97$), (FRAP $r^2 = 0.96$), (DPPH $r^2 = 0.94$), and (RP $r^2 = 0.92$). Thus, the antioxidant activity of our extracts can be attributed to polyphenols which would be endowed with antioxidant effects. Indeed, according to the study conducted by Marimoutou and collaborators on medicinal plants *A. borbonica* and *D. apetalum* and *G. mauritiana*, extracts of the plants rich in polyphenols decreased the production of ROS and the secretion of the proinflammatory markers IL-6 and MCP-1 induced by the mediators H_2O_2, TNFα, and LPS. Such a protective action was associated with increased gene expression of the antioxidant enzyme superoxide dismutase and decreased mRNA levels of the proinflammatory transcription factor NF-κB [116]. Flavonoids also show high correlation with DPPH ($r^2 = 0.88$) and ABTS ($r^2 = 0.75$) tests, which means that flavonoid compounds in our extracts possess much more antiradical power; in this sense, several researchers have reported the antioxidant effect of flavonoids [117, 118].

In addition, catechic tannins had a high correlation with DPPH ($r^2 = 0.97$) and ABTS ($r^2 = 0.88$), which indicates that tannins contained in the studied extracts are able to scavenge free radicals either by electron transfer or by hydrogen atom transfer, this is in agreement with what is announced in the literature by Okuda that tannins intervene by the antiradical mechanism for scavenging the DPPH free radical [119].

Through the results of the PCA, we note that polyphenols present a stronger correlation with tannins ($r^2 = 0.89$) than flavonoids ($r^2 = 0.75$); these results indicate that the polyphenols contained in the extracts of the leaves of *C. humilis* are mainly constituted by catechic tannins; these last ones could be responsible of the astringent taste of the plant. According to Gombau and colleagues, condensed and hydrolyzable tannins contribute to the astringency of wine [120].

Work conducted with other plants from the Taza region and reported by Boulfia and collaborators reveals that phenolic compounds, flavonoids, and tannins are strongly correlated with the antioxidant activity of *Juglans regia* and *Leopoldia comosa* [44, 49]. In addition, Senhaji and her collaborators demonstrated a good correlation between the total polyphenol content and DPPH, ABTS, FRAP, and RP tests performed on *Anabasis aretioides*. A positive correlation was also found between the polyphenol contents of *Ajuga iva* and the antioxidant capacity assessed by the ABTS test [47, 48].

5. Conclusion

The ethnopharmacological survey conducted among the population of the province of Taza has allowed us to know the frequent uses of different parts of the plant *C. humilis* by the local population to cure many diseases and ailments. According to the results of the survey, the diseases of the digestive system are the most treated by the different parts of the plant in question.

According to the mineralogical analysis, the leaves of *C. humilis* present an excellent source of minerals with therapeutic and pharmacological properties including Fe, K, P, Mg, Na, Cu, Ca, and Zn.

The quantitative analysis carried out revealed that the aqueous and organic extracts of the plant have highly elevated contents of total polyphenols, flavonoids, and tannins. All these compounds are endowed with a great therapeutic power.

The evaluation of the antioxidant activity of the aqueous and organic extracts of the leaves of *C. humilis* determined by five methods (DPPH, ABTS, H_2O_2, FRAP, and PR) showed that this part of the plant possesses potent antioxidant and antiradical activities, probably attributable to the chemical and mineral compounds that it contains.

The principal component analysis showed the presence of a strong positive correlation, on the one hand, between the four tests used for the study of the antioxidant activity of ABTS, DPPH, FRAP, and RP and, on the other hand, between these tests and the composition in total polyphenols, flavonoids, and tannins of the tested aqueous and organic extracts.

Ethanolic macerated had a strong inhibition against *Proteus mirabilis* (16 mm); ethanolic extract and ethanolic macerated had a bactericidal effect against *Proteus mirabilis*; these same extracts have a bactericidal effect against *Staphylococcus aureus* CECT 976 with 4 mg/ml each.

We believe and hope that this work can contribute to the improvement of traditional medicine practices and to better guide the use of this plant. In addition, the present study could open up new work perspectives, consisting on the one hand an in-depth study on the leaves of *C. humilis* by isolation, purification, and determination of the structures of the active compounds and subsequently conducting *in vivo* investigations based on the *in vitro* study of this part. On the other hand and based on the results of the ethnopharmacological survey, it would be interesting to study the pharmacological properties of the different parts of *C. humilis* used in traditional phytotherapy and also to carry out a correlation study between their chemical composition and their biological activities.

The ethnopharmacological survey is also a valuable source of data and information on the traditional use of *C. humilis* in several sectors, namely, the therapeutic and food sector for humans and animals, and also in the handicraft sector based on the dwarf palm leaves.

Abbreviations

ICP-AES: Inductively coupled plasma atomic emission spectrometry
C. humilis: *Chamaerops humilis* L. var. argentea Andre
Fe: Iron
K: Potassium
P: Phosphorus
Mg: Magnesium
Na: Sodium
Cu: Copper
Ca: Calcium
Zn: Zinc
Se: Selenium
Sr: Strontium
$AlCl_3$: Aluminum chloride
GAE: Gallic acid equivalent
QE: Quercetin equivalent
CE: Catechin equivalent
H_2O_2: Hydrogen peroxide
DPPH: 1,1-Diphenyl-2-picrylhydrazyl
ABTS: 2,2-Azino-bis-3-ethylbenzothiazolin-6-sulfonic acid
FRAP: Ferric-reducing ability of plasma
RP: Iron-reducing power
TE: Trolox equivalent
AAE: Ascorbic acid equivalent
BHT: Butylated hydroxytoluene
ANOVA: Analysis of variance
PCA: Principal component analysis
GA: Gallic acid
Q: Quercetin
IC_{50}: Concentration that causes 50% inhibition
DMSO: Dimethyl sulfoxide
CFU: Colony-forming unit
T: Tetracycline
AK: Amikacin
E. coli: *Escherichia coli*
Pm: *Proteus mirabilis*
Pa: *Pseudomonas aeruginosa* CECT 118
Sa: *Staphylococcus aureus* CECT 976
Bs: *Bacillus subtilis* DSM 6633
Lis: *Listeria innocua* CECT 4030
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MIC: Inhibitory concentrations
MBC: Minimum bactericidal concentrations.

Data Availability

The survey data was collected using an anonymous questionnaire; the datasets used and analyzed are included in the article in the form of tables and figures.

Conflicts of Interest

The authors declare no conflict of interest associated with this publication, and there has been no financial support for this work that could have influenced its outcome.

Authors' Contributions

Nacima Lachkar (NL) did the formal analysis and writing. Fatima Lamchouri (FL) did the formal analysis, data retention, writing, editing, and revision. Hamid Toufik (HT) did the guidance, writing, and revision. All the authors approved the final manuscript.

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Research Article

Determination of Phenolic Compounds and Bioactive Potential of Plum (*Prunus salicina*) Peel Extract Obtained by Ultrasound-Assisted Extraction

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Ultrasound-assisted extraction (UAE) of bioactive compounds from black plum peels was optimized by response surface methodology (RSM). Temperature (35-55°C), time (15-45 min), and ethanol concentration (50-90%) were selected as independent extraction parameters, whereas total anthocyanin content (TAC), total phenolic content (TPC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition were kept as response variables. The optimized extraction conditions were determined by RSM as extraction at 49°C for 37 min with 68% ethanol, which corresponded to TAC, TPC, and DPPH inhibition values of 5.42 ± 0.61 mg/g, 6.217 ± 0.76 mg GAE/g, and 89 ± 2.13%, respectively. Fourier-transform infrared spectrometer (FTIR), high-performance liquid chromatography (HPLC), and gas chromatography mass spectrometer (GCMS) were used for chemical characterization of optimized plum peel extract (PPE). Optimized PPE was further evaluated for antibacterial, antioxidant, anticancer, and food preservation potential. PPE showed 92.31% DPPH inhibition with IC_{50} value of 360.6 μg/ml. Optimized PPE extract was effective in the inhibition of cancer cell proliferation and migration, and IC_{50} values were in the range 1.85-3.96 mg/ml for different human cancer cell lines. Major phenolics identified in PPE were ferulic acid (47.87 mg/kg), sinapic acid (9.15 mg/kg), quercetin (7.44 mg/kg), gallic acid (3.24 mg/kg), m-coumaric acid (2.59 mg/kg), and vanillic acid (1.12 mg/kg). PPE extract inhibited the growth of various foodborne bacterial pathogens and increased the shelf life of PPE coated fresh grapes. PPE due to antibacterial, anticancer, antioxidant, and food preservation potential can be used in developing functional food and pharmaceutical products.

1. Introduction

Plum (*Prunus salicina*) is a popular fruit and shares the family Rosaceae with peach and apricot. Plum contains a broad variety of flavors (from sour to sweet) and colors (black, yellow, red, and purple) and its peels are comprised of approximately 10-20% of the total weight of plum fruit [1]. Plums are processed either by freezing, canning, juicing, or drying [2]. However, drying is the oldest and most common way of plum processing [3]. A significant amount of plum peel remains as a byproduct after drying and processing of plum, and due to the presence of bioactive compounds, plum peels can be used to develop nutraceuticals and functional food products [4, 5].

Phenolic compounds are well-known bioactive molecules for their health promoting ability and effectiveness

against various diseases [6]. Black plum peel (BPP) has wide variety of bioactive compounds such as phenolic acids, anthocyanins, carotenoids, flavanols, and various other aromatic compounds [7, 8]. Plums have a special attraction due to their color, taste, and nutritional value. Anthocyanins are a type of naturally occurring phenolic chemicals that give fruits their color [9]. Most of the anthocyanins are present in plum peel [10], which are responsible for their deep color [11]. Anthocyanins are known for their high antioxidant potential which makes them vital for human health. [12]. Plum peels are rich source of anthocyanins and various phenolic compounds (ferulic acid, chlorogenic acid, kaempferol, and p-hydroxybenzoic acid) which are associated with various health benefits such as antioxidant, anticancer, and antimicrobial potential [13, 14].

Free radicals are produced during metabolism, and their excessive production can result in multiple diseases of brain and bones [15]. Antioxidants protect cells by free radical scavenging and convert them into nonradical species [16]. Bioactive compounds from plant sources can be used as natural antioxidants due to their free radical scavenging capacity and safety in comparison to synthetic antioxidants [17]. Due to high content of bioactive compounds, plum fruit exhibits strong antioxidant, anticancer, antihyperglycemic, antihypertensive, and laxative properties, and its consumption has been linked to better cognitive function and bone health [10, 18]. Food preservation systems such as edible coatings can be developed based on natural preservatives from plants in combination with other edible polymers. Chitosan is nontoxic polysaccharide with good film forming capacity and can be used to develop biodegradable edible coatings [19].

Due to sensitive nature, extraction of bioactive compound from plant sources has always been a major concern. Conventional extraction techniques are time and solvent intensive and require a substantial amount of energy [20]. Ultrasonic-assisted extraction (UAE) is an innovative, green, and rapidly evolving technology that has a significant advantage over conventional extraction methods, including better extraction yield, less time, and solvent consumption [21, 22]. Due to variety of health benefits associated with fruit peels, the bioactive compounds extracted from peels can be used in the formulation of food preservation systems, nutraceutical, pharmaceutical, and medicinal products. In this study, UAE was used to extract bioactive compounds from plum peels and evaluated for their antioxidant, antimicrobial, and anticancer potential.

2. Materials and Methods

Black plum (*Prunus Salicina* L.) fruit samples were purchased from the local market of Sialkot city of Pakistan. The plums were washed with tap water and peeled manually. The peels were dried in hot air oven at 45°C for 72 h followed by mechanical grinding (Philips Co. Ltd., China). The powder plum peels were stored in zip lock bags covered with aluminum foil at 4°C until further use.

2.1. Optimization of Bioactive Compound Extraction. UAE of plum peels was optimized by RSM using the Design-Expert® software (Minneapolis, MN, USA) at a fixed frequency (20 kHz) and sample to solvent ratio (1 g/20 ml). Temperature (35, 45, and 55°C), time (15, 30, and 45 min), and ethanol concentration (50, 70, and 90%) were used as independent extraction variables. The different concentrations of ethanol were prepared by diluting ethanol with 0.1 N HCl (*v*/*v*). The powdered peels were placed in a beaker containing ethanol and subjected to ultrasonic processor (LSP-500, Industrial Sonomechanics, USA). Total phenolic content (TPC), total anthocyanin content (TAC), and DPPH radical scavenging activity were selected as the response variables.

2.2. Determination of TPC, TAC, and Antioxidant Activity. TPC of plum peel extract (PPE) was estimated by Folin-Ciocalteu reagent (Sigma-Aldrich, USA) as described by Sadiq et al. [17]. Gallic acid was used to develop a standard curve, and results were expressed as mg of gallic acid equivalent (GAE) per gram of sample. Antioxidant activity of PPE was evaluated by using standard DPPH inhibition assay [23].

TAC of PPE extract was estimated by pH differential method as described by Lee et al. [24] with slight modifications. PPE (1 ml) was added to 0.025 M potassium chloride buffer (9 ml, pH 1); similarly, PPE (1 ml) was added to 0.4 M sodium acetate buffer (9 ml, pH 4.5). The absorbance of resulting solutions was recorded within 20-50 min by UV-Visible spectrophotometer (Aurius 2000 series, Cecil Instruments, England) at 700 nm and 520 nm against blank. TAC was estimated by using equation (1) and expressed as mg of cyanindin-3-glucoside equivalent/g of sample.

$$\text{TAC (mg/g)} = \frac{\text{Abs} \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times L}, \quad (1)$$

where absorbance (Abs) $= [(A_{520} - A_{700})_{\text{pH 1.0}}] - [(A_{520} - A_{700})_{\text{pH 4.5}}]$, ε (cyanidin − 3 − glucoside molar absorbance) = 26,900 L mol^{-1} cm^{-1}, L is the path length of the cell (1 cm), MW (molecular weight of anthocyanins) = 449.2 D, and DF is the dilution factor.

2.3. Bioactive Potential of Optimized PPE

2.3.1. Antioxidant Activity. The optimized extraction conditions (49°C, 37 min, and 68% ethanol) were determined by using desirability function of Design-Expert. PPE obtained by using optimized extraction conditions was lyophilized (Christ Alpha 1-2 LDplus, Germany) and further evaluated for DPPH inhibition potential at different PPE concentrations (8000-31.25 μg/ml). For antioxidant potential, IC_{50} value of PPE was determined by nonlinear regression using GraphPad Prism® version 7 (San Diego, US).

2.3.2. Anticancer Activity. The anticancer activity of PPE was evaluated by using MTT cell proliferation assay and wound healing assay as described by Nelson et al. [25].

(1) Cell Culture. Four human cancer cell lines, DLD-1 (colon cancer), HCT-116 (colon cancer), MDA-MB-231 (breast cancer), and PC3 (prostate cancer) were cultured using DMEM cell culture medium supplemented with 10% heat

inactivated fetal bovine serum and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml). All cell culture media and reagents were purchased from Gibco, Thermo Fisher Scientific, USA. Cells were maintained in cell culture flasks with filtered caps for air exchange and kept at 37°C with 5% CO_2 in an incubator. Cells were maintained below confluence by passaging every two to three days.

(2) MTT Cell Proliferation Assay. MTT (methylthiazolyldiphenyl-tetrazolium bromide) cytotoxicity assay was used to assess the growth inhibition of human cancer cell lines in the presence of PPE. Vacuum dried samples of PPE were dissolved in DMEM at the final concentration of 20 mg/ml, and the solution was sterilized by passing through 0.22 μm syringe filter. For the assay, 8000 DLD-1 and HCT 116 and 4000 MDA-MB-231 and PC3 cells were seeded in 96-well flat bottom cell culture plates. Cells were grown in the final volume of 100 μl of complete DMEM in the presence of varying concentrations of PPE (10 μg/ml to 5 mg/ml) or doxorubicin as positive control. After 72 h of incubation, 10 μl of 5 mg/ml sterile solution of MTT was added in each well, and cells were further incubated for 4 h. Media was removed from wells, and 100 μl of isopropanol containing 0.04 N HCl was added in each well to dissolve the formazan crystals. Assay plates were read at 492 nm, and after subtracting background absorbance, data was normalized considering untreated wells as zero percent growth inhibition. Cell survival was plotted against log of PPE concentration, and by nonlinear regression curve fitting of normalized data, IC_{50} values were calculated using the GraphPad Prism software.

(3) Wound Healing Assay. MDA-MB-231 and PC3 cells were seeded in 6-well cell culture plates at 1×10^5 cells per well. Both cell lines were grown for a day to obtain confluent monolayers which were wounded by scratching with a sterile 200 μl tip. Cells were washed twice with serum free DMEM media to remove detached cells. Scratch wounds were allowed to heal in reduced serum DMEM medium (2.5% FBS) in the presence of different concentrations of PPE. Wound gaps were imaged using inverted phase contrast microscope at 0, 12, and 24 h. Wound healing was quantified by measuring wound areas using the ImageJ software. Percentage healing was calculated by normalizing wound areas after healing to areas at the start of experiment, presented as percentage of untreated controls.

2.4. Antibacterial Activity. Antibacterial activity of PPE was evaluated against *Escherichia coli* (ATCC# 8739), *Staphylococcus aureus* (ATCC# 25923), and *Salmonella typhimurium* (ATCC# 14028) by agar well diffusion method [26]. Each inoculum was adjusted to 0.5 McFarland standard and applied on the surface of nutrient agar (Oxoid, UK) plate using sterilized cotton swab. With sterilized cork borer, 10 mm wells were made in each plate, and 100 μl from different concentrations (100, 50, 25, and 12.5 mg/ml) of PPE was added into each well. The plates were incubated at 37°C for 24 h, and diameter of inhibition zone was recorded.

2.5. Food Preservation Potential of PPE. Preservation potential of PPE was evaluated in fresh grapes. Two different concentrations of PPE (25 mg/ml and 50 mg/ml) were added into 1% (*w*/*v*) chitosan (CS) solution and emulsified by the addition of glycerol (1%, *v*/*v*). The grapes were artificially inoculated with the microbial suspension of *S. aureus* (10^4 CFU/ml) for 90 s followed by draining. The grapes were kept at room temperature for 1 h to aid bacterial adherence and dipped into CS-PPE coatings for 1 min. The grapes were kept at 25°C, and microbiological analysis was performed daily for 5 days by following Mehmood et al. [27]. At each time interval, grapes were homogenized in peptone saline solution (0.1%, *w*/*v*), and total colonies were counted and reported as CFU/g of grapes.

2.6. FTIR Analysis of Optimized PPE. PPE extract was analyzed by FTIR spectrometer (Agilent Technologies, USA) in absorption mode (4000-650 cm^{-1}) with a resolution of 4 cm^{-1}.

2.7. Gas Chromatography Mass Spectrometer (GCMS) Analysis. PPE extract was analyzed by GC-MS system (GC-7890A/MS-5975C, Agilent Technologies, Santa Clara, CA, USA) with HP-5 MS capillary column. Sample injection was maintained at 200°C, and helium was used as mobile phase (1 ml/min). All the data were acquired by collecting the mass spectra in the range of 50–600 a.m.u.

2.8. High Performance Liquid Chromatography (HPLC) Analysis. Gradient HPLC (LC-10AT, SHIMADZU, JAPAN) was used for the separation of PPE components by using shim pack CLC-ODS (C-18), 25 cm × 4.6 mm, 5 μm column. Gradient mobile phase was used for separation of phenolic compounds: A (H_2O: acetic acid, 94 : 6; pH = 2.2) and B (acetonitrile 100%) (0-15 min = 15% B, 15-30 min = 45% B, 30-45 min = 100% B). UV–Visible detector (Shimadzu, SPD-10AV) was monitored at 280 nm, and phenolics were identified by comparing retention time and UV-Visible spectra with standards [28].

2.9. Statistical Analysis. Tukey's HSD test was used to determine significant differences ($p < 0.05$) among treatments by using SPSS statistical software package (SPSS, version 22.0, USA).

3. Results and Discussion

3.1. Optimized Extraction of Bioactive Compound. UAE of black plum peel was optimized by Box-Behnken design, and effects of independent extraction parameters (time, temperature, and solvent concentration) on response variables (TPC, TAC, and DPPH inhibition) are summarized in Table 1. UAE of plum peel was carried by different set of extraction conditions, and TAC, TPC, and DPPH inhibitions were observed in the ranges of 1.62-6.22 mg/g, 2.23-7.38 mg GAE/g, and 52-94%, respectively. The result indicated that the optimal extraction was at 45°C, 30 min, and 70% ethanol concentration which corresponded to TAC, TPC, and DPPH inhibition values of 5.78 mg/g, 5.53 mg GAE/g, and 94%, respectively. Traore et al. [29]

Table 1: Optimization of extraction of bioactive compounds from black plum peels.

Exp. no.	Independent variables			Response variables		
	Temperature (°C)	Time (min)	Solvent (%)	TAC (mg/g)	TPC (mg GAE/g)	DPPH inhibition (%)
1	35	30	50	4.69	5.88	73.03
2	55	30	90	3.94	2.48	84.92
3	45	45	90	3.51	2.41	89.31
4	45	30	70	5.78	5.53	94.0
5	45	15	90	3.27	3.79	70.86
6	45	30	70	4.94	6.01	93.06
7	45	15	50	1.62	3.57	86.41
8	35	30	90	5.03	2.23	84.58
9	35	15	70	4.13	2.67	91.71
10	55	15	70	5.37	5.90	92.62
11	55	45	70	4.89	6.30	93.0
12	45	30	70	5.27	6.15	84.68
13	45	45	50	6.22	7.38	52.59
14	55	30	50	5.76	6.092	63.98
15	35	45	70	4.94	4.375	88.74
16	45	30	70	2.05	6.63	88.44
17	45	30	70	4.19	5.025	91.98

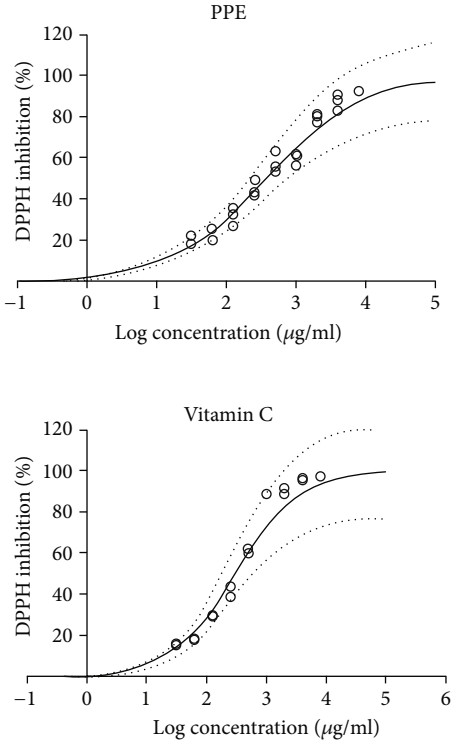


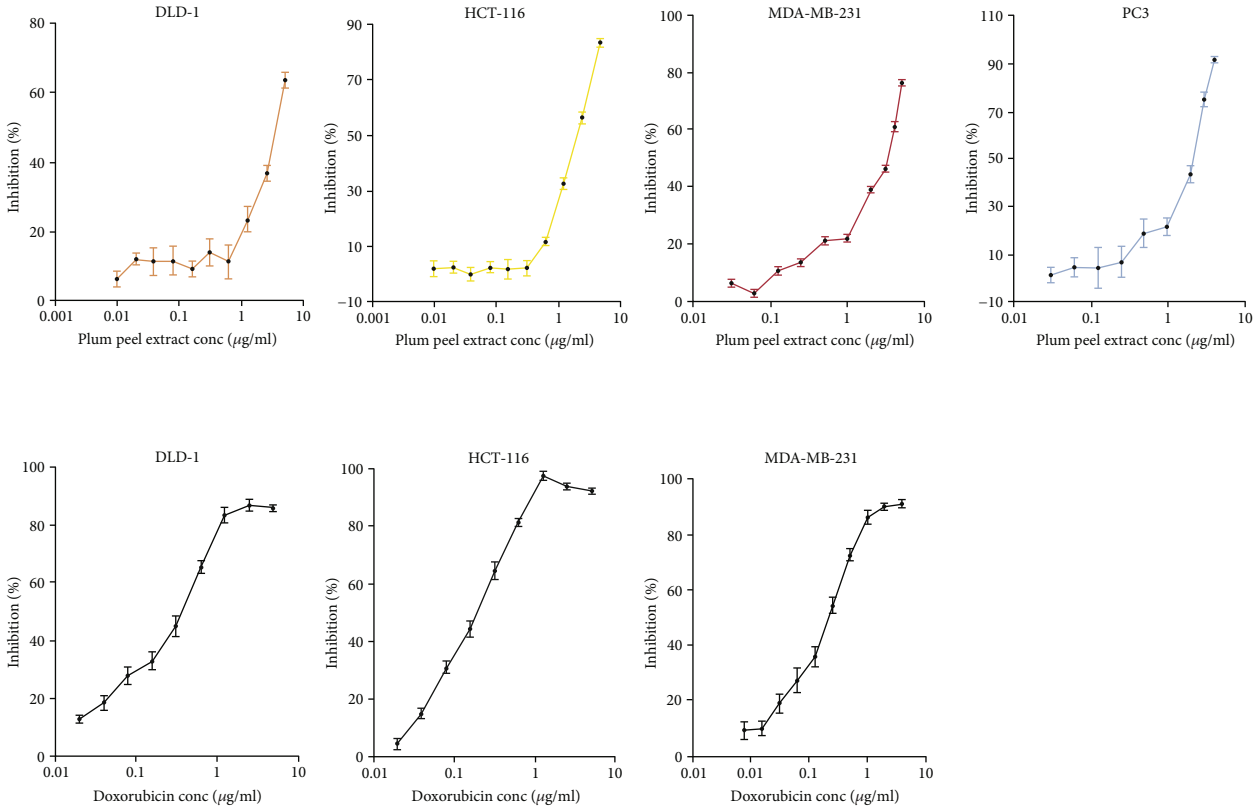
Figure 1: DPPH inhibition (%) of plum peel extract and vitamin C at different concentrations, using nonlinear regression.

reported that black plum peel contained significantly high TPC (383.03 mg GAE/100 g) than the flesh of plum (202.51 mg GAE/100 g). Anthocyanin content of plum varies with the variety, maturation stage, and environmental conditions. Wang et al. [30] reported TAC of content in Myrobalan plum peel in the range of 1.93-1986 mg/g of peel, which was similar to the findings of this study.

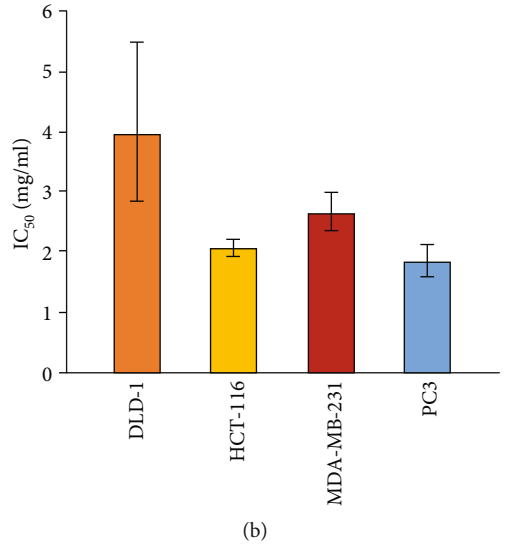
UAE is gaining considerable interest as a sustainable, green, and suitable technique for extraction of bioactive compounds [31]. Ethanol as extraction solvent is a viable option for extraction of natural and bioactive compounds due to its low cost and environment friendly nature [32]. The dilution of ethanol with water increases the polarity of extraction solvent, and acid addition facilitates to dissolve plant membranes, resulting in efficient extraction of target components [33].

The influence of extraction parameters on response variables was evaluated by quadratic model, and the model was well fitted to the data which was confirmed by the R^2 (coefficient of determination) values of 0.829, 0.887, and 0.946 for TAC, TPC, and DPPH inhibitions, respectively. Lack of fit was insignificant which indicated that data was well fitted. All the extraction parameters influence the response variables; however, the nature of influence was only significant for ethanol concentration which significantly ($p < 0.05$) influences all the response variables (Table S1). Previous studies reported an increase in TPC and antioxidant activity by increasing ethanol concentration and extraction time; however, after an optimum level, further increase in solvent concentration and extraction time was associated with decrease in the TPC and antioxidant activity of natural extracts [23, 34].

The extraction of peel sample was finally carried out at optimized extraction conditions (49°C, 37 min, and 68% ethanol), determined by using desirability function of Design-Expert, and experimental values of 5.42 ± 0.61 mg/g, 6.217 ± 0.76 mg GAE/g, and 89 ± 2.13% were obtained for TAC, TPC, and DPPH inhibitions, respectively.

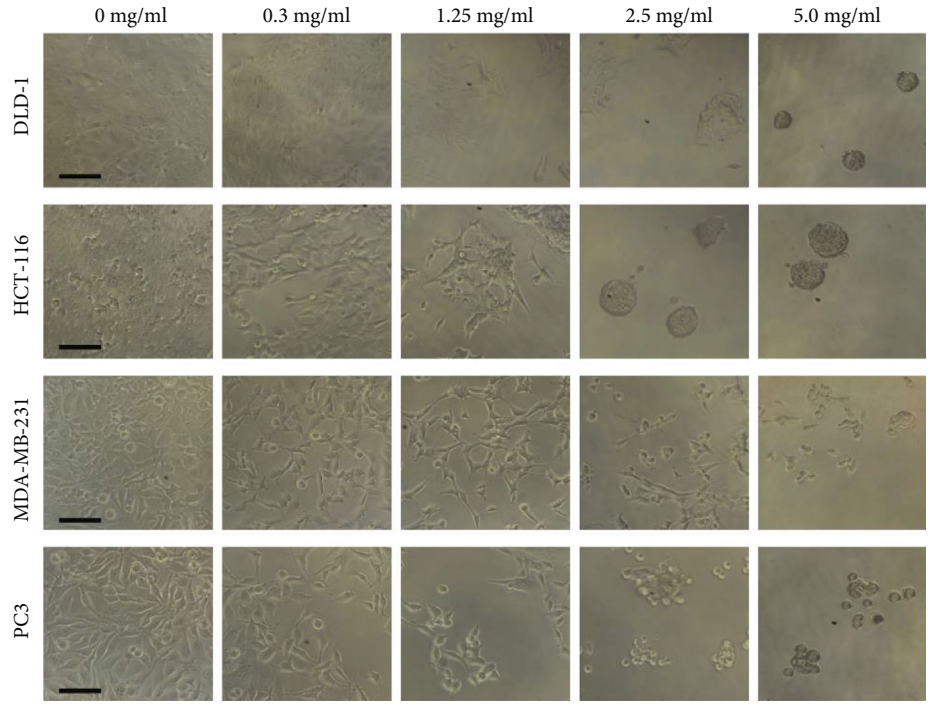


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: Cytotoxic effects of plum peel extract (PPE) on cancer cell lines in vitro. (a) Results of MTT cytotoxicity assay showing increase in growth inhibition of DLD-1, HCT-116, MDA-MB-231, and PC3 cells with increasing concentration of PPE. Doxorubicin was used as a positive control. Error bars show standard error of mean of four wells. (b) IC_{50} of PPE, values were obtained by nonlinear regression curve fitting of normalized cell survival; error bars represent 95% confidence interval. (c) Phase contrast microscopy, images were obtained after 72 hours of treatment with PPE; scale bar represents 100 μm.

3.2. Antioxidant Activity of PPE. Antioxidant activity of PPE increased with increase in concentration and at highest test concentration (8000 μg/ml), 92.31 ± 0.27% DPPH inhibition was observed; however, at the same concentration, vitamin C resulted in 97.44 ± 0.21% DPPH inhibition (Figure 1 and Table S2). IC_{50} values representing the concentration of test compound required for 50% DPPH inhibition were estimated by nonlinear regression and determined as 360.6 μg/ml for PPE and 298 μg/ml for vitamin C. Traore et al. [29] reported that the antioxidant activity of black plum peel was almost 3 times higher than the plum flesh and IC_{50} values of plum peel for DPPH inhibition were reported in the range of 0.48-0.91 mg/ml. Antioxidant activity and various other bioactive potential of fruits and their byproducts are associated with the presence of different phenolic compounds [30]. Phenolics scavenge the stable DPPH free radical by donating their hydrogen atoms, thus reducing it into the yellow-colored diphenyl-picryl-hydrazine [35].

3.3. Anticancer Activity

3.3.1. MTT Cell Proliferation Assay. To quantify the inhibitory effect of PPE on the proliferation of DLD-1, HCT-116, MDA-MB-231, and PC3, cells were cultured in the presence of PPE, concentration ranging from 10 μg/ml to 5 mg/ml. Results of in vitro MTT assay showed that PPE treatment significantly reduced cell proliferation in dose dependent manner (Figure 2(a)). All tested cell lines showed growth inhibition, and the IC_{50} values calculated were DLD-1, 3.96 mg/ml (95% CI 2.87-5.46 mg/ml); HCT-116, 2.06 mg/ml (95% CI 1.93-2.2 mg/ml); MDA-MB-231, 2.66 mg/ml (95% CI 2.36-2.99 mg/ml); and PC3, 1.85 mg/ml (95% CI 1.61-2.12 mg/ml). Doxorubicin a commonly used chemotherapeutic drug was used as positive control.

3.3.2. Wound Healing Assay. To quantify the impact of PPE on migration of cancer cells, we performed in vitro wound healing assay using PC3 and MDA-MB-231 cells. Results presented in Figures 3(a) and 3(b)showed statistically significant inhibition of migratory ability of cancer cells. The inhibitory effect was more noticeable for PC3 cells where 12 h of treatment with 0.5 and 1.0 mg/ml PPE resulted in reduction in their wound closure ability to 37 ± 1.7% and 31 ± 1.6% of untreated control cells, respectively. Similarly, 24 h of treatment with 0.5 and 1.0 mg/ml PPE reduced the healing ability to 25 ± 1.6% and 22 ± 1.0% of control cells, respectively. For breast cancer MDA-MB-231 cells, 24 h of incubation with 0.5 and 1.0 mg/ml PPE reduced the healing ability to 48 ± 7.3% and 41 ± 7.8% of untreated control.

Previous studies reported that plum extracts exhibited the ability to decrease the viability and proliferation of hepatocellular and colon cancer cell lines, and at a high

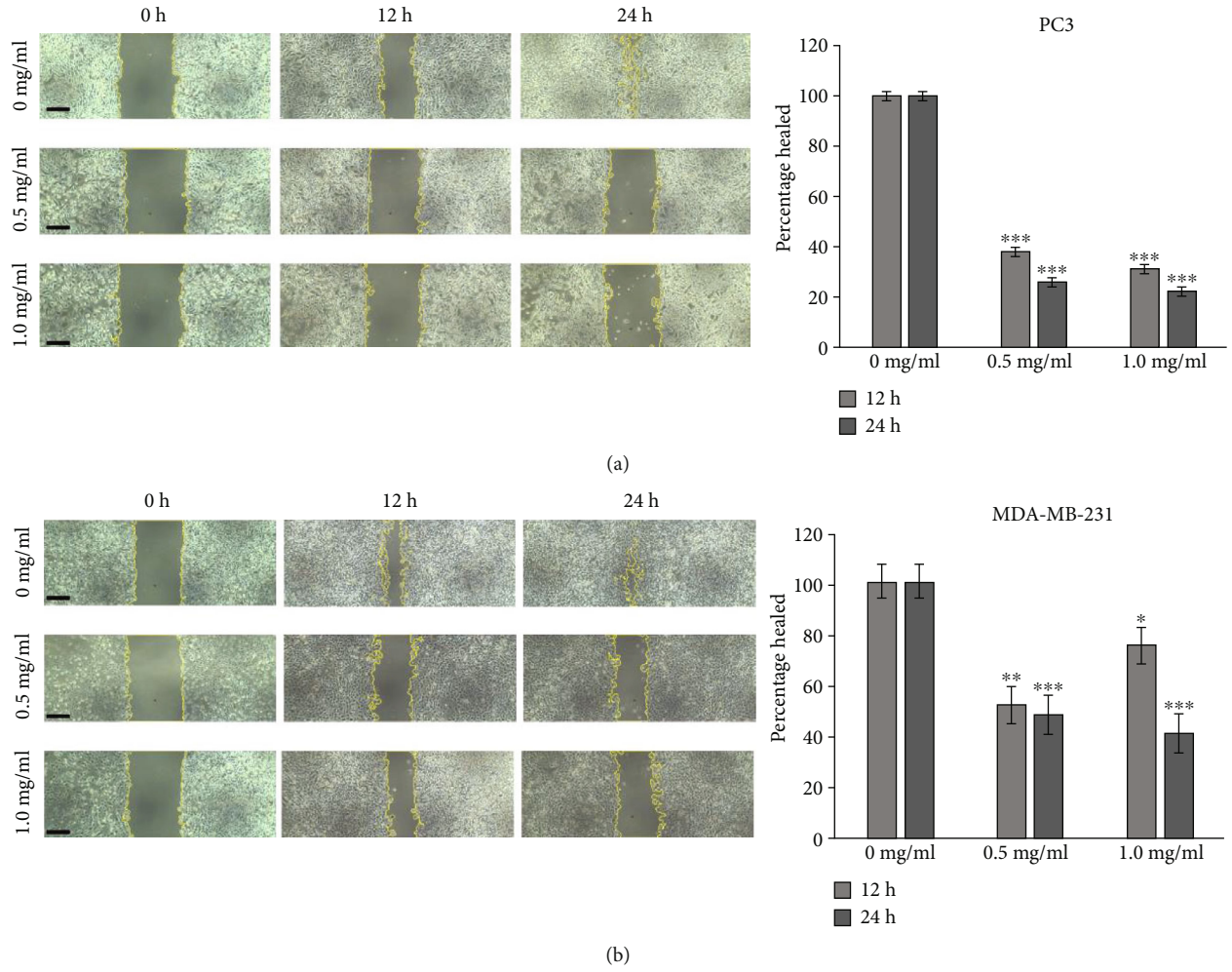


Figure 3: Reduction in migratory capacity of PC3 (a) and MDA-MB-231 (b) cells after PPE treatment. Left: phase contrast images of wound healing assay; cell monolayers were scratched and allowed to heal in the presence of PPE. Scratched areas were photographed at regular time intervals, yellow lines represent wound areas calculated using the ImageJ software, and scale bar represents 250 μm. Right: quantitative analysis of wound healing. Percentage healing was calculated by normalizing wound areas after healing to areas at start of experiment, shown as percentage of untreated controls. Error bars represent SEM. Student's t-test between treated and untreated cells. $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$.

Table 2: Antibacterial activity of optimized plum peel extract.

Extract conc. mg/ml	Diameter of zone of inhibition against pathogens (mm)		
	Escherichia coli	*Salmonella typhimurium*	*Staphylococcus aureus*
100	25 ± 1^{a}	21.33 ± 1.52^{a}	25.33 ± 0.57^{a}
50	22.66 ± 0.57^{b}	21.33 ± 2.08^{a}	23.66 ± 1.52^{a}
25	21.33 ± 0.57^{b}	19.66 ± 1.15^{a}	20.33 ± 0.57^{b}

Different superscript small letters indicate means which are significantly ($p < 0.05$) different.

concentration of plum extract (2 mg/ml), 87% of grown inhibition was observed ([36, 37]). Plum extract was reported to induce the cleaved caspase-8 protein expression which is responsible for inducing cancer cell apoptosis [38]. Plum extract was also reported to inhibit the cancer cell migration [39].

3.4. Antibacterial Activity and Food Preservation Potential of PPE. Antibacterial activity of PPE was increased in concentration-dependent manner, and at highest test concentration (100 mg/ml), inhibition zones of 25 ± 1, 21.33 ± 1.52, and 25.33 ± 0.57 mm were observed against *E. coli*, *S. typhimurium*, and *S. aureus*, respectively (Table 2).

PPE + CS-based coating restricted the growth of *S. aureus* in artificially inoculated grapes. After 5 days of storage, the bacterial count was 3.54 ± 0.04 and 4.86 ± 0.04 log CFU/g in grapes treated with CS + PPE (50 mg/ml) and CS alone, respectively (Table 3). The grapes treated with the combination of CS and PPE remained below 4 log CFU/g, which is maximum allowable limit recommended by Food

Table 3: Microbiological analysis of fresh grapes treated with PPE-based coating.

Days	Chitosan (CS) (log CFU/g)	Chitosan+ PPE 25 mg/ml (log CFU/g)	Chitosan +PPE 50 mg/ml (log CFU/g)	Control (log CFU/g)
1	3.04 ± 0.09^{a}	2.64 ± 0.18^{a}	2.49 ± 0.19^{a}	4.86 ± 0.04^{a}
2	3.19 ± 0.05^{ab}	3.01 ± 0.06^{b}	2.86 ± 0.08^{b}	5.15 ± 0.03^{b}
3	3.35 ± 0.08^{bc}	3.18 ± 0.04^{bc}	3.06 ± 0.09^{bc}	6 ± 0.08^{c}
4	3.48 ± 0.05^{c}	3.34 ± 0.05^{c}	3.25 ± 0.1^{c}	6.74 ± 0.07^{d}
5	4.86 ± 0.04^{d}	3.63 ± 0.07^{d}	3.54 ± 0.04^{d}	7.35 ± 0.05^{e}

Different superscript small letters indicate means which are significantly ($p < 0.05$) different.

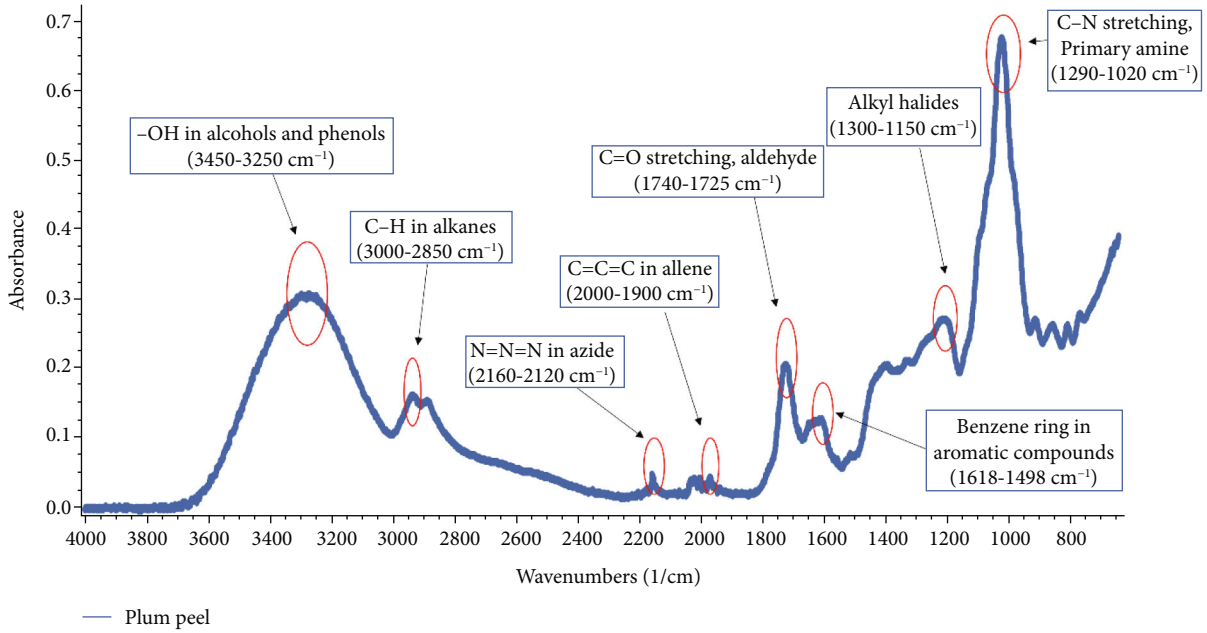


Figure 4: FTIR spectrum of plum peel extract.

Table 4: GCMS analysis of plum peel extract.

Serial #	Retention time	% of total	Compounds	Molecular formula
1	7.066	26.027	2-Cyclopenten-1-one, 2-hydroxy-	$C_5H_6O_2$
2	7.833	2.240	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$
3	8.123	4.782	2H-Pyran-2,6(3H)-dione	$C_5H_4O_3$
4	8.939	29.882	Butanoic acid, 3-methylbutyl ester	$C_9H_{18}O_2$
5	10.588	5.703	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$
6	11.881	19.261	5-Hydroxymethylfurfural	$C_6H_6O_3$
7	13.168	1.959	Tri(1, 2-propyleneglycol), monomethyl ether	$C_{10}H_{22}O_4$
8	20.364	4.027	n-Hexadecanoic acid	$C_{16}H_{32}O_2$
9	22.013	4.355	9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$
10	22.237	1.765	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	$C_{11}H_{16}O_4$

and Drug Administration (FDA) for fruits. However, control treatments (grapes without PPE) were found exceeding 4 log CFU/g after 1 day of storage.

Plums are rich in phenolic compounds which are associated with various bioactive potentials including antibacterial effect. Freeze dried plum powder extract was reported to inhibit the growth of various Gram positive and Gram negative bacteria [40]. Valtierra-Rodríguez et al. [41] reported that the plum fruit extract restricted the growth of *Campylobacter jejuni* and *Campylobacter coli* in poultry skin inoculated with these pathogens. Plum products have been also effective in inhibiting the growth of *Salmonella* and *E. coli* O157:H7 [42]. Nair et al. [43] used the pomegranate peel extract with CS and alginate to preserve. They found the guava fruit and found that peel extract in combination with CS was very effective in extending the shelf life of the fruit.

Table 5: HPLC analysis of plum peel extract.

Compound name	Retention time	Area (%)	Concentration (mg/kg)
Quercetin	2.773	2.4	7.44
Gallic acid	4.937	1.5	3.24
Vanillic acid	13.267	0.3	1.12
m-Coumaric acid	20.140	3.6	2.59
Ferulic acid	21.587	11.2	47.87
Sinapic acid	26.647	7.6	9.15

3.5. FTIR Analysis. The characteristic functional groups were assigned to corresponding peaks by following D'Angelo and Zodrow [44]. The assignment of functional group to corresponding functional groups is presented in Figure 4 and Table S3. The intense peak in the range of 1290-1020 cm^{-1} was attributed to C-N stretching of primary amines. The peak in the range of 3450–3250 cm^{-1} assigned to -OH functional group in alcohols and phenols [45]. Peaks in the range of 1300–1150 cm^{-1}, 1740-1725 cm^{-1}, 3000–2850 cm^{-1}, 1618–1498 cm^{-1}, and 2160-2120 cm^{-1} were assigned to alkyl halide, C=O stretching, C-H in alkanes, benzene ring in aromatic compounds, and N=N=N in azide, respectively.

3.6. GCMS and HPLC Analyses of PPE. The major phytoconstituents identified in PPE were 2-cyclopenten-1-one, 2-hydroxy-, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, 2H-pyran-2, 6(3H)-dione, butanoic acid, 3-methylbutyl ester, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 5-hydroxymethylfurfural, tri(1, 2-propyleneglycol), monomethyl ether, n-hexadecanoic acid, 9,12-octadecadienoic acid, methyl ester, and 9,9-dimethoxybicyclo[3.3.1]nona-2,4-dione (Table 4 and Figure S1). Plums were reported to exhibit varieties of volatile compounds such as esters, ethers, ketones, alcohols, and lactones [46]. 2-Cyclopenten-1-one, 2-hydroxy-, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, and 2H-pyran-2,6(3H)-dione were reported to exhibit antioxidant potential, whereas 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl was found to be associated with antipathogenic potential [47].

HPLC was used for the identification of bioactive phenolic compounds in PPE (Table 5). The major phenolics detected were ferulic acid (47.87 mg/kg), sinapic acid (9.15 mg/kg), quercetin (7.44 mg/kg), gallic acid (3.24 mg/kg), m-coumaric acid (2.59 mg/kg), and vanillic acid (1.12 mg/kg). Fanning et al. [13] identified phenolic compounds in different cultivars of Japanese plum and reported that quercetin was one of the dominating phenolic compound (2-27 mg/100 g) present in different varieties of plum. Ferulic acid, coumaric acid, and quercetin have been reported to exhibit strong antioxidant and anticancer activities [14, 48].

4. Conclusion

Natural food preservatives have been associated with various health benefits; however, high cost hinders their industrial applications. In this study, bioactive compounds with antibacterial, antioxidant, and anticancer potential were extracted from plum peels by optimizing UAE. Phytochemical analysis revealed the presence of high phenolic and anthocyanin contents in PPE. Optimized PPE was found rich in secondary metabolites which exhibit remarkable antibacterial, anticancer, and antioxidant potential. Due to the presence of high phenolic and anthocyanin content, PPE can be used in the development of functional foods, natural active ingredient-based pharmaceutical, and cosmetic products.

Data Availability

All the data has been incorporated within the study and supplementary file.

Conflicts of Interest

The authors declare no conflict of interest.

Supplementary Materials

Table S1: analysis of variance table for total anthocyanin content, total phenolic content, and antioxidant activity with 95% confidence level. Table S2: %age DPPH inhibition of optimized PPE extract and vitamin C. Table S3: assignment of FTIR peaks to the functional groups in the PPE. Figure S1: total ion chromatogram of plum peel extract. *(Supplementary Materials)*

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Research Article

Aqueous Ginger (*Zingiber officinale*) Extract Ameliorates the Harmful Effects of High-Dose Lornoxicam in Albino Male Rats

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Lornoxicam is a potent oxicam-class nonsteroidal anti-inflammatory drug (NSAID) with analgesic, anti-inflammatory, and antipyretic effects. Its impacts on many biological functions are not fully understood. We measured various biomarkers in male albino rats provided an oral aqueous ginger extract before IM administration of therapeutic and 2× the therapeutic doses of lornoxicam. The aqueous ginger plant extract was characterized by mass spectroscopy, and its effects were determined by examining free radical scavenging activity, blood parameters, renal and hepatic function, semen quality, proinflammatory cytokines, antioxidant markers, and histopathology. Rats administered lornoxicam had significantly higher liver and kidney function biomarker values, TNF-α, interleukin-6, and sperm abnormalities than the control rats. The overall erythrocyte count, packed cell volume, prostaglandin, and sperm counts were all considerably lower in the experimental animals. Histological changes were found in the liver, spleen, and testes of rats administered lornoxicam alone. In rats, pretreatment with ginger extract reduced the majority of the negative effects of conventional and high dosages of lornoxicam.

1. Introduction

Prevention and treatment of musculoskeletal illnesses are public health priorities in humans and other animals [1]. To treat musculoskeletal injuries, a large variety of anti-inflammatory medications from various chemical classes are commonly used [1], including nonsteroidal anti-inflammatory drugs (NSAIDs), which demonstrate anti-inflammatory and analgesic properties [2]. NSAIDs, including aspirin, ibuprofen, naproxen, and indomethacin, function by suppressing prostaglandin formation [3]. Unfortunately, NSAIDs are also well known to induce liver damage, annually affecting 3 to 23 patients per 100,000 [4]. Because of the identified hepatotoxic side effects, three specific NSAIDs—bromfenac, ibufenac, and benoxaprofen—were removed from the UK and/or US markets [5]. These NSAID-related side effects have been observed in children [6] and older adults [7].

The NSAID lornoxicam (chlorotenoxicam) is an oxicam derivative, suppressing polymorphonuclear (PMN) leukocyte motility, PMN leukocyte superoxide release, and macrophage nitric oxide release [8]. Used in both oral and parenteral forms [9], lornoxicam demonstrates many anti-inflammatory, analgesic [10], and antipyretic properties via the inhibition of two isoforms of the cyclooxygenase enzyme (COX-1 and COX-2) and suppression of prostaglandin (PG) and thromboxane [11]. It also promotes suppression of

endotoxin-induced IL-6 production in THP1 monocytes and lower TNF-α and IL-1β activity.

There are many variables that might lead to an increased risk of NSAID hepatotoxicity, including excessive intake, prescription overdose, drug interactions, and unique patient susceptibilities. [12]. In part to discover natural alternatives to toxic synthetic medications, researchers have investigated the potential of many plants and their extracts to treat various diseases [13]. For example, the active components in many herbs have been revealed as antioxidants that demonstrate low toxicity and high efficacy. One of these herbs is ginger (*Zingiber officinale*).

Medicinal herbs have been utilized to heal various infections since antiquity. According to the World Health Organization, 80 percent of the world's population uses diverse plant fractions and their dynamic ingredients as traditional remedies [14, 15]. The rhizomes of the ginger plant are the most medicinally significant part of the plant [16]. It bears antiarthritic, antiplatelet, antitumor, antioxidant, anti-inflammatory, antiviral, and antihepatotoxic properties [17, 18]. Ginger has been identified as protective against many toxic agents, such as cisplatin [19] and bromobenzene [20], attributed to its ability to increase antioxidant enzyme activity [21]. Researchers discovered a drop in plasma uric acid concentrations after giving ginger extract to broilers [22, 23]. Moreover, rats treated with 2% or 4% dietary ginger for one month demonstrated lower gentamycin-induced nephrotoxicity and renal oxidative stress [24–26]. Ginger ethanolic extracts given in single doses or for several days also protected the animals against nephrotoxicity induced by doxorubicin [27] and cisplatin [19], hepatotoxicity induced by acetaminophen [28] and bromobenzene [20], and testicular toxicity induced by cisplatin [29]. The primary antioxidants in ginger have been identified as 6-gingerol and 6-shogaol [30–32].

The effectiveness of ginger extracts in mitigating the harmful effects of lornoxicam administration has yet to be demonstrated. In addition, the effects of high dosages of lornoxicam (two times the therapeutic dose) on biochemistry, hematological, histology, oxidative stress, cytokine production, and sperm characteristics remain unknown. Therefore, we aimed to investigate the effect of an aqueous ginger extract on these parameters in male albino rats treated with therapeutic and 2× the therapeutic doses of lornoxicam.

2. Materials and Methods

2.1. Drug and Plant Aqueous Extract. Lornoxicam (16 mg, trade name: Xefo®; 6-chloro-4-hydroxy-2-methyl-N-2-pyridyl-2 H-thieno (2,3-e)-1,2-thiazine-3-carboxamide-1,1-dioxide) was obtained from Eva Pharma Pharmaceuticals Inc. (Cairo, Egypt) in powder form. The powder was diluted in 2 mL of distilled water before to injection, giving a final dosage of 8 mg/mL lornoxicam. Botanists from the Faculty of Agriculture, Benha University, obtained ginger rhizomes from a local market. The rhizomes were crushed in a blender and air-dried. A total of 125 g of air-dried powder was macerated in 200 mL of distilled water for 12 hours at room temperature before filtration to obtain a final aqueous extract concentration of 120 mg/mL for use in the experiment.

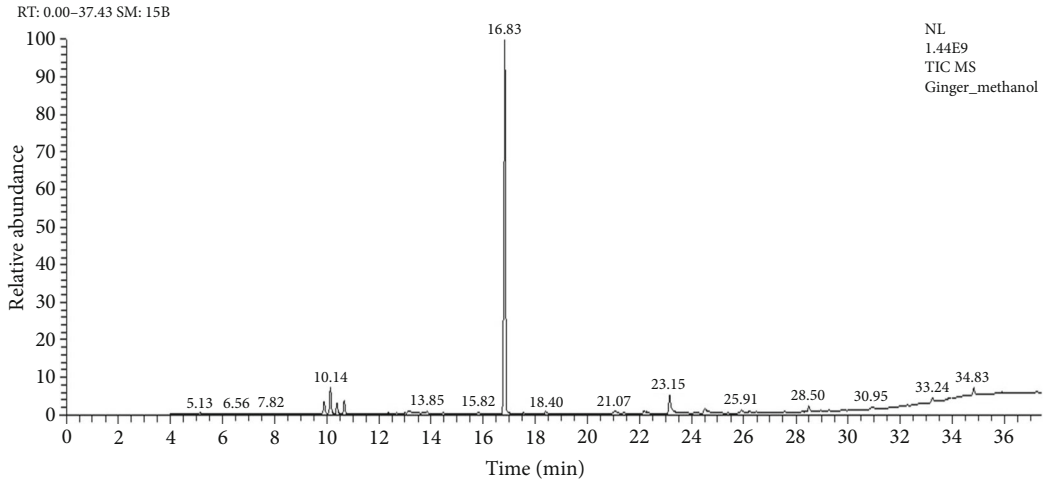
2.2. Gas Chromatography-Mass Spectrometry (GC–MS) Analysis of the Aqueous Ginger Extract. In the current study, the chemical composition of the aqueous ginger extract was determined using a Trace GC–TSQ mass spectrometer with a direct TG − 5MS 30 m × 0.25 mm × 0.25 μm (film thickness) capillary column (Thermo Scientific, Austin, TX, USA). The components were determined by comparing their relative mass spectra fractions to those of the WILEY 09 and NIST 14 mass spectral databases [33].

2.3. DPPH Free Radical Scavenging Activity. The radical-scavenging ability of aqueous ginger extract was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent according to previous studies [34]. To generate DPPH radicals, an ethanol solution of DPPH (1 mM) was generated shortly before the experiment, and 3.9 mL of the DPPH radical solution was mixed in triplicate with 100 L of aqueous ginger extract or 100 L of ethanol (control). The mixture was incubated for 30 min in darkness, and the absorbance was measured spectrophotometrically at 517 nm. Decreased sample absorbance indicates the capacity of the ginger extract to extract DPPH free radicals. The percentage scavenging activity was calculated as percentage inhibition of DPPH
(-
antioxidant capacity% = [absorbance of control – absorbance of sample/absorbance of control] × 100%).

2.4. Experimental Animals. Fifty male Wister rats (6–8 weeks old, 225–250 g body weight) were housed in separate cages. The laboratory environment was conventional (temperature 25°C, relative humidity 65%), with a 12-hour light/dark cycle. Throughout the experiment, rats were fed a typical diet of bread, barley and milk, and water ad libitum. For adaption and to prevent stress during the experimental period, the rats were acclimatized to the laboratory conditions for two weeks prior to treatment. The Institutional Animal Care and Use Committee for Research Ethics of Benha University (# BUFVTM3921) approved all study procedures, which followed the principles of the Declaration of Helsinki.

2.5. Experimental Design. The 50 rats were divided into five groups of 10 rats. The rats in the first group were given no therapy and served as a control. The rats in the second group were administered IM lornoxicam at a therapeutic dose (0.07 mg/kg/day; [35]) once a day for 10 days. The third group of rats received an oral aqueous extract of ginger (600 mg/kg/day at a concentration of 120 mg/mL; [17, 36]) two hours prior to receiving a therapeutic dose of IM lornoxicam once daily for ten days. The fourth group of rats received a daily IM injection of 2× the therapeutic dose of lornoxicam (0.14 mg/kg/day) for 10 days. The rats in the fifth group were given an aqueous ginger extract (600 mg/kg/day; 120 mg/mL; [17, 36] two hours before receiving an IM injection of double the therapeutic dose of lornoxicam once daily for 10 days.

2.6. Hematological Examination. After the end of the experiment, hematological assays were performed on whole blood samples taken from the retroorbital venous plexus. Total erythrocyte count (TEC), packed cell volume % (PCV%),



Retention time (min.)	Compound Name	Area %	MF	Molecular formula	Molecular weight	Library
9.90	α-curcumene	2.64	968	C15H22	202	replib
10.14	α-zingiberene	5.26	937	C15H24	204	Wiley registry 8e
10.39	2,6,10-dodecatrien-1-ol, 3,7,11-trimethyl-	2.18	897	C15H26O	222	Wiley registry 8e
10.39	α-bisabolene	2.18	884	C15H24	204	replib
10.67	α-sesquiphellandrene	2.69	910	C15H24	204	Wiley registry 8e
16.83	zingiberene	76.81	945	C15H24	204	Wiley registry 8e
23.15	6-Gingerol	4.13	940	C17H26O4	294	mainlib
28.50	shogaol	1.28	892	C21H32O3	332	mainlib
33.24	α-sitosterol	2.83	852	C29H50O	414	replib

Figure 1: The standard curve and components of ginger aqueous extract detected by gas chromatography mass spectrometry analysis.

Table 1: Effects of ginger (*Zingiber officinale*) aqueous extract on kidney function of lornoxicam treated male albino rats.

Groups	Uric acid (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)
1	3.13 ± 0.01^{e}	0.69 ± 0.02^{e}	20.89 ± 1.50^{e}
2	6.70 ± 0.03^{c}	1.15 ± 0.02^{c}	47.09 ± 1.16^{c}
3	5.99 ± 0.04^{d}	0.99 ± 0.02^{d}	44.25 ± 0.96^{d}
4	8.79 ± 0.03^{a}	2.01 ± 0.07^{a}	55.35 ± 1.69^{a}
5	7.44 ± 0.06^{b}	1.37 ± 0.03^{b}	50.18 ± 1.36^{b}

[a-e]The values represented as mean ± SD. Means within the same column followed by different letters are significantly different ($P \leq 0.05$).

hemoglobin concentration (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were detected using an automated cell counter (VetScan HM5 Hematology system, Abaxis, Inc., Union City, CA, USA).

2.7. Biochemical Examination. Serum samples were used to estimate the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) [37], catalase (CAT) [38] and glutathione peroxidase (GPX) [39], concentrations of creatinine, [40], urea [41], uric acid [42], and malondialdehyde (MDA) [43]. TNF-α and IL-6 concentrations were determined by a commercial ELISA kit [44].

2.8. Histopathological Examination. Liver, kidney, testes, and spleen tissue samples were taken from each group's slaughtered animals and processed for histopathological procedures that were performed using standard techniques [45]. Masson's trichrome staining was performed according to a standard protocol [46]. In addition, the epididymis heads were used to measure sperm count [47] and sperm abnormalities [48].

Table 2: Effects of ginger (*Zingiber officinale*) aqueous extract on liver function of lornoxicam treated male albino rats.

Groups	AST, U/mL	ALT, U/mL	ALP, U/mL
1	26.09 ± 1.60^{e}	104.31 ± 2.53^{e}	151.33 ± 5.58^{e}
2	44.42 ± 1.6^{c}	293.72 ± 2.72^{c}	228.67 ± 3.49^{c}
3	40.47 ± 1.5^{d}	273.0 ± 2.47^{d}	221.33 ± 2.62^{d}
4	57.71 ± 1.35^{a}	511.62 ± 5.08^{a}	404.67 ± 4.13^{a}
5	48.34 ± 1.87^{b}	397.04 ± 9.25^{b}	343.67 ± 3.84^{b}

The values represent mean ± SD. Means within the same column followed by different letters are significantly different ($P \leq 0.05$).

Table 3: Effects of ginger (*Zingiber officinale*) aqueous extract on hematological indices of lornoxicam treated male albino rats.

Groups	RBC×106/μL	HB gm/dl	PCV%	WBC×103 μL
1	7.69 ± 0.29^{a}	$13.86 \pm 2.79_{a}$	$38.57 \pm 1.28_{a}$	12.13 ± 3.2^{a}
2	7.02 ± 0.25^{a}	$11.43 \pm 1.47_{a}$	$35.89 \pm 1.47_{a}$	10.8 ± 2.82^{a}
3	7.66 ± 0.62^{a}	$12.03 \pm 1.97_{a}$	$36.20 \pm 1.93_{a}$	11.10 ± 1.15^{a}
4	3.10 ± 0.21^{b}	7.50 ± 0.58^{b}	$25.67 \pm 1.74_{b}$	3.50 ± 1.10^{b}
5	6.82 ± 1.28^{a}	$10.17 \pm 1.23_{a}$	$31.72 \pm 2.36_{a}$	10.58 ± 2.16^{a}

The values represent mean ± SD. Means within the same column followed by different letters are significantly different ($P \leq 0.05$).

Table 4: Effects of ginger (*Zingiber officinale*) aqueous extract on semen analysis and Prostaglandins concentration of lornoxicam treated male albino rats.

Groups	Sperm, million/gm	Sperm abnormalities %	Prostaglandins, ng/mL
1	6.23 ± 0.35^{a}	8.67 ± 0.88^{e}	2360.21 ± 4.89^{a}
2	4.18 ± 0.10^{c}	13.33 ± 0.45^{c}	948.90 ± 3.46^{c}
3	5.37 ± 0.07^{b}	11.33 ± 0.20^{d}	1017.3 ± 5.46^{b}
4	3.35 ± 0.07^{e}	25.10 ± 0.31^{a}	663.57 ± 7.64^{e}
5	4.50 ± 0.06^{d}	18.50 ± 0.32^{b}	891.67 ± 6.01^{d}

The values represent mean ± SD. Means within the same column followed by different letters are significantly different ($P \leq 0.05$).

2.9. Statistical Analysis. Data were statistically analyzed by one-way ANOVA with a post hoc Duncan multiple comparison test using a statistical software program (SPSS for Windows version 20, IBM, Armonk, NY, USA). Differences were considered significant at a P value ≤0.05.

3. Results

3.1. Characterization of the Aqueous Ginger Extract. GC–MS analysis of aqueous ginger extract indicated that this extract contained nine components: α-curcumene, α-zingiberene, β-sesquiphellandrene, β-bisabolene, zingiberene, shogaol, α-sitosterol, 6-gingerol, and 2,6,10-dodecatrien-1-ol, 3,7,11-trimethyl (Figure 1). In the DPPH assay, the aqueous ginger extract's antioxidant capacity was 42.79% ± 3.1%.

3.2. Liver and Kidney Function Tests. Treatment with lornoxicam at both the therapeutic (group 2) and 2× therapeutic doses (group 4) resulted in significantly higher levels ($P \leq 0.05$) of kidney function parameters (uric acid, creatinine, and urea; Table 1) and liver function parameters (AST, ALT, and ALP; Table 2) than the nontreated control group. Sera from rats given two times the therapeutic dose of lornoxicam had higher average estimated parameters than those from animals given the therapeutic dose. The increased blood liver and kidney functioning indicators were dramatically improved after treatment with aqueous ginger extract. The amelioration of lornoxicam pathology was observed in all treatment groups, in the following order: group 4 > group 5 > group 2 > group 3.

3.3. Hematological Parameters. Therapeutic lornoxicam treatment did not significantly alter the estimated hematological parameters (Table 3). However, rats receiving 2× lornoxicam (group 4) showed significantly reduced RBC count, Hb%, PCV, and WBC count. These changes were significantly improved by treatment with the aqueous ginger extract.

3.4. Semen Analysis. Compared with the nontreated control group, treatment with lornoxicam at both the therapeutic and 2× the therapeutic dose (groups 2 and 4, respectively) resulted in significantly lower sperm counts ($P \leq 0.05$; Table 4), higher % sperm abnormalities, and lower PG levels. Pretreatment with aqueous ginger extract resulted in significantly higher sperm count, lower % sperm abnormalities, and higher PG levels.

3.5. Proinflammatory Cytokines and Antioxidant Markers. Compared with the nontreated control group, treatment with lornoxicam at both the therapeutic and 2× the therapeutic doses (groups 2 and 4, respectively) resulted in significantly elevated TNF-α and IL-6 levels ($P \leq 0.05$; Table 5). Pretreatment with aqueous ginger extract significantly decreased TNF-α and IL-6 levels. Lornoxicam injection induced a significant reduction of CAT and GPX enzyme activity and increased levels of MDA (Table 5) in the sera of rats of all experimental groups compared to the control (group 1).

3.6. Histological Examination. Tables 6–9 showed the effects of ginger (*Zingiber officinale*) aqueous extract on the pathological grading of lornoxicam in renal, hepatic, splenic, and testicular tissues of different treated groups.

3.6.1. Kidney. Histological examination of kidney tissues by H&E staining revealed that control rats (Figure 2(a)) showed less blood vessel congestion than lornoxicam-treated rats (Figure 2(b)). Kidney architecture was restored to normal in rats treated with ginger extract two hours before injection of the therapeutic dose of lornoxicam (Figure 2(c)) and was more intact than that of rats treated with lornoxicam alone (Figure 2(b)). Severe congestion was observed in the kidney

Table 5: Effects of ginger (*Zingiber officinale*) aqueous extract on oxidative stress and cytokines of lornoxicam treated male albino rats.

Groups	MDA, nmol/gm	CAT, U/gm	GPX, U/gm	TNF-α, pg/mL	IL6, pg/mL
1	137.55 ± 5.3^{e}	628.29 ± 7.4^{a}	588.84 ± 4.5^{a}	43.84 ± 1.79^{e}	26.37 ± 0.33^{e}
2	182.61 ± 3.5^{c}	521.19 ± 5.2^{c}	422.14 ± 4.6^{c}	98.58 ± 1.58^{c}	33.97 ± 1.71^{c}
3	173.79 ± 2.8^{d}	534.32 ± 6.1^{b}	534.88 ± 5.1^{b}	88.93 ± 2.42^{d}	30.16 ± 0.68^{d}
4	283.98 ± 2.8^{a}	498.54 ± 3.4^{e}	369.08 ± 3.8^{e}	147.85 ± 5.67^{a}	49.60 ± 2.28^{a}
5	253.04 ± 2.6^{b}	502.69 ± 4.9^{d}	401.93 ± 5.4^{d}	123.19 ± 7.15^{b}	37.51 ± 1.27^{b}

The values represent mean ± SD. Means within the same column followed by different letters are significantly different ($P \leq 0.05$).

Table 6: Effects of ginger (*Zingiber officinale*) aqueous extract on the pathological grading of lornoxicam in renal tissue of different treated groups.

Groups	Number	Pathological grading			
		0	I	II	III
1	10	10	0	0	0
2	10	0	4	4	2
3	10	5	1	2	2
4	10	0	2	4	4
5	10	5	1	2	2

Table 7: Effects of ginger (*Zingiber officinale*) aqueous extract on the pathological grading of lornoxicam in hepatic tissue of different treated groups.

Groups	Number	Pathological grading			
		0	I	II	III
1	10	10	0	0	0
2	10	0	3	3	4
3	10	4	2	3	1
4	10	0	2	4	4
5	10	5	1	3	1

Table 8: Effects of ginger (*Zingiber officinale*) aqueous extract on the pathological grading of lornoxicam in splenic tissue of different treated groups.

Groups	Number	Pathological grading			
		0	I	II	III
1	10	10	0	0	0
2	10	0	5	4	1
3	10	5	2	2	1
4	10	0	2	4	4
5	10	5	3	1	1

tissues of rats treated with the 2× lornoxicam dose alone (Figure 2(d)) compared to the control group (Figure 2(a)). Hydropic degeneration was observed in the kidney tissues of rats pretreated with ginger extract two hours before injection of the 2× lornoxicam dose (Figure 2(e)) compared to control (Figure 2(a)). However, the kidney architecture was more intact than that of rats treated with the 2× lornoxicam dose alone (Figure 2(d)).

Table 9: Effects of ginger (*Zingiber officinale*) aqueous extract on the pathological grading of lornoxicam in testicular tissue of different treated groups.

Groups	Number	Pathological grading			
		0	I	II	III
1	10	10	0	0	0
2	10	0	4	4	2
3	10	5	1	2	2
4	10	0	2	4	4
5	10	5	1	2	2

3.6.2. Liver. H&E staining of the liver tissues revealed that, unlike the control (Figure 3(a)), lornoxicam alone resulted in a congested central vein (Figure 3(b)). The hepatic structure was normal in rats treated with ginger extract two hours before injection of the therapeutic dose of lornoxicam (Figure 3(c)) and was better than that of rats treated with lornoxicam alone (Figure 3(b)). Hepatic vascular degeneration, sinusoidal dilatation, picnotic and hyperchromatic cells, focal necrosis, mononuclear cellular infiltration, Von Kupffer cell hyperplasia, edema, ballooning, congested hepatic veins, and degenerative changes were observed in the hepatic tissues of rats treated with a 2× lornoxicam dose alone (Figure 3(d)). Only hepatic vein congestion was observed in the liver tissues of rats pretreated with ginger extract before injection of the 2× lornoxicam dose (Figure 3(e)). However, the liver architecture was more intact than in rats treated with 2× lornoxicam dose alone (Figure 3(d)). Results of the PAS reaction were positive in nelson capsules.

3.6.3. Spleen. Compared to the control group (Figure 4(a)), hemorrhage was observed by H&E staining in the splenic tissues of rats injected with a therapeutic dose of lornoxicam alone (Figure 4(b)). The splenic architecture was nearly normal in rats pretreated with ginger extract before receiving the therapeutic dose of lornoxicam (Figure 4(c)) and was more intact than that of rats treated with lornoxicam alone (Figure 4(b)). Hemorrhages with trabecular thickening and hemosiderosis were observed in the splenic tissues of rats treated with a 2× lornoxicam dose alone (Figure 4(d)). Hepatic vein hemosiderosis was observed in splenic tissues of rats pretreated with ginger extract before injection of the 2× lornoxicam dose (Figure 4(e)). However, the splenic architecture was more intact than in rats treated with a 2× lornoxicam dose alone (Figure 4(d)).

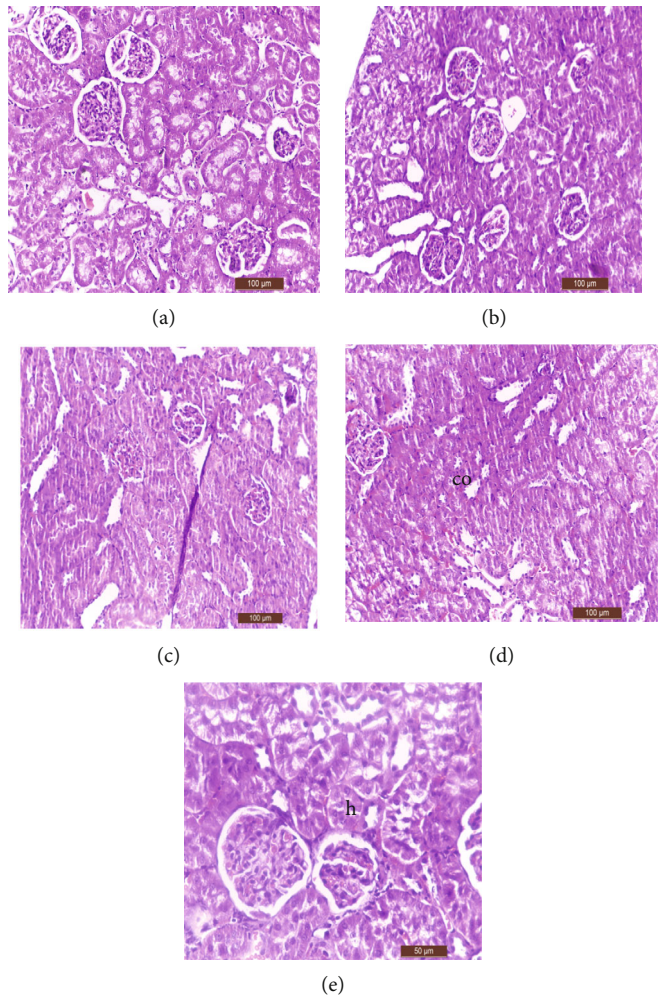


Figure 2: Histological examination of kidney tissues by H&E staining. (a) control (group 1) showed normal renal structure. (b) Group 2 showed congestion in blood vessels. (c) Group 3 showed nearly normal renal architecture. (d) Group 4 showed congestion with hydropic degeneration and desquamation of epithelial renal tubules. (e) Group 5 showed renal tissue return to normal structure except proximal convoluted tubules showed hydropic degeneration (h).

3.6.4. Testis. H&E staining of the testicular tissues revealed that, compared to the control (Figure 5(a)), administration of either the therapeutic or 2× lornoxicam dose alone resulted in desquamation in the spermatogonial cells and interstitial edema (Figures 5(b) and 5(d)). Furthermore, apoptosis was detected by Masson's trichrome staining in the testicular tissues of rats injected with either the therapeutic or 2× lornoxicam dose alone (Figures 5(b) and 5(d)) compared to control (Figure 5(a)). In rats given ginger extract two hours before receiving the therapeutic dose of lornoxicam, normal testicular architecture was seen (Figure 5(c)), and the architecture was more intact than in rats treated with lornoxicam alone (Figure 5(b)). Regeneration changes, including the disappearance of interstitial edema and intact spermatogenic cells, were detected in the testicular tissues of rats pretreated with ginger extract before injection of the 2× lornoxicam dose (Figure 5(e)), and the testicular architecture was more intact than in rats treated with a 2× lornoxicam dose alone (Figure 5(d)). A minced epididymis head solution stained with eosin and nigrosin revealed normal sperm heads and tails (Figure 6(a)) in the control group. The following sperm abnormalities were detected in rats administered lornoxicam: abnormal sperm head and tail structure (Figure 6(b)), abnormal sperm head and tail (Figure 6(c)), amorphous head (Figure 6(d)), abnormal head (Figure 6(e)), double head (Figure 6(f)), coiled tail (Figure 6(g)), and double tail (Figure 6(h)).

4. Discussion

In the current study, the antioxidant capacity (42.79% ± 3.1%) of the aqueous ginger extract was higher than that detected in a similar, previous study [49] in which the authors observed a DPPH scavenging activity of 16.2%. The highest antioxidant activity percentages detected previously were 79.83%, 70.43%, and 61.70%, observed in red ginger, emprit ginger, and elephant ginger, respectively [50].

Urea is a waste product of protein catabolism produced in the liver and discharged by the kidneys [51], which remove urea and creatinine from the blood through

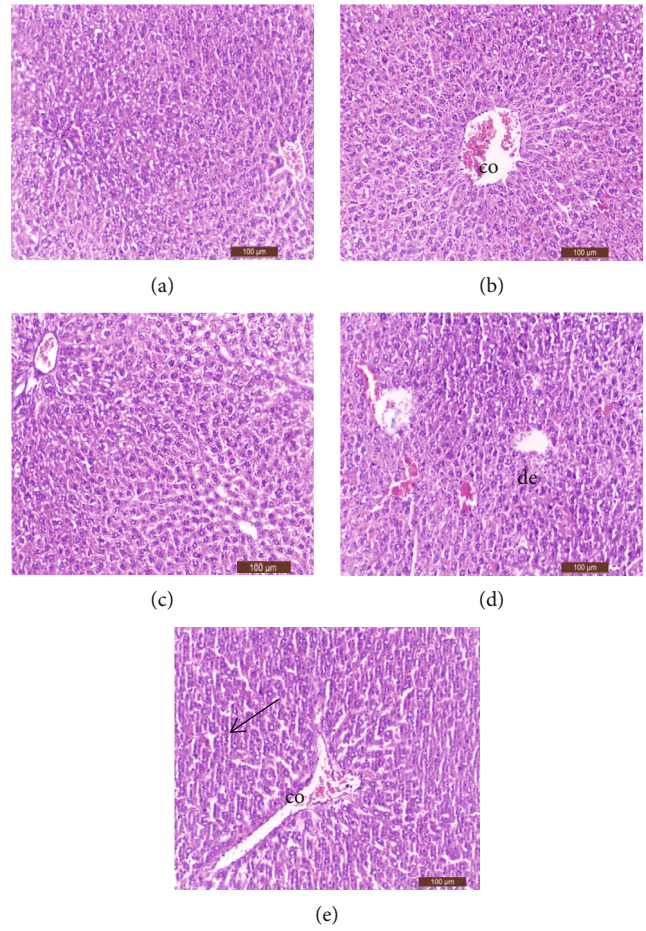


Figure 3: Histological examination of liver tissues by H&E staining: (a) control (group 1) showed normal hepatic structure; (b) group 2 showed congested central vein (co); (c) group 3 showed nearly normal hepatic cell architecture; (d) group 4 showed hepatic vascular degeneration, sinusoidal dilatation, picnotic and hyperchromatic cells, focal necrosis sites and mononuclear cellular infiltration, Von Kupffer cell hyperplasia, necrotic, apoptotic of hepatocelluar appearance, edema and ballooning degenerative changes (de), and congested hepatic veins; and (e) group 5 showed the hepatic architecture restored to normal except hepatic veins congestion (co). Positive PAS reaction (arrow).

glomerular ultrafiltration, measured by determining the glomerular filtration rate (GFR). Increased urea and creatinine levels in the blood occur from any abnormalities that diminish GFR [52]. We discovered that rats given conventional and high dosages of lornoxicam had higher uric acid levels in their blood than control rats, indicating that lornoxicam had a uricosuric impact. Previous studies similarly reported [53, 54] the uricosuric effect of the NSAID piroxicam, attributed by the authors to increased excretion of uric acid in the urine. The observed increase of serum creatinine after lornoxicam injection was similar to that observed after administration of meloxicam [51] and diclofenac sodium [55, 56] in rabbits. The authors examining the effects of diclofenac sodium proposed that the nephrotoxic effect of this drug was caused by inhibition of cyclooxygenases and subsequent suppression of PG production. Urea, uric acid, and creatinine are general renal biomarkers, and their increased levels indicate kidney injury [55, 56]. Our histopathological results in renal tissues supported the results of our biochemical analyses of kidney function. The improvement of kidney function parameters (uric acid, urea, and creatinine serum concentrations) in rats pretreated with ginger extract two hours before injection of lornoxicam in therapeutic and 2× the therapeutic doses indicates that ginger ameliorated lornoxicam's uricosuric effects [22]. Previously, it is reported that ginger extract reduced uric acid in the plasma of broilers [23]. Furthermore, 2% or 4% of dietary ginger ameliorated nephrotoxic and oxidative stress in rats [24–26]. In the current investigation, the ameliorative effect of ginger was observed in renal tissue histology. Except for hydropic degeneration in the proximal convoluted tubules, the tissue from pretreated rats displayed a more intact morphology. After lornoxicam administration without ginger, we found hydropic degeneration and desquamation of renal epithelial tubules in rat kidney tissues, particularly in rats injected with 2× the therapeutic dose. A previous study [35] similarly demonstrated renal vacuolization and decreased brush borders in proximal tubule epithelia of rats administered lornoxicam. NSAIDs were also found to induce renal vasoconstriction and decrease renal perfusion and acute renal abnormalities due to inhibition of PG biosynthesis [57].

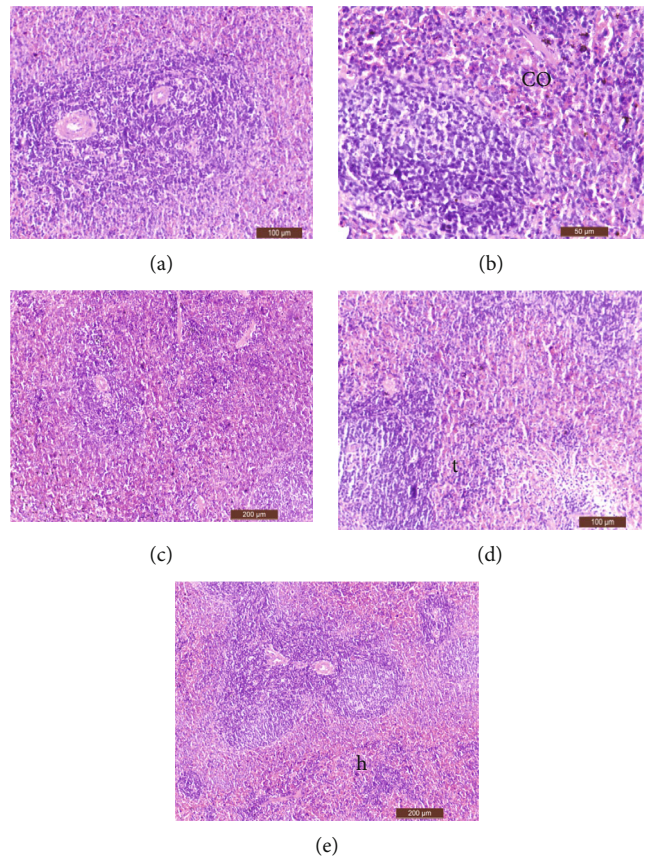


Figure 4: Histological examination of spleen tissues by H&E staining: (a) control (group 1) showed normal spleen structure, (b) group 2 showed hemorrhage (co), (c) group 3 showed nearly normal spleen architecture, (d) group 4 showed hemorrhage with trabecular thickening with hemosiderosis (t), and (e) group 5 showed the hepatic architecture restored to normal except decreased number of hemosiderosis (h).

The liver is the major target organ for drug metabolism. Hepatic biotransformation reactions induce hepatocyte apoptosis [58–60]. Therefore, AST, ALT, and ALP activities are commonly used as biomarkers of structural and functional alterations in the liver, which cause levels of these enzymes to increase in the blood [56]. As these enzymes are intracellular, their normal blood concentrations are very low. As a result, hepatocellular damage or necrosis induces an elevation in their blood serum levels [61]. Significant elevations in blood AST, ALT, and ALP were seen in rats injected with lornoxicam alone or in combination with ginger, indicating that the lornoxicam-treated groups had liver abnormalities. The role of ginger extract in improving the liver function enzymes AST, ALT, and ALP of rats injected with lornoxicam was reported previously [62].

In Swiss albino mice, hepatotoxicity, hepatitis, and elevated levels of the liver enzymes AST, ALT, and ALP had previously been documented [63] and rats [64] treated with diclofenac sodium. In addition, there is a high concentration of meloxicam (1 mg/kg/day for five days) and variable (sometimes extensive) necrosis with mild lymphocytic infiltration in rat liver and kidney tissues [65]. Our findings are similarly consistent with the previous research on meloxicam-induced liver injury in animals [66]. Furthermore, the histopathological picture (results of H&E, MTS, and PAS staining) of liver tissues supports the biochemical liver function results. The positive PAS reaction with mucopolysaccharide around the central vein of the liver tissues in nelson capsules indicated inflammation. Marked hepatic dysfunction (fibrotic, necrotic, and apoptotic hepatocytes) was also observed in a previous study of albino rats injected with lornoxicam [67]. Cytochrome P450 isozymes (CYP2C9, CYP2D6, and CYP3A4) metabolize lornoxicam to one of its major metabolites, 5′-hydroxy-lornoxicam, in the liver. This metabolite is responsible for lipid peroxidation and free radical production [68, 69]. This process will consume the GSH available for free radical scavenging. We propose that the consumption of GSH was responsible for the hepatic dysfunction observed in rats in the current study [67].

The protective effect of ginger has been attributed to its relatively high concentration of either vitamin C (35–38 mg per 100 g) [70] or shogaol [71, 72], which participate in antioxidant and antihepatotoxic activities. We identified nine ginger extract components that may contribute to ginger's antioxidant action. In a prior investigation, ginger aqueous extract was found to be protective against adriamycin-induced hepatotoxicity and cisplatin-induced hepatotoxicity [73]. We found that

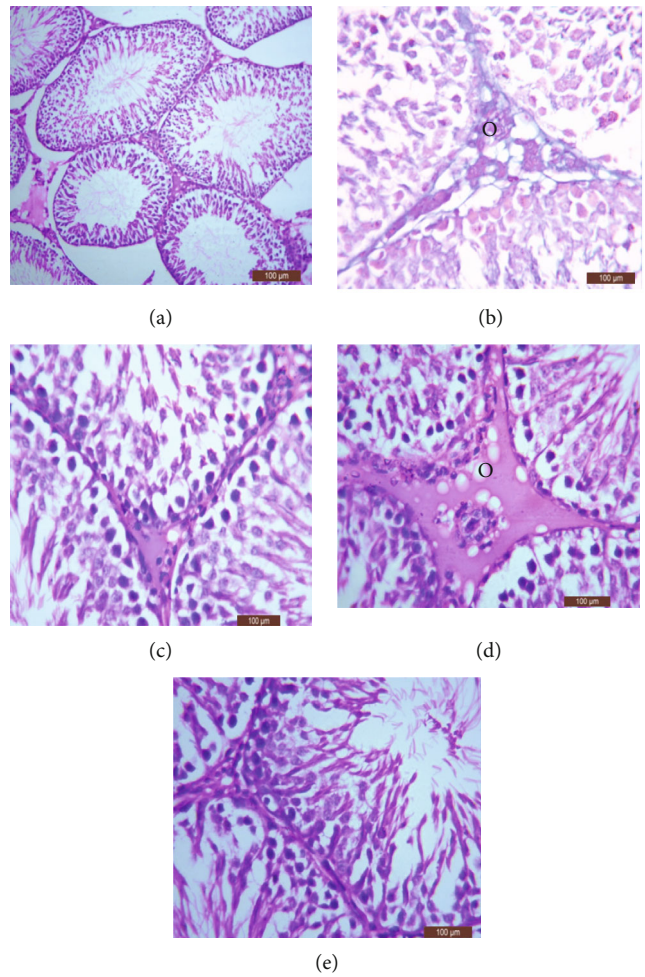


Figure 5: Histological examination of testicular tissues by H&E staining: (a) control (group 1) showed normal testes structure, (b) group 2 showed normal structure except desquamation in the spermatogonile cells and interstitial edema of some cells and sings of apoptosis (Masson technique; o), (c) group 3 showed nearly normal testicular architecture, (d) group 4 showed desquamation in the spermatogonile and interstitial edema and sings of apoptosis (Masson technique; o), and (e) group 5 showed regeneration changes include disappearance of interstitial edema and the spermatogenic cells returned to be intacted to the basement membrane.

administering ginger extract two hours before lornoxicam injection prevented many alterations in liver architecture in rats (the exception being hepatic vein congestion).

The significant changes in hematological parameters (TEC, Hb, and PCV %) observed in rats treated with lornoxicam at 2× the therapeutic dose may provide evidence of the drug's toxicity. The reduction of red blood cells with subsequent anemia was dose-dependent. This anemia results in reduced oxygen-carrying capacity in the blood and the amount of oxygen delivered to the tissues [74, 75]. Similar significant alterations of hematological parameters were observed in rats treated with diclofenac sodium [75] and paracetamol [74]. Moreover, the induction of anemia may be attributed to the loss of erythrocytes resulting from gastrointestinal bleeding and ulcers [74, 75].

H&E staining of the spleen showed hemorrhage with trabecular thickening and hemosiderosis in splenic tissue of rats injected with 2× the therapeutic dose of lornoxicam without ginger pretreatment. Similarly, N-acetyl-p-aminophenol (140 mg/kg) was found to induce overall splenic congestion, congested sinusoids, widened red pulp, and atrophy of lymphoid follicles [76]. Rats pretreated with ginger had nearly normal blood levels after injection with double the therapeutic dose of lornoxicam. This may be related to high gingerol and shogaol content in the ginger extract [71, 72, 77]. Gingerol has been found to have cardiotonic, analgesic, anti-inflammatory, and antipyretic effects [77], and shogaol acts as an antioxidant [71, 72].

A significant fall in PG levels in rats injected with lornoxicam without ginger pretreatment was previously recorded [78]. Lornoxicam was reported to be 100-fold more potent than tenoxicam in inhibiting PGD2 formation in rat polymorphonuclear leukocytes in vitro [78]. NSAIDs exert anti-inflammatory, antipyretic, and analgesic effects by suppressing PG and thromboxane synthesis [11] via COX enzyme inhibition [24]. In the current study, rats pretreated with ginger extract demonstrated improved prostaglandin levels after injection with lornoxicam, and this may be attributable to the antioxidant effect of ginger's free radical scavenging activity [79, 80].

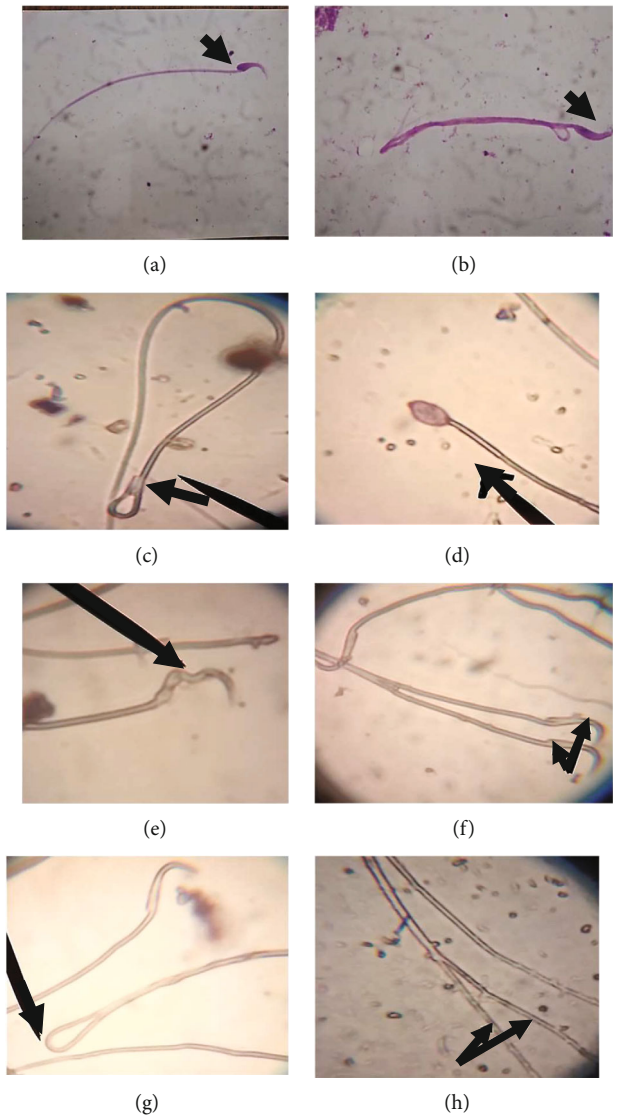


Figure 6: Microscopic examination of head of epididymis minced solution in rats stained with Eosin & Nigrosin stain: (a) normal sperm head and tail and (b) abnormal sperm head and tail, (c) abnormal sperm head and tail, (d) amorphous head, (e) abnormal head, (f) double head, (g) coiled tail, and (h) double tail.

Previously, it was discovered that aspirin causes hypercholesterolemia in rats via blocking PG production. This inhibition could result in altered cholesterol metabolism and androgen biosynthesis [81]. Androgens are essential for the survival and motility of spermatozoa in the epididymis [82]. In addition, PGs play substantial roles in regulating reproductive activity in males and females [82] and regulate sperm metabolism and function [83]. Aspirin may disrupt spermatogenic processes in the seminiferous tubules, epididymal function, or testosterone's effect on hypothalamic release factors and anterior pituitary gonadotropin production, potentially changing spermatogenesis [84]. In the current study, lornoxicam might have functioned similarly to aspirin [83] and induced morphological abnormalities in sperm cells, but additional studies are required to confirm this mechanism. The considerable decrease in sperm count in rats injected with lornoxicam without ginger pretreatment could be ascribed to cholesterol buildup in the testes. This buildup alters cholesterol metabolism, which impairs sperm dynamics, including spermatogenesis. Similarly, indomethacin was previously proposed to cross blood testis barriers and cause degeneration of seminiferous tubules, focal necrosis, and reduced spermatogenesis [85]. Additional studies are required to determine whether lornoxicam is similar to indomethacin in this regard.

In the current study, lornoxicam injection induced more sperm abnormalities and significantly fewer total sperm than the control. These findings are also reflected in testis histopathology. Desquamation in the spermatogonia and interstitial edema and apoptosis of some cells were the main features identified in rats injected with lornoxicam. A previous study [86] reported similar results in rats injected with indomethacin. We found that ginger extract pretreatment improved the sperm count and decreased sperm

abnormalities induced by lornoxicam injection in rats. The regeneration changes exhibited in the testis of rats treated with ginger extract two hours before lornoxicam injection may be attributed to ginger's antioxidant components (gingerol and shogaols) [71, 72, 77].

IL-6 and TNF-α are proinflammatory cytokines produced by activated macrophages. They help regulate various biological processes, including cell proliferation, differentiation, apoptosis, immune response, and activation of cell signaling proteins involved in systemic inflammation. The overproduction of TNF-α has been implicated in various diseases [87, 88]. Cytokine production may be affected by NSAID interaction with transcriptional factors. In addition, selective COX-2 inhibitors may cause an increase in TNF-α levels, probably by inhibiting prostaglandin production [89]. TNF-α and IL-6 are widely known to induce hepatorenal toxicity and tissue damage. In previous studies, intoxication with the NSAID paracetamol was found to induce oxidative stress, triggering a secondary inflammatory cascade associated with cytokine release from Kupffer cells [90–92]. Moreover, lornoxicam administration (1.3 mg/kg) in albino rats [93] and acetaminophen in mice [94] were previously associated with significantly increased MDA and cytokines IL-6 and TNF-α and significantly decreased CAT and GPX. Furthermore, hepatic GSH, antioxidant enzymes, and lipid peroxidation increased after lornoxicam injection in rats [67]. We propose that the protective effects observed in rats treated with ginger extract two hours before lornoxicam injection resulted from significant reductions of MDA and cytokines (IL-6 and TNF-α) and significant elevation of CAT and GPX. This observation may be attributed to the effect of the antioxidant components, gingerol and shogaols, of ginger extract [71, 72, 77, 95, 96]. In a previous study, rats pretreated with ginger (100 mg/kg/day for 14 days) experienced suppressed indomethacin-induced gastric ulceration (single dose, 20 mg/kg, IP). Ginger therapy improved biochemical and histological changes caused by indomethacin, most likely due to increased antioxidant defenses (GSH and SOD) and decreased lipid peroxidation (MDA) [97]. The key protective characteristic of ginger's natural compounds is their suppression of toxin-induced cytokine production (TNF-α, IL-6, IL-8, IL-2 and IL-1β, PLA2, iNOS, COX-2, and PGE2) [98]. High levels of polyphenolic and flavonoid compounds in ginger extracts might be responsible for the antioxidant and ameliorative activities [99, 100].

5. Conclusion

Administration of the therapeutic dose and 2× the therapeutic dose of the NSAID lornoxicam to rats without ginger extract pretreatment resulted in significant increases in the serum activities of ALT, AST, and ALP, concentrations of urea, creatinine, TNF-α, and IL-6, and sperm abnormalities. However, RBC, PCV, prostaglandin, and sperm counts were significantly decreased in rats administered lornoxicam without ginger extract pretreatment. Congestion, hydropic degeneration, desquamation, and focal necrosis were observed in the kidney, liver, spleen, and testes of rats administered either lornoxicam dose. All observed detrimental effects were more pronounced in rats receiving 2× the therapeutic dose than those administered the therapeutic dose of lornoxicam. These detrimental effects in rats administered 2× the therapeutic dose of lornoxicam were improved significantly by pretreatment with ginger. We recommend providing a ginger extract pretreatment in animals to avoid any possible hepatic, renal, splenic, and testicular injuries induced by administering a high dose of lornoxicam.

Data Availability

All data generated or analyzed during this study are included in this article.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization was contributed by S.M.E, R.R.E., and A.F.H. Methodology was contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H. Software was contributed by R.R.E. and A.F.H. Validation was contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H. Formal analysis was contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H. Investigation was contributed by S.M.E., R.R.E., M.A.A., and A.F.H. Resources were contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H. Data curation was contributed by R.R.E. and A.F.H. Writing–original draft preparation was contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H. Writing–review and editing was contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H. Supervision was contributed by S.M.E. Project administration was contributed by S.M.E. Funding acquisition was contributed by S.M.E., R.R.E., M.A.A., M.K., and A.F.H.

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Review Article

A Background Search on the Potential Role of *Scutellaria* and Its Essential Oils

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Scutellaria (Lamiaceae), which contains over 350 species, usually known as skullcaps, is found throughout Europe, the United States, and East Asia. In traditional Chinese medicine, several species are used to wipe out heat-evil and remove surface ills (TCM). The current study examines the ethnopharmacology, biological activity, and chemical substances associated with *Scutellaria* species. More than 295 chemicals, including flavonoids and diterpenes, have been identified. *Scutellaria* and its active principles have been shown in studies to have a wide range of pharmacological activities, including antioxidant, antimicrobial, antifeedant, phytotoxic, acaricidal toxicity, antibacterial, anti-inflammatory, and antianalgesic activities. Currently, effective monomeric compounds or active components from *Scutellaria* have been evaluated for pharmacological action *in vivo* and *in vitro*. More data facilitates applications and exploitation of novel medication development.

1. Introduction

Scutellaria is one of the largest genera within Lamiaceae. *Scutellaria* is a genus that seems to have 360–469 species that could be obtained in Europe, Latin America, Eastern Europe, and Central America. *Scutellaria* species have a strong tradition of usage as herbal remedies for chemotherapies, hepatic and intestinal disorders, asthma, neurological and cardiovascular ailments, and infectious diseases. Furthermore, contemporary pharmacotherapy has substantiated the traditional applications of *Scutellaria* medicinal species, including *Scutellaria lateriflora L*, *Scutellaria barbata D.* Don, and *Scutellaria baicalensis* Georgi [1–3]. This genus has extensive therapeutic potential, encompassing antioxidative, cytotoxic, anti-inflammation, and antivirus qualities, as well as hepatoprotective and neuroprotective abilities. The ongoing evolution of studies on the genus *Scutellaria* revealed parallels and variances in its range, pharmaceutical components, phytochemical constituents, and pharmacological usage. The diversity of *Scutellaria*'s active substance structures and abundant biological properties suggests that illustrative associations between phytochemical analysis, phylogeny, and therapeutic implications (conventional use and advanced pharmacology) are beneficial for investigators

to easily recognize the medicinal worth of this genus and investigate and exploit it [4–6].

The Lamiaceae (mint) family comprises 350 species of perennial flowering plants from the genus *Scutellaria*, which is endemic to Asian countries, North America, and Europe [1, 2]. This genus has about 300 species, many of which can be found throughout Asia [3–6]. There are 27 *Scutellaria* species in Iran, 12 of which are endemic [7]. From ancient times, *Scutellaria* genus has been utilized to treat hyperlipidemia, hepatitis, inflammation, allergies, arteriosclerosis, and hypertension [8]. *Scutellaria* has been used for over 2000 years in Asian medicine, notably Chinese medicine, to treat high fever, runny noses, influenza, and high blood pressure [9–12]. *Scutellaria* has anticancer, antibacterial, antioxidant, hepatoprotective, anti-inflammatory, and antiviral properties [8]. *Scutellaria* species are also valuable in the mitigation of nervous system conditions such as phenylethanoid glycosides, insomnia, anxiety, and hysteria [2]. Among the 295 compounds identified in this genus are flavonoids [12] and terpenes (diterpenes, monoterpenes, iridoid glycosides, and triterpenoids) [13].

Scutellaria has a high concentration of flavonoids (nearly flavones), which are beneficial chemicals [14]. *S. immaculata* Nevski ex Juz., S. *ramosissima* Popov, and *S. schachristanica* Juz. aerial parts are frequently used to diagnose hypertension, neurodegenerative disorders, and asthma in Uzbekistan. Secondly, they all contain flavonoids and essential oils. Essential oils are secondary plant metabolites that include a wide spectrum of synthetic compounds as well as therapeutic capabilities [15]. They may be harvested from plants in a variety of ways, both classic and cutting-edge [16]. Hydrodistillation, microwave, organic solvent extraction, steam distillation, supercritical CO_2, ultrasonic, and high-pressure solvent extraction are some of the procedures used to extract essential oils [17, 18]. The components of essential oils are regulated by extraction processes, geographic and climatic conditions, plant storage, physiological age, harvesting time, and drying type. Please keep in mind that the contents of several parts of the plant vary [19–22]. Terpenoids, which are classified as monoterpenes, sesquiterpenes, triterpenes, diterpenes, and tetraterpenes, are the most significant essential oil components [23, 24]. Essential oils protect plants from predators, environmental stress, parasites, and illness, and they also attract insects. They also help in attracting insects to provide an effective reproductive phase [25].

Scutellaria baicalensis may be safe to consume by mouth for the vast majority of individuals. It has the ability to induce sleep. Patients who took *Scutellaria baicalensis* had fever and lung irritation. However, there is insufficient information to determine if Scutellaria baicalensis is the cause of these adverse effects. A few also show that some products containing *Scutellaria baicalensis* may cause liver issues in some people. Flavocoxid, a unique combination drug, was determined to be safe in 12-week study studies. However, some people may develop liver issues as a result of this or other combination products. This negative impact does not appear to be prevalent, and it may only be felt by people who have an allergic reaction [11–15].

In this review, we have mentioned the antioxidant, antimicrobial, antifeedant, phytotoxic, acaricidal toxicity, antibacterial, anti-inflammatory, and antianalgesic activities of *Scutellaria* and its essential oils. The current review was compiled using content from the databases Web of Knowledge, Chemical Abstracts, Scopus, PubMed, ScienceDirect, and Google Scholar.

2. Traditional Uses

Per this study, around 50 species, 5 subspecies, and 17 variations of *Scutellaria* were utilized using natural remedies (aerial portion, rhizome, or whole plant) in countries like China, Nepal, India, North America, Nepal, Turkey, and Uzbekistan [26, 27]. *Scutellaria* medicinal herbs are being used to treat several diseases, which include peptic diseases (stomach pain, dysentery, bloating), liver function and gall diseases (jaundice, hepatitis), infections (carbuncles, furunculosis), neurological conditions (epilepsy, insomnia, chorea, spasm, hysteria), respiratory problems (respiratory infections, colds, scarlet fever), and traumatic injuries. Also, variability in the pharmacologic portions of *Scutellaria* may be attributed to morphological differences in *Scutellaria* and medication usage choices in various places [27–30]. Conventional herbal remedies in China are created from 32 species and 13 variations, the largest of which are found in the southern region. These medicinal plants' most commonly used sections are the root and, indeed, the entire plant. *Scutellaria* species with fleshy rhizomes are used as medicine in China including *Scutellaria rehderiana*, *Scutellaria baicalensis*, *Scutellaria amoena*, and *Scutellaria viscidula*. Additionally, as per traditional Chinese theory, certain species often have the quality of removing heat, moisture, and poisonous materials, as well as having a significant impact on treating upper respiratory tract infections and acute gastroenteritis [30–34]. *Scutellaria* plants are used whole as remedies; on the other hand, they often have short roots and little plants, such as *Scutellaria barbata*, *Scutellaria galericulata*, *Scutellaria indica*, and *Scutellaria sessilifolia*. These species frequently have the qualities of removing heat, wet, and poisonous elements, boosting blood circulation to alleviate blood stasis, and lowering inflammation to reduce pain, according to traditional Chinese medicine theory [34–36]. Furthermore, certain species, according to traditional Chinese thought, can remove heat, moisture, and harmful elements, as well as have a major influence on curing upper respiratory tract infections and acute gastroenteritis [37–40]. *Scutellaria* plants used as medicines contain short root systems as well as little plants, including *Scutellaria indica*, *Scutellaria barbata*, *Scutellaria galericulata*, and *Scutellaria sessilifolia*. According to the Chinese medicine concept, those species typically get the benefits of eradicating heat, dampness, and poisonous elements, increasing circulation of blood to decrease blood stasis, and decreasing inflammation to ease the pain. They have been shown to treat traumatic injuries, furuncles, and furunculosis and to also reduce inflammation derived from a variety of disorders. *Scutellaria baicalensis* and *Scutellaria barbata* D. are both frequently used natural medicines in China, and

they are also used in other Asian countries such as Japan and Korea [40–45].

3. Essential Oils

Aromatic plants produce natural volatile oil molecules with a strong odor as bioactive compounds. They are primarily twisted with the use of steam or hydro-distillation, which was initiated in the Middle Ages. Because of their antiseptic, i.e., virucidal, bactericidal, fungicidal, and therapeutic aspects, as well as their scent, they are utilized in embalming, food preparation, and as antibacterial, painkiller, sedative, anti-inflammatory, antispasmodic, and topically anesthetic therapies [45–48]. These characteristics have not altered substantially up to the present day, other than that so much is already understood about all of their modes of action, notably at the antimicrobial level. Essential oils are a crucial component in plant protection throughout nature as antibacterial, antifungal, pesticides, antivirals, and also against herbivores by limiting their hunger for those kinds of plants. They might even invite particular insects to aid in pollen and seed propagation while repelling others. Essential oils are extracted from several fragrant plants that are found in subtropical to warm climates such as those of the Mediterranean and tropical areas when compounds represent a large element of conventional pharmacopeia [48–50]. These were liquid, volatile, clear, but seldom colored, fat-soluble, and common organic solvents, with a density that is generally lower than water [50–55]. There are several approaches to extracting essential oils. The usage of liquid carbon dioxide or microwaves, as a greater or lower pressure distillation using boiling water or heated vapor, is an example of this. Healthcare and culinary usage are now becoming increasingly prevalent as compared to synthetic pharmaceutical goods to safeguard the natural balance due to their bactericidal and fungicidal capabilities. Extraction by steam distillation or expression, for instance, is favored in certain circumstances [55–60]. Extraction with lipid-soluble solvents and, in some cases, supercritical carbon dioxide is preferred for fragrance applications. Thus, the biochemical signature of essential oil products changes not only in the number of molecules extracted but also in the stereochemistry of molecules extracted depending on the mode of extraction, which is decided based on the aim of usage. Climate, age, plant organs, soil composition, and vegetative cycle phase can all improve the level and quantity, as well as proportions of the extracted product. To generate essential oils with a consistent constitution, they must be extracted under such conditions within the same organ of a plant that has grown in the same soil, location, and season. Gas chromatography and mass spectrometry studies are used to chemotype the majority of marketed essential oils. To certify the purity of essential oils, analytical monographs have already been issued (ISO, European pharmacopeia, WHO, Council of Europe) [60–65]. Essential oils have been used for qualities that have already been found in nature, such as antifungal, antibacterial, and insecticidal effects. Approximately 3000 essential oils are currently known, and approximately 300 of them would be economically relevant, particularly in the cosmetic, medicinal, food, agronomic, sanitary, cosmetic, and aroma sectors. Essential oils with their ingredients are applied in aromas and cosmetics, hygiene goods, medicine, horticulture, food custodians and supplements, and organic cures. D-Limonene, d-carvone, and geranyl acetate, for instance, are used in aromas, moisturizers, cosmetics, as fragrance enhancers in food, odors in-home cleaning chemicals, and as lubricating oils [65–68]. Further, essential oils are utilized in massaging as a combination with vegetable oil, in spas, and often in aromatherapy. Essential oils appear to have specific therapeutic characteristics that have been proposed to treat some organ dysfunctions or systemic disorders. Due to the high demand for natural, unadulterated substances in many industries, essential oils are utilized widely throughout the world and are continuing to expand in popularity. As a result, a lot of essential oils are produced around the world to support the cosmetics, aromatherapy, and phytomedicine businesses. Most of them have been efficient substitutes or supplements to added compounds used in the chemical process industries but generally do not get the same side effects [68–73].

4. Obtaining Evidence

Far more research reports on the essential oils of several *Scutellaria* species were studied [26–32]. Even though several assessment reviews on the *Scutellaria* genus have been performed [8, 13], knowledge regarding *Scutellaria* essential oils is lacking. As a result, this study focuses on the chemical components and biological activity of essential oils extracted from the *Scutellaria* genus.

5. Chemical Constituents

A review of the most current studies on the essential oils of various *Scutellaria* species was conducted [26–32]. While several overview aspects of the *Scutellaria* genus have been undertaken [8, 13], there is little research on *Scutellaria* essential oils. In conclusion, these studies were based on the chemical components and pharmacological effects of essential oils obtained from the genus *Scutellaria*. The concentration of essential oils varies according to the harvesting season, drying conditions, subspecies type, soil pH, geographic location, subspecies type, plant part, and extraction technique [19–22].

Essential oil compositions are depicted in supplementary file figures a to d; sesquiterpenes are the most abundant component in *Scutellaria* essential oils. The bulk of the essential oils in this species includes β-farnesene, hexadecanoic acid, β-caryophyllene, germacrene D, linalool, and eugenol. Figure 1 depicts the structures of these compounds. *Scutellaria* species have been shown to contain a variety of hydrocarbons and oxygenated terpenoid chemicals (Figure 1). Hexadecanoic acid, a saturated fatty acid, is found in plants, animals, and microbes [33]. Germacrene D is a sesquiterpene pioneer of cadences and selinenes [34, 35]. Germacrene D kills mosquitoes, aphids, and ticks [36–38]. β-Caryophyllene is a phytocannabinoid-rich sesquiterpene that may help with neuropathic pain, anxiety, endometriosis, ulcerative colitis, and renal safety [39–42]. Linalool is a

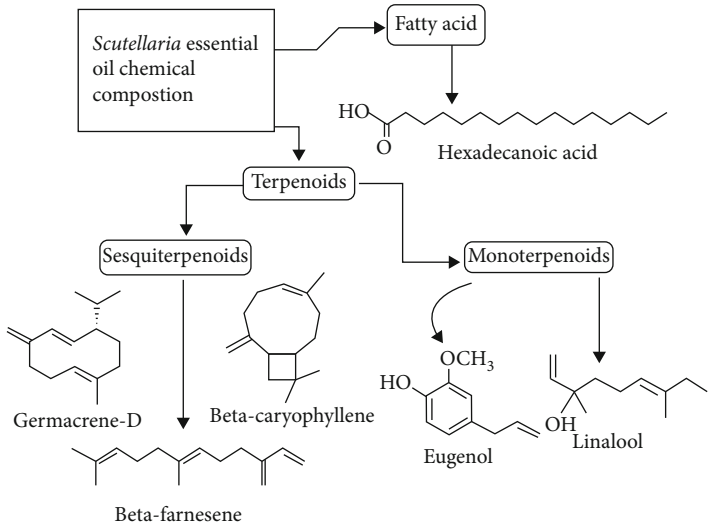

Figure 1: Chemical structures and among the major ingredients found in *Scutellaria* essential oils.

monoterpene component found in a wide variety of plants that has antinociceptive, antibacterial, and antihyperalgesic properties, but also antibacterial and antifungal action against a variety of pathogens and fungi [43]. Farnesene is a potent pheromone in the vast majority of aphid species [44]. Considering several publications on the essential oils of *Scutellaria* species (over 38), a wide variety of species remain unexplored. Additional investigation into the chemical properties of unreported *Scutellaria* essential oils is thus required [70].

The primary components of *S. diffusa* oil remained revealed to be hexadecanoic acid with 30% and caryophyllene oxide with 9%. Germacrene D with 21%, hexadecanoic acid with 16%, and β-caryophyllene with 13% were found identified to be critical elements in S. heterophylla oil. Germacrene D with 40% was perhaps the most important element of *S. salviifolia* oil, preceded by bicyclo germacrene with 14% and β-caryophyllene with 4% [70–75].

The essential oils extracted by hydrodistillation from extracts of three *Scutellaria* brevibracteata subspecies (subsp. subverting, subs. brevibracteata, and subsp. Pannosula) from their natural locations in Turkey were all studied simultaneously using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Depending on the chromatographic study and data analysis, the main determinants in S. brevibracteata subsp. brevibracteata oil were found to be β-caryophyllene (22.8%) and caryophyllene oxide (22.8%). The major components of S. brevibracteata subsp. subvelutina oil were 28.3% β-caryophyllene, 12.4% linalool, and 10.8% hexadecanoic acid. *S. brevibracteata* subsp. peninsula oil contains high levels of β-caryophyllene (36.4%), β-cadinol (9.8%), β-cadinene (7.0%), and linalool (5.3%). It was, to the best of our knowledge, the first research on the chemical properties of three *Scutellaria brevibracteata* essential oils. TLC, LSC, GLC, and GLC-MS methods were employed to analyze the essential oil *Scutellaria lateriflora* L. (Labiatae) generated by hydrodistillation in northern Iran. In the oil, there were at least 73 different substances. Low quantities of nonterpenoid components were discovered [75–80].

The Lamiaceae family includes *Scutellaria volubilis* and *Lepechinia paniculata*. In Ecuador, they are commonly utilized in the traditional system of medicine. After hydro distillation, the structural activity and chemical characteristics of essential oils derived from aerial parts of *Scutellaria volubilis* in its foliation-blooming period and *Lepechinia paniculata* in its propagation season remained explored. Gas chromatography/mass spectrometry (GC/MS) and gas chromatography/flame ionization detection (GC/FID) approaches were used to examine the composition of these essential oils. The essential oil of *Scutellaria volubilis* was found to have 37 components. Sesquiterpene hydrocarbons are also confirmed to be the primary elements: germacrene D with 20.4%, β-caryophyllene with 17.5%, β-humulene with 14.7%, and β-bisabolene with 5.8%. The essential oil of *L. paniculata* comprised 34 components, the overwhelming of which were sesquiterpene hydrocarbons such as aromadendrene (24.6%), viridiflorene with 12.4%, β-selinene with 7.4%, and valencene with 6.7%. Monoterpene hydrocarbons were identified in smaller concentrations, as was β-phellandrene with 6.9% and with 7.7%. Including both species, oxygenated monoterpenes and sesquiterpenes constitute less than 5%. This is the first description of this species' chemical characteristics [80–85].

Scutellaria albida L. subsp. albida, *Scutellaria albida* L. subsp. Colchica *[Rech.f.]J.R.Edm.*, *Scutellaria albida L.* subsp. Condensata [*Rech.f.]J.R.Edm.*, and *Scutellaria albida* L. subsp. Velenovskyi [Rech. Linalool with 20% and 29%, respectively, was found recognized as a prominent compound in the oils of S. albida subsp. albida and *S. albida* subsp. condensata. Hexadecanoic acid with 13% was discovered to be a significant constituent in the oil of S. albida subsp. colchica. The oil of *S. albida* subsp. velenovskyi has the highest proportion of -caryophyllene (20%) [85–90].

6. Essential Oils and Their Specificities

Essential oils are the main raw material for the aroma and fragrance, food, and pharmaceutical industries. Essential oils are concentrated plant extracts that retain the natural smell and flavor, or "essence," of their source. Some authors investigated the relationship between lavender essential oil production and gene expression during blooming, aiming to determine the optimum period for essential oil harvest [90]. Other researchers came to the conclusion that changes in chemical variety have a mosaic pattern, which is tied to changes in predator activity and may be influenced by geographic factors or natural enemies. In terms of antigenotoxicity, all of the essential oils assessed showed this preventive effect. Moreover, the manner of defense varied according to the mutagens, i.e., the kinds of defects generated and therefore the kinds of enzymatic identification and activation resulting in translational synthesis or late apoptosis/necrosis [90–95].

7. Biological Actions of Scutellaria Essential Oils

7.1. Antioxidant Capability. The antibacterial properties of *Scutellaria* genus essential oils are quite low relative to the antioxidant potential of *Scutellaria* extracts [70–72]. Mamadalieva et al. investigated the antioxidant properties of three Uzbek *Scutellaria* species' essential oils (*Scutellaria ramosissima*, *Scutellaria immaculata*, and *Scutellaria schachristanica*). These *Scutellaria* essential oils demonstrated significant antioxidant action due to the incorporation of eugenol, thymol, and carvacrol; however, it was less than ascorbic acid [57]. The antioxidant effect of a Scutellaria baicalensis trihydroxyflavone extract on oxidation generated by UV radiation was investigated using a phosphatidylcholine liposome membrane. The antioxidative activity of baicalin, baicalein, wogonin, and butylated hydroxytoluene (BHT) was also examined as standards [95].

The chemical confirmation of essential oils found from aerial sections of *Scutellaria immaculata Nevski ex Juz.*, *Scutellaria ramosissima* M. Pop., and *Scutellaria schachristanica* Juz. (Lamiaceae) rising wild in Uzbekistan was studied using GC and GC–MS. The foremost components of *S. immaculata* essential oils appear to be acetophenone with 30.39%, eugenol with 20.61%, thymol with 10.04%, but also linalool with 6.92%, whereas *S. schachristanica* essential oils appear to be acetophenone with 34.74%, linalool with 26.98%, but also eugenol with 6.92%. Germacrene D with 23.96%, β-caryophyllene with 11.09%, linalool with 9.63%, and hexadecanoic acid comprise the oil of *S. ramosissima* with 8.34%. In DPPH, ABTS, and FRAP experiments, the essential oils of *Scutellaria* species displayed lesser antioxidant activity. Especially eugenol showed a significant lowering power in the FRAP experiment (IC_{50} = 2476.9215.8 (mM Fe(II)/g)) [95–100].

7.2. Antimicrobial Activity. The physiological effects of *Scutellaria* essential oils have been examined, with the majority of the research focusing on antibacterial activity. Eugenol, linalool, and other long-chain alcohols may be responsible for the antibacterial properties of these oils [73]. Antibacterial action may also be aided by other essential oil components such as thymol and alpha-terpineol [74, 75]. Khotimchenko and Yakovleva explored the antibacterial properties of S. barbata essential oils contrary to 17 microorganisms (*Pseudomonas aeruginosa*, *Salmonella paratyphi-A*, *Klebsiella pneumonia*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Serratia liquefaciens*, *Escherichia coli*, *Staphylococcus haemolyticus*, *Candida tropicalis*, *Staphylococcus simulans*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Citrobacter freundii*, *Shigella flexneri*, and *Candida albicans*) utilizing the disc diffusion and broth microdilution approaches. Because the essential oil had such significant bacteriostatic activity, their data indicated that *S. epidermidis* was perhaps the furthermost sensitive microbe (29 mm inhibition zone and 0.77 mg/mL MBC), whereas *C. albicans* was possibly the most resistant (7-9 mm and 24.50 mg/mL MBC) [69]. The essential oils of *S. strigillosa*, according to Zhu et al., are substantially more efficient against Gram-positive bacteria and fungi than against Gram-negative germs [53]. Thus, according to Pant et al., essential oils of *S. grossa* exhibited antimicrobial properties toward *K. pneumonia*, *E. faecalis*, *B. subtilis*, and *S. enterica* [65]. As per Skaltsa et al., essential oils of *S. rupestris* and *S. sieberi* isolated in Greece had limited potency against S. aureus and B. cereus [28]. Skaltsa et al. discovered that perhaps the essential oil of S. albida subsp albida showed activity against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. cerevisiae* due to the huge amount of linalool and nerolidol [27]. Dereboylu et al. investigated the antibacterial properties *of S. aureus*, *E. coli*, *S. typhimurium*, *B. subtilis*, *E. faecalis*, and *P. aeruginosa* active ingredients against seven bacteria and one fungus (*S. aureus*, *S. typhimurium*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *B. subtilis*) [58]. The essential oil of *S. repens* being tested evaluated antibacterial activity on *A. tumefaciens*, *E. faecalis*, *K. pneumoniae*, *X. phaseoli*, *S. aureus*, *E. coli*, *S. enterica*, *E. chrysanthemi*, and *P. multocida* [67], where the zone of inhibition for *E. coli* was 23 mm, accompanied by *E. faecalis* at 18 millimeters, *K. pneumonia* at 15 millimeters, and then *B. subtilis* at 12 mm [67].

The plant *Scutellaria barbata* D. Don (Lamiaceae) is endemic to south China. This plant, identified as Ban-Zhi-Lian in Chinese traditional medicine, has been recognized as an antitumor, anti-inflammatory, and diuretic ingredient. *S. barbata* formulations have exhibited leads to growth inhibition in a variety of malignancies. The plant has been applied in the mitigation of digestive system malignancies, lung cancer, breast cancer, hepatoma, and chorioepithelioma in therapy. The plant is expected to possess alkaloids and flavonoids. Polyphenols (apigenin and luteolin) have been identified as bioactive components toward methicillin-resistant Staphylococcus aureus from just a 50% ethanolic leaf extract. E-1-(40-Hydroxyphenyl)-but-1-en-3-one was extracted from a methanolic extraction of the leaves and showed high cytotoxicity in K562 human leukemia cell lines. There is still no published evidence on the chemical components and antibacterial action of *S. barbata* essential oil.

Scutellaria barbata essential oil has been produced by hydrodistillation with a 0.3%(*v*/*w*) yield as well as examined by GC and GCMS. Hexahydrofarnesylacetone with 11.0%, 3,7,11,15-tetramethyl-2-hexadecane-1-ol with 7.8%, menthol with 7.7%, and 1-octen-3-ol with 7.7% were the primary components in the oil with 7.1%. The oil's antibacterial efficacy toward 17 microorganisms has been tested through disc diffusion and broth microdilution techniques. Gram-positive bacteria were just more tolerant to the oil than Gram-negative bacteria and yeasts, notably methicillin-resistant *Staphylococcus aureus*.

The aerial portions of three endemic *Scutellaria* species from Lamiaceae are being studied for glandular trichomes shape, volatile content, and antibacterial activity. The examined species have two kinds of glandular trichomes that are morphologically unique. Capitate trichomes were found in all three taxa studied. Peltate glandular trichomes featured a large secretory head with one central and three to eight periphery cells. Even in the petiole of *S. cypria* var. elatior could peltate trichomes found. Steam distillation in a Clevenger type system yielded 0.26-0.47% (*v*/*w*) volatiles and essential oil combinations from three species. The GC-FID and GC-MS methods were used for their studies. By comparing their relative retention indices, mass spectra, and certain literature records, these chemicals were identified. There was a total of 23 identified components. While overall volatile % ages were found to be 99.99%, essential oil yield varies from 34.64 to 92.25% for three species. Trans-caryophyllene with 22.58% and germacrene D with 42.01% were found to be key components of *S. sibthorpii*. Eugenol with 23.05% and palmitic acid with 27.00% were found considered to be significant components of *S. cypria* var. cypria. Furthermore, *S. cypria* var. elatior has a substantial fraction of linalool and palmitic acid, with 10.92% and 46.76%, respectively, where the MIC values of the volatiles towards Gram-negative and Gram-positive bacteria varied from 10 to 20 mg/mL. *C. albicans* was shown to be among the furthermost resistant yeast-like fungus, with MIC values found to be high than 20 mg/mL.

7.3. Antifeedant Activity. The essential oils of three *Scutellaria* species (*Scutellaria orientalis* ssp. Alpina, *Scutellaria brevibracteata*, and *Scutellaria hastifolia*) were tested in contradiction to the feeding and egg-laying activities of *Spodoptera littoralis* in research published by Formisano et al. Both *S. brevibracteata* and *S. hastifolia* extraction inhibited female moth egg laying on sheets; however, only *S. hastifolia* essential oil halted *Spodoptera littoralis* larvae from eating on mitigation discs [47]. As per Giuliani et al., the essential oil of *S. rubicunda* subsp. *linnaeana* has antifeedant action in contradiction of *Spodoptera littoralis* [26]. *S. littoralis* is a contraction for the letter S. The essential oil of the plant generated a dose-dependent positive feeding response in littoralis larvae. *Scutalbin* C, *Scutecyprol* B, and *scutecyprol B* remained initiate in aerial sections of *S. rubicunda subsp. linnaeana*. Linnaeana (FI at 100 ppm = 100) was antifeedant to larvae of five Lepidoptera species [1].

7.4. Phytotoxic Effect. Phytochemicals from various *Scutellaria* species displayed substantial cytotoxic effects on some human tumor cell lines in vitro. Flavonoids, neoclerodan diterpenoids, iridoids, phenyl alcohol glycosides, and alkaloids have all been identified from *Scutellaria* species. *S. strigillosa* essential oil has been studied for phytotoxicity to amaranth and bluegrass (amaranthus is a worldwide genus of annual or short-lived perennial plants, while bluegrass refers to numerous species of grasses of the genus Poa). Amaranthus seedling progress was completely inhibited by 3 μL/mL essential oil, although bluegrass growth was just slightly affected [53].

7.5. Acaricidal Toxicities. The acaricidal toxicities of 1-hydroxynaphthalene from *S. barbata* oil and its derivatives were determined and compared with those of benzyl benzoate. *S. barbata* essential oil has potent acaricidal action than the control sample (benzyl benzoate) [45].

7.6. Antibacterial Activity. S. grossa essential oil extracts had potent antibacterial effects on *Bacillus subtilis* and *Enterococcus faecalis* (MIC, 31.25–62.5 μL/mL), along with *Klebsiella pneumoniae* as well as *Salmonella enterica* (MIC, 125 μL/mL). *S. lindbergii* Rech.f. ethanolic had to have a considerable antibacterial property, with a MIC value of 6.25 mg/mL^{-1}. The steam volatile oil derived from *S. repens aerial* parts displayed identified potential towards *E. faecalis* (the MIC was found to be 125 μL/mL^{-1}), *Escherichia coli* (MIC, 31.25 μL/mL^{-1}), and *Klebsiella pneumoniae* (MIC, 250 μL/mL^{-1}) [56].

The chemical constituents of the steam volatile oil produced by steam distillation of this aerial portion of *Scutellaria grossa* Wall ex Benth. (Lamiaceae) were investigated using capillary GC and GC-MS. Oxygenated monoterpenes were shown to be abundant in the oil (88.6%). There must have been 50 elements discovered, accounting for 94.4% of the overall oil content. Linalool (37.0%) and 1-octen-3-ol (32.0%) were shown to have main components. The oil's antibacterial efficacy towards ten bacterial strains was established by measuring the growth inhibitory zones. The oil had substantial antibacterial action toward Gram-positive *Bacillus subtilis* and *Enterococcus faecalis* bacteria as well as Gram-negative *Klebsiella pneumoniae* but also *Salmonella enterica enterica* bacteria. The minimal inhibitory concentration against *E. faecalis* was reported at 31.25 μL mL^{-1} [56–60].

7.7. Anti-inflammatory Action. Many combinations of *Scutellaria baicalensis* hot water extract (SB-HW) and Chrysanthemum morifolium ethanol extract (CM-E) were tested for anti-inflammatory activity. SB-HW (80 g/mL)/CM-E (120 g/mL) or SB-HW (40 g/mL)/CM-E (160 g/mL) substantially decreased LPS-stimulated NO and IL-6 levels in RAW 264.7 cells. SB-HW (80 g/mL)/CM-E (120 g/mL) was shown to be the most effective combination for suppressing MUC5AC secretion in PMA- and LPS-induced NCI-H292 cells. In PMA-induced A549 cells, the active combination also reduced PGE2 and IL-8 production. According to LC-MS/MS tests, the active combination had a high concentration of flavone glycosides such as baicalin and cynaroside. The active combination inhibited phosphorylation of ERK,

JNK, and p38 on Western blots, showing that MAPK signaling was inhibited. The active combination, according to our findings, might be employed as a new anti-inflammatory herbal medication [101].

7.8. Antianalgesic Activity. *S. edelbergii* crude extract with subfraction analgesic effectiveness was tested in Swiss albino mice at various dosages to treat acetic acid-induced writhes. In this investigation, aspirin was utilized as a control. EtOAc was found to be the most active fraction, with inhibition rates of 37% and 55% at dosages of 50 and 100 mg/kg body weight, respectively, followed by chloroform, with inhibition rates of 29% and 48% at doses of 50 and 100 mg/kg body weight, respectively [102].

8. Additional Information

The cytotoxic potential of essential oils found in this prooxidant behavior can make this effective disinfectant and microbial mitigation for personal use, including air purification, personal hygiene, and even internal use via oral ingestion, and also the pesticide approval process for agricultural or supply product preservation [70].

Essential oils have a considerable advantage in that they can be free of long-term genotoxic effects. Furthermore, several of them still have strong antimutagenic potential, which could also be connected to anticarcinogenic action. Current findings have shown that the prooxidant activities of essential oils or certain of their ingredients, as well as those of many polyphenols, are particularly efficient in reducing either tumor volume or tumor growth using apoptotic and/or necrotic activities. Myrica gale essential oil possesses an anticancer effect on lung and colon cancer cell lines, per Carson and Riley [75]. *Nigella sativa* has been established to have an antiproliferative effect and suppressed 1,2-dimethylhydrazine-induced malignancy in rats by Sun et al. Because essential oils have the potential to interact with mitochondrial activity, they would add prooxidant effects and even become true antitumor agents. Several radical-producing drugs are used in anticancer therapies. In the case of essential oils, oxidative generation may be strictly controlled and tailored while representing no harmful or mutagenic risks to healthy cells [44]. Essential oils or related bioactive constituents might be integrated into vectorized liposomes, allowing for more precise quantification. As a result, essential oils may force their way from the classical to the pharmaceutical sphere [76].

Scutellaria is a genus of seldom shrubs, herbs, or subshrubs, with a broad range of structures. Many plants in this genus are being used as conventional remedies to cure a variety of ailments all over the world, with their most popular medicinal portions including the root, aerial part, and complete plant. As per the reports, aerial parts of around 12 species, 5 subspecies, and 1 variation have been used in conventional ways. The majority of them will be capable of treating the neurological disorder, trauma, allergies, peptic, cerebrovascular illnesses, hepatic cardiovascular and gallbladder disorders, and malignancy. Likewise, the majority of the therapeutic plants of the genus *Scutellaria* are tiny plants, have short roots, and are present mostly in Europe, Latin America, Asia, and Southwest Asia. The roots of around 16 species and 4 variations are also used to treat a variety of diseases, including respiratory disorders, miscarriage, liver and gall problems, gastric diseases, hypertension, insomnia, and trauma, and are primarily found in China. This species was distinguished by its well-developed rhizome features. Cold symptoms, infectious diseases, snake bites, hepatitis, peptic disorders, gynecologic inflammation, internal injury, renal vacuousness, lumbar discomfort, pyelonephritis, migraine, toothache, major trauma, tinea of the feet and hands, and miscarriage are among the ailments treated with the whole plant. These species, which are mostly perennial plants and subshrubs, are found across East and South Asia. As a result, the identification of therapeutic components may be influenced by vegetation structure and regional medical practices. Moreover, due to its several flavonoids, *Scutellaria* has been deemed a distinct genus of *Scutellarioideae*, and while several chemicals, specifically a number of diterpenes, have already been discovered in *Scutellaria* in recent times. There are also variances in the dispersion of the chemicals based on established chemistry research on these therapeutic plants in the genus *Scutellaria*. Is there a link between many medical components, organic compounds, and applications? As we investigated *S. baicalensis* and *S. barbata*, the two largest studied species in terms of nutrients, we determined the difference in chemical structure, pharmacological components, and therapeutic properties [66]. *Scutellaria baicalensis* is an herbaceous plant with a dense and meaty rhizome, and the dried root of *Scutellaria baicalensis* has an abundance of 4′-deoxyflavones, which lead to *Radix Scutellariae*'s clarifying warmth and detoxing properties. *S. barbata*, on hand, is a perennial herb with a small and thick rhizome that comprises flavonoids and is high in neo-clerodane diterpenoids, and the entire plant has been used to treat cancer, major injuries, and carbuncles [55–60]. Neo-clerodane diterpenoids are recognized to be reflective components of *S. barbata*. Moreover, they are the existing research area of *S. barbata* with varied geometries and strong carcinogenic and antifeedant actions. Hence, it is suggested that every species' different molecular components coincide with unique medical value and that their pharmacological purposes correlate with their morphological features. Ignoring the fact that so much research on their chemical characteristics is concentrated on the pharmacological parts, and various bioactive components have been separated from *Scutellaria* medicinal plants, their impact on the plants' claimed medical benefit or demonstrable pharmacological properties has not yet been satisfactorily studied. Furthermore, phytochemistry research on nearly 50% of those phytochemical constituents has not yet been completed. As a result of insufficiently defined studies on these herbs and shrubs, the links between the therapeutic component chemical characteristics and traditional usage in other medicinal belonging to the genus *Scutellaria* require future investigation. More studies need to be done to look for particular bioactive components in *Scutellaria* plants [44]. *Scutellaria* is a Lamiaceae genus that has been used as medicine for countless generations [76, 77]. Multiple

investigations into the essential oils of several *Scutellaria* species have now been conducted [8, 13, 26–32]. Nonetheless, because several species of the *Scutellaria* genus will not be researched, many studies on the composition and biologically active compounds of underresearched *Scutellaria* essential oils may be completed. The present investigation highlights the chemical characteristics and bioactivities of the *Scutellaria* genus (antioxidant, antifeedant, antibacterial, phytotoxic, and acaricidal activities). The essential characteristics comprised β-farnesene, hexadecanoic acid, β-caryophyllene, germacrene D, linalool, and eugenol (any of these oil constituents contain therapeutic capabilities). This article can be used as a guide in the disciplines of essential oils and ethnopharmacology.

9. Conclusion

Essential oils are secondary metabolites with a variety of organic structures that have therapeutic activity depending on their content. With about 250 species, *Scutellaria* is a Lamiaceae genus of perennial plant species. For a long time, it has been used to treat hypertension, arteriosclerosis, allergies, hyperlipidemia, inflammatory illnesses, hypertension, and hepatitis. Several substances, particularly essential oils, have been discovered in numerous studies on the chemical constituents of the *Scutellaria* genus. Various compounds have been discovered in studies on the chemical compositions of essential oils from the *Scutellaria* genus. Chemical constituents and biological activities of *Scutellaria* essential oils were identified. The main components of this genus' essential oils are hexadecenoic acid, farnesene, caryophyllene, germacrene D, linalool, and eugenol. Although 38 studies on the essential oils of *Scutellaria* species are still available, there appears to be a large number of species that need to be investigated. As a result, more research is needed into the various elements and pharmacological actions of unstudied *Scutellaria* essential oils. Based on available data, this study examined studies on the chemistry and biochemical processes of *Scutellaria* essential oils, including phytotoxic, antioxidant, antimicrobial, antifeedant, and antiparasitic toxic effects.

Data Availability

No associate data is present in this review. Although if necessary, corresponding author will update.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Md. Habibur Rahman was responsible for the conceptualization. Mehrukh Zehravi and Chenmala Karthika wrote the original draft. Abul Kalam Azad, Zubair Ahmad, Farhat S. Khan, Md. Sohanur Rahman, Rokeya Akter, and Md. Habibur Rahman reviewed and edited the manuscript. Finally, all authors approved for submission of final version of the manuscript.

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Supplementary Materials

Figure 1: the relative abundance of sesquiterpene hydrocarbons in *Scutellaria* species essential oils. Figure 2: the relative abundance of monoterpene hydrocarbons in *Scutellaria* species essential oils. Figure 3: the relative abundance of oxygenated sesquiterpenes in *Scutellaria* species essential oils. Figure 4: the relative abundance of oxygenated monoterpenes in *Scutellaria* species essential oils. *(Supplementary Materials)*

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Research Article

Identification of Novel and Safe Fungicidal Molecules against *Fusarium oxysporum* from Plant Essential Oils: In Vitro and Computational Approaches

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Phytopathogenic fungi are serious threats in the agriculture sector especially in fruit and vegetable production. The use of plant essential oil as antifungal agents has been in practice from many years. Plant essential oils (PEOs) of *Cuminum cyminum*, *Trachyspermum ammi*, *Azadirachta indica*, *Syzygium aromaticum*, *Moringa oleifera*, *Mentha spicata*, *Eucalyptus grandis*, *Allium sativum*, and *Citrus sinensis* were tested against *Fusarium oxysporum*. Three phase trials consist of lab testing (MIC and MFC), field testing (seed treatment and foliar spray), and computer-aided fungicide design (CAFD). Two concentrations (25 and 50 μl/ml) have been used to asses MIC while MFC was assessed at four concentrations (25, 50, 75, and 100 μl/ml). *C. sinensis* showed the largest inhibition zone (47.5 and 46.3 m^2) for both concentrations. The lowest disease incidence and disease severity were recorded in treatments with *C. sinensis* PEO. *Citrus sinensis* that qualified in laboratory and field trials was selected for CAFD. The chemical compounds of *C. sinensis* PEO were docked with polyketide synthase beta-ketoacyl synthase domain of *F. oxysporum* by AutoDock Vina. The best docked complex was formed by nootkatone with -6.0 kcal/mol binding affinity. Pharmacophore of the top seven *C. sinensis* PEO compounds was used for merged pharmacophore generation. The best pharmacophore model with 0.8492 score was screened against the CMNP database. Top hit compounds from screening were selected and docked with polyketide synthase beta-ketoacyl synthase domain. Four compounds with the highest binding affinity and hydrogen bonding were selected for confirmation of lead molecule by doing MD simulation. The polyketide synthase-CMNPD24498 showed the highest stability throughout 80 ns run of MD simulation. CMNPD24498 (FW054-1) from *Verrucosispora* was selected as the lead compound against *F. oxysporum*.

1. Introduction

The secondary metabolites produced by plants play an important role in plant defense mechanism [1]. Most of the plant essential oils (PEOs) are secondary metabolites and found to be involved in plant defense system as antioxidant, antifungal, or antimicrobial [2]. Very large contribution of the PEOs has been reported in traditional medicine manufacturing for the last many decades [3]. PEOs have been commercially used in pharmaceutical, cosmetics, and beverage industry [4].

The need for use of biological agents in pest management has evolved due to irreversible and drastic effects of synthetic pesticides in the environment and human health [4]. The conventional biological pest control agents are parasitoid, predators, microbe, and fungi, but PEOs have also been assigned in this category because of their origin as plant secondary metabolites. They have become popular as an integral part of integrated pest management (IPM) because of the antimicrobial, antioxidant, and antifungal properties [5]. The antimicrobial properties of PEOs are rendered by their terpenoid and phenol constituents [6]. These compounds have been found safe for humans, animals, and environment, when used in food medicines and pesticides [7]. The use of PEOs, as a substitute of synthetic chemicals, has been recommended in the European Union directive vide 2009/128/CE [8].

More than 30% of the crops, from sowing till harvesting even in stored conditions, are caused by phytopathogenic fungi [9]. Various chemical products have been in use for controlling fungal plant disease. But the use of PEOs as an antifungal agent is getting popular in crop protection sector from the last two decades [10]. The *in vitro* assay of PEO can be performed by using various parameters. The efficacy of PEOs against phytopathogenic fungi can be tested in laboratory by evaluating, as lethal concentration (LC50), minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) [11].

The genus Citrus of Rutaceae family includes about seventeen species distributed throughout the tropical and temperate regions [12]. Its fruit is used as deserts, and the unique aroma of this plant is due to its essential oils present in the leaf, peel, and bark [13]. Various investigations reveal the antimicrobial, antifungal, antioxidant, and radical-scavenging properties of the biological active molecules found in these PEOs [14]. The essential oils from *Citrus limon* and *Citrus aurantifolia* have been reported as strong inhibiter of phytopathogenic fungi growth in different crops [15].

Tomato (*Lycopersicon esculentum*), belonging to the family Solanaceae, is an important crop grown worldwide. It is an important crop of summer and usually regarded as the pole of kitchen gardening, used in sauces and different food stuff compositions [16]. Tomato cultivation is affected by various kinds of pathogenic disease caused by viruses, fungi, bacteria, and mycoplasma. Various kinds of fungal diseases are responsible for affecting the tomato production worldwide which are early blight of tomato, late blight of tomato, and tomato wilt disease. According to an estimate, late blight and early blight of tomato are responsible for almost 49-91% yield losses in Pakistan [17].

Fusarium wilt is a soil-borne fungal disease of solanaceous plant caused by *Fusarium oxysporum* fungus. Fusarium wilt is very common in tropical southern areas during warm-to-hot weather [18]. Pathogen blocks the xylem vessels due to which plant wilts and dies off. Fungal pathogens can cause 80% of plant diseases [19]. Since antiquity, *F. oxysporum* f. sp. lycopersici, as with all phytopathogenic fungus, has posed a hazard to agriculture [20]. Chemical fungicides, while commonly employed, are costly and polluting and provide a danger of toxicity to the planters and hence do not form a crop management technique for long-term development [21].

The use of computational approaches, of computer-aided drug design (CADD), is getting popular in pesticide development research and industry from the end of the last decade [22]. The increasing rate of pesticide resistance development, human health hazards, and environment pollution by synthetic pesticides tends to an urge of development of novel effective and safe molecules for agricultural industry. The pesticide development in the wet labs is a tedious expensive and time-consuming job. The pipeline of techniques used in CADD is based on the initial screening of chemical compounds which leads to narrowing down the dataset consisting of the effective and potential molecules [22]. This regime changes in pesticide development from conventional methods to combining with computational technology have been proved a progressive trend [23]. These fast and smart strategies of computer-aided pesticide design (CAPD) can be useful in lead molecule identification. To avert the emergence of pesticide resistance, CADD techniques deliver baseline knowledge regarding potentially safer pesticide compounds and their target site [8].

The currently planned study was targeted to detect *in vitro* antimicrobial effectiveness of five medicinal PEOs against *Fusarium oxysporum*. In the first step of the study, MIC and minimum fungicidal concentrations were evaluated for ten essential oils against *F. oxysporum*. Secondly, three PEOs showing good results in the first step were tested as foliar application and seed treatment; lastly, one of the qualified essential oils was used for computer-aided fungicide design. In the current study, we not only have identified a new, safe, and effective antifungal molecule against *F. oxysporum* but also new target site. This can become helpful in pesticide resistance management due to target site insensitivity. These findings are recommended to be confirmed in wet lab experiment and can be used in novel, safer, and effective fungicide against *F. oxysporum*.

2. Materials and Methods

The seeds (*Allium sativum*, *Trachyspermum ammi*, *Cuminum cyminum*, and *Syzygium aromaticum*), peel (*Citrus sinensis*), and leaves (*Azadirachta indica*, *Moringa oleifera*, *Mentha spicata*, and *Eucalyptus grandis*) of nine plants were collected locally for PEO extraction. The PEO extraction was done by following methods described. The extracted PEOs, following methods recommended by Odak et al. [24], were

stored at room temperature. The fungal culture of *F. oxysporum* used in the experiment was provided by the PCSIR, Lahore (Pakistan). The fungus was subcultured at room temperature (25°C) for 120 hours using potato dextrose agar (PDA) slants to prepare spore suspension for subsequent experimental use.

2.1. Laboratory Evaluation of Plant Essential Oil Activity

2.1.1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) Evaluation of Plant Essential Oils. The agar well diffusion method was used to evaluate MIC for two concentrations (25 and 50 μl/ml) of PEOs. The size of inhibition zone produced by PEO application reflects the inhibitory concentration of the PEO. The lowest concentration of essential oil producing the largest inhibition zone reflects it potential to inhibit fungal growth, and this concentration is referred as the MIC. Each treatment was replicated three times in CRD experiment layout.

The test tubes containing culture media broth and essential oil (at three concentrations 50, 75, and 100 μl/ml) to be tested were inoculated with 1×10^6 cfu/ml fungal spore load. Broth tubes used without essential oils were assigned control. The fungal growth was observed in test tubes 48 hours after incubation at 25°C. A volume of 100 μl, from tubes showing no visible fungal growth, along with agar was poured in petri plates after the incubation duration. The lowest PEO concentration showing no fungal growth in petri plates, after 48 hours of incubation, was considered as MFC.

2.2. Field Trials

2.2.1. Foliar Application of Plant Essential Oils. Two concentrations, 60 and 80 μl/ml, of *E. grandis*, *C. cyminum*, and *C. sinensis* PEO were selected to be applied as foliar spray in the field experiment. Treatments (Supplementary Table1) were planned under completely randomized design with three replications. A fungal spore load of 10^6 cfu/ml was inoculated in soil as pathogen inoculum at 15 days posttransplant stage. The foliar applications of PEOs were done four times with 15 days interval.

(1) Data Recording.

(1) Percent disease incidence: the plants showing symptoms of Fusarium wilt were counted to record percent disease incidence using following formula [25]

$$\text{Percent disease incidence} = \frac{\text{Number of infected plant}}{\text{Total number of plants assessed}} \times 100. \quad (1)$$

(2) Percent disease severity: a scale of 0-5 was kept as standard to calculate the disease severity as suggested by Rahman et al. [26]. A standard formula by Chester [27] was followed for the determination of percent disease index (PDI)

$$\text{PDI} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of plants (sample)}} \times \text{highest rating} \times 100. \quad (2)$$

Percent disease severity (PDS) was calculated to assign the disease severity scales/ratings to the treatment. Ten infected leaves were randomly selected from ten infected plants each. Total leaf area was measured by using regression equation (R^2, 98) developed by Blanco and Folegatti [28].

$$\text{Total leaf area} = 0.347 \cdot (L.W) - 10.7. \quad (3)$$

Percent disease severity (PDS) for each treatment was calculated as under, by observing and measuring the infected leaf area [29].

$$\text{PDS} = \frac{\text{Infected leaf area}}{\text{total leaf area}} \times 100. \quad (4)$$

The following formula was used to calculate percent efficacy of disease control (PEDC) of each treatment [30].

$$\text{PEDC} = \frac{\text{Infection index in control} - \text{Infection index in treatment}}{\text{Infection index in control}} \times 100. \quad (5)$$

2.2.2. Tomato Seed Treatment. Twenty preinoculated seeds were treated with 1 ml of 60 μl/ml concentration of PEOs (*C. sinensis, E. grandis*, and *C. cyminum*). Treatments with fungicide and sterile distilled water served as controls. The experiment was performed using Randomized Complete Block Design (RCBD) with five treatments and three replications (Supplementary Table 2).

Land preparation and agronomic field practices were done as directed by the Punjab Agriculture Department, Pakistan. A fungal spore load of 10^6 cfu/ml was inoculated in soil as pathogen inoculum at 15 days posttransplant stage.

(1) Data Recording and Analysis. The data for PDI and PDS were recorded fortnightly as mentioned in foliar application experiment. Fruit was harvested and weighed from randomly selected ten plants for each treatment. Plant height was recorded from randomly selected 10 plants from each treatment. Fruit parameters, fruit weight, pericarp thickness, and fruit volume of randomly selected 20 fruits from each treatment were recorded. Fruit volume was measured by using the regression equation (R^2, 98) developed by Concha-Meyer et al. [31] as

$$\text{Tomato fruit volume } \left(\text{cm}^3\right) = 7.3 + 0.92\ \text{Weight (g)}. \quad (6)$$

Analysis of variance (ANOVA) was done to find significant difference among treatments. Multiple comparison among the treatments to find statistical differences or similarities among the treatments was done by using Tukey's HSD test.

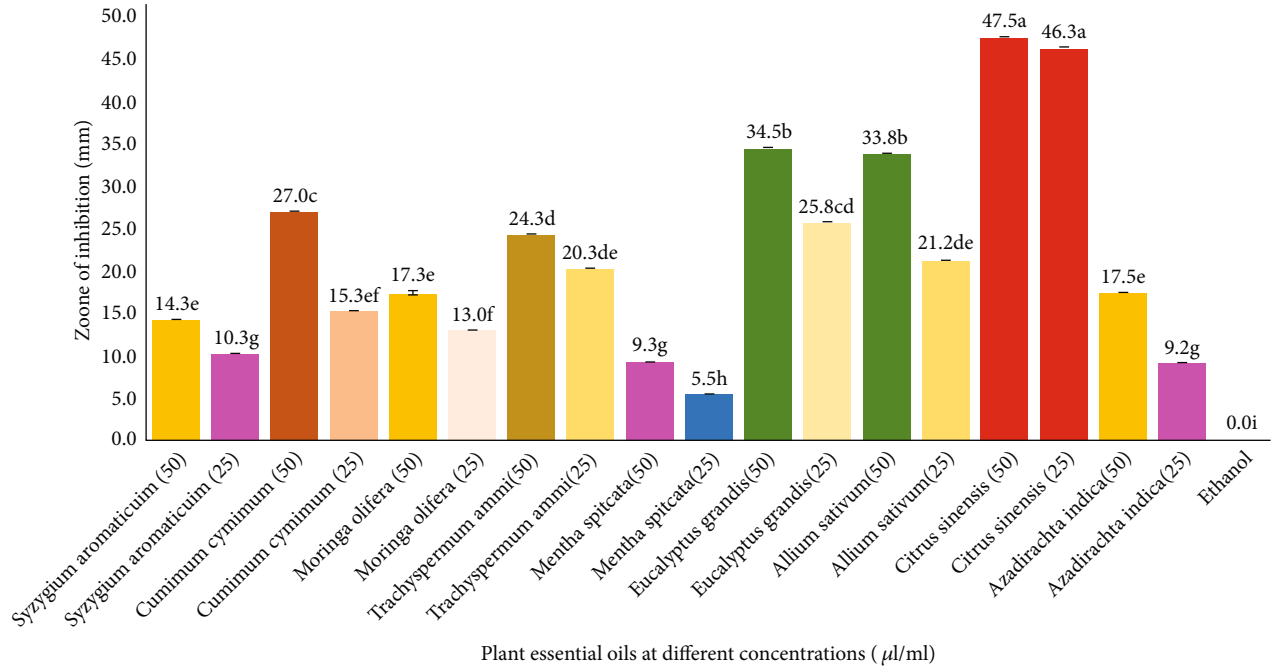


Figure 1: Minimum inhibitory concentration of different concentrations of plant essential oils against *Fusarium oxysporum*.

2.3. Computer-Aided Fungicide Designing (CAFD). Laboratory- and field test-qualified essential oil was selected for identification of potential lead molecule against *F. oxysporum*.

The chemical compounds of essential oil of orange *Citrus sinensis* L. were retrieved from literature, and their structure were retrieved from PubChem (Supplementary Table 3). The pesticide likeness [32] and nontoxicity of the compounds were predicted by DruLiTo 2.0 and DataWarior. The qualified compounds were selected for further use in docking.

2.3.1. Protein 3D Structure Prediction and Molecular Docking. The amino acid sequence of polyketide synthase, an important toxin-producing enzyme in *F. oxysporum* [33], was retrieved from UniProt (UniProt ID: A0A0D2YG10). The toxin-producing domain of enzyme was predicted by InterPro-EMBL-EBI. Three-dimensional structure/model of the selected domain was predicted by using online server Robetta. The predicted 3D model was refined by an online available server GalaxyWEB, and the refined structure was evaluated by SAVES server.

The molecular docking of polyketide synthase domain and selected *C. sinensis* PEO compounds/ligands was done by using AutoDock Vina.

2.3.2. Pharmacophore Modeling and Virtual Screening. The docked ligand-receptor complexes showing lower binding energies and hydrogen bond interaction were selected to be imported, as training set, to Ligand Scout 4.4 for pharmacophore generation. The best pharmacophore model was selected for virtual screening against Comprehensive Marine Natural Products Database (CMNPD). The matching compounds were evaluated and screened as in the first docking.

2.3.3. Molecular Dynamics (MD) Simulations. Four protein-ligand docked complexes having lower binding energy were used for MD simulation. The software package, Amber v18, was used for MD simulations at 80-nanosecond time period.

3. Results

3.1. Laboratory Evaluation of Essential Oil Activity

3.1.1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) Evaluation of Plant Essential Oils. The significantly largest but not significantly different from each other inhibition zone, 47.5 and 46.3 mm, was recorded for *C. sinensis* 50 μl/ml and *C. sinensis* 25 μl/ml, respectively (Figure 1). No fungal colony growth after 72 hours was observed in *A. sativum* 100 μl/ml, *E. grandis* 75 μl/ml, *C. cyminum* 75 μl/ml, and *C. sinensis* 50 μl/ml treatments (Figure 2).

3.2. Field Trials

3.2.1. Foliar Application of Plant Essential Oils

(1) Percent Disease Incidence. The percent disease incidence after the first application was significantly the lowest for the plant essential of *C. sinensis*; 80 μl/ml showed significantly the lowest (2.2) percent disease incidence (Figure 3). In the mean percent disease incidence after four applications of essential oil, only *C. sinensis* 80 μl/ml showed the significantly lowest (1.9) percent disease incidence. A not significantly different percent disease incidence was observed among the rest of the treatments. A not significantly different trend of disease incidence was observed among different application intervals.

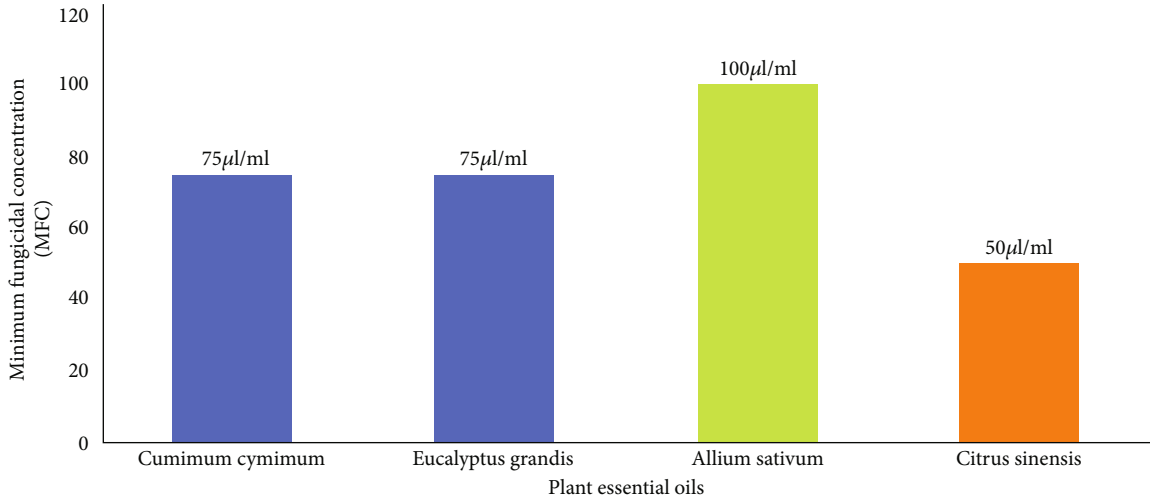


Figure 2: Minimum fungicidal concentration activity of different plant essential oils against *Fusarium oxysporum*.

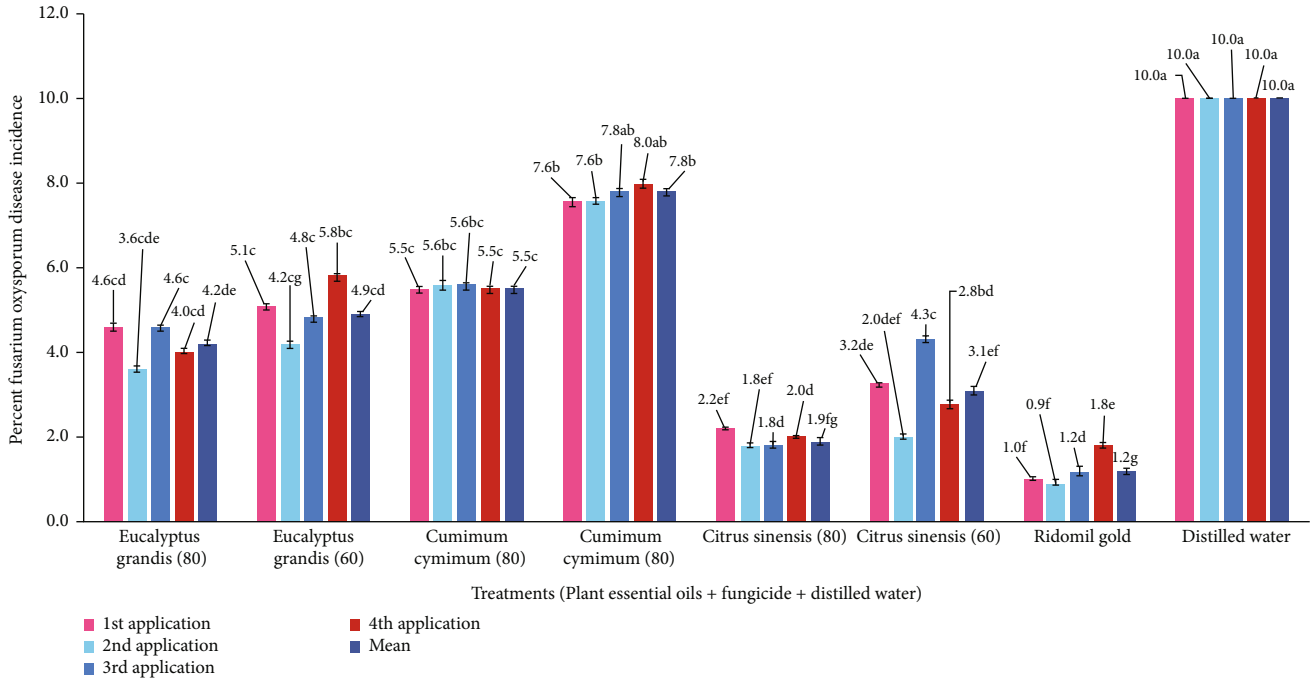


Figure 3: Effect of foliar application of different concentrations of plant essential oils on percent disease incidence of *Fusarium oxysporum* in tomato.

(2) Percent Disease Severity. The data for percent disease severity were recorded two times after PEO application, i.e., after the first and fourth applications (Table 1). The significantly lowest percent disease severity (3.1), after the first PEO application, was recorded for *C. sinensis* (80 μl/ml), which was not significantly different from those for *C. sinensis* 60 μl/ml, *E. grandis* (80 μl/ml), and Ridomil Gold. Two treatments (*C. sinensis* 80 μl/ml and *C. sinensis* 60 μl/ml) fell in class 1 of disease severity scale with PEDC value 80 and PDI 20. A similar trend for percent disease severity was observed after application.

3.2.2. Tomato Seed Treatment. The efficacy of essential oil was tested as seed treatment. As the PEOs are volatile in nature, it might be possible that they act more effectively as seed treatment than foliar spray application [34].

(1) Percent Disease Severity and Percent Disease Incidence. The data were recorded from ten randomly selected plants (Table 2). All the three treatment effects on disease severity scale fell in class 1. The lowest (2.11) percent disease severity, not significantly different from that by *E. grandis*, was recorded for *C. sinensis*. Both treatments fell in DSS class

Table 1: Effect of foliar application of different concentrations of plant essential oils on percent disease severity of *Fusarium oxysporum* in tomato.

No.	Plant essential oil concentrations (μl/ml)	Total leaf area (cm^2)	Infested leaf area (cm^2)	PDS**	Infested leaf area (cm^2)	PDS**	Reduction	DSS***	PDI****	PEDC*****
T1	*Eucalyptus grandis* (80)	23.2	1.5	$5.7 \pm 0.01^{cd*}$	0.9	$3.7 \pm 0.02^{c*}$	2.6	2-1	40-20	60-80
T2	*Eucalyptus grandis* (60)	22.7	1.3	6.5 ± 0.01^{c}	0.9	3.9 ± 0.02^{c}	2.0	2-1	40-20	60-80
T3	*Cuminum cyminum* (80)	23.1	2.3	10.0 ± 0.03^{b}	2.6	11.3 ± 0.03^{b}	-1.3	3-4	20	80
T4	*Cuminum cyminum* (60)	23.3	3.0	12.9 ± 0.01^{b}	2.8	12.0 ± 0.02^{b}	0.9	4	60	40
T5	*Citrus sinensis* (80)	22.6	0.7	3.1 ± 0.01^{d}	0.6	2.7 ± 0.01^{d}	0.4	1	20	80
T6	*Citrus sinensis* (60)	22.6	1.0	4.4 ± 0.02^{cd}	0.8	3.5 ± 0.02^{cd}	-1.8	1	20	80
T7	Ridomil Gold WG	22.8	0.5	2.2 ± 0.02^{d}	0.3	1.5 ± 0.03^{de}	0.7	1	20	80
T8	Ethanol	23.1	17.0	73.6 ± 0.00^{a}	16.0	69.3 ± 0.02^{a}	4.3	5	100	0
LSD				**1.505**		**1.812**				

*Values sharing the same letters in column are not significantly different. **Percent disease severity. ***Disease severity scale. ****Percent disease index. *****Percent efficacy of disease control.

Table 2: Effect of seed treatment of different plant essential oils on percent disease severity of *Fusarium oxysporum* in tomato.

No.	Plant essential oils (60 μl/ml)	Total leaf area (cm^2)	Infected area (cm^2)	PDS**	DSS***	PDI****	PECD*****	%incidence
1	*Eucalyptus grandis*	22.0	1.1	$3.64 \pm 0.1^{cd*}$	1	40	60	$2.6.\pm0.1^{c}$
2	*Cuminum cyminum*	21.7	2.7	12.44 ± 0.0^{b}	3	60	40	4.5 ± 0.2^{b}
3	*Citrus sinensis*	21.6	0.8	2.11 ± 0.3^{d}	1	20	80	1.5 ± 0.2^{d}
4	Ridomil Gold WG	22.0	0.3	1.36 ± 0.1^{d}	1	20	80	1.0 ± 0.2^{d}
5	Distilled water	23.0	19.0	82.61 ± 0.1^{a}	5	100	0	9.6 ± 0.0^{a}
			LSD	**1.8725**				**0.4245**

*Values sharing the same letters in column are not significantly different. **Percent disease severity. ***Disease severity scale. ****Percent disease index. ******Percent efficacy of disease control.

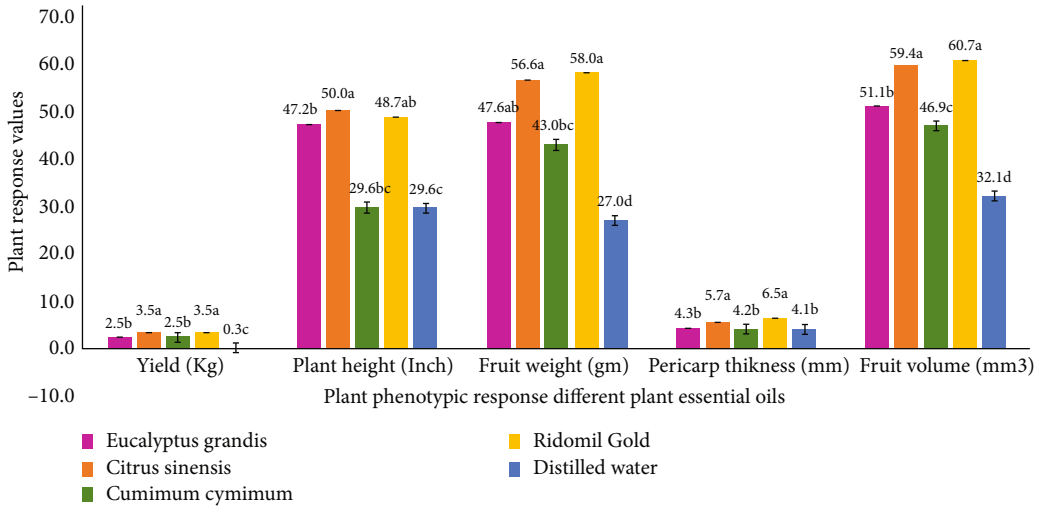


Figure 4: Tomato plant phenotypic response to the seed treatment by plant essential oils.

1, while percent disease incidence was significantly the lowest (1.5) for *C. sinensis*.

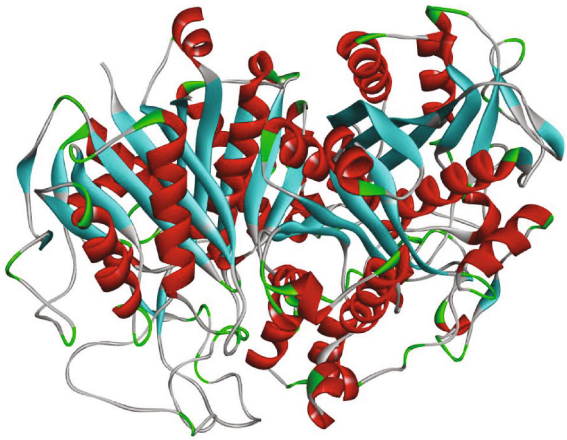
(2) Effect of Seed Treatment on Yield, Plant Height, and Fruit Characteristics of Tomato. The significantly highest fruit yield per plant (3.8 kg) was harvested from the plants treated with *C. sinensis* oil (Figure 4). The fruit yield per plant was not significantly different among the treatments *E. grandis* and *C. cyminum*. Plant height was significantly the highest for *C. sinensis*. The significantly highest value (59.4 m^3) for fruit volume was recorded in plants treated with *C. sinensis* PEO.

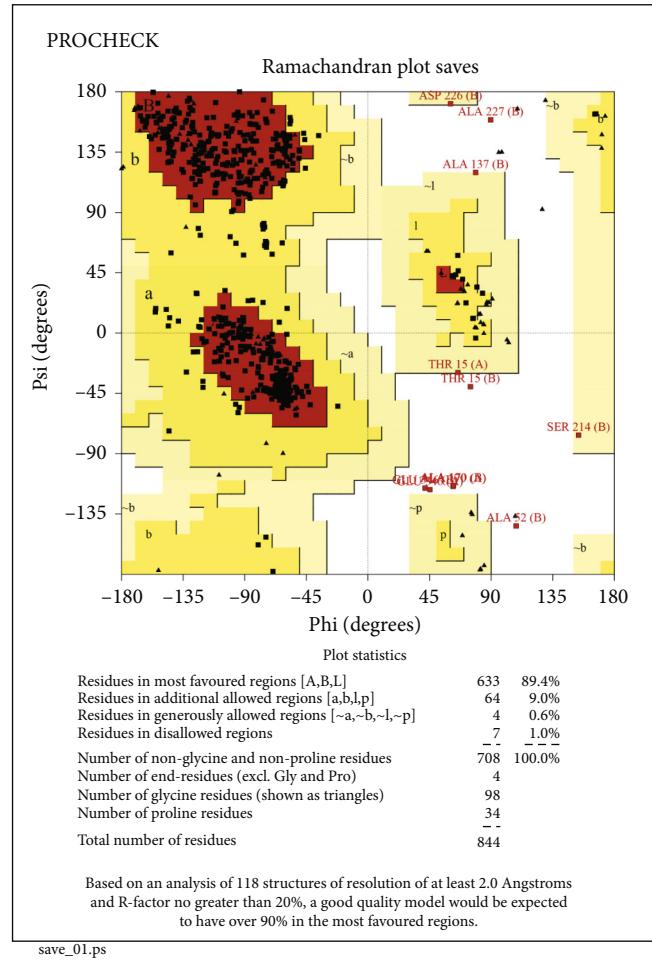
3.3. Computer-Aided Fungicide Design (CAFD)

3.3.1. Chemical Compound Structure Acquisition and Screening. Only thirty-three compounds were qualified to have pesticide likeness and nonhazardous for humans (Supplementary Table 4). Active domain beta-ketoacyl synthase (IPR020841) of polyketide synthase was selected from domain scanning results. The refined 3D model passed the quality checks and was saved for further analysis (Figure 5(a)). The overall Ramachandran score of the refined 3D model of domain was 98.4% (Figure 5(b)). Among which, 89.4% amino acid residues were found in the most favored region while only 9% residues lied in the additional allowed region. ERRAT quality factor was 85.803 Figure 5(c). The residues passed the verification 3D check with 90.40% residues having averaged 3D-1D score ≥ 0.2 Figure 5(d).

3.3.2. Molecular Docking. Only sixteen compounds having hydrogen bond donor (HBD)/hydrogen bond acceptor (HBA) sites were selected to perform ligand-receptor docking. The overall binding affinity among docked complexes ranged from -6.2 to -4.3 kcal/mol (Table 3). The highest binding affinity (-6.2) resulted for two compounds: caryophyllene oxide and germacrene. But no hydrogen bond was found in both cases. Only seven ligand molecules showed hydrogen bonding with the protein. One hydrogen bond was formed in six docked complexes while nootkatone formed two hydrogen bonds with -6.0 kcal/mol binding affinity.

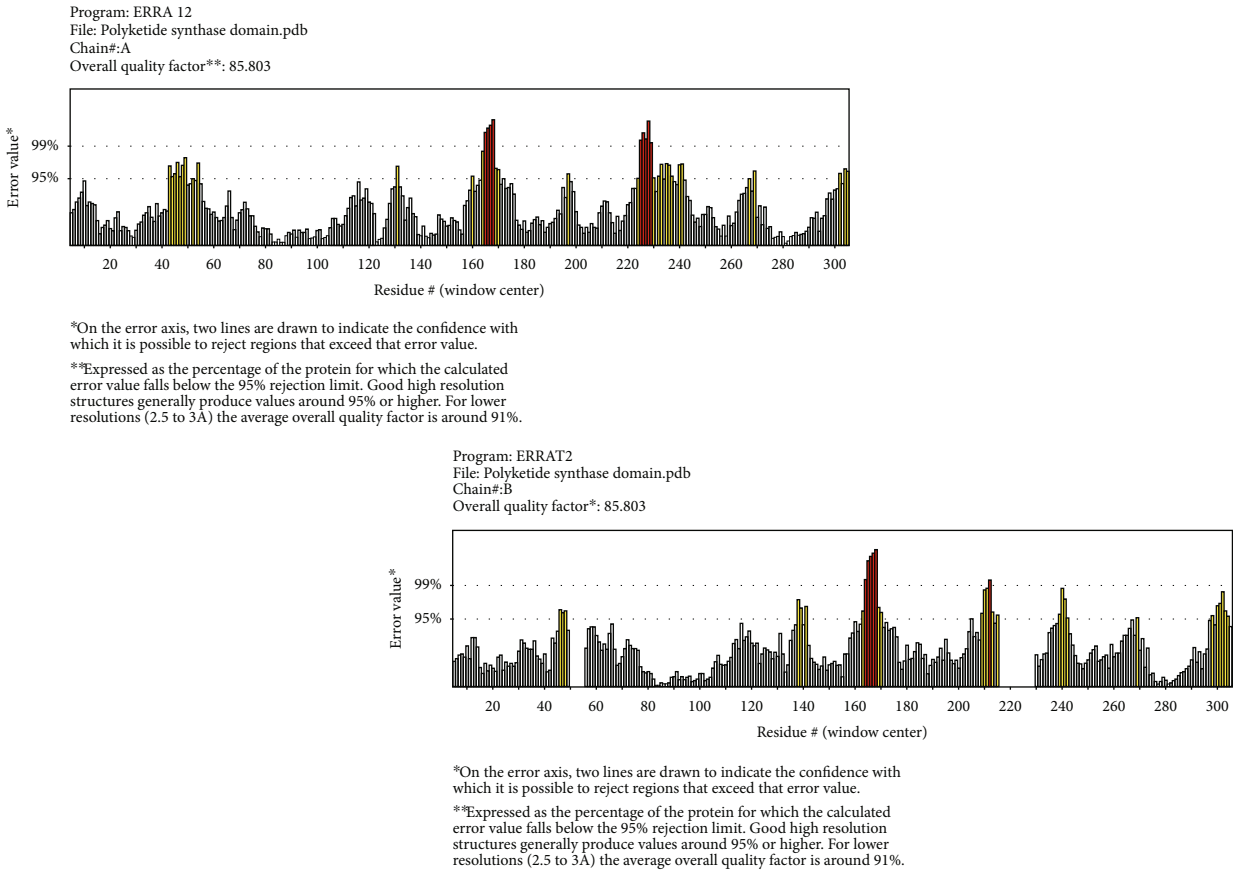
3.3.3. Pharmacophore Modeling, Virtual Screening, and Molecular Docking. Seven chemical compounds showing low binding energies and hydrogen bond interaction, α-terpineol, (Z)-p-menth-2-en-1-ol, neral, elemol, nootkatone, and citronellyl acetate, were selected for merged feature pharmacophore generation. The best pharmacophore model (score: 0.8492) was selected for virtual screening. The selected pharmacophore had two HBA, two HBD, three hydrophobic hydrogens, and one aromatic ring (Figure 6). The hit rate 20.35% was obtained in virtual screening of



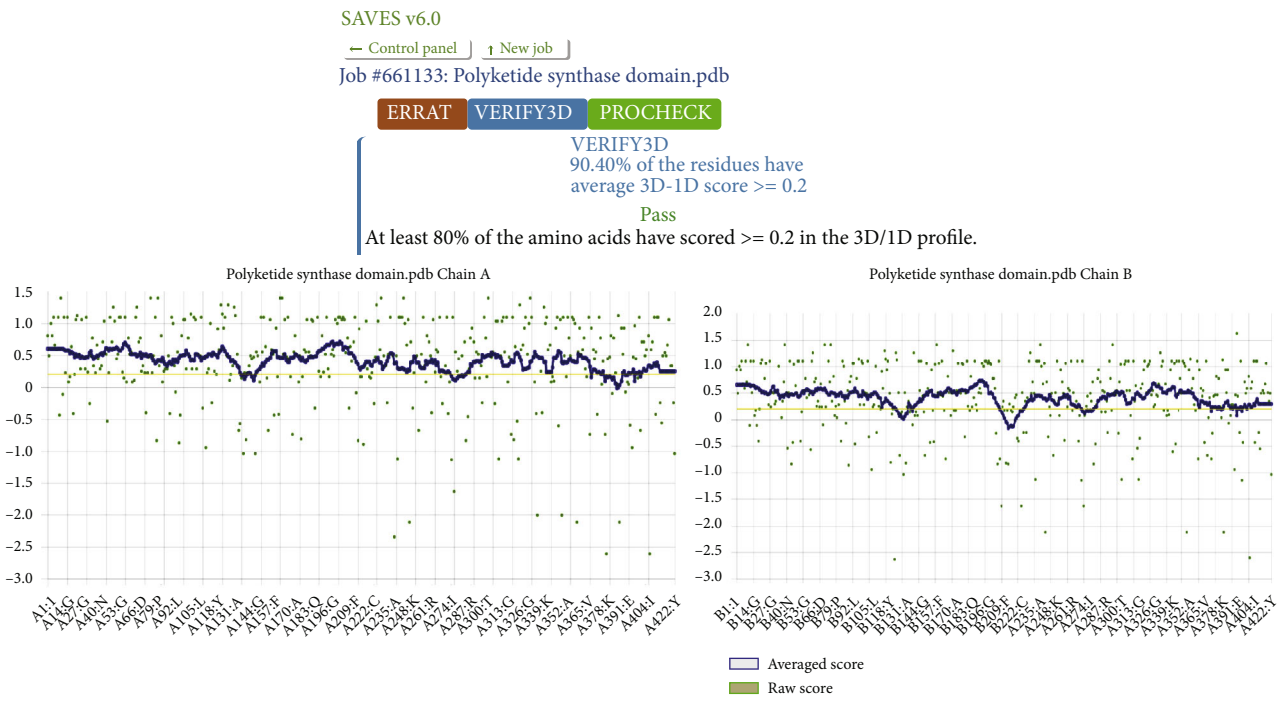


(a) (b)

Figure 5: Continued.



(c)



(d)

Figure 5: Refined 3D model of polyketide synthase beta-ketoacyl synthase domain in *Fusarium oxysporum* and model evaluation results: (a) refined 3D model, (b) Ramachandran plot, (c) ERRAT score, and (d) verify 3D score.

Table 3: Properties of chemical compounds of *Citrus sinensis* essential oil and results of docking with polyketide synthase beta-ketoacyl synthase domain in *Fusarium oxysporum*.

No.	Compounds	PubChem CID	MW (g/mol)	HBD	HBA	nRTB	Logp	BA (kcal/mol)	H-bond
Monoterpenes									
1	β-Pinene	14896	136.23	1	0	0	3.1	-4.3	0
2	β-Myrcene	31253	142.27	1	0	0	4.3	-3.8	0
3	Limonene	22311	136.23	2	0	1	3.4	-5.8	0
4	Sabinene	18818	136.23	1	0	1	3.1	-4.8	0
Oxygenated monoterpenes									
5	α-Terpineol	17100	154.25	1	1	1	1.8	-4.7	1
6	(Z)-p-Menth-2-en-1-ol	13918681	154.25	1	1	1	2.3	-6.1	1
7	Neral	643779	152.23	0	1	4	3.0	-5.9	1
Sesquiterpenes									
8	δ-Cadinene	92313	204.35	2	0	1	4.3	-5.7	0
9	β-Farnesene	5281517	204.35	1	0	7	6.2	-5.0	0
10	α-Cyperone	6452086	218.33	0	1	1	3.8	-5.8	0
11	Caryophyllene oxide	1742210	220.35	0	1	0	3.6	-6.2	0
12	Germacrene B	5281519	204.35	2	0	1	4.7	-6.2	0
Oxygenated sesquiterpenes									
13	Elemol	92138	222.37	1	1	3	4.4	-5.3	1
14	Nootkatone	1268142	218.33	0	1	1	3.9	-6.0	2
Other oxygenated compounds									
15	Citronellyl acetate	9017	198.3	0	2	7	3.8	-4.8	1
16	Neryl acetate	1549025	196.29	0	2	6	3.5	-4.5	1

MW = molecular weight; HBD = hydrogen bond donor; HBA = hydrogen bond acceptor; nRTB = number of rotatable bonds; BA = binding affinity.

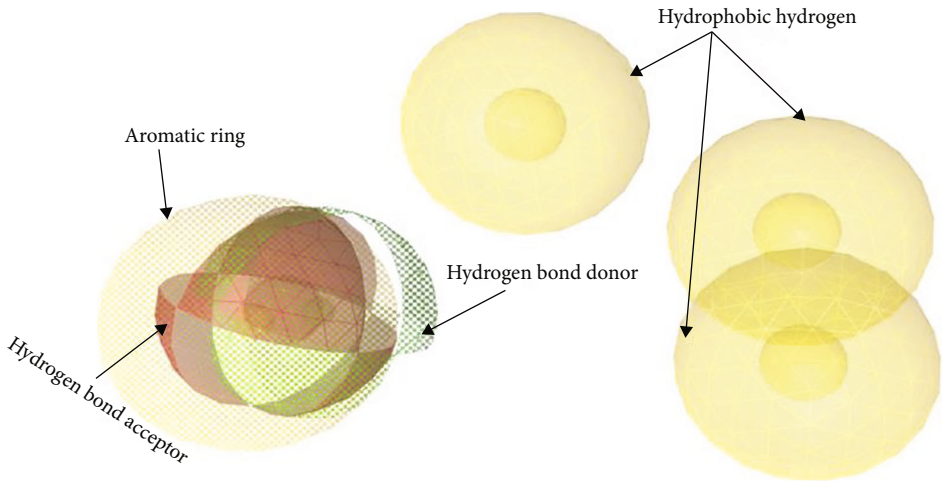


Figure 6: Pharmacophore features of selected, polyketide synthase beta-ketoacyl synthase domain in *Fusarium oxysporum* inhibitor, compounds from *Citrus sinensis* plant essential oils.

the pharmacophore against CMNPD. Only 27 compounds, after screening, were qualified to be docked against query protein. The binding affinity of ligand-protein docked complex ranged from -6.9 to -4.1 (Table 4).

3.3.4. Molecular Dynamics Simulation. Four protein-ligand docked complexes with lower binding energy, i.e., beta-ketoacyl synthase-CMNPD91 (-6.9 kcal/mol), beta-ketoacyl synthase-CMNPD19958 (-6.7 kcal/mol), beta-ketoacyl synthase-CMNPD1118 (-6.5 kcal/mol), and beta-ketoacyl synthase-CMNPD24498 (-6.2 kcal/mol), were simulated in an explicit water environment for a total of 80 ns. The beta-ketoacyl synthase-CMNPD24498 (-6.2 kcal/mol) complex showed stable interactions throughout the run. Three hydrogen bonds were found originally in the complex which was retained till 80 ns. At 30, 40, 60, and 70 ns, only one hydrogen bond was observed (Figures 7(a)–7(h)). Phenylalanine was found, most of the time, to be involved in hydrogen bond formation. To test the simulation system reliability, the backbone atom deviation was measured using

Table 4: Properties of marine life-derived compounds and results of docking with polyketide synthase beta-ketoacyl synthase domain in *Fusarium oxysporum*.

No.	CMNPD ID	IUPAC name	MW (g/mol)	Logp	HBA	HBD	nRB	nAR	BA (kcal/mol)	H-bond	Source species
1	91	2-[(1R,2S)-1,2-Dimethyl-3-methylidenecyclopentyl]-5-methylphenol	195.99	3.792	1	0	1	1	-6.9	4	*Laurencia subopposita*
2	538	(1S,2R,3R,5R,6R,7R,8R)-5-[(4E)-6-Hydroxy-6-methylhepta1,4-dien-2-yl] -2,8-dimethyltricyclo[5.3.0.02,6]decan-3-ol	275.01	4.488	2	0	4	0	-4.1	3	*Stoechospermum polypodioides*
3	1118	(3bR,6S,7S,9aR)-7-Hydroxy-6-(hydroxymethyl)-3b,6,9a-trimethyl -4,5,5a,7,9,9b,10,11-octahydronaphtho[2,1-e][2]benzofuran-8-one	303.98	3.25	4	0	1	1	-6.5	2	*Spongia* sp.
4	2024	(1Z)-1-[(3S,3aS,7S,7aS)-3,7-Dimethyl-1,2,3,3a,5,6,7,7a-octahydroinden -4-ylidene]-2-methylpropan-2-ol	198.01	4.752	1	0	1	0	-5.4	1	*Unidentified* sp.
5	2365	(1R,2R,4S)-4-Methyl-1-propan-2-yl-3,4-dihydro-2H-naphthalene-1,2,6-triol	215.98	1.167	3	0	1	1	-5.1	2	*Lemnalia cervicornis*
6	2924	2-[(1R,3E,7E,11E)-4,8,12-Trimethylcyclotetradeca-3,7,11-trien-1-yl] propane-1,2-diol	271.99	4.881	2	0	2	0	-5.8	2	*Sinularia mayi*
7	4785	3-Hydroxy-3,5,5-trimethyl-7-propan-2-yl-2,4-dihydroinden-1-one	211.99	2.328	2	0	1	0	-5.8	1	*Sarcophyton trocheliophorum*
8	5394	[(1R,2aR,3S,4aR,5R,6S,7R,8R,8aR)-3,6-Dibromo-7-hydroxy -2a,4a,5,8-tetramethyl-1,2,3,4,5,6,7,8-octahydrocyclobuta[i]inden-1-yl] acetate	409.82	4.625	3	0	2	0	-5.5	0	*Laurencia tenera*
9	15648	(9Z,11E)-Tricyclo[12.3.1.12,6]nonadeca-1(17),2,4,6(19),9,11,14(18), 15-octaene-3,8,17-triol	275.98	2.135	3	0	0	2	-6.7	0	*Cymodocea nodosa*
10	15845	6,7,9a-Trimethyl-1,3,5,5a,6,8,9,10a-octahydrofuro[3,4-b][1] benzoxepin-7-ol	227.98	1.386	3	0	0	0	-5.6	0	*Laurencia luzonensis*
11	18682	(1S,4R,4aS,8aS)-6-(Hydroxymethyl)-4-(2-hydroxypropan-2-yl)-1-methyl- 3,4,4a,7,8,8a-hexahydro-2H-naphthalen-1-ol	230	0.841	3	0	2	0	-5.7	3	*Streptomyces* sp.
12	19958	[(1S,3aS,4R,8S,8aR)-8-Ethyl-1,4-dimethyl-3a,5,6,7,8,8a-hexahydro -1H-azulen-4-yl] methanol	198.01	4.216	1	0	2	0	-6.7	1	*Ulva lactuca*
13	20001	(1S,3aR,4S,8aS)-1,4-Dimethyl-7-propan-2-yl-2,3,3a,5,6,8a -hexahydroazulene-1,4-diol	214.01	2.613	2	0	1	0	-5.5	0	*Grateloupia turuturu*
14	20352	(1S,3aS,4S,7S)-1,4-Dimethyl-7-propan-2-yl-3,4,5,6 -tetrahydro-2H-azulene-1,3a,7-triol	227.98	0.744	3	0	1	0	-5.8	7	*Sinularia leptoclados*
15	20360	(3S,4E,6S,8Z,11S,16R)-6,16-Dihydroxy-8-(hydroxymethyl) -4,15,15-trimethyltricyclo [9.3.1.13,14] hexadeca-1(14),4,8-trien-2-one	306	0.45	4	0	1	0	-6.3	1	*Cespitularia hypotentaculata*
16	22108	(1S,4S,5S,9R)-8-(Hydroxymethyl)-4-(1-hydroxypropan-2-yl) -1-methylspiro [4.5] dec-7-en-9-ol	228.99	2.351	3	0	3	0	-5.1	5	*Lemnalia cervicornis*
17	**24498**	**2-Hydroxy-3,6-dimethyl-N-(4,5,6-trihydroxy-4-methyloxan-3-yl) benzamide (FW054-1)**	**289.97**	**0.733**	**7**	**0**	**3**	**1**	**-6.2**	**3**	***Verrucosispora* sp.**
18	24574	[(1S,2S,4E,6R,7E,9R)-1,6-Dihydroxy-8-(hydroxymethyl) -4,11,11-trimethyl-2-bicyclo [7.2.0] undeca-4,7-dienyl] acetate	284.98	0.006	5	0	3	0	-5.6	5	*Ascotricha* sp.
19	26841	(1R,2S,5R,8R,9R)-1,4,4,8-Tetramethyl-12-oxatricyclo [6.3.1.02,5] dodecan-9-ol	214.01	3.563	2	0	0	0	-5.2	1	*Rumphella antipathes*
20	26845	(3aS,5S,5aR,6S,8S)-5a,6,8-Trihydroxy-3a-methoxy-1,5,8 -trimethyl-4,5,6,7-tetrahydroazuleno[6,5-b] furan-2-one	287.97	-0.833	6	0	1	0	-6.0	0	*Menella kanisa*

Table 4: Continued.

No.	CMNPD ID	IUPAC name	MW (g/mol)	Logp	HBA	HBD	nRB	nAR	BA (kcal/mol)	H-bond	Source species
21	28987	9-Hydroxy-3-(1-hydroxyethyl)-3H-2-benzoxepin-1-one	207.98	0.862	4	0	1	1	-5.5	4	*Pestalotia heterocornis*
22	30788	(1S,2R,3aR,8aS)-1-(2-hydroxypropan-2-yl)-3a,6-Dimethyl-2,3,4,7,8,8a-hexahydroazulene-1,2-diol	228.99	3.074	3	0	1	0	-5.7	2	*Trichoderma virens*
23	30789	(1S,2R,3aR,4R,8aS)-3a,6-Dimethyl-1-propan-2-yl-2,3,4,7,8,8a-hexahydroazulene-1,2,4-triol	228.99	1.947	3	0	1	0	-6.2	2	*Trichoderma virens*
24	20359	(3S,4E,6R,7S,11R,16R)-6,7,16-Trihydroxy-4,15,15-trimethyl-8-methylidenetricyclo [9.3.1.13,14] hexadeca-1(14),4-dien-2-one	306	1.197	4	0	0	0	-5.4	6	*Cespitularia hypotentaculata*
25	11537	[(1S,8aS)-5,5,8a-Trimethyl-2-methylidene-3,4,4a,6,7,8-hexahydro-1H-naphthalen-1-yl] methanol	195.99	3.067	1	0	1	0	-5.6	0	*Cadlina luteomarginata*
26	11370	(1R,3aR,4R,8aR)-1-Methoxy-1,4-dimethyl-7-propan-2-yl-2,3,3a,5,6,8a-hexahydroazulen-4-ol	226.01	3.132	2	0	2	0	-5.8	0	*Sarcophyton buitendijki*
27	5390	2-[(1R,3R,4S)-4-Chloro-1,3-dimethylcyclohexyl]-5-methylphenol	230.96	3.789	1	0	1	1	-6.7	0	*Laurencia dendroidea*

MW = molecular weight; HBD = hydrogen bond donor; HBA = hydrogen bond acceptor; nRTB = number of rotatable bonds; BA = binding affinity; nAR = number of aromatic rings.

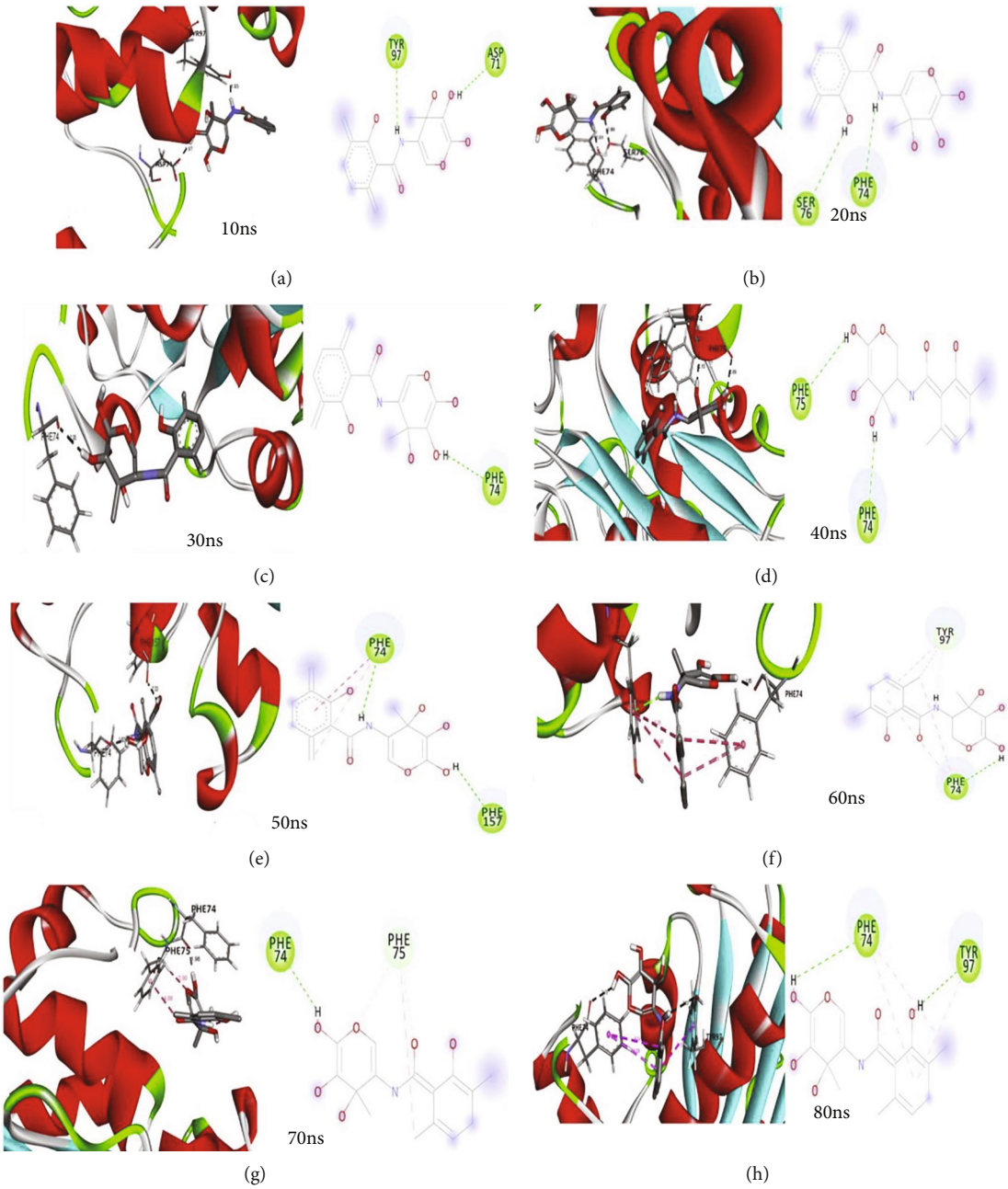


FIGURE 7: (a–h) Three-dimensional (3D) and two-dimensional (2D) interactions of polyketide synthase beta-ketoacyl synthase domain-CMNPD24498 at different time slots during MD simulation.

the RMSD (root-mean-square deviation). In particular, the RMSD result revealed that the graph exhibited a progressive increase starting at 1 Å and oscillating at 2.5 Å to 3.2 Å (Figure 8(a)). A peak 3.7 Å was observed at 45 ns after that started declining and 3.2 Å was recorded at 80 ns, which favors the stability and reliability of the complex. Root-mean-square-fluctuations (RMSF) were calculated in order to understand the fluctuation of individual residue in the docked complex. The results revealed fluctuation peaks for glycine 50, aspartic acid 125, and threonine 275 at 4.8 Å, 4.3 Å, and 4.7 Å, respectively. The high fluctuation in the docked complex residues might be due to the free movement of the residues. Most of the residue in docked protein showed a steady behavior which might indicate the stable interaction with the ligand (Figure 8(b)). B-factor and RMSF are interconvertible and related to each other [35]. The amino acid fluctuations shown by B-factor were similar to RMSF results (Figure 8(c)). The result revealed a very consistent behavior in terms of Rg value between 21.5 and 21.3 Å throughout the MD simulation time (Figure 8(d)).

4. Discussion

The phytopathogenic fungi are a serious threat for agriculture sector worldwide [18]. The indiscriminate use of fungicides especially on vegetable and fruit is very harmful for

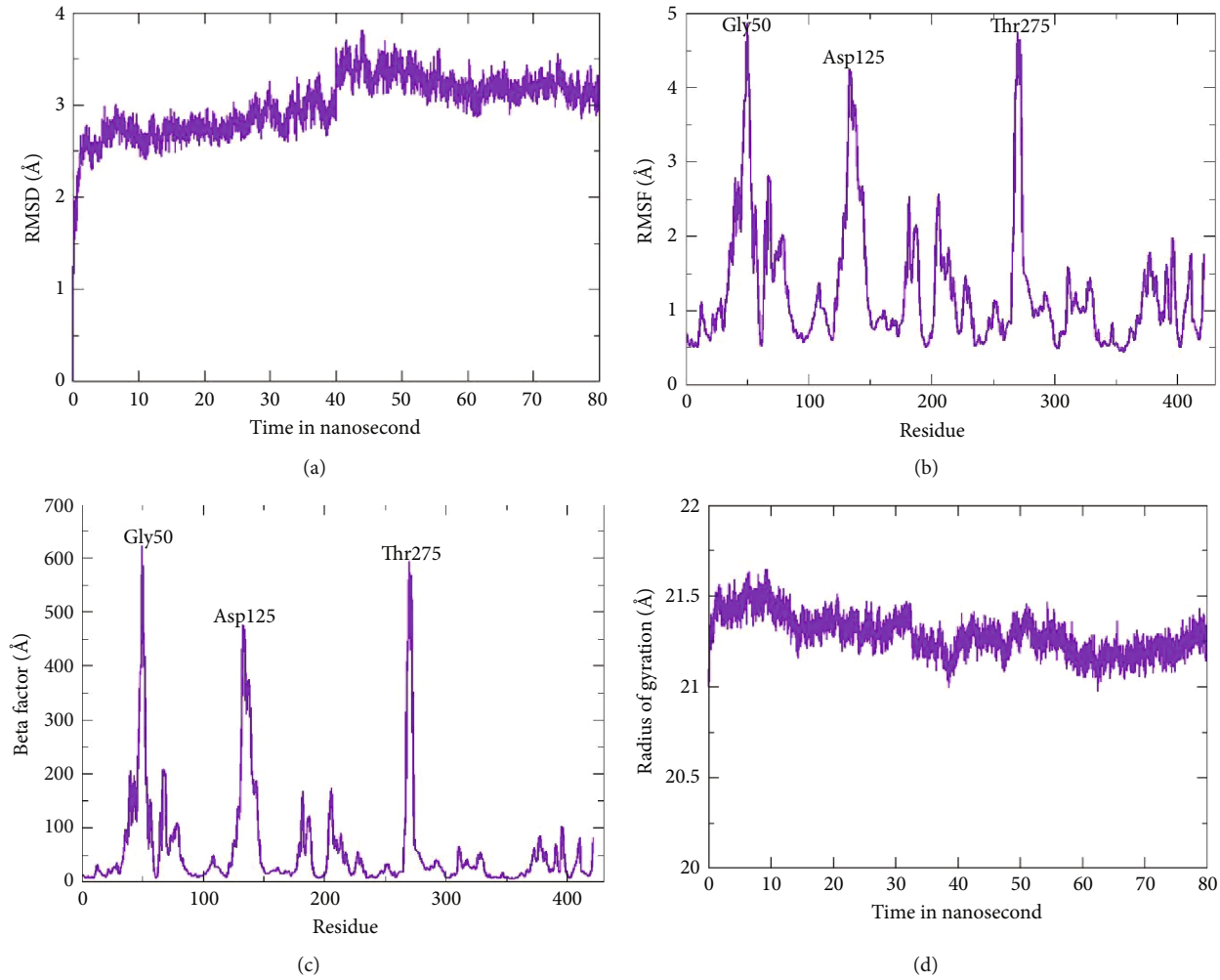


Figure 8: MD simulation results of polyketide synthase beta-ketoacyl synthase domain-CMNPD24498 complex.

human health [36]. Moreover, it causes environmental pollution and pesticide resistance but these issues are least addressed for fungicides as compared to insecticides [37, 38]. The use of alternate plant fungal disease management methods is need of time. PEOs are volatile molecules produced by plants as secondary metabolites having antifungal potential [39]. The biodegradable nature of PEOs makes them potential candidate for fungicide development [40].

The effectiveness of PEOs, i.e., *Syzygium aromaticum*, *Azadirachta indica*, *Mentha spicata*, *Trachyspermum ammi*, *Moringa oleifera*, *Cuminum cyminum*, *Eucalyptus grandis*, *Allium sativum*, and *Citrus sinensis*, has been tested, against *Fusarium oxysporum*, in the present study. The study was conducted in three trials, i.e., laboratory testing of PEO, field evaluation, and computer-aided fungicide design. MIC and MFC of PEOs were evaluated in laboratory. The MIC of a chemical is its lowest concentration required to inhibit the substantial growth of a pathogen [41]. The lowest MIC and MFC against *F. oxysporum* have been recorded for *Citrus sinensis* PEO. The second effective PEOs were of *E. grandis* and *A. sativum*. Shafique et al. reported good potential of *E. grandis* against different fungi but least effective against other *F. oxysporum* [42]. *Eucalyptus grandis* essential oil was found effective against *F. oxysporum* and *Botrytis cinerea* in banana [43]. Antifungal activity of essential oil from *A. sativum* has been reported from many recent studies [44]. The third runner-up of the treatments was *C. cyminum* (50 μl/ml). Romagnoli et al. and Mohammadpour et al. reported antifungal potential of *C. cyminum* [45, 46]. Three qualified PEOs, *C. sinensis*, *C. cyminum*, and *E. grandis*, from lab experiment were selected to be tested on tomato plants in field conditions. Two trials for field testing were carried out, i.e., foliar application of PEO and seed treatment with PEO. In foliar application trial, the lowest percent disease incidence and percent disease severity were observed for *C. sinensis* followed by *E. grandis*. Percent disease severity was calculated to find out the PEDC of PEO suggested by Jadon et al. [47]. This parameter is used to test the effectiveness of a chemical against pathogenic disease [45]. PEDC of *C. sinensis* was found the highest in our study. The efficacy of PEO was found to be more persistent as seed treatment than as foliar application. It might be possible that when exposed to sunlight the essential oil degrades rapidly [48].

CADD became a popular method for developing new pharmaceutical drugs [49]. Nevertheless, while the pharmacodynamics and techniques used in CADD and pesticide design (CAPD) are similar, this technique is not used in agricultural pesticides [50]. In the subject of pesticide chemistry, research into new target locations and innovative pharmacological compounds is quite limited. A little work has been done in CAPD against phytopathogenic fungi control [51]. Novel, more effective, and least toxic drug molecules and new target sites in the pathogen can be identified more efficiently by using CAPD approaches. This may help to overcome the problem of environment pollution, human health hazards, and pesticide resistance issues in a smart, least expensive, and rapid manner.

The target enzyme of *F. oxysporum* polyketide synthase was selected to be inhibited. It is involved in mediating fusaric acid biosynthesis which is a mycotoxin with low to moderate toxicity to humans and animals but highly phytotoxic [33]. Protein domain is the conserved sequence of protein which controls its function independently [52]. It is better to identify the toxin-producing domain in protein for inhibition by ligand molecules so beta-ketoacyl synthase domain (IPR020841) was selected for inhibition. This domain was found be involved in a number of enzymatic systems, including fatty acid synthetase, which catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH which is involved in the biosynthesis polyketide synthase. Top seven compounds from *C. sinensis* PEO showing minimum binding energy and good hydrogen bonding with target protein were selected for merging their features to construct the pharmacophore. The pharmacophore defined by IUPAC is "a collection of steric and electronic characteristics that is essential to make sure the optimal supramolecular interactions with a particular biological target and to activate (or block) its biological reaction." It is generally done by retrieving widely used chemical characteristics from 3D structures of a set of known ligands that are reflective of the ligands' crucial interactions with certain macromolecular targets [53]. This can be used as a query for retrieving potential lead identification from structural databases, for designing molecules with specific desired characteristics [54]. This approach has been effectively used in drug designing from novel and human safe chemical compounds [55]. These common features among the top compounds shown by pharmacophore can be used to design a novel fungicide molecule effective against *F. oxysporum*.

High-throughput screening (HTS) has become an integral part of CADD procedure pipeline for identification of hits of effective compounds against target protein [56]. The first step of CADD procedure pipeline is the identification of molecular targets (natural or synthetic) for our protein of interest, and after identification and validation, the selected compound is referred as lead molecule. The molecules found in natural products play a highly significant role in the drug discovery and development process. The chemical compounds that originated from marine organisms are getting attention and becoming popular to be selected as drug molecules [57]. In the current study, we did virtual screening of the pharmacophore, against Comprehensive Marine Natural Products Database (CMNPD). This database contains >38000 marine organism-originated chemical compounds.

Molecular dynamics (MD) simulations were used to check the stability of docked complexes. This analysis of MD simulation is used to evacuate the movements of the highly complexed macromolecular systems [58]. The estimation of structural fluctuations, in terms of RMSD and RMSF, of docked complex is the most crucial feature of this analysis which reflects the stability and flexibility of the complex. The stability interaction profile is reflected in the RMSD value. The average ligand-receptor RMSD in this investigation was 1 nm, showing that the system was stable. The dislocation of a single atom, or a group of atoms, relative to the reference structure is estimated using RMSF, which is averaged across the number of atoms [59].

The compound CMNPD24498 (FW054-1) has been selected as lead molecule for fungicide development against *F. oxysporum*. This compound has been derived from Verrucosispora genus of Micromonosporaceae family [60]. Micromonosporaceae belongs to the gram-positive Actinobacteria. This genus is getting attention in the field of drug development due to antibiotic nature of some species [61]. Some important antibiotic products of *Verrucosispora* sp. are gifhornenolones A and B from *Verrucosispora gifhornensis* [62], thiocoraline A from *Verrucosispora sp.* WMMA107, proximicins A-C from *Verrucosispora fiedleri* MG-37, brevianamide F from *Verrucosispora sp.* MS100047, and butrepyrazinone from *Verrucosispora sp.* K51G [63–65].

5. Conclusion

The fungal plant diseases are a very serious threat to fruit and vegetable industry. Complete eradication of disease from plants is required for good market value of fruit and vegetable. Synthetic fungicides are being extensively used for controlling fungal disease. This indiscriminate and extensive use of synthetic chemical compounds causes very serious threat to human health and environment. Alternative and safer pest management methods have been recommended to be tested against phytopathogenic fungi, especially in fruits and vegetables. The use of plant essential oil (PEO) is considered a safe and environment-friendly plant disease control method. In the current study, different plant essential oils have been tested, in the laboratory and field, against *F. oxysporum* in tomato. The plant essential oil of *Citrus sinensis* has been found most effective among all the PEOs tested. But the use of plant essential oil on a large scale for plant disease control is not a cost-effective method. The computer-aided fungicide design (CAFD) technique has been employed to identify safer and effective chemical molecules to be used in fungicide development against *F. oxysporum*. The shared features of the top seven compounds of *C. sinensis* PEO against toxin-producing enzyme, polyketide synthase, of *F. oxysporum* were screened against the CMNPD database. One compound CMNPD24498 (FW054-1) from *Verrucosispora* sp. (bacteria) showed the highest rank of similarity for shared features

of selected effective *C. sinensis* PEO compounds. This biological originated compound fulfilled the pesticide likeness criteria and nontoxic in nature. FW054-1 can be used for the development of an effective and safe fungicide against *F. oxysporum*.

Data Availability

The authors declare that all the data supporting the findings of this study are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

The supplementary file S1 is available online along with the manuscript. Other supplementary material will be provided by the corresponding author on request. *(Supplementary Materials)*

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Research Article

High Throughput Screening for Bioactive Components of *Berberis baluchistanica* Ahrendt Root and Their Functional Potential Assessment

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Berberis baluchistanica Ahrendt is a medicinal plant potentially known for the treatment of different diseases. The bioactive, antioxidant, nutritional components, and antimicrobial properties of crude ethanolic root extract of *Berberis baluchistanica* were evaluated in this study. The extract was analyzed for total phenolic, flavonoid, DPPH (2, 2-diphenyl-1-picryl-hydrazyl) scavenging ability, FRAP (ferric reducing antioxidant power), nutritional, and antimicrobial potentials. The alkaloids, tannins, cardiac glycosides, anthraquinones, coumarin, saponins, phenolics, flavonoids, steroids, and terpenoids were confirmed. The extract possessed DPPH radical inhibition with the IC_{50} of 1.125 mg/mL and FRAP % reduction activity with IC_{50} (0.912 mg/mL). Total phenolic 19.897 ± 4.8141 mg GAE/g and flavonoid 12.9876 ± 0.8388 mg QE/g contents were confirmed in the root. The extracts exhibit good antibacterial activity against a broad spectrum of food borne pathogens *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*. The highest inhibitory activity was against *Escherichia coli* 23.30 ± 1.16 mm and lowest against *Klebsiella pneumoniae* 7 ± 0.01 mm. Furthermore, the presence of various phytochemical constituents (plant secondary metabolites) was also confirmed with gas chromatography and mass spectroscopy analysis. Results disclosed the occurrence of more than 70 compounds possessing various medicinal properties supporting the traditional uses of root of *Berberis baluchistanica* in various medical complications indigenously.

1. Introduction

Therapeutic plants have significant positive role in individual and communities health [1, 2]. These plants and their components are traditionally in use for the treatment of many diseases such as fever, cough, internal injury, eye disease, removal of kidney stones, wound healing, rheumatism, and other infections of human beings and livestock [3, 4]. Herbal medicines contain an intricate combination of various significant bioactive components such as alkaloids, polyphenols, flavonoids, terpenoids, minerals, and vitamins. These components have considerable antimicrobial, anti-

inflammatory, cytotoxic, and antioxidant potential to reduce free radicals. It decreases the production of lipid peroxidation in human bodies, which are responsible for a variety of human diseases. The herbal medicines containing these phytochemical constituents (plant secondary metabolites) are used as alternate of synthetic drugs which are hazardous for both human and environment [5]. The presence of these bioactive and antioxidant components in plants have increased their therapeutic and nutritional values and overall demand as a folk medicines [1].

The genus Berberis belongs to family Berberidaceae, with 15 genera and 650 species [6]. It is among the primitive angiosperm having great economic and medicinal values [7]. Different phytochemicals such as glycosides, steroids, anthraquinones, anthraquinones, saponins, alkaloids, phlobatannins, tannins, reducing sugars, flavonoids, terpenoids, oleanolic acid, palmatine, and stigmasterol have also been confirmed in Berberis [8]. The antioxidant, antidiabetic, antibacterial, anti-inflammatory, heptoprotective, and hypotensive properties of berberine found in different Berberis species have also been described [9].

Berberis baluchistanica Ahrendt is wild medicinal plant locally known as Zralag in Pashto, Archin in Brahvi, and Korae in Balochi languages. It is endemic to Balochistan and belongs to family *Berberidaceae*. It is an evergreen shrub three meter tall with red brown to red stem and thick rigid leaves, harboring yellow color flowers between the months of March to May. The plant is distributed in Harboi, Kalat, and Zarghun area of Quetta and Ziarat [10]. This medicinal plant is considered as nontoxic and used as decoction or powder form. Due to the presence of berberine, the plant is utilized locally to cure different diseases like cold, cough, fever, internal injury, diseases of eyes, kidney stones removal, and wound [10, 11]. Berberisinol, oleanolic acid, berberine, gallic acid, 8-oxoberberine, palmatine, polyphenols, and vitamins were obtained with its notable antioxidant and antimicrobial [12]. Previously, [13] analyzed that the extract of root has strong antibacterial effects against a variety of pathogenic bacteria. The presence of these valuable components makes this plant medicinally important. Though the plant is generally used in local medicines, limited work has been reported on its phytochemicals and pharmacological activities. In recent years, much consideration has been paid to evaluate the bioactive components of medicinal plants, their chemical nature, antioxidant, and antimicrobial potentials. Similar studies are needed to investigate the curative potential of *Berberis baluchistanica* an indigenous plant for its actual health application and potential assessments. Hence, the current study was aimed at analyzing the bioactive components, total phenolics and flavonoids contents, antioxidant potential, and antimicrobial power of crude ethanolic extracts of root of *Berberis baluchistanica* Ahrendt. Furthermore, the biologically active compounds of the root extract were also analyzed by GC-MS for bioactive chemical profiling.

2. Materials and Methods

2.1. Collection of Plant Samples. The plant was collected from district Ziarat Balochistan, identified by taxonomists Dr. Shazia Saeed Assistant Professor Botany and banked in the Herbarium, Department of Botany University of Balochistan Quetta, Pakistan.

2.2. Sample Preparation. The root was separated from the plant, washed off to make it dust and contaminations free, and was dried at room temperature for 3-4 weeks consecutively in controlled environment. Electrical grinder was used to crushed the root into powdered and stored in desiccators at room temperature for further analysis [14].

2.3. Extraction of Compounds. One liter ethanol was used for the maceration extraction of root fine powder (100 g) keeping 1 : 10 ratio following [14]. Light exposures were avoided during the maceration process by doing the experiments in dark room. The mixture was shaken with specific frequency for better extraction of compounds. Whatman filter No. 1 filter paper was used to filter the ethanolic mixture. The mixture was dried in rotary evaporator (LEV400T-L), and the thick extracts was used for biological and functional activities determination.

2.4. Bioactive Component Analysis. The ethanolic extract of root was analyzed for the identification and occurrence of alkaloids, tannins, glycosides, anthraquinones, saponins, flavonoids, coumarin, quinones, steroids, terpenoids, and phlobatannins through phytochemical analysis following the standard methods [14, 15].

2.5. Total Phenolic Content. For total phenolic contents of root extract, the Folin Ciocalteu reagent method described by [16] was followed with slight modification. A stock solution (1 mg/mL) of dried crude plant extract was diluted with deionized water to make 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL. For extract, 0.5 mL was mixed with freshly prepared (2 mL) Folin Ciocalteu reagent and mixed properly followed by 5 min incubation at room temperature and further neutralized with 2 mL 10% Na_2CO_3 and mixed followed by 30 min incubation. The absorbance was calculated at 750 nm using T60 UV-visible spectrophotometer (PG, UK). Ethanol (95%) was used as blank. For the quantification of the extract, a calibrations curve was made from diverse dilutions of gallic acid. The findings were articulated as milligram of gallic acid equivalents (GAE)/g of sample dried weight.

2.6. Total Flavonoid Contents. Aluminum chloride colorimetric method as explained by [17] was used for the total flavonoids content determination. Simply 0.5 mL (1 mg/mL) of the extract was mixed up with 95% ethanol and 0.5 mL $NaNO_2$ (5%) solution, 0.1 mL (10% *w*/*v*, 0.1 mL) of $AlCl_3.6H_2O$, (0.5 mL of 1 M) NaOH and 2 mL of deionized water was added and incubated for 40 min at 25°C. Absorbance at 415 nm was measured by using T60 UV-visible spectrophotometer (PG, UK) against blank. Quantifications were made on the basis of a standard curve from different concentrations of quercetin. Results were denoted as mg quercetin equivalents per gram of sample (mg QE/g sample). Each step was carried in triplicates.

2.7. Protein Determination by Lowry's Method. Lowry's method was used for protein contents analysis [18]. Simply 4 mL of reagent 1 [2%Na_2CO_3 (48 mL) in 0.1 N NaOH + 1% $KNaC_4H_4O_6.4H_2O$ (1 mL) + 0.5%$CuSO_4.5H_2O$ (1 mL)] was added to 0.5 mL of each extract followed by 15 min incubation. After that, 0.5 mL of freshly prepared reagent 2 (2 mL Folin Ciocalteu reagent, 4 mL water with 1 : 2 ratio) was mixed rapidly and incubated for 30 min in dark. Standard reagent Bovine Serum Albumin was used for standard curve preparing and deionized water as blank. Subsequently, the absorbance of the standard solutions and sample extract was calculated at 660 nm. The quantification was completed in triplicate, and the amount of protein was mentioned as mg BSAE/g of sample [19].

2.8. DuBois Carbohydrate Assay. Carbohydrates were estimated by phenol sulphuric reagent method. Phenol 80% (0.05 mL) was added to extract (1 mL), and then, concentrated H_2SO_4 (5 mL) was added and kept for 10 min. The mixture was put in a water bath at 25° to 30° C for 10 to 20 minutes, and change in color was observed. The absorbance of the characteristic yellow orange color was measured at 510 nm. Deionized water was used as blank, and glucose was used as a standard. The results were mentioned as mg GE/g [19].

2.9. Quantitative Assay for DPPH Free Radical Scavenging Activity. The hydrogen donating capability of root extract was carried out in the presence of DPPH stable radical. Different concentrations (1-0.0625 mg/mL) of root extract were made from stock solution, and 0.1 mM DPPH solution (0.5 mL) (for making DPPH 0.1 mM, add 0.0039432 g of DPPH in absolute ethanol 100 mL) was mixed up with 50 μL of the extract. The reaction mixture was shaken and incubated at room temperature for reaction at dark place for 30 min using ascorbic acid as standard. Decolorization of DPPH was observed by measuring the decrease in absorbance at 517 nm. The ethanol, ascorbic acid, and DPPH solution without plant extract were used as a blank, standard, and control correspondingly [14, 17].

The following equation was used for % inhibition calculation:

$$\%\ \text{inhibition of DPPH} = \frac{(\text{AC} - \text{AS})}{\text{AC}} \times 100. \quad (1)$$

Here, AC is absorbance of control (DPPH), and AS is extract absorbance.

The IC_{50} values describe the concentration of sample extract used for scavenging 50% of the DPPH free radicals by characterizing the antioxidant ability of the extracts. The relationship curve was made by plotting the scavenging activities against different concentrations of extract and expressed in mg/mL.

2.10. Ferric Reducing Antioxidant Power (FRAP) Assay. For the FRAP assay, the procedure from [20] was followed with little modifications. Stock solution comprised of acetate buffer (300 mM) with pH 3.6, TPTZ (10 mM) solution, prepared in HCL (40 mM), and ferric chloride hexahydrate (20 mM) solution. The solution was made by mixing acetate buffer (25 mL), TPTZ solution (2.5 mL), and ferric chloride hexahydrate solution (2.5 mL) with 10 : 1 : 1 (v/v), respectively. Briefly, 0.5 mL of the extract (1 mg/mL) was taken in test tubes and 2 mL of FRAP solution and 1 mL distilled water was added. The extract was allowed to react with FRAP solution in the dark for 30 minutes. Absorbance of the colored product was checked at 593 nm. Distilled water was taken as blank, and $FeSO_4$ was taken as standard. The analysis was carried out in triplicates for the extract, and % reduction was determined by the following equation:

$$\text{FRAP \% Reduction} = \frac{(\text{AC} - \text{AS})}{\text{AC}} \times 100, \quad (2)$$

where AC is the absorbance of control, and AS is the absorbance of the extract.

2.11. Antibacterial Activity. The antibacterial potential of *Berberis baluchistanica* root extract was evaluated by using agar well diffusion method. Fresh sterile Mueller Hinton agar was inoculated with the target bacterial strains *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*. Wells were made through 6 mm sterilized cork borers in the plates and extracts dissolved in dimethyl sulfoxide (100 μL) were added into the agar wells. Dimethyl sulfoxide and doxycycline (DO 30 μg) were used as negative and positive controls. The results were observed after 16-24 h of incubation at 37°C, diameter of the inhibition zones was measured in millimeter (mm) [21].

2.12. Gas Chromatography-Mass Spectrometry Analysis. The GC-MS study was executed using a (Shimadzu GC-MS QP2020) gas chromatograph mass spectrometer supplied with an HP-INNOWAX capillary column (30 m length × 0.25 mm id 0.25 mm film thickness) (PaloAlto, CA, USA). The mass spectrum was obtained by electron ionization at 70 eV with a mass scan mode range of 35-500 amu (atomic mass units). The carrier gas helium was used at a flow rate of 1 mL/min, about 2 μL volume of the sample was injected, and the injection temperature was 255°C with a split ratio 1 : 10. The oven temperature for the GC was initially organized at 50°C for 5 min then increased at the rate of 250°C at 5°C/min and finally to 300°C at 5°C/min for 10 min. The start temperature was 50°C and increased at the rate of 8°C/min to 250°C followed by 5°C/min till 300°C. The identification and composition of the targeted compounds were validated by comparing their mass spectral record with those of NIST 14 and 14 s (National Institute of Standards and Technology) Libraries. Mass spectrum was used to identify the name, molecular formula, molecular weight, and structure of the components.

2.13. Statistical Analysis. The measured data in each experiment comprised of 3 replications and the results were expressed as the average ± standard deviations (SD). The magnitude of the means, standard curve, and standard deviations were calculated by using MS Excel 2010 Software.

Table 1: Phytochemical constituents of the *Berberis baluchistanica* root extracts.

S. no	Phytochemical test	Results
1.	Alkaloids	+ve
2.	Cardiac glycosides	+ve
3.	Tannins	+ve
4.	Steroids	+ve
5.	Terpenoids	+ve
6.	Flavonoids	+ve
7.	Saponins	+ve
8.	Coumarin	+ve
9.	Quinones	-ve
10.	Anthraquinones	+ve
11.	Phlobatannins	-ve

Note: +ve: present and –ve: absent.

3. Results

3.1. Phytochemical Analysis. The phytochemical evaluation in root of *Berberis baluchistanica* indicated the presence of alkaloids, cardiac glycoside, anthraquinones, saponins, tannins, flavonoids, coumarin, steroids, and terpenoids. However, quinones and phlobatannins were not present in root (Table 1).

3.2. Phenolic Contents. Total phenolic contents of *Berberis baluchistanica* root extract were evaluated by Folin Ciocalteu method by using gallic acid as standard. Total phenolic content of the extract was determined from the regression equation and expressed as milligram gallic acid equivalents (GAE) per gram in dry weight of sample. TPC value was 19.897 ± 4.8141 mg GAE/g for root extract (Table 2).

3.3. Flavonoid Contents. Total flavonoid content was determined, and results were calculated from the calibration curve and expressed as mg quercetin equivalents (QE) per gram of sample in dry weight. The total flavonoids in root extract were 12.9876 ± 0.8388 mg QE/g as presented in (Table 2).

3.4. Total Protein and Carbohydrates. Total protein contents in root of *Berberis baluchistanica* were calculated by Lowry's method ,and the absorbance values were obtained at various concentrations of BSA for the construction of calibration curve. Total proteins of the extract were 4.675 ± 0.1696 (mg BSAE/g) for root extract (Table 2).

Carbohydrate estimation was carried out by phenol sulphuric reagent method using glucose as standard. A linear trend was observed between the glucose standards concentration and the absorbance at 510 nm. The carbohydrate contents in root extract were 3.696 ± 0.2958 (mg GE/g) as presented in (Table 2).

3.5. Quantitative Assay for DPPH Free Radical Scavenging Activity. The hydrogen donating capability of the root extract of *Berberis baluchistanica* was determined in the presence of DPPH stable radical, and their reducing potential was calculated on the basis of their concentration showing 50% inhibition that is the concentration of extract needed to scavenge 50% DPPH free radicals. The results were obtained by the linear regression equation formed by the concentration of extract against their % scavenging ability. In present study, the DPPH radical scavenging activity was recorded with $IC_{50} = 1.125$ mg/mL in comparison with the standard ascorbic acid having $IC_{50} = 0.325$ mg/mL as shown in (Table 3). The radical scavenging activity increased with increased in concentration is shown in (Table 4 and Figure 1(a)).

3.6. Ferric Reducing Antioxidant Power Assay. The antioxidant capacity of *Berberis baluchistanica* root extract was determined by ferric reducing antioxidant power (FRAP) assay. Results were expressed on the basis of their concentration providing 50% inhibition (IC_{50}) that is the concentration of extract required to reduce Fe3+ into Fe2+. The results were obtained by the linear regression equation formed by the concentrations of extracts against their percent of reducing ability. Obtained results showed that the extract has FRAP % reduction activity with $IC_{50} = 0.912$ mg/mL as presented in Table 3. The higher IC_{50} value indicates lower antioxidant potential and same for ferric reducing activity. In comparison with the standard having IC_{50} value 0.472 mg/mL, the root extract showed lowest antioxidant potential with higher IC_{50} value 0.912 mg/mL. The current findings revealed that the radical scavenging activity of the extract increased with increase in concentration as shown in Table 4 and Figure 1(b).

3.7. Antibacterial Activity. The root extract of the *Berberis baluchistanica* plant was evaluated for its antibacterial properties against different bacteria. The diameter of the inhibition zones against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhi* were 23.14 ± 1.16, 13.38 ± 0.41, 20.21 ± 0.06, 7 ± 0.01, and 14.84 ± 1.06. The extract was found to be active against selected bacteria except *Klebsiella pneumoniae* that showed minimum inhibition, and the activity has been shown in (Figure 2) comparing to the positive control applied in the study. The negative control DMSO was found inactive against all the targeted bacteria.

3.8. GC-MS Analysis. The GC-MS analysis of root extract is given in (Table 5), and the characteristic chromatogram is shown in (Figure 3). The extract is found to contain more than 70 different compounds belonging to different chemical classes with high therapeutic potential. The major compounds included 4-hydroxy-3-(4-hydroxy-3-nitrocinnamoyl)-, 1,3-dioxane-5,5-dimethanol, 2-methyl-, 4-[5-(4-fluoro-phenyl)-tetrazol-2-yl]-butyramide, bicyclo -3-en-2-one, 3,8-dihydroxy-1-methoxy-7-, allyloxydi(tert-butyl)silane, imidazo[1,2-a]pyridin-2(3H)-one, 8-(dimethylamino)-1-hydroxynaphthalene-2-carbonitrile, bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, 7,7-dimethoxy-, 4-allyl-3-(dimethylhydrazono)-2-methylhexane-2, 5-diol, carbonic acid, 2-chloroethyl 2-pentyl ester, carbonic acidbut-3-yn-1-yl octyl ester, acetonitrile, 2,2′-iminobis-, acetic acid, cyano-, 2-

Table 2: Total phenolic contents, total flavonoid contents, total protein, and carbohydrates.

Samples	Total phenolic (mg GAE/g) ± SD	Total flavonoid (mg QE/g) ± SD	Total proteins (mg BSAE/g) ± SD	Total carbohydrates (mg GE/g) ± SD
Root	19.897 ± 4.8141	12.9876 ± 0.8388	4.675 ± 0.1696	3.696 ± 0.2958

Results are expressed as Mean ± S.D for three readings.

Table 3: Estimated IC_{50} values of ethanolic root extract of *Berberis baluchistanica*.

Samples	DPPH assay (IC_{50} mg/mL)	FRAP assay (IC_{50} mg/mL)
Root	1.125	0.912
Ascorbic acid (standard)	0.325	—
$FeSO_4$ (standard)	—	0.472

Ascorbic acid and $FeSO_4$ = standard.

Table 4: DPPH percentage inhibitions and FRAP percent reduction of *Berberis baluchistanica* root extract at different concentration.

Concentrations in (mg/mL)	DPPH % inhibition	FRAP % reduction
1	44.91 ± 0.67	68.92 ± 0.81
0.5	36.43 ± 1.07	56.72 ± 0.24
0.25	17.86 ± 0.94	41.13 ± 1.01
0.125	12.47 ± 1.22	29.12 ± 0.56
0.0625	0.10 ± 0.073	12.01 ± 0.63

hexynoic acid, pyrimidin-4(3H)-one, 2-amino-6-hydroxy-5-nitro-, 3-chlorophenyl pent-4-en-2-yl ester, isoquinoline, decahydro-, 1-nitrobiuret, 6-chloromethyl-N,N-dimethyl-[1,3,5]triazine-2,4-diamine, methyl 3-hydroxyoctadecanoate, TMS derivative, cephaloridine, 3,6,9,12-tetraazatetradecane-1,14-diamine, N(2),N(6)-bis(dimethylaminomethy lene)lysine, cholestan-3-ol, TMS derivative, 3-methyl-4-nitro-5-(1-pyrazolyl)pyrazole, 2-naphthalenecarboxylic acid, 4,4′-methylenebis[3-methoxy-, p-undecyloxybenzoic acid methyl ester, 4-penten-2-ynylamine, N,N,4-trimethyl-, acetamide, N-(aminocarbonyl)-2-chloro-, piperazine, 2-methyl-, cyclopentane, 1-methyl-3-(2-methyl-2-propenyl)-, ethanone, 1-(4-pyridinyl)-, oxime, thiourea, dimethylaminomethyl, histamine-2-carboxylic acid, 9,12-octadecadienoic acid (Z,Z)-, pentanedioic acid, 2,4-dimethyl-, dimethyl ester, bis(sec-butoxo)(methyl)oxovanadium, methyl 4-(2,4-dinitrophenylhydrazono) valerate, dihydroartemisinin, 6-deshydro-5-deshydroxy-3-deoxy, lavandulyl butyrate, butanoic acid, 2,3-dichloro-, silane, (dotriacontyloxy)trimethyl-, 6-methyl-1-(2-thenylidene)furo[3,4c]pyridine, N-heptyl-2-(2-hydroxyethoxy)-N methylpropionamide, urea, N,N′-diethyl-, erythritol, acetamide, 2-chloro-N-1H-purin-6-yl-benzamide, 4-methoxy-N-[4-(1-methylcyclopropyl)phenyl]-,glycylglycine ethyl ester, 4-methyl-2,4-bis(p-hydroxyphenyl)pentene, (3-methylthiopropylideneamino)acetonitrile, androst-5-en-17-one, 16-(1,1-dimethylethyl)-3-hydroxy-, aminohippuric acid, 3TMS derivative, 7-methyl-6,8 bis(methylthio) pyrrolo [1, 2] pyrazine, cyclotetrasiloxane, octamethyl-,pentanedioic acid, 3,3-dimethyl-, dimethyl ester, N,N-dimethylsuccinamic acid, silicic acid, diethyl bis(-trimethylsilyl)ester, p-phenylenediamine, N,N-dimethyl-N′-,benzestrol, 2TMS derivative, 6-chlorohexanoic acid, 4-cyanophenyl ester, pentasiloxane, dodecamethyl-,cyclotrisiloxane, hexamethyl-, 4-methyl-2,4-bis(p-hydroxyphenyl)-pent-1-ene, trisiloxane, 1,1,3,3,5,5-hexamethyl-, cyclooctyl N,N-di isopropyl phosphoramidocyanidate, N-trifluoroacetyl-3-methoxytyramine, cyclotrisiloxane, hexamethyl-, 4-methyl-2,4-bis (p-hydroxyphenyl) pentene, 6-methoxypurine, and TBDMS derivative. These identified compounds are known to have several pharmacological potentials.

4. Discussion

The current study was conducted to analyze the bioactive components and antimicrobial activity of root extract of the *Berberis baluchistanica* plant. Different bioactive compounds such as alkaloids, tannins, cardiac glycosides, anthraquinones, saponins, flavonoids, phenolics, coumarin, steroids, and terpenoids were detected. Many compounds such as phenolics, berberine, alkaloids, pakistanamine, gallic acid, and flavanols have already been reported [11, 13]. All the identified compounds are known to be biologically active possessing antibacterial, antifungal, antiviral, antiparasitic, and antioxidant potentials [10, 22].

The presence of these phytochemicals provides credibility to its use by the local community, and the discovery of new drugs will lead to understand the therapeutic effects of valuable compounds in medicinal plants [23].

Medicinal plants possess phenolic and flavonoid contents that are known to have antimicrobial, antispasmodic, antitumor, antioxidant, anti-inflammatory, and antidepressant activities [23]. Phenolic compounds are good reducing agents and are capable for scavenging of free radicals [24] and also regulate cell division, growth, and metabolic pathways in plants [22]. Flavonoid also reduces a wide range of enzymes like alkaline phosphatase, hyaluronidases, hydrolases, arylsulphatase, cAMP phosphodiesterase, lipase, α-glucosidase, and kinases [25]. The higher phenolic content supports the antioxidant property of plants. Therefore, the occurrence of total phenolics and flavonoid contents was investigated (Table 4).

Previously, the total phenolic contents of *Berberis baluchistanica* whole plant were determined by using different fractions [26]. The obtained values of TPC and TFC in current study were propositionally lower than previously reported [26]. Recently, phenolic and flavonoids contents in bark extract were reported as 48.2 mg GAE/g and 141.3 mg QE/g [10]. These variations could arise from variations in plant species, genetic backgrounds, growing

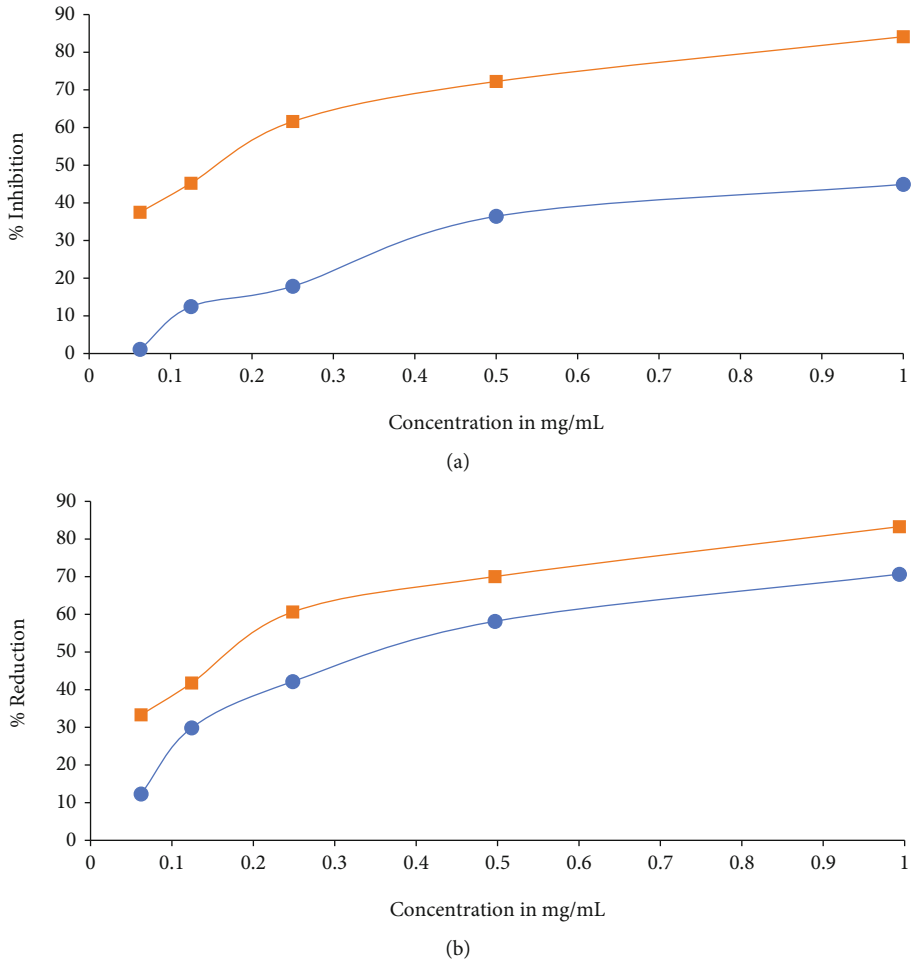

Figure 1: (a) Free radical scavenging activity (DPPH). (b) Ferrous reducing capacity (FRAP) of root ethanolic extract of *Berberis baluchistanica*, (■) represent standard, while (●) represent root sample. Ascorbic acid and ferrous sulfate ($FeSO_4$) were used as a standard in DPPH and FRAP.

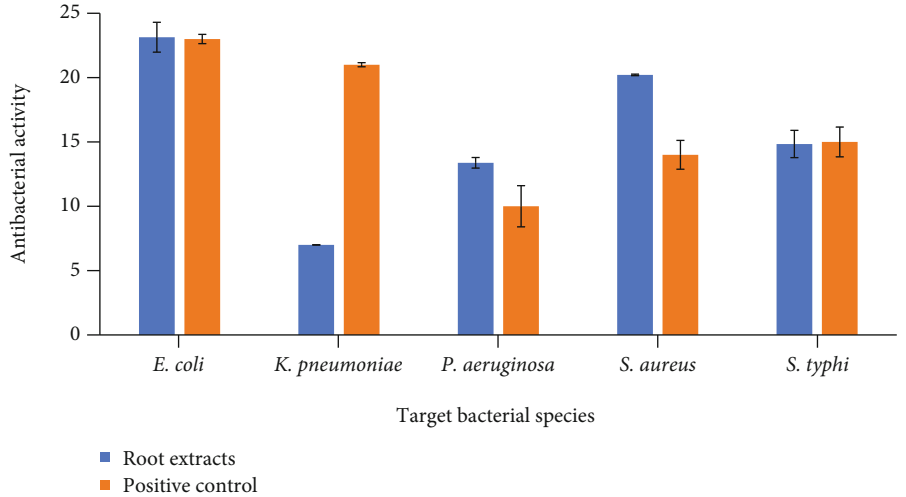

Figure 2: Antibacterial activity of *Berberis baluchistanica* root extract against various bacterial strains *E. coli* (*Escherichia coli*), *K. pneumoniae* (*Klebsiella pneumoniae*), *P. aeruginosa* (*Pseudomonas aeruginosa*), *S. aureus* (*Staphylococcus aureus*), and S. typhi (Salmonella typhi).

Table 5: The major bioactive compounds analyzed in the ethanolic root extract of *Berberis baluchistanica* by GCMS analysis.

S.#	Retention time (min)	Area (%)	Name of the compound	Mol. formula	Mol. Weight
1.	4.728	0.15	4-Hydroxy-3-(4-hydroxy-3-nitrocinnamoyl)-	$C_{15}H_{11}NO_7$	317
2.	4.855	0.08	1,3-dioxane-5,5-dimethanol, 2-methyl-	$C_7H_{14}O_4$	162
3.	5.559	0.47	4-[5-(4-Fluoro-phenyl)-tetrazol-2-yl]-butyramide	$C_{11}H_{12}FN_5O$	249
4.	6.255	0.25	Bicyclo -3-en-2-one, 3,8-dihydroxy-1-methoxy-7-	$C_{21}H_{24}O_7$	388
5.	6.523	0.17	Allyloxydi(tert-butyl)silane	$C_{11}H_{23}OSi$	199
6.	7.531	0.08	Imidazo[1,2-a]pyridin-2(3H)-one	$C_7H_6N_2O$	134
7.	8.045	0.14	8-(Dimethylamino)-1-hydroxynaphthalene-2-carbonitrile	$C_{13}H_{12}N_2O$	212
8.	9.737	0.25	Bicyclo[2.2.1]hept-5-ene-2-carboxylic acid, 7,7-dimethoxy-	$C_{10}H_{14}O_4$	198
9.	10.834	0.09	4-Allyl-3-(dimethylhydrazono)-2-methylhexane-2, 5-diol	$C_{12}H_{24}N_2O_2$	228
10.	11.628	0.11	Carbonic acid, 2-chloroethyl 2-pentyl ester	$C_8H_{15}ClO_3$	194
11.	12.961	0.02	Carbonic acidbut-3-yn-1-yl octyl ester	$C_{13}H_{22}O_3$	226
12.	13.083	0.07	Acetonitrile, 2,2′-iminobis-	$C_4H_5N_3$	95
13.	13.143	0.14	Acetic acid, cyano-	$C_3H_3NO_2$	85
14.	14.409	0.1	2-Hexynoic acid	$C_6H_8O_2$	112
15.	14.524	0.11	Pyrimidin-4(3H)-one, 2-amino-6-hydroxy-5-nitro-	$C_4H_4N_4O_4$	172
16.	16.05	0.1	3-chlorophenyl pent-4-en-2-yl ester	$C_{15}H_{17}ClO_4$	296
17.	16.685	0.2	Isoquinoline, decahydro-	$C_9H_{17}N$	139
18.	17.821	0.2	1-Nitrobiuret	$C_2H_4N_4O_4$	148
19.	18.09	0.1	6-Chloromethyl-N,N-dimethyl-[1,3,5]triazine-2,4-diamine	$C_6H_{10}ClN_5$	187
20	19.025	0.02	Methyl 3-hydroxyoctadecanoate, TMS derivative	$C_{22}H_{46}O_3Si$	386
21.	20.409	0.1	Cephaloridine	$C_{19}H_{17}N_3O_4S_2$	415
22.	20.554	0.1	3,6,9,12-Tetraazatetradecane-1,14-diamine	$C_{10}H_{28}N_6$	232
23.	21.79	0.08	N(2),N(6)-Bis(dimethylaminomethylene)lysine	$C_{13}H_{26}N_4O_2$	270
24.	21.445	0.08	Cholestan-3-ol, TMS derivative	$C_{30}H_{56}OSi$	460
25.	22.688	0.15	3-Methyl-4-nitro-5-(1-pyrazolyl)pyrazole	$C_7H_7N_5O_2$	193
26.	22.963	0.06	2-Naphthalenecarboxylic acid, 4,4′-methylenebis[3-methoxy-	$C_{25}H_{20}O_6$	416
27.	24.365	0.3	p-Undecyloxybenzoic acid methyl ester	$C_{19}H_{30}O_3$	306
28.	25.712	0.17	4-Penten-2-ynylamine, N,N,4-trimethyl-	$C_8H_{13}N$	123
29.	26.77	0.12	Acetamide, N-(aminocarbonyl)-2-chloro-	$C_3H_5ClN_2O_2$	136
30.	27.49	0.1	Piperazine, 2-methyl-	$C_5H_{12}N_2$	100
31.	28.31	0.16	Cyclopentane, 1-methyl-3-(2-methyl-2-propenyl)-	$C_{10}H_{18}$	138
32.	28.404	0.16	Ethanone, 1-(4-pyridinyl)-, oxime	$C_7H_8N_2O$	136
33.	29.301	0.09	Thiourea, dimethylaminomethyl	$C_4H_{11}N_3S$	133
34.	29.865	0.12	Histamine-2-carboxylic acid	$C_6H_9N_3O_2$	155
35.	30.364	0.03	9,12-Octadecadienoic acid (Z,Z)-	$C_{21}H_{40}O_2Si$	352
36.	32.825	0.07	Pentanedioic acid, 2,4-dimethyl-, dimethyl ester	$C_9H_{16}O_4$	188
37.	33.235	0.06	Bis(sec-butoxo)(methyl)oxovanadium	$C_9H_{21}O_3V$	228
38.	34.605	0.19	Methyl 4-(2,4-dinitrophenylhydrazono) valerate	$C_{12}H_{14}N_4O_6$	310
39.	35.223	0.06	Dihydroartemisinin, 6-deshydro-5-deshydroxy-3-deoxy	$C_{15}H_{22}O_3$	250
40.	35.725	0.07	Lavandulyl butyrate	$C_{14}H_{24}O_2$	224
41.	36.241	0.12	Butanoic acid, 2,3-dichloro-	$C_4H_6Cl_2O_2$	156
42.	36.837	0.1	Silane, (dotriacontyloxy)trimethyl-	$C_{35}H_{74}OSi$	538
43.	36.935	0.11	6-Methyl-1-(2-thenylidene)furo[3,4c]pyridine	$C_8H_{10}ClNO_2$	187
44.	37.53	0.2	N-Heptyl-2-(2-hydroxyethoxy)-N-methylpropionamide	$C_{13}H_{27}NO_3$	245
45.	37.265	0.2	Urea, N,N′-diethyl-	$C_5H_{12}N_2O$	116
46.	37.392	0.17	Erythritol	$C_4H_{10}O_4$	122
47.	37.892	0.19	Acetamide, 2-chloro-N-1H-purin-6-yl-	$C_7H_6ClN_5O$	211
48.	38.91	0.27	Benzamide, 4-methoxy-N-[4-(1-methylcyclopropyl)phenyl]-	$C_{18}H_{19}NO_2$	281

Table 5: Continued.

S.#	Retention time (min)	Area (%)	Name of the compound	Mol. formula	Mol. Weight
49.	38.121	0.14	Glycylglycine ethyl ester	$C_6H_{12}N_2O_3$	160
50.	38.215	0.15	4-Methyl-2,4-bis(p-hydroxyphenyl)pentene	$C_{24}H_{36}O_2Si_2$	412
51.	38.395	0.13	(3-Methylthiopropylideneamino)acetonitrile	$C_6H_{10}N_2S$	142
52.	39.985	0.2	Androst-5-en-17-one, 16-(1,1-dimethylethyl)-3-hydroxy-,	$C_{23}H_{36}O_2$	344
53.	40.2	0.14	Aminohippuric acid,3TMS derivative	$C_{18}H_{34}N_2O_3Si_3$	410
54.	40.58	0.24	7-Methyl-6,8 bis(methylthio) pyrrolo [1, 2] pyrazine	$C_{10}H_{12}N_2S_2$	224
55.	40.82	0.18	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	296
56.	40.151	0.23	Pentanedioic acid, 3,3-dimethyl-, dimethyl ester	$C_9H_{16}O_4$	188
57.	41.006	0.34	N,N-Dimethylsuccinamic acid	$C_6H_{11}NO_3$	145
58.	41.86	0.25	Silicic acid, diethyl bis(trimethylsilyl)ester	$C_{10}H_{28}O_4Si_3$	296
59.	41.106	0.18	p-Phenylenediamine, N,N-dimethyl-N′-	$C_{11}H_{11}F_5N_2O$	282
60.	41.148	0.27	Benzestrol, 2TMS derivative	$C_{26}H_{42}O_2Si_2$	442
61.	42.295	0.27	6-Chlorohexanoic acid, 4-cyanophenyl ester	$C_{13}H_{14}ClNO_2$	251
62.	42.889	0.45	Pentasiloxane, dodecamethyl-	$C_{12}H_{36}O_4Si_5$	384
63.	43.25	0.24	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222
64.	43.915	0.44	4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene	$C_{24}H_{36}O_2Si_2$	412
65.	44.194	0.33	Trisiloxane, 1,1,3,3,5,5-hexamethyl-	$C_6H_{20}O_2Si_3$	208
66.	44.423	0.27	Cyclooctyl N,N-di isopropyl phosphoramidocyanidate	$C_{15}H_{29}N_2O_2P$	300
67.	45.865	0.28	N-Trifluoroacetyl-3-methoxytyramine,	$C_{17}H_{28}F_3NO_3Si_2$	407
68.	46.145	0.36	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	222
69.	46.456	0.48	4-Methyl-2,4-bis (p-hydroxyphenyl) pentene	$C_{24}H_{36}O_2Si_2$	412
70.	50.655	0.13	6-Methoxypurine, TBDMS derivative	$C_{12}H_{20}N_4OSi$	264

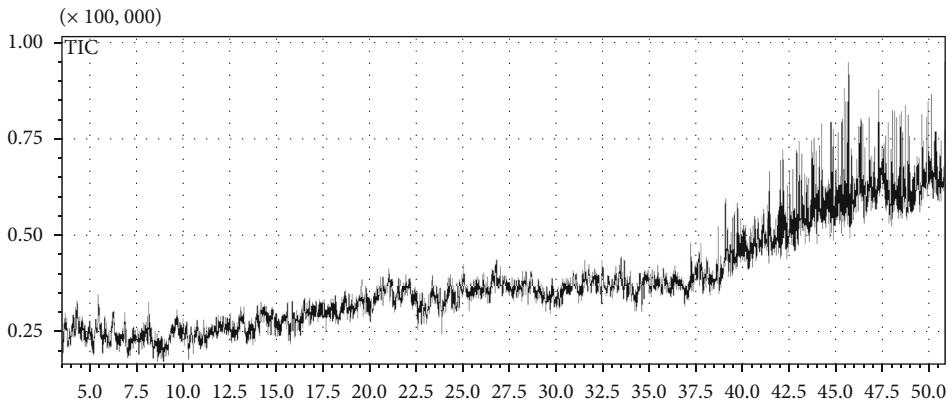

Figure 3: GC-MS chromatograph of ethanolic root extract of *Berberis baluchistanica*.

conditions, environmental factors, and agronomic practices polarity of the solvents [27].

The phenolic and flavonoid contents are responsible for the biological effects of the crude extract. Flavonoids are highly efficient in scavenging of oxidizing molecule, including different free radicals involved in various diseases [28]. Phenolic contents contribute the stress tolerance in plants. Extracts of herbs, vegetables, fruits, and grains being rich in phenolics are increasingly used in the food industries for their antioxidative potentials and medical advantages. Similarly, flavonoid suppresses the formation of reactive oxygen involved in production of free radicals and scavenging of reactive species for antioxidant defense.

The DPPH assay is considered as one of the most valid used tools to identify the scavenging potential of free radical by plant extracts. The IC_{50} value is basically concentration of substrate which inhibits 50% of DPPH radicals. In the current study, the obtained results of DPPH radical scavenging activity were higher than the ethyl acetate soluble fraction IC_{50} value ($15.96 \pm 1.5\ \mu g/mL$) of the whole plant of *Berberis baluchistanica* reported by [26]. Current findings were higher than the recently reported antioxidant activity IC_{50} ($52.86\ \mu g/mL$) of CME of *Berberis baluchistanica* [12]. The lower IC_{50} value indicates higher antioxidant potential and same for radicals scavenging activity. The antioxidant power of plants extracts is generally

associated with the concentration of phenolic and flavonoid compounds present in the sample. Higher quantity of polyphenols and flavonoids describes the higher antioxidant activity [14]. Literature reveals that berberine an alkaloid and berberisinol flavones obtained from Berberis species exhibit antioxidant potential. Berberine and berberisinol also present in *Berberis baluchistanica* [29] might be a causative factor for hydrogen donor capacity of *Berberis baluchistanica* ethanolic root extract.

FRAP % reduction activity was done to measure the antioxidant capacity of *Berberis baluchistanica* root extract against reactive oxygen species. Antioxidants have capability to give electrons and reduce Fe3+ into Fe2+. The complexes of Fe2+ and tripyridyltriazine give an intense blue color with high absorbance at wavelength of 593 nm. The antioxidant capacity of the extracts was related to the IC_{50} values. The higher IC_{50} values indicate lower reducing activity or lower antioxidant potential. The present study revealed that the ethanolic root extract exhibited lower FRAP % reduction with IC_{50} (0.912 mg/mL). Obtained results were in conformity with previous data [26]. Phenolic and flavonoids have made significant antioxidant power as they act as suitable scavenging agents. According to [6], the total antioxidant activity was directly related to phenolic compounds. Moreover, comparing our results of antioxidant activity showed strong correlation between DPPH and ferric reducing antioxidant power assay. Results revealed that total antioxidant activity of root extract is due to its higher association with antioxidant compounds such as phenols. Current findings were in agreement with previous reported literature [30].

The antibacterial potential of *Berberis baluchistanica* root extract was assessed by using agar well diffusion method. The extract showed effective antibacterial potential against bacterial strains of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Salmonella typhi*. Gram negative bacteria, *Escherichia coli* proved to be the most sensitive with maximum zone of inhibition (23.14 ± 1.16) followed by *Staphylococcus aureus* (20.21 ± 0.06). Results revealed that all selected pathogens were susceptible to the extract except *Klebsiella pneumoniae* that turned out to be the most resistant strain and showed minimum inhibition. The results of current study were linked with the previously reported [10] and relatively higher than those found in literature [13]. However, [11] documented higher results. Recently, [12] reported the crude methanolic extract of *Berberis baluchistanica* against *Pseudomonas aeruginosa* (Gram negative bacterium) and *Micrococcus luteus* and *Bacillus subtilis* (gram positive strains) at three different concentrations. All concentrations were found with better inhibitory profile *Micrococcus luteus* 16.81, 17.93, and 20.22 mm and *Bacillus subtilis* and 12.18, 16.72, and 18.37 mm, respectively. Similar trends were observed with the previously documented results of other species of *Berberis* [23, 29, 31]. The results suggested that root extract is a powerful source of wide-ranging antimicrobial agent. The strong antibacterial effect of the obtained extract is probably due to the presence of secondary metabolites such as alkaloids, steroids, coumarin, saponins, and terpenoids and high contents of phenolics and flavonoids which seemed to be associated in nucleic acid biosynthesis inhibition and new metabolic practices [25].

The total proteins and total carbohydrates contents were determined by Lowry's method and phenol sulphuric reagent method, respectively. Total proteins of the extract were 4.675 ± 0.1696 (mg BSAE/g) and carbohydrate contents in root extract were 3.696 ± 0.2958 (mg GE/g). Current results of protein and carbohydrates estimation of *Berberis baluchistanica* root extract showed that it contains a reasonable amount of proteins and carbohydrates, so root can be used as a natural source of low cost and easily available proteins and carbohydrates enriched food material for nutritional purposes. Current findings were in accordance with previous findings [32].

The results of the GC-MS analysis revealed that a total of 70 different known compounds belonged to different chemical classes were identified. The active principles of the identified compounds with their retention time, concentration (area %), molecular formula, and molecular weight are presented in Table 5. The representative chromatograms of the ethanolic root extract of *Berberis baluchistanica* are presented in Figure 3.

GC-MS study was executed to recognize the biologically active components present in root of *Berberis baluchistanica* due to the fact that the plant has many phytochemicals possessing several therapeutic activities. From the results, it was observed that 70 different compounds with the mass spectra have been identified. The results revealed that the detected compounds were as traces (below 0.5%). Among major compounds, 9,12-octadecadienoic acid (Z,Z)- is known to have anti-inflammatory, hypocholesterolemic, and antiarthritic property [19]. Recently, [33, 34] reported the strong antioxidant, anti-inflammatory, cytotoxicity, nematicidal, and antibacterial potential of hexadecanoic acid and pentanedioic acid methyl ester. Naphthalene is also reported to possess strong antioxidant, antifibrinolytic, and antimicrobial activity [19]. Strong antioxidant, anti-inflammatory, and cytotoxicity of acetamide were reported by [35]. Presence of silicic acid, diethyl bis(trimethylsilyl) ester in relatively higher quantity might be accountable for the biological strength of the extract against human pathogen [36]. Erythritol is a sweet antioxidant releases oxidative stress and has unique nutritional properties [37]. Some other significant medicinal compound p-andecyloxybenzoic acid methyl ester, benzamide, benzestrol, thiourea, and dimethylaminomethyl were also reported by the earlier workers [38–40]. The principle identified compounds in the sample are 4-hydroxy-3-(4-hydroxy-3-nitrocinnamoyl)-,1,3dioxane-5,5-dimethanol,2-methyl-bicyclooct-3-en-2-one,3,8-dihydroxy-1-methoxy-7-3,8-dihydroxy-1-methoxy-7, allyloxydi(tert-butyl)silane,7,7-dimethoxy-,5-diol, carbonic acid, 2-chloroethyl 2-pentyl ester, carbonic acid but-3-yn-1-yl octyl ester, acetonitrile, 2,2′-iminobis-, acetic acid, cyano-, 2-hexynoic acid, pyrimidin-4(3H)-one, 2-amino-6-hydroxy-5-nitro-, succinic acid, isoquinoline, decahydro-, 2-naphthalenecarboxylic acid, 4,4′-methylenebis[3-methoxy-, p-undecyloxybenzoic acid methyl ester, 4-penten-2-ynylamine, N,N,4-trimethyl-, acetamide, N-(aminocarbonyl)-2-chloro, piperazine,2-methyl-, cyclopentane, 1-methyl-3 (2-

methyl-2-propenyl), cyclotrisiloxane, hexamethyl-, N-trifluoroacetyl-3-methoxytyramine, and methoxypurine derivative and were also present. GC-MS analysis of *Berberis baluchistanica* root extract showed a mixture of acetamides, glycosides, and methyl esters, and its derivatives that are known to have significant antioxidant, inflammatory, hypocholesterolemic and antiarthritic and cytotoxicity and antibacterial properties [41].

5. Conclusion

The obtained results clearly showed that *Berberis baluchistanica* root extract is rich in bioactive compounds with strong antioxidant potential. The extract was found to have significant antimicrobial potential against different bacterial pathogens. The protein and carbohydrate estimation shows that it contains low cost and easily available proteins and carbohydrates enriched food material for nutritional purposes. GC-MS analysis established the existence of various bioactive compounds in root extract having the potential to act as antimicrobial, antioxidant, anti-inflammatory, cytotoxicity, and nematicidal substances. From this study, it can be concluded that the *Berberis baluchistanica* root may provide a potential source of medicines due to the presence of biologically active components with strong antioxidant and antibacterial activities.

Data Availability

Most of the data is part of the manuscript, and the remaining data will be made available on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Retraction

Retracted: Unani Formulation Habb-e-Suranjan: A Treasure of Biological Activities

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] A. I. A. T. Ahemad, Q. M. A. Aejazuddin, G. J. Khan et al., "Unani Formulation Habb-e-Suranjan: A Treasure of Biological Activities," *BioMed Research International*, vol. 2022, Article ID 1640193, 11 pages, 2022.

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Research Article

Unani Formulation Habb-e-Suranjan: A Treasure of Biological Activities

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Habb-e-Suranjan (HES), an Unani formulation, has been studied for its anti-inflammatory properties in both *in vitro* and *in vivo* experiments. HES is recommended for arthritis, gout, and joint pain. The current endeavor is an attempt to put it to the test and verify its efficacy scientifically. It was tested for DPPH, hydroxyl, and nitric oxide scavenging activities. It was shown that HES had the greatest TAC and FRAC values when compared to catechin and ascorbic acid. HES exhibited DPPH and hydroxyl radical scavenging activity that was dose-dependent. Incubation of sodium nitroprusside solutions in PBS at 25°C for 150 min resulted in the production of nitric oxide. Nitric oxide production was effectively decreased by HES. Anti-inflammatory medications boosted the migration of PMN cells toward the chemoattractant FMLP in an agarose experiment of PMN chemotaxis. In carrageenan-induced rat paw edema, in the HES-treated group, paw thickness was 3.021 ± 0.084 at $t = 0$, but it showed an increase in paw inflammation after one hour, i.e., 3.195 ± 0.082 cm which again showed a decrease in paw thickness up to 4th hour, i.e., 3.018 ± 0.078, 2.98 ± 0.032, and 2.684 ± 0.061 at $t = 2$, 3, and 4, respectively. It showed again getting back to the normal thickness of paw at $t = 24$ hrs, i.e., 3.029 ± 0.118 cm. It is concluded that the formulation is potent enough and can be used effectively for the treatment of inflammation and associated health issues. Moreover, there is much scope to evaluate its effectiveness using different *in vitro* and *in vivo* models.

1. Introduction

Unani System of Medicine is Graeco-Arabic medicine founded by Hippocrates and Galen and refined by Arabian and Persian doctors such as Rhazes (al Razi) and Avicenna (Ibn-e-Sina), Al-Zahrawi, and Ibn Nafis throughout the Middle Ages. Buqrat (460-377 BC), also known as Hippocrates, was a descendant of Aesculapius and is regarded as the "Father of Unani Medicine" [1, 2]. It began around 2500 years ago in Greece, and it has been employing drugs that are 90% herbal, 4-5 percent animal, and 5-6 percent mineral in nature. It is not only the original science of medicine but also a vast repository of medical ideas and philosophies that can be extremely beneficial to both medicine and science in general. Diseases are thought to disrupt the body's normal temperament and humor equilibrium. So regimental therapy (Ilaj Bil-Tadabeer) and pharmacology (Ilaj Bil-Dawa) have been used since ancient times with the express

purpose of restoring humor equilibrium and correcting aberrant temperament. When this method of treatment fails, surgery (Ilaj Bil-yad) is recommended as a last resort [3, 4].

Many researchers are working on herbal medicine to prove its effectiveness in a variety of diseases including COVID-19 [5–14]. It is well documented that herbal medicine has proven its role in the treatment of inflammation, obesity, cancer, diabetes mellitus, etc. [15–19]. Although it is thought that the usage of herbal remedies dates back to 1550 BC in Egypt, many of its pharmacological effects are still unknown. Only approximately a quarter of the estimated 800,000 plant species on the planet have been classified, and only a small percentage of these have been tested for pharmacological efficacy. More plant-based drugs were sought to aid in the treatment of the various ailments that still afflict society [20–23].

For the promotion and maintenance of health, the Unani system of medicine places a strong focus on lifestyle management. Diet, lifestyle, emotions, interactions with the environment, and even spiritual considerations are all taken into account [24]. It takes into account both external elements such as seasons, air quality, food and beverages, and internal aspects such as sleep and wakefulness, movement and rest, evacuation and retention, and so on. The Unani system of medicine is extremely relevant to modern healthcare because of its ability to promote health and disease prevention through nondrug lifestyle variables [7, 25].

Elwa/Sibr (*Aloe barbadensis*), Halela (*Terminalia chebula*), and Suranjan (*Colchicum luteum*) were combined in equal amounts in the test formulation, Habb-e-Suranjan (HES), which was made under the supervision of a GMP-certified pharmacist at CRIUM, Hyderabad, and sent to the research site. Three times a day, patients were instructed to apply 5 ml of lukewarm oil to the afflicted joints in the morning and evening twice a day for two weeks after taking HES (500 mg each) for two weeks [26].

Subchronic oral toxicity studies of HES in albino Wistar rats were investigated by Ghazanfar et al. [3]. The antiarthritic impact of Unani pharmacopoeial formulations of HES and Raughan-e-Suranjan in Waja-al-Mafasil was researched and assessed by Ahmed et al. [26]. Suhail et al. [27] studied and explained Habb/pill (plural: Huboob) is a solid medicinal preparation, made by mixing powdered drugs in a suitable binder (water/oil/resin of plant) and made into a round and uniformly shaped balls of the required size. Habb-e-Suranjan is one of the common Huboob or pills used in the Unani system of medicine from ancient times. This polyherbal formulation is being used as an analgesic for the treatment of various types of joint disorders and nerve pains like Waja'-al-Mafaasil (Arthralgia), Waja'-al-A'saab (Neuralgia), Irq-al-Nasa (Sciatica), and Niqris (Gout). It is also used in Qabz (constipation). A comparative study of the therapy of primary gout with HES and allopurinol was conducted by Akhtar and Siddiqi [28]. HES contains different potential ingredients such as Elwa (Musabbar)-Aloe vera (Indian Aloe) extract (11.29%), Tuhkm Soya (11.29%), Turbud Safed (Turpeth)-Ipomoea Turpethum (30.64%), Habb-ul-Neel-Indigo (11.29%), Suranjan Shirin (Colchicum Corm)-Colchicum Luteum (25.80%), Guggulu-Gum Guggul-Commiphora Mukul (4.83%), and Mastagi-Mastiche (4.83%) [29].

A review of the literature revealed that the *in vivo* and *in vitro* anti-inflammatory potential activity of Unani formulation HES was not previously conducted to evaluate the formulation's traditional claim as anti-inflammatory and antioxidant. Therefore, present work was aimed at performing phytochemical screening and *in vivo* as well as *in vitro* anti-inflammatory activities of HES.

2. Material and Methods

2.1. Drugs and Chemicals Used. Habb-e-Suranjan (HES) was purchased from the local Unani medical store of Nandurbar, Maharashtra, India. Oxycodone (Sigma-Aldrich, St. Louis, MO, USA) and 0.9% sodium chloride (Hospira, Lake Forest, IL, USA), Folin-Ciocalteu reagent, 2,2-diphenyl-1-picryl hydroxyl radical (DPPH), phenazine methosulfate, nicotinamide adenine dinucleotide, sodium nitroprusside (SNP), trichloroacetic acid (TCA), thiobarbituric acid (TBA), and L-ascorbic acid were purchased and procured from Lab Trading Laboratory, Aurangabad. Catechin and rutin were purchased from Sigma Chemicals, India.

2.2. Physicochemical Analysis and Preliminary Phytochemical Screening of Habb-e-Suranjan. The specification of the HES mixture was evaluated by performing physicochemical analyses such as appearance, color, taste, odor, pH value, friability, hardness, weight change, and disintegration time. Initial phytochemical testing of the powder of HES tablets was carried out following previously documented procedures [12].

2.3. Animals Used and Ethical Approvals. 36 male Wister rats and 30 male mice weighing about 150-200 g and 25-30 g each were used for the study. They were fed with a standard pellet diet and were supplied with water ad libitum, housed less than 12 h light/dark cycles, with controlled temperature (22-25°C). At least one week before the trial began, the animals were acclimated to the new environment. Care of the animals and experimental procedures were done according to the guidelines of the Institutional Animal Ethics Committee (IAEC) having approval number CPCSEA/IAEC/JLS/16/07/21/11.

2.4. In Vivo Anti-inflammatory Activity

2.4.1. Carrageenan-Induced Rat Paw Edema. The carrageenan-induced rat paw edema method was used to test the anti-inflammatory properties of HES. The 24 rats were split up into four equal groups, each with six rats. Group 1 (control group) was injected with saline and provided with the vehicle, group 2 (carrageenan control) was injected with carrageenan and was orally treated with the vehicle, group 3 (treatment 1) was treated with HES 100 mg/kg p.o., and group 4 (standard group) was treated with diclofenac sodium 100 mg/kg. Carrageenan, a substance that causes inflammation, and the conventional and experimental medications were all given intravenously via the use of sterile saline water.

This research began by treating the animals with the medications stated above (vehicle, standard, and three treatments). Carrageenan solution was injected into the subplantar area of the right hind paw one hour after the previous treatment to produce edema. Carrageenan injections increased the thickness of the paws, indicating edema. After carrageenan injection, measurements were taken at 0, 1, 2, 3, 4, and 24 hrs using the Plethysmometer. The difference between the paw thickness at "0 hour" and the paw thickness at the corresponding hours was used to calculate the increase in paw thickness [30, 31].

2.4.2. Paw Withdrawal Threshold (Von Frey Test). Mechanical allodynia: the rat was placed individually on an acrylic cage elevated maze and adopted for the test environment for a minimum of 15 min. Von Frey filament was attached to the rat's plantar aspect of the hind paw from the mesh floor's base. When enough filament force was given to the paw, it bent slightly and held there for a brief period. Withdrawal of the paws was seen as a favorable reaction. Oxycodone was used as a standard drug; it was given at a dosage of 150 g/0.1 ml/kg after being dissolved in 0.9 percent sodium chloride [32, 33].

2.4.3. Acetic Acid-Induced Vascular Permeability. Both HES and diclofenac were given to rats at doses of 100 mg/kg intravenously, and the vehicle was used as a control. 2 percent Evan's blue solution was administered i.v. into the tail vein of each rat 1 hour after the treatments. A solution of 6.5% acetic acid (in saline) was administered i.p. into each mouse 10 minutes later at a dose of 10 ml/kg body weight into each animal. The rats were euthanized and their peritoneal cavity was cleaned three times with saline (10 ml) after 30 minutes of acetic acid administration. The saline washes were centrifuged at 3500 rpm for five minutes. A plate reader was used to measure the absorbance at 590 nm of the collected supernatant. In micrograms, Evan's blue extravasation was counted using a standard curve [34, 35].

2.4.4. Tail Immersion Test. Rats were divided into 6 animals in each group. The lower part of the tail (5 cm) was immersed in a beaker containing water maintained at 55 ± 0.5°C. The time taken for the withdrawal of the tail from the water was recorded as a reaction time, with 10 sec as a cut-off time. The reaction time was noted one hour before the administration of drugs and as well one hour after the administration. The control group was provided with saline, whereas the treatment groups were provided with HES (100 mg/kg) p.o, and dextropropoxyphene (65 mg/kg) was administered as a standard drug subcutaneously, 30 min before the test [36].

2.4.5. Adjuvant-Induced Arthritis (AIA) in Rats. A kind of arthritis was induced in rats after they were injected with Freund's complete adjuvant (CFA). On day 0, rats were anesthetized with an 80 : 10 mg/kg i.p. ketamine and xylazine mixture before being injected intradermally with 0.1 ml CFA 1 mg/ml heat-inactivated Mycobacterium tuberculosis in 85 percent paraffin oil and 15 percent mannide monooleate at the tail base. Animals in the control group received a saline injection with the same volume as the experimental group rats. Grouping was done as follows: control (no adjuvant, saline), AIA (adjuvant, no treatment), HES (adjuvant, 100 mg/kg HES), and 0.1 mg/kg methotrexate (MTX); it is the most utilized antirheumatic drug and hence was used as a standard drug administered p.o. The injections were administered every day for 27 days [37]. Blood was drawn from the retroorbital plexus to conduct laboratory testing on hematological parameters. Red blood cell (RBC), white blood cell (WBC), platelet count, and erythrocyte sedimentation rate (ESR) are among the hematological characteristics measured [38].

Table 1: Organoleptic character.

Sr. no.	Parameters	Observation
1	Size	Oval
2	Color	Light olive brown
3	Taste	Agreeable
4	Odor	Astringent
5	Appearance	Habb (pills)

Table 2: Physiochemical character.

Sr. no.	Parameters	HES
1	Friability test	0.83%
2	Hardness test	5.5 kg/cm^2 (Monsanto)
3	Weight variation	0.112
4	Disintegration time	32 min
5	A pH of 1% solution	6.5
6	10% solution	5.6

2.5. In Vitro Anti-inflammatory Activity

2.5.1. Determination of Total Antioxidant Capacity (TAC). Rahman et al. [39] presented a technique for determining sample TAC. This test is primarily dependent on the drugs/samples reducing Mo (VI) to Mo (V), resulting in the development of a green-colored phosphate/Mo (V) complex at acidic pH. 3 ml of a mixture comprising 0.6 M sulphuric acid, 28 mM sodium phosphate, and 1 percent ammonium molybdate was combined with 0.5 ml of samples/standards at varied concentrations of 12.5-150 μg/ml. The reaction was completed by incubating the test tubes containing the above mixtures at 95°C for 10 minutes. After allowing the reaction mixture to cool to ambient temperature, the absorbance at 695 nm was measured using a spectrophotometer using a blank solution as a control. Catechin was utilized as a point of comparison. A blank solution was prepared using 3 ml of the reaction mixture, and the same amount of solvent as was used for the samples and standard. The blank was also incubated for 10 minutes at 95°C before being measured at 695 nm. Absorbance increases indicate a higher total antioxidant capacity. For each antioxidant assay, standard/samples were used at five different concentrations ranging from 12.5 to 150 μg/ml.

Table 3: Effect of HES on carrageenan-induced paw edema.

Treatment	Paw thickness of rats (cm)					
	0 hr	1 hr	2 hr	3 hr	4 hr	24 hrs
Group I: normal control	3.026 ± 0.89	3.026 ± 0.073	3.026 ± 0.082	3.026 ± 0.092	3.026 ± 0.074	3.026 ± 0.109
Group II: carrageenan control	3.031 ± 0.076	3.189 ± 0.069	3.392 ± 0.087	3.628 ± 0.093	3.732 ± 0.061	4.261 ± 0.123
Group III: HES	3.021 ± 0.084***	3.195 ± 0.082***	3.018 ± 0.078***	2.98 ± 0.032***	2.684 ± 0.061***	3.029 ± 0.118***
Group IV: diclofenac sodium (standard)	3.035 ± 0.086***	3.098 ± 0.087***	2.961 ± 0.069***	2.92 ± 0.077***	2.899 ± 0.088***	3.034 ± 0.139***

Values are expressed as mean ± SEM. $^{*}p < 0.001$ compared to control group and $^{***}p < 0.001$ compared to carrageenan control group.

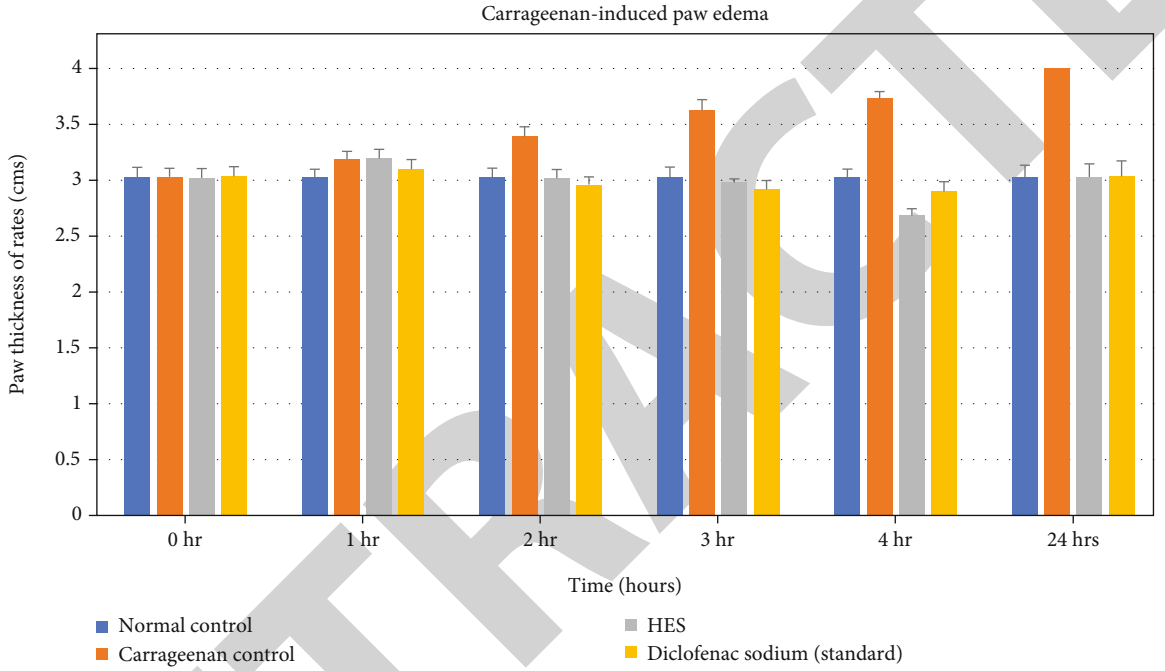

Figure 1: Paw edema caused by carrageenan treatment with HES.

Several different concentrations were tested to see which one best reflected the reasonable rise in antioxidant activity as the concentration of the sample rose.

2.5.2. Ferrous Reducing Antioxidant Capacity Assay (FRAC). The FRAC of samples was determined using Rahman et al.'s technique [39]. The development of Perl's Prussian blue at 700 nm may be used to monitor Fe^{2+}. The test tubes were filled with 0.25 ml of standard/sample solutions ranging from 12.5 to 150 g/ml, 0.625 ml of potassium buffer (0.2 M), and 0.625 ml of 1 percent potassium ferricyanide solution. It took around 20 minutes of incubation at 50 degrees Celsius to complete the reaction. The test tubes were then filled with 0.625 ml of a 10% trichloroacetic acid solution. The aforesaid combination was centrifuged for 10 minutes at 3000 rpm, after which 1.8 ml of the supernatant was removed from the test tubes and mixed with 1.8 ml of distilled water and 0.36 ml of 0.1 percent ferric chloride solution. The absorbance at 700 nm was measured using a spectrophotometer, and the results were compared to a blank. The absorbance of a blank solution containing the same

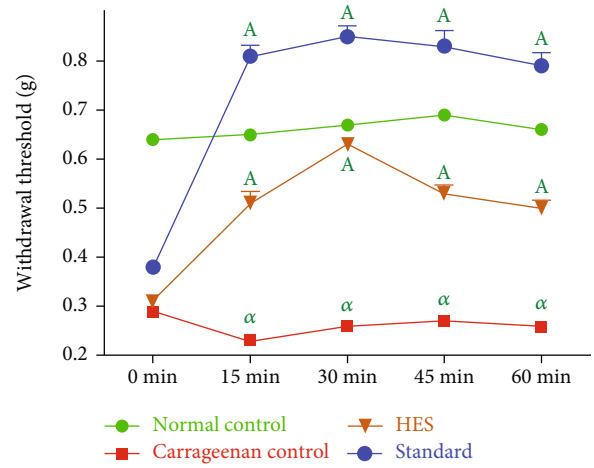

Figure 2: Effect of HES on paw withdrawal threshold.

reaction mixture but without the sample/standard was measured at 700 nm after incubation under the same circumstances. Higher reaction mixture absorption indicates a

Table 4: Effect of HES on paw withdrawal threshold.

Treatment	Paw withdrawal threshold (g)				
	0 min	15 min	30 min	45 min	60 min
Normal control	0.64 ± 0.02	0.65 ± 0.02	0.67 ± 0.02	0.69 ± 0.02	0.66 ± 0.02
Carrageenan control	0.29 ± 0.01	0.23 ± 0.03 $^{\alpha}$	0.26 ± 0.03 $^{\alpha}$	0.27 ± 0.02 $^{\alpha}$	0.26 ± 0.02 $^{\alpha}$
HES	0.31 ± 0.03	0.51 ± 0.02^{a}	0.63 ± 0.02^{a}	0.53 ± 0.03^{a}	0.50 ± 0.03^{a}
Standard	0.38 ± 0.02	0.81 ± 0.01^{a}	0.85 ± 0.01^{a}	0.83 ± 0.01^{a}	0.79 ± 0.02^{a}

Values are expressed as mean ± SEM. $^{\alpha}$ $p < 0.001$ compared to control group and $^{a}p < 0.001$ compared to carrageenan control group.

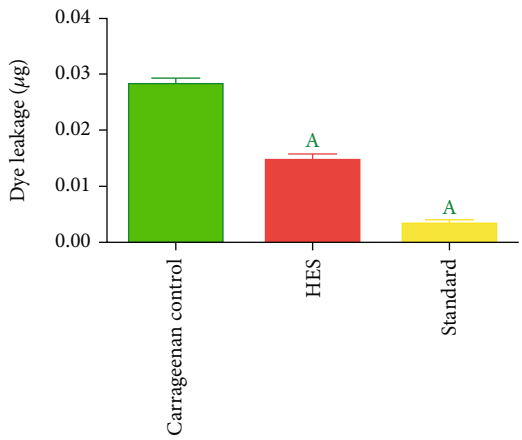

Figure 3: The impact of HES on Evans Blue dye extravasation into the rat peritoneal cavity.

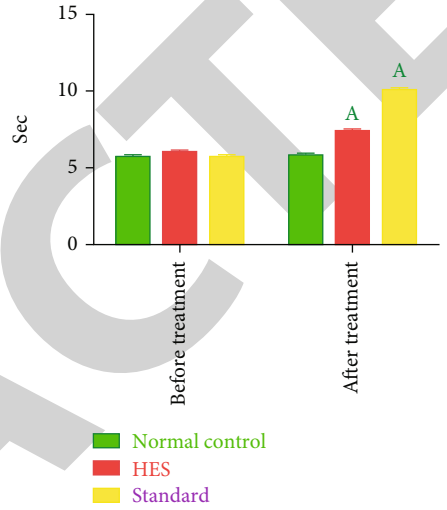

Figure 4: Tail withdrawal reflexes elicited by the tail immersion technique in rats are affected by HES.

Table 5: Tail withdrawal reflexes elicited by the tail immersion technique in rats are affected by HES.

Drug (dose)	Before treatment (sec)	After treatment (sec)
Control (saline)	5.7 ± 0.082	5.8 ± 0.099
HES (100 mg/kg)	6.0 ± 0.053	7.3 ± 0.19^{a}
Dextropropoxyphene (65 mg/kg)	5.7 ± 0.066	10 ± 0.16^{a}

greater capacity to reduce. At each concentration, the experiment was performed three times.

2.5.3. Radical Scavenging Activity

(1) DPPH Radical Scavenging Assay. The compounds' capacity to scavenge free radicals was assessed using the DPPH radical scavenging assay, as reported by Rahman et al. [39]. The capacity of the compounds to donate hydrogen atoms was assessed by decolorizing a methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). In methanol solution, DPPH produces a purple/violet hue that fades to shades of yellow in the presence of antioxidants. A 0.1 mM DPPH in methanol solution was produced, and 2.4 ml of it was combined with 1.6 ml of extract in methanol at concentrations ranging from 12.5 to 150 μg/ml. The solution combination was completely mixed before being stored in the dark at room temperature for 30 minutes. At 517 nm, the absorbance was determined spectrophotometrically. As a control, butylated hydroxytoluene (BHT) was utilized. The percentage of DPPH radical scavenging activity was determined by this equation: %DPPH radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$.

Here, A_0 is the absorbance of the control, whereas the drug's/absorbance standard is A_1. The IC_{50} was then determined by plotting the percentage of inhibition versus the concentration. At each concentration, the experiment was carried out three times.

(2) Hydroxyl Radical Scavenging Activity. By using the technique of Rahman et al., the hydroxyl radical scavenging activity of the medicines was determined [39]. This system created hydroxyl radicals by combining Fe^{3+}-ascorbate-EDTA-H_2O_2 (Fenton reaction). In this test, the 2-deoxy-D-ribose breakdown product, which generates a chromogen when heated with TBA at low pH, is quantified. For this experiment, the reaction mixture comprised the following components: 50 mmol l^{-1} of phosphate buffer solution, 12.5-150 μg/ml of drug/standard, 10.0-40.0 mol l^{-1} of sodium azide, 10.0-10.0 mg/ml of sodium deoxycholate, and 20.0-20.0 mg/ml of 2-deoxy-D-ribose (28 mmol l^{-1}). At 370 degrees Fahrenheit, the mixtures were held in a water bath and the reaction was initiated by adding 0.2 ml of ascorbic acid, H_2O_2 (2 mmol l^{-1}), and AA (2 mmol l^{-1}) (10 mmol l^{-1}). 1.5 ml of HCl is added after 1 hour of incubation at 370°C.

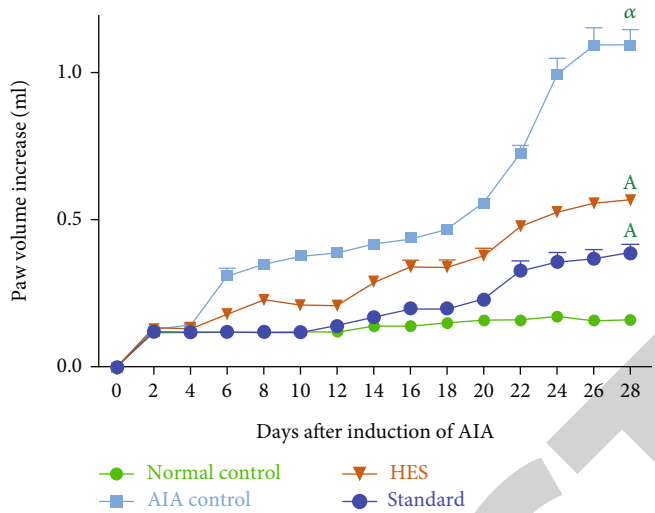

Figure 5: In rats, HES has an anti-inflammatory impact on AIA.

Table 6: The hematological parameters of rats treated with Freund's complete adjuvant and the effect of HES on these parameters.

Group	WBC count (10^3/mm^3)	RBC ($\times 10^6/\mu$l)	Platelet count (10^5/mm^3)	ESR (mm/hr)
Normal control	8.7 ± 0.25	6.0 ± 0.15	2.4 ± 0.16	3.7 ± 0.19
AIA control	$12 \pm 0.31^{\alpha}$	$4.3 \pm 0.28^{\alpha}$	$3.5 \pm 0.11^{\alpha}$	$5.6 \pm 0.15^{\alpha}$
HES	10 ± 0.34^{b}	5.5 ± 0.18^{c}	2.9 ± 0.07^{c}	5.2 ± 0.16
Standard	9.5 ± 0.26^{a}	6.0 ± 0.21^{a}	2.5 ± 0.12^{a}	4.4 ± 0.09^{c}

For 15 minutes, the reaction mixture was heated to 100°C and then cooled with a stream of cold water. The solution's absorbance was measured at 532 nm. With the suppression of the percentage of 2-deoxy-D-ribose oxidation on hydroxyl radicals, the scavenging capability of hydroxyl radicals was determined. The proportion of hydroxyl radical scavenging activity may be calculated using the following equation: %hydroxyl radical scavenging activity = $[A_0 - (A_1 - A_2)] \times 100/A_0$.

In the equation, A_0 is the control's absorbance without a sample present. Sample and 2-deoxy-D-ribose were added to A_1 and the absorbance was measured. When 2-deoxy-D-ribose is omitted from the sample, its absorbance is equal to A_2. The IC_{50} was then determined by plotting the percentage of inhibition versus the concentration. At each concentration, the experiment was carried out three times.

(3) In Vitro Activity of HES on Human PMN Cell. Samples of fresh, anticoagulated blood taken from healthy human volunteers were used to isolate PMN cells. In order to extract PMN cells, 5 ml of blood was combined with 1.75 percent Dextran T-500 solution in saline and placed for 45 minutes at room temperature for sedimentation. The remaining RBCs were washed three times with PBS before being suspended in a 10% fetal bovine serum-containing RPMI-1640 medium, which was then centrifuged. The PMN cell density was set using RPMI-1640 media containing 10% fetal calf serum. As part of the in vitro testing, dilutions of the active ingredients in each of the assays were made in RPMI-1640 medium by adding 10 μl of the dilution to 990 μl medium. Effect on the chemotaxis of PMN cells/human PMN cell chemotaxis produced by formyl-N-methyl-leucyl-phenylalanine (FMLP) was studied in vitro using the chemotaxis test technique under agarose. The agarose solution (1.2 w/v) was prepared in Eagle's minimal essential medium (MEM) and heated to 56°C. The microscopic slides (50 × 75 mm) were produced by putting 6.0 cc of the aforementioned solution over each slide and allowing this to settle at room temperature. The slides were kept at 2-8°C for six hours. After that, 5 mm diameter wells were drilled into the settled gel, each one 4 mm in diameter. The wells were added with 20 μl FMLP solution in RMPI-1640 medium whose concentration was 1×10^{-8} for determining the chemotaxis in response to FMLP. The surrounding wells contained 10 μl PMN (1×10^8 cells/ml in minimum essential medium) and 10 μl of each drug solution. The slides were then incubated at 37°C in the presence of 5% CO_2 for 2 h. The neutrophils were fixed for 30 min using methanol, after the process of incubation. The gels were gently removed after fixation, and the neutrophils were stained with Giemsa stain.

The slides were then examined at a 100x magnification using a digital microscope. Motic-3.2 software was used to measure the distance traveled by the leading front of cells toward the FMLP-containing wells [40].

(4) Nitric Oxide Scavenging Assay. Borra and Gurumurthy explained the procedure in detail, in which a solution of 10 mM sodium nitroprusside in phosphate-buffered saline

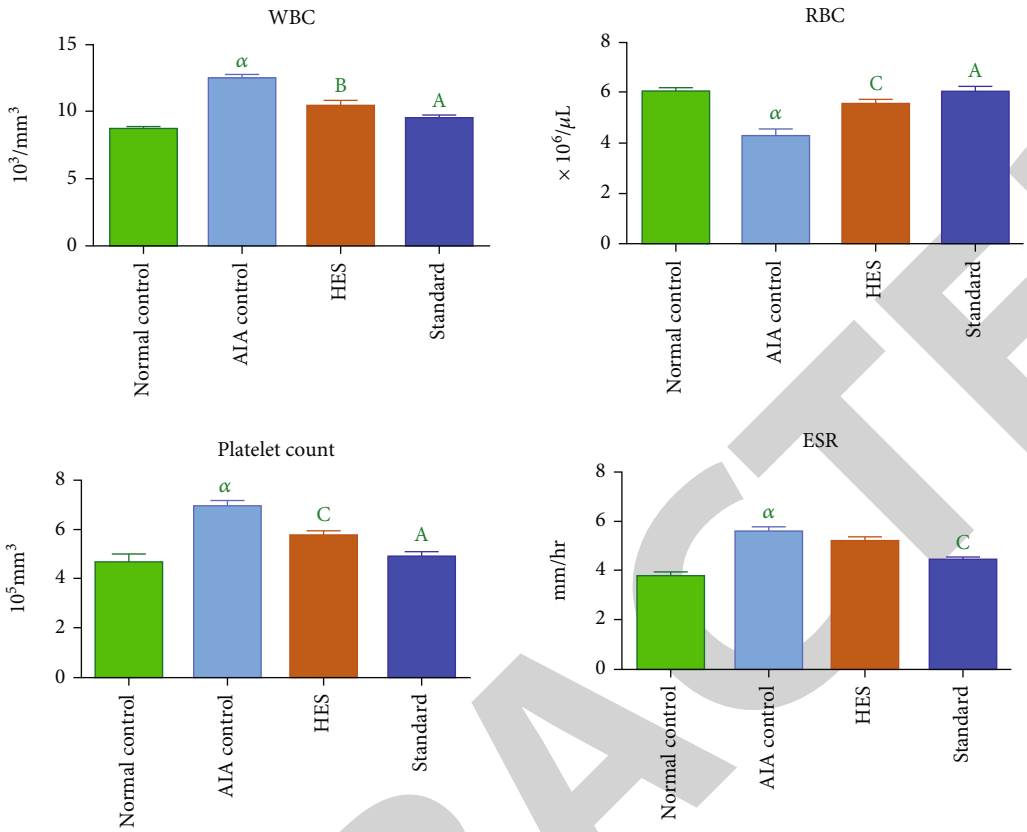

Figure 6: The hematological parameters of rats treated with Freund's complete adjuvant and the effect of HES on these parameters.

(PBS) pH 7.4 was combined with 0.5 ml of drug solutions at different concentrations ranging from 10 to 50 μg/ml and ascorbic acid at concentrations of 25 to 200 μg/ml. This combination was kept at a temperature of 25°C. 0.5 ml of the incubation solution was collected after 150 minutes and mixed with 0.5% of the Griess reagent, 0.5% of the sulfanilic acid reagent (0.33 percent prepared in 20% glacial acetic acid at room temperature for 5 minutes), and 0.1 percent w/v of the naphthyl ethylene diamine dihydrochloride (1.0 ml). After 30 minutes of incubation at room temperature, the mixture's absorbance at 540 nm was recorded [41].

3. Results and Discussion

3.1. Physicochemical Analysis and Preliminary Phytochemical Screening of HES. The HES pills were light olive brown and oval (solid) with a distinctive agreeable taste and astringent smell. The pills were kept in a cold, dark area in firmly covered containers, shielded from moisture, light, and temperature. Physicochemical examination found a slightly basic pH of 6.5 as stated in Tables 1 and 2.

The presence of numerous secondary metabolites like alkaloids, tannins, flavonoids, proteins, and the mucilage may explain the therapeutic benefits of HES tablets. As a result, preliminary screening assays can aid in the detection of bioactive components, which can lead to medication development and discovery. Furthermore, these tests make it easier to estimate the quantity of pharmacologically active chemical substances and to separate them qualitatively. In the HES extract, preliminary phytochemical screening with several qualitative chemical assays revealed the presence of reducing sugars, protein, amino acid, alkaloids, and tannins, whereas carbohydrates, glycosides, flavonoids, saponins, fats and oils, and steroids were lacking.

Table 7: Absorbance HES at two different concentrations.

Drugs	TAC		FRAC	
	At 100 μg/ml	At 150 μg/ml	At 100 μg/ml	At 150 μg/ml
HES	0.934 ± 0.098	1.284 ± 0.095	1.576 ± 0.032	1.874 ± 0.056
CA	1.89 ± 0.076	2.394 ± 0.064	—	—
AA	—	—	3.409 ± 0.065	3.225 ± 0.067

CA: catechin; AA: ascorbic acid. $n = 3$, mean ± SD.

3.2. In Vivo Anti-inflammatory Activity

3.2.1. Experimental Method (Carrageenan-Induced Rat Paw Edema). In a carrageenan-induced paw edema test, rats received subplantar injections of carrageenan that displayed increased and decreased paw thickness with time. In the normal control group at $t = 0$, paw thickness was observed at 3.026 ± 0.89 cm which remained the same after 24 hrs. In the carrageenan control group, paw thickness showed an exponential increase and displayed significance at $p < 0.001$. It was 3.031 ± 0.076 cm at $t = 0$, which was increased

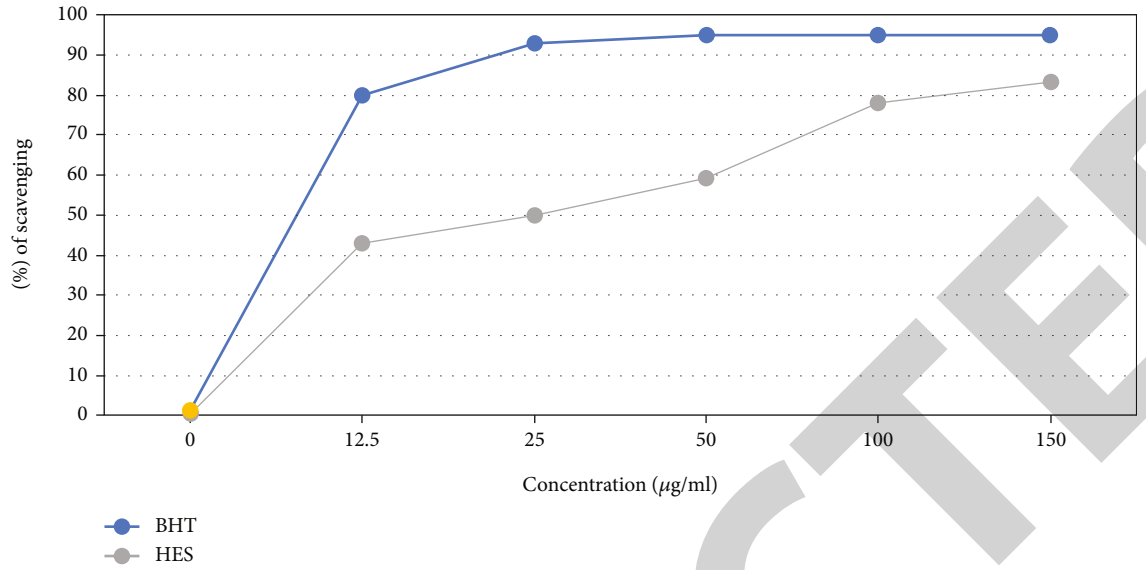

Figure 7: Evaluation of the DPPH radical scavenging activity of HES.

Table 8: Evaluation of the DPPH radical scavenging activity of HES.

Concentration (μg/ml)	BHT	HES
0	0	0
12.5	80	43
25	93	50
50	95	59
100	95	78
150	95	83

after 24 hrs to 4.261 ± 0.123 cm. In the HES-treated group, paw thickness was 3.021 ± 0.084 at $t = 0$, but it showed an increase in paw inflammation after one hour, i.e., 3.195 ± 0.082 cm which again showed a decrease in paw thickness up to 4th hour, i.e., 3.018 ± 0.078, 2.98 ± 0.032, and 2.684 ± 0.061 at $t = 2$, 3, and 4, respectively. It showed again getting back to the normal thickness of paw at $t = 24$ hrs, i.e., 3.029 ± 0.118 cm. In diclofenac sodium-treated groups, it displayed the same pattern as demonstrated by the HES-treated group. All the results obtained were significant with the control group. The values are tabulated in Table 3 and the graph paw thickness Vs time is illustrated in Figure 1.

3.3. Paw Withdrawal Threshold (Von Frey Test)

3.3.1. Effect of HES on Paw Withdrawal Threshold. In Figure 2 and Table 4, the Von Frey test reporting pain threshold measurement is displayed. A decreased withdrawal response was observed through a nonnoxious mechanical stimulation of the paw (allodynia-like measure) in carrageenan-treated animals, which maintained a plateau at 2 and 3 hours after treatment. Treatment with HES ($p < 0.001$) showed a significant decrease in pain from 0 to 60 min, and similar treatment with standard oxycodone also showed a significant decrease ($p < 0.001$) in pain from 0 to 60 min. Thus, HES and the standard drug showed complete blockade of carrageenan-induced hypersensitivity.

3.4. Acetic Acid-Induced Vascular Permeability

3.4.1. The Impact of HES on Evans Blue Dye Extravasation into the Rat Peritoneal Cavity. Standard reduced the dye leakage into the peritoneum more effectively when compared to HES and control. HES has an anti-inflammatory effect because it reduces the permeability of blood vessels, which results in less dye leakage. In the present method, HES ($p < 0.001$) and metformin ($p < 0.001$) have shown significant inhibition of dye leakage (Figure 3).

3.4.2. Effects of HES on Tail Withdrawal Reflexes in Rats Induced by Tail Immersion. All three samples have shown significant inhibition concerning control (HES, $p < 0.001$). From Table 5 and Figure 4, it is evident that all the formulation had shown significant analgesic activity. Dextropropoxyphene had a significantly higher concentration than this.

3.4.3. Rats' Resistance to Adjuvant-Induced Arthritis after Treatment with HES. From day 8 to day 28, the AIA group's paw volume was considerably higher than that of the regular control group ($p < 0.001$). Paw edema was significantly reduced in the HES and MTX-treated groups compared to the untreated controls ($p < 0.001$) (Figure 5).

3.4.4. Effect of HES on Rats Treated with Freund's Complete Adjuvant on Hematological Parameters. Arthritis-related hematological alterations are shown in Table 6 and Figure 6. Levels of WBC, platelet count, and ESR were increased in arthritic rats, while the level of RBC was decreased. These levels were observed to be near normal on treatment with standard drugs, whereas HES has shown less significant or no significance when compared to the AIA group.

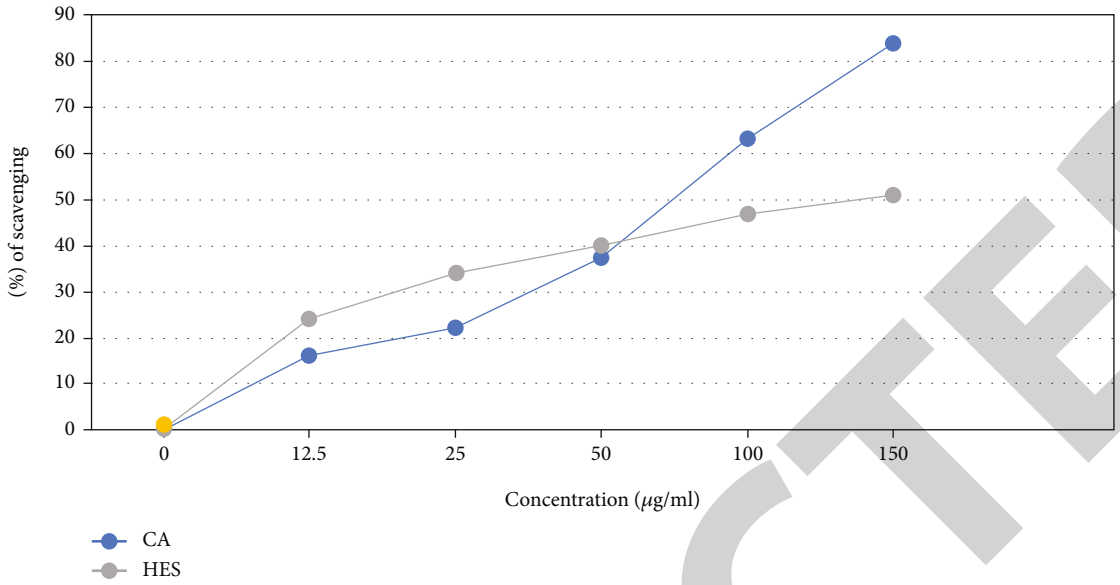

Figure 8: Hydroxyl radical scavenging capabilities of HES and CA.

Table 9: Hydroxyl radical scavenging capabilities of HES and CA.

Concentration (μg/ml)	CA	HES
0	0	0
12.5	16	24
25	22	34
50	37	40
100	63	47
150	84	51

3.5. In Vitro Models

3.5.1. Determination of TAC and FRAC. The TAC and FRAC of HES are shown in Table 7. When compared to normal catechin, HES had significantly higher antioxidant activity. At the concentration of 100 μg/ml, the absorbance of HES was in the range of 0.584 ± 0.053 to 1.89 ± 0.076, while at 150 μg/ml, the range was 0.874 ± 0.043 to 2.394 ± 0.064. An increase in therapeutic dosage resulted in an increase in total antioxidant activity.

A moderate to high FRAC was seen in HES when drug concentrations were boosted in the animal. At 100 μg/ml, the absorbance was in the range of 0.975 ± 0.056 to 3.409 ± 0.065, while at 150 μg/ml, the range was 1.345 ± 0.086 to 3.225 ± 0.067. Results show that HES has the greatest TAC and FRAC values, which is similar to the catechin and ascorbic acid standards.

3.5.2. Evaluation of the DPPH Radical Scavenging Activity of HES. Figure 7 and Table 8 illustrate the free radical scavenging activity of both HES and BHT. At a concentration of 100 μg/ml, the scavenging activity of HES and BHT was 78 ± 0.64 and 95 ± 0.94 μg/ml. By suppressing the DPPH radical with a high inhibition value, the extract demonstrated concentration-dependent antioxidant activity. The approach relies on the creation of the nonradical form DPPH-H by the reaction, which reduces the DPPH solution in the presence of a hydrogen donor antioxidant. The extract was able to convert the DPPH radical into yellow diphenyl picrylhydrazine, which was stable. Because of its propensity to contribute hydrogen, ascorbic acid has been discovered to decrease and discolor 1,1-diphenyl-2-picrylylhydrazyl. HES extract appears to be able to give hydrogen while also acting as an antioxidant. As the concentration of the extract rises, so does the effect of elimination.

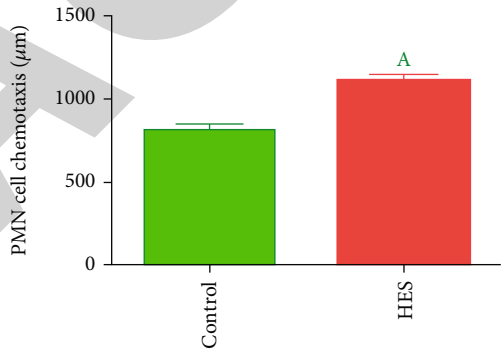

Figure 9: Effect of HES on FMLP-induced chemotaxis of PMN cells. Values are expressed as mean ± SEM. $^{a}p < 0.001$ when compared to the control group.

3.6. Hydroxyl Radical Scavenging Capabilities of HES. HES has exhibited a dose-dependent hydroxyl radical scavenging activity as illustrated in Figure 8 and tabulated Table 9. The scavenging activity of HES at a concentration of 100 μg/ml was 47 ± 0.75, while that of catechin was 63 ± 0.84 μg/ml.

3.7. In Vitro Activity of HES on Human PMN Cell. In an agarose experiment of PMN cell chemotaxis, the drugs stimulated the migration of PMN cells toward the chemoattractant FMLP. According to Figure 9, all groups' stimulation of

Table 10: IC_{50} value for *in vitro* nitric acid scavenging activity.

Sr. no.	Sample	Nitric oxide (μg/ml)
1	HES	41.59 ± 2.45
2	Ascorbic acid	186.34 ± 3.28

PMN cell chemotaxis (measured in micrometres) was significant (HES $p < 0.001$).

3.8. Nitric Oxide Scavenging Assay. In tests, the formulation had just a little ability to reduce levels of nitric oxide (NO). Drug concentration raises the percentage of inhibition. After 150 minutes of incubation at 25°C with sodium nitroprusside solutions in PBS, NO production was detected. As shown in Table 10, the IC_{50} for HES was 41.59 ± 2.45 μg/ml, while the IC_{50} for ascorbic acid was 186.34 ± 3.28 μg/ml.

4. Conclusion

One of the most frequently prescribed Unani formulations for the treatment of inflammation and arthritis is Habb-e-Suranjan. In the present investigation, we tried a scientific attempt to evaluate its anti-inflammatory potential using different *in vitro* and *in vivo* models. A battery of tests evaluating a range of activities such as carrageenan-induced rat paw edema, Von Frey test, adjuvant-induced arthritis (AIA) in rats, hydroxyl radical scavenging activity test, nitric oxide scavenging activity test, free radical scavenger DPPH, and *in vivo* and *in vitro* anti-inflammatory activities have been demonstrated. The inclusion of diverse polyherbal components, which is a recognized powerful antioxidant and anti-inflammatory, could explain Habb-e-Suranjan's effectiveness. Overall, the current study shows the anti-inflammatory and antioxidant ability of formulation, which not only rationalizes some ethnomedicinal claims but also identifies a promising candidate for further research, particularly in chronic inflammatory disorders like rheumatoid arthritis. More study is needed to be done to find the biomolecules that are responsible for the anti-inflammatory and antioxidant effects. In the future, we are aiming to investigate its effectiveness using more quality *in vivo* models and by estimating more precise biochemical parameters followed by histological examination.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Toxicity Mitigation of Textile Dye Reactive Blue 4 by Hairy Roots of *Helianthus annuus* and Testing Its Effect in *In Vivo* Model Systems

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An anthraquinone textile dye, Reactive Blue 4 (RB4), poses environmental health hazards. In this study, remediation of RB4 (30-110 ppm) was carried out by hairy roots (HRs). UV-visible spectroscopy and FTIR analysis showed that the dye undergoes decolourization followed by degradation. In addition, toxicity and safety analyses of the bioremediated dye were performed on *Allium cepa* and zebrafish embryos, which revealed lesser toxicity of the bioremediated dye as compared to untreated dye. For *Allium cepa*, the highest concentration, i.e., 110 ppm of the treated dye, showed less chromosomal aberrations with a mitotic index of 8.5 ± 0.5, closer to control. Two-fold decrease in mortality of zebrafish embryos was observed at the highest treated dye concentration indicating toxicity mitigation. A higher level of lipid peroxidation (LPO) was recorded in the zebrafish embryo when exposed to untreated dye, suggesting a possible role of oxidative stress-inducing mortality of embryos. Further, the level of LPO was significantly normalized along with the other antioxidant enzymes in embryos after dye bioremediation. At lower concentrations, mitigated samples displayed similar antioxidant activity comparable to control underlining the fact that the dye at lesser concentration can be more easily degraded than the dye at higher concentration.

1. Introduction

The textile industry is one of the major industries using dyes of various kinds. Nearly 80,000 tonnes of dyestuff are produced in India, and approximately 10,000 textile dyes are manufactured commercially on a global scale. Globally, the production of textile dye is 7×10^5 metric tons, of which 30% of dyes are used superfluously [1]. 2% of these dyes fail to adhere to fibre and consequently discharge into effluents, implying that the textile dyeing process generates harmful wastewater [2]. The most frequently used dyeing compounds are azo dyes followed by anthraquinone dyes owing to their stability against microbial degradation and photolysis [3, 4]. Azo groups and aromatic groups of anthraquinone dyes are resistant to chemical degradation and are liable to accumulate in the environment with a very high degree of persistence [1]. Anthraquinone dyes are the second most abundantly produced dyes on a global scale. While numerous groups have investigated the remediation of azo dyes, less attention has been dedicated to the degradation of anthraquinone dyes [5]. Industrial effluents containing these substances have a detrimental influence on natural water bodies and aquatic life, while also offering an implicit hazardous and even carcinogenic risk to humans [6]. Therefore, amelioration of these effluents is imperative and research on it is a pressing priority.

Existing methods for textile wastewater treatment include physical and chemical, in addition to certain engineered techniques such as adsorption, electrolysis, oxidation, and photoionization. The majority of the procedures discussed above have significant disadvantages, including high costs, low efficiency, and the generation of hazardous intermediates [7]. Adsorption, on the other hand, requires less land space, offers more flexibility in the design and operation, generates fewer toxic elements, and extends increased removal of contaminants. However, it leads to concentration of pollutants from textile effluents to the adsorbent and does not ensure complete removal of the pollutant. Therefore, it is of importance to determine if the plant species are capable of transforming or degrading the dye into simpler, nontoxic products. Hairy roots (HRs) from various plant species are extensively used to assess tolerance, accumulation, and/or elimination of environmental pollutants [8, 9]. Additionally, HR crops also serve as *in vivo* model systems to investigate phytoremediation processes and mechanisms [10]. Amidst all the pollutants that endanger biodiversity, industrial dye-based effluents present the most serious threat [11]. Textile colours in extremely low quantities in effluent and their byproducts are likewise hazardous to the ecology [12]. Hence, only the adsorption process is not enough, and degradation of the dyes also becomes critical. Numerous researchers have described the use of HRs to degrade dyes; however, there is limited data investigating the safe nature of the bioremediated dyes using in vitro models. Among the plants used for remediation of pollutants, sunflower (*Helianthus annuus*) is well known for remediation of pollutants like phenol and heavy metals [13]. Hence, the present study is aimed at evaluating the bioremediation potential of HRs towards an anthraquinone textile dye RB4, and toxicity analysis of the degraded dye products was performed using *in vivo* model systems. The outcomes from this study are expected to further standardize the use of HRs as an effective bioremediation agent for a broad range of textile dyes.

2. Materials and Methods

2.1. Raw Materials and Chemicals. Hairy roots were obtained from explants of *Helianthus annuus*. The textile dyes RB4, Direct Black B (DBB), Reactive Green 19 (RG19), Reactive Orange 84 (RO84), Reactive Yellow 17 (RY17), Reactive Red 35 (RR35), Reactive Red M8B (RRM8B), Reactive Red M5B (RRM5B), Reactive Violet 5R (RV5R), and Reactive Violet 13 (RV13) were obtained from Appex Industries, Ahmedabad, India. All other chemicals used were of high analytical grade.

2.2. Preparation of Hairy Roots. *Agrobacterium rhizogenes* MTCC532 was used for HR induction in leaf explants of *Helianthus annuus*, and molecular confirmation was done by PCR [14].

2.3. Decolourization of Textile Dyes. The capacity of HRs to decolourize 10 textile dyes for 120 h was performed, and percent decolourization was calculated by the method of Jha et al. [14] using the following formula:

$$\%\text{Decolourization} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100. \quad (1)$$

2.4. Effect of Different Parameters on Decolourization of Dyes. The study was carried out by incubating HRs and selected dye (RB4) solution of varying pH (1.8, 2.8, 3.8, 4.8, 5.8, 6.8, and 7.8), temperatures ($27 \pm 2^\circ$C, $35 \pm 2^\circ$C, and $45 \pm 2^\circ$C), initial dye concentrations (30, 50, 70, 90, and 110 ppm), and biomass dosages (10-50 g/L). The decolourization (%) was calculated (see Section 2.3) for each sample with varying parameters.

2.5. Phytodegradation Analysis of Dyes by HRs

2.5.1. UV-Visible Spectroscopy. The absorbance of the supernatant obtained at 0 h and during decolourization of RB4 by HRs was taken in a UV-Vis spectrophotometer (Shimadzu UV-Vis Spectrophotometer 2800) at 595 nm wavelength.

2.5.2. Fourier Transform Infrared Spectroscopy (FTIR). The metabolites extracted, after decolourization of RB4, were mixed with spectroscopically pure KBr in the ratio of 5 : 95. The analysis was performed in the mid-IR region of 400-4000 $cm^{-1}$with 16 scan speeds using the PerkinElmer 783 Spectrophotometer and compared with control [14].

2.6. Toxicity Analysis

2.6.1. Allium cepa Test. The first set of bulbs was exposed to water (control), the second set to untreated dye (110 ppm), and the third set to treated dye (110 ppm) for 120 h. The cells were checked for different types of chromosomal aberrations [15].

2.6.2. Zebrafish Maintenance and Fish Embryo Toxicity (FET) Test. The embryos of wild-type zebrafish were used and maintained as per Westerfield 2000. Fertilized eggs in the cleavage period until the blastula stage were selected under an inverted microscope (Nikon ECLIPSE TS100) for subsequent experiments. An *in vivo* toxicity test was performed as per the Organization for Economic Co-operation and Development (OECD) test guideline no. 236. For this study, healthy zebrafish embryos were placed in 6-well culture plates (15 embryos per well). 30 mL per well samples of each untreated and treated dye was used in the range of 30, 50, 70, 90, and 110 ppm, respectively. Embryos in sterile distilled water were used as a control. The plates were then kept in the dark at 26 ± 2°C. The embryos exposed from 24 to 96 h (every 24 h) were used for toxicity and biochemical analyses. The embryos exposed to the untreated and treated dye were evaluated for hatching, mortality, tail malformations, heartbeat, coagulation, malformation of somites, development of eyes, pigmentation, and edemas [16, 17]. All the experiments were conducted in triplicate.

2.7. Biochemical Assays. The embryos were homogenized in an ice-cold buffer (0.1 M Tris-HCl, 0.1 mM EDTA, and 0.1% Triton X-100 (*v*/*v*), pH 7.8). The homogenates were centrifuged, and the supernatants were used for the measurement of total protein and malondialdehyde (MDA) content as per Rajneesh et al. [18], superoxide dismutase (SOD) activity as per Bewley et al. [19], succinate dehydrogenase (SDH) as per Singh et al. [20], catalase (CAT) assay as per Bhori et al. [21], and peroxidase (POX) assay as per Bhunia et al. [22].

2.8. Statistical Analysis. Each analysis was performed using GraphPad Prism 8.4.2 in triplicate, and the results were represented as mean ± SD. The significance of the difference among the groups was assessed using a two-way analysis of variance (ANOVA) test followed by Tukey's post hoc test of the difference between all group means. Symbols used for significance are $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results and Discussion

3.1. Screening of Textile Dyes. As the dyes used in the textile processing industry are of varying chemical structures, the effluents from the industry significantly vary to a large extent in composition. Therefore, it was important to evaluate the decolourization efficiency of HRs for different dyes. All the dyes were screened, and decolourization was observed in the range of 10% to 90% (Figure 1) after 24 h, 48 h, and 120 h of incubation. The dye, which showed maximum decolourization, was RB4, i.e., 90% after 120 h (Figure S1). The absorbance of supernatants at 120 h of decolourized RB4 was taken in a UV-visible spectrophotometer and compared with that of an untreated sample (Figure 2(b)). The decolourization percentage varied with different dyes, which might be attributed to their structural differences [23], increased structural complexities due to high molecular weight, and occurrence of inhibitory groups such as NO_2 and SO_3Na [24].

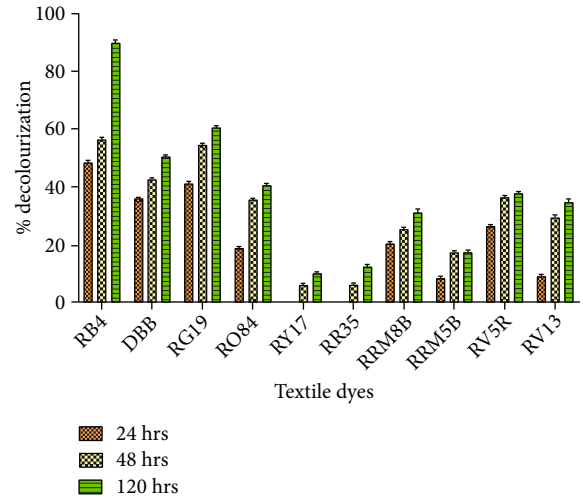


Figure 1: Decolourization of different dyes by hairy roots: untreated dye (U) and treated dye (T).

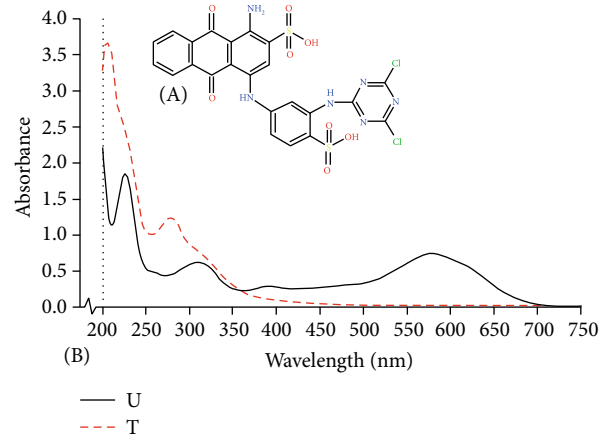


Figure 2: (a) Structure of RB4 and (b) UV-visible spectrum of RB4 untreated dye (U) and treated dye (T).

3.2. Effect of Different Parameters on Dye Decolourization by HRs. Maximum decolourization was observed at pH 4.8-5.8 (Figure S2), while temperature from 25 to 45°C (Figure S3) did not substantially affect decolourization. Therefore, all further experiments were conducted at 25°C and medium pH 5.8. Maximum decolourization (>90%) was observed at a biomass dosage of 40 g/L and 50 g/L (Figure S4). Decolourization was observed to be inversely proportional to the dye concentration ranging 30-110 ppm. The lowest concentration decolourized to 99%, and the highest was 51% (Figure S5).

3.3. FTIR. The FTIR spectrum of untreated and treated samples showed a variation in the molecular structure which is due to biodegradation of dye (Figure 3). In untreated dye, band at 3430.10, 2132.05, 1020-1220, and 691.45-548.19 cm^{-1} represents O–H stretching vibration of hydrogen-bonded hydroxyl groups in polymeric association, C=C stretching bond of alkynes molecule, alkyl amine, and halogen compound (chloro compound) (C–CI), respectively. Similar broad bands at 3420.87 and 3442.87 cm^{-1},

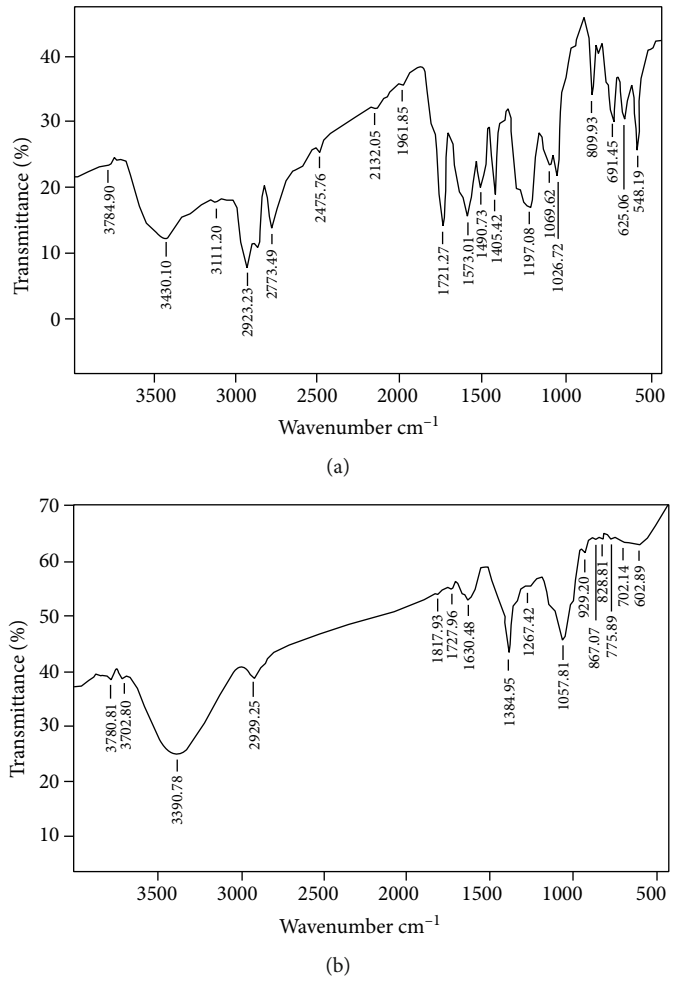


Figure 3: FTIR spectrum of RB4: (a) untreated dye and (b) treated dye.

respectively, representing –NH– and hydroxyl (–OH) extensions in the RB4 spectrum were also reported by Afreen et al. [25] and Atteke et al. [26]. In our study, FTIR spectrum of treated dye showed bands at 3390.78, 1727.96, 1630.48, and 702.14-602.89 cm^{-1} which are attributed to the presence of bonded N–H/C–H/O–H stretching of amines and amides, ketones, aromatic ring (C=C in plane) stretching symmetric, and halogen compound (C–CI), respectively. In another study, the difference in bands obtained in FTIR spectra of Reactive Red 198 and after its decolourization by HRs of *Tagetes patula* also indicated the degradation of the dye [27]. In a previous study, the FTIR spectra of another azo dye, Reactive Green 19A, also indicated similar functional groups like sulfonic groups and azo groups at the same wavenumbers as shown in our results [11]. Also, our previous study had reported the presence of azo groups in parent dye Acid Red 114 and the absence of these groups along with the emergence of new bands in treated dye by HRs of *Ipomoea carnea*, thereby suggesting the degradation of dye [28].

3.4. Toxicity Analysis

3.4.1. Allium cepa Test. Higher plants are recognized as excellent genetic models to detect environmental mutagens and are frequently used in monitoring studies. Among the plant species, *Allium cepa* has been used to evaluate DNA damage, such as chromosome aberrations and disturbances in the mitotic cycle. Meristematic mitotic cells of *A. cepa* are established as capable constituents for cytotoxicity analysis [29]. In the present study, cytotoxic implications of treated and untreated dye were analyzed based on the mitotic index (MI) and chromosomal aberrations (Table 1). The MI of bulbs grown in 110 ppm untreated dye was found to be 6.5 ± 0.15, which is statistically lower than cells in distilled water, whereas the MI of bulbs grown in 110 ppm treated dye was found to be improved and significantly closer to distilled water samples. Likewise, the percentage of aberrant cells at 110 ppm untreated dye was statistically higher ($p < 0.001$) than that of distilled water cells and significantly decreased to 10% in the case of cells exposed to the treated dye. In another study, toxicity analysis of the treated textile dye, RR35, using *A. cepa* root cells demonstrated improvement in cell viability, root length, mitotic index, and chromosomal aberrations when compared to untreated dye [30]. Different types of chromosomal abnormalities in treated and untreated dye samples like sticky metaphase, disturbed metaphase, anaphasic bridge, disturbed anaphase, and laggards have been previously

Table 1: Phytodegradation analysis of RB4 and its degradation products.

	Number of cells examined	Average number of roots	Mitotic index (MI)	% aberrant
Distilled water	510	14 ± 1.16	9.8 ± 0.52	4.0 ± 1.15
Untreated (110 ppm)	470	8 ± 1.15^{ns}	6.5 ± 1.10^{ns}	$38 \pm 0.55^{a***}$
Treated (110 ppm)	480	12 ± 1.15^{ns}	8.5 ± 0.55^{ns}	$10 \pm 1.15^{aNS,b**}$

The data represent mean ± SD. Symbols in the figure represent that comparisons are made between [a]sample dye vs. control ($p < 0.001$) and [b]treated dye vs. untreated ($p < 0.01$). Statistical significance: $^{***}p < 0.001$ and $^{**}p < 0.01$. NS: nonsignificant.

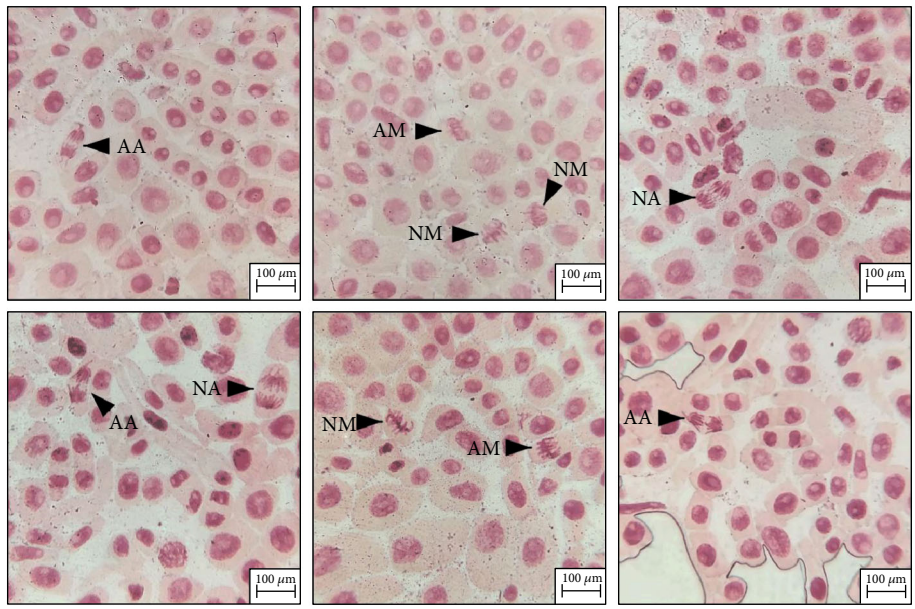


Figure 4: Stages of mitosis in root tips of *Allium cepa*: NM: normal metaphase; AM: abnormal metaphase; NA: normal anaphase; AA: abnormal anaphase.

reported [31]. Our microscopic results showed a significantly higher number of cell alterations in untreated than in control, with the most common being binucleate cells (Figure 4). In addition, untreated dye cells are shown to have more laggards than the cells exposed to treated dye, which is indicative of the genotoxic nature of the nonremediated dye.

3.4.2. FET. The zebrafish (*Danio rerio*) has immense advantages like small size, short life cycle, ease of breeding and maintenance, genetic similarities with humans, and high fecundity as a model system. It has been widely used as an effective biomarker in environmental toxicology. Zebrafish embryo serves as an alternative to the higher vertebrate model for which ethical consideration has become more contentious [32], thus gaining immense popularity in revealing the repercussion of natural or man-made chemicals [17]. In the FET test, control embryos showed a normal growth pattern, while a constant abnormal hatching pattern was observed in the case of test samples from 30 ppm to 110 ppm. Embryos reared in treated dye showed equivalent hatching rate as control whereas the ones in untreated dye exhibited a slightly delayed hatching at all concentrations under consideration (Figures 5(a) and 5(b)). At 96 hpf, embryos in untreated dye showed around 58% hatching (30, 50, 70, and 90 ppm) and 53% hatching for 110 ppm dye, whereas embryos at all the concentrations of treated dye showed 78% hatching, which accounts for nearly 26% amelioration in toxicity posttreatment of dye using HRs. This delayed hatching of the eggs can be attributed to abridged expression of hatching-specific enzymes and embryonic movements that reduce the ability of the embryo to break the egg envelope as reported for other RB dyes [7]. Heart rate was also measured after 24 hpf till 96 hpf, but no significant difference was observed between control and both types (untreated and treated) of test samples (data not shown).

Zebrafish mortality is another important parameter to account for the dye toxicity. The untreated dye sample was observed to affect embryo survival percentage, even at the lowest concentration, but a sharp decline was observed at 70 and 90 ppm, i.e., 71.1% and 5%, respectively. Least survival percentage was noted in 110 ppm of untreated dye which seems to be quite close to the survival percentage of 90 ppm dye-exposed embryos. This major drop of the survival curve in 70 and 90 ppm of untreated dye seems to get flattened at 110 ppm. Upon bioremediation, improvement in the embryo survival was observed as shown in Figures 5(c) and 5(d). The survival percentage in 70 and 90 ppm of treated dye was recorded to be 88.9 and 82.2%, whereas the highest concentration of 110 ppm treated dye showed 77.8% survival. Therefore, the toxicity induced by untreated dye seems to be significantly

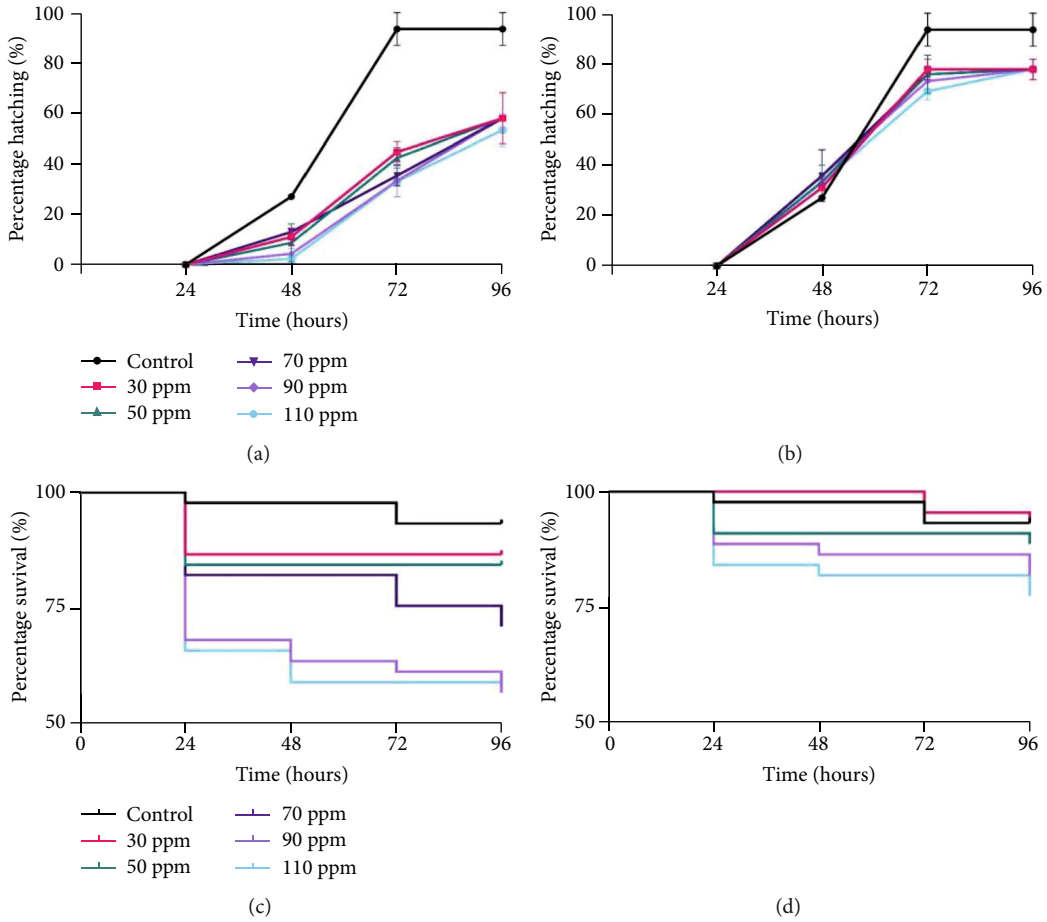


Figure 5: Ramification of RB4 dye and bioremediated dye on embryo hatching and survival rates. (a, b) Percentage hatching of embryos reared at different concentrations of untreated dye and treated dye, respectively. (c, d) Kaplan-Meier survival curves for zebrafish embryos reared at different concentrations of untreated dye and treated dye, respectively.

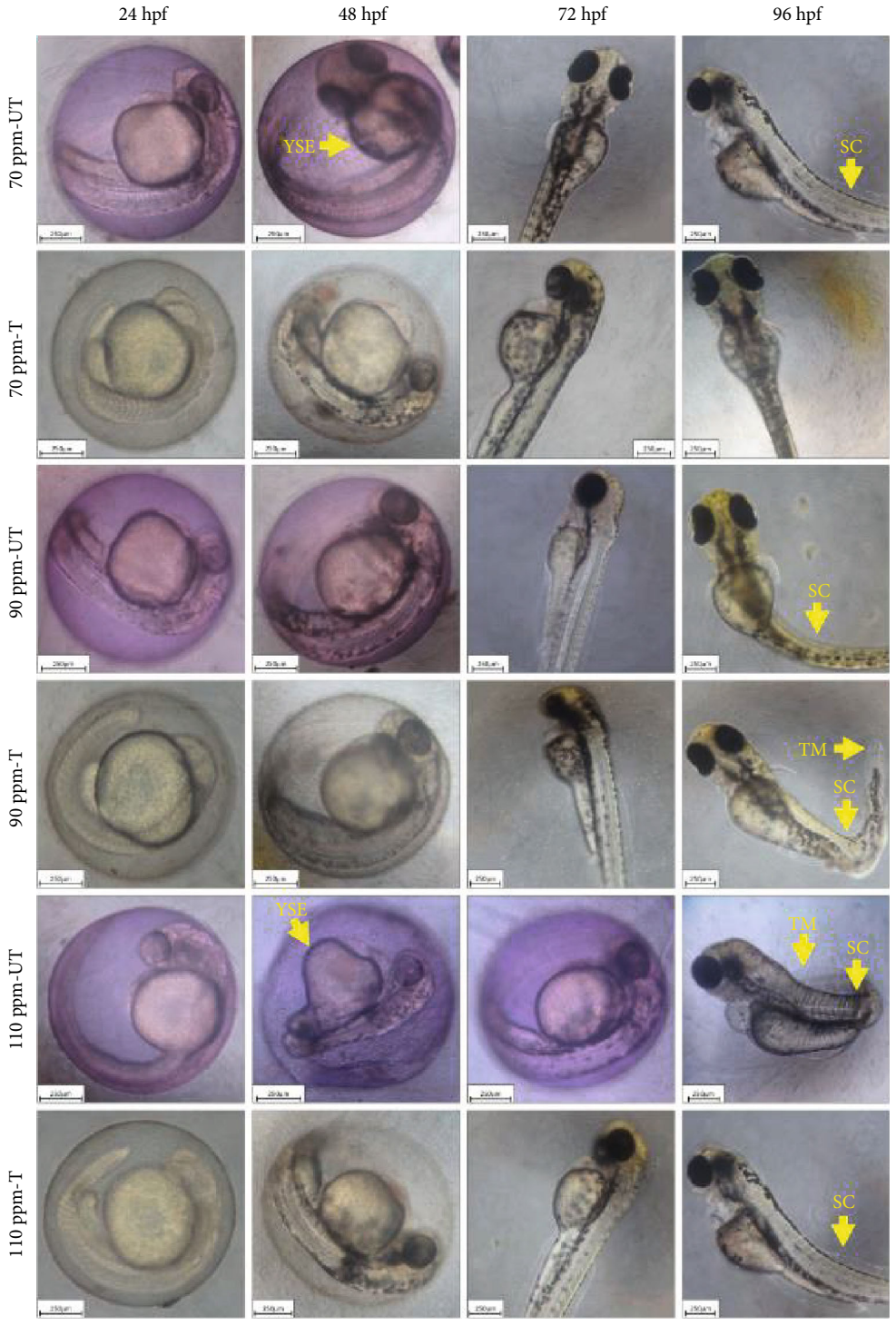
mitigated indicating the promising nature of HRs in the bioremediation of textile dyes.

Zebrafish embryos were monitored at every 24 h intervals till 96 hpf. A striking difference between the growth patterns was observed between the embryos reared at all the concentrations of untreated dye and treated dye (Figures 6(a) and 6(b)). Embryos raised at 50 ppm dye and above showed delayed hatching and other morphological deformities both before and after the bioremediation of RB4. 90% of the embryos displayed hyperpigmentation at 72 hpf and 96 hpf, respectively, for untreated dye samples (90 ppm and 110 ppm), while the extent of hyperpigmentation was reduced to around 50% of embryos after dye treatment. Though yolk sac edema (YSE) was prevalent in the embryos in untreated dye samples (except for 70 ppm), pericardial edema was absent. Bioremediation of RB4 ameliorated YSE, as YSE was absent in the embryos in treated dye samples. No deformity germane to somite formation was observed. A prominent spinal curvature (SC) was visible in embryos at 72 hpf and 96 hpf of 50 ppm and 70 ppm (96 hpf) untreated samples. This SC was reversed upon dye bioremediation. But SC observed at 96 hpf of 90 ppm and 110 ppm was irreversible. Lastly, tail malformation (TM) was observed in 50% embryos reared at 70 ppm at 96 hpf and in 90% embryos reared at 90 ppm and 110 ppm of untreated dye samples. At 110 ppm and 96 hpf, the embryo displayed entangling of the tail around the embryo axis. This may happen due to the defects at the molecular level which involve malformation of the tail and motor proteins required for the tail movement. Bioremediated dye samples displayed better results while nullifying TM for 70 ppm at the same time interval and reducing the deformity to 10% in the case of 90 ppm and 110 ppm samples. To the best of our knowledge, the toxicity of RB4 on the molecular cell signaling of zebrafish is not available. It is previously known that zebrafish T-box genes namely *spadetail* (*spt*) and *no tail* (*ntl*) are involved in the formation of the medial floor plate that in turn gives rise to the tail where *pipetail* (*ppt*) and *kugelig* (*kgg*) play an important role [33, 34]. Other textile azo dyes, namely, DB38, RO16, and DR28, induce toxicity in the early developmental stages of zebrafish that includes the curved tail, delayed hatching, and YSE, respectively [16]. Therefore, in the same context, RB4 could be attributed to interfering with the tail formation pathway in zebrafish embryos by influencing the expression of these proteins.

3.5. Biochemical Assays. If the rate of reactive oxygen species (ROS) formation is greater than the rate of their elimination,

(a)

Figure 6: Continued.



(b)

Figure 6: (a) Photomicrograph representing morphological abnormalities in zebrafish caused by RB4 dye and bioremediated dye exposure at various concentrations (0-50 ppm). The dashed yellow line represents the normal spinal axis. YSE: yolk sac edema; SC: spinal curvature. (b) Photomicrograph representing morphological abnormalities in zebrafish caused by RB4 dye and bioremediated dye exposure at various concentrations (70-110 ppm). The dashed yellow line represents the normal spinal axis. YSE: yolk sac edema; SC: spinal curvature; TM: tail malformation.

then the inactivation of enzymes, damage of the DNA, and peroxidation of unsaturated fats can further destroy the integrity of the cell [35]. The activities of enzymes like SOD, CAT, and glutathione peroxidase (GPx) are used as redox biomarkers under oxidative stress to measure the status of scavenging capacities that can nullify ROS generated

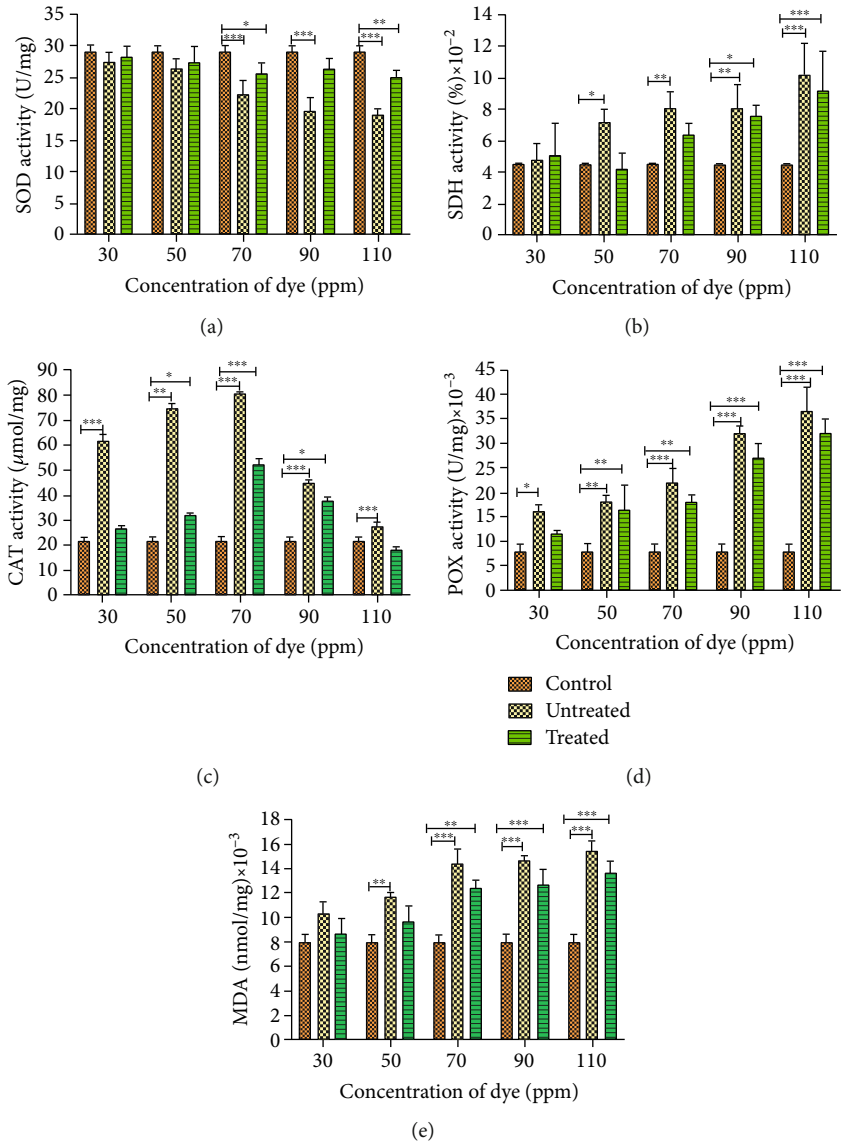


Figure 7: Oxidative stress profile of zebrafish larva exposed to untreated and treated dye: (a) SOD; (b) SDH; (c) CAT; (d) POX; (e) LPO.

by environmental contaminants [36]. In this study, activities of SOD, SDH, CAT, POX, and MDA levels in zebrafish larvae were investigated in treated and untreated dye samples (Figure 7). The SOD activity in untreated samples showed a declining trend with a significant decrease of ~1.2-fold at 70 ppm ($p < 0.001$) which continued to decline by ~1.5-fold at 110 ppm, which may be attributed to a high level of oxidative stress generated beyond the preventive potential of the existing SOD level [37]. Similar decreasing trends in SOD activities are reported wherein zebrafish larvae were exposed to increasing dimethyl phthalate concentration [38] or pesticide endosulfan [39]. In treated samples, SOD activity was found to be relatively closer to the control for initial concentrations and marginal decline at the maximum concentration, i.e., 110 ppm ($p < 0.01$). The higher levels of SOD in treated samples (i.e., 70-110 ppm), compared to untreated samples, suggest that to protect cells from free radicals, more proteins are required that bolster the enzymatic activity against oxidants [40]. SDH activity was found to increase in both untreated and treated dyes with respect to the control (Figure 7(b)). However, samples treated with 110 ppm of untreated and treated dye showed an increased SDH activity by 2.38-fold and 2.26-fold, respectively, as compared to control. In untreated samples, the relative concentration of H_2O_2 was slightly higher as compared to the treated samples ($p < 0.01$). Due to low levels of SDH in the untreated dye, there was an escalated production of H_2O_2. Excess H_2O_2 buildup due to SOD and SDH activity becomes toxic to cells. As CAT and POX enzymes catalyze the conversion of H_2O_2 to molecular oxygen and water, the activity of these enzymes was evaluated. A change in CAT activity after exposure to dye is shown (Figure 7(c)). At lower concentrations of untreated dye (30-70 ppm), there was a significant increase ($p < 0.001$) in CAT activity by 3-, 3.75-, and 4-fold, respectively, in comparison to control, in order to reduce or counterbalance the excessive ROS production. But at 90 and

110 ppm of dye exposure, there was a significant reduction in CAT activity ($p < 0.05$). After a threshold concentration of dye exposure, the CAT could no longer eliminate the increased oxidative stress generated, and thus, CAT activity reduces [38]. In another study, Meireles et al. reported a decline in the CAT activity to 1.55-, 1.44-, and 1.25-fold when Red Disperse dyes, i.e., DR60, DR73, and DR78, were used, respectively [41]. According to Cong et al., exposure to a higher concentration of dye can cause oxidative damage leading to reduced CAT activity, consequentially depleting CAT and SOD enzymes [38]. In our study, at the lowest concentration of treated dye exposure (30 ppm), there was a negligible increase in CAT, and at a higher concentration, i.e., 70 ppm, activity was found to have increased significantly ($p < 0.001$) by ~2.5-fold as compared to control. With a further increase in dye concentration (>70 ppm) of treated samples, a significant decline ($p < 0.001$) was observed. This investigation showed remarkably low levels of CAT activity in treated samples when compared to untreated samples from 30 to 110 ppm. This can occur in view of SOD enzyme dysfunction caused by stress-induced inactivation of its active site [42] in untreated dye. In Figure 7(a), SOD levels in untreated samples were reduced as compared to treated samples, leading to curtailed H_2O_2 production, and may thus result in deficiency of CAT synthesis in untreated dye. POX catalyses the removal of H_2O_2 by oxidising a substrate pyrogallol to purpurogallin [43]. POX activity in both treated and untreated samples produced a rising trend as compared to control (Figure 7(d)). For any given concentration, the POX activity of untreated dye was distinctly higher than that of treated dye. A higher POX activity is a consequence of high H_2O_2 levels. Increased POX is associated with tissue damage and can lead to disruption in larval development [44]. The lower POX activity in treated samples may occur due to minimized oxidative stress in treated samples. It is therefore noted that these antioxidant enzymes are adept at capturing H_2O_2 and superoxide anions which lead to the protection of organisms from oxidative stress conditions [45].

Free radicals induce lipid peroxidation, wherein degradation of lipid peroxides leads to the production of many subproducts including MDA, the levels of which can be used to determine the severity of oxidative damage evoked in larvae [46]. The untreated dye exhibits a rising trend in MDA levels with a significant increase of ~1.7-fold ($p < 0.001$) at 70 ppm with respect to control (Figure 7(e)). At lower concentrations (i.e., 30-50 ppm), the level of MDA was relatively closer to control, but at a slightly higher concentration, i.e., 70 ppm, ~1.5-fold rise in MDA levels was observed. In the presence of high dye toxicity (i.e., 90 ppm onwards), the ability of antioxidant enzymes to eliminate ROS reduces, and therefore, the residual free radicals attack unsaturated fatty acids inciting an increase in MDA content of larvae [46]. Analogous results were observed using naphthalene sulfonic acid (NSA), metanilic acid (MA), and acid blue 113 (AB113) textile dye [47] and fungicide azoxystrobin [48], where an apparent rise in MDA content of zebrafish was paralleled with an increase in concentration. A similar observation was reported by Mao et al. using pesticides [49]. In our study as well, the content of MDA in treated samples is fairly reduced as compared to that in untreated RB4 samples. This suggests the occurrence of minimized MDA formation or the ability to scavenge them as an important measure for preventing cell damage in hairy root bioremediated samples. Similar results were proclaimed in a study by Cong et al., where oxidative damage in fish, treated with a low concentration of DMP for 24 h, was effectively exterminated by the cause of its antioxidant mechanisms [38].

In the untreated samples, the concentration of dye, i.e., 50 ppm onwards, causes severe damage in embryos. But in the treated samples of 50 and 70 ppm, the activity of antioxidant enzymes such as SOD, SDH, CAT, POX, and LPO content (Figure 7) indicates toxicity amelioration after treatment with HRs as there was delayed hatching, morphological deformities, and the absence of YSE in zebrafish embryos. These enzymes have been known to play an important role in early stress even at relatively low concentrations of dye, helping to reduce ROS [50]. However, at a further increase in the concentration of dye, i.e., 90 and 110 ppm, the ROS produced is also high, which cannot be remediated by HRs, in the severity of stress like embryo hyperpigmentation. These may be due to stress-induced inhibition or changes in the subunit of the antioxidant enzymes [51]. Several reports have shown the toxic nature of different anthraquinone dyes, but to the best of our knowledge, this is the first report on comprehending the repercussion of untreated and bioremediated RB4 dye on zebrafish embryos thereby contributing to the rudimentary understanding of the impact of remediated RB4 on ecosystems.

4. Conclusion

In summary, we emphasize the need to develop nontoxic dyes or efficient methods to treat industrial effluents having synthetic dyes. The HRs used in this study were found to be effective in the degradation of potentially toxic textile dyes, and treated dye was found to be less toxic in comparison to untreated, after in vivo toxicity assessment. We also hypothesize that RB4 might be hindering the normal cellular pathway for tail formation further contributing to the increased mortality in embryos. The expression of different antioxidant enzymes during dye exposure corroborates the synchronous activity of antioxidant machinery to protect developmental toxicity in zebrafish embryos. This study thus suggests that HRs can be accounted as viable candidates for the treatment of RB4-contaminated effluents. This study has added novel approaches of remediation-based investigation to understand the activity of enzymes under dye stress in a process to develop a system for degradation of high dye concentration with minimal or no residual toxicity.

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 is no conflict of interest regarding the publication of this article.

Authors' Contributions

KT performed zebrafish experiments and wrote the manuscript. RS performed experiments of hairy root induction and antioxidant experiments. VZ wrote the original draft, prepared figures, and reviewed and edited the manuscript. PA and RJ wrote the original draft, did editing, and prepared a graphical abstract. NN, NA, and FS prepared the manuscript, edited the paper, and funded the work. PJ and SS helped in analysis of results and draft preparation. MS helped in data analysis and draft editing. PJ conceptualized the study, did standardization of methods, wrote original drafts, and reviewed raw data.

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Supplementary Materials

Figure S1: (A) untreated dye, (B) treated dye (with HRs) at 0 h, and (C) treated dye (with HRs) at 120 h. Figure S2: effect of different pH on % decolourization by HRs. Figure S3: effect of different temperatures on % decolourization by HRs. Figure S4: effect of different biomass dosages on % decolourization by HRs. Figure S5: effect of different concentrations of dye on % decolourization by HRs. *(Supplementary Materials)*

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Research Article

In-Depth Study of *Thymus vulgaris* Essential Oil: Towards Understanding the Antibacterial Target Mechanism and Toxicological and Pharmacological Aspects

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Questions have been raised apropos the emerging problem of microbial resistance, which may pose a great hazard to the human health. Among biosafe compounds are essential oils which captured consumer draw due to their multifunctional properties compared to chemical medication drugs. Here, we examined the chemical profile and the mechanism(s) of action of the *Thymus vulgaris* essential oil (TVEO) against a Gram-negative bacterium *Salmonella enterica* Typhimurium ATTCC 10028 (*S. enterica* Typhimurium ATTCC 10028) and two Gram-positive bacteria *Staphyloccocus aureus* ATCC 6538 (*S. aureus* ATCC 6538) and *Listeria monocytogenes* ATCC 19117 (*L. monocytogenes* ATCC 19117). Findings showed that TVEO was principally composed of thymol, o-cymene, and γ-terpinene with 47.44, 16.55, and 7.80%, respectively. Molecular docking simulations stipulated that thymol and β-sesquiphellandrene (a minor compound at 1.37%) could target multiple bacterial pathways including topoisomerase II and DNA and RNA polymerases of the three tested bacteria. This result pointed plausible impairments of the pathogenic bacteria cell replication and transcription processes. Through computational approach, the VEGA quantitative structure–activity relationship (QSAR) model, we revealed that among twenty-six TVEO compounds, sixteen had no toxic effects and could be safe for human consumption as compared to the Food and Drug Administration (FDA) approved drugs (ciprofloxacin and rifamycin SV). Assessed by the SwissADME server, the pharmacokinetic profile of all identified TVEO compounds define their absorption, distribution, metabolism, and excretion (ADME) properties and were assessed. In order to predict their biological activity spectrum based on their chemical structure, all TVEO compounds were subjected to PASS (Prediction of Activity Spectra for Substances) online tool. Results indicated that the tested compounds could have multiple biological activities and various enzymatic targets. Findings of our study support that identified compounds of TVEO can be a safe and effective alternative to synthetic drugs and can easily combats hazardous multidrug-resistant bacteria.

1. Introduction

In recent years, antimicrobial-resistant (AMR) bacteria have been admitted as a public health risk that could cause an increase in the global burden of infectious disease [1–4]. For instance, each year, more than 670,000 infections and 700,000 deaths worldwide were provoked by AMR [5, 6]. Mutually controlled by host immune condition, microflora organization, and antimicrobial interventions, AMR evolution occurs with the multidrug resistance [7, 8]. While AMR could not be pragmatically eradicated, antimicrobials will endure to miss their potency, and, in the close future, more people may die from infections [9–11]. Therefore, it is crucial to explore original effective and broad-spectrum antibacterial agents to control bacteria, which can be antibiotic resistant, highly virulent, and high costs for the medication. Among the leading strategies for the exploration and detection of new targets in pathogens are the masterfully

using bioinformatics tools. These tools can provide useful information for better comprehension of the interactions between targets and biomolecules and therefore help to anticipate new treatment targets for pathogenic microorganisms [12, 13].

Extracted from natural plants, essential oils (EOs) can be exploited as a practical alternative. More specifically, thyme (*Thymus vulgaris* L.), appertained to the family of *Lamiaceae* and the genus of *Thymus*, was consumed for centuries because of its medicinal properties and was generally recognized as safe (GRAS) by the FDA [14–17]. In addition, essential oil acquired from *Thymus vulgaris* L. had an extensive range of biological activities [17]. Thyme EO contains high levels of phenolic compounds, like thymol, carvacrol, p-cymene, and γ-terpinene [17, 18]. Interestingly, numerous studies reported that thymol, the major antibacterial component occurring in TEO, can destroy the bacterial cell membrane [19, 20]. Liu et al. revealed that thymol powerfully inhibit *Pseudomans aeruginosa* and directly change the cell structure [15]. *P. aeruginosa* cell membrane integrity is destroyed as evidenced by an increase in permeability of the inner/outer membranes. In other studies, Wang et al. and Lade et al. noted that thymol disrupts the *Staphylococcus aureus* membrane integrity to achieve the inner structure of the bacterial cell and joints to the minor groove of bacterial DNA, ensuring in a destabilization of the DNA secondary structure [21, 22].

In extension of our research to disclose the potential of natural therapeutic agents [13], this current investigation is aimed at elucidating the molecular docking interactions of all components of *Thymus* EO with bacterial DNA and RNA polymerases, as well as the topoisomerase II of *S. aureus*, *S. enterica* Typhimurium, and *L. monocytogenes* were profoundly reviewed *in silico*. As a part of our endeavor to increase the potential and exploration of these activities, a pharmacokinetics and computational toxicological studies were well discussed.

2. Materials and Methods

2.1. Plant Material, Essential Oil Extraction, and Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

2.1.1. Plant Material Collection and EO Extraction. *Thymus vulgaris* L. plant was collected from the region of Sfax, Tunisia (N: 34.4426°, E: 10.4537°) which is characterized by a semiarid climate. Aerial parts were harvested during the flowering stage in April 2022 and were air-dried in obscurity at room temperature. The EO of dried samples of *T. vulgaris* (TVEO) aerial parts was hydrodistilled for 3 h using a Clevenger apparatus.

2.1.2. EO Analysis Using Gas Chromatography–Mass Spectrometry (GC–MS). The analysis of TVEO was accomplished using a GC/MS HP model 6980 inert MSD, equipped with an Agilent Technologies capillary HP-5MS column (60 m × 0.25 mm, 0.25 mm film thickness) coupled to a mass selective detector (MSD5973, ionization voltage 70 eV, all Agilent, Santa Clara, CA, USA). The carrier gas was helium and has been maintained at 1.2 mL/min flow rate. The oven temperature program was as follows: 1 min at 100°C ramped from 100 to 280°C at 5°C/min and 25 min at 280°C. The chromatograph was equipped with a split/split less injector used in the split less mode. Identification of TVEO components was achieved by matching their mass spectra with Wiley Registry of Mass Spectral Data 7th edition (Agilent Technologies) and National Institute of Standards and Technology 05 MS (NIST) library data.

2.2. Antibacterial Activity

2.2.1. Microorganisms and Growth Conditions. In order to evaluate TVEO antibacterial activity, two Gram-positive bacteria: *S. aureus* ATCC 6538 and *L. monocytogenes* ATCC 19117, and two Gram-negative bacteria: *S. enterica* Typhimurium ATCC 14028 and *E. coli* ATCC 8739, were selected. Bacterial cultures were deposited in 3 mL of Luria-Bertani (LB) agar medium composed of (g/L): peptone, 10; yeast extract, 5; NaCl, 5; and agar, 20 at pH 7.2; then, the bacterial strains were incubated at 37°C according to the method described by [23].

2.2.2. Agar Diffusion Method. Antimicrobial activity of TVEO was evaluated by agar-well diffusion assay according to a method proposed by Güven et al. [24]. Fifteen milliliters of the molten agar (45°C) were flowed into sterile Petri dishes (Ø 90 mm). Bacterial cell suspensions were prepared, and 100 μL was evenly deposited onto the surface of plates containing LB agar medium. Plates were aseptically dried, and then, 5 mm wells were punched into the agar with a sterile Pasteur pipette. TVEO was dissolved in DMSO, water (1/9; *v*/*v*) to a final concentration of 1 mg/mL and then filtered through 0.22 μm pore size black polycarbonate filters. 100 μL of this filtered solution was placed into each well, and the plates were incubated at 37°C. Gentamicin (10 μg/wells) was used as a positive control.

2.2.3. Minimal Inhibitory Concentration (MIC). MIC is defined as the lowest concentration that could inhibit the visible growth of the tested microorganism. In this context, MIC of TVEO was tested against four pathogenic bacteria which are as follows: two Gram-positive bacteria: *S. aureus* ATCC 6538 and *L. monocytogenes* ATCC 19117, and two Gram-negative bacteria: *S. enterica* Typhimurium ATCC 14028 and *E. coli* ATCC 8739, using the microdilution method with serial dilution described by Chandrasekaran and Venkatesalu [25]. Then, bacterial suspension was added with a final inoculum concentration of 10^6 CF/mL. The contents of the tubes were mixed by pipetting and were incubated for 24 h at 37°C. For the antibacterial activity determination (inhibition zones and CMIs), each experiment was carried out simultaneously in triplicate under same conditions. The obtained diameters of inhibition zones were measured in mm and the MIC values were reported in mg/mL.

2.3. Interaction Study between TVEO Compounds and Selected Bacterial Targets by Molecular Docking.

Receptor targets Fasta sequences of *S. aureus* ATCC 700699, *S. enterica* Typhimurium ATCC 700720, and L. *monocytogenes*

Table 1: Chemical composition of TVEO.

Compound	Molar mass (g/mol)	Molecular formula	Retention time (min)	EO (%)
α-Pinene	136.23	$C_{10}H_{16}$	5.99	1.74
α-Thujene	136.23	$C_{10}H_{16}$	6.15	1.61
Camphene	136.23	$C_{10}H_{16}$	6.66	1.60
β-Pinene	136.23	$C_{10}H_{16}$	7.39	0.29
β-Myrcene	136.23	$C_{10}H_{16}$	7.85	2.36
α-Fellandrene	136.23	$C_{10}H_{16}$	8.17	0.34
3-Carene	136.23	$C_{10}H_{16}$	8.31	0.16
D-Limonene	136.23	$C_{10}H_{16}$	8.52	1.61
O-Cymene	134.22	$C_{10}H_{14}$	8.88	16.55
Cymol	134.22	$C_{10}H_{14}$	9.46	0.09
γ-Terpinene	136.23	$C_{10}H_{16}$	9.79	7.80
Terpinolene	136.23	$C_{10}H_{16}$	10.56	0.10
Linalool	154.25	$C_{10}H_{18}O$	11.03	4.41
Camphor	152.23	$C_{10}H_{16}O$	12.14	1.15
Borneol	152.23	$C_{10}H_{18}O$	12.84	2.02
4-Terpinenol	152.23	$C_{10}H_{18}O$	13.23	0.92
α-Terpineol	152.23	$C_{10}H_{18}O$	13.90	0.10
Thymol methyl ether	164.24	$C_{11}H_{16}O$	14.99	0.43
Thymol	150.22	$C_{10}H_{14}O$	16.48	47.44
Caryophyllene	204.35	$C_{15}H_{24}$	19.59	2.09
α-Humulene	204.35	$C_{15}H_{24}$	20.43	0.03
α-Amorphene	204.35	$C_{15}H_{24}$	20.99	0.10
α-Curcumene	202.33	$C_{15}H_{22}$	21.17	0.71
α-Zingibirene	204.35	$C_{15}H_{24}$	21.49	3.40
α-Bisabolene	204.35	$C_{15}H_{24}$	21.79	1.12
β-Sesquiphellandrene	204.35	$C_{15}H_{24}$	22.17	1.34
Monoterpenes hydrocarbons				34.25%
Oxygenated monoterpenes				56.47%
Sesquiterpens				8.79%
Total				99.51%

ATCC 19115 were obtained from UniProt database [26] and NCBI database [27]. Protein models were projected to SWISS-MODEL server [28] for molecular homology modeling approach [29]. Validation of the obtained models was performed by checking Ramachandran plots and the QMEAN values [30], using PROCHECK analysis tool integrated in Profunc server ([31]. SMILES (simplified molecular input line entry systems) strings of TVEO compounds were obtained from the PubChem database [32] and controls (rifamycin SV and ciprofloxacin). SMILES structures were downloaded from the DrugBank database [33], and all were converted into 3D structure using CORINA demo webserver [34] and then saved in pdb file format. Molecular docking was performed using the AutoDock Vina software (version 1.2.0) to calculate free energy of binding (kcal/mol) scores according to the methodology proposed by [35]. The docking position results were visualized using Discovery Studio version 16.1.0 (Dassault Systemes BIOVIA, 2016).

2.4. Toxicity Prediction of Compounds by VEGA HUB Software Using QSAR Method. All TVEO compounds and the two controls (rifamycin SV and ciprofloxacin) were subjected to 8 toxicity measurements in a view to assess carcinogenicity, mutagenicity, developmental/reproductive toxicity, endocrine disrupting ability, and genotoxicity. All tests were performed by VEGA software version 1.1.5 using the QSAR (quantitative structure–activity relationship) approach [36].

2.5. ADME Analysis. Pharmacokinetics, drug-likeness, and medicinal chemistry properties of TVEO compounds were predicted using SwissADME which is an open access software for ADME parameters evaluation and profiling [37].

2.6. In Silico Prediction of Possible Bioactivities. The PASS software was used to predict bioactivity of molecules based on the structural similarity to the large data base of known

Table 2: Antibacterial activity of TVEO and the control gentamicin. Zones of growth inhibition was expressed in mm, and minimum inhibitory concentrations (MICs) were expressed in mg/mL.

Bacterial strains	Inhibition zone diameters (mm)		MIC (mg/mL)	
	TVEO	Gentamicin	TVEO	Gentamicin
S. aureus ATCC 6538	23 ± 1.00^{b}	20 ± 0.83^{a}	0.097 ± 0.00^{b}	0.013 ± 0.00^{b}
L. monocytogenes ATCCC 19117	22 ± 1.00^{b}	20 ± 0.75^{a}	0.097 ± 0.00^{b}	0.013 ± 0.00^{b}
S. enterica Typhimurium ATCC 14028	23 ± 1.00^{a}	25 ± 1.25^{b}	0.097 ± 0.00^{b}	0.005 ± 0.00^{b}
E. coli ATCC 8739	21 ± 0.83^{a}	25 ± 1.25^{b}	0.195 ± 0.00^{b}	0.005 ± 0.00^{b}

A Student t-test was used to determine the significant differences between inhibition zones and MIC.

Table 3: Receptors models of the selected pathogenic bacteria used in molecular docking simulation with TVEO constituents.

Bacterial Strain	Bacterial Target	Receptor	Template	Identity (%)	Ramachandran Plot		QMEAN
					Favored regions (%)	Additional allowed regions (%)	
S. aureus ATCC 700699	DNA polymerase	P63979 (UniProt)	4IQJ.1.L	36.77	87.9	10.1	−3.26
	RNA polymerase	Q932F8 (UniProt)	6WVK.1.C	81.09	85.5	12.7	−2.36
	Topoisomerase II	P66936 (UniProt)	6GAV.1.A	54.42	88.4	10.7	−1.69
S. enterica Typhimurium ATCC 700720	DNA polymerase	P14567 (UniProt)	5FKU.1.A	96.72	88.0	10.0	−2.35
	RNA polymerase	P06173 (UniProt)	4LLG.2.C	98.66	88.0	11.0	−1.11
	Topoisomerase II	P0A213 (UniProt)	4TMA.2.B	95.41	90.2	9.2	−1.82
L. monocytogenes ATCC 19115	DNA polymerase	WP031669548 (NCBI)	6VDE.1.A	38.30	81.6	16.4	-3.91
	RNA polymerase	GAT39567 (NCBI)	6WVK.1.C	86.10	87.1	11.4	-2.24
	Topoisomerase II	GAT39106 (NCBI)	2XCR.2.A	69.59	87.4	10.4	-2.01

active substances in order to find new TVEO molecule targets [38, 39].

2.7. Statistical Analysis. All tests were assayed in triplicate and expressed as the mean ± standard deviation of the measurements. The statistical program SPSS version 21.00 for Windows (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Variance was analyzed by one-way ANOVA and Student's t-test was applied to compare each parameter at $p < 0.05$.

3. Results and Discussion

3.1. Chemical Composition Analysis of TVEO. GC-MS analysis of TVEO revealed the existence of 26 different components (Table 1). The main components were thymol, the most abundant compound (47.44%), followed by o-cymene (16.55%), γ-terpinene (7.80%), and linalool (4.41%). Moreover, it detected the presence of 4 compounds which their percentages (EO %) were more than 2%. These later are the α-zingibirene (3.40%), β-myrcene (2.36%), caryophyllene (2.09%), and borneol (2.02%).

Thymol, known also by the chemical name 2-isopropyl-5-methylphenol, is a natural phenol monoterpene [40] importantly detected in *Lamiacaeae* family [41] including many plant species such as *Thymus vulgaris L.* [42], *Ocimum gratissimum L.* [43], *Origanum L.* [44], and *Trachyspermum ammi L.* [45] and other species of the genus *Satureja L.* [46] and *Monarda L.* [47]. This volatile monoterpenoid is largely used by nutraceutical, pharmaceutical, and cosmeceutical industries due to its multiple potential therapeutic properties [48–58]. In addition, thymol was globally recognized-as-safe food additive according to US department of Food and Drug Administration (FDA) [59].

O-cymene, known as 1-isopropyl-2-methylbenzene, is an acyclic monoterpene which belongs to p-cymene isomers and has an orthosubstituted alkyle group [60]. Previous studies demonstrated that o-cimene has several therapeutic effects [61–66]. Several studies indicated the existence of a remarkable synergetic effect between ocimene and other terpenes such as α-pinene and myrcene which were noticed to produce more beneficial effects when combined [67, 68].

γ-Terpinene, renowned also as p-mentha-1,4-diene, is a naturally occurring monoterpene hydrocarbon [69] that has been isolated from many botanical sources including *Origanum vulgare*, *Citrus limon* L, *Melaleuca alternifolia*, and *Eucalyptus obliqua* [70, 71]. This monoterpene is largely employed in food, cosmetics, and pharmaceutical industries [72]. Previous research showed that γ-terpinene has potential biological activities such as antioxidant [73], anti-inflammatory [74], and antimicrobial activities [75, 76].

On the other hand, linalool, known as 3,7-dimethyl-1,6-octadien-3-ol [77], is a naturally occurring acyclic monoterpenoid and tertiary alcohol which is commonly found as a major active component in the essential oil of several aromatic plant species [78] principally in *Lamiaceae* and

Table 4: Molecular docking results for complexes of *T. vulgaris* EO compounds against DNA and DNA polymerases of *S. aureus* ATCC 700699, *S. enterica* Typhimurium ATCC 700720, and *L. monocytogenes* ATCC 19115 using Autodock Vina (kcal/mol).

Bacterial target	*S. aureus* ATCC 700699			*S. enterica* Typhimurium ATCC 700720			*L.monocytogenes* ATCC 19115		
Compound	DNA polymerase	RNA polymerase	Topoisomerase II	DNA polymerase	RNA polymerase	Topoisomerase II	DNA polymerase	RNA polymerase	Topoisomerase II
α-Pinene	-5.1	-6.1	-5.3	-5.4	-5.4	-5.2	-5.9	-6.0	-5.4
α-Thujene	-4.9	-5.4	-5.5	-5.3	-5.2	-5.3	-5.5	-5.3	-5.6
Camphene	-5.2	-6.0	-5.6	-5.2	-5.2	-5.0	-6.1	-5.9	-5.3
β-Pinene	-5.2	-5.7	-5.2	-5.5	-5.7	-5.3	-5.9	-6.0	-5.2
β-Myrcene	-4.9	-5.0	-5.2	-5.1	-5.1	-5.0	-5.2	-5.0	-5.0
α-Fellandrene	-5.2	-5.6	-5.4	-5.1	-5.1	-5.1	-5.5	-5.5	-5.6
3-Carene	-5.2	-5.6	-5.2	-5.6	-5.6	-5.5	-5.6	-5.5	-5.7
D-Limonene	-5.1	-6.0	-5.4	-5.4	-5.4	-5.1	-5.5	-5.6	-5.6
O-Cymene	-5.3	-5.5	-5.6	-5.7	-5.4	-5.4	-5.8	-5.5	-5.5
Cymol	-5.2	-5.7	-5.6	-5.5	-5.5	-5.2	-6.0	-5.9	-5.7
γ-Terpinene	-5.3	-6.0	-5.6	-5.8	-5.6	-5.7	-5.8	-5.8	-5.6
Terpinolene	-5.4	-5.8	-5.6	-5.6	-6.0	-5.6	-5.8	-5.6	-5.8
Linalool	-5.0	-5.2	-5.2	-5.5	-5.6	-5.4	-5.8	-5.1	-5.2
Camphor	-5.3	-6.0	-5.4	-5.4	-5.2	-5.3	-6.0	-5.6	-5.2
Linderol (borneol)	-5.4	-5.7	-5.5	-5.3	-5.3	-5.2	-5.8	-5.7	-5.3
4-Terpineol	-5.4	-6.2	-5.8	-5.8	-5.8	-5.5	-5.9	-6.0	-5.8
α-Terpineol	-5.4	-6.0	-5.6	-5.6	-5.8	-5.0	-5.8	-5.6	-5.8
Thymol methyl ether	-5.5	-5.8	-5.6	-5.3	-5.4	-4.9	-5.6	-5.5	-5.7
Thymol	-6.3	-6.2	-6.6	-6.4	-6.6	-6.2	-6.8	-6.7	-6.6
Caryophyllene	-6.0	-6.4	-6.7	-6.4	-6.5	-6.4	-6.5	-6.3	-6.4
α-Humulene	-6.3	-6.3	-6.9	-6.4	-7.1	-6.1	-6.5	-6.8	-6.4
α-Amorphene	-6.1	-6.2	-6.3	-6.5	-6.0	-6.1	-6.6	-6.6	-6.2
α-Curcumene	-6.3	-6.6	-6.7	-6.5	-6.5	-6.0	-6.8	-6.4	-6.3
α-Zingibirene	-5.4	-6.5	-6.4	-6.0	-5.5	-5.6	-5.7	-6.0	-6.2
α-Bisabolene	-5.6	-6.8	-6.6	-6.4	-6.4	-5.8	-6.4	-5.9	-6.2
β-Sesquiphellandrene	-5.9	-7.0	-7.1	-6.8	6.8	-6.7	-7.1	-6.9	-6.9
Rifamycin SV	-8.7	-9.8	—	-8.7	-8.6	—	-9.6	-10.2	—
Ciprofloxacin	—	—	-7.4	—	—	-6.3	—	—	-6.5

Table 5: Interactions details of thymol and β-sesquiphellandrene with the selected bacterial targets.

Bacteria	Compound	Targets	Number of Residues Interacting	Residues with H-bond
S. aureus ATCC 700699	Thymol	DNA polymerase	4	LYS228
		RNA polymerase	4	—
		Topoisomerase II	6	GLU609, ASP610
	β-Sesquiphellandrene	DNA polymerase	1	—
		RNA polymerase	7	—
		Topoisomerase II	8	—
S. enterica Typhimurium ATCC 700720	Thymol	DNA polymerase	5	—
		RNA polymerase	1	—
		Topoisomerase II	6	ASN588
	β-Sesquiphellandrene	DNA polymerase	8	—
		RNA polymerase	5	—
		Topoisomerase II	6	—
L. monocytogenes ATCC 19115	Thymol	DNA polymerase	8	—
		RNA polymerase	5	—
		Topoisomerase II	3	VAL113
	β-Sesquiphellandrene	DNA polymerase	6	—
		RNA polymerase	4	—
		Topoisomerase II	5	—

Lauraceae botanical families. It has been reported that this monoterpenol is broadly used in food industry as an aromatic and preservative agent, in cosmetic as a fragrance and antiseptic constituent [79–83].

It should be noted that TVEO composition may depend on many biotic and abiotic factors including seasonal variations of temperature and humidity [84], phenological stages and different vegetation cycles [85], geographic location [86], environmental stress [87], and extraction technique [88]. Aljabeili et al. reported that the TVEO collected from KSA showed a significant composition variation and the major compounds were thymol (41.04%), 1,8-cineole (14.26%), γ-terpinene (12.06%), and p-cymene (10.50%) [89]. Another study revealed that Turkish TVEO has different components amounts such as thymol (49%), β-cymene (19.99%), carvacrol (7.63%), and *trans*-caryophyllene (6.79%) [90]. In addition, Moghaddam et al. reported that Iranian TVEO contains thymol (36.81%), ρ-cymene (30.90%), and carvacrol (3.16%) [91].

3.2. Antibacterial Activity. As represented in Table 2, TVEO displayed an interesting antibacterial activity against the four tested bacterial strains with inhibition zones ranging from of 21 to 23 mm against Gram-negative and Gram-positive bacteria, respectively. In addition, it is important to note that monoterpenes exhibit a broad-spectrum antibacterial activity against pathogenic bacteria [92, 93]. This fact could be explained by the presence of a lipophilic character which provides to monoterpenes the ability to adhere to bacterial cell membrane lipids and to deploy their antibacterial action [94]. In our study, the obtained MIC values mentioned in Table 2, indicated that TVEO was more potent ($p < 0.05$) against Gram-positive bacteria (MICs = 0.097 mg/mL) than Gram-negative bacteria (MICs = 0.195 mg/mL). These findings were in agreement with previous studies which reported that Gram-positive bacteria are susceptible to be more sensitive to plant EOs than Gram-negative bacteria due to the existence of lipopolysaccharides which acts as a hydrophobic barrier [95, 96].

3.3. Interactions between TVEO Molecules and Bacterial Topoisomerase II and DNA and RNA Polymerases. Computational modeling is a 3R-based approach and an attractive alternative to experiments in a view to understand bioactive compounds mechanism of action and their antibacterial inhibitory process [97]. Molecular docking was performed to predict different TVEO components that could bind specifically to select bacterial receptors active sites responsible for DNA replication and transcription processes. In this respect, we investigated TVEO antibacterial inhibitory effect on topoisomerase II (DNA gyrase) and DNA and RNA polymerases of three pathogenic bacteria *S. aureus* ATCC 700699, *S. enterica* Typhimurium ATCC 700720, and *L. monocytogenes* ATCC 19115. Molecular homology results indicated that selected templates can be used for molecular modeling (Table 3); their identities (%) are higher than 30% [98], and Ramachandran plot values of favored regions and allowed regions were over 90% [99].

Moreover, it is necessary to remind that DNA and RNA polymerases are crucial enzymes involved in the DNA replication, transcription, and translation as well as nucleic acid formation in bacterial cells [100]. On the other hand, topoisomerase II (DNA gyrase) is also implicated in DNA

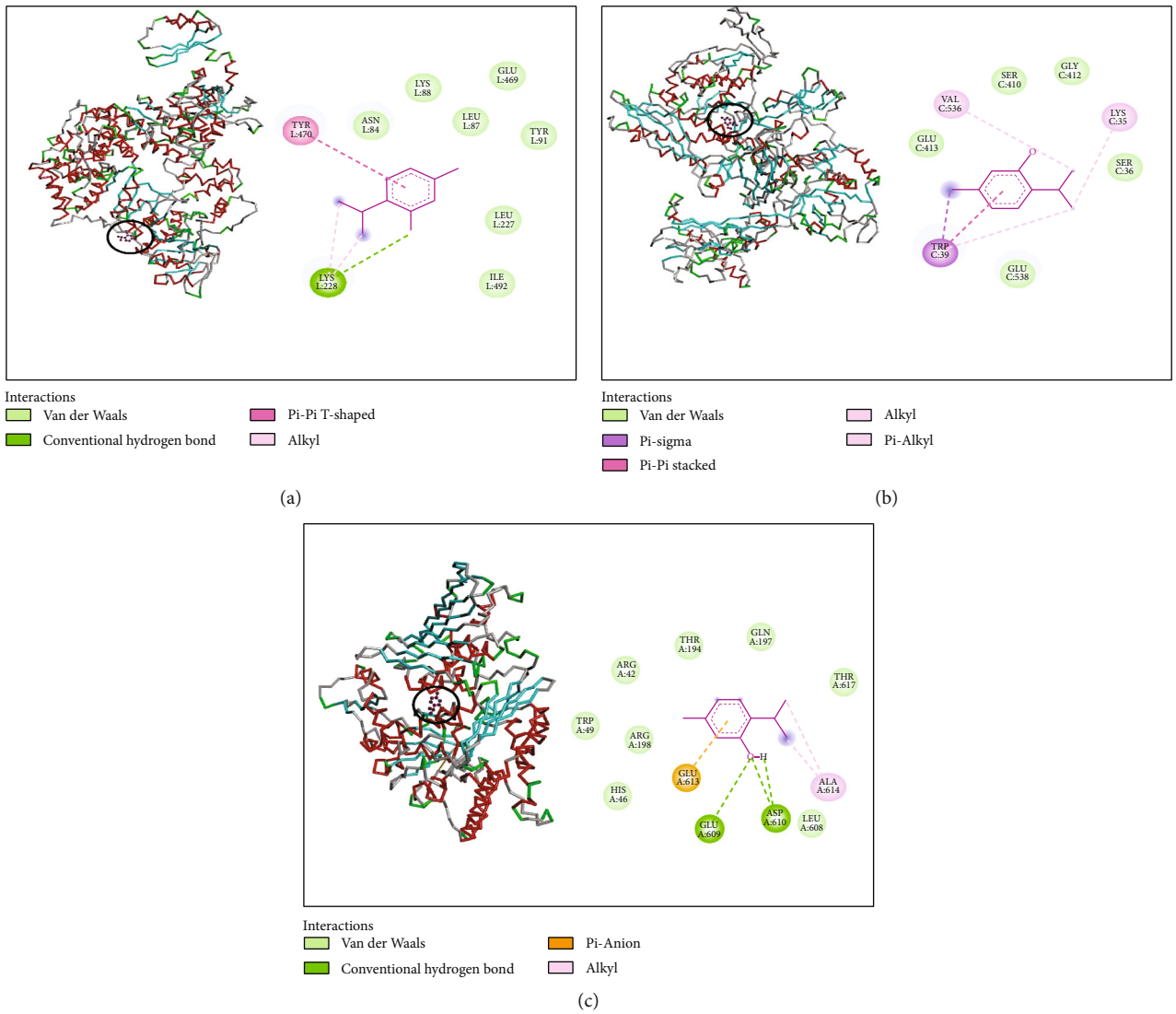

Figure 1: Thymol complexed with DNA polymerase (a), RNA polymerase (b), and topoisomerase II (c) of *S. aureus*.

replication and transcription processes and has an imperative role characterized by its ability to catalyze the unwinding of supercoiled DNA strands [101]. These imperative enzymes constitute attractive and validated targets for antibacterial agents [102]. Molecular docking simulation results are elucidated by (Table 4). These later displayed that TVEO major compound thymol (47.44%) showed a good inhibitory effect on topoisomerase II, RNA polymerase, and DNA polymerase of the selected pathogenic bacteria.

A previous study conducted by Liu et al. reported that thymol has a potential antibacterial activity against *P. aeruginosa* [15]. This monoterpenoid can affect bacterial DNA normal function. It could block gene expression processes including replication, transcription, and expression by intercalation with bacterial DNA leading to bacterial death. The same study indicated that thymol could destroy bacterial membrane integrity by affecting its permeability and it could also hinder biofilm formation. Another research paper displayed that thymol could bind to bacterial DNA, modulate its structure, and prohibit its biological function [103]. Furthermore, dos Santos Barbosa et al. showed that the antibacterial inhibitory activity of *Origanum vulgare* EO was effective against *Salmonella Enteritidis* due to the presence of thymol which caused interference in protein regulation as well as DNA synthesis [104]. On the other hand, the minor compound β-sesquiphellandrene (1.34%) showed the lowest free energy of binding (Kcal/mol) and the highest inhibitory potential on topoisomerase II and DNA and RNA polymerases of the selected pathogenic bacteria (Table 4). A previous study reported that β-sesquiphellandrene was responsible for the high antibacterial and antioxidant activities of some pomelo varieties' EOs [105]. Another study indicated that the presence of β-sesquiphellandrene presented in ginger (*Zingiber officinale*) essential oil could make this later as a potential

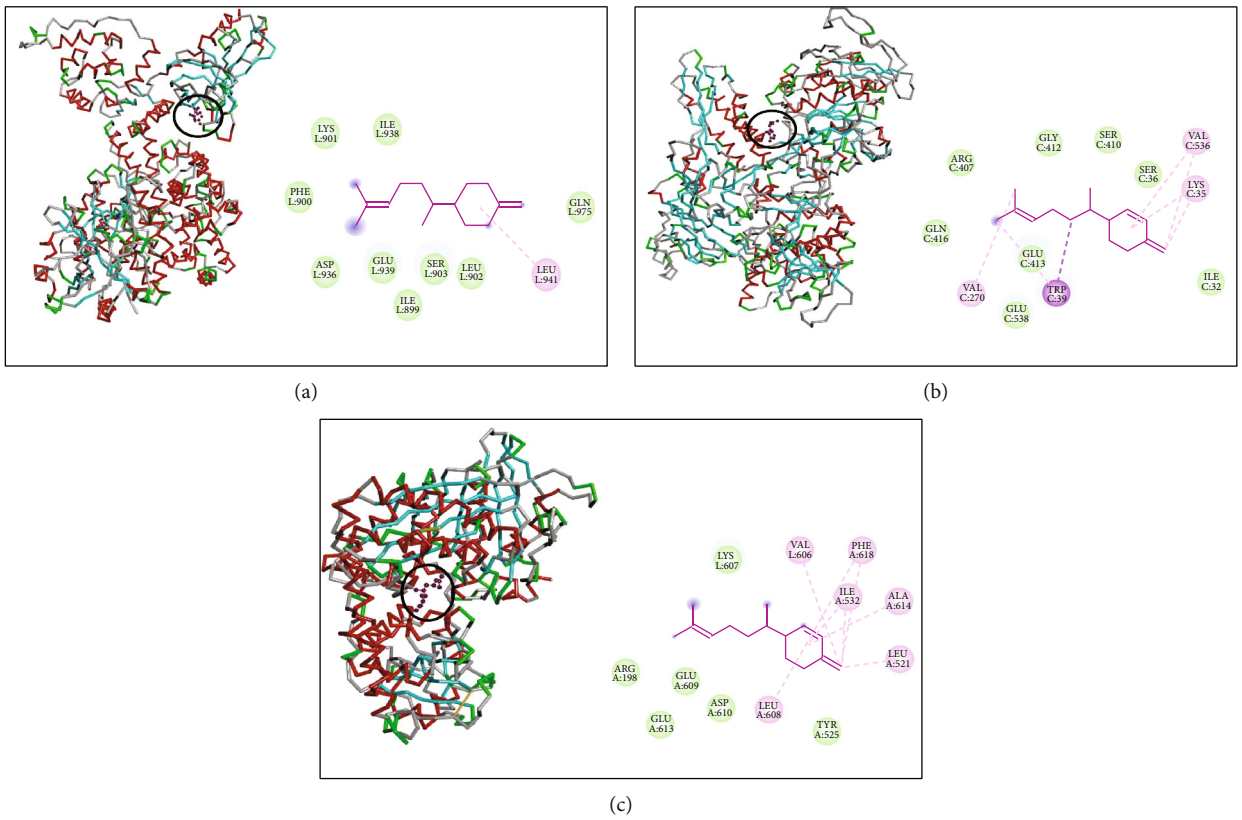

Figure 2: β-Sesquiphellandrene complexed with DNA polymerase (a), RNA polymerase (b), and topoisomerase II (c) of *S. aureus*.

antimicrobial agent by inhibiting mycobacterial acyl carrier protein reductase enzyme and Enoyl acyl carrier protein reductase activities [106].

A recent study showed that *Cupressus sempervirens* EO had a great inhibitory effect on DNA gyrase and DNA and RNA polymerases of *S. aureus* and *S. enterica* owing to the presence of α-pinene, δ-3 carene, and borneol [13]. Moreover, a previous study conducted by [107] evaluated the inhibitory effect of *Litsea cubeba* EO on topoisomerase and DNA and RNA polymerases of *E.coli*. Another study revealed that the germacrene B, a minor compound in *Siparuna guianensis* EO, had an effective inhibitory activity against bacterial DNA and RNA polymerases of multiple pathogenic bacteria including *E.coli*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes* [108]. Therefore, these findings confirmed that TVEO has a powerful inhibitory effect on pathogenic bacteria based on the inhibition of DNA replication and transcription processes. Consequently, we project in subsequent work to perform further in vitro assays based on the evaluation of protein-molecule binding assays.

Interaction profiles details of thymol and β-sesquiphellandrene with the active sites of the selected targets of *S. aureus* ATCC 700699, *S. enterica* Typhimurium ATCC 700720, and *L. monocytogenes* ATCC 19115 are summarized in Table 5.

Interaction profile results of thymol and β-sesquiphellandrene with topoisomerase II and DNA and RNA polymerases of *S. aureus* are presented by Figures 1 and 2. Thymol made a complex with DNA polymerase receptor via alkyl interaction and conventional hydrogen bond with LYS228 and van der Waals interactions with ASN84, LYS88, LEU87, GLU469, TYR91, LEU227, and ILE492 (Figure 1(a)). Additionally, RNA polymerase complexed with thymol had alkyl and Pi-alkyl interaction with VAL536 and LYS35; van der Waals interactions with GLU413, SER410, GLY412, SER36, and GLU538; and Pi-sigma interaction with TRP39 (Figure 1(b)). Likewise, it interacted with topoisomerase II via alkyl interaction with ALA614; van der Waals interactions with HIS46, ARG198, TRP49, ARG42, THR194, GLN197, THR617, and LEU608; and two conventional hydrogen bonds with ASP610 and GLU609 (Figure 1(c)).

β-Sesquiphellandrene complexed with DNA polymerase showed Pi-alkyl interaction with LEU641 and van der Waals interactions with ILE938, LYS901, PHE900, ASP936, GLU939, ILE899, SER903, LEU902, and GLN975 (Figure 2(a)). When complexed with RNA polymerase, it made Pi-alkyl interaction with VAL270, LYS35, and VAL536; van der Waals interactions with SER36, SER410, GLY412, ARG407, GLN416, GLN413, GLU538, and ILE32; and Pi-sigma interaction with TRP39 (Figure 2(b)). Concerning topoisomerase II, β-sesquiphellandrene displayed alkyl and Pi-alkyl interactions with VAL606, PHE618, ILE532, ALA614, LEU521, and LEU608 and van der Waals interactions with LYS607, ARG198, GLU609, GLU613, ASP610, and TYR525 (Figure 2(c)).

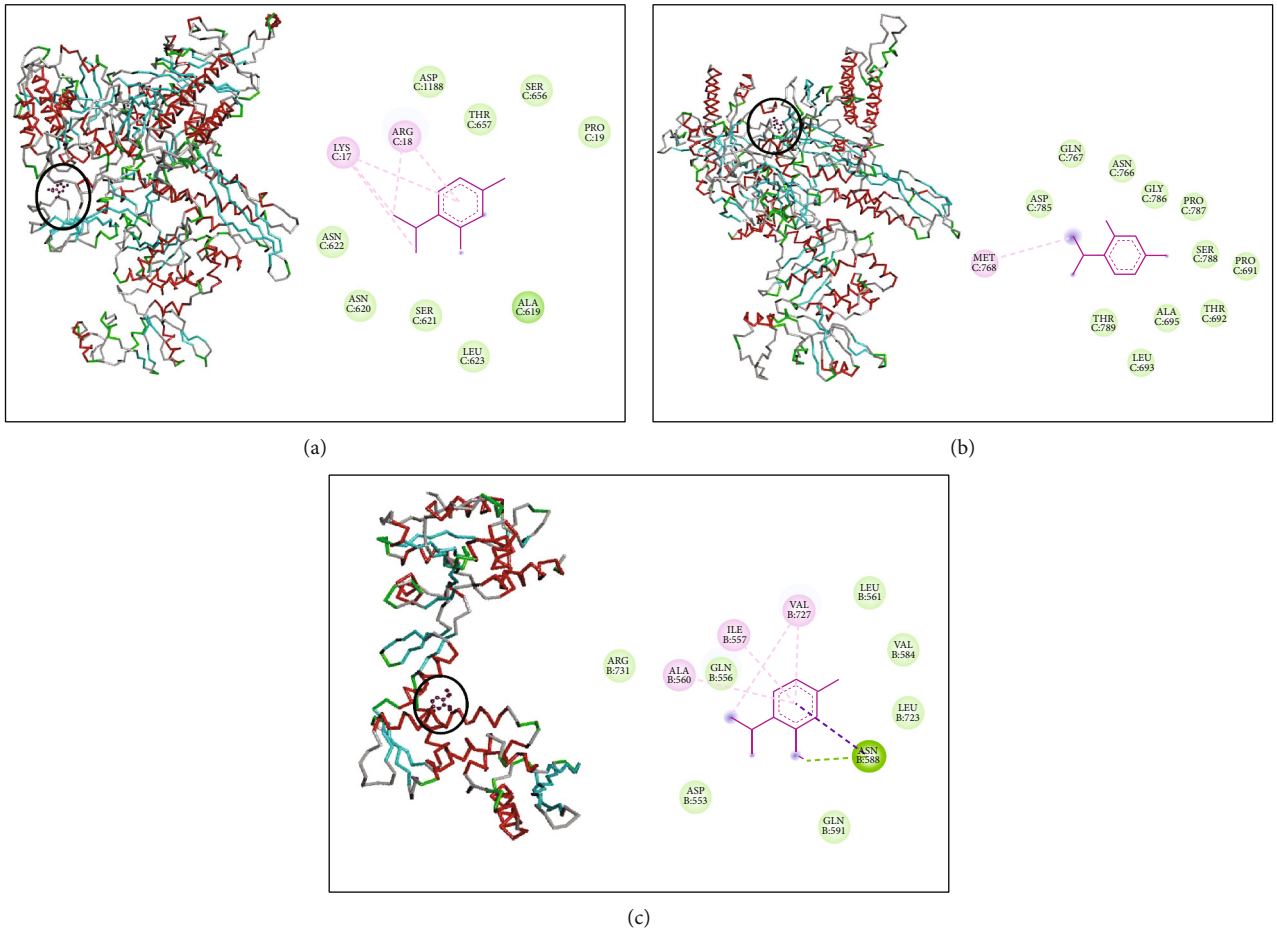

Figure 3: Thymol complexed with DNA polymerase (a), RNA polymerase (b), and topoisomerase II (c) of *S. enterica* Typhimurium.

On the other hand, interaction profiles between thymol and β-sesquiphellandrene with topoisomerase II and DNA and RNA polymerases of *S. enterica* Typhimurium are outlined in Figures 3 and 4. DNA polymerase complexed with thymol showed alkyl and Pi-alkyl interactions with ARG18 and LYS17 and van der Waals interactions with ASP1188, THR657, SER656, PRO19, ASN622, ASN620, SER621, LEU623, and ALA 619 (Figure 3(a)). Thymol complex with RNA polymerase indicated the presence of alkyl interaction with MET768 and van der Waals interactions with ASP785, GLN767, ASN766, GLY786, PRO787, SER788, PRO691, THR692, ALA695, THR789, and LEU693 (Figure 3(b)). This monoterpene also made interactions with topoisomerase II via alkyl interactions with VAL727, ILE557, and ALA560; van der Waals interactions with LEU561, VAL584, LEU723, GLN591, and ASP553; and one conventional hydrogen bond with ASN588 (Figure 3(c)).

The complex of β-sesquiphellandrene and the DNA polymerase of *S. enterica* Typhimurium revealed the existence of alkyl and Pi-akyl interactions with LEU75, ALA94, LEU129, LEU32, PHE35, and ILE39 and van der Waals interactions with GLN41, GLN36, GLY76, and MET130 (Figure 4(a)). This sesquiterpene complexed with RNA polymerase showed alkyl and Pi-alkyl interactions with MET130, LEU32, LEU129, PHE35, ALA94, and LEU75 and van der Waals interactions with ILE39, GLN36, and ASP32 (Figure 4(b)). In addition, it made interactions with topoisomerase II via alkyl and Pi alkyl interactions with VAL467, PHE513, PHE777, ARG516, and LEU462 and van der Waals interactions with MET461, LEU509, and THR512 (Figure 4(c)).

DNA polymerase receptor of *L. monocytogenes* complexed with thymol displayed the existence of alkyl and Pi-alkyl interactions with MET389, LEU345, LEU394, ILE392, and PHE367 and van der Waals interactions with GLU359, THR357, LYS358, ILE343, THR360, and SER364 (Figure 5(a)). It also showed when complexed with RNA polymerase alkyl interactions with LYS283 and LYS284 and van der Waals interactions with VAL150, GLY149, TYR151, ASN410, ASP403, ASP571, and ASN289 (Figure 5(b)). Further, thymol made interactions with topoisomerase II via alkyl interaction with PHE97; van der Waals interactions with GLN95, SER98, GLN267, TYR266, THR220, VAL268, ASN269, GLY115, and SER112; and one conventional hydrogen bond with VAL113 (Figure 5(c)).

Finally, β-sesquiphellandrene complex with DNA polymerase of *L. monocytogenes* indicated the existence of Alkyl interactions with MET386, ALA382, ILE399, and LEU488

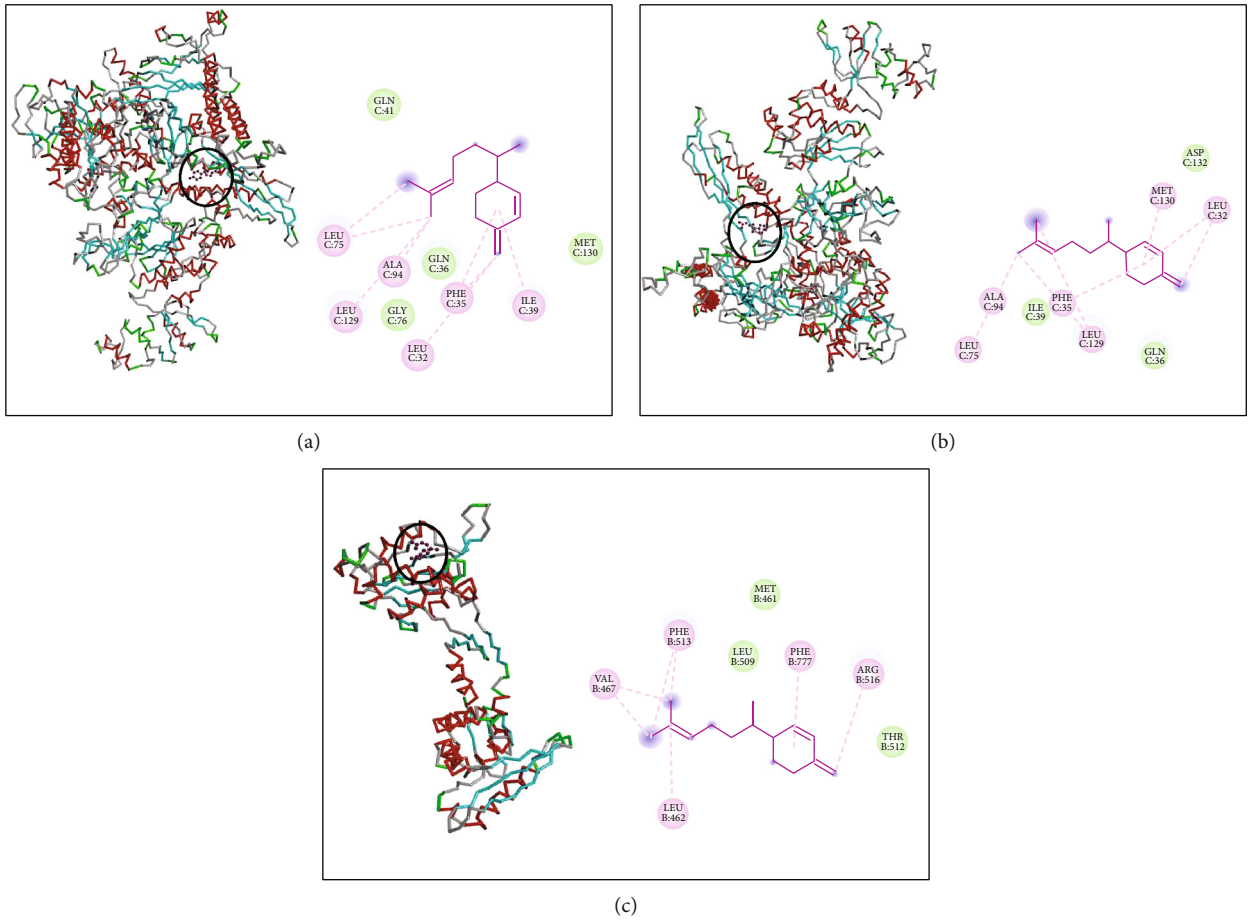

Figure 4: β-Sesquiphellandrene complexed with DNA polymerase (a), RNA polymerase (b), and topoisomerase II (c) of *S. enterica* Typhimurium.

and van der Waals interactions with SER397, PHE492, PRO489, GLU484, THR400, and THR491 (Figure 6(a)). It showed also with RNA polymerase alkyl interactions with LYS280, LYS284, and TYR151 and van der Waals interactions with VAL150, GLY149, ASP403, ASN410, ASP571, ASP404, ASP401, ASN289, and LYS283 (Figure 6(b)). In addition, β-sesquiphellandrene made a complex with topoisomerase II via alkyl and Pi-alkyl interactions with VAL113, ILE264, and PHE97 and van der Waals interactions with GLY115, SER112, GLN95, ARG92, GLY111, PHE88, SER98, ASN269, VAL268, THR220, PRO265, and TYR266 (Figure 6(c)).

3.4. In Silico TVEO Compound Toxicity Evaluation by VEGA QSAR Model. Toxicity evaluation of different TVEO compounds and the two selected FDA-approved antibiotics, used as controls (rifamycin SV and ciprofloxacin), was performed by the help of QSAR (quantitative structure–activity relationship) approach and using VEGA HUB software. We chose to assess compounds toxicity based on 8 different toxicity measurements. Results are represented by (Table 6) and revealed that FDA-approved drugs could be toxic in several assays. In this context, rifamycin SV and ciprofloxacin are found to be mutagenic in the mutagenicity test/model (Ames test) and predicted to engender developmental/reproductive toxicity. These two antibiotics were also predicted to be genotoxic according to the in vitro micronucleus activity model. However, many TVEO compounds such as α-pinene, α-thujene, camphene, β-pinene, 3-carene, D-limonene, γ-terpinene, terpinolene, linalool, borneol, 4-terpineol, α-terpineol, thymol, caryophyllene, α-bisabolene, and β-sesquiphellandrene showed nontoxic effects. Computational toxicity results confirmed that TVEO molecules could be used as a safe antimicrobial agents and economically low-cost alternative as compared to synthetic antibiotics. It is important to mention that research related to toxicological profiles of different essential oil compounds are poorly studied due to experiments complexity, expensive cost, and difficulty to detect toxicity variation because of the chemical function and factor variability [109]. However, previous studies indicated that toxicity is a dose-/concentration-dependent manner and thymol could have a certain limit of toxicity ranging from 36 mg/mL to 49 mg/mL with less risks of accumulation in body tissues and suggested to replace synthetic drugs, which has more side effect [110]. In addition, Schönknecht et al. confirmed the safety and

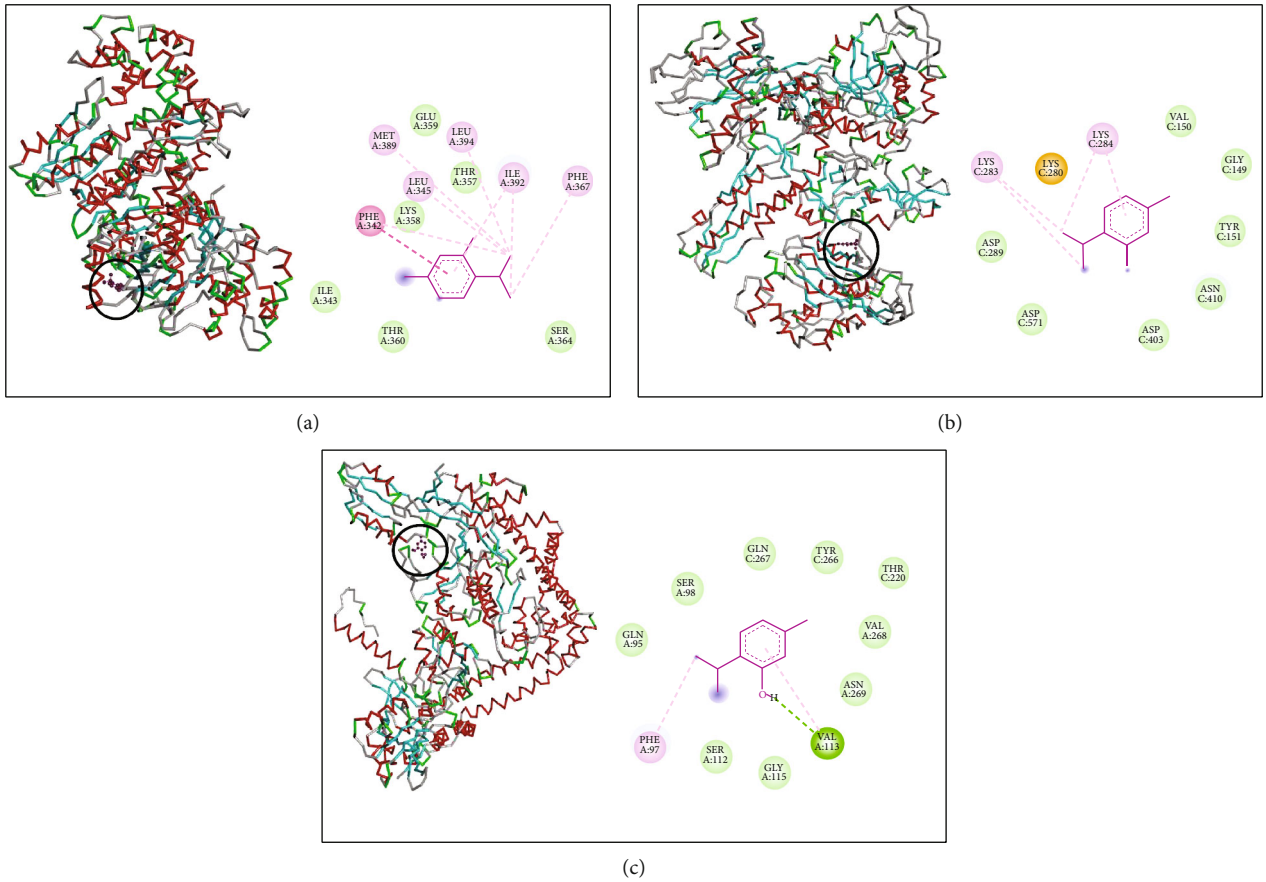

Figure 5: Thymol complexed with DNA polymerase (a), RNA polymerase (b), and topoisomerase II (c) of *L. monocytogenes*.

the effectiveness of the drug containing the extracts of thyme with the addition of thymol (Bronchosol®) in cough treatment instead of using the synthetic drug ambroxol [111]. Concerning β-sesquiphellandrene, it was reported that this sesquiterpenoid could have a great anticancer activity and can be safe to use as compared to synthetic chemotherapeutic agents velcade, thalidomide, and capecitabine [112]. Nevertheless, more toxicological *in vitro*/*in vivo* data are needed to validate the safety of these phytochemicals. Thus, computational toxicity assessment could be the best alternative that would give robust data, avoid unnecessary waste of reagents, and minimize cruelty and sacrifices of lab animal testing.

3.5. TVEO Component ADME Analysis. In the present study, SwissADME server was used to determine some pharmacokinetics parameters included in absorption, distribution, metabolism, and excretion (ADME), drug-likeness, and medicinal chemistry characteristics for all TVEO compounds as represented in (Table 7). Results revealed that all TVEO components possess slow passive gastrointestinal absorption (GI) except linalool, camphor, borneol, 4-terpineol, α-terpineol, thymol methyl ether, and thymol which were predicted to have high GI permeability. Additionally, only sesquiterpenes including caryophyllene, α-humulene, α-amorphene, α-curcumene, α-zingibirene, α-bisabolene, and β-sesquiphellandrene were found not to be blood-brain barrier (BBB) permanent due to their heavy molecular weight. However, rest of compounds could easily cross the blood-brain barrier for that reason it could be suggested as potent central nervous system antioxidants and effective drug candidates in the treatment of neurodegenerative diseases like Alzheimer's and Parkinson's [113]. On the other part, none of the tested compounds was predicted to be P-gp transporter substrate. Concerning Cytochrome p450 (CYP) isoenzymes which are involved in 50-90% of therapeutic molecules biotransformation processes in a view to reduce metabolites accumulation in blood/tissues and drug-drug interaction risks [114], thymol methyl ether and thymol were predicted to be CYP1A2 inhibitors. Many TVEO molecules were predicted to inhibit CYP2C9 and CYP2D6 inhibitors. However, none of the compounds showed an inhibitory effect towards the CYP3A4. Moreover, skin permeation coefficient (log Kp) indicated that all compounds were impermeable through the skin barrier. Interestingly, TVEO drug-likeness score was acceptable with good bioavailability score (>10%) and the absence of violations related to known rules such as Lipinski's rule of five that predicts drug permeability and absorption based on H-bond

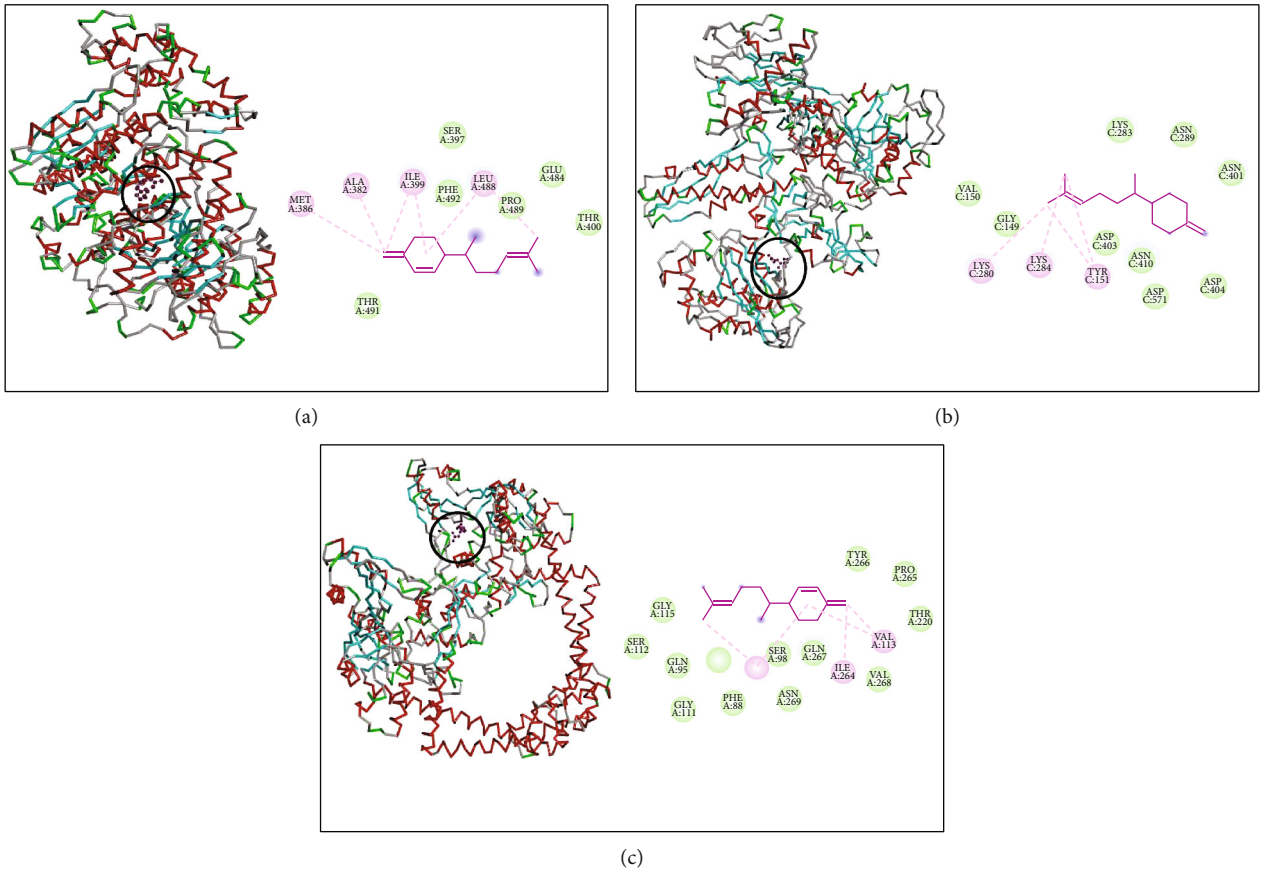

Figure 6: β-Sesquiphellandrene complexed with DNA polymerase (a), RNA polymerase (b), and topoisomerase II (c) of *L. monocytogenes*.

donors, H-bond acceptors, molecular weight (MW > 500), and a calculated log p) [115], as well as Veber. Both rules are a mainstay of decision-making in drug design and development and in the present study; both were validated. Furthermore, medicinal chemistry parameters revealed that none of the selected molecules returns any pan-assay interference compounds (PAINS) alert. The synthetic accessibility values of TVEO compounds indicated that these later could be synthesized for pharmaceutical uses.

3.6. Prediction of Possible Activity Spectra of TVEO Components. All TVEO compounds were subjected to PASS (Prediction of Activity Spectra for Substances) online tool intending to predict their biological activity spectrum based on their chemical structure. Results indicated that the tested compounds could have multiple biological activities and various enzymatic targets (Table 8). We have selected the top three activities which showed a Pa ≥ 0.7. Pa and Pi values indicated the probability of the selected molecule to be active/ inactive towards the targeted receptor. Mojumdar et al. reported that the probability of experimental pharmacological action is high when Pa > 0.7; however, the chance of finding the activity experimentally is less when Pa < 0.5 [116]. Interestingly, TVEO major compounds such as thymol which were predicted, in earlier section by molecular docking, to have a potent antibacterial activity on pathogenic bacteria by inhibiting DNA replication and transcription processes, displayed the existence of other biological activities including the ability to inhibit bacterial membrane permeability (Pa = 0.876) and it was anticipated to enhance AP0A1 expression (Pa = 0.830) involved in the cellular synthesis of beneficial HDL [117]. In addition, O-cymene was predicted to be mitochondria ubiquinol-cytochrome-c reductase inhibitor (Pa = 0.924) (antifungal activity), mucomembranous protector (Pa = 0.842), and a fibrinolytic agent (Pa = 0.778) which could stimulate the dissolution of blood clots. Concerning γ-terpinene, it was predicted to treat skin eczema (Pa = 0.854) and phobic disorders (Pa = 0.803) owing to its ability to cross the blood-brain barrier (BBB). Finally, PASS prediction revealed that the minor compound β-sesquiphellandrene has other possible biological activities including antineoplastic effect (Pa = 0.827) and antipsoriatic (Pa = 0.750) and could be also used as immunosuppressant agent during organ transplant (Pa = 0.702) instead of using synthetic compounds such as cyclosporin A which was demonstrated to cause severe cholestatic liver disease [118]. These findings could provide more insights towards further in vivo and in vitro assays to validate the computational predictions.

Table 6: Toxicity predictions of TVEO compounds using the VEGA QSAR model.

Toxicity measurements Compound	Mutagenicity (Ames Test) Model (CAESAR) 2.1.13	Carcinogenicity model (CAESAR) 2.1.9	Developmental/ Reproductive Toxicity Library (PG) 1.1.0	Estrogen receptor relative binding affinity model (IRFMN)	Androgen receptor-mediated effect (IRFMN/COMPARA) 1.0.0	Thyroid receptor alpha effect (NRMEA) 1.0.0	Thyroid receptor beta effect (NRMEA) 1.0.0	In vitro micronucleus activity (IRFMN/ VERMEER) 1.0.0
α-Pinene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
α-Thujene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Camphene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
β-Pinene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
β-Myrcene	Nonmutagenic	Carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Active
α-Fellandrene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Active
3-Carene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
D-Limonene	Nonmutagenic	Carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
O-Cymene	Nonmutagenic	Noncarcinogen	Toxicant	Inactive	Nonactive	Inactive	Inactive	Not predicted
Cymol	Nonmutagenic	Noncarcinogen	Toxicant	Inactive	Nonactive	Inactive	Inactive	Not predicted
γ-Terpinene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Terpinolene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Linalool	Nonmutagenic	Non carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Camphor	Nonmutagenic	Noncarcinogen	Toxicant	Inactive	Nonactive	Inactive	Inactive	Active
Borneol	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
4-Terpineol	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
α-Terpineol	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Thymol methyl ether	Nonmutagenic	Carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Active
Thymol	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Caryophyllene	Nonmutagenic	Carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
α-Humulene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Active
α-Amorphene	Nonmutagenic	Carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
α-Curcumene	Nonmutagenic	Noncarcinogen	Toxicant	Inactive	Active	Inactive	Inactive	Active
α-Zingibirene	Nonmutagenic	Carcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Active
α-Bisabolene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
β-Sesquiphellandrene	Nonmutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Inactive
Rifamycin SV	Mutagenic	Noncarcinogen	Nontoxicant	Inactive	Nonactive	Inactive	Inactive	Active
Ciprofloxacin	Mutagenic	Noncarcinogen	Toxicant	Inactive	Nonactive	Inactive	Inactive	Active

Table 7: TVEO compounds pharmacokinetic properties prediction using SwissADME.

Compound	Pharmacokinetics								Drug-likeness and Medicinal chemistry				
	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log K_p (cm/s)	Lipinski	Veber	Bioavailability score	PAINS	Synthetic accessibility
α-Pinene	Low	Yes	No	No	Yes	No	No	-3.95	Yes	Yes	0.55	0 alert	4.44
α-Thujene	Low	Yes	No	No	No	No	No	-5.11	Yes	Yes	0.55	0 alert	3.99
Camphene	Low	Yes	No	No	Yes	No	No	-4.13	Yes	Yes	0.55	0 alert	3.50
β-Pinene	Low	Yes	No	No	Yes	No	No	-4.18	Yes	Yes	0.55	0 alert	3.73
β-Myrcene	Low	Yes	No	No	No	No	No	-4.17	Yes	Yes	0.55	0 alert	2.85
α-Fellandrene	Low	Yes	No	No	No	No	No	-4.85	Yes	Yes	0.55	0 alert	4.15
3-Carene	Low	Yes	No	No	Yes	No	No	-4.02	Yes	Yes	0.55	0 alert	3.84
D-Limonene	Low	Yes	No	No	Yes	No	No	-3.89	Yes	Yes	0.55	0 alert	3.46
O-Cymene	Low	Yes	No	No	No	Yes	No	-4.01	Yes	Yes	0.55	0 alert	1.00
Cymol	Low	Yes	No	No	No	Yes	No	-4.21	Yes	Yes	0.55	0 alert	1.00
γ-Terpinene	Low	Yes	No	No	Yes	No	No	-3.94	Yes	Yes	0.55	0 alert	3.11
Terpinolene	Low	Yes	No	No	Yes	No	No	-3.96	Yes	Yes	0.55	0 alert	2.98
Linalool	High	Yes	No	No	No	No	No	-5.13	Yes	Yes	0.55	0 alert	2.74
Camphor	High	Yes	No	No	No	No	No	-5.67	Yes	Yes	0.55	0 alert	3.22
Borneol	High	Yes	No	No	No	No	No	-5.31	Yes	Yes	0.55	0 alert	3.43
4-Terpineol	High	Yes	No	No	No	No	No	-4.93	Yes	Yes	0.55	0 alert	3.28
α-Terpineol	High	Yes	No	No	No	No	No	-4.83	Yes	Yes	0.55	0 alert	3.24
Thymol methyl ether	High	Yes	No	Yes	No	Yes	No	-4.64	Yes	Yes	0.55	0 alert	1.09
Thymol	High	Yes	No	Yes	No	No	No	-4.87	Yes	Yes	0.55	0 alert	1.00
Caryophyllene	Low	No	No	No	Yes	No	No	-4.44	Yes	Yes	0.55	0 alert	4.51
α-Humulene	Low	No	No	No	Yes	No	No	-4.32	Yes	Yes	0.55	0 alert	3.66
α-Amorphene	Low	No	No	No	Yes	No	No	-4.65	Yes	Yes	0.55	0 alert	4.35
α-Curcumene	Low	No	No	No	No	Yes	No	-3.71	Yes	Yes	0.55	0 alert	2.31
α-Zingibirene	Low	No	No	No	Yes	No	No	-3.88	Yes	Yes	0.55	0 alert	4.81
α-Bisabolene	Low	No	No	No	Yes	No	No	-3.03	Yes	Yes	0.55	0 alert	3.90
β-Sesquiphellandrene	Low	No	No	No	Yes	No	No	-3.71	Yes	Yes	0.55	0 alert	4.42

Table 8: PASS prediction of TVEO compounds activity spectrum.

Compound	Pa	Pi	Possible biological activities
	0.821	0.004	Cardiovascular analeptic
α-Pinene	0.746	0.010	Antidyskinetic
	0.706	0.006	Carminative
	0.866	0.008	Antieczematic
α-Thujene	0.807	0.006	Anti-inflammatory
	0.729	0.063	Phobic disorder treatment
	0.882	0.006	Antieczematic
Camphene	0.782	0.040	Phobic disorder treatment
	0.738	0.015	Alkylacetylglycerophosphatase inhibitor
	0.902	0.005	Antieczematic
β-Pinene	0.735	0.004	Ovulation inhibitor
	0.729	0.013	Respiratory analeptic
	0.941	0.004	Mucomembranous protector
β-Myrcene	0.892	0.004	Antineoplastic (breast cancer)
	0.756	0.002	Antiviral (Rhinovirus)
	0.869	0.012	Ubiquinol-cytochrome c reductase inhibitor
α-Fellandrene	0.753	0.009	Fibrinolytic
	0.727	0.005	Adenomatous polyposis treatment
	0.815	0.005	Antidyskinetic
3-Carene	0.718	0.034	Antiseborrheic
	0.713	0.004	Transplant rejection treatment
	0.961	0.001	Carminative
D-Limonene	0.743	0.004	Acetylcholine neuromuscular blocking agent
	0.740	0.003	Chemoprotective
	0.924	0.004	Ubiquinol-cytochrome c reductase inhibitor
O-Cymene	0.842	0.010	Mucomembranous protector
	0.778	0.005	Fibrinolytic
	0.831	0.015	Polyporopepsin inhibitor
Cymol	0.822	0.005	Omptin inhibitor
	0.796	0.004	Tpr proteinase (*Porphyromonas gingivalis*) inhibitor
	0.854	0.009	Antieczematic
γ-Terpinene	0.803	0.033	Phobic disorders treatment
	0.756	0.023	Sugar-phosphatase inhibitor
	0.927	0.004	Glutamate-5-semialdehyde dehydrogenase inhibitor
Terpinolene	0.848	0.003	Carminative
	0.715	0.014	Venombin AB inhibitor
	0.913	0.003	Cell adhesion molecule inhibitor
Linalool	0.803	0.005	Lipid metabolism regulator
	0.725	0.004	Gastrin inhibitor
	0.922	0.004	Respiratory analeptic
Camphor	0.877	0.006	Antiseborrheic
	0.745	0.002	Pediculicide
	0.872	0.003	Vasoprotector
Borneol	0.822	0.002	Peptidoglycan glycosyltransferase inhibitor
	0.781	0.004	Alopecia treatment

Table 8: Continued.

Compound	Pa	Pi	Possible biological activities
	0.842	0.019	Ubiquinol-cytochrome c reductase inhibitor
4-Terpineol	0.796	0.020	Antiseborrheic
	0.729	0.014	Fibrinolytic
	0.825	0.014	Antieczematic
α-Terpineol	0.763	0.023	Alkenylglycerophosphocholine hydrolase inhibitor
	0.750	0.048	Aspulvinone dimethylallyltransferase inhibitor
	0.891	0.005	Mucomembranous protector
Thymol methyl ether	0.790	0.019	Antineurotic
	0.723	0.006	Anesthetic general
	0.913	0.003	Antiseptic
Thymol	0.876	0.004	Membrane permeability inhibitor
	0.830	0.003	APOA1 expression enhancer
	0.915	0.005	Antineoplastic (lung cancer)
Caryophyllene	0.847	0.005	Apoptosis agonist
	0.722	0.002	NF-E2-related factor 2 stimulant
	0.818	0.003	MMP9 expression inhibitor
α-Humulene	0.769	0.002	Interleukin agonist
	0.741	0.011	Anti-inflammatory
	0.850	0.003	Carminative
α-Amorphene	0.821	0.009	Antineoplastic
	0.726	0.059	Ubiquinol-cytochrome c reductase inhibitor
	0.942	0.004	Mucomembranous protector
α-Curcumene	0.757	0.003	Vitamin-K-epoxide reductase (warfarin-insensitive) inhibitor
	0.723	0.003	BRAF expression inhibitor
	0.842	0.010	Mucomembranous protector
α-Zingibirene	0.785	0.008	Fibrinolytic
	0.711	0.002	Antiviral (rhinovirus)
	0.920	0.004	Antieczematic
α-Bisabolene	0.867	0.003	Carminative
	0.760	0.017	Antineoplastic
	0.827	0.009	Antineoplastic
β-Sesquiphellandrene	0.750	0.004	Antipsoriatic
	0.702	0.016	Immunosuppressant

Pa* represents probability to be active; Pi* represents probability to be inactive.

4. Conclusion

The dramatical increase of antibiotic resistance urged scientists to diverge towards the use of aromatic medicinal plants essential oils to tackle the spread of superbugs. In that regard, the present study is aimed at investigating the TVEO chemical composition and antibacterial mechanism of action against *S. aureus* ATCC 6538, *S. enterica* Typhimurium ATCC 14028, and *L. monocytogenes* ATCC 19117. In addition, chemocomputational toxicological profile and pharmacological proprieties were developed. Interestingly, molecular docking simulations revealed that TVEO compounds such as thymol and β-sesquiphellandrene had an effective antibacterial activity against the tested bacteria by inhibiting topoisomerase II and DNA and RNA polymerase functions leading to vigorous impairment of bacterial DNA replication and transcription processes. Additionally, through VEGA QSAR, we demonstrated that TVEO could be a safe resource for potential antibacterial agents. Moreover, ADME analysis showed that both compounds fulfill the Lipinski's rule of five and could be used as potential candidate to overcome antibiotic resistance. Likewise, the *in silico* PASS prediction studies disclosed the presence of other useful bioactivities and possible enzymatic targets of TVEO which would be applied in the future to reduce the impact of several lethal diseases.

Data Availability

All the relevant data have been provided in the manuscript. The authors will provide additional details if required.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Laticifer Identification, Rubber Characterization, Phenolic Content, and Antioxidant Activity of *Pergularia tomentosa* Latex Extract

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Pergularia tomentosa is a perennial twining herb widely spread out arid and semiarid Tunisian regions. It is searched for its richness in enzymes, secondary metabolites, antifungal activity, and milk-clotting activity. Traditional use implies the fresh latex in wounding heals. The present study was aimed at identifying laticifer distribution in *Pergularia tomentosa* stems, leaves, and petioles. In the present study, the identification of latex extract's components and its valorisation by estimation of phenolic content, flavonoids, and antioxidant activity are conducted. Anatomical structures proved the presence of laticifers in the aerial parts of *Pergularia tomentosa*. They are particularly observed along the pith and cortical parenchyma in stem, in leaf mesophyll, and in petiole phloem. Identified laticifers were characterized as nonarticulated. FTIR spectroscopy proves the presence of several functional groups in the latex and mainly the *cis*-1-4-isoprene monomer. Results suggested that *Pergularia tomentosa* latex contributes significantly as a source of phenol content (62.3 mg Eq GAE/g) and flavonoids (24.8 mg Eq QE/g). Scavenging free radicals of DPPH exhibited IC_{50} value of 12 μg/ml. In conclusion, latex extracted from *Pergularia tomentosa* can be implied in industry as a natural rubber. It can be used, also, in medicine as a therapeutic agent.

1. Introduction

Pergularia tomentosa L. called as Bouhliba due to its latex belongs to Asclepiadaceae family and sub family of Asclepiadoideae. The species known by a lactiferous twinner presents follicles generally assembled together as twins and secreting at the slightest touch a sticky white fluid called latex [1]. In Tunisia, *P. tomentosa* represents the only species of the genus *Pergularia*. The plant is widespread in different countries of Mediterranean basin and Sahara (Algeria, Morocco, Egypt, Jordan, and Saudi Arabica) and often grown in Tunisia [2, 3]. In our country, traditional habit implies its integration to heal feet wounds, in tanning some clothes, and for depilatory procedure. Ethnopharmacological survey showed the implication of *Pergularia tomentosa* during the treatment of diarrhea, bronchitis, constipation, wound, and related injuries [4, 5]. Latex extracted from leaves was used to treat skin infections like *Tinea capitis* [6]. It is commonly administered as a poultice, abortifacient, laxative, and anthelmintic. Several important compounds screened in *P. tomentosa* like cardiotonic glycosides, uzarigenin, pergularoside, and *D*-glucopyranosylcalactin [7] correlate to this plant an important medicinal interest.

Castelblanque et al. [8] reported that around 12,500 plants belonging to 22 families secreting latex. This latex may contain enzymes, phenolic compounds, cardenolides, lipids, polysaccharides, triterpenes, alkaloids, and other components [9, 10]. The occurrence and amount of chemical compounds in each species led to an inconstant latex colour [11]. Thus, the appearance of this latex depended on the plant species-organ and can differ from clear to translucent [12]. In some family, it can be yellow, orange, brown, red, and colourless. In Apocynaceae, it is generally fluid and sticky with a milky-white colour [10]. *Pergularia tomentosa*

latex was revealed as a stimulator agent on milk-clotting process [13].

A poisonous substance linked to lactucone and having a resinous character called aslepione was detected on the Asclepia latex [14]. The valorisation of *Pergularia tomentosa* as a source of natural latex bioinsecticide and the toxicity bioassay of its extract against *Locusta migratoria* showed that it can cause significant mortality by disturbing the respiratory rhythm and the hemocyte number [15]. In addition, due to their alkaloids localised in the aerial part of the plant, an important larvicidal potentiality was detected against the same insect [16]. Cardiotonic glycosides of *Pergularia tomentosa* were reported to have toxic effects against *Monacha obstructa*, land snail. This molluscidal activity can reach 96.6% [17].

Antifungal activity against *Fusarium oxysporum f.* sp. *Lycopersici*, a harmful pathogen of tomato vascular wilt, was recorded [18]. Indeed, this plant contained antibacterial, cytotoxic, and allelopathic compounds [6, 19, 20]. It was considered as antirheumatic and antitumor agent [21] and has hypoglyceaemic effects [22].

Latex is distributed throughout the plant in a special internal secretory system constituted by a series of elongated cells, called laticifers. By releasing their latex from laticifer cell combined to rubbers from parietal cytoplasm, latex-bearing plants construct a protective mechanism against herbivores and microorganisms as well as wound cicatrisation [10, 23]. A fast coagulation of latex revealed secretion efficiency to protect the plant [23, 24].

Latex production was combined to a distortion of cell contents leading, thus, a supply of required energy and an abundance of mitochondria, lipid bodies, starch grains, and osmiophilic structure transferred to the central vacuole [25]. The discontinuity of transverse cell walls associated to cell accumulation in protoplast is responsible of laticiferous cell creation [24, 26]. Latex secretion implies protoplasm degeneration, restriction of the cytoplasm, and fusion of small vacuoles with some vesicles into the central vacuole [27].

Pergularia tomentosa can be found in the arid lands of Tunisia, a main source of natural agent leading to hydrolysis of κ-casein and therefore milk-clotting procedure.

In order to valorise the species, the identification of cells excluding latex as well its chemical composition, phenolic content, and antioxidant activity of the secreted latex is very important. The present study identifies laticifers occurring in the stem, leaf, and petiole and even latex characterization.

2. Materials and Methods

2.1. Plant Material. Fresh plant of *Pergularia tomentosa* was collected in early morning in March from a Tunisian site belongs to subarid climate, Chebba. This site is a coastal town in the Tunisian Sahel located about sixty kilometers north of Sfax and about thirty kilometers south of Mahdia (Weather: 11°C, NW wind at 23 km/h, 63% humidity). Collection was done manually, and wearing plastic gloves was indispensible during the plant handling as a precaution from skin irritation due to the secreted latex at the slight touch. Plant material was authenticated by Pr. Mohamed Chaeib. Voucher specimen was deposit at the herbarium of the Laboratory of Ecosystems and Biodiversity in Arid Environment, Department of Biology, University of Sfax, LR18ES-29.

2.2. Extraction of Bioactive Compounds from Latex. At the moment of dripping stem extremities, latex was collected directly in sterile glass and kept at 4°C. The coagulable content identified as the percentage of dry rubber of latex was estimated according to the following formula [28]: dry rubber content (%) = (mass of dry coagulum/mass of latex) $\times$ 100. Following this method, latex sample will be used for Fourier transform infrared (FTIR) spectroscopy test.

By cutting stem apices, the latex was dropped swiftly and directly into the sterile glass tubes. The tubes already contained an equal volume of petroleum ether. The mixture was precipitated until the separation of petroleum ether layer. Aqueous layer containing pure latex were dried at 50°C for 48 h. The sample was then dissolved in 80% methanol with continuous agitation in shaker at 32°C and 140 rpm overnight to extract bioactive compounds from *Pergularia* latex [29]. Filtered solution considered as latex preparation was used for phenolic content and antioxidant activity tests.

2.3. Anatomical Study. After sampling, fresh leaves and stems were fixed in a Formalin-Acetic-Alcohol (FAA) mixture according to the Sass (1958) fixation procedure [30]. The fixed material was dehydrated by successive submersions in different solutions of distilled water and ethanol 95%. Serial transversal and longitudinal sections were made in a manual microtome (Leica RM2235). All sections were subsequently stained with aceto-carmin [31]. Anatomical observations were observed under a light Reichert LKB microscope and photographed with a digital camera (Olympus).

2.4. Fourier Transform Infrared (FTIR) Spectroscopy. Latex solution was pressed into pellets for the estimation of the Fourier transform infrared spectra with the scanned wave ranging from 4000 to 500 cm^{-1}. Spectra were recorded on a PerkinElmer 1750 spectrophotometer equipped with a diamond attenuated total reflection (ATR) device.

2.5. Determination of Total Phenolic and Flavonoid Content. The total phenolic content was measured according to the Folin-Ciocalteu method based on the reduction of a phosphotungsten-phosphomolybdate complex [32]. 800 ?l of latex solution was added to 50 ?l of Folin-Ciocalteu reagent (2 N) and incubated for 3 min at room temperature. Then, 150 ?l of sodium carbonate solution was added. Absorbance was measured at 765 nm after 2 h of incubation in the dark. Gallic acid (GA) was used as a standard.

The total flavonoid content of the extracts was determined by the method of Lamaison and Carnat based on the formation of the complex flavonoid-aluminum [33]. The aliquot of latex solution (0.25 ml) was mixed with 0.5 ml of 2% aluminum chloride and incubated for 15 min.

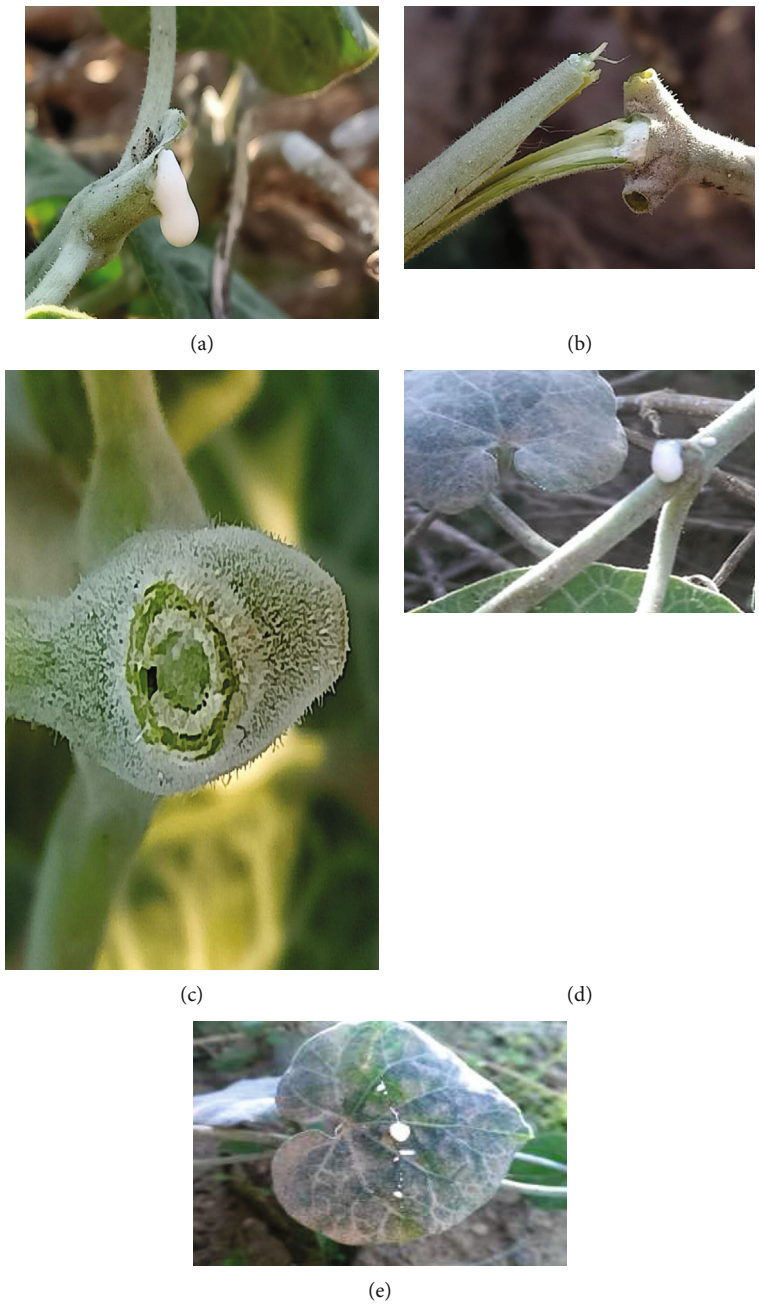

Figure 1: Photographs of latex exudation from vegetative aerial parts of *Pergularia tomentosa*. (a) Profuse latex exudation in stem (GX 10). (b) Latex exudation in stem (GX 10). (c) Detail of laticifers at stem level (GX 40). (d) Profuse latex exudation in petiole (GX 10). (e) Sectioned leaf showing latex droplets (GX 0).

Absorbance was recorded at 430 nm. Quercetin was used as a standard.

2.6. Evaluation of Antioxidant Activity. The radical scavenging assay of methanolic extracts was measured as equivalent of hydrogen donor, according to the DPPH method [34]. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method was used for the determination of the antioxidant activity. 1 ml of freshly prepared DPPH solution (10−4 M) was added to 1 ml of latex solution at different concentrations (0.0625–2 mg/ml). Incubation was for 20 min at room temperature and dark. Absorbance was measured at 517 nm. The inhibition percent was calculated according to the following equation: radical scavenging effect (%) = [1 − (optical absorbance of the latex solution/optical absorbance of the control)] × 100 . BHT and α-tocopherol were used as positive control.

3. Results and Discussion

3.1. Morphological. In a warm period from spring to the beginning of autumn, the perennial herb shows greener and more compact with new shoots of leaves and stems. Leaves are opposite, cylindrical, and pubescent measuring about 10 ∗ 45 mm. Green-white petioles reached 22 mm of

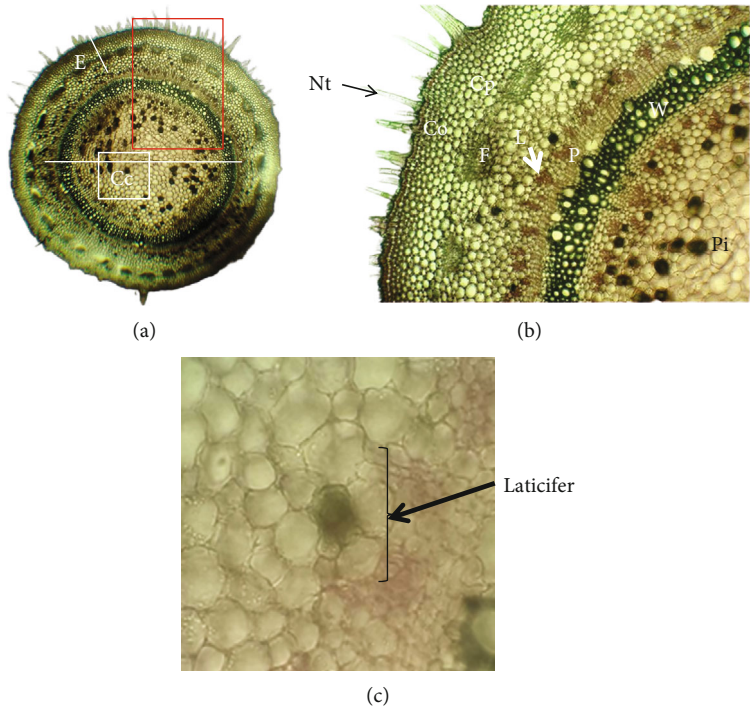

Figure 2: Stem cross sections of *Pergularia tomentosa*. Fixation FAA, double staining (aceto-carmin). (a) Overview of fresh stem transverse section (GX 40). C: cortex; Cc: central cylinder. (b) Details of (a), showing the anatomical structure of the stem (GX 120). Pi: pith; Nt: nonglandular trichome; Ec: epiderm with thick cuticule; F: fiber; Cp: cortical parenchyma; Co: collenchyma; X: xylem II; P: phloem; L: laticifer.(c) Details of (b) (rectangle), high magnification of a laticifer (GX 1000).

length for the adult plant. The young climbing stems of 3 mm of diameter twined around the old ones. Milky-white biological fluid named as latex was profusely exuded with a consistent dense at the slow injury of stem and leaf of *Pergularia tomentosa* (Figures 1(a), 1(b), 1(d), and 1(e)). All the aerial parts including stems, leaves, and petioles highlighted the latex exudation at the slight touch and a physical damage of the plant. The leaked latex was sticky and gives a small burning sensation when it is left on the skin for a long time. Stem showed saturated and fulfilled laticifers with a disposition in the pith and extremely in the cortex with a clear cylindrical arranging (Figure 1(c)).

3.2. Anatomical Study. Previous studies on laticifers focused on their distribution in plants and organs, their localisation, and identification of their types. In fact, they are considered as the main focus of herbivores and more exposed to microorganism attacks; the stems and leaves were the common organs of latex-bearing plant tissues [24]. Laticifer distribution patterns in plant species were very extent, and their types were various [35]. Their cell localisation depends on the vegetative organ and species genus [8, 36]. Nevertheless, there are few researches on anatomical structure at distribution on Asclepiadaceae (Asclepiades, Apocynaceae), and to our knowledge, there is no any anatomical previous research concerning laticifers in *Pergularia tomentosa*.

Our results revealed that, in stems, laticifers were located in the pith, in the collenchyma, and mainly in the cortical parenchyma (Figure 2). While, it was demonstrated that laticifers were identified in Apocynaceae stems and depending on the plant species, they could be observed in medullar radiuses, pericycle, phloem and pith. [37]. However, they can occur in the vascular tissue and cortical parenchyma in other species sections [26].

In leaves, laticifers were scattered in the mesophyll (spongy and palisade) and localised also in the central cylinder (Figure 3). Obtained results were in accordance to those found previously for other Asclepiadaceae [12, 27, 36, 38]. While in other study, secretory cells were found towards leaf extremities [26].

Cross section of *Pergularia* petiole showed two smaller and rounded lateral vascular bundles (Figures 3(c) and 3(d)). Laticifers located particularly in the phloem. In *Vinca sardoa* petiole, authors showed that laticifer cells were localised in the epidermis and the phloem parenchyma [39]. But in the case of *Euphorbia lathyris*, they were localised along the midrib of the petiole [8]. Laticiferous cell localisation can be considered as valuable criteria for taxonomical classification other than it differs within vegetative organs it is correlated to species nature.

Further studies described Asclepiadaceae laticifers as nonarticulate. They were dispersed in all parts of the plant from the pith to cortex and in vascular tissues and closely associated with the leaf phloem [26, 40]. Laticifers of *Vinca* [29], *Calotropis* [25], *Periploca* [28], and *Asclepias* [41] were identified as nonarticulated structures. Nevertheless, few

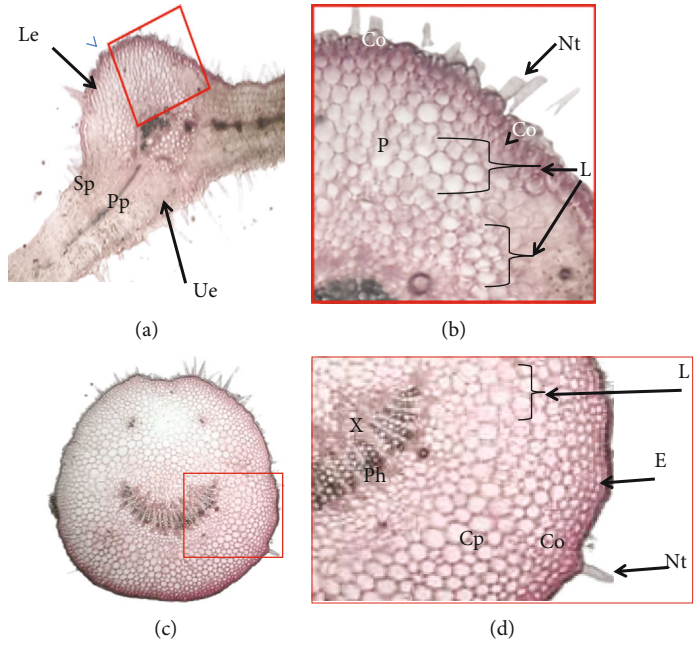

Figure 3: Cross sections of leaf and petiole of *Pergularia*, stained with aceto-carmin and fixed with FAA. (a) Transverse sections at the midrib level of a leaf (GX 40). (b) Details of (a) (rectangle) showing laticifer (GX 100). Co: collenchyma; Le: lower epidermis; P: phloem; Sp: spongy parenchyma; Pp: palisade parenchyma; Ue: upper epidermis; X: xylem; L: laticifer; Nt: nonglandular trichome. (c) Overview of cross section of *Pergularia* petiole (GX 40). (d) Details of (c) (rectangle) showing laticifers (GX 120). Co: collenchyma; Cp: cortical parenchyma, Ph: phloem; X: xylem; L: laticifer; Nt: nonglandular trichome.

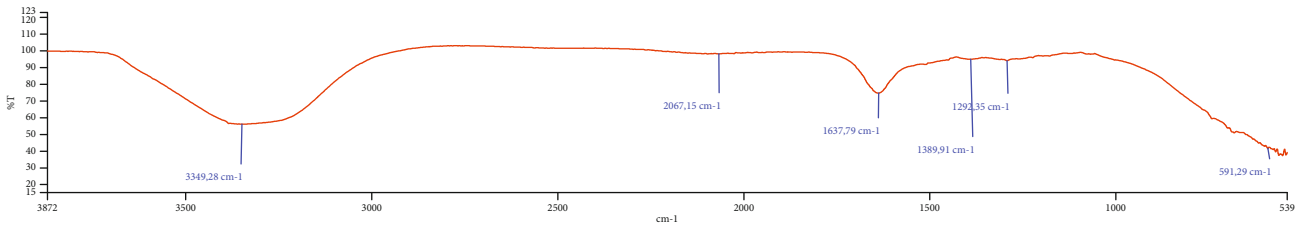

Figure 4: FTIR spectra of *Pergularia tomentosa* latex extract.

species within Apocynaceae family were recognized as articulated anastomosing type like *Blepharodon bicuspidatum* belonging to Asclepiadoideae [24, 42]. Nonarticulate laticifers existed in families other than Asclepiadoideae, like *Asclepias speciosa* [41]; several species belonging to Euphorbiaceae, Moraceae, and Urticaceae have also single long laticifer cells branched without reconnection called nonarticulated laticifers [11].

Articulated laticifers have several interconnected simple and long cells, with intact or perforated transverse and lateral walls [36, 43]. Therefore, *Pergularia tomentosa* can be considered as nonarticulated laticifers.

3.3. FTIR Analysis of Latex. Latex sample was pressed into pellets for the estimation of the infrared spectra with the scanned wave ranging from 4000 to 500 cm^{-1}. Spectra were recorded on a PerkinElmer Universal ATR Sampling Accessory (Figure 4). This method led to define the organic material functional groups and characterize their bonding information [44].

Several compound functional groups could be identified by Fourier transform infrared spectrum studies as alkenes, aromatic compounds, amines, carboxylic acids, amides, esters, alcohols, phenols, and nitro compounds [45]. The band 3349.28 cm^{-1} was probably related to the overlap of free O-H and N-H side groups descendant from amino group. Rubber oxidation may also be related. Vibration at 2067.15 cm^{-1} corresponds to carboxyl compound frequency, transition metal carboxyls, and nitrile compounds as expected phytocompound is identified. A vibrational mode characteristic from rubber main chain was defined at 1637.79 cm^{-1} related to C=C stretching. Bands at 1389.91 cm^{-1} and 1292.35 cm^{-1} indicate the presence of C-H bending, CH_2 and CH_3, respectively, corresponding to *cis*-1-4-isoprene monomer, while the peak at 591.28 cm^{-1}, of low intensity, reveals predominantly C-C-C deformation

vibrations. The mentioned functional groups present in the *Pergularia* latex are mainly correlated to the constituent mixture of sterols, fatty acids, proteins, sugars, gums, enzymes, etc. [18, 46].

3.4. Phenolic Content. A previous study highlighted the presence of phenolic compounds in stems, leaves, roots, and fruits of *Pergularia tomentosa* extracted following an increased gradient of polarity, with successively hexane, chloroform, ethyl acetate, and *n*-butanol [18]. Following the extraction method of active compounds from latex of *Pergularia tomentosa* described previously, the total phenolic and flavonoid contents of the latex extract were 62.3 mg Eq GAE/g latex and 24.8 mg Eq QE/g latex, respectively.

Furthermore, low contents in total phenolics and flavonoids were recorded in *Ficus carica* (50.2 mg GAE/g and 12.5 mg CE/g, respectively), *Euphorbia tirucalli* (10.5 mg GAE/g and 4.3 mg CE/g, respectively), and *Euphorbia dendroides* latex extracts (4.75 mg GAE/100 g and 1.46 mg RE/100 g FW, respectively) [29, 47].

Plants synthesize secondary metabolites like phenolic compounds and flavonoids occasionally for growth, essential physiological processes, and adaptive mechanism. Their production depended on the genotype and physiology of the species, environmental factors (temperature, sunlight exposure, dryness, and salinity), and growth stage of the plant. Abiotic and stress factors stimulate the plant to produce more of those compounds [48].

Consequently, they were implied in defence response to herbivore attacks and pathogenic microbial infection [49]. Moreover, researches proved that flavonoids possessed an important pharmacological interest and a significant antiproliferative effect [50]. In addition to phenolic, latex can contain carboxylated polysaccharides and lipophilic and hydrophilic substances as it was revealed in the case of *Tabernaemontana ventricosa.* Terpenoids, alkaloid neutral lipids, proteins, resin acids, acidic substances, mucilage, and pectin were also detected in latex of the vegetative organs [26].

3.5. Antioxidant Activity. Consuming natural antioxidants extracted from plants was recorded to boost the immunity system against danger system as cancer by their sweeping effect on the free radicals [51]. Antioxidant power can depend on many factors such as plant species and the reactivity and structure of the antioxidant. A strong positive correlation was established between phenolic content and antioxidant of *Pergularia tomentosa* fruits, stems, leaves, and root extracts [18].

In order to evaluate the antioxidant activity of *Pergularia tomentosa* latex, its ability to scavenge free DPPH was tested. The exhibited IC_{50} value was 12 μg/ml. It corresponded to the extract concentration which is able to inhibit 50% of the free radicals. A low value indicates a high antioxidant activity. Among the latex samples recorded as source of natural antioxidants, *Ficus carica*, *Euphorbia tirucalli*, *and Euphorbia dendroides*, evaluated on the basis of their ability to scavenge DPPH, IC_{50} values were estimated, 13.6, 7.0, and 6.0 μg/ml, respectively [29]. Moreover, the latex of *Pergularia tomentosa* can be considered as a promising source for several bioactive compound antioxidants. Other than its phenolic content, antioxidant ability of the latex extract might be related to the type of the solvent used for extraction and interaction between sample components [51, 52]. Also, phenolic content of the latex sample can have a different susceptibility toward free radicals of the selected solvent, they present the same antioxidant ability [29], and each phenolic compound contained in the latex extract can have different interactions during antioxidant assay [53].

4. Conclusion

The present study for the first time provides screening and identification of laticifers in vegetative organs of *Pergularia tomentosa*, including stem, leaf, and petiole. Anatomical studies revealed that the species is a nonarticulated laticifer type. By its functional group variety, latex composition was estimated as a complex of proteins, glucides, sterols, enzymes, lipids, etc. Rubber mainly contains *cis*-1-4-isoprene monomer as a major component.

From the above results, it can be concluded that the latex extract is a promising natural source of total phenolics and flavonoids. The low IC_{50} confirmed a higher ability to scavenge DPPH, and therefore, latex has an interesting antioxidant activity. The correlation between secondary metabolites and antioxidant activity of *Pergularia tomentosa* latex encourages researchers to prove the same therapeutic properties revealed with roots, leaves, stems, and fruit extracts mainly including antifungal, antimicrobial, antimolluscicidal, antiapoptic, and anti-inflammatory activities. The latex is often searched for industry application as protective gloves, shoes, and hoses and for medical equipment like gloves, tubing, and straps. The described properties led to more study and investigate the composition in order to its valorisation as a natural and an eco-friendly extract with a low cost.

Data Availability

The data is available upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Enzymes Inhibition and Antioxidant Potential of Medicinal Plants Growing in Oman

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The recent study was designed to explore *Dodonaea viscosa*, *Juniperus excelsa*, *Helianthemum lippii*, and *Euryops pinifolius* using methanolic (MeOH) extract. Their subfractions were examined against urease, carbonic anhydrase II (CA-II), α-glucosidase enzymes, and free radicals scavenging significance based on local practices via standard methods. Significance potential against the urease enzyme was presented by ethyl acetate fraction (EtOAc) of *D. viscosa* with ($IC_{50} = 125 \pm 1.75$ μg/mL), whereas the *H. lippii* ($IC_{50} = 146 \pm 1.39$ μg/mL) in the EtOAc was found efficient to scavenge the free radicals. Besides, that appreciable capacity was observed by the *J. excelsa*, *D. viscosa*, *J. excelsa*, and *E. pinifolius* as compared to the standard acarbose ($IC_{50} = 377.24 \pm 1.14$ μg/mL). Maximum significance was noticed in methanolic (MeOH) extract of *J. excelsa* and presented carbonic anhydrase CA-II ($IC_{50} = 5.1 \pm 0.20$ μg/mL) inhibition as compared to the standard (acetazolamide). We are reporting, for the first time, the CA-II inhibition of all the selected medicinal plants and α-glucosidase, urease, and antioxidant activities of the *E. pinifolius*. Thus, further screening is needed to isolate the promising bioactive ingredients which act as an alternative remedy to scavenge the free radicals, antiulcer, and act as a potential source to develop new antidiabetic drugs for controlling postprandial blood sugar as well as carbonic anhydrase inhibitors.

1. Introduction

Phytomedicines are made up of medicinal plants and their chemical ingredients and have a key therapeutic role in various health-related complications, for instance, gastrointestinal infections, free radicals scavenging, and antidiabetic properties [1]. In this context, plant extracts are made up of a variety of chemical elements and are well-known for their wide range of clinical applications. They are derived from plants using both traditional and other modern approaches [2].

Urease enzymes play a leading role to catalyze the hydrolysis of urea, thus gaining substantial attention regarding human health and their life qualities [3–5]. It maintains optimum pH and treats the NH_3 to balance its medium level due to which they own an incredible medical position [6, 7]. It is the main public health matter related to the bacterium *H. pylori*, which can endure in an acidic environment of the stomach having pH 2 range [6, 8]. The high prevalence of *H. pylori* in the human population indicates that such microbes have developed mechanisms for resistance against host defenses [8]. Marketed available urease drugs (phosphorodiamidate, hydroxamic acid derivatives, and imidazoles) are much more toxic with less efficacy rate and thus influenced by their limited clinical use [9, 10]. Thus, the quest for innovative urease inhibitors with enhanced stability and minimal toxicity is needed to improve the life quality of human beings and animals. Therefore, plant-based drugs are the alternative basis for having the ability to overcome the mentioned complications.

Diabetes mellitus (DM) is a common metabolic illness that has become a serious worldwide health issue. When DM is left untreated, it can harm the nerves, eyes, kidneys, and other organs. Increased urination, impaired vision,

weariness, hunger, and thirst are among the symptoms of T2DM [11]. Controlling postprandial hyperglycemia by delaying carbohydrate digestion and absorption is one of the therapy options for T2DM. α-Glucosidase (EC 3.2.1.20) is an enzyme found on the small intestine's brush edge. *Inhibition of the α-glucosidase can limit the digestion of carbohydrates resulting in declined postprandial blood sugar levels* [12]. As a result, α-glucosidase inhibitors (AGIs) can be used as first-line therapy for T2DM [13–15].

Natural antioxidants can improve food quality including color, taste, flavor, and stability and also act as standardized nutrients (nutraceuticals) to end up the attack of free radicals in biological systems [16, 17], that might deliver additional health benefits to consumers [18, 19] and reduce the risk of disorders caused by free radicals [20]. Recently, considerable attention focused on the use of natural antioxidants to defend the human body against brain tissues and neurological disorders associated with free radical damage [20, 21]. This research is focused on searching for new sources of natural antioxidants and urease inhibitors that can be used directly or in combination with other official drugs as a lead compound for drug discovery.

Carbonic anhydrase (CA) is a metalloenzyme that contains zinc and is mainly used to catalyze CO_2 hydration into bicarbonate and hydrogen ions [22]. The CA inhibitors control the enzymatic actions and prevent bicarbonate reabsorption which leads to numerous adverse effects such as potassium and bicarbonate retention in the human urine and decreased sodium absorption as a diuretic [23]. The intake of synthetic drugs for longer use to release this complication might be harmful. Therefore, searching for plant-based alternative remedies can be useful to cope with these disorders [24].

Juniperus excelsa M. Bieb (JE, Cupressaceae) is used mainly for lowering blood pressure [25], jaundice, bronchitis, tuberculosis, common cold [26], diabetes, stomachache, grazing, and wood harvesting [27]. Literature surveys documented its positive effects in treating colds, cough, dysmenorrheal, persuading menses, and expelling fetuses [28, 29]. *Dodonaea viscosa* Linn (DV, Sapindaceae) was reported to possess antiviral, anti-inflammatory, laxative, spasmolytic, antimicrobial, hypotensive agents [30], smooth muscle relaxant, anesthetic, throat infection, malaria, and antiulcerogenic [31, 32]. Traditionally, it is used to treat many illnesses like malaria, cold, aches, fever, toothaches, rheumatism, headaches, ulcers, diarrhea, dysmenorrhea, irregular menstruation, and constipation [33].

Helianthemum lippii (HL) belongs to the genus *Helianthemum*, which is a widely distributed and most taxonomically complex genus of the Family Cistaceae. Alsabri et al. [34] reported anti-inflammatory and analgesic activity of *H. lippii* against carrageenan-induced paw edema and hotplate-induced pain in rats. Previous studies showed that the plant is a rich source of polyphenols and flavonoids [35, 36] with antioxidant, antiulcer, and antimicrobial [37], as well as cytotoxic [38]. *Euryops pinifolius* A. Rich belongs to the genus *Euryops* (family: Asteraceae) [39], mostly cultivated in southern Africa, with a few species in other parts of Africa and on the Arabian Peninsula [28]. The local uses of *E. pinifolius* are least known, however, in some places of the Arabian Peninsula (Yemen, Oman, and Saudi Arabia), are used to wound healing [40].

To devise innovative plant-derived drugs, four important medicinal plants were collected from Al Jabal Al Akhdar (Northern Oman) and evaluated for antioxidant and enzyme inhibition activities. To the best knowledge, this is the first report on the enzyme inhibition study of these plants. In addition, we are also reporting the antioxidant activity of *H. lippii* and *E. pinifolius* for the first time.

2. Materials and Methods

2.1. Collection and Identification of the Medicinal Plants. Aerial parts of the plant species ,viz., *J. excelsa* (3.5 Kg), *H. lippii* (3.5 Kg), *E. pinifolius* (4.0 Kg), and *D. viscose* (5.5 Kg), were collected from Al Jabal Al Akhdar, Oman, identified by a plant taxonomist (Dr. Syed Abdullah Gillani) at the Department of Biological Sciences and Chemistry, University of Nizwa, Oman. After documentation, voucher specimens (HL-01/2012, EP-02/2012, DV-03/2012, and JE-04/2012) were kept at the herbarium of Natural and Medical Sciences Research Center, University of Nizwa, Oman, for further processing.

2.2. Extraction and Fractionation. The whole aerial parts of the *Dodonaea viscosa* were dried, chopped, and soaked in methanol at room temperature for 15 days three times as reported earlier by Shah et al. [16]. Evaporation of the MeOH in vacuo at 45°C yielded a crude methanol extract, which after suspension in water was successively fractionated into *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), butanol (n-BuOH), and aqueous (H_2O) (Figure 1). The same procedure was used for the extraction and fractionation of other medicinal plants. The details of quantity of crude extracts and the different fractions of the selected plants are given in Table 1. The crude extracts and their fractions were subjected to biological screening to determine their potential effect.

2.3. Antioxidant Activity. The antioxidant bioassay of the crude extracts and subfractions of the selected plants was evaluated using α, α-diphenyl-β-picrylhydrazyl (DPPH) assay [41]. About 100 μL of methanol was mixed with 150 μL of DPPH solution as a negative control. For the sample, about 150 μL of DPPH was added with 100 μL of three concentrations of extract (100, 500, and 1000 μg/mL). The absorbance was taken at 517 nm by spectrophotometer. All the results were compared to a control containing 50 μL of methanol. The positive control used was ascorbic acid (Table 1). Each test was repeated three times, and % inhibition was calculated as:

$$\text{Percent (\%) inhibition of DPPH activity} : A_c - A_s/A_c \times 100,$$
$$A_c = \text{Absorbance of control (ascorbic acid)};$$
$$A_s = \text{Absorbance of sample.} \quad (1)$$

2.4. Urease Enzyme Inhibition. Urease enzyme inhibition

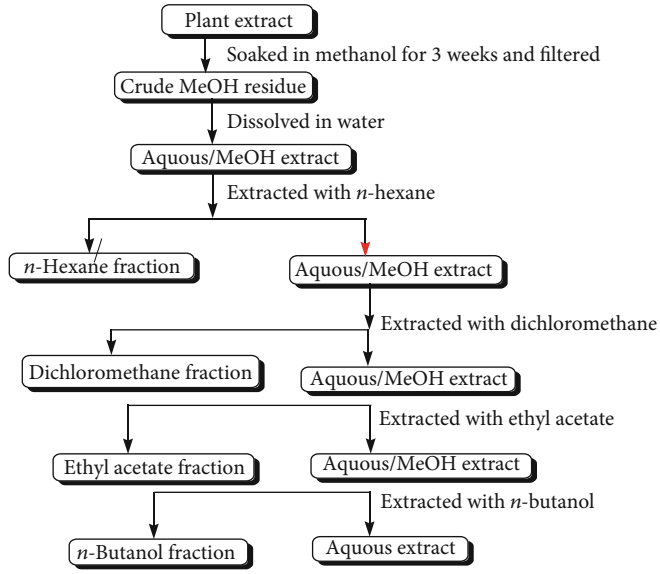

Figure 1: General fractionation scheme for the solvent-solvent extraction of medicinal plants.

Table 1: Crude extracts and fractions of the selected medicinal plants.

Plant's name	Crude (g)	Fractions (g)				
	MeOH	*n*-hexane	CH_2Cl_2	EtOAc	n-BuOH	H_2O
D. viscosa	135	16	45	28	14	28
J. excelsa	115	11	23	20	24	35
H. lippii	95	12	18	25	12	28
E. pinifolius	90	13	19	21	18	17

assay was performed using available literature [42, 43]. About 25 μL solution of Jack bean urease was mixed with 50 μL urea dissolved in phosphate buffer (pH 8.20) with 20 μL of three concentrations (100, 500, and 1000 μg/mL) of different extract fractions and then incubated at 37°C for 15 min. Then, 50 μL of solution B (phenol reagent (1% w/w phenol) own expect 3 mg in 30 mL + 0.005% w/v sodium nitroprusside) and 70 μL of solution A (alkali reagent (0.5% w/v NaOH +1% active chloride NaOCl)) were added and then incubated again for 15 min. Thiourea was used as a positive (standard) control, while methanol was used as a negative control (Table 2). The absorbance was recorded at 630 nm with a total volume of 215 μL.

$$\mathrm{OD}_{\text{test compound}} = \text{Optical Density of control (thiourea)};$$

$$\mathrm{OD}_{\text{control}} = \text{Optical Density of the sample.} \quad (2)$$

2.5. α-Glucosidase Assay. All the twenty-four samples of crude extract and subfractions were evaluated in vitro against α-glucosidase enzyme (E.C.3.2.1.20) as described earlier by Shah et al. [11], by using (50 mM) phosphate buffer of pH (6.8). The enzyme was properly dissolved in the phosphate buffer; 1 U/2 mL, 20 μL/well of the enzyme, and 135 μL/well phosphate buffer was used as reaction buffer, 20 μL/well of the tested samples were solubilized in DMSO (0.5 μg/mL), in 96-wells plates incubated for 15 min at 37 °C. After the incubation period, the substrate para nitro phenyl-D-glucopyranoside was added at a concentration of 0.7 mM, and the change in absorbance was measured at 400 nm for 30 minutes. The positive control used was acarbose, and DMSO was used as negative control.

$$\mathrm{OD}_{\text{test compound}} = \text{Optical Density of control (acarbose)};$$

$$\mathrm{OD}_{\text{control}} = \text{Optical Density of the sample.} \quad (3)$$

2.6. Carbonic Anhydrase II Inhibition Assay. The total reaction mixture comprised 20 μL (0.5 mmol/well) of test compounds (10% DMSO in total), and then add HEPES–Tris buffer 140 μL (20 mmol, pH =7.4), 20 μL of purified bovine erythrocyte CA-II (1 mg/mL, 0.1 units/well) prepared in buffer, and finally substrate 4-nitrophenyl acetate (4-NPA, 0.7 mmol) 20 μL to attain final volume 200 μL/well [44, 45]. An enzyme (EC 4.2.1.1, Sigma-Aldrich, St. Louis, MO, USA) along with the tested compounds was incubated for 15 min in a 96-well plate. Then, the reaction was started with the addition of 20 μL of the substrate (4-nitrophenyl acetate) and continuously monitored the rate (*velocities*) of product formation for 30 min with the intervals of 1 min, at 25°C by using a microplate reader (Bio-Rad, Molecular Devices, CA, USA). Acetazolamide and DMSO were used as positive and negative controls, respectively.

$$\mathrm{OD}_{\text{test compound}} = \text{Optical Density of control (acetazolamide)};$$

$$\mathrm{OD}_{\text{control}} = \text{Optical Density of the sample.} \quad (4)$$

2.7. Statistical Analysis. The SoftMax Pro package and Excel were utilized.

Table 2: Antioxidant activity (%) of four Omani medicinal plants.

Plant Species	Fractions	Antioxidant activity IC_{50} ± S.E.M (μg/mL)
J. excelsa	n-Hexane	Nd
	DCM	Nd
	EtOAc	402 ± 2.15
	n-BuOH	428 ± 1.54
	MeOH	Nd
	Aqueous	Nd
E. pinifolius	n-Hexane	Nd
	DCM	Nd
	EtOAc	378 ± 1.56
	n-BuOH	904 ± 2.68
	MeOH	Nd
	Aqueous	Nd
H. lippii	n-Hexane	788 ± 2.68
	DCM	658 ± 1.54
	EtOAc	146 ± 1.39
	n-BuOH	561 ± 1.34
	MeOH	368 ± 2.18
	Aqueous	460 ± 1.21
D. viscosa	n-Hexane	Nd
	DCM	Nd
	EtOAc	386 ± 1.65
	n-BuOH	467 ± 1.84
	MeOH	914.±2.61
	Aqueous	Nd
Ascorbic acid		6.25 ± 0.56

Ascorbic acid∗= μM; DCM: dichloromethane; EtOAc: ethyl acetate; BuOH: n-butanol; MeOH: methanol; SEM: standard error mean; Nd: not determined (Conc. = 1 mg/mL).

The given formula below was used to calculate percent inhibition.

$$\%\text{Inhibition} = 100 - \left(\frac{\text{O.D}_{\text{test compound}}}{\text{O.D}_{\text{control}}}\right) \times 100. \quad (5)$$

EZ-FIT (Perrella Scientific, Inc., USA) was used for IC_{50} calculations of all tested samples. To overcome on the expected errors, all experiments were performed in triplicate, and variations in the results are reported in standard error of mean values (SEM).

$$\text{SE} = \frac{\sigma}{\sqrt{n}}\#. \quad (6)$$

3. Result and Discussion

Herbs have been used as a source of medicine since the dawn of human civilization, and they continue to play an important role in clinical use and quality control for a variety of health issues [46].

3.1. Antioxidant Capability. The antioxidant significance of the selected plants is determined to evaluate the free radical scavenger capacities of the selected plant species utilizing ascorbic acid as a standard inhibitor as presented in Table 2. In recent years, increasing attention has been paid to antioxidant compounds (flavones, anthocyanin, flavonoids, catechin, isoflavones, and other phenolics) derived from plants due to their valuable role in reducing various disorders such as immune system, brain dysfunction, heart disease, decline, cardiovascular disease, aging, and cancer [47]. The free radicals produced due to human metabolism affect the cellular membrane to overcome these complications [47]. The investigation reveals that among the screened four plant species, *H. lippii* fractions offered a significant ability to scavenge the free radicals and act as an antioxidant agent. The EtOAc fraction of *H. lippii* exhibited the highest potential to act as an antioxidant agent with IC_{50} of 146 ± 1.39 μg/mL followed by the MeOH (IC_{50} = 368 ± 2.18 μg/mL) and aqueous extract (460 ± 1.21 μg/mL), respectively. This significance is attributed due to the presence of an affluent basis of polyphenolic constituents as documented by Benabdelaziz et al. [48]. Alali et al. [49] investigated *H. lippii* from Jordan and reported methanol (IC_{50} = 176.1 μmol TE g^{-1}dry weight) and aqueous (IC_{50} = 176.1 and 274.2 μmol TE g^{-1}dry weight) extracts in comparison to the standard via ABTS assay. However, in the current study, the geographical location, collection, habitat, harvesting season, screening approach, and standard used are different from earlier studies. Therefore, our finding reveals that the *H. lippii* has a significant ability to neutralize the free radicals. Belyagoubi et al. [36] collected *H. lippii* from Algeria as a plant habitat influenced the quality and quantity of bioactive compounds responsible for promising pharmacological potentials [50]. However, moderate capability was observed in the *n*-hexane and CH_2Cl_2 fractions (Table 2). In the case of *E. pinifolius*, the EtOAc fraction displayed significance inhibition (IC_{50} = 378 ± 1.56 μg/mL) followed by the n-BuOH fraction (IC_{50} = 904 ± 2.64 μg/mL). Moreover, the EtOAc fraction of *D. viscosa* also produced promising findings with (IC_{50} = 386 ± 1.65 μg/mL) ensued by the n-BuOH (IC_{50} = 467 ± 1.84 μg/mL), while normal activity was examined by the MeOH extract (Table 2). The current findings consented to the data stated for some Yemeni traditional medicinal plants by Mothana et al. [51] that *D. viscosa* was one of the most active plants that showed promising antioxidant activity. In addition to that, the current outcome also supports the results reported by Singh et al. [52] for *Rhus aucheri* as the understudy plant was collected from Oman. Recently, Muhammad et al. [53] isolated some flavonoids from the EtOAc fraction of *D. viscosa* showed higher antioxidant activity further stringent our findings. It was also observed that the free radicals scavenging significance of

some medicinal plants from Iran was dissimilar from our recorded data as described by Boroomand et al. [54] due to the variation of their habitat, climatic, topographic, and edaphic factors influenced the content and quality of the metabolites accountable to act as an antioxidant agent. The data obtained from these *in vitro* models demonstrated the strong antioxidant potential of EtOAc and n-BuOH fractions of the selected medicinal plants, which might be a concern with its high medicinal and pharmaceutical use as a functional food in the treatment of different diseases.

3.2. Antiulcer Potential. The urease enzyme inhibitory activity of crude extracts/fractions of the plants was determined using a concentration of 1.0 mg/mL. Ethyl acetate fraction of *D. viscosa* exhibited significantly promising urease inhibition ($IC_{50} = 125 \pm 1.75$ μg/mL), followed by *n*-hexane ($IC_{50} = {}_{1}42 \pm 2.00$ μ/mL) and *n*-BuOH ($IC_{50} = 410 \pm 2.50$ μg/mL) fractions. The data of crude extract and fractions of *J. excelsa* revealed that only the EtOAc fraction exhibited significant inhibition ($IC_{50} = {}_{1}73 \pm 2.50$ μg/mL) as compared to other fractions. In the case of *H. lippii*, the EtOAc fraction showed significantly strong inhibition ($IC_{50} = 257 \pm 1.25$ μg/mL), followed by n-BuOH ($IC_{50} = 435 \pm 2.75$ μg/mL), while MeOH and aqueous fractions of the same plant did not show activity (Table 3). The EtOAc fraction of E. pinifolius attributed promising inhibition (IC50 = 390 ± 2.50 μg/mL), followed by the n-BuOH (IC50 = 430 ± 2.25 μg/mL), while other fractions did not show urease inhibition (Table 3).

These findings provide crucial information about the biologically active constituents present in medicinal plants truly responsible for the inhibition of the urease enzyme. Thus, our finding matched with the data reported by Rauf et al. [55] for *Diospyros lotus* roots and in favor of the outcomes presented by Maherina et al. [56] as the use of the same approach to determine the urease significance in the medicinal plants. Moreover, our current findings do not agree with the data reported by Tahseen et al. [57] due to their variability in their habitat. In the future, bioassay-guided isolation of these secondary metabolites might be exciting and interesting to know the chemical constituents responsible for inhibition and to understand their basic mechanism against these enzymes.

3.3. Antidiabetic Significance. Crude extract and subfractions of the four plant species (*D. viscosa*, *J. excelsa*, *H. lippii*, and *E. pinifolius*) were tested to analyze their antidiabetic potential by targeting the key carbohydrate digestive enzyme α-glucosidase. Furthermore, the aqueous and n-hexane fractions of *J. excelsa* showed below 50% inhibitory activity and were found to be inactive. While other samples displayed several folds of potent inhibitory potential in the range of 1.30-20.75 μg/mL, compared with acarbose ($IC_{50} = 377.24 \pm 1.14$ μg/mL). Moreover, the n-BuOH and n-hexane fractions of *D. viscosa* exhibited significant inhibitory activity with IC_{50} (1.30 ± 0.05 and 2.04 ± 0.06 μg/mL), respectively. Thus, our data is supported by the literature explained by Assefa et al. [58] and VVM et al. [59]. It might be due to the presence of active chemical ingredients having the ability to cure diabetes. On the other hand, the EtOAc, DCM, aqueous, MeOH, n-hexane, and n-BuOH fractions of *J. excelsa* exhibited potent α-glucosidase inhibitory potential with IC_{50} (1.31 ± 0.02, 3.65 ± 0.12, 2.48 ± 0.13, 3.11 ± 0.14, 2.78 ± 0.11, and 2.05 ± 0.08 μg/mL), respectively. Thus, our current screening consented to the findings of Bhatia et al. [60], which depicted a little variation in outcomes reported by Sancheti et al. [61] and Gok et al. [62]. Due to differences in the chemical ingredients influenced by environmental factors and the solvents and methods used in our studies. in addition to that, the *E. pinifolius* offered variations in the anti-α-glucosidase potential. For instance, the MeOH extract was found to be the most potent and displayed $IC_{50} = 2.86 \pm 0.03$ μg/mL. A slight decrease in the α-glucosidase inhibitory activity was observed in the other fraction samples, such as EtOAc having $IC_{50} = 7.85 \pm 0.16$ μg/mL. Likewise, a slightly further decline in the antidiabetic capability was observed, in the DCM and n-BuOH fractions depicted ($IC_{50} = 16.72 \pm 0.15$ μg/mL and 22.12 ± 0.15 μg/mL), respectively. So, these outcomes also reflect that our data agrees with the findings of Khatib et al. [63].

Furthermore, our investigation exhibited a little variation as compared to the data stated by Ibrahim et al. [64] as mentioned previously that the habitat variation can also be led to variability among the chemical ingredients among the different and same plant species. Interestingly all the fractions of *H. lippii* displayed several fold potent inhibitory activities with almost the same potency comprises of EtOAc, DCM, aqueous, n-hexane, and BuOH with IC_{50} of 5.12 ± 0.18, 5.73 ± 0.21, 5.47 ± 0.13, 5.73 ± 0.21, and 6.45 ± 0.11 μg/mL, respectively, as compared to MeOH extract ($IC_{50} = 10.48 \pm 0.26$ μg/mL). Our current study consented to the literature described by Zarei et al. [65]. However, our current result does not agree with the findings of Rungprom et al. [66]. The similarity of the antidiabetic significance presented by the medicinal plants might be due to the presence of the phenols and flavonoids. As we know in the current era plant extractions are becoming increasingly popular in medicinal therapies, and they are an alternative and valuable herbal medicinal medicine because of their broad usage and lower adverse effects, the current results insight into the crucial therapeutic importance of the *D. viscosa*, *J. excelsa*, *H. lippii,* and *E. pinifolius* in their crude and subfractions. Hence, these promising findings might be used as a therapeutic approach for the management of type 2 diabetes (*T2DM*) displayed in Table 3.

3.4. Carbonic Anhydrase II Significance. The selected plants are profiled for their carbonic anhydrase activity as shown in Table 3. Among the subfractions, EtOAc fraction of the *D. viscosa* presented significanse activity with IC50 of 27.5 ± 3.12 ± µg/mL trail by the MeOH extract IC50 = 50.4 ± 2.03 µg/mL, while other subfractions were found inactive. The *D. viscosa* contains phenols and polyphenols having the capability to act as carbonic anhydrase inhibitors; thus, our findings agree with the data stated by Karioti et al. [67]. The current finding presented that our data do not match with the data recorded by Rudenko et al. [68] because environmental stress influences the quality and quantity of

Table 3: α-Glucosidase CA-II and urease activities of the selected medicinal plants.

Sample code	Fractions	α-Glucosidase $IC_{50} \pm SEM$ (μg/mL)	Urease $IC_{50} \pm SEM$ (μg/mL)	CA-II $IC_{50} \pm SEM$ (μg/mL)
D. viscosa	EtOAc	5.99 ± 0.20	125 ± 1.75	27.5 ± 3.12
	DCM	10.86 ± 0.17	416 ± 1.50	NA
	Aqueous	5.34 ± 0.14	NA	NA
	MeOH	3.18 ± 0.10	NA	50.4 ± 2.03
	n-Hexane	2.04 ± 0.06	142 ± 2.00	NA
	n-BuOH	1.30 ± 0.05	410 ± 2.50	NA
J. excelsa	EtOAc	1.31 ± 0.02	173 ± 2.50	38.4 ± 2.52
	DCM	3.65 ± 0.12	NA	46.3 ± 1.95
	Aqueous	2.48 ± 0.13	NA	51.3 ± 1.35
	MeOH	3.11 ± 0.14	NA	5.1 ± 0.20
	n-Hexane	2.78 ± 0.11	NA	NA
	n-BuOH	2.05 ± 0.08	NA	66.8 ± 3.19
E. pinifolius	EtOAc	7.85 ± 0.16	390 ± 2.50	47.0 ± 3.99
	DCM	16.72 ± 0.15	NA	45.4 ± 2.08
	Aqueous	N/A	NA	98.2 ± 4.84
	MeOH	2.86 ± 0.03	NA	NA
	n-Hexane	N/A	NA	NA
	n-BuOH	22.12 ± 0.15	430 ± 2.25	41.5 ± 0.82
H. lippii	EtOAc	5.12 ± 0.18	257 ± 1.25	35.6 ± 1.32
	DCM	5.73 ± 0.21	NA	NA
	Aqueous	5.47 ± 0.13	NA	9.9 ± 0.35
	MeOH	10.48 ± 0.26	NA	NA
	n-Hexane	5.73 ± 0.21	NA	18.8 ± 3.13
	n-BuOH	6.45 ± 0.11	435 ± 2.75	59.4 ± 2.33
Acarbose		608.21 ± 1.74		
Thiourea			1.58 ± 0.95	
Acetazolamide				4.04 ± 1.63

DCM: dichloromethane; EtOAc: ethyl acetate; BuOH: butanol; MeOH: methanol; N/A (nonactive); concentration =0.5 mg/mL; SEM: standard error mean; ND: not determined.

bioactive ingredients responsible for numerous biological activities. Furthermore, the MeOH extract of *J. excelsa* followed by the EtOAc fraction of *J. excelsa* displayed significant potential an $IC_{50} = 5.1 \pm 0.20$ and $IC_{50} = 38.4 \pm 2.52$ μg/mL significance in comparison to other tested fractions. The n-BuOH fraction of *E. pinifolius* presented appreciable significance having $IC_{50} = 41.5 \pm 0.82$ μg/mL, followed by the DCM fraction with $IC_{50} = 45.4 \pm 2.08$, and the EtOAc fraction exhibited an $IC_{50} = 47.0 \pm 3.99$ μg/mL potential, whereas the MeOH and n-hexane extract were found inactive for carbonic anhydrase activity. The *H. lippii* fractions also displayed appreciable potential except for the DCM and MeOH extracts, while aqueous extract was most potent presented $IC_{50} = 9.9 \pm 0.35$ μg/mL, proceeded by the n-hexane with $IC_{50} = 18.8 \pm 3.13$ μg/mL and $IC_{50} = 35.6 \pm 1.32$ μg/mL in comparison to the standard acetazolamide having an $IC_{50} = 4.04 \pm 1.63$ μg/mL. The current results also match up with the outcomes of Aydin et al. [69] for *Satureja cuneifolia* and dodoneine by Carreyre et al. [70] which was found effective for the carbonic anhydrase activity. However, the study reported for the bioactive ingredient dodoneine by Carreyre et al. [70] was significant as compared to our current findings because might be bioactive compounds are responsible compounds as compared to our selected plants and tested fractions

4. Conclusion

In conclusion, the selected medicinal plants (D. viscosa, J. excelsa, H. lippii, and E. pinifolius) possess significance anti-ulcer, antioxidant, antidiabetic and carbonic anhydrase-II inhibition and can be considered as essential source of bioactive ingredients. Additionally, up to now, no such scientific data were reported for the enzyme inhibition potential, whereas the two plant species were reported for the first time in a recent study. Therefore, it could be

contended that the medicinal plants have significant potential to serve as an antioxidant and own enzyme inhibitory attributes. However, further investigations are considered necessary for the isolation and identification of the chemical ingredients accountable for the antioxidant and enzymatic significance of the selected plants.

Data Availability

All datasets on which the conclusion of the manuscript relies are presented in the paper.

Conflicts of Interest

The authors declare that they have no conflict of interest regarding this manuscript.

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Retraction

Retracted: Targeting Streptomyces-Derived Streptenol Derivatives against Gynecological Cancer Target PIK3CA: An In Silico Approach

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] H. Jemmy Christy, S. Vasudevan, S. Sudha et al., "Targeting Streptomyces-Derived Streptenol Derivatives against Gynecological Cancer Target PIK3CA: An In Silico Approach," *BioMed Research International*, vol. 2022, Article ID 6600403, 15 pages, 2022.

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Research Article

Targeting Streptomyces-Derived Streptenol Derivatives against Gynecological Cancer Target PIK3CA: An In Silico Approach

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Streptomyces is amongst the most amenable genera for biotechnological applications, and it is extensively used as a scaffold for drug development. One of the most effective therapeutic applications in the treatment of cancer is targeted therapy. Small molecule therapy is one of them, and it has gotten a lot of attention recently. Streptomyces derived compounds namely streptenols A, C, and F–I and streptazolin were subjected for ADMET property assessment. Our computational studies based on molecular docking effectively displayed the synergistic effect of streptomyces-derived compounds on the gynecological cancer target PIK3CA. These compounds were observed with the highest docking scores as well as promising intermolecular interaction stability throughout the molecular dynamic simulation. Molecular docking and molecular dynamic modeling techniques were utilized to investigate the binding mode stability of drugs using a pharmacophore scaffold, as well as physicochemical and pharmacokinetic aspects linked to alpelisib. With a root mean square fluctuation of the protein backbone of less than 0.7 nm, they demonstrated a steady binding mode in the target binding pocket. They have also prompted hydrogen bonding throughout the simulations, implying that the chemicals have firmly occupied the active site. A comprehensive study showed that streptenol D, streptenol E, streptenol C, streptenol G, streptenol F, and streptenol B can be considered as lead compounds for PIK3CA-based inhibitor design. To warrant the treatment efficacy against cancer, comprehensive computational research based on proposed chemicals must be assessed through in vitro studies.

1. Introduction

Recent clinical studies have revealed that worldwide cancer incidence and associated mortality rates are raising an alarm in the field of healthcare and medicine [1]. Chemotherapy and radiotherapy are the conventional therapeutic strategies for treating cancer, but in many situations, a focused strategy is lacking, leaving patients exposed to drug resistance. Novel concepts have emerged in recent years to improve existing therapy options for malignancies with poor survival results. [2]. In recent times, the collecting of genomic, transcriptomic, and proteomic data for the structural and functional organization of regulatory processes in a cell has enabled the detection of disease-related traits and the selection of highly promising targets for prospective drug development [3, 4]. Anticancer therapeutics that target apoptosis inhibitor proteins and cancer cell indicators are currently being employed as criterion. Anticancer medicines that are suitable for cancer targets are commonly

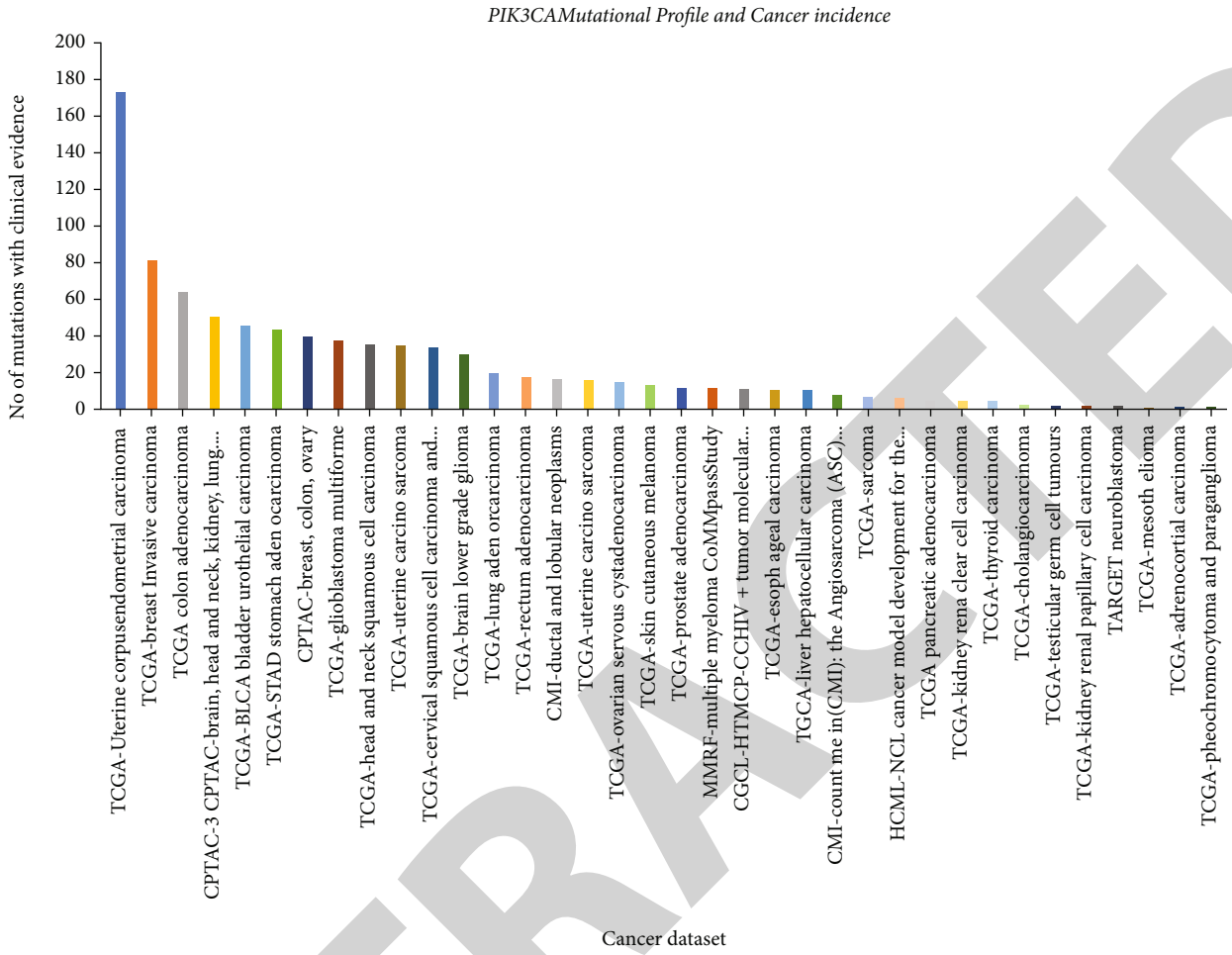

Figure 1: PIK3CA mutational profile and their prevalence among various cancers.

discovered through in silico investigations [5]. Actinomycetes produce many structurally varied secondary metabolites, many of which have pharmaceutically significant biological activities [6]. Researchers have concentrated their efforts on investigating marine actinomycetes as a source for the search for novel secondary metabolites since these organisms have the most genetic and metabolic diversity [7–9]. Among Actinobacteria phylum, Streptomyces is the most prevalent and productive drug-producing genus [10, 11]. The species can be found in a wide range of environments, including arctic regions, deserts, mountains, insects, plant stems, and marine sediments. This species can be found in a wide range of environments, including arctic regions, deserts, mountains, insects, plant stems, and marine sediments [12]. Streptomyces species have shown a phenomenal potential to deliver secondary metabolites, many of which are used to treat human illnesses. Differential expression studies on a variety of malignancies, as well as allied in silico methodologies, are promising strategies for identifying treatment targets [13]. Polyketides, peptides, and polyketide-peptide hybrids were secondary metabolites of Streptomyces species that have been demonstrated to have therapeutic potential against cancer cells [14]. The cytotoxic activity of streptenol derivatives A, C, F, G, H, and I against cancer cell lines were assessed in the earlier studies. Based on the literature survey, we defined our systematic study to explore the structural scaffolding of the proposed streptenol derivatives. Streptomyces misionensis-BAT-10-03-123, marine-derived bacterium-based stretenol derivatives were tested in cell lines and revealed anticancer activity [15] PI3K elevated activity is often associated with various human cancers and anticancer drug resistance [16, 17]. Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) complexed with alpelisib and the receptor-based pharmacophore model were employed for further virtual screening of the streptenol derivatives as inhibitors for PIK3CA. Streptenol derivative binding mode towards the promising cancer target PIK3CA was studied in the present study. PIK3CA heterodimer catalytic subunits are tightly controlled by the associated regulatory subunits. Although the same p85 regulatory subunits associate with all class IA PI3Ks, the functional outcome depends on pocket specificity of catalytic subunit. In addition, physicochemical and drug-likeness property assessment of compounds and subsequent intermolecular studies to screen their therapeutic efficacy was done using the ligand fit algorithms.

2. Material and Methods

2.1. PIK3CA as Promising Player of Cancer Cell Signaling Pathway. The Cancer Genome Atlas (TCGA) project is a

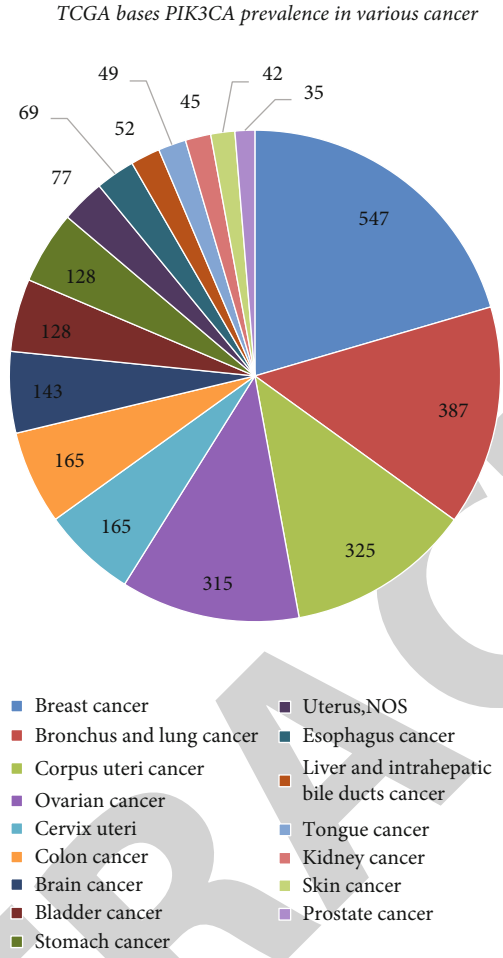

Figure 2: TCGA project-based PIK3CA prevalence among various cancers.

Table 1: Differentially expressed PIK3CA retrieved from GEO dataset.

GEO accession number	Platform	Control	Sample
GSE6791 (cervical cancers and head/neck)	GPL570	56	76
GSE39001(cervical cancer)	GPL201	12	12
GSE22035 (breast cancer)	GPL570	15	15

collective effort to compile data from a variety of cancer types and make it accessible to researchers across the world. TCGA enabled the comprehensive and coordinated process to accelerate our understanding about the PIK3CA and their molecular level involvement in cancer induction [18]. PIK3CA mutation prevalence was also assessed among the various cancer types and was also recorded. Differential expression analysis was carried out using the GEO2R platform to screen the PIK3CA upregulation among the top listed cancer categories like breast, cervical, and ovarian cancers [19].

2.2. Metastasis Profile of PIK3CA among the Cancer. The principal reason for cancer-related death is metastasis, which seems to be the spread of cancer from one organ to another without being directly associated with it. When considering effective treatment techniques for cancer patients, it is vital to evaluate the prevalence of metastasis. At the transcriptional level, a variety of techniques have been used to discover and define genes involved in cancer metastasis. By pooling several data sources, it is crucial to characterize the complex molecular mechanism. Our comprehension of cancer metastasis requires an integrated assessment of multidimensional transcriptome data. CMDB was created to bring these data together and make studying gene expression deregulation in metastasis easier [20].

2.3. PIK3CA Functional Network Assessment Using Gene Multiple Association Network Integration Algorithm. GeneMANIA uses a repository of organism-specific weighted networks to build the consistent set, generate hypotheses

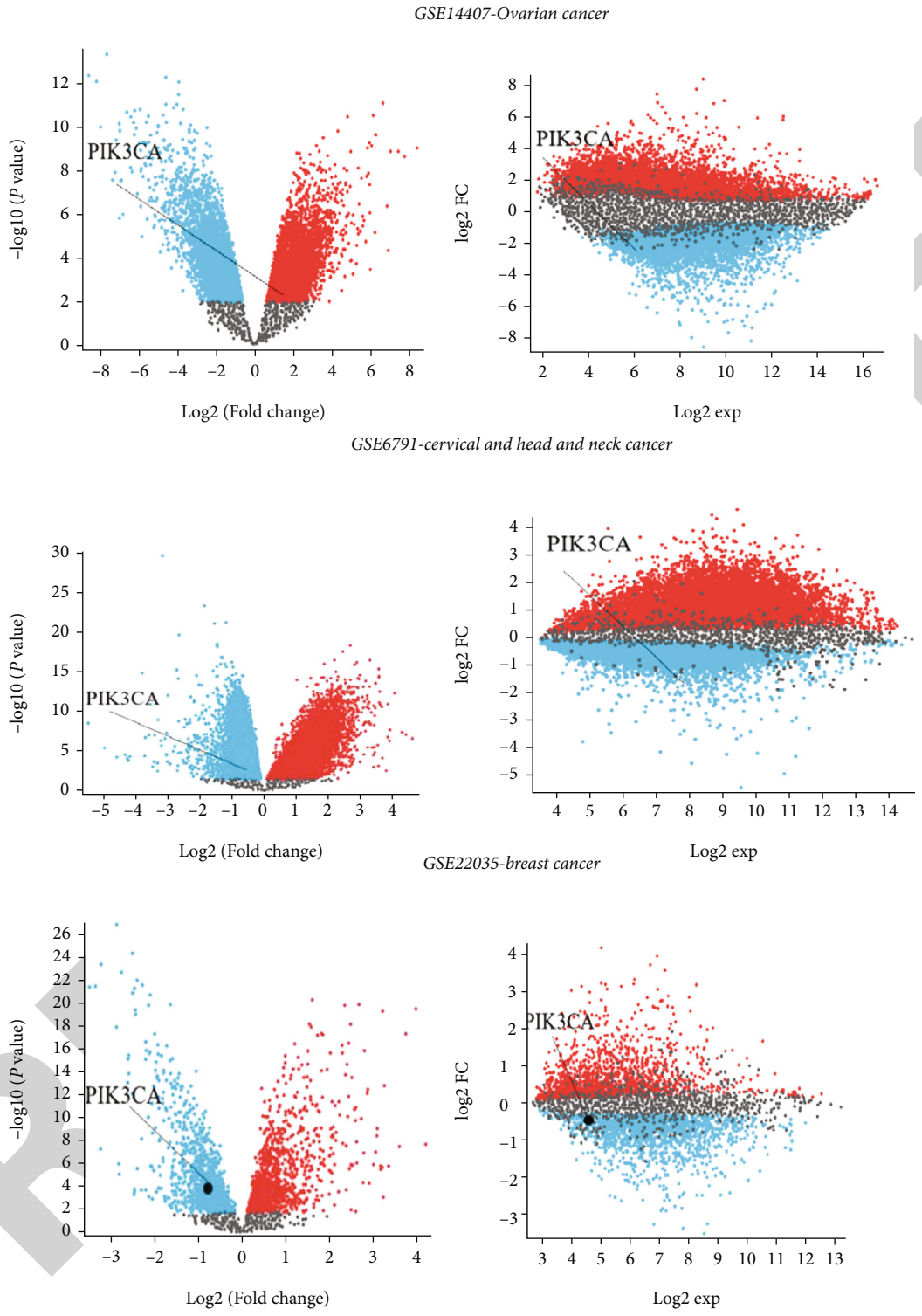

Figure 3: Differential expression profile of PIK3CA in various cancers.

regarding gene functions, evaluate gene lists, and prioritize genes for functional studies. PIK3CA Gene MANIA uncovers functionally related genes for the query gene using a multitude of genomics and proteomics data. In this mode, it weights each functional genomic dataset according to its prediction value for the query. Gene MANIA was utilized in this study to depict molecular network analysis to investigate probable PIK3CA linked gene interaction networks and mechanisms [21].

2.4. Selection of Streptomyces-Derived Compounds and Structure Retrieval. Based on earlier studies on crude extract

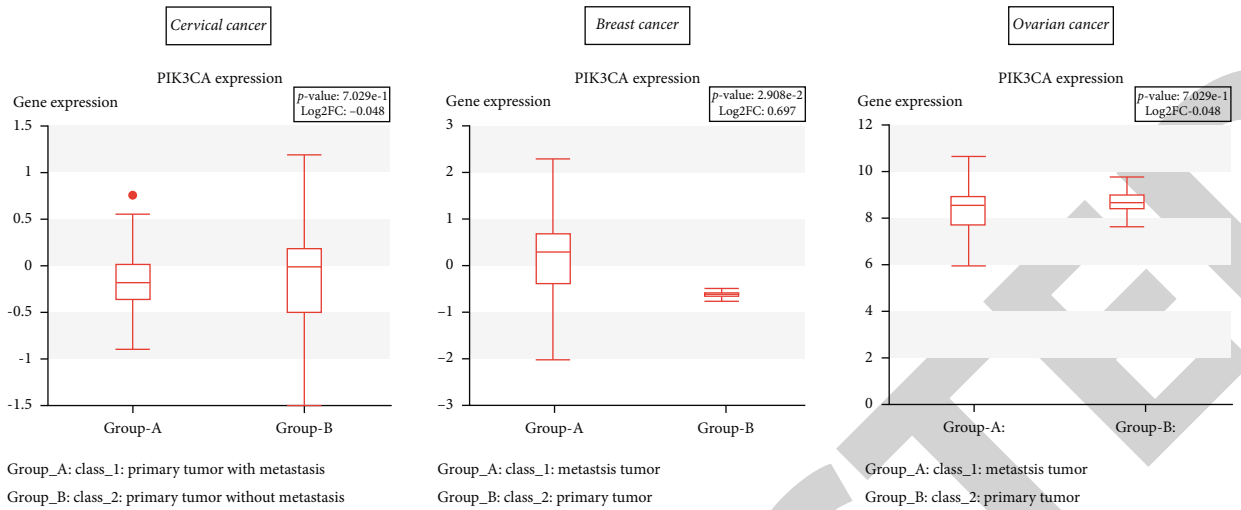

Figure 4: TCGA derived PIK3CA metastasis-related expression data of various cancers.

Table 2: Annotated functions of PIK3CA with its interacting proteins/genes based on the protein/gene-protein/gene interaction network.

Function	FDR	Genes in network
Regulation of protein kinase B signaling	7.29*E*-13	10
Protein kinase B signaling	7.29*E*-13	10
Phosphatidylinositol-mediated signaling	1.12*e*-12	9
Inositol lipid-mediated signaling	6.57*e*-12	9
Phosphatidylinositol 3-kinase signaling	3.36*e*-09	7
Regulation of phosphatidylinositol 3-kinase signaling	7.63*e*-08	6
Phosphatidylinositol metabolic process	2.02666*e*-06	6
Receptor tyrosine kinase binding	5.74532*e*-05	3
Phosphatidylinositol 3-kinase complex	5.74532*e*-05	3

isolation from the potent *Streptomyces sps*-based streptenol derivatives that were included for the present study, open-source repository PubMed was used to retrieve the three-dimensional structure and physicochemical attributes of the proposed compounds [22]. The prepare ligand module was used to determine the atom's coordinates and bond order, and then hydrogen was added to the proposed compounds and optimized using CHARMM force fields [23]. Streptenol derivatives were further reduced with the smart minimizer, which used conjugate gradient algorithms and 2000 steps of steepest descent to keep the RMS gradient at 0.001 kcal/mol. For pharmacophore hypothesis creation, fitting of the chemical into the model hypothesis, and affinity evaluation against cancer-related protein targets, minimal conformers were used [24].

2.5. Physicochemical and Drug-Likeness of Streptomyces-Derived Compounds. Intermolecular interaction studies of proposed compounds with promising cancer therapeutic target PIK3CA started with three-dimensional structure retrieval of compounds in SDF format from the PubChem database [25]. The SMILES (Simplified Molecular-Input Line-Entry System) notation has been used to analyze the chemical properties and drug-likeness score of three compounds. Lipinski's filter takes into account molecular weight, Clog*P*, polar surface area, number of hydrogen bond donors and acceptors, number of atoms, violations, rotational bonds, and volume [26]. Lipinski rule-based assessment for the derived compounds listed as bioavailability radar in the Figure 1. Drug-likeness is a qualitative notion that describes how a drug reacts to elements like bioavailability and assesses the compound's toxicity. The drug-likeness score of the bioactive compounds was computed using SwissADME, a web resource for efficiently assessing several drug likeness features using a few high-quality prediction models [26].

2.6. Modeling of Pharmacophores Based on Ligands. The Auto Pharmacophore Generation applied on the DS was started to critically probe into the main chemical properties imbibed inside the proposed streptenol derivatives to generate the most feasible pharmacophore models. The information rendered by the above was exploited in the generation of the pharmacophore. The genetic function approximation (GFA) model was used to find the pharmacophore with the best selectivity. Compounds with pharmacophore characteristics were created to test for essential properties that are important for intermolecular interactions with cancer

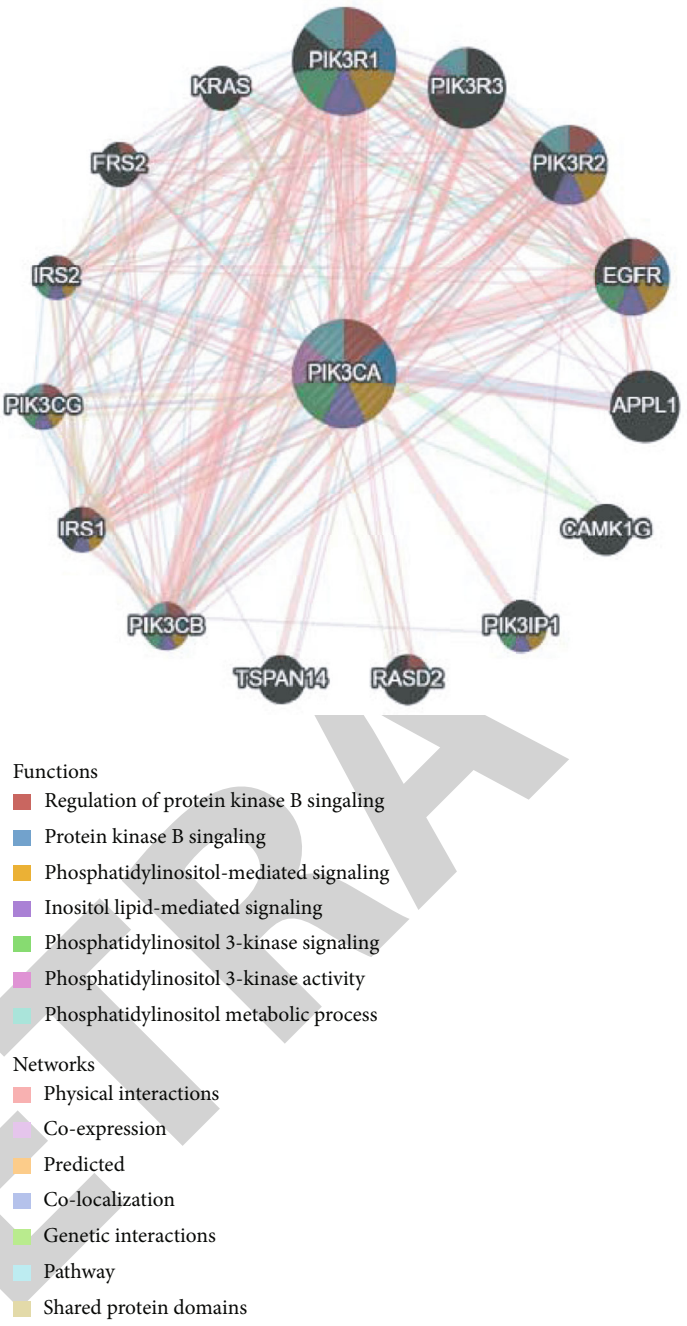

Figure 5: PIK3CA protein-protein interaction network.

targets [27]. The HypoGen approach, which incorporates the biological activity values of the compounds in the training set and creates the hypothesis on the discovery studio platform, was used to create pharmacophores. To create compound conformers per molecule, the "BEST" algorithm was used.

2.7. Receptor-Ligand Pharmacophore Generation (Structure-Based Approach). To get insight about the receptor-based pharmacophore model generation for PIK3CA-inhibitor complex, experimentally resolved structure (4JPS) was retrieved from the PDB [28]. The structure of PIK3CA was generated by removing the heteroatoms. The presence of PIK3CA residues near the FDA inhibitor alpelisib was investigated and reported. Meanwhile, all other PIK3CA proposed inhibitors and their residue preference were also recorded. Biovia Discovery studio module *Receptor-ligand Pharmacophore Generation* model was created with the help of Pharmacophore Generation. The best pharmacophore model was chosen based on a high selectivity score and appropriate interactions with catalytic active residues of the ATP-binding site PIK3CA. The selectivity score categorizes pharmacophore models based on the sensitivity and specificity of novel ligands against the receptor, with the best models

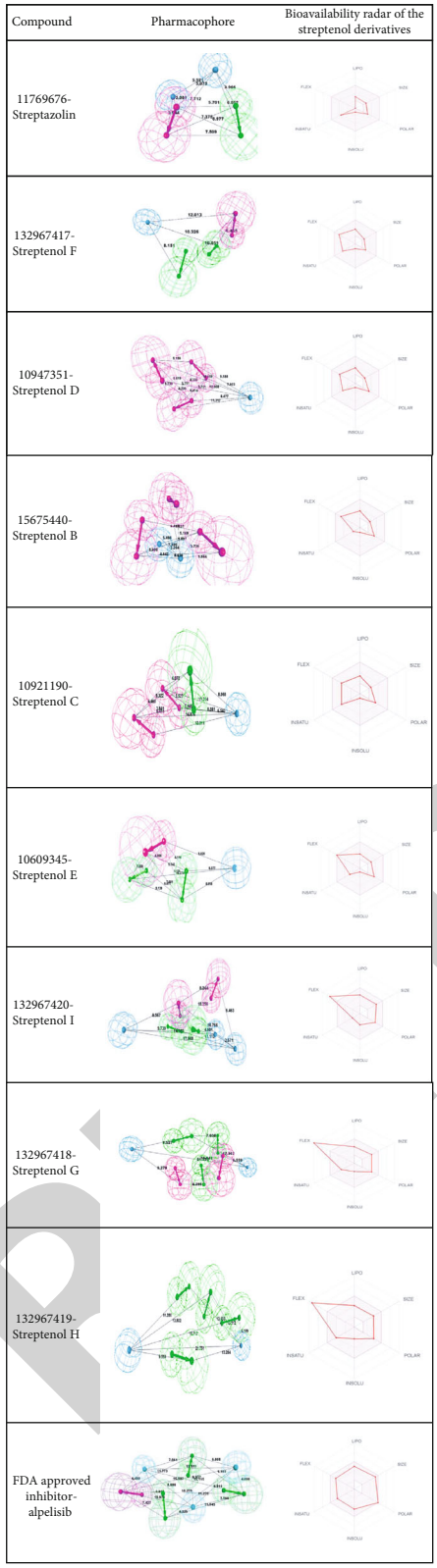

Figure 6: Streptenol derivative pharmacophore scaffold and geometrical constraints. The pharmacophore features were colored as follows: green hydrogen bond acceptor (HBA), red hydrogen bond donor (HBD), orange ring aromatic (RA), and cyan hydrophobic (HY).

being returned. During the selectivity score computation, the generated pharmacophore models are evaluated to a comprehensive 3D database of drug-like compounds available in DS, and the selectivity is measured using the genetic function approximation (GFA) method. A training set of 1544 pharmacophore models was used to develop the GFA model in DS. Each pharmacophore contains between two and eight pharmacophoric features, which are used to evaluate the CapDiverse database in DS. Descriptors derived from the total number of features in pharmacophore models and interfeatures distance bin values are used to train the GFA model.

2.8. Intermolecular Interaction Studies of Bioactive Compounds of Streptomyces. Molecular docking of Streptomyces-derived compounds used to identify their low energy binding mode with the binding cavities of the cancer target receptors. In general, small molecular interaction with the appropriate receptor or target could create an antagonist effect and can be used as a therapeutic agent [29]. In our computational studies, we used the ligand fit algorithm to screen the *Streptomyces*-derived compounds intermolecular interactions [30]. LigandFit docking algorithms used to screen out incompatible ligands and quickly create shape-based alignments of compound poses. They evaluate the volume and shape of the receptor's binding site pattern for screening. The receptor patterning on a grid was used to initiate the binding site analysis for cancer targets. Binding site identification includes two processes like receptor shape-based screening using the Eraser algorithm [31] and volume-based occupancy of ligand posed within the binding cavity [32]. The LigandFit algorithm-based docking approach is divided into two parts: identifying the cavities inside the cancer target receptor as the docking site and docking Streptomyces-derived compounds to the site. Multiple orientations/variation dataset of the ligand key moments with the prime position of the site yield a suitable ligand conformation for the given binding site. Finally, the dock score energy function is used to rank the promising ligand interaction pose with the specified receptor protein.

2.9. Molecular Dynamic (MD) Studies of Streptenol Derivative and PIK3CA Complex. The molecular dynamic simulations have been performed on the CABS Flex 2.0 platform. In recent days, this coarse-grained simulation-based model for assessing protein motion has evolved as a key tool. CABS Flex-based fluctuation plot listed the RMSF values using the Monte Carlo dynamics. Parameters set for 50 trajectory frames within the time span of 10 ns. Distance restraints with global weight of 1.0, and Poisson-Boltzmann/generalized born (PB/GB) molecular mechanics were considered for conformational stability of the PIK3CA-streptenol derivative complex system [33, 34].

3. Results

3.1. PIK3CA-Key Player of Cancer Cell Signaling Pathway. The high incidence of PI3K pathway mutations in cancer has inspired lots of new therapeutic development efforts

Table 3: Molecular docking study between streptenol derivatives along with PIK3CA revealed their intermolecular docking scores.

Compound ID	Ligscore Dreiding 1	Ligscore Dreiding 2	PLP1	PLP2	JAIN	PMF	Dock score
11769676-streptazolin	1.34	3.66	50.37	37.05	-0.56	40.84	47.889
132967417-streptenol F	2.34	4.34	68.81	67.67	-1.03	56.76	66.014
10947351-streptenol D	2.89	4.4	70.14	72.76	-0.82	38.44	66.355
15675440-streptenol B	3.21	4.28	71.24	83.57	0.23	44.59	67.926
10921190-streptenol C	1.97	3.53	71.93	77.56	-0.48	34.43	68.057
10609345-streptenol E	2.75	4.43	81.81	78.14	0	58.16	76.359
132967420-streptenol I	1.88	4.8	94.65	93.04	-0.77	68.86	88.275
132967418-streptenol G	1.41	3.49	94.71	95.23	-2.04	69.49	88.432
132967419-streptenol H	2.36	4.49	100.65	95.99	-1.3	76.04	96.081
FDA-approved inhibitor-alpelisib	2.16	4.32	98.59	91.62	-1.1	72.08	95.917

addressed in the present study. Despite the fact that numerous robust and specific inhibitors have been developed and tested in preclinical models, clinical licensing has limitations. Mutational landscape of PIK3CA and expression profile data PIK3CA among the various cancers was retrieved from TCGA and depicted in Figures 1 and 2. Differentially expressed PIK3CA profile was retrieved from the GEO dataset and tabulated in Table 1. PIK3CA was listed as top genes among the upregulated expression data of gynecological cancer-related datasets, and the volcano plot depicted their localization in the dataset as in Figure 3.

3.2. Metastasis Profile of PIK3CA among the Cancer. According to the HCMDB, metastasis expression profile data of PIK3CA its revealed that differential expression for PIK3CA is associated with regional lymph node metastasis among the cervical and ovarian cancer patients; in addition, PIK3CA mutation was also associated with metastasis progression among the breast cancer patients and reported data represented as Figure 4.

3.3. PIK3CA Gene Network Assessment. The gene-gene interaction network of PIK3CA was assessed using the GENEMANIA platform. Proposed PIK3CA interaction network framed by the GENEMANIA gene function prediction program was based on the Multiple Association Network Integration Algorithm (MANIA), and this systematic algorithm integrates a set of functional features of PIK3CA, like coexpression, pathways, physical interactions, colocalization, genetic interactions, and protein domain similarity. It has been observed that this algorithm is more precise and computationally efficient than other gene function prediction methods as in Table 2. Finally, gene-gene interaction network was visualized in GENEMANIA platform, and the features of the created network were given in Figure 5.

3.4. Protein Target Retrieval and Feature Assessment. Experimentally resolved three-dimensional structure of PIK3CA bound with FDA-approved inhibitor alpelisib was downloaded from PDB. In discovery studio platform, the Cavity algorithm was used to rank the listed binding site. Specific amino acids V851, I848, S854, I932, I800, P778, Y836, Q859, M772, D933, and K802 localized in ATP binding pocket 3D structure of PIK3CA were considered as region of interest. The prepare protein module was used to fix common issues such as inserting missing loop regions based on SEQRES data or user-defined loop definitions and calculating the pK and protonation of the supplied PIK3CA structure.

3.5. Drug-Likeness and Pharmacophore Feature Assessment. The calculation of pharmacokinetic parameters such as ADMET is an important aspect of the drug discovery process. We used two web resources, namely, SwissADME and Discovery studio ADMET screening, in the currently proposed work and assessed data that was shown in Figure 6. SwissADME is a web platform that estimates physicochemical attributes of streptenol derivatives and includes the pharmacokinetics and drug-likeness assessment of proposed compounds. For lipophilicity prediction, it used numerous extrapolative models and a consensus technique to assess the streptenol derivatives. In this study, ADME analysis was used to see if suggested compounds might make the designated targets, and it was discovered that all the compounds on the list met the drug similarity requirements.

3.6. Receptor–Ligand Interaction Analysis Using Molecular Docking. In gynecological cancers, PIK3CA is the most often mutated oncogene, and somatic mutations in the PIK3CA gene result in enhanced PI3K activity. PIK3CA mutations have been linked to increased cell proliferation and decreased apoptosis in cervical cancer. Five PI3K inhibitors have been approved by the US Food and Drug Administration so far (copanlisib, idelalisib, umbralisib, duvelisib, and alpelisib) (FDA). This has persuaded clinicians and researchers to investigate different PI3K inhibitors in preclinical and clinical settings to find a powerful PI3K inhibitor with significant clinical efficacy, minimal toxicities, and optimal bioavailability. Streptenol derivatives streptenols A, C, and F–I and streptazolin bound resolutely at the PIK3CA cavity by forming conventional hydrogen bonds, C-H bonding, and series of pi-stacked bonds with residues Val 851, Ile 848, Ser 854, Ile 932, Ile 800, Pro 778, Tyr 836, Gln 859, Met 772, Asp 933, and Lys 802 (Tables 3 and 4). Stable conformer of streptazolin mediated the interaction through Pi-stacked bonds with val 850, Met 922, and TRP 780, and

Table 4: Molecular docking study between streptenol derivatives along with PIK3CA revealed the kind of interactions and bonding distance and the interacting residues.

Streptenol derivatives	Interacting atoms	Bond distance (A^0)	Bond type
Streptazolin-11769676	Streptazolin:H19-A:VAL851:O	2.56512	Carbon hydrogen bond
	Streptazolin:H19-A:SER854:OG	2.99151	Carbon hydrogen bond
	A:VAL850-streptazolin	5.09434	Alkyl
	A:MET922-streptazolin	5.21562	Alkyl
	A:TRP780-streptazolin	5.02045	Pi-alkyl
Streptenol D-10947351	A:SER854:OG-streptenol D:O1	3.22236	Conventional hydrogen bond
	Streptenol D:H26-A:GLU849:O	2.0072	Conventional hydrogen bond
	Streptenol D:H14-A:SER854:OG	3.05414	Carbon hydrogen bond
	A:TRP780-streptenol D:C13	5.43126	Pi-alkyl
10921190-streptenol C	A:SER854:OG-streptenol C:O1	2.83465	Conventional hydrogen bond
	Streptenol C:H21-A:VAL851:O	2.55821	Conventional hydrogen bond
	Streptenol C:H23-A:GLU849:O	2.69578	Conventional hydrogen bond
	Streptenol C:H20-A:GLU849:O	1.83534	Carbon hydrogen bond
	A:ARG852-streptenol C:C13	4.48454	Alkyl
132967418-streptenol G	A:VAL851:N-streptenol G:O2	2.88574	Conventional hydrogen bond
	A:SER854:OG-streptenol G:O3	2.75294	Conventional hydrogen bond
	Streptenol G:H42-A:SER854:OG	2.02097	Conventional hydrogen bond
	Streptenol G:H31-A:SER854:OG	2.32419	Carbon hydrogen bond
	Streptenol G:H38-A:SER854:O	2.2941	Carbon hydrogen bond
	Streptenol G:H38-A:SER854:OG	2.89904	Carbon hydrogen bond
	Streptenol G:C22-A:ILE848	4.59251	Alkyl
	Streptenol G:C22-A:ILE932	4.14713	Alkyl
132967420-streptenol I	Streptenol I:H37-A:GLU849:O	2.75816	Carbon hydrogen bond
	Streptenol I:C22-A:MET922	5.35331	Alkyl
	Streptenol I:C22-A:ILE932	5.4146	Alkyl
	A:TRP780-streptenol I:C25	5.1071	Pi-alkyl
132967419-streptenol H	Streptenol H:H40-A:VAL851:O	2.94788	Conventional hydrogen bond
	A:ARG852-132967419:C24	3.50435	Alkyl
	Streptenol H:C25-A:ILE932	4.20838	Alkyl
132967417-streptenol F	A:ASN853:N-streptenol F:O2	3.20635	Conventional hydrogen bond
	Streptenol F:H26-A:SER854:O	2.42357	Conventional hydrogen bond
	Streptenol F:H26-A:SER854:OG	2.42369	Conventional hydrogen bond
	Streptenol F:H15-A:SER854:O	2.32294	Carbon hydrogen bond
	Streptenol F:H23 A:GLN859:OE1	2.83208	Carbon hydrogen bond
	Streptenol F:C14-A:VAL851	4.754	Alkyl
	Streptenol F:C14-A:ILE932	5.02592	Alkyl
	A:TYR836-streptenol F:C14	3.37793	Pi-alkyl
	A:PHE930-streptenol F:C14	5.43637	Pi-alkyl
15675440-streptenol B	A:SER854:OG-streptenol B:O2	3.20635	Conventional hydrogen bond
	Streptenol B:H26-A:VAL851:O	2.42357	Conventional hydrogen bond
	Streptenol B:H26-A:SER854:OG	2.42369	Conventional hydrogen bond
	Streptenol B:H30-A:GLU849:O	2.83208	Conventional hydrogen bond
	Streptenol B:C13-A:MET772	4.754	Alkyl
	A:TRP780-15675440:C13	5.02592	Pi-alkyl
10609345-streptenol E	A:SER854:OG-streptenol E:O1	2.68539	Conventional hydrogen bond
	Streptenol E:H28-A:VAL851:O	2.72975	Conventional hydrogen bond
	Streptenol E:H28-A:SER854:OG	3.04873	Conventional hydrogen bond

Table 4: Continued.

Streptenol derivatives	Interacting atoms	Bond distance (A^0)	Bond type
	Streptenol E:H29-A:VAL851:O	2.39476	Conventional hydrogen bond
	Streptenol E:C16-A:ILE848		4.50287
	Alkyl		Streptenol E:C16-A:ILE932
4.13859	Alkyl		

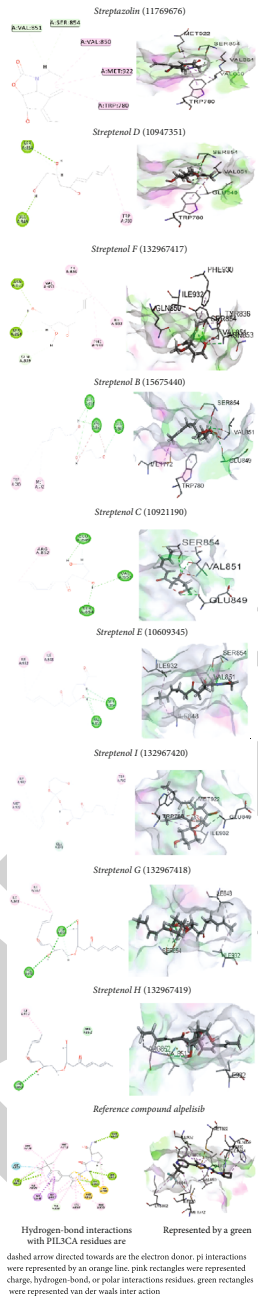

Figure 7: Proposed streptenol derivative intermolecular interaction with the ATP binding pocket residues of PIK3CA.

key residues like Val 851 and Ser 854 conferred the C-H bonding. Streptenol D similarly conferred a conventional hydrogen bond with Ser 854 and Glu 849 and Trp 780. PIK3CA binding residue Asn 853 and Ser 854 mediated the H-bonding, and Val 851, Tyr 836, Phe 930, Ile 932 conferred Pi stacked interactions with proposed streptenol F, and streptenol B conferred series two Pi stacked interactions with residues Trp 780 and Met 772 and conventional hydrogen bond with Ser 854, Val 8851, and Glu849. PIK3CA residue positions Val 851, Glu 849, and Ser 854 conferred three stable hydrogen bond with streptenol C; similarly, streptenol E also mediated the H-bonding with Val 851 and Ser 854 residues of PIK3CA, and interaction details were depicted in Figure 7. Streptenol I conferred the interactions with Glu 849, Met922, Ile932, and Trp 780 residues of PIK3CA interaction types that are specified in Figure 7. Streptenol G mediated the conventional H-bond with Val 851 and Ser 854 residues and two pi stacked interactions with Ile 848 and Ile 932 of PIK3CA.Streptenol H conferred e hydrogen bond with Val 851 and Arg 852 and Pi-alkyl bond with Ile 932. Reference FDA-approved inhibitor alpelisib also confirms the similar residue preference with PIK3CA, and the interaction details were illustrated in Figure 7. Proposed streptenol derivatives mediated the hydrogen bonding with the druggable cavity residues of PIK3CA. Ser854, Val 851, and Glu 849 were the residues conferred the maximum number h-bond interactions. PIK3CA drug target residue preference towards streptenol derivatives mediates hydrophobic interactions and was shown in Figure 8. PIK3CA drug target ATP binding pocket residues preference towards streptenol derivatives was depicted in Figures 9 and 10. Q849 is not conserved within the PI3K family. Isoforms b, d, and c have an aspartic acid, an asparagine, and a lysine residue, respectively, at this position. The aspartic acid and lysine residues of the b and c isoforms are obviously not able to establish the same hydrogen bond donor–acceptor interactions with the primary amide group of the inhibitor as a glutamine. Q849 mediated hydrogen bonding formed a favorable interaction of the inhibitor with the ATP pocket, but hinge region residue Val 851 is highly conserved among the PIK3 family proteins, and this promising residue mainly mediated the streptenol-based compound interaction; in general, kinase inhibitors target these "hinge-binders" localized in the ATP binding site sits at the interface between the two lobes of the kinase domain.

3.7. Molecular Dynamic Simulation (MDS) Evaluation of the Docked Complex. The receptor employed in this work was PIK3CA, and we examined the binding pocket as well as druggable residues using the cavity method based on literature data. MDS of PIK3CA was carried out using CABSfex2.0 server by 50 ns simulation. Simulation results revealed a fluctuation plot representing the PIK3CA amino

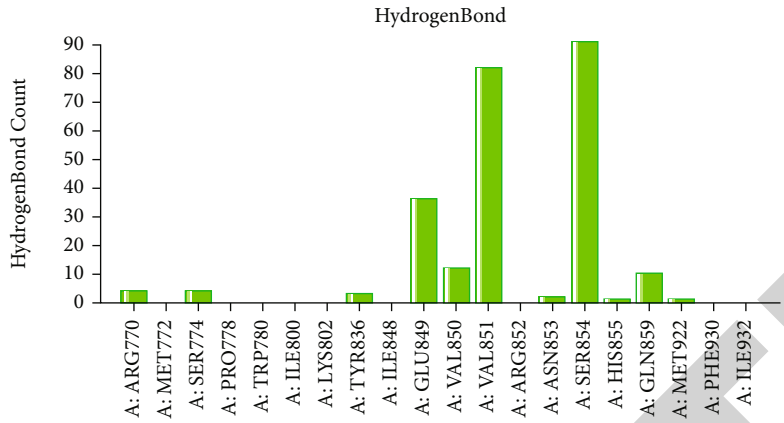

Figure 8: PIK3CA drug target residue preference towards streptenol derivatives by mediating hydrogen bond interactions.

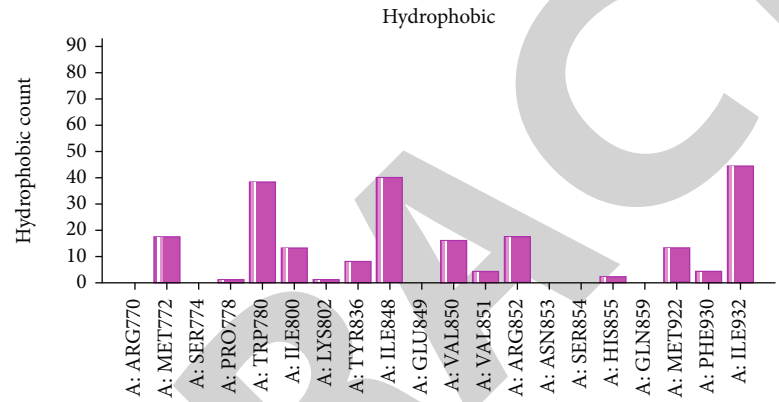

Figure 9: PIK3CA drug target residue preference towards streptenol derivatives by mediating hydrophobic interactions.

acid level fluctuations observed in the phase of simulation. Both svg formatted graphics data and numerical data were recorded for analysis. Residues with higher RMSF values were shown to have greater flexibility, and the details were depicted in Figure 11. Larger RMSF values indicated greater flexibility, whilst smaller RMSF values indicated restricted mobility during simulations. When utilizing effective restraints in the all-atom molecular dynamics' technique, a famous simulation methodology for proteins, the findings demonstrate that there are acceptable secondary structure residues with the -helix and -sheet of the protein that present with little fluctuation. Under all these conditions, proposed streptenol derivatives were displayed to provide their molecular connections with the focus on protein, confirming their potential interaction. Overall, the findings clearly imply that streptenol derivatives could be used as a lead candidate in the development of PIK3CA inhibitors.

4. Discussion

Kinase proteins are implicated in a wide range of biological functions, such as metabolism, cell signaling, protein regulation, cell trafficking, secretion, and many more. Nearly 497 protein kinases were identified in eukaryotes and in those 58 regular kinases well studied for their physiological role. Functional kinases represent 2% of the overall human genome construct, and kinases were first enzymes assessed for their function in oncogenic evolution and considered as potential drug target in cancer therapy [35]. The essential players in gynecological cancer pathways are PIK3CA, BRAF, and epidermal growth factor receptor (EGFR), which are key members in tumor microenvironment. PIK3CA is the second most highly mutated protein reported in cancer studies next to p53. PIK3CA related mutation was observed in 14% of cancers studies and seems to be amplified in 6% of all cancers in their Pan-Cancer Proteogenomic Atlas [36]. Their key physical importance is reflected in pharmacological research, with kinase-related preparations accounting for almost all new drugs discovered today. Kinase domain is localized between the 797 and 1068 residue positions of PIK3CA and was considered as key element and catalyzes substrate phosphorylation; thus, we assessed the proposed compound interaction pattern with this site. Streptomycetes produce a multitude of bioactive secondary metabolites with significant pharmacological potential. More than 60% of today's anticancer/antitumor medications come from these natural sources [37, 38]. Cancer chemoprevention is just as vital as carcinogenesis intervention. Antagonist agents stop the neoplastic process or suppressing agents that inhibit cancer cells from acquiring a malignant phenotype. As a corollary, attempts to find exceptionally accurate and efficient chemotherapy/chemo preventive medicines from other sources such as microbes are progressing [39]. In

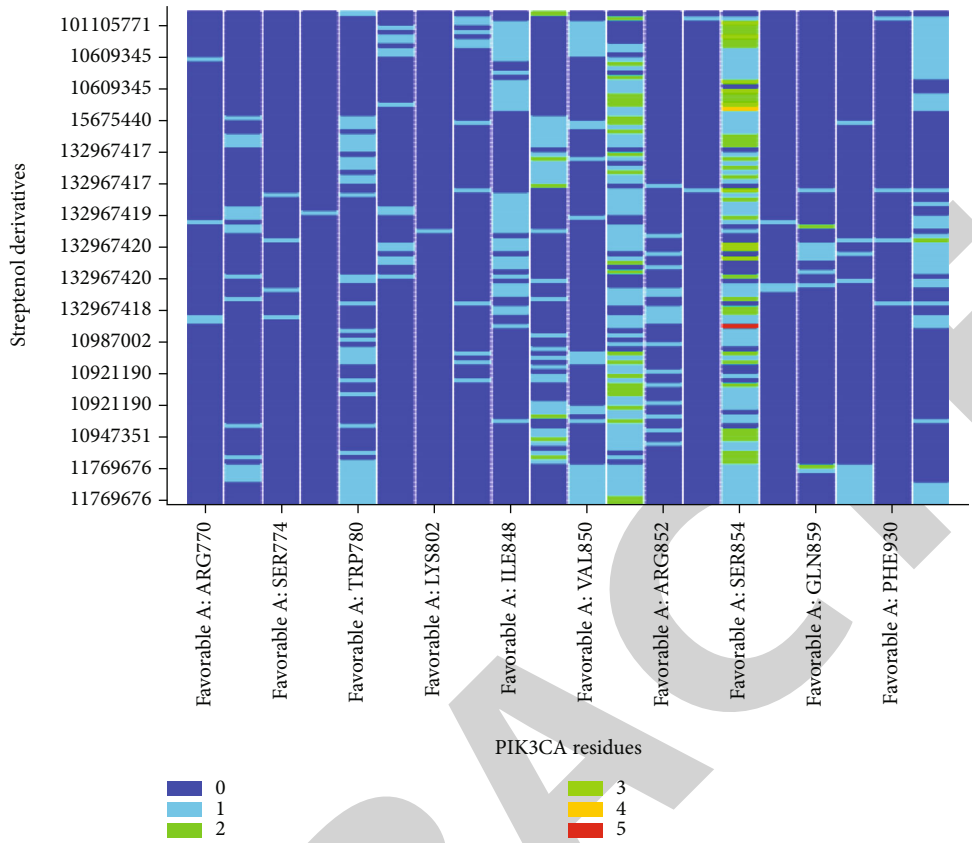

Figure 10: PIK3CA drug target ATP binding pocket residues preference towards streptenol derivatives.

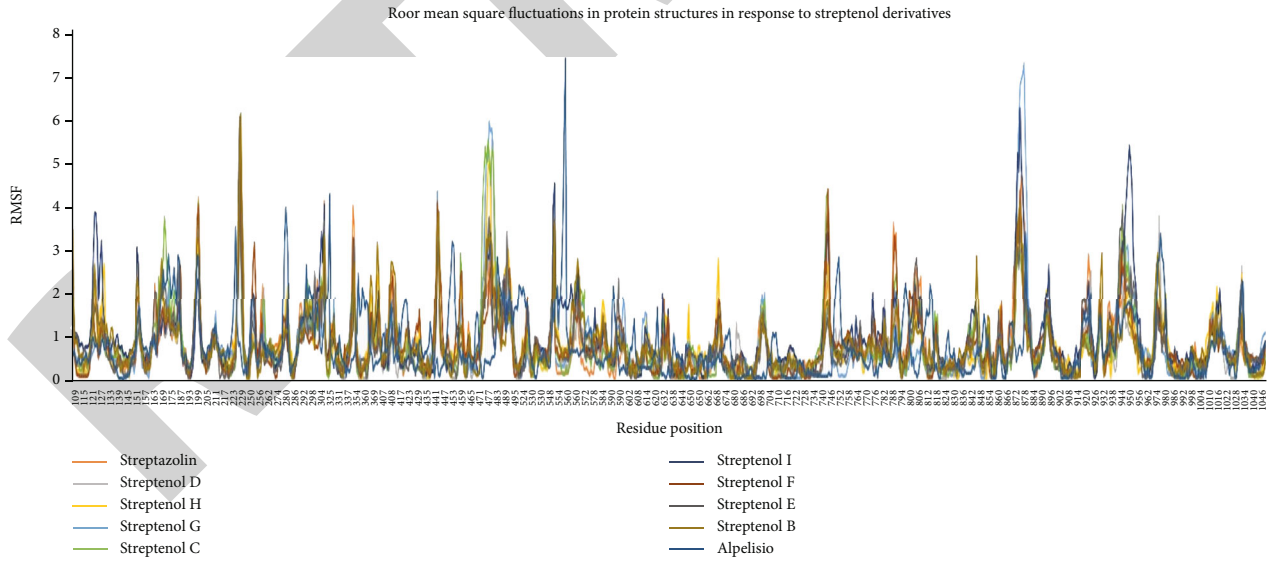

Figure 11: Root mean square fluctuations in protein structures in response to streptenol derivatives:

this research paper, study was designed to find the novel selective allosteric inhibitor for PIK3CA from streptenol derivatives. Anticancer effect of Streptomyces derivatives has a long-term success history, and it started with doxorubicin which has an antibiotic made by the bacteria *Streptomyces peucetius* [40]. Since the 1960s, it has been commonly used as a chemotherapeutic agent. Doxorubicin is a prescription medicine that belongs to the anthracycline group. Methods based on receptors and ligands can be used to create pharmacophore models. The structure of the PIK3CA target complexed with an approved inhibitor was used in our investigations [41]. We analyzed all the experimentally

resolved PIK3CA structure complexed with inhibitor before constructing the multiple receptor-ligand based pharmacophore model of PIK3CA inhibitors and further employed the receptor ligand interaction-based pharmacophore technique to reconnoitre the structure activity relationship of proposed cancer drug target PIK3CA and streptenolderivatives. Hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (HY), negative ionizable (N), positive ionizable (P), and ring aromatic (RA) were among the properties that were considered for the study. Streptenol compounds' projected physicochemical and ADMET properties were within the acceptable optimum standards for medication development [42]. The ADME parameters suggested that proposed compounds with good quality attributes, such as water solubility, human intestine absorption, plasma protein binding ability, and others, were discovered. Furthermore, the molecules streptenol D, streptenol E, streptenol C, streptenol G, streptenol F, and streptenol B were projected to be safe with minimal or no toxicity. Multiple human malignancies have somatic mutations in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), which encodes the p110 catalytic subunit of PI3K [43, 44]. The ATP pocket is located at 772, 780, 800, 836, 922, 930, 851, 848, 770, 778, 850, 932, and 933, while the non-ATP pocket is located at 954, 955, 956, 957, 1043, 1044, 984, 1047, 1051, 960, 964, 977, 980, and 981 [45]. Alpelisib makes hydrophobic contact with three spine residues (RS4, CS6/8) and the gatekeeper residue (Sh2). The proposed compounds also conferred hydrophobic contact with the first residue of the P-loop M772 and W780 of the β2-strand, which make up the specificity pocket [46]. In PIK3CA, hinge residues localized between 849 and 851 were considered as prime region because of their h bonding with ATP especially adenine. Thus, the main objective of present study focused to assess the streptenol derivative interaction with this residue. Proposed streptenol derivatives are found between p110 residues Ile800, Val850, and Val851 on one side and Met922, Phe930, Ile932, and and Asp932 on the other side on of PIK3CA.The docking results revealed that H-bonds and hydrophobic interactions with specific residues such as Val 851, Ile 848, Ser 854, Ile 932, Ile 800, Pro 778, Tyr 836, Gln 859, Met 772, Asp 933, and Lys 802 may play an vital part in the molecular contacts between the streptenol derivatives streptenol D, streptenol E, streptenol C, streptenol G, streptenol F, and streptenol B and the PIK3CA. Ligand binding underpins a wide range of recognition mechanisms that are generally understudied due to a lack of studies using Streptomyces-derived compounds. The application of trustworthy computational approaches to investigate protein–ligand interactions can substantially improve our perception of such systems and advantage to the development of innovative streptenol-based lead molecules for cancer treatment. Proposed compounds as well as reference compound interaction pattern with PIK3CA binding pockets especially hinge region, specificity region, affinity region, and nonconserved region were considered in the present study to support the reference compounds. PIK3CA is a lipid kinase composed of N- and C-lobes, which are connected by a hinge region, and ATP binds to a small pocket between these lobes. The hinge is conserved among PI3Ks, and the ATP-binding pocket residues are key to design the lipid kinase inhibitors. Further in vitro studies will support the present findings.

5. Conclusion

Compared to standard chemotherapy and surgery listed for gynecological cancer, molecular targeted therapies were considered as more specific as well as lesser side effects. Marine-derived bacteria comprise a promising source of new secondary metabolites with diverse chemical structures and interesting biological activities for drug development and particularly, marine actinobacteria represent an attractive resource for anticancer compounds. We performed a comprehensive computational study to identify potential effective inhibitors of PIK3CA from Streptomyces-derived compounds. Spine and shell residues of active PI3Kα as well as surrounding residues mainly mediate the intermolecular interactions with streptenol derivatives. Our study revealed that streptenol D, streptenol E, streptenol C, streptenol G, streptenol F, and streptenol B can be used as lead compounds in the development of PIK3CA inhibitors. However, further in vivo and in vitro studies are warranted before these drug candidates could enter the market for clinical applications.

Data Availability

The datasets used and/or investigated during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors have declared no conflict of interest.

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Research Article

A Comparative Study of *Diospyros malabarica* (Gaub) Extracts in Various Polarity-Dependent Solvents for Evaluation of Phytoconstituents and Biological Activities

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Keeping in mind the ascribed repute of *Diospyros malabarica* (*D. malabarica*), this investigation was commenced to assess the effect of diverse solvents on extraction yields, phytochemical components and antioxidant capability, and *in vitro* biological activities of *D. malabarica* for pharmaceutically active constituents to combat various infections. To screen phytochemicals both qualitatively (flavonoids, terpenoid, saponins, tannins) and quantitatively like total phenolic and flavonoid contents, *Diospyros malabarica* parts include the following: root, leaves, bark, stem, ripe, and unripe fruit were sequentially extracted with organic solvents such as petroleum ether, dichloromethane, ethyl acetate, ethanol, methanol, and water in increasing order of polarity from less polar to more polar solvents. Furthermore, biological activities such as antibacterial, antifungal, anticancer, antidiabetic, and anti-inflammatory were explored. The results revealed that all the tested solvents displayed a vital role in the extraction yield, the content of phytochemicals, and the studied biological activities. Methanol was found as the best solvent followed by the ethanol for the extraction, representing the highest extraction yield (18.3%), rich diversity of phytochemicals, and the highest total phenolic contents ($602 \pm 0.001\ \mu$g EAG/mg of extract) and total flavonoid contents ($455 \pm 0.6\ \mu$g EQ/mg of extract) in bark extract. Furthermore, methanol bark extract showed high *in vitro* antibacterial activity ($30.25\ \text{mm} \pm 0.9$), antifungal activity ($18.25\ \text{mm} \pm 0.2$), anticancer activity (48%), antidiabetic activity (68%) and anti-inflammatory activity (62%) followed by ethanol amongst other extracts of *D. malabarica*. Accordingly, methanol might be as an ideal solvent to get maximum content of phytochemicals, promising antioxidants, and *in vitro* biological activities from bark extract amongst other extracts of *D. malabarica* compared to pet ether, ethyl acetate, and dichloromethane and may act as free radical rummager because phytochemical constituents exhibit antioxidant capability. Our findings suggest that phytochemical compounds (flavonoids, tannins, phenols, saponins, and terpenoids) found in the bark extract of *D. malabarica* may be attributed to evaluate potent anti-inflammatory, anticancer, antidiabetic, antibacterial, and antifungal activities.

1. Introduction

Herbal medicines and extracts are a rich source of unprocessed drugs with medicinal properties. According to the World Health Organization, 80% of the worldwide people uses diverse plant components and their active ingredients as traditional therapies [1–4]. Therapeutic plants have tremendous healing sound effects due to the presence of several biologically active compounds such as flavonoids, terpenoids, phenols, saponins, resins, and steroids [5]. Phytochemical investigations are underway to find out the pharmacologically active compounds from plants possessing secondary metabolites which provide a complete defense system and are the main factors of therapeutic efficacy for

curing many chronic diseases [6]. Free radicals are formed during metabolic processes as necessary intermediates through diverse endogenous roots (breathing, mitochondria, incitement of granular leukocyte, peroxisomes and phagocytic cells, etc.) and exogenous causes (pollution, radiation, smoking, certain drugs, toxins, insecticides, and heavy metals and natural solvents) [7]. An increased number of free radicals and inadequate antioxidant defense system causes oxidative damage. To suppress the hazard of a variety of free radicals and ROS, potent antioxidants possessing the capability to rummage free radicals by neutralizing them are required [8]. Though both natural (plant-based) and man-made (chemically synthesized) antioxidants are helpful to promote health and protect the biological system by suppressing free radicals, plant-based antioxidants are reported best as they inhibit the formation of free radicals without any side effects [9, 10].

Many studies stated that man-made antioxidants as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have toxic effects on human health through lipid, protein, and DNA damaging [11]. The harmful effects of these artificial antioxidants have required the exploration of new natural products having antioxidant characteristics. Recent investigations indicated that the plant-based antioxidants have been known to shield humans from a few prolonged ailments, for example, aggravation, immune system sicknesses, malignancy, and tumor arrangement by preventive the production of free radicals [12]. The research on plants has been increasing worldwide, and scientific data collected so far reflects the use of medicinal plants in many traditional systems [13].

D. malabarica, "Gaub plant," is an evergreen tree with ornamental usage in Pakistan, well adopted in this climate and producing flowers and fruits. It is one of the indigenous medicinal plants granted with strong antioxidant activity [14]. The various parts of *Diospyros kaki L.* (Ebenaceae) like leaves and fruit have historically being used to treat high blood pressure and atherosclerosis owe to their antiproliferative and anti-inflammatory attributes [15, 16]. As a rich source of pharmacologically active bioconstituents, *D. malabarica* could be anticipated for its use as a phytomedicine. It has been reported previously that all organs of this plant, especially fruits, bark, and leaves, are used in medicinal preparation due to its antioxidant potential as possessing potency to combat various disorders in many traditional medicinal systems of the world [14]. Various extraction techniques with different optimization conditions [17] for natural products have been given in Table 1.

Moreover, the leave extracts are used for the treatment of burning, diabetes, atherosclerosis, intermittent fever, and cancer and reflect potent antidiabetic [18] antimicrobial, anti-inflammatory [1], and antipyretic activities [19]. The alcoholic extract of the stem revealed the anticancer and antidiarrheal activities. The methanol extract of *D. malabarica* ripe fruits has shown antibacterial, antitumor, antioxidant, hepatoprotective, antidiabetic, and antidiarrhoeal activities [20, 21]. Hypoglycemic and antihyperglycemic, antibacterial, and antiurolithiatic activities [22] were as follows. The traditional uses of *D. malabarica* have been reported as continued onset in reduction of gastrointestinal motility, inhibition of prostaglandin synthesis, and diarrhea [23]. Subsequently, extensive investigations on diverse organs of *D. malabarica* regarding comparative phytochemical and biological investigations have not been executed up till now.

In this study, we prepared different extracts of *D. malabarica* organs using organic solvents in order of increasing polarity from less to more polar solvents to extract both polar and nonpolar phytoconstituents. The phytochemicals of extracts were explored both qualitatively and quantitatively for biological activities. These extracts were further studied for their *in vitro* anticancer, anti-inflammatory, antibacterial, antifungal, and antidiabetic properties to figure out the most significant and ideal plant part as a potent future source of herbal drugs by virtue of its antioxidant competency.

2. Experimental Data

2.1. Sample Collection. Different organs including young fresh green leaves, unripe fruit and stem, dark black bark, and fully ripe fruit of only one sample of *D. malabarica*, "Gaab plant," were collected from the plant nursery of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, and were identified by the botanist. From July 2017 to Sep. 2017, plastic baskets saved in labeled containers in freezer at 10°C to maintain the integrity were carried to the laboratory for investigation. The samples were cleaned with water to get rid of dust and then rinsed with deionized water for analysis. The analysis was conducted at NIAB (MAB Lab-1), Faisalabad, Pakistan.

2.2. Drying and Grinding. The selected and carefully washed plant organs (leaves, bark, and stem, ripe and unripe fruit) were air-dried for 72 hours at 25°C to 30°C. The dried samples were then finely grounded by mortar and pestle into a powder and kept in labeled clean airtight bottles at room temperature for further analysis.

2.3. Preparation of Extracts. To extract various polarity-based chemical constituents, the extracts using different solvents were prepared by sequential extraction from less polar solvents to more polar solvents [24]. 50 g of powdered plant organs was dissolved in 500 mL solvent and petroleum ether (40-60°C) and kept in an airtight container. After that, it was supported to keep for 72 hours at room temperature having continuous shaking until soluble material was broken down to form a solution. Whatman filter paper No. 42 was used to filter extract. The marc left after extraction was air-dried and again extracted with solvent, dichloromethane for another 72 hours. This was followed by the extraction with ethyl acetate, ethanol, methanol and finally, water. The five extracts of each organ of *D. malabarica* were well-found. After complete evaporation of solvent, all extracts were solubilized in 10% dimethyl sulphoxide (DMSO) to get final concentration of 50 mg/mL and kept at 5°C in sanitized covered labelled bottles till further experimentation [25].

Table 1: Various extraction techniques with different optimization conditions for natural products.

Method	Solvent	Temperature	Time	Volume of solvents used
Maceration	Water, aqueous, nonaqueous solvents	Room temperature	Long	Large
Soxhlet extraction	Organic solvents	Under heat	Long	Moderate
Supercritical fluid extraction	Supercritical fluid (usually S-CO_2), sometimes with modifier	Near room temperature	Short	None or small
Ultrasound-assisted extraction	Water, aqueous, and nonaqueous solvents	Room temperature or under heat	Short	Moderate
Microwave-assisted extraction	Water, aqueous, and nonaqueous solvents	Room temperature	Short	None or moderate
Reflux extraction	Aqueous and nonaqueous solvents	Under heat	Moderate	Moderate

2.4. Extraction Yield. The ratio between the obtained mass of the dry plant extract and the total mass of plant material processed in the experiment are called the performance of crude extract [26]. This yield can be calculated with the help of the following formula (Figure 1):

$$\%\text{Yield} = \text{weight of extract/weight of dry powder} \times 100. \quad (1)$$

2.5. Phytochemical Screening. The phytochemical screening was conducted on the basis of precipitation reactions or coloring. According to the Houghton and Raman method [27], plant organs were converted directly into powder form. Phytochemical analyses along with antibacterial and antifungal activities were conducted in the Applied Chemistry Hi-Tech Laboratory of Govt. College University, Faisalabad, Pakistan. Likewise, biological activities such as anti-inflammatory and antidiabetic activities were executed at plant breeding and genetics division (MAB Lab-1) in Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan while anticancer activity was investigated at Hussain Ebrahim Jamal Research Institute of Chemistry, Karachi, Pakistan.

2.5.1. Qualitative Screening of Phytochemicals of Extracts. Following standard protocols were executed in order to find out the presence of tannins, terpenoids, saponins, flavonoids, and total phenol and flavonoid contents [28].

(1) Tannins. 500 mg of each dry sample was heated in 5 mL of H_2O. Then, after filtration few drops of ferric chloride ($FeCl_3$) solution was mixed, the development of bluish-black color confirmed the tannin [28].

(2) Saponins. 200 mg of the powdered sample was boiled in 500 mL deionized H_2O and filtered. Then, 10 mL of filtrate and 5 mL of deionized H_2O were mixed and agitated extensively to get a strong constant lather. Then, the lather was mixed with olive oil (3 drops) and blended extensively until the formation of emulsion [28].

(3) Flavonoids. 5 mL of 1.0 M diluted NH_3 solution was mixed in 10 mL of each filtered aqueous sample, and then 5 to 6 drops of concentrated H_2SO_4 were added to this solution. The resulted yellow color was an indication of flavonoids [28].

(4) Terpenoids. 5 mL plant sample and 2 mL of $CHCl_3$ were mixed vigorously. After that, 3 mL of concentrated sulphuric acid was added with care to get a layer. The appearance of the reddish-brown color on the inner face was a sign of terpenoids [29].

2.5.2. Quantitative Screening of Phytochemicals

(1) Test for Total Flavonoid Contents (TFC). Following the established colorimetric method with few modifications, the TFC was estimated in different extracts of *D. malabarica* using quercetin as standard described by Hossain et al. (2019). 5 mg of each plant extract was added in 4 mL of methanol in volumetric flask (5 mL). After this, 4 mL of distilled H_2O was added in 1 mL of each plant sample. To this reaction solution, 0.15 mL of 10% $NaNO_3$ was added. After five minutes, 0.15 mL of 10% $AlCl_3$ was added. Then, this mixture was incubated for six minutes followed by the addition of 1 mL of NaOH. At the same time, different concentrations (450, 400, 350, 300, 250, 200, 150, 100, and 50 μg/mL) of quercetin as standard were prepared in the same practice as defined in sample extracts. Then, after incubation, the absorbance of sample and standard solutions was recorded against methanol blank at 510 nm by UV-visible spectrophotometer. Total flavonoid contents of all tested plant extracts were determined by calibration curve of quercetin standard, and the attained results of TFC were expressed as quercetin equivalents (μg quercetin/mg dry sample). All the estimation for TFC investigation in the extracts was done in triplicates [30].

(2) Total Phenolics Contents (TPC). For the estimation of phenolic compounds in different plant extracts of *D. malabarica*, an established FCR (Folin-Ciocalteau (*F-C*) reagent) method was followed with few modifications by using gallic acid as standard stated by Al-Saeedi et al. (2016) [31]. For evaluation, an ice-cold pestle and mortar were used to homogenize 5 mg each plant sample in 0.5 mL ice-cold methanol (95%) in volumetric flask of 5 mL. Then, for incubation, plant samples were kept for 48 hours at ambient temperature in darkness. At room temperature, the plant samples were rotated at 14,462 × g for 5 minutes. The clear liquid that lies above was then separated for TPC analysis. At first, 100 μL supernatant and 10% (v/v) Folin–Ciocalteu

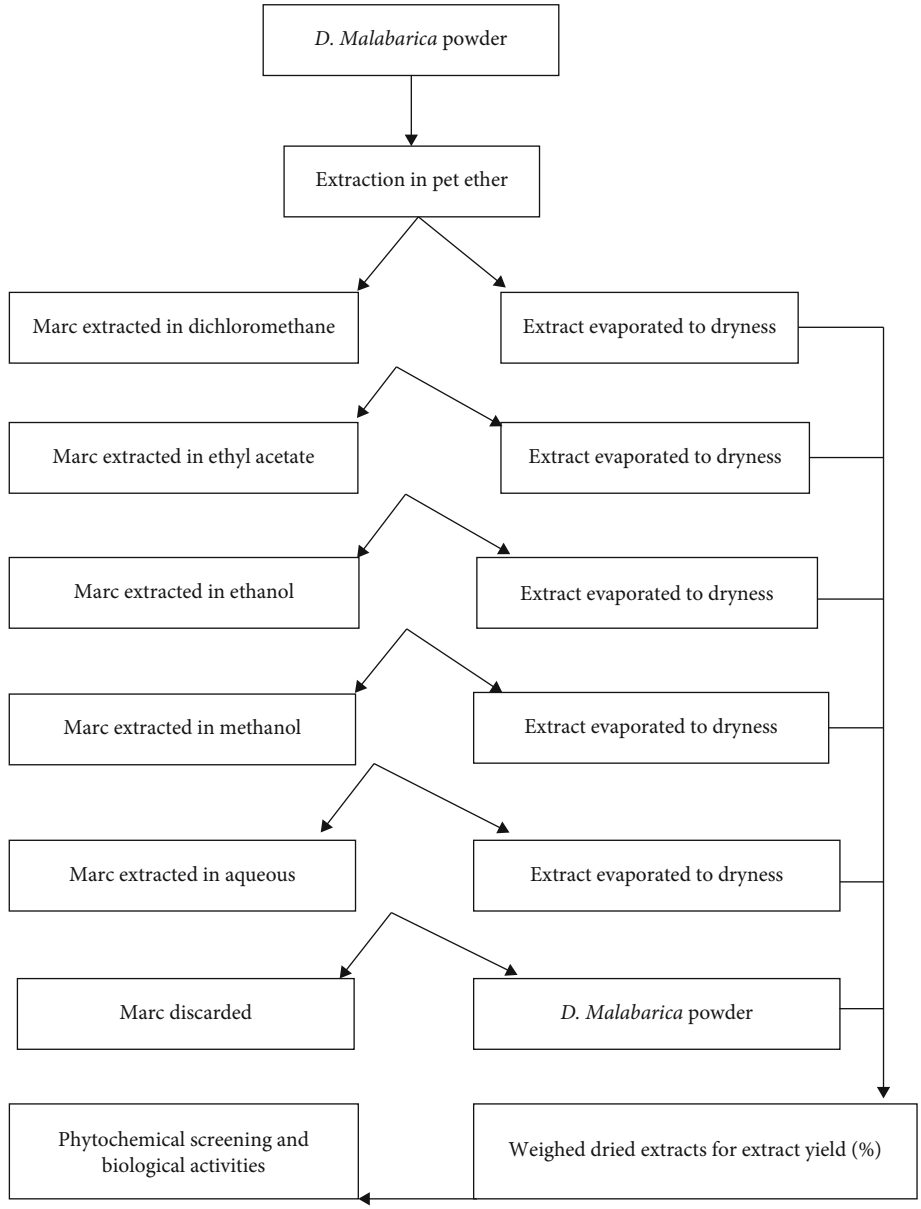

Figure 1: Schematic representation for extraction of phytochemicals.

reagent (FCR) (100 μL) were mixed and vortexed vigorously, and 700 mM sodium carbonate (800 μL) was taken in test tubes and incubated for 1 hour at lab temperature. At 765 nm, the absorbance of blank corrected sample solutions was measured. With the help of different gallic acid concentrations (450, 400, 350, 300, 250, 200, 150, 100, and 50 μg/mL), a standard curve was drawn, and a linear regression equation was measured. By using the linear regression equation, phenolic contents of samples equivalent to gallic acid were determined (μg EAG/mg of the dried sample) [31].

2.6. Biological Activities

2.6.1. Anti-Inflammatory Activity (In Vitro Inhibition of Albumin Denaturation). The inhibition technique of albumin denaturation was pursued to evaluate anti-inflammatory action. Method of [32] was used to execute tests with few modifications. 10 mg of diclofenac sodium was used as a standard drug. An aqueous solution of 1% bovine albumin serum was prepared and adjusted at pH 6 using 1 M HCl. The reaction solution was involving test extracts at a concentration of 5 mg/mL of 10% DMSO to obtain stock solutions. These reaction mixtures were further used to produce two final concentrations of 100 and 400 μg/mL of 10% DMSO of test substances to find plant activity at low and high concentration. 450 μL of 1% BSA was added to 50 μL of test extract, and its volume was increased to three times. Then, all the sample solutions and standards were incubated for 20 min. at 25°C and then heated at 70°C in a water bath for 5 minutes to denature the protein. The turbidity was measured using a spectrophotometer after cooling the reaction mixtures at 660 nm. Three concordant readings were taken [32]. % inhibition of

protein denaturation was calculated as follows:

$$\%\text{inhibition} = \frac{Abs\ \text{control} - \text{Abs sample}}{\text{Abs control}} \times 100, \quad (2)$$

where $\text{Abs}_{\text{control}}$ is the absorbance without sample, and $\text{Abs}_{\text{sample}}$ is the absorbance of plant extract/standard.

2.6.2. Anticancer Activity. For the evaluation of anticancer action of composites in 96-well level-bottomed microplates, standard 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) colorimetric measure was utilized [33]. Minimal Essential Medium Eagle was utilized as the culture for HeLa cells, enhanced with 5% of fetal bovine serum (FBS), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin in flasks (75 cm^2) and kept in 5% carbon dioxide incubator at 37°C. Then, harvested exponentially developing cells were tallied with a hemocytometer and diluted with a specific [34] medium. The cell culture was prepared with a concentration of 6×10^4 cells/mL and brought into 96-well plates (100 μL/well). After keeping on incubation the whole night, the medium was detached, and a fresh medium of 200 μL was added having (1-30 μM) different concentrations of compounds. After two days, 200 μL of MTT (0.5 mg/mL) was added to wells and again incubated for four hours. Afterward, dimethyl sulfoxide (100 μL) was added to all wells. The plant extract was prepared at concentration of 100 μg/mL in DMSO (1%). Doxorubicin as standard drug was also prepared in the same way and was also utilized as positive control. The extent of MTT reduction to formazan in cells was recorded by taking absorbance at 570 nm, utilizing μplate per user (Spectra Max Plus, Molecular Devices, CA, and USA). Anticancer activity was calculated as fixation causing development hindrance of half (IC_{50}) for HeLa cell lines. The % inhibition was determined with the following formula:

$$\%\text{inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100. \quad (3)$$

%inhibition = the results was organized by using SoftMax Pro software (Molecular Device, USA).

2.6.3. Antidiabetic Activity (In Vitro α-Amylase Inhibitory Activity). The 3,5-dinitrosalicylic acid (DNSA) method was followed to assay α-amylase inhibition [35] with few modifications. 5 mg of each plant extract of *D. malabarica* was dissolved in a minimum amount of 10% DMSO and was further dissolved in 20 mM sodium phosphate buffer and 6 mM NaCl at pH 6.9 to prepare two concentrations of 100 and 400 μg/mL. The reaction mixture was consisting of 200 μL α-amylase solution (2 units/mL) with 200 μL of the plant extract and was allowed to stand in an incubator at 30°C for ten minutes. Then, 200 μL of 1% starch solution in distilled H_2O (w/v) was added in all extracts and incubated for three minutes. 200 μL DNSA reagents (12 g of Rochelle salt in 8 mL of 2 M NaOH and 20 mL of 0.096 M of 3, 5 DNSA solutions) were further added to terminate the reaction and then boiled in a water bath for ten minutes at 90°C. After cooling all the reaction mixtures were diluted with the addition of 5 mL distilled H_2O, the absorbance was measured by UV-visible spectrophotometer at 540 nm. 200 μL of buffer was used as a substitute for plant extract to prepare blank with 100% enzyme activity. Another blank reaction was also prepared in the absence of the enzyme solution at each concentration using the plant extract. In the same way, positive control was prepared by using acarbose (100 μg/mL–2 μg/mL) as a standard drug, and reaction was performed similarly to the reaction with plant extract as mentioned above.

The α-amylase inhibition was calculated as % inhibition using the equation as follows:

$$\%\text{inhibition} = \frac{\text{Abs}_{\text{control}}\,(100\%) - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}(100\%)} \times 100. \quad (4)$$

2.7. Antibacterial Activity

2.7.1. Bacterial Strains. The antibacterial activity of plant extracts was assessed by using two bacterial strains, *Escherichia coli* (MG1655), the gram-negative bacteria, and *Streptococcus* (ATCC25925), the gram-positive bacteria. The bacterial strains were provided from the culture collection of Botany Dept. Government College University Faisalabad, Pakistan.

(1) Preparation of Inoculum and Test Solutions. The bacterial strains were separately subcultured for 24 hours at 37°C in nutrient agar pates to get well-settled confined colonies of the same morphological nature that were chosen from the cultured media. Every colony was contacted with a blazed wire-loop. Overnight hatching was done at rotating shaker at 37°C. The strain growth moved into a sanitized test tube containing 5 mL sterile saline H_2O. The test tubes containing the bacterial suspension were vortex to be blended well consistently. At that point, the bacterial suspension was attuned with 0.5 barium sulfate turbidity norms. The attunement and evaluation of turbidity of inoculum tubes were detected by observing them visually with nude eye against a 0.5 barium sulfate turbidity equivalence stock with grey background and distinct blue lines in passable light. The attuned bacterial suspensions would be used as inoculum within fifteen minutes; if not, they cannot be used for analysis [36].

(2) Disc Diffusion Method. For the determination of the antibacterial property of aqueous and solvent extracts, the disc diffusion method was adopted with few modifications [37]. Inoculum of each bacterial culture to be tested with concentration of 10^6 CFU/mL was spread on nutrient agar dishes with sterilized gauze saturated with the bacterial suspension. In this manner, discs with diameter of 9 mm were perforated into the agar medium and loaded up with concentration of 100 μL (10 mg/mL of deionized H_2O) of all plant extracts and antibiotic disc and permitted to diffuse at ambient temperature for two hours. The nutrient agar plates were then incubated in the upstanding situation at 37°C for overnight. Ciprofloxacin (250 mg), the standard antibiotic, was utilized

as the positive control. After incubation of 24 hours, the diameters of inhibition zones were recorded in mm with the help of Caliber, and all plant samples were verified in triplicates. The data was mentioned as mean ± SD.

2.8. Antifungal Activity. Two fungal strains including *Aspergillus niger (A. niger)* (ATC 1688) and *Aspergillus flavus* (*A. flavus)* (IL 152) were experienced for antifungal efficiency of root, stem, bark, leaves, unripe fruit, and ripe fruit extracts of test plant. These two fungal strains were attained from the Department of Botany, Govt. College University, Faisalabad, Pakistan, and these strains were sustained at 4°C on potato dextrose agar for further experiment. An inoculum of fungal strains of *A. niger* and *A. flavus* was suspended in 5 mL potato dextrose agar and hatched at 37°C for 48 hours. The antifungal potency was evaluated by the disc diffusion method [37] with some modifications. In this method, the inoculum was spread evenly over medium of potato dextrose agar with sterilized glass diffuser. Small round paper discs with diameter of 9 mm were perforated into the agar medium and loaded up with concentration of 100 μL (10 mg/mL of deionized H_2O) of each plant extract and antibiotic standard and permitted to diffuse in medium at ambient temperature for absorption of plant extracts and then kept in the incubator for 24 to 48 hrs.at 37°C. The antifungal activity was assessed by estimating the diameter of zone of inhibition by using Caliber. Novidate (500 mg), antibiotic standard, was utilized as positive control. Triplicate values were measured for all tested extracts.

2.8.1. Minimum Inhibitory Concentration (MIC). MIC is the lowermost concentration observed in maintaining the capability of inoculums. Based on the primer screening, ethanol and methanol removes that uncovered intense antimicrobial action were additionally tried to select the minimum inhibitory concentration (MIC) for each bacterial example (Table 2). The serial dilution method was used to find the MIC of plant extracts against both gram-positive and gram-negative bacteria. To obtain a stock solution by following the disc diffusion method [38], the sample extract was dispersed in 1 mL of deionized H_2O. Afterward, it was diluted to 10 folds by the sequential dilution method in which 9.5 mL of H_2O and 0.5 mL of sample were added in the test tube. Then, 0.5 mL was taken from this test tube and added into another test tube having 9.5 mL water. This process was repeated 10 times, and then solutions of 10%, 40%, 70%, and 90% were prepared for the antibacterial and antifungal tests.

2.9. Statistical Analysis. Values were accounted for as the mean ± S.D. of three different experiments. For analysis and organization of resulting data, descriptive statistics were applied. For the analysis of data, two-route ANOVA with replications was used. Significance of information was tried by examination of fluctuation and Turkey (HSD) test at $p < 0.05$ and where appropriate at $p < 0.01$ utilizing XLSTAT programming. Information was additionally exposed to principal component analysis utilizing PC programming Microsoft Excel alongside XLSTAT Version 2012.1.02, Copyright Add in soft 1995-2012 (http://www.xlstat.com).

Table 2: Estimation of TPC and TFC of *D. malabarica* extracts.

Extracts	Solvents	Total phenols (μg EAG/mg)	Total flavonoids (μg EQ/mg)
Root	Methanol	299 ± 0.004	285 ± 0.7
	Ethanol	246 ± 0.002	204 ± 0.8
Stem	Methanol	208 ± 0.005	196 ± 0.2
	Ethanol	191 ± 0.005	106 ± 0.1
Bark	Methanol	602 ± 0.001	455 ± 0.6
	Ethanol	534 ± 0.002	432 ± 0.5
Leaves	Methanol	440 ± 0.001	379 ± 1.2
	Ethanol	383 ± 0.001	368 ± 1.1
Unripe fruit	Methanol	467 ± 0.04	211 ± 1.2
	Ethanol	416 ± 0.07	220 ± 1.7
Ripe fruit	Methanol	450 ± 0.002	301 ± 1.5
	Ethanol	397 ± 0.08	237 ± 1.0

EAG: equivalent gallic acid; EQ: equivalent quercetin.

3. Results and Discussions

3.1. Extraction Yield. The yield percentage shows the extract amount gained from the extraction method stated in gram (g) of extracts found from per 100 gram (g) of crude plant powder and shown in Table 3.

The maximum yield was achieved in methanol bark extract (18.8 g/50 g of crude powder) followed by ethanol bark extract (14.2 g/50 g) while the petroleum ether extract indicated the lowest yield (4.2 g/50 g of crude powder) amongst all plant solvent extracts. In present investigations, polarity dependent intensification in extraction yield of different solvent extracts may be ascribed to high affinity for antioxidant components towards more polar solvents as compare to nonpolar solvents. The greater yield in polar solvent (methanol and ethanol) extracts specifies the polar nature of most of the phytochemicals in *D. malabarica* plant extracts. The lower yield in nonpolar solvent extracts showed a lower amount of nonpolar compounds in *D. malabarica* and the following order: methanol > ethanol > water > ethylacetate > dichloromethane > petroleum ether extract (Table 3). So, these outcomes were found in partial agreement with those investigated earlier [39].

The obtained results indicated that polar solvents as methanol followed by ethanol could be the best and effective solvents to extract more phytochemicals as compared to petroleum ether, ethyl acetate, and dichloromethane and can act as free radical scavengers because phytochemical contents determine their antioxidant capacity. On the other hand, the highest yield obtained in bark extracts amongst other plant parts demonstrated that *D. malabarica* bark extracts are a superb source of phytochemicals. Accordingly, the methanol, most polar solvent after water as testified formerly due to exhibiting more extraction yield, might be responsible to reflect more presence of phytochemicals and effective solvent in evaluation of potent biological activities [40].

Table 3: Extraction yield (% w/w) of *D. malabarica* extracts prepared using different solvents.

Solvents	Extract	Powdered mass (g)	Extracted mass (g)	Yield (%)
Petroleum ether	Bark	50	2.1	4.2
Dichloro methane		50	2.3	4.6
Ethyl acetate		50	2.6	5.2
Ethanol		50	7.1	14.2
Methanol		50	8.9	17.8
Aqueous		50	2.3	4.6
Petroleum ether	Stem	50	1	2
Dichloro methane		50	1.2	2.4
Ethyl acetate		50	1.3	2.6
Ethanol		50	2.2	4.4
Methanol		50	3.3	6.6
Aqueous		50	1.4	2.8
Petroleum ether	Leave	50	1.3	2.6
Dichloro methane		50	1.5	3
Ethyl acetate		50	1.9	3.8
Ethanol		50	6.6	13.2
Methanol		50	6.9	13.8
Aqueous		50	1.9	3.8
Petroleum ether	Unripe fruit	50	1.3	2.6
Dichloromethane		50	1.5	3
Ethyl acetate		50	1.7	3.4
Ethanol		50	4.3	8.6
Methanol		50	4.9	9.8
Aqueous		50	2.2	4.4
Petroleum ether	Ripe fruit	50	1.4	2.8
Dichloro methane		50	1.6	3.2
Ethyl acetate		50	1.8	3.6
Ethanol		50	3.8	7.6
Methanol		50	4.4	8.8
Aqueous		50	2	4
Petroleum ether	Root	50	0.5	1
Dichloro methane		50	0.6	1.2
Ethyl acetate		50	0.9	1.9
Ethanol		50	3.4	6.8
Methanol		50	4.9	8.1
Aqueous		50	1.9	3.8

3.2. Qualitative Phytochemical Screening. *D. malabarica* root, stem, bark, leaves, unripe fruit, and ripe fruit extracts were prepared by using more polar solvents (i.e., water, methanol, and ethanol) and less polar solvents (i.e., ethyl acetate, dichloro methane, and petroleum ether). The phytochemical constituents such as tannins, saponins, terpenoids, and flavonoids, of all tested plant extracts, were determined by qualitative analysis while total phenol content (TPC) and total flavonoid content (TFC) were determined through quantitative analysis.

Qualitative phytochemical screening of different extracts of *D. malabarica* in different solvents affirmed the presence of terpenoids, tannins, saponins, and flavonoids as shown in Table 4. The phytochemical screening (Table 3) revealed tannins with the affirmation of blue-black color while saponins and flavonoids confirmed their presence by the froth formation and yellow color, respectively. The subsequent appearance of the reddish-brown color on the inner face indicated the presence of terpenoids in the plant. In our study, the phytochemical screening showed that different tested plant organs of *D. malabarica* are a good source of flavonoids which could be supported by previous report [41] which displayed efficacy of each phytochemical for various biological action, like flavonoids show a vital role in antioxidant capability. Likewise, other phytochemicals as terpenoids, saponins, and tannins also demonstrate the potential of plants towards antibacterial and antifungal activities while recent studies also provide an overview of saponins about antiobesity healing prospective of saponins separated from therapeutic plants [42]. Plants enclose phytochemical compounds that were extracted and mostly utilized to heal some sorts of health-linked ailments and also exploited in production of food supplement and other nutrients. Each phytochemical reflects innovative biological actions that may possibly enhance the probabilities in detecting new antibiotic components against microbes [43]. In general, phytochemicals have substantial antioxidants, antimicrobial, anti-inflammatory, antiviral and resistant function, purification, and other functions of cell [44]. So, our present investigations were in agreement with earlier report which demonstrated the presence of phytochemicals like flavonoids, phenolic, saponins, alkaloids, sterols, tannins, and triterpenoids in *D. malabarica* plant extracts. Moreover, strong *in vitro* antioxidant potential due to the abundance of phytochemicals as terpenoids and flavonoids in ethanol extract of *D. malabarica* bark has been reported. The phytochemicals are being explored on priority for nutritive and herbal medicinal products [45]. Accordingly, in our current study, all tested parts of *D. malabarica* especially bark extract due to the greatest presence of tannins, saponins, terpenoids, and flavonoids could be a new addition in the production of phytomedicines and may be used for above reported similar trials as well as methanol may be considered as the optimal solvent to obtain high content of phytochemicals.

Accordingly, for the further quantitative determination of TPC and TFC and evaluation of biological activities, only methanol and ethanol solvent extracts of *D. malabarica* were screened due to reflecting their highest extraction yield and greatest presence of phytochemicals only in polar solvent as methanol and ethanol than nonpolar solvents.

3.3. Quantitative Phytochemical Analysis

3.3.1. Determination of TPC and TFC. Total phenolic and flavonoid content of *D. malabarica* plant extracts has been expressed as μg EAG/mg and μg EQ/mg, respectively. All

Table 4: Comprehensive view of phytochemical constituents in *D. malabarica*.

Solvents	Extract	Tannin	Saponin	Flavonoid	Terpenoid
Petroleum ether	Bark	+	+	+	+
Dichloro methane		+	+	+	+
Ethyl acetate		+	+	+	+
Ethanol		+	++	+	+
Methanol		++	++	++	++
Aqueous		+	+	+	+
Petroleum ether	Stem	+	+	+	—
Dichloro methane		+	+	—	—
Ethyl acetate		+	+	+	—
Ethanol		—	+	+	—
Methanol		+	—	+	—
Aqueous		+	+	—	—
Petroleum ether	Leave	+	—	+	+
Dichloro methane		+	+	+	+
Ethyl acetate		+	—	+	+
Ethanol		+	—	+	+
Methanol		+	—	+	+
Aqueous		+	—	+	+
Petroleum ether	Unripe fruit	+	+	+	+
Dichloro methane		+	+	+	+
Ethyl acetate		+	+	+	+
Ethanol		+	+	+	+
Methanol		+	+	+	+
Aqueous		+	+	+	+
Petroleum ether	Ripe fruit	—	+	+	+
Dichloro methane		—	—	+	—
Ethyl acetate		+	+	+	+
Ethanol		—	+	+	+
Methanol		+	+	++	++
Aqueous		+	—	—	+
Petroleum ether	Root	—	+	+	—
Dichloro methane		+	+	—	+
Ethyl acetate		—	+	+	—
Ethanol		+	+	+	+
Methanol		+	+	+	+
Aqueous		+	+	—	+

+: low color intensity, +++: high color intensity; -: absence of coloration.

tested plant extracts demonstrated certain amount of TPC and TFC. The maximum level of phenols ($602 \pm 0.001\ \mu$g EAG/mg of extract) and flavonoids ($455 \pm 0.6\ \mu$g EQ/mg of extract) was found in the methanol bark extract, and minimum was in ethanol stem extract (Table 2). This concludes the fact that most of the phenolic, flavonoids in all tested parts of plant were taken out by more polar solvent, methanol than ethanol as stated in previous report [46]. So, highest values of TPC and TFC in methanol indicated that most of the phenolic compounds found in *D. malabarica* might be polar in nature. On the other hand, the bark extract exhibited the highest amount of TPC and TFC amongst other tested extracts of *D. malabarica*. The results showed that methanol bark extract amongst other extracts could be more effective and fight against free radicals because antioxidant capability is determined by phytochemical contents as it was found in partial agreement of earlier report [47, 48] which demonstrated that antioxidant potency of methanol bark extract of *D. malabarica* might be due to the greatest presence of well-known natural antioxidants such as polyphenol, tannins, and flavonoids. Of all phytochemicals, polyphenols are greatly identified as an antioxidant anti-inflammatory, antiviral, and antimicrobial agents [49]. TFC and TPC are also reported to prevent DNA from oxidative

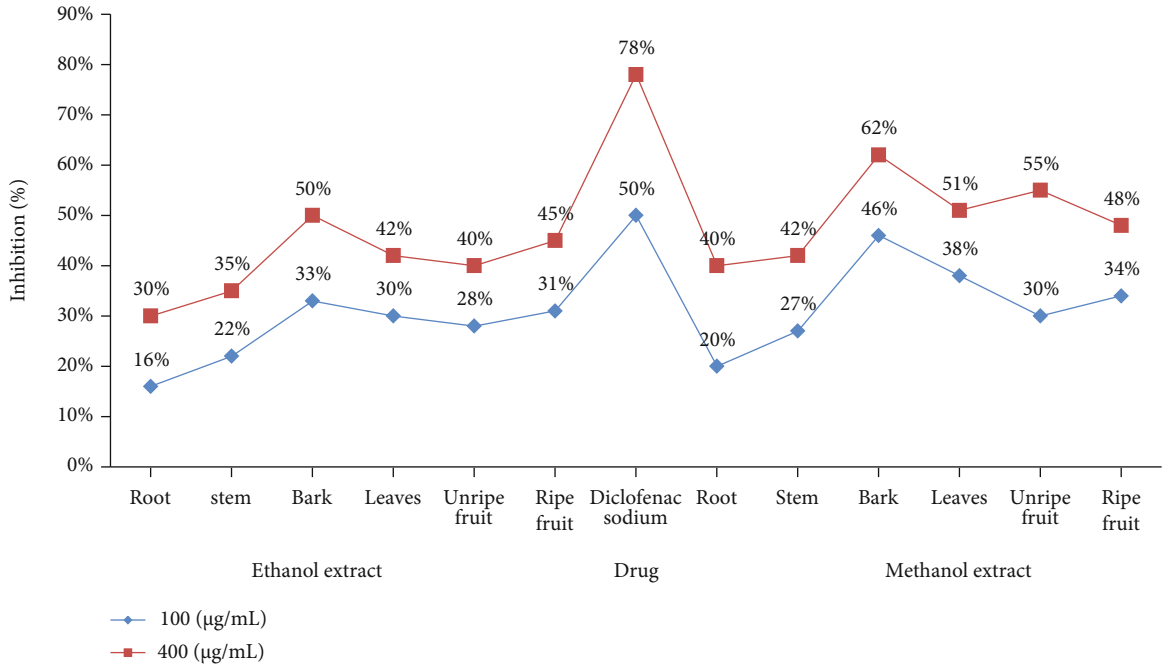

Figure 2: Albumin denaturation inhibitory activity of different *D. malabarica* extracts.

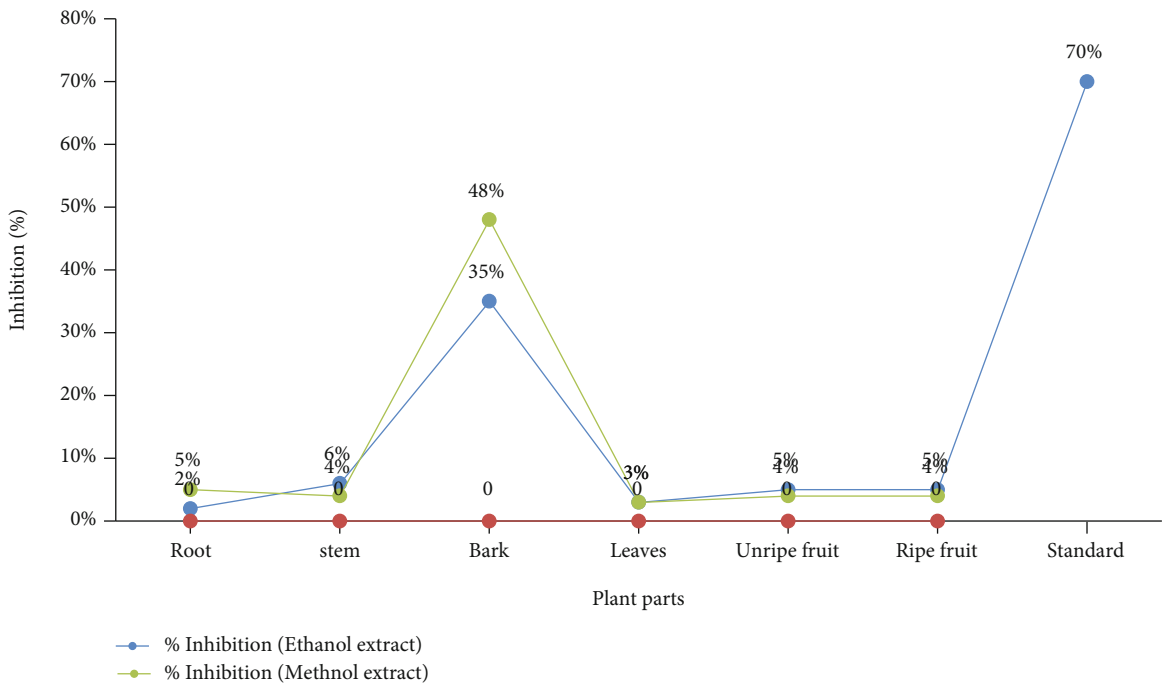

Figure 3: Anticancer activity of different *D. malabarica* extracts (30 μg/mL).

stress including inhibition of tumor cell growth and exhibit antimicrobial and anti-inflammatory activities. The previous investigations also revealed the role of phenolic compounds as a great source of antioxidant activity in *D. malabarica* [50], and the flavonoids are polyphenols which play a vital role in antibiotics action [51] because only flavonoids are involved in making complexes with microbial proteins, cell wall, and many other components that are responsible for biological function.

3.4. Biological Activities

3.4.1. Anti-Inflammatory Activity (Inhibition of Albumin Denaturation). Protein denaturation is a method through

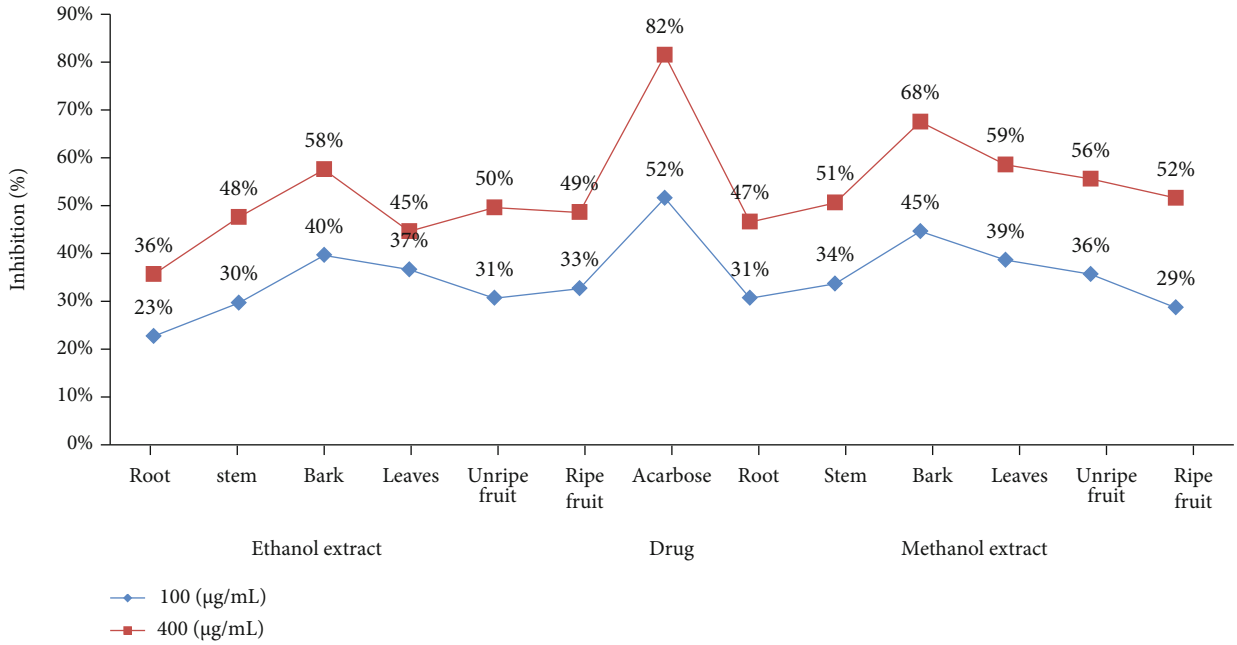

Figure 4: α-Amylase inhibitory activity of different *D. malabarica* extracts.

which protein structure is demolished due to the presence of external stress, oxidative stress, other compounds, or heat; so, it becomes responsible to fail their biological activity. Hence, the denaturation of tissue proteins due to oxidative damage is documented as a symbol of inflammation. Here, the *in vitro* anti-inflammatory activity of *D. malabarica* extracts was evaluated for hindrance against protein denaturation. Figure 2 shows the inhibitory influence of different *D. malabarica* extracts on protein denaturation. *D. malabarica* methanol bark extract amongst other extracts exposed significantly 62% greater protein protection near around diclofenac sodium (78%), a standard anti-inflammatory drug, at a concentration of 400 μg/mL which may be attributed due to the rich diversity of phytochemical constituents (tannins, phenols, flavonoids, saponins and terpenoids) in greater amounts as well as the solvent type used to extract bioactive components completely found within *D. malabarica* plant as reported formerly [52]. The three triterpenoid compounds such as betulin, betulic acid, and ursolic acid isolated from *D. malabarica* have been reported to exert pronounced anti-inflammatory activity [5]. Similarly, another reported significant anti-inflammatory activity in *D. malabrica* bark extract [53] encouraged our present findings.

3.4.2. Anticancer Activity. The cytotoxic results of ethanol and methanol extracts of *D. malabarica* against the HeLa cell line are shown in Figure 3. All tested extracts presented a bit anticancer activity but methanol bark extract of *D. malabarica* showed a potent anticancer effect (48%) while doxorubicin (70%) was used as a standard anticancer drug. Although resulted from anticancer activity in *D. malabarica* bark extract was quite less than standard but found not to be inactive even at very low concentration (100 μg/mL) which could be the result of remarkable polyphenols including total flavonoids and tannins consisting of antioxidant properties that may act as anticancer agents as these phytochemicals were reported earlier as anticancer compounds [34] and may be the substitute of conventional chemotherapy or however reduce its side effects, our finding may be in agreement with earlier investigations which revealed an extensive optimistic connection between diverse secondary metabolites like flavonoids, phenolics, tannins, and saponins with antioxidant and anticancer potency. It is reported that a person's diet involving polyphenols being natural antioxidants can improve health and reduce the risk of cancers [54].

3.4.3. Antidiabetic Activity (α-Amylase Inhibition). The methanol bark extract of *D. malabarica* followed by ethanol extract at concentrations of 100 and 400 μg/mL revealed substantial antidiabetic activity. The methanolic bark amongst other extracts exhibited 68% α-amylase inhibition (Figure 4) which was not very close to acarbose (82%), a standard antidiabetic drug but reflected significant antidiabetic activity even at a very low concentration 400 μg/mL. This antidiabetic potential of bark extract may be attributed to the presence of flavonoids and terpenoids as reported by Kavatagimath and Jalalpure [55] reinforcing our findings. Previously, methanolic leaf extract was reported to exhibit good antihyperglycemic activity in glucose tolerance tests and alloxan-induced diabetic rats [55]. Consequently, the methanol bark extract of *D. malabarica* might be used as a potential *in vivo* antidiabetic agent. The α-amylase inhibitory activity in methanol extract is most likely to be due to polar compounds and is worth investigating further and isolating pure active compounds [56].

3.4.4. Antibacterial Activity. In the course of evaluation for antibacterial activity by the disc diffusion method, all the *D. malabarica* extracts presented different degrees of antibacterial activity ($9.50 \pm 1.2 - 19.25 \pm 1.9$) mm against *E.coli*, the

Table 5: Antimicrobial activity of different *D. malabarica* solvent extracts.

Extracts	Solvents		*Mean ± S.D.	MIC Concentration	Mean		*Mean ± S.D.	MIC Concentration	Mean
Ciprofloxacin (250 mg)	Standard		39.50 ± 2.3				45.50 ± 0.9		
Root	Methanol		11.50 ± 2.0	90%	9.25		11.25 ± 0.9	90%	10.00
				70%	Nil			70%	Nil
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
	Ethanol		9.50 ± 1.2	90%	9.25		13.25 ± 0.9	90%	12.00
				70%	Nil			70%	9.00
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
Stem	Methanol		11.75 ± 1.7	90%	9.75		15.25 ± 1.2	90%	9.75
				70%	Nil			70%	Nil
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
	Ethanol		10.25 ± 1.5	90%	9.25		9.75 ± 0.9	90%	9.25
				70%	Nil			70%	Nil
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
Bark	Methanol		12.00 ± 1.8	90%	9.50		30.25 ± 0.9	90%	12.75
				70%	Nil			70%	9.50
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
	Ethanol	E. coli (gram-negative bacteria)	11.50 ± 2.0	90%	9.25	*Streptococcus* (gram-positive bacteria)	15.75 ± 1.7	90%	14.00
				70%	Nil			70%	10.75
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
Leaves	Methanol		11.75 ± 1.5	90%	9.5		17.00 ± 1.4	90%	11.75
				70%	Nil			70%	Nil
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
	Ethanol		11.5 ± 1.9	90%	9.25		15.75 ± 0.9	90%	12.50
				70%	Nil			70%	9.25
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
Unripe fruit	Methanol		10.50 ± 1.5	90%	14.25		18.25 ± 1.7	90%	12.75
				70%	12.25			70%	9.25
				40%	9.75			40%	Nil
				10%	Nil			10%	Nil
	Ethanol		19.25 ± 1.9	90%	9.75		14.25 ± 0.9	90%	9.50
				70%	Nil			70%	Nil
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil
Ripe fruit	Methanol		13.25 ± 1.2	90%	10.5		14.25 ± 1.2	90%	9.75
				70%	Nil			70%	Nil
				40%	Nil			40%	Nil
				10%	Nil			10%	Nil

Table 5: Continued.

Extracts	Solvents	*Mean ± S.D.	MIC Concentration	Mean	*Mean ± S.D.	MIC Concentration	Mean
	Ethanol	13.50 ± 1.9	90%	10.25	10.25 ± 1.5	90%	9.25
			70%	9.25		70%	Nil
			40%	Nil		40%	Nil
			10%	Nil		10%	Nil

*Values are mean of duplicates of zone of inhibition (mm).

Table 6: Antifungal potential of different *D. malabarica* solvent extracts.

Extracts	Solvents	*A. niger*	*Mean ± S.D	*A. flavus*	*Mean ± S.D
Novidate (500 mg)	Standard		20.25 ± 0.5		20.50 ± 0.5
Root	Methanol		11.75 ± 0.2		9.75 ± 0.2
	Ethanol		10.50 ± 0.1		9.25 ± 0.5
Stem	Methanol		9.50 ± 0.5		10.00 ± 0.8
	Ethanol		12.75 ± 0.5		13.00 ± 1.8
Bark	Methanol		17.50 ± 0.3		18.25 ± 0.2
	Ethanol		12.25 ± 0.7		15.00 ± 0.8
Leaves	Methanol		10.00 ± 0.8		12.25 ± 0.2
	Ethanol		14.75 ± 0.5		15.00 ± 0.1
Unripe fruit	Methanol		13.50 ± 0.5		15.25 ± 1.7
	Ethanol		10.00 ± 0.8		13.75 ± 0.7
Ripe fruit	Methanol		11.75 ± 0.7		12.75 ± 0.2
	Ethanol		14.25 ± 0.2		16.25 ± 0.1

*Values are mean of duplicates of zone of inhibition (mm).

Table 7: Conclusive view of biological activities of *D.malabarica* extracts.

Extracts	Solvents	Biological activities				
		Anti-inflammatory	Anticancer	Antidiabetic	Antibacterial	Antifungal
Root	Methanol	++	++	++	++	++
	Ethanol	+	+	+	+	+
Stem	Methanol	++	++	++	++	+
	Ethanol	+	+	+	+	++
Bark	Methanol	++++	++++	++++	++++	++++
	Ethanol	++	++	++	++	++
Leaves	Methanol	++	+	++	++	+
	Ethanol	+	+	+	+	++
Unripe fruit	Methanol	++	+	++	+	+
	Ethanol	+	+	+	++	++
Ripe fruit	Methanol	++	+	++	++	+
	Ethanol	+	+	+	+	++

(+) and (+++) signs indicate the low intensity and high intensity of biological activities, respectively.

gram-negative bacteria, and (9.75 ± 0.9 – 30.25 ± 0.9) mm against *streptococcus*, the gram-positive bacteria (Table 2). The methanol bark extract displayed the strongest zone of inhibition against *streptococcus* (30.25 mm) while ethanol unripe fruit extract exhibited (19.25 mm) against *E.coli* amongst all extracts while the zone of inhibition reflected by standard ciprofloxacin 250 mg was 45.5 mm. Antibacterial action was discovered to be more articulated against gram-positive microscopic organisms contrasted with gram-negative. This discloses itself to the earlier investigations demonstrating that plant extracts have greater ability against gram positive microorganisms than gram-negative [57]. In this overall

antimicrobial study, the methanolic extract exhibited strong activity than petroleum ether extract as investigated previously [58]. Methanol showed more reliable and protuberant antibacterial activity as compared to ethanol extracts (Table 5). The minimal antibacterial potency in ethanol extracts might be due to presence of lower concentration of antibacterial components in these plant extracts [39]. A number of studies have been executed that ascribe the phenolics to kill microbes. It can be ascribed from the fact that high content of phytochemicals present in plant extracts might be reflected as the source for antimicrobial potency as appealed earlier that plants rich in tannin and saponin have intense antimicrobial action [59]. Antibacterial activity of medicinal plants was reported by several investigations [60] which supported our research finding. The antibacterial activity has been reported earlier in *D. malabarica* leaves against various diseases [5] while our study revealed significant antibacterial activity in the methanol bark extract which may be attributed for similar trials. The MIC values of different parts of *D. malabarica* are shown in Table 5. MIC of all extracts was found in 90%, 70%, 40%, and 10% concentrated sample against *E. coli* and *Streptococcus.* The inhibitory zone was shown at 90% while no inhibition was found at a concentration of 10% which may be due to absence of antibacterial components at lower concentratins in plant extracts. Accordingly, our current study is supported by earlier literature conclusions that antibacterial activities are directly related to increasing the concentration (%) of extracts [61]. Although various classes of phytochemical constituents were described showing antimicrobial capabilities, however, they are not recognized as tonic by medical community. In current finding, worthy antibacterial action was displayed by methanol bark extract against the gram-positive bacteria which is as per the previous investigation which revealed the methanol extract to have the most extreme flavonoid and phenolic levels and showing more articulated antibacterial activity contrasted with other solvent extracts [62].

3.4.5. Antifungal Activity. The current study assesses the antifungal activity of *D.malabrica* extracts. Ethanol and methanol extracts of *D. malabarica* plant parts were screened for their antifungal activities against fungal strains *A. niger* and *A. flavus* (Table 6). Here, methanol bark extract showed the highest 18.25 mm zone of inhibition against *A. flavus* and 17.50 mm against *A. niger* (Table 7) which was found very close to novidate 500 mg (20.25 mm), the standard antifungal drug. The obtained results indicated that extracts of *D. malabarica* were found not to be inactive against *A. niger* and *A. flavus* even at low concentrations of 10 mg/mL and showed antifungal activity but methanolic bark extract amongst other extracts of *D.malabarica* comprises strong antifungal activity against microorganisms that may be due to the presence of tannins, saponins, flavonoids, terpenoids, and phenolic compounds in bark extract as indicated in the previous study that saponins might be attributed to fight fungal microbes [63]. The phytochemicals including flavonoids and phenolic compounds found in plants as secondary metabolites may be responsible of antimicrobial activity [64]. Accordingly, earlier reports supported our research finding regarding potent antifungal activity possessed by methanol bark extract *D. malabarica* [65]. This may be because of the used type of solvent for extraction as portrayed earlier that methanol is the more effective solvent for extraction of bioactive compounds particularly antimicrobial components from therapeutic plants when contrasted with different solvents as well as water [61].

4. Conclusions

D. malabarica bark extract attained from the methanol, a polar solvent, showed a significant measure of phytochemicals than less polar or nonpolar solvents. Methanol is the more effective solvent for extraction of bioactive compounds particularly antimicrobial components from therapeutic plants when contrasted with different solvents. In this way, it is predicted that in the future drugs emerging from these phytochemicals will be entity of a developing interest for infection and oxidative stress related illnesses. We believe that the recognizable proof of the potential medical advantages of phytochemicals might be a vital factor to give further bits of knowledge into the revelation of medications or effective nutrition. So, *D. malabarica* bark being rich in various phytochemical compounds with various healing tendency can be utilized as a potent source of natural antioxidants with other curative applications including antihepatotoxicity. *D. malabarica* bark might be screened further against different infection causing microorganisms and can be an expected source of biologically significant medication competitors.

Data Availability

All the data relevant to this study is mentioned in the manuscript. There is no supplementary data.

Conflicts of Interest

All the authors of this paper declare no conflict of interest.

Acknowledgments

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Retraction

Retracted: Toxicological and Teratogenic Effect of Various Food Additives: An Updated Review

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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Review Article

Toxicological and Teratogenic Effect of Various Food Additives: An Updated Review

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Scientific evidence is mounting that synthetic chemicals used as food additives may have harmful impacts on health. Food additives are chemicals that are added to food to keep it from spoiling, as well as to improve its colour and taste. Some are linked to negative health impacts, while others are healthy and can be ingested with little danger. According to several studies, health issues such as asthma, attention deficit hyperactivity disorder (ADHD), heart difficulties, cancer, obesity, and others are caused by harmful additives and preservatives. Some food additives may interfere with hormones and influences growth and development. It is one of the reasons why so many children are overweight. Children are more likely than adults to be exposed to these types of dietary intakes. Several food additives are used by women during pregnancy and breast feeding that are not fully safe. We must take specific precaution to avoid consuming dangerous compounds before they begin to wreak havoc on our health. This study is intended to understand how the preservatives induce different health problem in the body once it is consumed. This review focuses on some specific food additives such as sodium benzoate, aspartame, tartrazine, carrageenan, and potassium benzoate, as well as vitamin A. Long-term use of food treated with the above-mentioned food preservatives resulted in teratogenicity and other allergens, according to the study. Other health issues can be avoided in the future by using natural food additives derived from plants and other natural sources.

1. Introduction

Food additives are used in the product and processing of nearly all types of food to give desirable rates. Simply said, it is a material that is added to food to ameliorate its flavor, appearance, or other desirable characteristics. Some of the food additives are listed in Table 1. Food additives are defined by the US Public Exploration Council's food protection commission as "a substance or a mixture of substances that is present in a food as a result of an aspect of product, processing, storage, or packaging." Teratogens are chemicals that, when exposed to a pregnant woman, can cause physical or functional defects in a human embryo or fetus. Its examples include alcohol and cocaine. The length of exposure, the amount of teratogenic material present, and the stage of development of the embryo at the moment of exposure all have an effect on the embryo [1].

Teratogens can affect an embryo in a variety of ways, including physical deformities and behavioural or mental disorders and a reduction in the child's intellectual quotient. It can also lead to problems including unseasonable labor, robotic revocations, and deliveries and detriment embryo. Physical agents, metabolic circumstances, infection, and incipiently drugs and chemicals are the four orders of teratogens [2].

Food complements are composites that food manufacturers add to food in small quantities during product or processing to ameliorate the food's organoleptic parcels similar as flavor, appearance, and taste [3]. They contribute to the food's shelf life by icing product thickness, healthiness, and

Table 1: Safety evaluation of certain food additives with examples and INS number.

S. no.	Functional class	Description	Example of food additive	INS no.	Acceptable daily intake (ADI)	Median lethal dose (LD_{50})
1.	Preservative	Prolong the shelf life of foods by protecting them against deterioration caused by microorganisms.	(a) Sodium benzoate (b) Potassium benzoate	211 212	0-5 mg/kg bw 0-5 mg/kg bw	Oral rat >4070 mg/kg [92] Rat >10,000 mg/kg [93]
2.	Sweeteners	Impart a sweet taste to foods or in tabletop sweeteners.	Aspartame	951	40 mg/kg bw	Oral rat >10,000 mg/kg [94]
3.	Stabilizers	Maintain the physicochemical state of a food stuff.	Carrageenan	407	75 mg/kg bw	Oral rat >5,000 mg/kg [95]
4.	Colouring agents	Add or restore colour in a food and include natural constituents of foods and natural sources.	Tartrazine	102	0-10 mg/kg bw	Mouse >6250 mg/kg [96]

newness. They make a variety of accessible foods available without the stress of grocery shopping or cuisine on a diurnal base. The complements must be added in precise quantities and should be within the diurnal consumption limits.

Some complements, similar as those used to save food, have been used for glories pickling (preservation with ginger) and salting, the preservation of sweets, and the usage of sulphur dioxide in wines. With the preface of reused foods in the alternate half of the twentieth century, numerous further complements were introduced in the alternate part of the twentieth century. Both natural and artificial origins have been added [4].

Food complements can be employed in a number of ways, both directly and laterally. Direct complements are those that are added to foods for a definite purpose, whereas circular complements are those that are added after the fact during manufacturing, puddings, yoghurt, and biting goods [5].

Numerous food complements are listed on the component marker of a product. Some food complements may enter the food in trace quantities during quilting, storehouse, or handling that are known as circular food complements. Some colourings, similar as erythrosine, are exemplifications (red); Colourants like erythrosine (red) and canthaxanthine (unheroic orange) give dishes a nice appearance that attracts guests despite the fact that they do not provide any nutrients [6]. Some food complements that show teratogenicity are described below.

2. Methodology

Required data were searched/collected from the online databases as described by researcher [7] including Wiley, Google, PubMed, Google Scholar, ScienceDirect, and Scopus. Keywords used in search are in toxicological evaluations, food additives, and teratogenicity. Latest published data were selected.

3. Results and Discussion

3.1. Preservatives as Teratogens. A preservative is any material able to prevent, slow down, or stop the growth of microorganisms, as well as any food deterioration caused by microorganisms. Preservatives can be antimicrobial, inhibiting the growth of bacteria or fungi, or antioxidants, which operate similarly to oxygen absorbers by inhibiting the oxidation of food constituents. Traditional preservatives also include natural compounds such as sugar, ginger, alcohol, and diatomaceous earth. Calcium propionate, sodium nitrate, sodium benzoate, sodium nitrite, sulfites (sulphur dioxide, sodium bisulfite, potassium hydrogen sulfite, etc.), and disodium are examples of common chemical preservatives [8]. The preservatives that cause teratogenicity are noted below.

3.1.1. Sodium Benzoate. Sodium benzoate (Figure 1) is a synthetic food preservative, which is extensively used in food, medicinal, and cosmetics diligence. The chemical is a sodium salt of benzoic acid that is safe to eat and apply to the skin. It should not be used in some acidic products because it can interact with other chemicals to induce dangerous composites, but it is not poisonous or irritable to tissue. It dissolves easily in water, and its inclusion in food is approved as it is able to prevent the growth of molds and other microbes. The negative effects of sodium benzoate on health have been established, including cellular damage. Numerous research has been conducted in recent years on the use of natural elements with various purposes in food, with some success, but there is still a need to study in the food sector. The teratogenic impact of sodium benzoate is discussed below with the assistance of a study of fetal deformations due to long-term consumption of sodium benzoate in pregnant BALB/c mice [9].

(1) Mechanism of Action. A high dosage of sodium benzoate can induce histamine to be released from the body. H1

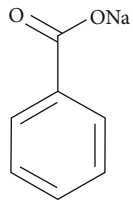

Figure 1: Chemical structure of sodium benzoate.

receptors are affected by mast cell granules and histamine accessible in endothelial cells, leading to a rise in artery diameter leaking of elements of blood plasma and its permeability in the tissues. As a result, it is suitable to assign hemorrhages, and physical tissue damage reported in sodium benzoate affects the skin of embryos by this way. Sodium benzoate enters into blood vessels and interfere with genes, which is invloeved in the synthesis of blood clotting factors [10]. The studies have also shown the cytogenetic goods of benzoic acid sodium salts in lymphocytes [11]. In mice, exposure to methylnitrosourea (MNU) caused proliferating cell damage via macromolecule alkylation and the production of reactive oxygen species (ROS). Elevated ROS levels in mice lowered the severity of retinal abnormalities and, though unknown mechanisms, inhibited fetal Pax-3 gene expression, which is crucial in neural tube development blockage [12]. In proliferating embryonic tissues of rats, increased ROS suppresses the expression of the bcl-2 (antiapoptotic) gene. Rajadurai and Prince (2006) have reported that elevated production of ROS in the biological system showed adverse effects on nuclei acids [13].

Some benzoate derivations, such as SB, appear to be free radical scavengers in humans [14], whereas other mechanisms possibly causing embryonic hemorrhage and eye tissue disorders after PB exposure may result from the induction of potentially harmful ROS situations in embryonic tissues (such as the eye) and inhibit embryonic gene expression, such as Pax-3 or alternative genes required for blood clotting. Our study's findings are related to hyperkalemia, which has been associated to intraventricular hemorrhage in premature neonates. Researchers hypothesized that low systemic blood flow, as measured by lower urine output and K+ secretion, could contribute to the development of hyperkalemia in premature neonates [15]. It is also believed that the hemolytic activity of the limited intraventricular blood clot contributes to an intraventricular K^+ load surplus [16].

(2) Adverse Effects. The current study's findings on the teratogenic effects of sodium benzoate on embryo revealed a variety of defects, including craniofacial deformities. Mandibular hypoplasia and other forms of mandibular hypoplasia are the most common calvarias deformity and vertebral column deformation. Scoliosis and neural tube defects (NTDs) are two examples of this. It had been argued in a study that administering high doses of antibiotics on a daily basis sodium benzoate has the potential to be genotoxic and teratogenic modifications in the neurological system [17]. The other severe impacts also include on growth factors, cell cycle, and gene expression, as well as the fact that it can cause deformations during birth. Food additives in general must be reevaluated as needed in light of new information shifting usage situations and fresh scientific findings information.

3.1.2. Potassium Benzoate. Potassium benzoate (Figure 2) is an odorless white powder made by heating benzoic acid and potassium salt together. Benzoic acid is a naturally occurring chemical found in plants, animals, and fermented foods. It was once made from the benzoin resin of particular tree species, but it is now largely made in factories. Salt beds or minerals are the most common sources of potassium salts. PB is used as a preservative because it prevents bacteria, yeast, and mold from growing. As a result, it is frequently used to increase the shelf life of food, beauty, and skin care goods. The teratogenicity of potassium benzoate is defined by an experiment "Teratogenic Effects of Long-term Consumption of Potassium Benzoate on Eye Development in BALB/c Fetal Mice" [18].

(1) Mechanism of Action. It has also been observed that potassium benzoate suppresses intracellular protein and DNA synthesis at doses below 100 pg/ml and over 500 pg/ml. The study found that benzoic acid (200 and 500 pg/ml) increases chromosomal abnormalities, sister chromatid exchanges, and micronucleus prevalence in human cells without changing the medium pH [19]. There is some suggestion that a mutation in the homeobox gene, OTX2, may also be implicated in the development of these symptoms. The expression pattern of the OTX2 gene in human embryo is also linked to bilateral anophthalmia to certain retinal consequences and pigmentary retinopathy [20]. Because PB can be mutagenic, various studies have been done to precisely investigate the potential teratogenic effects of the food preservative, PB, on the embryonic eye development in pregnant mice. The findings showed that pregnant mice exposed to PB experienced severe bleeding of the embryonic eye, a malformed lens, and retinal folds with underdeveloped layers. Because of the complexity of eye development, many congenital anomalies occur, but most of them are not common [21].

Congenital eye abnormalities can be caused by a variety of environmental teratogens. Some studies have found a link between the use of some antiepileptic medicines during pregnancy and congenital eye malformations such as anophthalmia, microphthalmia, or coloboma of the iris or optic disc [22]. Defects in the PAX6 gene expression cause aniridia-like iris abnormalities [23], corneal opacities, and lens-corneal adhesions similar to Peters' anomaly [24]. Detailed histologic examinations of neonatal mice with Peters-like abnormalities indicated that the lens commonly fails to detach from the cornea [25]. Furthermore, the study found that OTX2 loss-of-function mutations are linked to a wide range of ocular phenotypes, from bilateral anophthalmia to mild microphthalmia with retinal abnormalities [26].

Alqahtani et al. have investigated the cytogenetic effects of potassium salts of 1-p-(3-methyltriazeno) benzoic acid

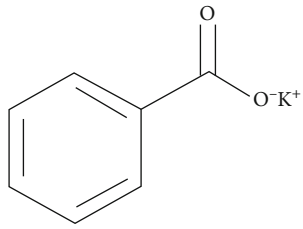

Figure 2: Chemical structure of potassium benzoate.

in human lymphocytes [7]. There was also a dose-dependent increase in chromosome breakage in human cells after exposure to 1-p-(3-methyltriazeno) benzoic acid potassium salts [27]. Dose-dependent administration of toxicant showed increased toxicity and reaches the saturation level at certain doses. As a result, PB and reduced cell proliferation, as well as PAX6 and OTX2 mutation, delayed normal eye development.

(2) Adverse Effect. The potential teratogenic effects of PB on embryonic eye development were investigated in the study. As a result of the exposure of pregnant mice to PB, severe bleeding of the embryonic eye, malformed lenses, and retinal folds with underdeveloped layers were observed. Many congenital defects occur as a result of the intricacy of eye development; however, the majority of them are uncommon [28]. Congenital eye abnormalities can be caused by a variety of environmental teratogens. Some researchers have discovered clear evidence of a link between antiepileptic drug use during pregnancy and congenital eye malformations including anophthalmia and microphthalmia [29].

Mice treated to methyl nitrosourea (MNU) during pregnancy suffered harm to proliferating cells due to macromolecule alkylation and reactive oxygen generation. Increased ROS in mice reduced the severity of retinal problems and blocked fetal Pax-3 gene expression, which is important for neural tube development, through unknown processes [30]. Other mechanisms that may cause embryonic bleeding and eye tissue abnormalities after PB exposure include the generation of potentially harmful ROS levels in embryonic tissues (such as the eye) and the inhibition of embryonic gene expression such as Pax-3 or alternative blood clotting genes. The above findings are comparable to those of hyperkalemia, which has been linked to intraventricular hemorrhage in premature babies. In our research, we discovered that PB-exposed mouse fetuses have significantly reduced weight and crown-rump lengths. Adult mice subjected to 280 and 560 mg/kg/day of PB for 20 days showed no adverse effects. As a result, it is thought that mouse embryos are more vulnerable to PB than adults. The above findings imply that PB has teratogenic effects on mouse fetuses' ocular development. Thus, further detailed investigations on its specific and general impacts are required [31].

3.2. Sweeteners as Teratogens. Sweeteners are defined as food additives that are used or intended to be used either to conduct a sweet taste to food or as a tabletop sweetener. They are substances of low energy value that give sweet taste but do not have the calories of carbohydrates or their cariogenic or glycemic goods. Sweeteners are classified as either high intensity or bulk. High-intensity sweeteners retain a sweet taste but are noncaloric, give basically no bulk to food, have lesser agreeableness than sugar, and are thus used at veritably low situations [32].

On the other hand, bulk sweeteners are generally carbohydrates, furnishing energy (calories) and bulk to food. Other sweeteners are used to keep the food's energy (calories) low, and they are typically suggested for diabetic's people, dental decay, and diarrhoea so that the blood sugar situations will not rise. Sweeteners are classified as natural and synthetic. The natural ones are the most nutritional salutary sweeteners like sucrose, fructose, lactose, and maltose. The synthetic sweeteners because of their violent agreeableness are called high energy sweeteners (HPS), e.g., certain proteins and terpenes glycosides like saccharin, cyclamates, aspartame, and acesulfame-K [33].

Artificial sweeteners are substantially man-made chemicals that are not found in nature. Such chemicals can be dangerous to human body. It can cause ingestion and other health-related issues. The most contraversial artificicial sweetner, aspartame (Figure 3) discussed below, may cause neurological damage especially in younder children where brain is still developing. It breaks down in the body to phenylalanine, and it may also contribute to obesity. Its metabolites can be toxic to many organs, although there have been a few investigations on the use of aspartame during pregnancy, which may result in deformities. Using chick embryos as a model, this study investigated how aspartame serves as a teratogenic agent on development. It is noncaloric (4 kcal/g) and not suited for diabetics. Aspartame is also teratogenic and is used as an addition in baking items. The research Teratogenic effect of Aspartame Exposure on Chick Embryonic Development possibly describes the teratogenic effect of aspartame [34].

3.2.1. Mechanism of Action. Despite the Food and Drug Administration's approval of L-aspartyl-L-phenylalanine methyl ester (aspartame), there are still worries about the safety of this commonly used low-calorie sweetener. One source of worry is the potential impact of aspartame on embryo and child development. Because the placenta can concentrate amino acids in fetal plasma, almost twice the concentration seen in the mother's plasma, developing fetuses may be especially vulnerable to the effects of aspartame [35]. Aspartame is rapidly degraded after ingestion into its two main amino acids, phenylalanine (about half of aspartame by weight) and aspartate (about forty percent). Because children have relatively lower body weights, their dose levels in terms of body weight could be significantly higher than adults ingesting equivalent amounts of aspartame-containing meals [36]. Children and infants may be more sensitive to the effects of aspartame than adults due to variations in their physiological responses to its constituent amino acids. Protein synthesis has been demonstrated to be reduced by high amounts of phenylalanine.

During infancy, when brain protein synthesis is critical, the phenylalanine component of aspartame's potential

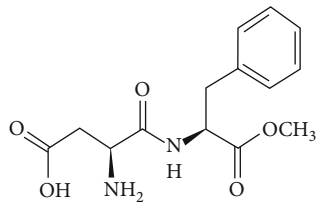

Figure 3: Chemical structure of aspartame.

harmful effects may be amplified. When given intraperitoneally at 1000 mg/kg body weight, phenylalanine was demonstrated to reduce protein synthesis in the brains of baby rats [37]. Intraperitoneal injection of phenylalanine (2000 mg/kg body weight) affects protein synthesis in newborn mice, but not in older mice [38]. The reduction in brain protein synthesis has also been linked to high amounts of other amino acids. During brain development, hyperphenylalaninemia can diminish myelin production, as seen by decreased incorporation of methionine into myelin proteins [39], decreased total lipid content, decreased myelin yield, [40], and overall lower brain weight [41]. Aspartame and its constituent amino acids have been studied for their impact on baby development. Infant mice were demonstrated to have brain damage after being intragastrically intubated with high doses of aspartate [42].

The women are deficient in an enzyme that permits them to digest the amino acid phenylalanine, which is a component of aspartame. If they consume aspartame, they may develop excessive levels of phenylalanine in their blood, which can result in birth abnormalities. The cytotoxicity of aspartame-derived methanol and its metabolite formaldehyde adducts was exerted through functional change of proteins and DNA mutations, resulting in brain damage, growth retardation, abnormalities, and death of cells. Further, oxidative stress may be the cause of nuclear damage [43].

3.2.2. Adverse Effect. The total amount of chick embryos in all experimental groups exhibited retardation of brain formation in all three sections, as well as brain flexure (only cephalic flexure and microphthalmia and no branchial flexure), anencephaly, anophthalmia, aberrant cardiac looping, tail degeneration, and node degeneration. Limb buds and somites slow down only half of the body's development [44]. At high aspartame concentrations, there were obvious adverse effects such as growth retardation, shrinkage, tail deformities in developing embryos, and physiological changes [45]. The administration of aspartame to rats implies that aspartame administration during pregnancy slows fetal growth due to cell damage during this time [46]. The outcomes of this study clearly demonstrated that aspartame concentrations increase, resulting in many observable defects and deformities in chick embryonic development at high concentrations. As a result, pregnant women should avoid consuming aspartame for the sake of their own safety.

3.3. Stabilizers as Teratogens. These substances help to improve and stabilize food texture, help crystallization (sugar and ice), and keep emulsions and foams stable to make icings on baked goods less sticky, and flavors are encapsulated. Polysaccharides, similar as Arabic gum, are thickeners. Thickeners include polysaccharides such as Arabic gum, agar-agar, alginic acid, starch and its derivatives, carrageenan, and pectin. One noncarbohydrate material that is constantly employed for this purpose is gelatin. Hydrophilic stabilizers and thickeners are diffused in water colloids as a solution [47]. These thicken meals by swelling in hot or cold water. Gravies, pie fillings, cutlet condiments, jellies, puddings, and salad dressings are just the many examples of what you can make. Thickeners are added to make the mix thicker density without affecting its other characteristics significantly. In milk and dairy products, the food additives that cause teratogenicity are discussed below. The stabilizers used in dairy product cause teratogenicity. Carrageenan is one of the examples for stabilizers used in dairy products and its teratogenic effect is noted below.

3.3.1. Carrageenan. Carrageenan (Figure 4) is a type of natural linear sulfated polysaccharide found in red edible seaweeds. Chondrus crispus (Irish moss) is a dark red parsley-like plant that grows adhering to the rocks and is still most important red seaweed utilized for creating the hydrophilic colloids necessary to make carrageenan. Because of their gelling, thickening, and stabilizing qualities, carrageenan is frequently employed in the food business. Because of their high binding to dietary proteins, they are mostly used in dairy and meat products. Carrageenan which mimics the native glycosaminoglycans (GAG) has emerged as a viable candidate in tissue engineering and regenerative medicine applications in recent years [48]. Tissue engineering, wound covering, and medication delivery are the most common applications. Carrageenan is a biopolymer derived from algae that is widely utilized in the food industry to generate gels and emulsions in order to stabilize fat in milk, ice cream, and milkshakes.

(1) Mechanism of Action. In the current study's experimental conditions, lambda carrageenan has a teratogenic effect on chicken embryos. These findings are consistent with those of Hunt ('51), who discovered that injecting sucrose into albumen caused a variety of abnormalities of the hen's egg. Some sugars (mono-, di-, and trisaccharides) can cause abnormalities in chicken embryo. Carrageenan induced defects in chick embryos that were similar to those induced by a number of other teratogenic substances. The current findings reinforce the notion that the neural tube and embryonic axis are extremely vulnerable to teratogenic chemicals, while it is still in the early phases of development [49]. Furthermore, because lambda carrageenan elicited anomalies that were comparable to or identical to those provoked by a variety of other chemical and physical agents, it can be assumed that suggested that the pathophysiology of most, if not all, of them is governed by a similar mechanism.

The teratogenic impact of lambda carrageenan on the chick embryo can be explained by at least two mechanisms: (1) carrageenan can bind to cell surfaces, preventing regular

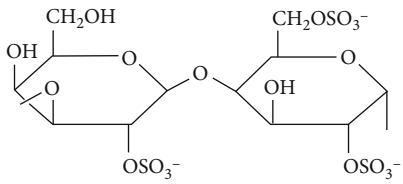

Figure 4: Chemical structure of lambda carrageenan.

cell activity and morphogenesis; or (2) this polygalactoside could be broken down into simple sugars like galactose, which has teratogenic properties. Both hypotheses are supported by evidence. In terms of carrageenan's direct influence on cell surfaces, cell-surface components play a role in cell-to-cell contacts and embryonic morphogenesis.

In terms of carrageenan's direct influence on cell surfaces, cell-surface components are involved in cell-to-cell contacts and embryonic morphogenesis [50]. On the other hand, erythrocytes have been shown to bind sulfated polysaccharides like carrageenan [51]. There is evidence that surface glycosyltransferases catalyse the glycosylation of the terminal end of the matching sugar chain [52]. Carrageenan may interfere with the linking of the terminal sugars of glycoconjugates on the cell surface.

(2) Adverse Effects. Strong acid phosphatase activity has been related to a process mediated by carrageenan degradation products in the chick's blastoderm border, with distinct enzymatic activity in yolk globules, implying that the latter corresponds to specific structures [53]. Galactose was likely released as a result of the breakdown of carrageenan in the embryo or in cells of the area vacuoles, or both, causing the abnormalities reported therein. The lambda carrageenan-injected group had partial duplication of the body, aberrant trunk flexures, anencephaly, a severely deformed brain, thickening of the neural tube wall, and an irregular neural tube lumen with segmentary occlusion [54]. Carrageenan caused abnormalities in chick embryos that were similar to those caused by a variety of other teratogenic chemicals. The current findings back up the theory that the neural tube and embryonic axis are extremely vulnerable to teratogenic chemicals at early stages of development [55].

3.4. Colouring Agents as Teratogens. Colour additives are any dye, pigment, or material that can be used to colour food and cosmetics (either alone or in combination with other substances) [56]. Colourants are compounds added to colour food or correct the colour of food, according to the Codex Alimentarius Commission (CAC). Furthermore, colourants are used to restore the natural colour of the food that has been lost during processing and storage, to improve the current colour, to strengthen the weak colour, to colour food that is truly colourless, and to win customers by concealing substandard quality [57]. Natural and synthetic sources are used to categorise them. Plant and animal animals, as well as microbes, produce natural colouring substances, for example annatto, anthocyanin, carotenes, and lycopenes. Synthetic colourants are compounds that are not found in nature and are created through chemical synthesis: Allura red, tartrazine, erythrosine, sunset yellow, and more colours [58].

Synthetic food colourants outperform natural food colourants in terms of colouring ability, colour tone, colour distribution, brightness, stability, and ease of use [59]. Synthetic food colourants, on the other hand, have long been thought to have a negative impact on human health and children's behaviour, such as behavioural disorders, hyperactivity, and attention impairments, all of which exhibit significant individual differences in children [60]. The teratogenic effect of tartrazine is discussed below.

3.4.1. Tartrazine. Tartrazine (Figure 5) is a chemical dye having the formula 3-carboxy-5-hydroxy-1(p-sulfophenyl)-4-carboxy-5-hydroxy-1(p-sulfophenyl)-4-carboxy-5-hydroxy-1(p-sulfophenyl)-4-carboxy-5-hydroxy-1(p-sulfophenyl)-4-carboxy-5-hydroxy-1(p-sulfophenyl)-4-carboxy-5- hydroxy-1(p-sulfophenyl)-4-carboxy-5-hydroxy-1((sulfophenylazo) pyrazolone E 102, FD & C Yellow No. 5 [61]. To produce a yellow colour, it is commonly used as a food colourant in sweets, juices, jams, mustard, and sodas [62]. The work "Embryotoxic and Teratogenic Effects of Tartrazine in Rats" describes tartrazine's teratogenicity [63].

(1) Mechanism of Action. TAZ is the most commonly used food dye. TAZ causes hepatonephrotoxicity and changes many metabolic characteristics in experimental animals at doses several times greater than its ADI for humans, according to past studies [64]. Furthermore, TAZ causes leucocyte DNA damage in rat liver and kidneys, as well as severe cellular changes, which could have negative health consequences [65]. The stage of embryonic development is crucial in defining the embryo's susceptibility to the drug and, as a result, its response pattern.

A recent study found that nearly all medicines given during pregnancy enter the fetal blood via passive diffusion to some extent [66]. Lipid solubility and molecule size are the two most important elements that influence placental diffusion. The larger the molecular size and solubility of the lipid, the better is the placental transfer [67]. Substances delivered during the cleavage and blastula phases of mammalian embryonic development, as well as before implantation, which happens on the sixth day of gestation in rats, often elicit minimal teratogenic reaction, despite the fact that the same agents evoke noticeable responses when administered at higher dosages later in embryo development [68].

The embryo's vulnerability to teratogenesis decreases as tissue differentiation develops, and once organogenesis is complete, the embryo as a whole is teratogen-resistant [69]. As a result, TAZ administration in this investigation was limited to the critical period of organogenesis, which is the 6th–15th day of gestation, when cellular differentiation takes place and the rat embryo is most vulnerable to external and internal stimuli. The current study examined the effects of TAZ on growing embryo during pregnancy to the control group. It has been suggested that a drug's teratogenic effect can be predicted if it can bypass the placental barrier [70]

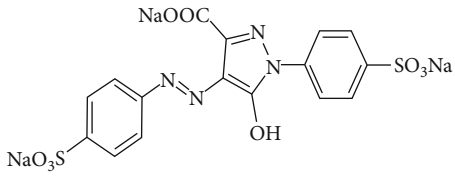

Figure 5: Chemical structure of tartrazine.

or inhibit protein synthesis [71], as well as reduce the activity of the material enzymes [72].

Several factors may contribute to TAZ's effect on fetal growth and development. TAZ is metabolised by intestinal microorganisms into two metabolites: sulfanilic acid and aminopyrazolone [73]. These metabolites are destroyed slowly or not at all, and they can produce reactive oxygen species, which can cause abnormalities in the developing embryo [74]. Many xenobiotics cause oxidative stress, which contributes to their toxicity [75]. Furthermore, synthetic food colouring compounds, such as TAZ, have been shown to decrease mitochondrial respiration in rat liver and kidneys [76] and alter mitochondrial membrane integrity, which is critical for sustaining mitochondrial activities and causing cellular death. As a result, TAZ-induced embryonic deformities could be linked to an increase in apoptosis. This could be the reason for disruption of the mitochondria and inactivation of certain enzymes, which is concerned with the energy metabolism [77].

(2) Adverse Effect. [78]. Tartrazine is considered as a cheaper substitute for natural food colorants like curcumin and saffron and is the second most used food dye, generally derived from coal tar. Cardiomegaly, hepatorenal damage, and splenic pigmentation were also found in the TAZ-treated groups. In this view, dietary additive intake is strongly associated to mutagenicity in the form of gene mutation and chromosomal abnormalities [79]. In this study, the effects of tartrazine on developing embryo during the gestation period were compared to the control group. It has been proposed that a drug's teratogenic effect can be predicted if it can breach the placental barrier, diminish protein synthesis, and inhibit material enzyme function. Administration of toxicants produces multiorgan toxicity, even though it is organ specific [80].

The effect of tartrazine on fetal growth and development could be due to a number of variables [81]. In the fetuses, TAZ increased fetal resorptions and mortality, as well as cardiomegaly, hepatorenal damage, and splenic pigmentation. Missing coccygeal vertebrae, missing sternebrae, missing hind limbs, and unequal ribs were among the skeletal malformations caused by the treatment. TAZ was found to be embryotoxic and teratogenic in rats as a result of these findings [82].

3.5. Fortifying Agents as Teratogens. The practice of adding vitamins and minerals to regularly consumed foods during processing to increase their nutritional value is known as food fortification. It is a tried-and-true, safe, and affordable technique for improving diets and preventing and controlling micronutrient deficiencies. Cereals and cereal-based products, milk and dairy products, fats and oils, accessory food items, tea and other beverages, and newborn formulae are the most commonly fortified foods, according to the FAO. This procedure is used by food manufacturers to increase micronutrients and vitamins in their goods by adding food fortification agents. Food strengthening agents are utilized in staple foods to reduce dietary deficit. To boost the nutrients in dietary food and cereals, food-fortifying agents are used [83]. The increased demand for healthful foods is the primary driver of food-fortifying agents. Because of their changing lifestyles, consumers are becoming more conscious of healthy food products. There are numerous issues that function as roadblocks to the market expansion of food-fortifying agents.

3.5.1. Vitamin A. Vitamin A is an essential component for pregnant mothers and their developing fetus [84]. Vitamin A fortification is thought to function by boosting daily intake and absorption of preformed vitamin A also called retinol (Figure 6) to levels adequate that close the current consumption gap and greatly increase liver reserves, hence correcting vitamin A deficiency and its health and survival implications is important. Since it is involved in cell differentiation, eye integrity maintenance, and the prevention of xerophthalmia, vitamin A plays a critical role in ocular function. Its absence is the leading cause of night blindness in the world. Vitamin A is necessary for the regular development of the embryo, in addition to its crucial role in numerous body tissues [85].

(1) Mechanism of Action. *In vitro* rat embryo studies have revealed that retinoids act directly on the embryo, causing abnormal development [86]. The quantity of active retinoid that accumulates over time (concentration-time relationship) during phases of organ development is important for developing organs. The rate at which retinoids are absorbed by the maternal intestine, maternal retinoid metabolism, the half-life of the retinoid in maternal plasma, and the rate at which retinoids are transferred from the pregnant female to her embryos are all factors that influence the amount of active retinoid in the embryo [87]. The effects of one retinoid may differ from one species to the next; each species has its unique animal tissue; thus, even when exposed to the same amount of retinoid, distinct developmental events may occur. By activating gene transcription in numerous areas of the embryo, retinoic acid aids in the regulation of embryonic development. The cells will only respond to retinoic acid if they have the appropriate receptors and retinoic acid concentrations are within the receptors' tolerable range. Many doctors and pathologists research the precise control of retinoic acid concentrations because varying levels of retinoic acid activate different genes. In the early phases of human embryonic development, notably the fourth week, retinoids help to promote the expression of Hox genes.

Hox genes are involved in the development of the embryo's body plan [88]. There are thirty-eight Hox genes

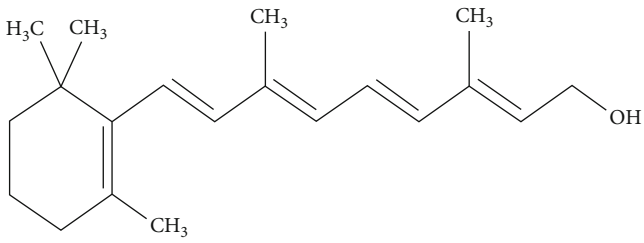

Figure 6: Chemical structure of retinol.

in humans. The Hox gene malfunctions in embryos exposed to too much retinoic acid, disrupting the genetic control of body shape (axial patterning) during development [89]. Developmental abnormalities can result from these changes, notably in the embryonic spinal cord, central nervous system, and spinal cord, where retinoic acid production and catabolism enzymes are found. In 1953, Sidney Q. Cohlan discovered that high vitamin A doses in pregnant rats were linked to teratogens in the progeny.

(2) Adverse Effects. Vitamin A (retinol) is a vital vitamin for human health that aids in the regulation of epithelial tissue cellular development. Deformities in the fetuses' skulls, faces, limbs, eyes, and central nervous system can be observed in cases where pregnant women consume too much vitamin A and retinoid. Retinoids, which are vitamin A derivatives, are also extensively used to treat a range of skin problems, including carcinomas, which are the most common type of cancer in humans. Embryos from a range of animals, including monkeys, rabbits, rats, mice, and hamsters, were studied [90]. Central nervous system abnormalities such as an abnormally small head (microcephaly), incomplete development of the medulla spinalis (spina bifida), an encephalopathy in which some of the brain is found outside of the skull (exencephaly), and brain swelling due to fluid build-up were the most common defects found across all species (hydrocephaly). Facial nerve paralysis, underdevelopment of the upper jaw, cleft palate, cleft lip, nonexistent or malformed ears, and shortened limbs were among the other prevalent abnormalities, according to research [91].

4. Conclusion

The above-mentioned additives have teratogenic effect, so it must be replaced from our daily routine for better health. It can cause various health issues, so it is better to minimize the use of chemical food additives. In contrast to synthetic additives, natural food additives have gained wider acceptance due to their many advantages. According to the present study, chemical food additives can trigger a slew of serious health issues. The troubles that it creates will vary depending on the amount and duration of the preservatives used. Even if some additives do not act directly, they might nevertheless cause or contribute to health problems. The study also revealed how additives work, their mechanism of action, why they cause various health issues, and which health issues are caused by each synthetic food additives. Natural food additives help to extend the shelf life of a product while reducing the possibility of harmful side effects. Hence, natural food additives should be preferred over synthetic food additives.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Cardioprotection by Citrus grandis (L.) Peel Ethanolic Extract in Alloxan-Induced Cardiotoxicity in Diabetic Rats

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Diabetic cardiomyopathy (DCM) pathogenesis is multifarious, and there are insufficient therapeutic options to treat DCM. The present research explored the effects of Citrus grandis peel ethanolic extract (CGPE) in alloxan-induced DCM in rats. Diabetes was triggered by intraperitoneal (i.p.) injection of alloxan (150 mg/kg) in Wistar rats (200-250 g). CGPE (100, 200, and 400 mg/kg) or glibenclamide (Glib, 10 mg/kg) were administered orally for 2 weeks. After the treatment schedule, prooxidants (thiobarbituric acid reactive substances), antioxidants (glutathione, catalase, and superoxide dismutase), and inflammatory markers (tumor necrosis factor-α) were determined in cardiac tissues. Biomarkers of cell death, viz., lactate dehydrogenase (LDH), creatine kinase MB (CK-MB) activity, glucose levels, total cholesterol (TC), and high-density lipoproteins (HDL), were assessed in the blood. Rats administered with alloxan showed a consistent increase in blood glucose level (days 7 and 14) that was lowered considerably ($p < 0.001$) by CGPE or Glib. Alloxan-induced increase in LDH, CK-MB, TC, and decline in HDL was attenuated ($p < 0.001$) in rats that were treated with CGPE or Glib. Alloxan significantly ($p < 0.001$) elevated oxidative stress, inflammation, and reduced antioxidants in the cardiac tissue of rats, and these pathogenic abnormalities were ameliorated ($p < 0.001$) by CGPE. Histopathological studies showed a decrease in morphological disruptions by alloxan in CGPE-treated rats. CGPE (400 mg/kg) significantly ameliorated biochemical parameters in comparison to the lower doses against alloxan cardiotoxicity. Citrus grandis peel extract can be an alternative in the management of DCM.

1. Introduction

At an early point, diabetic cardiomyopathy (DCM) is primarily described by myocardial fibrosis and abnormal remodeling, is linked as diastolic dysfunction and then systolic dysfunction, and results in the progression of acute heart failure [1]. DCM is the major cause of diabetes-induced mortality. While the number of diabetes cases

continues to grow, new approaches to defend against DCM, which are marked by cardiac hypertrophy, accelerated apoptosis, fibrosis, and impaired insulin metabolism, are desperately required [2]. DCM's exact pathogenesis is complicated and multifaceted. However, early causes contributing to diabetic heart cell mutilation are known to be a consequence of different triggers, i.e., persistently increased blood sugar level, reactive oxygen species (ROS), reactive nitrogen species (RNS), extracellular matrix protein upregulation, myocardium fibrosis, protein kinase C instigation, cytokines, and renin-angiotensin system activation [3]. An imbalance between endogenous ROS as well as the antioxidant system has been reported to contribute to the advancement or progression of DCM [4]. Too much ROS generation, i.e., superoxides can contribute to cardiovascular system (CVS) problems [5]. Because the usage of synthetic medicines to treat this disease and its consequences involves tremendous costs for developing nations' economies with unintended side effects, new approaches are desperately needed. It was also concluded by a study that a diet rich in fruits, vegetables, and spices lowers the risk of cardiovascular death [6].

Another study concluded that proinflammatory cytokines are released in conditions that are associated with myocardial inflammation, such as myocardial ischemia, myocarditis, and idiopathic cardiomyopathies. These cytokines are found to be strong inducers of nitric oxide (NO) synthesis in cardiac myocytes. An increased level of NO has been found to be combined with cell death [7]. It has been reported that the advancement of DCM involves disparities between endogenous ROS as well as the antioxidant system [4].

Novel medications for diabetes management that may link the development of hyperglycemia and diabetic disorder are on the lookout. The World Health Organization (WHO) diabetes expert committee has suggested investigating traditional diabetes management treatments and their complication. In Western countries, too, the use of herbal products is booming, as these are known to be safer [8]. Another consideration is that it is time-intensive and extraordinarily costly to identify new chemical entities. WHO reports that 80 percent of Asian and African communities are already utilizing herbal medicines for their health care needs. Around 28 percent of new chemical entities were found to be natural products or their derivatives between 1987 and 2002 [9]. Recent research findings revealed that dieting loaded with fruits, vegetables, and also spice reduces the cardiovascular risk of death [6]. In the discovery of new medicinal agents from plants, phytochemical research based on ethnopharmacological information is generally considered a sound approach [9].

Research findings had shown that several edible plants (e.g., Embelia ribes and Rooibos) [10], phytoproducts (e.g., green tea), and phytoconstituents (e.g., taxifolin and aspalathin) [11, 12] attenuate cardiac dysfunction [13]. Citrus fruits are often identified for their economic value and pharmaceutical properties. The title Citrus is the common floral genus of Rutaceae owing to the tropical and subtropical kinds of Southeast Asia. The most versatile citrus fruits are mandarins (C. reticulate Blanco), pomelos (C. grandis Osbeck), sour oranges (C. aurantium L.), sweet orange (C. sinensis Osbeck), lime (C. aurantifolia Christm), and lemon (C. limon L.). Citrus fruits are famed for their scent, owing in part to flavonoids and limonoids (which in effect are terpenes) found in the peel, and are recognized as being abundant in many phytonutrients that are important to wellbeing endorsement and illness deterrence [14]. Several investigators conclude that the citrus florae exhibit an array of flavonoid components which are the plants' secondary chemical metabolites. Whilst a wide range of bio-like activities existed for some flavonoids, their protective role in living structures was mainly an outcome of their antioxidant potentials owing to transferring ROS, metal catalyst chelation, antioxidants, and colon cancer [15, 16].

Citrus grandis peel extracts have the properties to be a possible therapeutic mediator for the management of DCM owing to their flavonoids, hypoglycemic function, antioxidant activity, and antiapoptosis mechanisms [17]. In the current experiments, the potency of ethanol extract from Citrus grandis peel in alloxan-induced DCM in rats was explored.

2. Methods

2.1. Drugs and Chemicals. Glibenclamide (Glib) was procured from Ind-Swift Pvt. Ltd. (India). Alloxan and sodium carboxymethyl cellulose (CMC) were procured from SD Fine Chem Ltd. (SDFCL), Mumbai. Dibasic sodium phosphate, monobasic sodium phosphate, and diethyl ether (A.B. Enterprises, Mumbai); formalin, Folin-Ciocalteu reagent, thiobarbituric acid (TBA), glutathione (GSH), and dimethylsulfoxide (DMSO) (Hi-Media Lab Pvt. Ltd., Mumbai); ethylenediaminetetraacetic acid (EDTA) (Loba Chemie Pvt. Ltd; Mumbai); ethanol (Indian Chemical Co., Delhi); and aluminium trichloride ($AlCl_3$), sodium cyanide, sodium hydroxide (NaOH), sodium nitroprusside, sodium nitrite ($NaNO_2$), and sodium carbonate (Na_2CO_3) (Sisco Research Laboratory, Mumbai) were procured. Total cholesterol kit, HDL kit, and total protein kit were procured from Avecon Healthcare Pvt. Ltd., Saha (Ambala). The lactate dehydrogenase kit was procured from Reckon Diagnostics Pvt. Ltd., Gorwa, Vadodara. Creatine kinase isoenzyme (CKMB) was procured from Coral Clinical Systems, Industrial Estate, Verna, Goa.

2.2. Investigational Animals. Male Wistar rats (200-250 g; age 5-6 months) were acquired from the National Institute of Pharmaceutical Education and Science, Mohali (Punjab). All the animals were quarantined and their health was monitored for one week. The rats were housed under standard laboratory conditions at the institute's Central Animal Facility (CAF), viz., temperature, $23 \pm 2^{\circ}C$; humidity, $40 \pm 10\%$; and artificial light-dark period, 12 hours each (8 : 00 AM to 8 : 00 PM). The Institutional Animal Ethics Committee (SSP/IAEC/17/02) authorized the investigational procedure of this research, and experiments were conducted as per the guiding principles of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Forests and Climate, Government

of India, and Indian National Science Academy. All the animals were given an acclimatization period of 14 days before the initiation of the experiments. According to the guidelines of CPCSEA, animals were nourished with a normal rodent pellet diet (Ashirwad Industries, Mohali) and clean drinking water ad libitum during the whole duration (56 days) in institutional CAF until euthanasia.

2.3. Plant Material and Formulation of the Extract. Citrus grandis (L.) Osbeck (grapefruit) fruit was collected from Saharanpur (Uttar Pradesh, India) and subsequently used for obtaining the peel. The plant material was validated by Dr. Sunita Garg, Emeritus scientist, Council of Scientific and Industrial Research - National Institute of Science Communication and Policy Research (CSIR-NISCAIR), New Delhi (India), and a specimen was submitted and preserved in this institute for future reference (Ref. voucher no. RHMD/2016/2998-25-1). The plant sample (peel of Citrus grandis) was shade dried and ground. The samples were examined for not containing any of the white flesh under the peel. The ground material (60 g) was extracted for 72 hours using aqueous ethanol (80%) in the Soxhlet apparatus. The extract was sieved (Whatman paper) and concentrated to aridity under low compression in a revolving evaporator at 45-55°C yielding 12.5% (*w*/*w*) peel extract. The extract yield was 18.5%. The extract was kept at -20°C until usage. Peel extract was suspended in 0.5% sodium CMC (doses 100, 200, and 400 mg/kg).

2.4. Total Phenolic Content. The Folin-Ciocalteu test was intended to measure phenolic content from the extract. In the first instance, 3.5 ml of distilled water (H_2O) and 0.25 ml of Folin-Ciocalteu mixture were mixed with 0.25 ml of the extract diluted, and 20% Na_2CO_3 was added after 3 minutes. The test tubes were set at 40°C for 40 minutes before a double-beam spectrophotometer measured the absorbance at 685 nm. The overall phenolic content was measured in mg per g of extracts as an equivalent of gallic acid [18].

2.5. Total Flavonoid Content. In brief, 0.05 ml of crude extract was added with 1 ml of ethanol, 4 ml of distilled H_2O, and 0.3 ml of 5% $NaNO_2$ solution and then incubated. After 5 minutes, 0.3 ml of aluminium trichloride ($AlCl_3$) reagent (10%) was added, and the assay blend was permitted to stand for 6 minutes. Subsequently, 2 ml of NaOH (1 M) was added. With distilled H_2O, the final volume was made 10 ml. The assay blend was permitted to stand for 15 minutes. Optical density was assessed at 510 nm wavelength, and the outcome was articulated as mg rutin equivalent per g extract [19, 20].

2.6. Acute Oral Toxicity Study. To evaluate LD50 (median lethal dose), the Karber technique was used [21]. Different clusters of Wistar rats of either sex ($n = 5$, 200-220 g) were given a single C. grandis peel ethanolic extract (CGPE) dose (10, 100, 300, 2000, 5000, and 6000 mg/kg) by oral gavage. For acute toxicity study, Guideline 420 of the Organisation for Economic Cooperation and Development (OECD): Fixed-Dose Chemicals Test Procedure [22] was followed. Animals were independently monitored after dosing within the first 30 minutes at least once, regularly over the first 24 hours (with extra emphasis provided within the first 4 hours), and activity was observed for 48 hours. Observations involved modifications in skin and hair, eyes, and mucous membrane, as well as gastrointestinal, circulatory, autonomic, and central nervous (CNS) processes, and variations in somatomotor behavior and neurological behavior. Attention was guided to tremors, convulsions, salivation, vomiting, lethargy, fatigue, and coma monitoring.

2.7. Experimental Protocol. Thirty rats were randomly (single-blind method) separated into 2 collections: control ($n = 6$) and diabetic ($n = 24$). Control animals were given 0.5% sodium CMC vehicle and normal saline (5 ml/kg) only for 14 days daily. To induce diabetes, the animals fasted overnight, and a solitary intraperitoneal (i.p.) injection of freshly arranged alloxan (150 mg/kg in 0.9% w/v normal saline) was given on day 1 [23]. After 6 hours of administration of alloxan, glucose solution was provided orally in a feeding tube for 24 hours to resolve the early hypoglycemia arising from immediate major insulin release. After 3 days, 12 hours of fasting blood glucose (FBG) was measured by glucometer (ACCU-CHEK). The second drop of blood collected from the tail vein was used in ACCU-CHEK. Animals with glucose levels > 250 mg/dl were deemed diabetic. Diabetic animals were randomly distributed into following groups: diabetic control (DC), CGPE-L (low dose 100 mg/kg), CGPE-M (medium dose 200 mg/kg), CGPE-H (high dose 400 mg/kg), and Glib (5 mg/kg). C. grandis peel ethanolic extract (CGPE) or glibenclamide (Glib; 5 mg/kg) [24] was administered orally for 14 consecutive days to the diabetic rats. Diabetic control animals received an equivalent volume of 0.5% sodium CMC for 14 days daily. Blood glucose level was checked on days 1, 3, 7, and 14. Initial and final body weights were analyzed.

2.8. Biochemical Parameters. The blood sample was collected under light anesthesia (diethyl ether) by the sinocular puncture method in EDTA-containing tubes. The serum was detached by centrifugation of the blood sample at 2500 × g for 5 minutes at 4°C. Clear serum was used for the measurement of total cholesterol (TC), high-density lipoproteins (HDL), lactate dehydrogenase (LDH) rate, and creatine kinase (CK) rate. Lactate dehydrogenase (LDH) and creatine kinase MB (CK-MB) were determined as per the procedure given in kit booklets. Rats were sacrificed by cervical dislocation, and the heart was dissected out and rinsed in ice-cold isotonic saline. A 10% *w*/*v* cardiac tissue homogenate was made using freezing 50 mM potassium phosphate buffer and centrifuged at 10,000 × g for 15 minutes at 4°C to obtain clear supernatant. This supernatant was used to measure biochemical parameters. Thiobarbituric acid reactive substance (TBARS) was measured (nmoles/mg protein) by the method of Ohkawa et al. [25]. Ellman's [26] procedure was used for reduced glutathione (GSH) measurement (nmoles/mg protein). Superoxide dismutase (SOD) activity (IU per mg protein) was measured by following the method of Misra and Fridovich [27]. Catalase (CAT) activity (moles of H_2O_2

decayed per minute per mg protein) was assessed by the method given by Aebi [28]. ELISA kit was used for the evaluation of tumor necrosis factor-α (TNF-α). Total protein was computed by the technique of Lowry et al. [29]. For histological analysis, the cardiac tissues were preserved in a 10% formalin solution.

2.9. Histopathological Examinations. For histopathological evaluation, 4 μm sections were developed by microtome and stained for 15 minutes with hematoxylin (H), followed by eosin (E) counterstain for 2 minutes. The slides were examined using a pathological microscope (Magnus) attached to a digital camera and software system.

2.10. Statistical Analysis. The statistical measurements used GraphPad Prism 5.01 (USA). The results were represented as mean ± standard mean error (SEM). A one-way ANOVA and Tukey's HSD tests were used to analyze the data. Time-course data were assessed using repeated measures two-way ANOVA and Bonferroni post hoc test. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Phenolic and Flavonoid Contents. The total phenolic content of CGPE was 46.23 ± 1.67 gallic acid equivalents/g (calibration curve $R^2 = 0.995$) and the total flavonoid content 36 ± 1.56 rutin equivalents/g ($R^2 = 0.998$).

3.2. Acute Oral Toxicity Investigation. The LD50 of CGPE was examined up to 6000 mg/kg dose. No symptoms of toxicity or death were evident at these doses. The observations revealed the nontoxic nature of the extract as all the animals were alive, healthy, and active during the observation period. CGPE had no influence on the change in behavior or general activity of the animals, and no death was observed at this dose. It can be inferred that the LD50 value of CGPE was more than 6000 mg/kg and is in the nontoxic range.

3.3. Effect of C. grandis Peel Ethanolic Extract on Body Weight and Blood Glucose Levels. The initial body weight and blood glucose level had no significant alteration. However, the mean body weight of the DC group on day 14 was considerably low in contrast to the control group. Oral administration of CGPE for 14 days daily attenuated the alloxan-induced decline in body weights of rats relative to DC rats (Figure 1(a)). Glib managed to attenuate the decrease in body weight in alloxan-treated rats when juxtaposed to rats that received alloxan and vehicle only. Injection of alloxan caused an increase in FBG on day 3 (>250 mg/dl). Rats treated with alloxan only showed a substantial rise in blood glucose levels on days 7 and 14 relative to the vehicle treated control rats. Treatment with CGPE or Glib significantly attenuated the alloxan-induced rise in blood glucose in rats when juxtaposed to rats that were given alloxan and vehicle only. Interestingly, we observed a dose-dependent decrease in blood glucose levels as shown in Figure 1(b).

3.4. Effect of C. grandis Peel Ethanolic Extract on Blood Biomarkers. DC group, administered with alloxan and vehicle only, showed a significant increase in serum TC levels, LDH activity, and CK activity and decline in HDL relative to the vehicle-treated control group (Figure 2). Oral administration of CGPE or Glib for 14 days in separate groups caused a noteworthy decrease in TC, LDH, and CK and a rise in HDL content in alloxan-treated rats when juxtaposed to rats that were given alloxan and vehicle only. The higher dose of CGPE (400 mg/kg) showed a conspicuous amelioration of blood lipid parameters and LDH and CK activities in comparison to the lower dose (100 mg/kg) in alloxan administered rats.

3.5. Effect of C. grandis Peel Ethanolic Extract on Oxidative Stress Biomarkers in Cardiac Tissue. The current findings depicted a substantial upsurge in lipid peroxidation (TBARS) and a diminution in cardiac antioxidants (GSH, SOD, and catalase) in retort to alloxan treatment when compared to the vehicle-treated controls (Figure 3). Treatment with CGPE or Glib attenuated alloxan-induced increase in TBARS and decline in antioxidants with respect to alloxan and vehicle alone treatments. Oral administration of CGPE-H dose caused a consistent decrease in TBARS and also enhanced the antioxidant levels steadily in the cardiac tissue in comparison to the lower dose, i.e., CGPE-L in alloxan-treated rats.

3.6. Effect of C. grandis Peel Ethanolic Extract on TNF-α in Cardiac Tissue. Analysis of ELISA results showed a significant rise in TNF-α (inflammatory biomarker) in cardiac tissue of alloxan-treated rats with respect to the vehicle-treated control rats. CGPE or Glib treatments for 14 days in alloxan treated rats significantly lowered the TNF-α levels relative to rats that received alloxan and vehicle only (Figure 4). The higher dose of CGPE (400 mg/kg) showed a conspicuous amelioration of inflammatory biomarkers in comparison to the lower dose (100 mg/kg) in alloxan administered rats.

3.7. Histopathology of Cardiac Tissue. Alloxan-induced diabetes caused significant pathogenic alterations in the heart of rats. DC group showed prominent changes in the plasma membrane, cell swelling, and chromatin condensation that highlighted the irreversible cell damage symptoms. Treatment with CGPE abrogated this alloxan-induced pathogenicity and showed a dose-dependent decline in cell deterioration. Glib also protected cell structure against alloxan-induced diabetes in rats (Figure 5).

4. Discussion

The research was aimed at determining the ameliorative effect of CGPE on DCM caused by alloxan. Alloxan is the most widely used entity for the induction of human insulin-dependent diabetes mellitus (IDDM) in laboratory diabetic animal models. When given intravenously, intraperitoneally, or subcutaneously, alloxan imposes its hyperglycemic actions. Alloxan has been reported to suppress the glucose-induced secretion of insulin by adaptive glucokinase deactivation and to cause IDDM. Due to a spurt in ROS

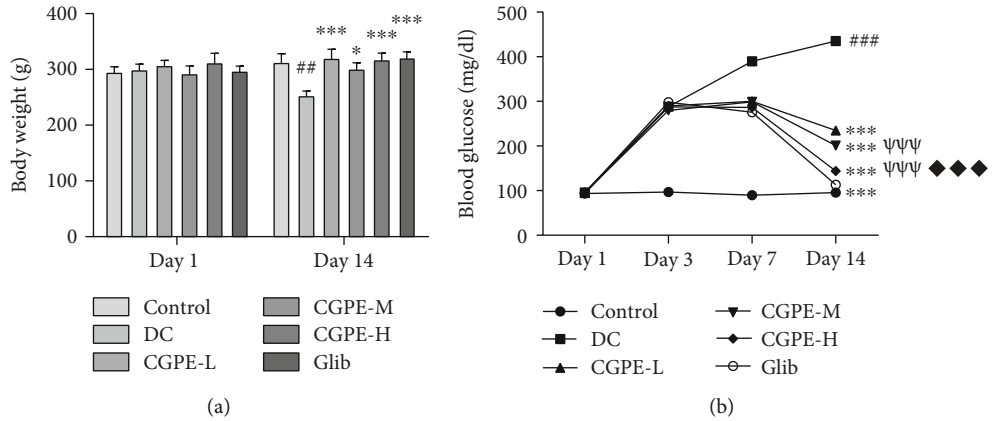

Figure 1: Effect of C. grandis peel ethanolic extract (CGPE) on (a) body weight and (b) blood glucose level. Repeated measures two-way ANOVA and Bonferroni post hoc test were used to analyze the data. Values are mean ± SEM ($n = 6$). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. control group; $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. DC group; $^{\Psi\Psi\Psi}p < 0.001$ vs. CGPE-L group; $^{\blacklozenge\blacklozenge\blacklozenge}p < 0.001$ vs. CGPE-M group.

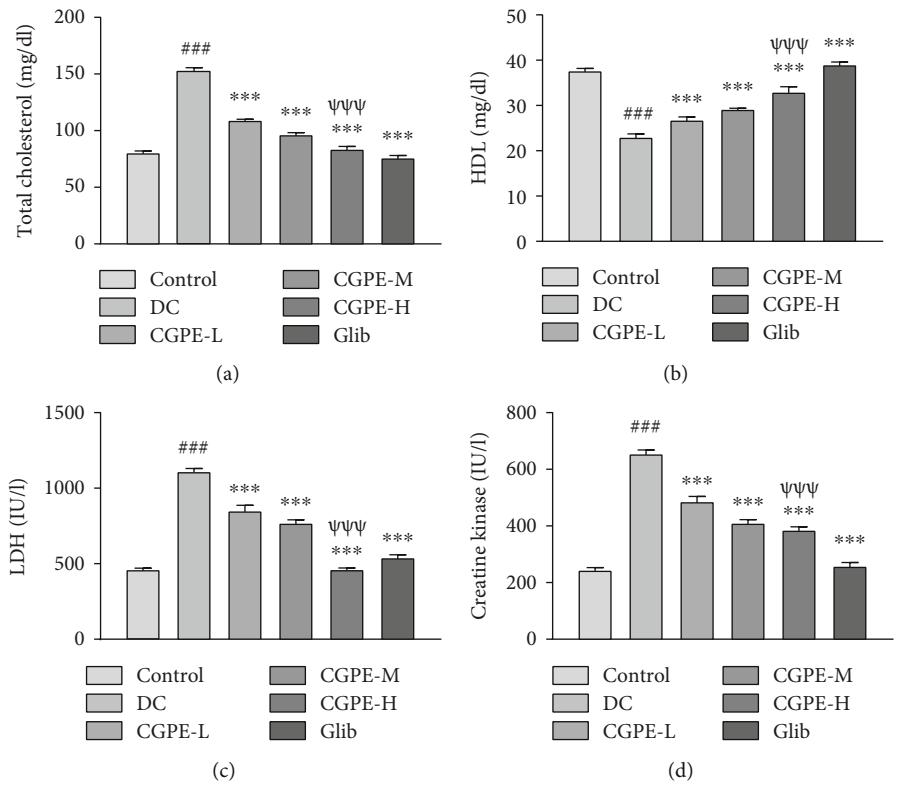

Figure 2: Effect of C. grandis peel ethanolic extract (CGPE) on blood biomarkers. (a) Total cholesterol level, (b) high-density lipoprotein (HDL), (c) lactate dehydrogenase (LDH) activity, and (d) creatine kinase MB activity. One-way ANOVA and Tukey's HSD post hoc test were used to analyze the data. Values are mean ± SEM ($n = 6$). $^{\#\#\#}p < 0.001$ vs. control group; $^{***}p < 0.001$ vs. DC group; $^{\Psi\Psi\Psi}p < 0.001$ vs. CGPE-L group.

(oxidative stress) and peroxides [30], alloxan has also been reported to induce a cytotoxic effect on pancreatic cells. Such free radical species cause oxidative and induced late inflammatory damage to the living systems [31]. In this investigation, a single-dose administration of alloxan (200 mg/kg, i.p.) caused persistent hyperglycemia that was attenuated by a 2-week CGPE or Glib treatment in rats.

In the diabetic control rats, the blood TC levels were increased, whereas HDL levels were reported to decrease. Earlier reports also indicate the level of TC in alloxan-induced diabetic rats to be increased [32]. However, treatment with CGPE or Glib (standard) was found to significantly decrease the TC and surge HDL. CGPE-H (400 mg/kg) showed the highest lipid-ameliorating activity among all CGPE treatment groups. The absence of flexibility between energy sources results in reduced heart efficiency and contractile dysfunction, which is a feature of cardiomyopathy in diabetics. The leakage of the cytoplasmic LDH

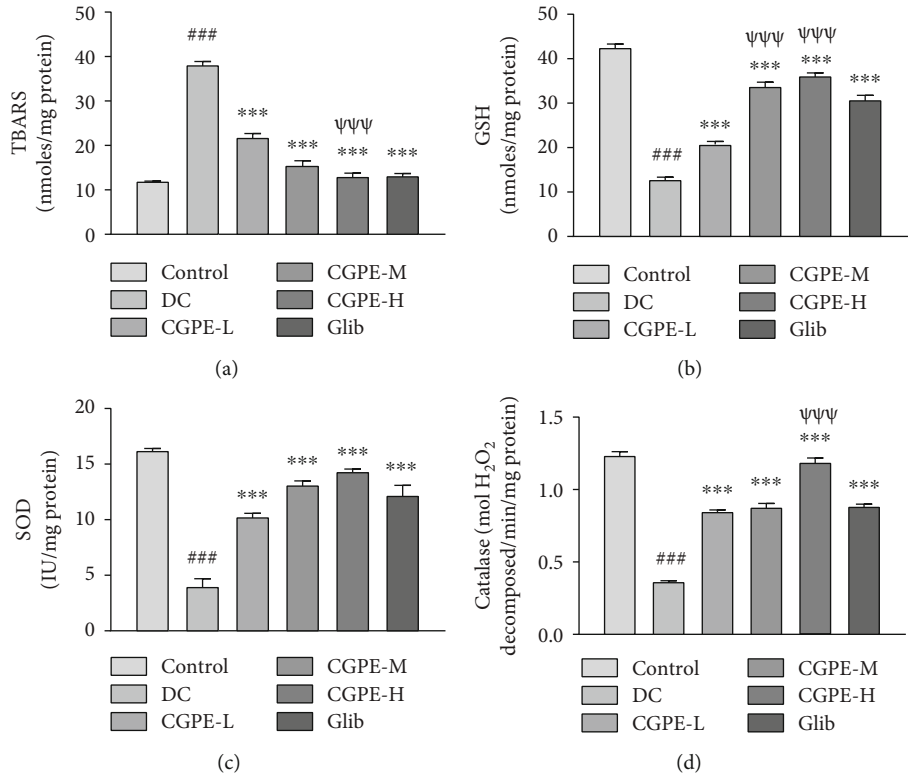

Figure 3: Effect of C. grandis peel ethanolic extract (CGPE) on oxidative stress biomarkers in cardiac tissue. (a) TBARS level, (b) glutathione (GSH) level, (c) superoxide dismutase (SOD) activity, and (d) catalase activity. One-way ANOVA and Tukey's HSD post hoc test were used to analyze the data. Values are mean ± SEM ($n = 6$). $^{\#\#\#}p < 0.001$ vs. control group; $^{***}p < 0.001$ vs. DC group; $^{\Psi\Psi\Psi}p < 0.001$ vs. CGPE-L group.

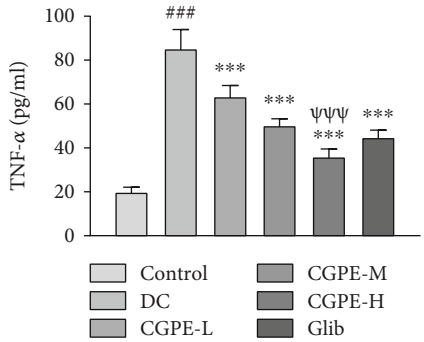

Figure 4: Effect of C. grandis peel ethanolic extract (CGPE) on TNF-α in cardiac tissue. One-way ANOVA and Tukey's HSD post hoc test were used to analyze the data. Values are mean ± SEM ($n = 6$). $^{\#\#\#}p < 0.001$ vs. control group; $^{***}p < 0.001$ vs. DC group; $^{\Psi\Psi\Psi}p < 0.001$ vs. CGPE-L group.

caused by the damage to cell membrane integrity is reported as a reliable indicator of cell death and has been used to estimate the cytotoxicity [33]. It has been observed that an increase in the serum LDH levels indicates the nonspecific damaging response of any tissue [34]. The serum LDH levels were found to increase in the diabetic control rats. However, treatment with different doses of CGPE was found to attenuate the LDH levels significantly. Among all the different doses of C. grandis extract, CGPE-H was found to attenuate the LDH levels more significantly than other doses of it. An increase in serum levels of creatine kinase MB (CK-MB) has been used as a specific indicator of myocardial damage. This increase in CK-MB levels indicates cardiac muscular damage which results in leakage of enzymes from the heart and hence their elevated levels in the serum [10]. The CK-MB concentrations in the diabetic control rats were reported to be elevated. However, therapy with different doses of CGPE was found to greatly attenuate the CK levels. CGPE-H (400 mg/kg) has been found to ameliorate the CK-MB level more significantly among other CGPE dose groups. The present findings are in line with previous results that indicated amelioration of lipid profile, glucose metabolism, and attenuation of insulin resistance and cell death in different animal models [35]. Citrus fruits including C. grandis target diverse set of molecules such as PPARs, nuclear factor-κB, platelet-derived growth factor (PDGF), transforming growth factor-1 (TGF-1), and genes, such as FAS, Progastricsin (PGC-1α and PGC-1β), and GLUT4 to prevent the progression of pathogenic mechanisms [35–37].

It has been confirmed that citrus phenolic compounds particularly flavonoids have significant antioxidant effects against radicals and anti-inflammatory properties. The citrus flavonoids are capable of trapping electrons and suppressing and/or scavenging the radicals. The citrus flavonoids create a tautomeric dislocation that prohibits certain oxygen-free radicals from propagating chain reactions [38, 39]. The heart disruption induced by hyperglycemia results in myocardial necrosis that triggers cardiac impairment, elevated lipid

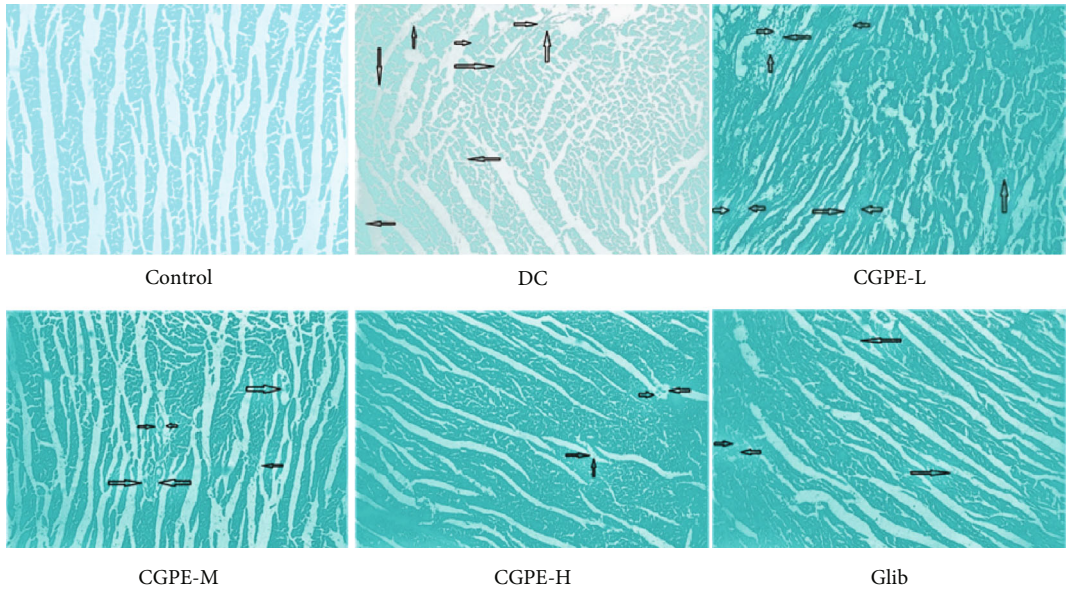

Figure 5: Histology of cardiac tissue. Cardiac photomicrograph (45x) showing usual anatomy with well-preserved cytoplasm. Diabetic control cardiac tissue shows clear penetration of inflammatory cells, the existence of lipid accumulation, swelling, ruptured, edema, and necrosis as indicated by arrows. Treatment with CGPE or Glib showed reduced, absence of lipid accumulation, inflammatory cells, and inflammation in cardiac tissue, the inflammatory cells decrease considerably, and partial cardiac tissue recovery is observed as shown by the arrows but shows some swollen and ruptured cells with lipid accumulation. There is a significant decrease in lipid accumulation, necrosis, and partial recovery of inflammatory cells, swollen, ruptured, and edema.

peroxidation, decreased myocardial lipid concentrations, and altered heart enzyme and antioxidant activity. As this research reveals that the amount of TBARS and TNF-α in diabetic control groups was observed to be increased. Nonetheless, treatment with 400 mg/kg of CGPE was found to significantly decrease the TBARS and TNF-α levels. Of all the doses of CGPE, the 400 mg/kg concentrations were observed to decrease lipid peroxidation and inflammation more than other doses and this disparity was significant. In diabetic control groups, the SOD activity, reduced glutathione level, and catalase rate were also observed to decrease. However, therapy with CGPE has been shown to dramatically improve these antioxidants.

Histopathological alterations arise in DCM characterized by necrosis; focal accumulation of chronic inflammatory cells comprised of lymphocytes, plasma cells, and macrophages; and edema [23]. In this research, the diabetic control group demonstrated the existence of granular cardiomyocyte degeneration, necrosis, perivascular edema, and extreme inflammatory cell infiltration. Treatment with CGPE or Glib attenuated necrosis and inflammatory cell infiltrations against DCM. CGPE was observed to reduce the inflammatory cells and recover the architecture of cardiomyocytes in the hearts of diabetic rats.

5. Conclusion

Present research indicates that chronic hyperglycemia is liable for heart muscle damage via the production of free radicals and inflammation. CGPE attenuated oxidative stress and inflammation in cardiac tissue and also lowered glucose levels and ameliorated lipid levels against alloxan-induced DCM in rats. The enhanced free radical generation acts as a chief position in the progression of DCM. Current insulin therapies that are used to control insulin resistance do not offer much protection against comorbidities, such as DCM. The use of crude phytochemicals as an adjuvant to current therapies continues to show benefits in various epidemiological and clinical studies. Hence, it may rule out some noxious side effects of synthetic drugs and widens the scope of a novel alternative therapy such as C. grandis peel extract in DCM.

Data Availability

The data is available from the corresponding author upon a suitable request.

Conflicts of Interest

The authors report no conflicts of interest.

Acknowledgments

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Retraction

Retracted: Dioscorea nipponica Makino Relieves Ovalbumin-Induced Asthma in Mice through Regulating RKIP-Mediated Raf-1/MEK/MAPK/ERK Signaling Pathway

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] W. Wang, L. Xu, L. Zhou, S. Wan, and L. Jiang, "Dioscorea nipponica Makino Relieves Ovalbumin-Induced Asthma in Mice through Regulating RKIP-Mediated Raf-1/MEK/MAPK/ERK Signaling Pathway," *BioMed Research International*, vol. 2022, Article ID 8077058, 11 pages, 2022.

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Research Article

Dioscorea nipponica Makino Relieves Ovalbumin-Induced Asthma in Mice through Regulating RKIP-Mediated Raf-1/MEK/MAPK/ERK Signaling Pathway

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Purpose. Dioscorea nipponica Makino (DNM) is a traditional herb with multiple medicinal functions. This study is aimed at exploring the therapeutic effects of DNM on asthma and the underlying mechanisms involving RKIP-mediated MAPK signaling pathway. *Methods*. An ovalbumin-induced asthma model was established in mice, which was further administrated with DNM and/or locostatin (RKIP inhibitor). ELISA was performed to detect the serum titers of OVA-IgE and OVA-IgG1, bronchoalveolar lavage fluid (BALF) levels of inflammation-related biomarkers, and tissue levels of oxidative stress-related biomarkers. The expression of RKIP was measured by quantitative real-time PCR, Western blot, immunohistochemistry, and immunofluorescence. HE staining was used to observe the pathological morphology of lung tissues. The protein expression of MAPK pathway-related proteins was detected by Western blot. *Results*. Compared with the controls, the model mice exhibited significantly higher serum titers of OVA-IgE and OVA-IgG1, BALF levels of IL-6, IL-8, IL-13, TGF-β1, and MCP-1, tissue levels of MDA and ROS, lower BALF levels of IL-10 and IFN-γ, and tissue level of GSH. DNM relieved the allergic inflammatory response and oxidative stress in the model mice. DNM also recovered the downregulation of RKIP and the pathological injury of lung tissues in asthma mice. In addition, the Raf-1/MEK/MAPK/ERK pathway in the model mice was blocked by DNM. Silencing of RKIP by locostatin weakened the relieving effects of DNM on asthma through activating the Raf-1/MEK/MAPK/ERK pathway. *Conclusion*. DNM relieves asthma via blocking the Raf-1/MEK/MAPK/ERK pathway that mediated by RKIP upregulation.

1. Introduction

Asthma is a respiratory disease characterized by airway inflammation and hyperresponsiveness [1]. Asthma is mostly occurred in childhood at a prevalence of 495 per 100,000 in comparison to that in adults at 92 per 100,000, which will affect about 400 million people worldwide by 2025 [2–4]. In clinical, asthma is accompanied by nonspecific symptoms, including recurrent wheeze, shortness of breath, cough, and variable expiratory airflow limitation [5]. To avoid symptom burden and exacerbation risk, anti-inflammation and bronchodilation are dominant for the treatment of asthma [6]. Inhaled corticosteroids (ICS) and bronchodilators are the most commonly used drugs for asthma; nevertheless, the responsiveness varies among individuals [7]. The discovery of more effective drugs against asthma without side effect is still needed.

In China, there is a long-term history of traditional Chinese medicine (TCM) use in the treatment of respiratory diseases including asthma [8]. A diversity of TCMs have been

determined to have therapeutic potential for asthma, such as Cordyceps sinensis [9], Astragalus membranaceus [10], Carthamus tinctorius [11], Atractylodis rhizome [12], and Cimicifugae Rhizoma [13]. Dioscorea nipponica Makino (DNM) is a perennial twining herb that exhibits a variety of medicinal functions, such as anti-inflammation, antitussive, antitumor, antipain, panting-calming and phlegm-dispelling, immune regulation, and cardiovascular system protection [14]. DNM has been widely used to protect against rheumatoid arthritis, Kashin-Beck disease, sprains, bruises, bronchitis, and cough in China [14, 15]. It is noted that DNM may also contribute to the remission of asthma. Ou-Yang et al. concluded the pharmacological profile of DNM and demonstrated that DNM possesses the favorable antiasthmatic effect that was validated in mice with asthma [14]. Junchao et al. have shown that diosgenin from DNM inhibits the inflammation in asthmatic mice [16]. Wang et al. have found that saponins from DNM mitigate the effects of IL-17A on the proliferation, migration, and cytoskeleton remodeling of human airway smooth muscle cells in vitro, indicating a candidate for treating asthma-associated airway hyperresponsiveness [17]. However, the specific effects of DNM and the underlying molecular mechanisms in asthma have not been fully revealed.

MAPK signaling pathway is a convergent node responses to diverse stimulus, such as metabolic stress, DNA damage, protein dysregulation, external growth factor signaling, cell-matrix interaction, and cell-cell communication [18]. By regulating the transcription of target genes, MAPK pathway plays a critical role in fundamental cellular functions (cell proliferation, apoptosis, differentiation, and migration), tumorigenesis, extrinsic stress response, and metabolic reprogramming [19–21]. Notably, MAPK pathway is also the action target of many TCMs against asthma. For example, hydroxysafflor yellow A attenuates airway resistance, inflammation, and pathologic changes of OVA-induced asthma in guinea pigs through inhibiting the MAPK pathway [11]. Icariin alleviates pulmonary inflammation, airway remodeling, and the proliferation of airway smooth muscle cells through inhibiting the MAPK/ERK pathway in a mouse model of OVA-induced asthma [22]. Ginkgolide B inhibits OVA-induced eosinophilia in lung tissues and mucus hypersecretion in the airway in a mouse model [23]. In addition, previous studies have also determined that DNM exhibits a great potential in the treatment of gouty arthritis via regulating MAPK pathway [24, 25]. However, the action mechanisms of DNM in asthma involving the MAPK pathway are not entirely understood.

In this study, the therapeutic potential of DNM was analyzed in a mouse model of ovalbumin- (OVA-) induced asthma. The action mechanisms of GNV involving RKIP-mediated MAPK signaling pathway were further determined. The objective of this study is to investigate the therapeutic efficacy and mechanism of DNM on asthma, thereby providing promising natural drug and target therapeutic strategy for asthma treatment.

2. Methods

2.1. Model Establishment and Grouping. Animal experiments were approved by the ethical committee of The First Affiliated Hospital of Zhejiang Chinese Medical University. Female BALB/c mice (HFK Bio, Beijing, China) at 8 weeks old were used to establish OVA-induced asthma model as previously described [26]. All mice were raised in a laboratory room with $24 \pm 2^{\circ}C$, $60 \pm 5\%$ humidity, and 12 h light/dark cycle. Simply, mice were sensitized by intraperitoneal injection of 200 μL OVA inducer (50 μg OVA and 0.8 mg aluminum hydroxide dissolved in 0.9% physiological saline; Sigma-Aldrich, MO, USA) for twice with an interval of 14 days (days 1 and 14). After injection, mice were exposed to 2% OVA (dissolved in 3 mL 0.9% physiological saline) for 20 min every 3-4 days (days 14, 17, 20, 23, and 27). DNM (a natural herb) with an active ingredient of Dioscin (0.2505 ± 0.0003 ng/uL, $3.074 \pm 0.053\%$, HPLC) was purchased from Tongrentang (Beijing, China). The OVA-sensitized and OVA-challenged mice (model mice) were randomly divided into 5 groups ($N = 6$ each group) and received different administrations for 14 days following OVA injection. Model group: it is a group of model mice without administration; model+DNM: model mice were orally administrated with 1.95 g/kg DNM; model+locostatin (RKIP inhibitor; Darmstadt, Germany): model mice were intraperitoneally injected with 0.5 mg/kg locostatin; model +DNM+locostatin: model mice were orally administrated with 1.95 g/kg DNM and intraperitoneally injected with 0.5 mg/kg locostatin; model+prednisone acetate (PA): model mice were gavaged with 5 mg/kg PA. Normal mice without OVA injection and oral administrated with physiological saline were used as the control group ($N = 6$). After the last intervention (day 27), mice were anaesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection) and sacrificed by cervical dislocation.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA). The blood samples were collected from mice via the retroorbital plexus. The serum titers of OVA-IgE and OVA-IgG1 were measured using commercial ELISA kits (Mlbio, Shanghai, China). The bronchoalveolar lavage fluid (BALF) was collected by 3 times of intratracheal instillation with PBS, and the supernatants were used for detecting inflammation-related biomarkers, including the IL-6, IL-8, IL-10, IL-13, IFN-γ, TGF-β1, and MCP-1. In addition, the resected lung tissues were collected for measuring the contents of GSH, MDA, and ROS. The above parameters were all measured using corresponding commercial ELISA kits in accordance with the manufacturer's instructions (IL-6/IL-8/IL-10/IL-13/IFN-γ, Mlbio; TGFβ1/MCP-1, Thermo Fisher Scientific, CA, USA; GSH, Jiancheng, Nanjing, China; MDA, Solarbio, Beijing, China; and ROS, QualitYard, Beijing, China). The levels of related parameters were finally calculated according to established standard curves.

2.3. Quantitative Real-Time PCR (qRT-PCR). Total RNAs were extracted from lung tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). By using the template of cDNAs that reversely transcribed by FastKing First-strand cDNA Synthesis Mix (Tiangen, Beijing, China), qRT-PCR was performed on MX3000P Real-Time PCR instrument (Agilent, Santa Clara, CA, USA). The qRT-PCR program

Table 1: Primer sequences for qRT-PCR.

Gene	Species	Forward primer (5′-3′)	Reverse primer (5′-3′)
RKIP	Mice	AGGTTATGAACAGGCCCAGC	AGACATAGCGGTGGAGACCT
GAPDH	Mice	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT

was an initial 95°C for 3 min and 40 cycles of 95°C for 15 s and 62°C for 40 s. The relative expression of RKIP was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as the internal control. The primers used in qRT-PCR are listed in Table 1.

2.4. Hematoxylin-Eosin (HE) Staining. The resected lung tissues were fixed with 4% formalin for 24 h, embedded in paraffin, and sectioned into 5 μm. After dewaxing, the sections were stained with hematoxylin for 5 min and then with eosin for 1 min. The stained sections were finally observed under an optical microscope (Olympus, Japan).

2.5. Immunohistochemistry (IHC) and Immunofluorescence (IF). The paraffin sections of lung tissues were further used in IHC and IF for detecting the expression of RKIP. After dewaxing, the sections were microwave irradiated in citrate buffer for 15 min at 95°C, soaked in 3% H_2O_2 for 20 min, blocked with 5% goat serum for 15 min, and incubated with anti-RKIP (1 : 100, Abcam) for 12 h at 4°C. Then, the sections received 1 h of incubation with HRP-IgG (1 : 500, Abcam) for IHC and with Alexa Fluor® 488-IgG for IF (1 : 500, Abcam) at 25°C. Sections used in IHC were visualized with diaminobenzidine and observed under an optical microscope (Olympus). Sections used in IF were counterstained with DAPI and observed under a confocal microscope (ULTRAVIEW VOX, Perkin Elmer, USA).

2.6. Western Blot. The protein samples were isolated from lung tissues by lysing in RIPA buffer (Beyotime) and quantified using BCA Protein Assay Kit (Beyotime). The proteins were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. After blocked with 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies (anti-RKIP (ab76582) and anti-GAPDH (ab181602), Abcam, 1 : 1,000; anti-(p)-Raf-1 (Cat# AP3922a) and -(p)-ERK1/2 (Cat#AP3906a), Abcepta, 1 : 1,000; and anti-(p)-MEK1/2 (#9154S), -(p)-p38 MAPK (#4511), Cell Signaling Technology; 1 : 1,000) at 4°C overnight. Subsequently, the membranes were further incubated with secondary antibody (HRP, 1 : 5,000, ab205718, Abcam) for 1 h at 25°C. The protein bands were visualized using an ECL kit (Thermo Fisher Scientific, CA, USA) and quantified by ChemiDoc imaging system (Bio-Rad, CA, USA). GAPDH served as an internal control.

2.7. Statistical Analysis. Statistical analysis was performed using the software of GraphPad Prism 7. All data were expressed as the mean ± standard deviation. Comparisons among different groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test. A P value less than 0.05 represented significantly different.

3. Results

3.1. DNM Relieves the Allergic Response in Asthma Mice through Upregulating RKIP. To reveal the effects of DNM in asthma, a mouse model of OVA-induced asthma was established. As shown in Figures 1(a) and 1(b), the serum titers of OVA-IgE and OVA-IgG1 were both significantly higher in the model mice than those in the controls ($P < 0.001$). qRT-PCR showed that the mRNA expression of RKIP was significantly decreased in the model mice compared with the controls ($P < 0.001$, Figure 1(c)). In consistent with PA, DNM significantly decreased the serum titers of OVA-IgE and OVA-IgG1 and increased RKIP expression in the model mice ($P < 0.001$, Figures 1(a) and 1(c)). RKIP was then silenced by locostatin to determine the action mechanisms of DNM relating with RKIP. The results showed that the intervention of locostatin increased the serum titers of OVA-IgE and OVA-IgG1 and downregulated RKIP in the model mice ($P < 0.05$, Figures 1(a) and 1(c)). In addition, locostatin weakened the inhibitory effects of DNM on the allergic response of asthma via downregulating RKIP ($P < 0.05$, Figures 1(a) and 1(c)). In consistent with the mRNA expression of RKIP, constant changes were also determined on the protein expression of RKIP in lung tissues by IHC and IF (Figures 2(a) and 2(b)).

3.2. DNM Relieves the Inflammatory Response in Asthma Mice through Upregulating RKIP. Since asthma is a chronic inflammatory disease, some inflammation-related biomarkers were detected in the BALF. As shown in Figures 3(a)–3(e), the BALF levels of IL-6, IL-8, IL-13, TGF-β1, and MCP-1 were significantly higher in the model mice than those in the controls ($P < 0.001$). On the contrary, significantly lower levels of IL-10 and IFN-γ were revealed in BALF of the model mice compared with the controls ($P < 0.001$, Figures 3(f) and 3(g)). The intervention of DNM significantly decreased the IL-6, IL-8, IL-13, TGF-β1, and MCP-1 levels and increased the IL-10 and IFN-γ levels in BALF of the model mice ($P < 0.01$). Consistent results with DNM were observed in a positive drug PA in the model mice ($P < 0.01$). In addition, locostatin showed opposite effects on the above biomarkers with the DNM via inhibiting RKIP ($P < 0.01$). Notably, locostatin weakened the effects of DNM on reducing IL-6, IL-8, IL-13, TGF-β1, and MCP-1 and on elevating IL-10 and IFN-γ in BALF of the model mice ($P < 0.05$, Figures 3(a)–3(g)).

3.3. DNM Relieves the Oxidative Stress in Asthma Mice through Upregulating RKIP. Oxidative stress is closely associated the asthmatic inflammation and severity. Here, ELISA determined significantly higher contents of MDA and ROS and lower content of GSH in lung tissues of the model mice

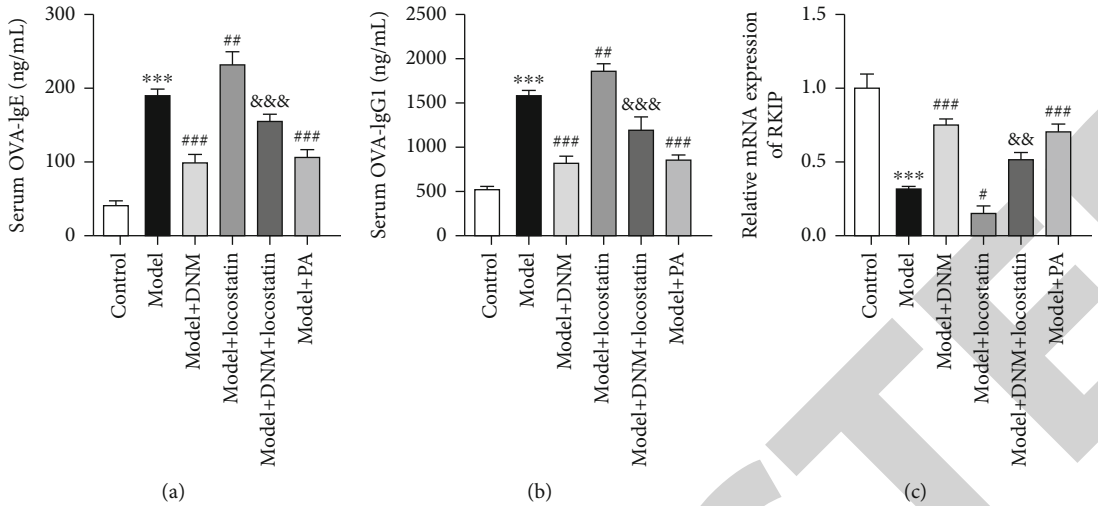


Figure 1: DNM inhibits the allergic response via downregulating RKIP in a mouse model of OVA-induced asthma. (a) Serum titers of OVA-IgE; (b) serum titers of OVA-IgG1; (c) the mRNA expression of RKIP. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. model; $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ vs. model+DNM. ELISA and qRT-PCR were performed in triplicate in six independent mice.

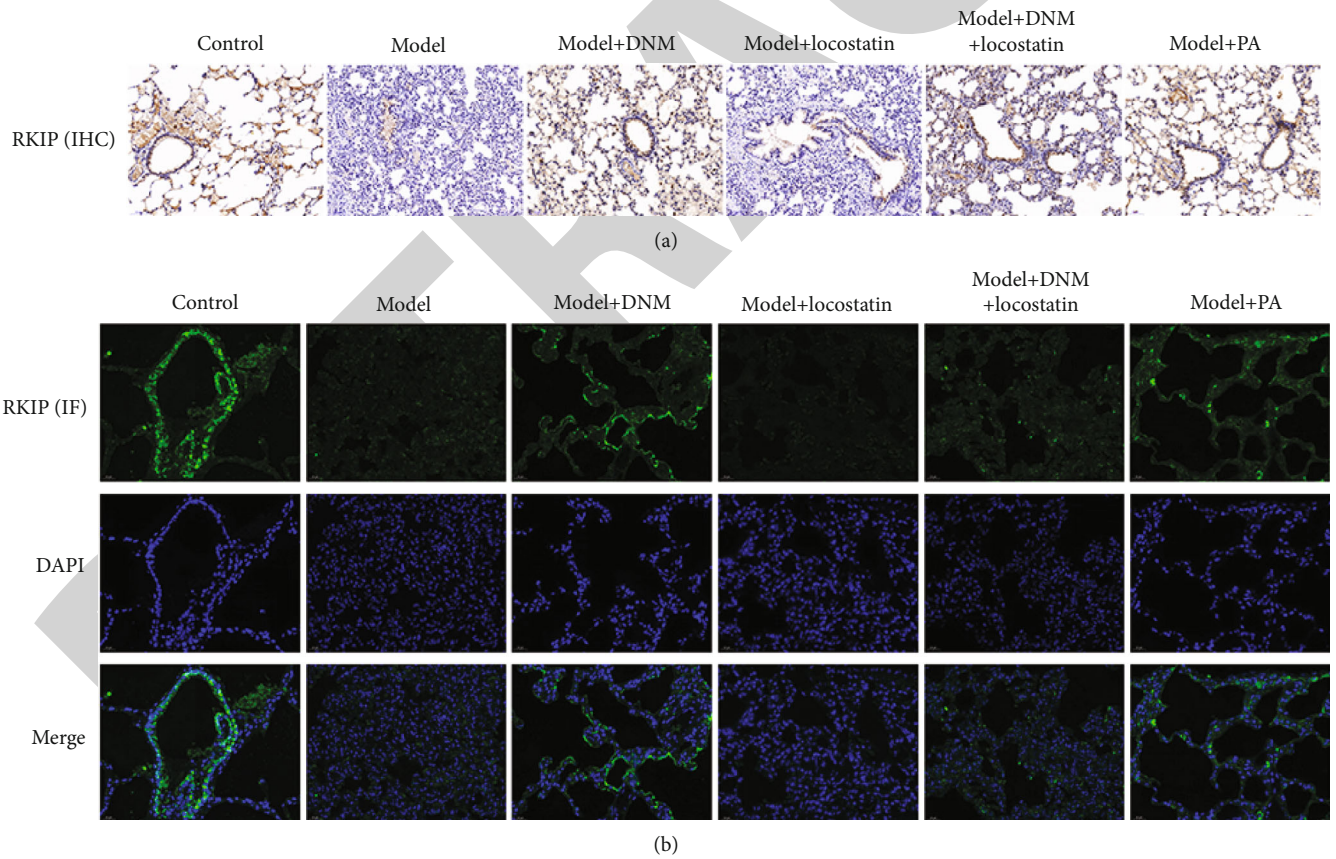


Figure 2: The protein expression of RKIP in lung tissues of OVA-induced asthma mice. (a) IHC; (b) IF. IHC and IF were performed in six independent mice.

compared with the controls ($P < 0.001$). Both DNM and PA could decrease the MDA and ROS contents and increase the GSH content in the model mice ($P < 0.01$). Via inhibiting RKIP, locostatin presented contrary effects on the above biomarkers with DNM ($P < 0.01$). In addition, locostatin weakened the effects of DNM on decreasing MDA and ROS and on increasing GSH in lung tissues of the model mice ($P < 0.05$, Figures 3(h)–3(j)).

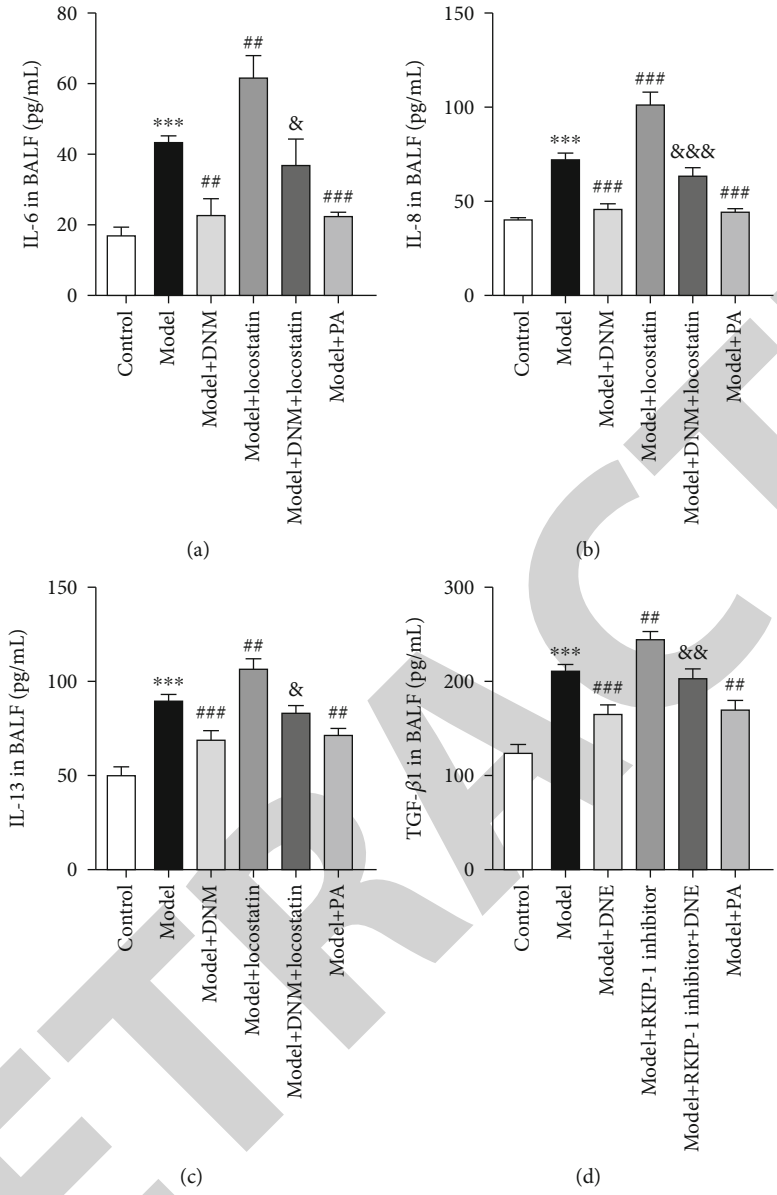
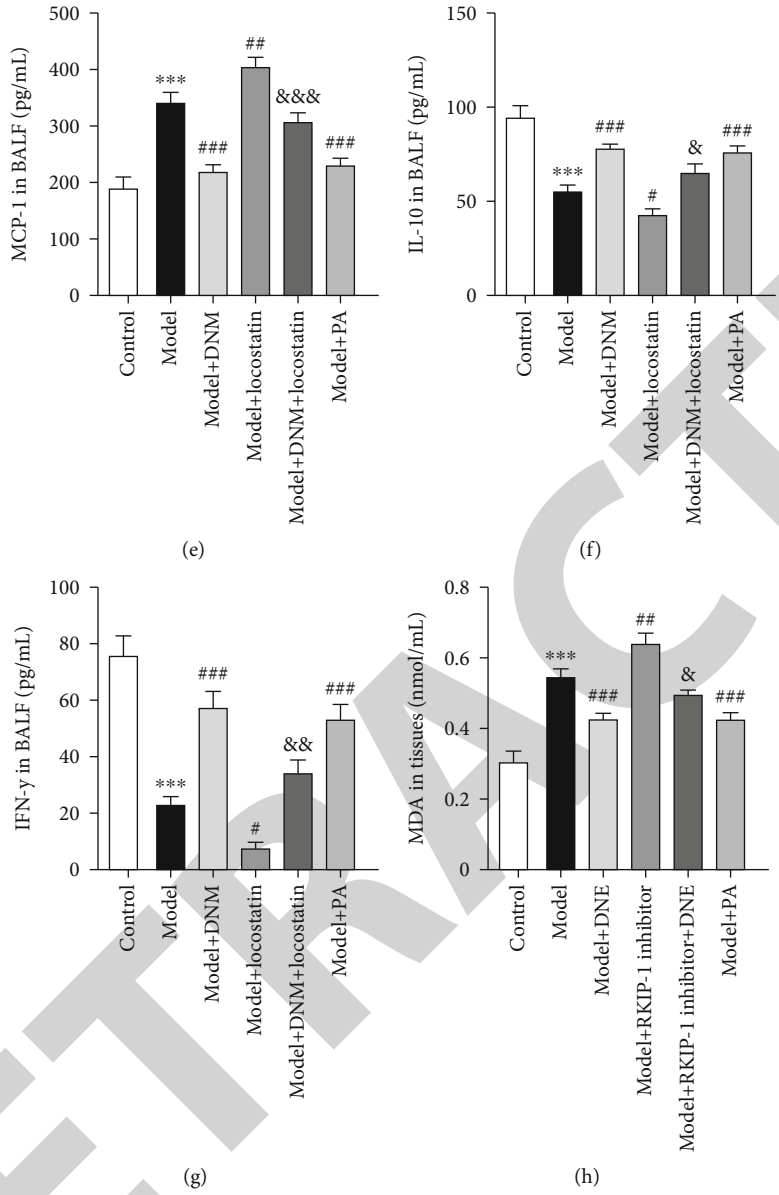


Figure 3: Continued.



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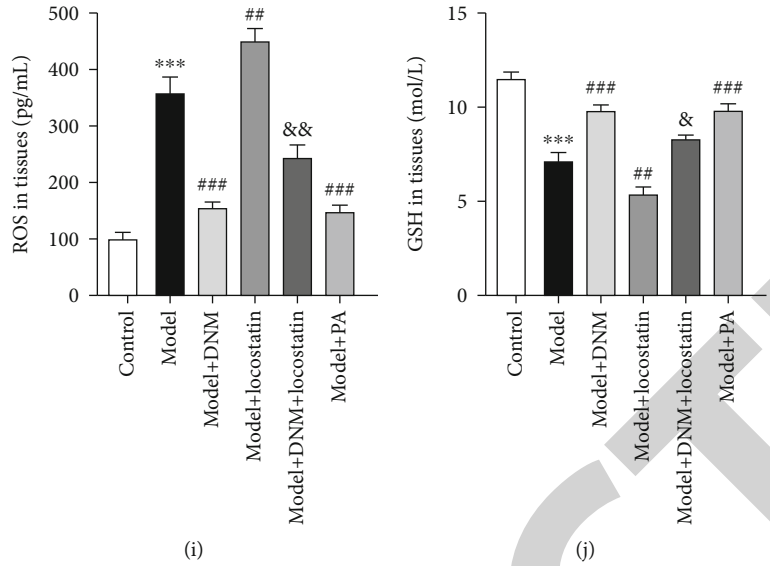

Figure 3: DNM inhibits the allergic inflammatory response and oxidative stress via downregulating RKIP in a mouse model of OVA-induced asthma. (a) IL-6 in BALF; (b) IL-8 in BALF; (c) IL-13 in BALF; (d) TGF-β1 in BALF; (e) MCP-1 in BALF; (f) IL-10 in BALF; (g) IFN-γ in BALF; (h) MDA in lung tissues; (i) ROS in lung tissues; (j) GSH in lung tissues. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. model; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, and $^{\&\&\&}P < 0.001$ vs. model+DNM. ELISA was performed in triplicate in six independent mice.

3.4. DNM Relieves the Pathological Injury of Lung Tissues in Asthma Mice through Upregulating RKIP. The pathological morphology of lung tissues was observed by HE staining. As shown in Figure 4, normal mice (control group) exhibited clear alveolar texture, regular bronchial lumen, intact airway mucosal epithelium and alveolar wall, and no inflammatory cell infiltration. However, obvious pathological injury was observed in lung tissues of the model mice, presenting bronchial lumen stenosis, capillary edema, partial alveolar fusion, airway epithelium abscission, and massive inflammatory cell infiltration. The pathological injury in the model mice was relieved by DNM or PA but was aggravated by locostatin to some degrees. Locostatin also weakened the relieving effects of DNM on the pathological injury of lung tissue in the model mice (Figure 4).

3.5. DNM Blocks the Raf-1/MEK/MAPK/ERK Signaling Pathway in Asthma Mice through Upregulating RKIP. Because RKIP is involved in the inhibition of the MAPK signaling pathway by mediating Raf-1, the action mechanisms of DNM involving the Raf-1/MEK/MAPK/ERK pathway were further analyzed. As shown in Figure 5, the protein expression of p-Raf-1, p-MEK1/2, p-p38 MAPK, and p-ERK1/2 was significantly higher in the model mice than that in the controls ($P < 0.001$). In consistent with PA, DNM inhibited the activation of the Raf-1/MEK/MAPK/ERK pathway in the model mice ($P < 0.001$). In addition, locostatin-induced inhibition of RKIP further enhanced the activation of the Raf-1/MEK/MAPK/ERK pathway in the model mice, evidenced by upregulated p-Raf-1, p-MEK1/2, p-p38 MAPK, and p-ERK1/2 ($P < 0.05$). Locostatin also reversed DNM-induced inhibition of the Raf-1/MEK/MAPK/ERK pathway ($P < 0.05$).

4. Discussion

Asthma is an inflammatory condition of the respiratory tract that driven by T-helper 2- (Th2-) immune response [27]. So far, a variety of TCMs have been widely used for the treatment of asthma, including the DNM. However, fundamental researches on the function and underlying molecular mechanisms of DNM in asthma are limited. In this study, an OVA-induced asthma was established in mice to determine the role of DNM. Firstly, we found that DNM significantly decreased the serum titers of OVA-IgE and OVA-IgG1 in the asthma mice. IgE and IgG are known to be central in the immunopathogenesis of allergic asthma, which are positively associated with the phenotypes of bronchial airway inflammation and hyperreactivity and eosinophil enrichment [28–30]. Our findings indicate that the intervention of DNM inhibited the allergic response of asthma mice against OVA. In addition to the increased immunoglobulins, asthma is also characterized by increased Th2 type cytokines [31]. IL-13 is one of the most important Th2-type cytokines that exerts a critical role in eosinophilic inflammation, mucus hypersecretion, airway hyperactivity, and airway remodeling [32]. In this study, DNM-induced decreasing of IL-13 level in BALF of model mice illustrates that DNM is able to inhibit Th2-type response in asthma. In addition, DNM also significantly decreased IL-6, IL-8, MCP-1, and TGF-β1 and increased IL-10 and IFN-γ in BALF of the model mice. These results further indicate that the intervention of DNM inhibited the allergic inflammatory response in asthma. On the other hand, oxidative stress is also involved in asthmatic inflammation and severity, and targeting oxidizing molecules combined with inflammatory mediators has become a novel therapeutic strategy for asthma [33, 34]. In this study, the MDA and ROS levels were decreased

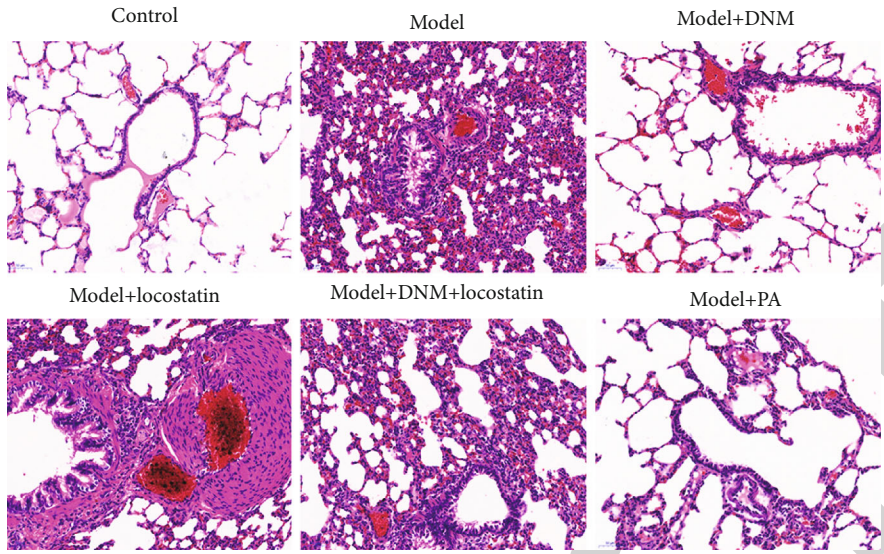

Figure 4: The pathological morphology of lung tissues in a mouse model of OVA-induced asthma was assessed by HE staining. HE was performed in six independent mice.

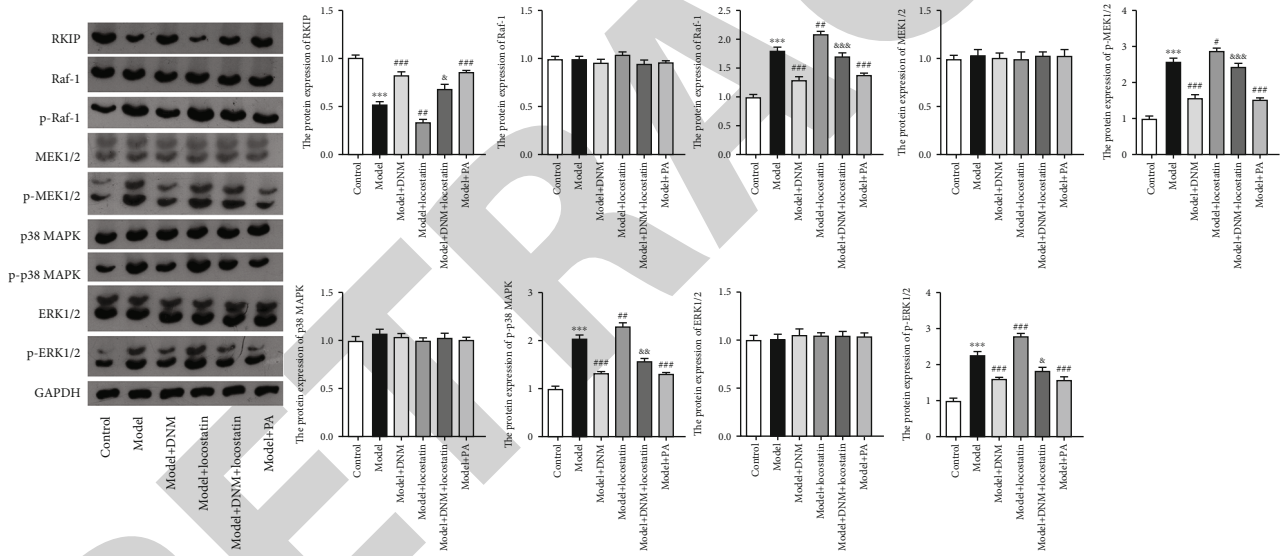

Figure 5: The activation of the Raf-1/MEK/MAPK/ERK signaling pathway in mice with OVA-induced asthma was detected by Western blot. Western blot was performed in six independent mice. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. model; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, and $^{\&\&\&}P < 0.001$ vs. model+DNM.

and the GSH level was increased by DNM in lung tissues of asthma mice. These findings indicate that DNM may be benefit for the treatment of asthma through inhibiting oxidative stress. Furthermore, the HE staining directly confirmed the alleviating effect of DNM on asthma, evidenced by a mild pathological injury in lung tissues of the model mice.

Furthermore, the potential molecular mechanism by which DNM exerts the therapeutic effect on asthma was investigated. RKIP, a member of the phosphatidylethanolamine-binding protein family, is a pivotal modulator in the inflammatory and immune system via interacting with multiple signaling molecules [35, 36]. It has been reported that RKIP plays a substantial role in asthma via interacting with diverse functional proteins, such as Raf-1, MUC5AC, GRK2, and 15LO1 [37]. Therefore, RKIP is a promising target for the therapeutic intervention of DNM on asthma. In this study, a downregulated RKIP was observed in OVA-induced asthma mice. This result is consistent with a previous study that the expression of RKIP was decreased in the peripheral blood of patients with asthma [38]. Lin et al. have also revealed that the RKIP deficiency in mast cells render mice more sensitive to IgE-FcεRI-mediated allergic response and OVA-induced airway inflammation [38]. Our findings showed that DNM significantly increased the expression of RKIP in OVA-induced asthma mice. We suspect that the upregulation of RKIP may contribute to the alleviating effect of DNM in asthma. Our subsequent results further determined this hypothesis, evidenced by that the inhibition of RKIP by

locostatin weakened the relieving effects of DNM on the allergic inflammatory response, oxidative stress, and pathological injury in asthma mice.

The function of RKIP is inseparable from its interaction with multiple signaling molecules involving inflammatory processes [37]. Under homeostatic condition, RKIP appears to exhibit anti-inflammatory role through inhibiting the ERK/MAPK pathways [37]. Evidence has proved that RKIP can inhibit Raf-1-mediated activation of the MAPK/ERK signaling pathway by suppressing the phosphorylation of Raf-1 [39]. Consistently, we found that the inhibition of RKIP by locostatin enhanced the activation of the Raf-1/MEK/MAPK/ERK pathway in asthma mice. MAPK pathway is a classical inflammatory pathway that involved in the pathology of diverse human inflammatory diseases, including cancer, neurodegenerative disorders, diabetes, cardiovascular diseases, and inflammatory bowel diseases [40]. There many potential drugs have been determined to treat asthma through inhibiting the MAPK pathway, such as hydroxysafflor yellow A [11], icariin [22], ginkgolide B [23], caffeic acid phenethyl ester [41], dehydrodieugenol [42], and paeonol [43]. In this study, the activated Raf-1/MEK/MAPK/ERK pathway in asthma mice was also inhibited by DNM, indicating a potential action mechanism of DNM. Zhou et al. have shown that the saponin fraction from DNM exerts anti-inflammation effects on gouty arthritis by inhibiting MAPK pathway [24]. To combine with the promoting role of DNM on RKIP, we suspect that DNM may upregulate RKIP to block the Raf-1/MEK/MAPK/ERK pathway, thereby alleviating asthma.

5. Conclusion

In conclusion, DNM relieves OVA-induced asthma in a mouse model, evidenced by decreased allergic inflammatory response, oxidative stress, and pathological injury of lung tissues. In addition, DNM blocks the Raf-1/MEK/MAPK/ERK pathway through upregulating RKIP, presenting an underlying action mechanism of DNM against asthma. This study provides a promising natural drug with low side effects for the treatment of asthma. Meanwhile, the molecular mechanism of DNM treating asthma was revealed, which provides new guidance for the target therapy of asthma. However, this study is limited by the unclear bioactive components in DNM. Further, we can explore the potential bioactive components of DNM against asthma, which may exert the better therapeutic efficacy on asthma. Thus, other potential molecular mechanisms of DNM against asthma can be deciphered based on bioactive compounds. In-depth researches on the action mechanisms of DNM in asthma are also needed.

Data Availability

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

Additional Points

Highlight. (1) DNM alleviates the allergic response in asthma mice. (2) DNM relieves the inflammation and oxidative stress in asthma mice. (3) GNV relieves the pathological injury of lung tissues in asthma mice. (4) GNV upregulated RKIP and blocked Raf-1/MEK/MAPK/ERK pathway

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Crocus sativus L. Tepal Extract Induces Apoptosis in Human U87 Glioblastoma Cells

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Crocus sativus (*C. sativus*) is considered as the costliest spice and an important medicinal plant. Herein, we investigated the effects of tepal extract (TE) of *C. sativus* on the viability of the human glioblastoma cells. Results revealed that TE significantly ($P < 0.05$) inhibited the proliferation of U87 glioblastoma cells in a dose-dependent manner with comparatively lower toxic effects against normal astrocytes. The IC_{50} of TE against U87 glioblastoma cells was found to be 130 μg/mL as compared to 600 μg/mL against normal astrocytes. TE also inhibited the colony formation of U87 cells significantly ($P < 0.05$). The AO/EB and Annexin V/PI staining assays indicated that TE stimulated apoptosis in U87 cells dose dependently. The early and late apoptotic U87 cells increased from 0.66% and 2.3% at control to 14.2% and 21.4% at 260 μg/mL of TE. Moreover, TE caused upregulation of Bax and suppression of Bcl-2. Wound healing assay showed that migration of the U87 cells was suppressed significantly ($P < 0.05$) at 80 μg/mL of TE. Taken together, these results suggest that TE exhibits antiproliferative effects against U87 glioma cells and may prove to be an important source of natural anticancer agents.

1. Introduction

Crocus sativus L. (*C. sativus*) belonging to family *Iridaceae* is an important medicinal plant [1]. Medicinal properties of *C. sativus* are generally attributed to the presence of a specific group of compounds. These compounds are formed by the oxidative cleavage of carotenoids and are commonly known as apocarotenoids. These apocarotenoids include crocin, safranal, and picrocrocin in considerable quantities [2]. In addition to apocarotenoids, *C. sativus* is also a rich source of flavonoids such as kaempferol, taxifolin, and naringenin, to name a few [3, 4]. *C. sativus* has array of medicinal properties, for instance, antidiabetic, anti-inflammatory, cardioprotective, and anticancer [5]. Samarghandian et al. showed that *C. sativus* extract inhibits pulmonary tumor suppression via activation of apoptosis [6]. Bakshi et al. showed that crocin from *C. sativus* induces apoptosis and cell cycle arrest of the pancreatic cancer cells [7]. In yet another study, D'Alessandro et al. showed that *C. sativus* stigma extract stimulates apoptosis to suppress proliferation of the human prostate cancer cells [8]. Nonetheless, there is little information on the antiproliferative effects of tepal extracts of *C. sativus*. The present study was therefore undertaken to determine the anticancer properties of *C. sativus*

tepal extract against the human U87 glioma cells and to unravel the possible underlying molecular mechanisms.

Gliomas are prevalent type of tumors of central nervous system and responsible for significant number of human mortalities [9]. As per the American Brain Tumor Association, gliomas constitute 24.7% of all the primary brain tumors and 74.6% of all malignant tumors [10]. Unfortunately, the survival rate of the glioma patients is very poor. The average survival period of the patients with the currently available treatment strategies is less than 60 months for low-grade glioma and below 15 months for the advanced stage disease [11]. Therefore, there is urgent need to identify efficient treatment strategies for the management and improvement of survival rates of the glioma patients. Consistently, the present study was undertaken to examine the effects of tepal extract (TE) of *C. sativus* against the human glioma cells. This study will form basis for the identification of lead molecules from *C. sativus* for anticancer chemotherapy.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions. The U87 glioblastoma cells were cultured in DMEM/F12 containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. Astrocyte growth medium from an AGM-Astrocyte Medium Bullet kit (Lonza) was used for the maintenance of normal human astrocytes.

2.2. Preparation of the C. sativus Tepal Extract. The extract was prepared from shade dried tepals (100 g) of *C. sativus* by maceration with ethanol as described previously [12]. The tepal extract (TE) was then filtered and concentrated at 50°C under reduced pressure on a Rotavapor®. The resultant extract was used for further experiments.

2.3. Cell Viability Assay. The glioma U87 and the normal astrocytes were added in 96-well plates with of 5×10^3 cells/well. Then, treatment of the cells with different concentrations (0 to 640 μg/L) of TE for 24 h at 37°C was followed. Next, 10 μL of MTT (5 mg/mL) was followed by an additional 4 h incubation at 37°C. Thereafter, DMSO (10%) was used to solubilise the formazan crystals. Finally, OD_{570} was determined by using spectrophotometer to estimate cell viability.

2.4. Colony Formation Assay. Around 5000 U87 glioma cells were seeded in 12-well plates and treated with varied concentrations of TE. The plates were incubated for 14 days at 37°C, and the colonies so developed were fixed in paraformaldehyde for 20 minutes, PBS washed, and subjected to staining for with 0.1% crystal violet for 35 minutes. The visible colonies were PBS washed and photographed under a microscope. The colonies were finally counted using the Image J software.

2.5. Acridine Orange and Ethidium Bromide (AO/EB) Staining. AO/EB staining was carried out for the detection of apoptosis in U87 glioma cells. Briefly, the U87 cells were seeded in coverslips with 5×10^5/wells and cultured for 24 h at 37°C. Then, the culture medium was replaced with a fresh medium containing different concentrations of TE. The cells were then again incubated at 37°C for 24 h. Subsequently, AO/EB staining of monolayer cells was performed. Finally, the cells were examined under a fluorescence microscope (×200) magnification, and images were captured.

2.6. Annexin V/PI Assay. The U87 glioma cells were collected and then suspended in 500 μL of 1X binding buffer. Then, Annexin V-FITC (5 μL) was added to the suspension followed by PI (10 μL) (Annexin V-FITC detection Kit, Beyotime Biotechnology). The cells were mixed evenly and refrigerated at 4°C for 10 min in the dark. Flow cytometer (Accuri C6, Becton-Dickinson, US) was used to detect the apoptotic cells.

2.7. Wound Healing Assay. U87 cells were cultured in 6-well plates with density of 1×10^5 cells/wells to confluent monolayers and subsequently subjected to overnight starvation in serum-free media. Wound scratching was done by sterile pipette tips. The plates were washed to remove the debris, treated with TE, and subjected to incubation for 24 h at 37°C. The wound gaps were photographed at 0 and 24 h time intervals to determine cell migration.

2.8. Western Blotting Assay. Treated and control U87 cells were subjected to washing with PBS lysed in ice cold lysis buffer. The protein concertation of each sample was determined by Bio-Rad protein Assay kit. Whole-cell lysates (30 μg) were separated by 12% SDS–polyacrylamide gel electrophoresis and subsequently moved to a polyvinylidene difluoride membrane. Afterwards, western blot analysis was used for specific primary antibodies Bax (sc-7480, Santa Cruz, CA, USA), Bcl-2 (sc-23960, Santa Cruz, CA, USA), and Actin (sc-58673, Santa-Cruz, CA, USA). The membranes were washed three times with TBST and incubated for 2 h with appropriate HRP-conjugated secondary antibody anti-rabbit IgG HRP (Cell Signaling Technology, 7074s, 1 : 2000). Signals were visualized by using the Enhanced Chemiluminescence Plus (ECL Plus) detection system (GE Healthcare).

2.9. Statistical Analysis. Each experiment was done with three replicates, and data was shown as mean ± SD. Statistical analysis was carried out by using t-test through GraphPad prism 7, and $P < 0.05$ indicated statistically significant difference.

3. Results

3.1. TE Inhibits the Proliferation of Glioblastoma Cells. The effects of TE were evaluated against the U87 glioma and normal human astrocytes by MTT cell viability assay (Figure 1). It was revealed that the viability of U87 cells diminished significantly ($P < 0.05$) in a dose-dependent manner. At 320 μg/mL of the extract, the cell viability was inhibited by 96.5%. However, the antiproliferative effects of TE were milder against the normal astrocytes. The IC_{50} of TE was found to be 130 μg/mL against the U87 cells as comparted to an I C_{50} of 600 μg/mL against the normal astrocytes suggestive of cancer specific antiproliferative effects of TE. Next, the

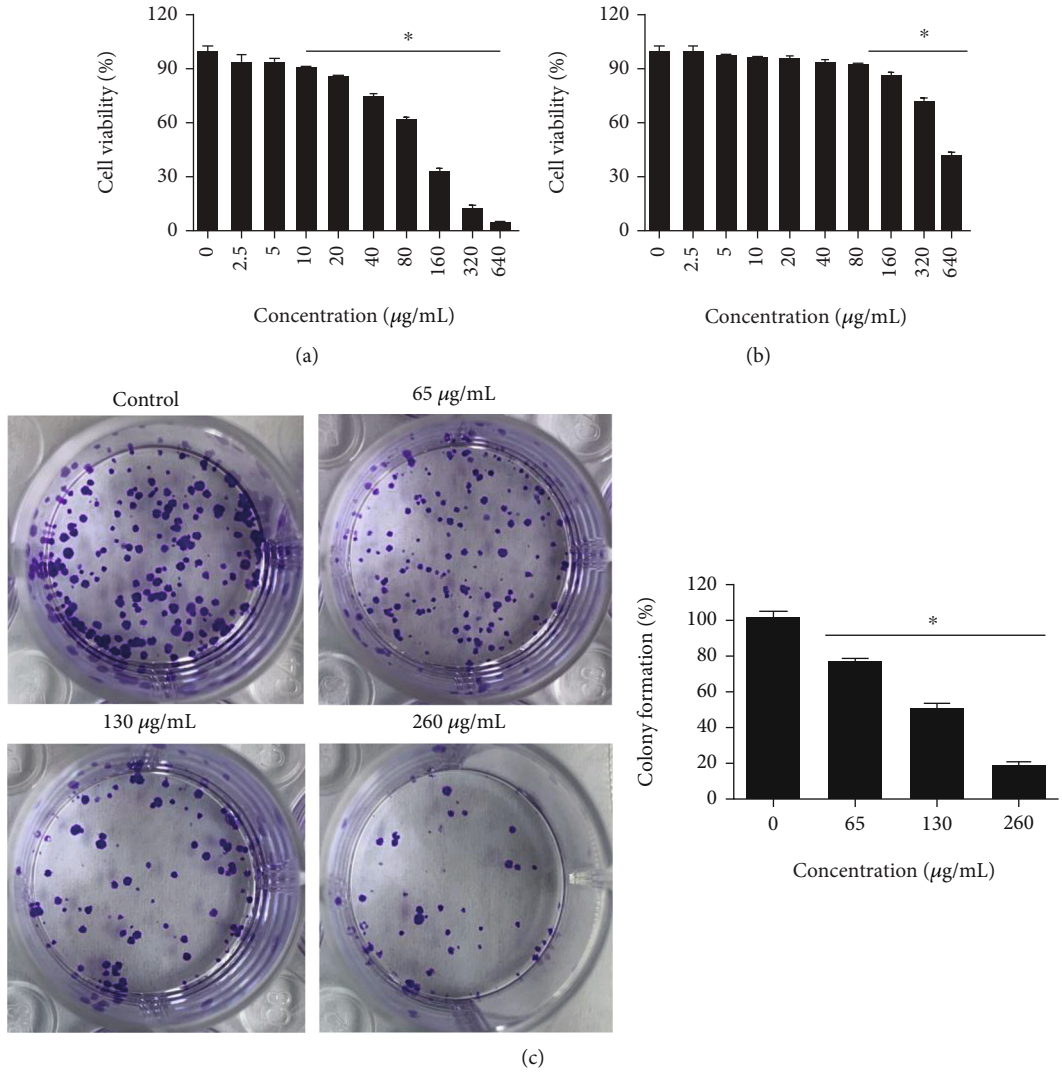
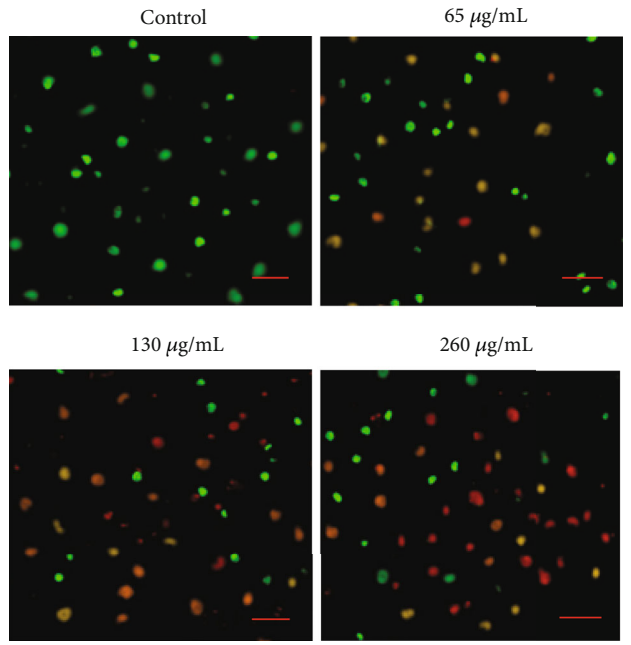


Figure 1: TE suppresses viability of human glioblastoma cells. (a) MTT assay showing the viability of the U87 cells at indicated concentrations of TE. (b) MTT assay depicting the viability of normal astrocytes at indicated concentrations of TE. (c) Colony formation of the U87 cells at indicated concentrations of TE. Data is shown as mean ± SD ($^*P < 0.05$).

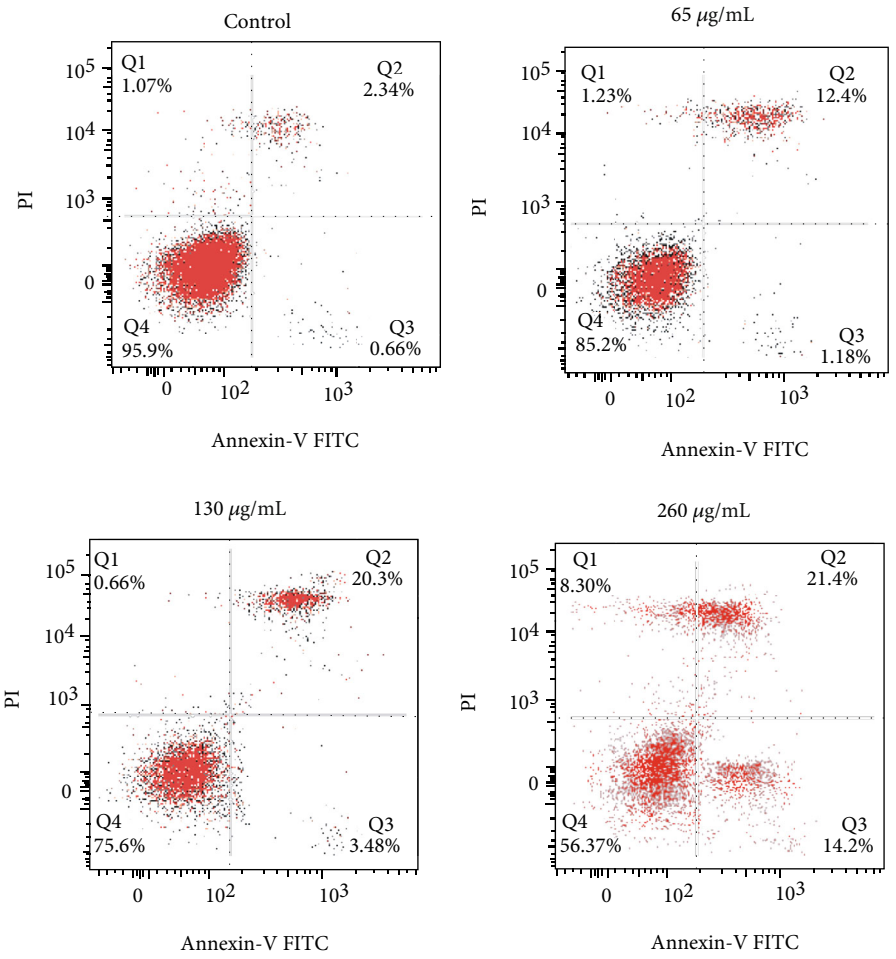
effects of TE were assessed on the colony-forming potential of U87 cells. Results indicated that the colony formation ability of the U87 cells decreased significantly ($P < 0.05$). Compared to control, the colony formation potential of the U87 cells decreased by 85% at 320 μg/mL of TE. These findings clearly suggest the antiproliferative effects of TE against U87 glioblastoma cells.

3.2. TE Induces Apoptosis in Glioblastoma Cells. To find out how TE inhibits the proliferation of the U87 cells, AO/EB staining was performed. AO/EB staining revealed apoptosis to be responsible for the inhibition of proliferation by TE. Fluorescence microscopy showed the presence of different types of cells. The bright green color cells indicate the normal viable cells, the yellowish cells represent early apoptotic, orange cells represent late apoptotic, and the red color cells represent the necrotic cells (Figure 2(a)). Next, Annexin V/PI assay was performed to find out the percentage of apoptotic U87 glioblastoma cells at each concentration of TE. The early and late apoptotic U87 cells increased from 0.66% and 2.3% at control to 14.2% and 21.4% at 260 μg/mL of TE respectively (Figure 2(b)). Western blotting revealed that the expression of Bax increased and that of Bcl-2 decreased upon treatment of the U87 cells with TE (Figure 2(c)). These findings indicate that TE stimulates apoptosis in U87 glioblastoma cells.

3.3. C. sativus Extract Suppresses the Migration of Glioblastoma Cells. Next, the effects of TE were also assessed on the migration of the U87 glioblastoma cells by wound healing assay. To overrule the antiproliferative effects, lower dosage of TE (80 μg/mL) was used in wound healing assay. The results showed that TE extract significantly ($P < 0.05$) inhibited the migration of the U87 cells (Figure 3).



(a)



(b)

Figure 2: Continued.

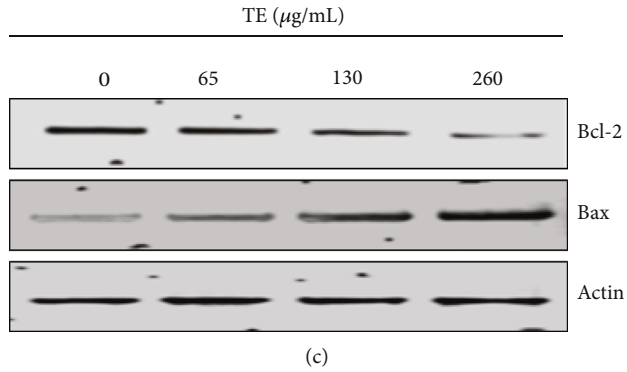


Figure 2: TE induces apoptosis in human glioblastoma cells. (a) AO/EB staining of the U87 cells treated with different concentrations of TE. (b) Annexin V/PI staining showing U87 cells treated with different concentrations of TE. (c) Western blots showing the effect of TE on the expression of Bcl-2 and Bax in U87 cells.

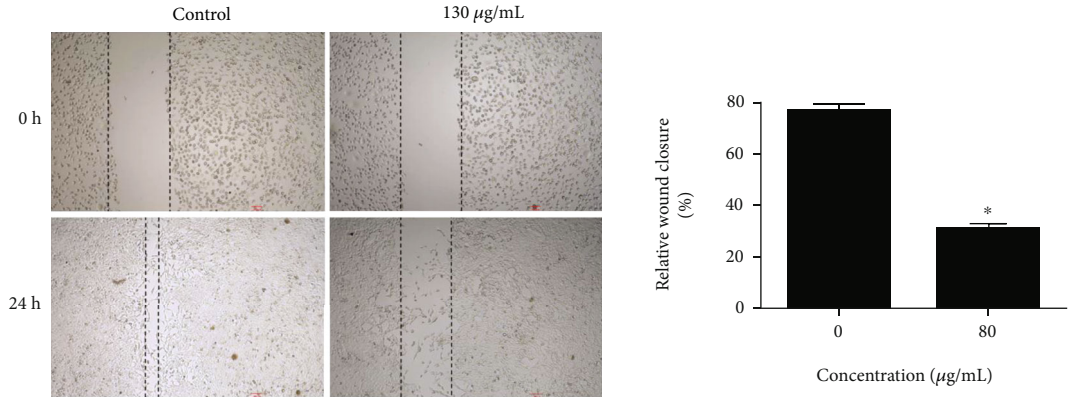


Figure 3: TE inhibits the migration of glioblastoma cells. Wound heal assay depicting the effect of TE extract on the migration of the U87 glioblastoma cells. Data is shown as mean ± SD ($^*P < 0.05$).

4. Discussion

Plants have served and will continue to serve as an important repository of bioactive compounds [13, 14]. The compounds or their derivatives have been used as drugs or lead molecules for the development of drugs to treat human ailments [15–17]. Several well-known drugs currently used for the treatment of deadly disease have their origin in plants [18]. *C. sativus* is a well-known medicinal plant, and a wide array of pharmacological properties have been attributed to different parts of this plant [5]. Nonetheless, effects of TE have not been evaluated against human glioblastoma cells. Consistently, herein, we report the anticancer effects of TE against the human glioblastoma cells. It was found that TE inhibited the viability of U87 cells more profoundly as compared to the normal astrocytes. This selective inhibition of U87 cells by TE could be attributed to the fact that several signalling pathways show dysregulation in cancer cells [19], and TE extract might modulate any of these pathways. However, more studies are needed to infer the exact mechanism for the selective inhibition of the U87 cells. Our findings are supported by previous finding wherein *C. sativus* extracts have been found to suppress the growth of different cancers such as colorectal cancer [20]. Chryssanthi et al. reported that the extracts of different *Crocus* species exhibit the potential to inhibit the growth of the breast cancer cells [21]. Similarly, Bathaie et al. reported that crocetin from *C. sativus* could inhibit the growth of the human adenocarcinoma gastric cancer growth *in vivo [22]*. In yet another study, Mir et al. showed that crocetin beta-d-glucosyl ester from *C. sativus* could inhibit the growth of the breast cancer cells via ER-alpha/HDAC2 axis [23]. Apoptosis is an essential process which eliminates the defective cancerous cells from the body of an organism and helps to maintain homeostasis [24]. In the present study, we found that TE inhibited the proliferation of the U87 cells by inducing apoptosis. However, at IC_{50} of TE, the percentage of both early and late apoptotic cells was 23.78% indicating that apoptosis alone could not been responsible for the antiproliferative effects of TE. Therefore, more research endeavours are needed to identify other molecular routes through which TE exerts its effects. Nonetheless, our results are supported by a previous study wherein Samarghandian et al. showed that *C. sativus* extract induces apoptosis in pulmonary tumor [6]. Next, TE also suppressed the migration of the glioblastoma cells indicating that TE may also exhibit antimetastatic potential given that migration is an initial step in the metastasis of cancer [25]. Although the present study revealed the

anticancer potential of TE extract, more studies including *in vivo* studies are needed. Additionally, the active phytochemical constituents of TE extract need to be identified by natural product chemistry approaches.

5. Conclusion

Collectively, the results of this study revealed that *C. sativus* tepal extract suppresses the proliferation of human glioblastoma cells via stimulation of apoptosis. Additionally, it could also suppress migration of the glioblastoma cells. These findings suggest that *C. sativus* tepal extract may prove to be an important source of anticancer agents or anticancer lead molecules. However, further research endeavours involving identification, isolation, and mechanism of action of each component of tepal extract are urgently needed.

Data Availability

All 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.

Acknowledgments

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Review Article

Flavonoids a Bioactive Compound from Medicinal Plants and Its Therapeutic Applications

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Plants generally secrete secondary metabolites in response to stress. These secondary metabolites are very useful for humankind as they possess a wide range of therapeutic activities. Secondary metabolites produced by plants include alkaloids, flavonoids, terpenoids, and steroids. Flavonoids are one of the classes of secondary metabolites of plants found mainly in edible plant parts such as fruits, vegetables, stems, grains, and bark. They are synthesized by the phenylpropanoid pathway. Flavonoids possess antibacterial, antiviral, antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic properties. Due to their various therapeutic applications, various pharmaceutical companies have exploited different plants for the production of flavonoids. To overcome this situation, various biotechnological strategies have been incorporated to improve the production of different types of flavonoids. In this review, we have highlighted the various types of flavonoids, their biosynthesis, properties, and different strategies to enhance the production of flavonoids.

1. Introduction

Flavonoids are naturally occurring compounds that are present in the nascent parts of a plant. These are an abundant pigment along with chlorophylls and carotenoids that are present in almost all plants. They are known to provide fragrance and taste to fruits, flowers, and seeds. Flavonoids are low molecular weight polyphenolic phytochemicals secreted as a secondary metabolite in plants [1]. Secondary metabolites are produced in secondary pathways that synthesize compounds that are needed in trace amounts [2]. The secondary metabolites regulate primary pathways such as hormones, and coenzymes. During specific stress conditions, these act as toxins and antibiotics. In addition to their significant role in plants, they are important for human health because of various pharmacological activities. Some flavonoids are known as flower pigments that impart color aroma in flowers [3]. Flavonoids have been identified to have broad-spectrum effects in both microorganisms and animals with varied structures and functions. Since ancient times, the presence of flavonoids in plants has been identified, but their chemical structure was not known until the end of the nineteenth century [4]. In the early twentieth century, flavonoids and associated substances were chemically characterized in different plants and synthesized under laboratory conditions. Most of the interest was on their role as pigments,

and the studies were mainly focused on the family of anthocyanins. Since then, more than 9,000 derivatives of flavonoids have been reported. A wide range of derivatives play an essential role in the integrity of plant structure, photoprotection from UV rays, reproduction, regulation of cell signaling, and physiology. Synthesis of flavonoids occurs from phenylalanine and malonyl coenzyme A. They are all structurally derived from parent compound flavones that are usually found in the cell sap of young tissues of plants.

Flavonoids are biologically active phytochemicals that are ubiquitous in the plant kingdom which are being used in various herbal medicines for many years now. They constitute an essential part of our daily diet. They accumulate mainly in the edible parts of plants [5, 6]. Flavonoids are generally found in fruits, vegetables, grains, bark, stems, tea, and wine. There are multiple factors that are required to target the treatment of a complex chronic disorder, and in conventional treatment, this refers to polypharmacy. Therefore, it has to be emphasized that herbal medicines are a chemically complex blends containing multiple major and minor elements with multiple potent targets and processes. They are synthesized by the phenylpropanoid pathway and are derivatives of 2-phenylbenzyl-pyrone [7]. Flavonoids are a hydroxylated phenolic substance and are synthesized by plants in response to microbial infections. Many studies have been conducted on medicinal plants to explore their therapeutic potential to treat numerous diseases. Treatment with medicinal plants has been considered as safer due to their minimal to no side effects compared to conventional drugs. Medicinal plants show various potentials for the treatment of several diseases that are considered difficult to cure. Ayurveda has been using herbal drugs successfully for the prevention and suppression of various tumors for many years. Medicinal herbs have a positive impact as there are about half a million plants around the world and most of them have not yet been studied for medical purposes. Therefore, current and future studies on therapeutic activities can be effective in treating diseases

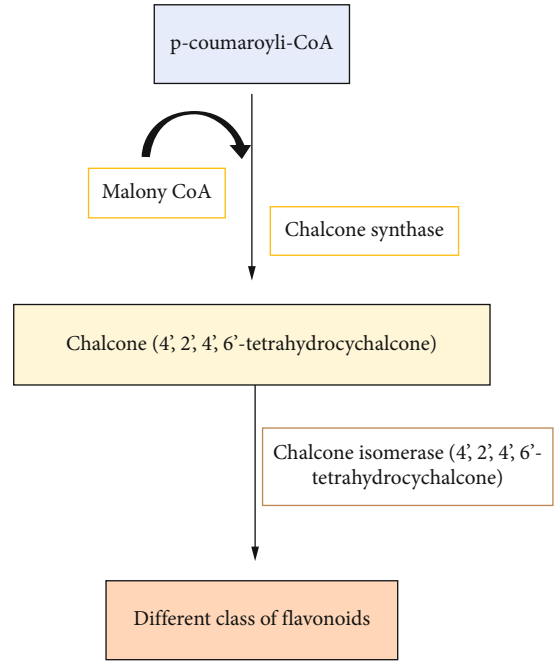

Figure 1: Flavonoid synthesis.

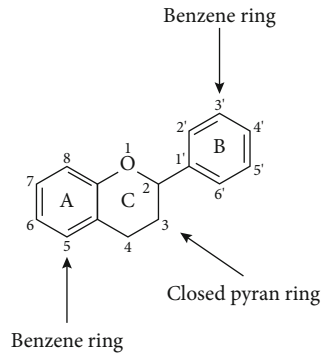

Figure 2: Basic structure of flavonoids.

2. Methodology

For this review paper, data has been collected by using different search engines which includes PubMed, ScienceDirect, Google Scholar, and ResearchGate.

3. Biosynthesis of Flavonoids

Biosynthesis of flavonoids starts with condensation of one molecule with three molecules of malonyl-CoA which yields chalcone by using chalcone synthase (CHS) (Figure 1). Then chalcone isomerization by using chalcone isomerase (CHI) leads to the formation of flavanone [8]. Then, it forms different classes of flavonoids.

4. Basic Structure and Type of Flavonoids

The basic structure of flavonoids (Figure 2) has a skeleton of diphenyl propane, which contains 15C atoms. Two 6-membered rings A and B linked with 3 carbon units which may or may be the part of the third ring. This 3-carbon ring is the heterocyclic oxygen-containing pyrene ring [9]. This basic structure of the flavonoid ring is also referred to as C6-C3-C6 structure [10]. Flavonoids are divided into various subgroups, which depend on the carbon of the C ring and the degree of oxidation of the B ring. Isoflavones are those in which the B ring is attached at position 3 of the C ring (Figure 3). Neoflavonoids have B rings attached to C rings at position 4 (Figure 4). And the others are where the B ring is attached to the C ring at position 2 and are further divided into different classes. And the other has opened the C ring [9].

4.1. Isoflavonoid. Isoflavonoids are a subgroup of flavonoids. They have a limited occurrence in plants and are mainly found in soya beans, in other leguminous plants, and in some microbes [11]. These compounds are able to prevent cancer and show an effect on cardiovascular and menopausal health [12]. They also have antioxidant effects on

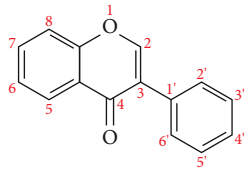

Figure 3: Structure of isoflavones.

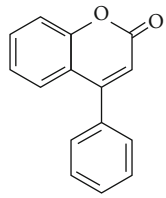

Figure 4: Structure of neoflavonoids.

blood vessels. Isoflavonoids are also used as phytoestrogens because of the estrogenic activity shown by them in animal models [3].

4.2. Neoflavonoid. Neoflavonoids has a backbone of 4-phenylchromen with the absence of substituted hydroxyl group at the 2nd position. First, neoflavonoid, *Calophyllum inophyllum* was used in the year 1951. *Calophyllum inophyllum* was named and also found in the bark *of Mesua thwaitesii* [3].

4.3. Flavones. In flavones, the B ring is joined at 2 C to the C ring. The main flavonoids of this group are rutin, leutein, and luteolin glycosides. Other examples of flavonoids found in this group are apigenin and chrysin. The major dietary sources of flavones are fruits skin, red wine, buckwheat, red pepper, and tomato skin. They are found in several plants such as *Aloe vera*, *Bacopa moneirra* of the family Sarophulariaceae, *Mentha longifolica* of Lamiaceae family, and *Momordica charantia* of Cucurbitaceae [7].

4.4. Flavonols. In flavonols, the B ring is attached to the C ring at 2 positions, and a hydroxyl group is attached to 3 positions of the C ring. The main examples of flavonols are quercetin, kaempferol, and galangin. Other ingredients are myricetin and tamarixetin. The main sources of flavonols are onion, red wine, olive oil, berries, and grapefruit [13]. They are found in plants such as *Acalypha indica*, *Azadirachta indica*, *Betula pendula*, and *Cannabis sativa* [14].

4.5. Flavanones. Flavanone is a significant class of flavonoids. Lemons, oranges, grapes, and citrus fruits are the main sources of flavanone (Felgines C, 2000). Naringin, naringenin, and hesperidin are some types of flavanones. Hesperidin is found in *Citrus medica* of the Rutaceae family [7].

4.6. Flavanonols. Flavanonols are the other classes of flavonoids. They have their basic structure in which the B ring is attached to the C ring at position 2 and the hydroxyl groups are attached at the 3rd position of the C ring and the 3rd and 4th positions of the B ring. An important compound of flavanonols is taxifolin [15].

4.7. Flavanols. Flavanols are also known as flavan-3-ols due to the presence of a OH group at position 3rd of C ring [16]. Like many flavonoids, there is an absence of double bonds between positions 2 and 3. Sources of flavanols are apples and tea. Catechin, epicatechin, epigallocatechin, glausan-3-epicatechin, and proanthocyanidins are included under flavanols [17]. Plant source of flavanols includes *Brysonima crassa* [18].

4.8. Chalcones. Chalcones include phloretin and chalconaringenin [3]. They are characterized by the absence of C ring, or we can say there the C ring has an open structure. Therefore, they are also known as open-chain flavonoids. Different structure of different flavonoids are given in Figure 5.

5. Function of Flavonoids in Plants

Flavonoids play a wide number of important roles in plants (Figure 6). Flavonoids acts as signaling molecules, detoxifying agents, phytoalexins and hepls in the stimulation of seed germination, temperature acclimatization, and provide drought resistance. Flavonoids also reduce reactive oxygen species in plant tissue, which are generally generated due to infection or UV irradiation. Another important role they play is in the fragrance, color, and taste of fruits, flowers, or seeds. This fragrance and color attract pollinators that help pollination and dispersal of seeds [20].

5.1. Protect against Radiation. Flavonoids have the property of UV absorption, which is why they are considered to play a role in the protection of plants from UV radiation. Some of them act as free radical scavengers such as reactive oxygen species (ROS) and chelating metals [21]. UV light induces the formation of flavonol due to the presence of OH in the third position of the flavonoid backbone, and this is the main reason that is responsible for its chelating property of metal ions like iron, zinc, aluminum, and copper, and this property of them inhibits the formation of free radicals and reduces ROS that have already been produced; this was evident in some plants like *Petunia*, *Arabidopsis*, grapevine, and wild privet.

5.2. Protect against Infection. Flavonoids give protection to plants from pathogens and herbivores. They secrete substances such as phytoalexins and lignins that act as a barrier to prevent the spread of pathogens and regulate the expression of these genes that produce protective metabolites like flavonols. Legumes such as soya bean and chickpea produce isoflavonoids which play an important role in defense against pathogens. Vesitol, which comes from the class of isoflavans, is the main phytoalexin synthesized by lotus species. The C-glycosyl flavones maysin and other compounds such as apimaysin and methoxymaysin are secreted by maize in response to its pest corn earworm and interfere with its amino acid metabolism in the guts and are converted into very toxic quinones. These quinones bind to the -SH and -NH2 of proteins and amino acids and hence reduce their availability [21].

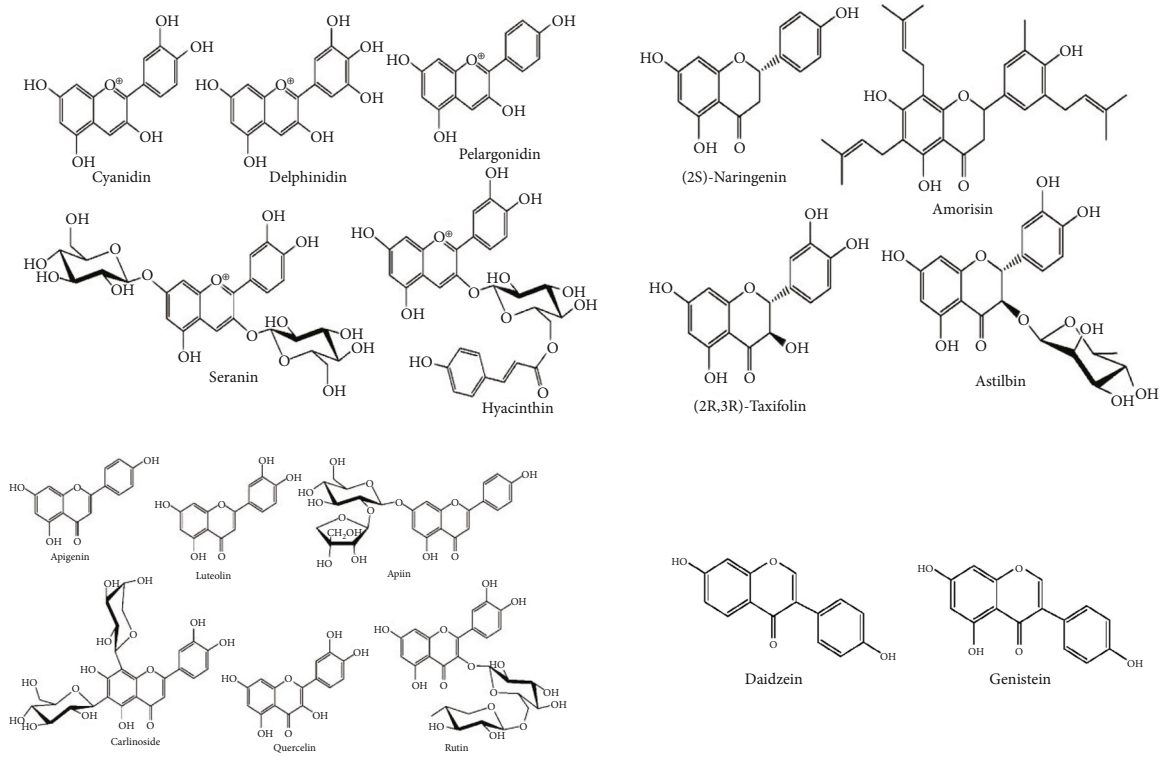

Figure 5: Structures of flavonoids [19].

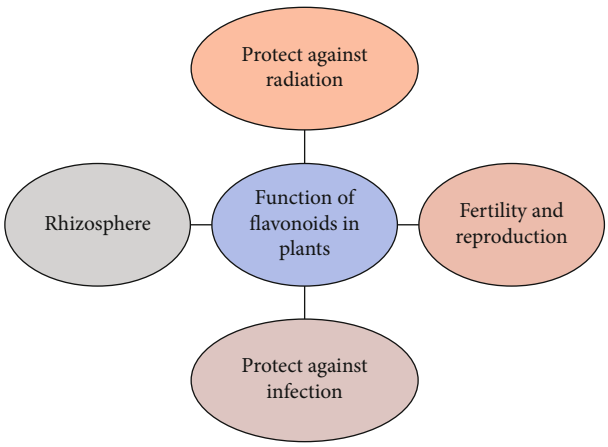

Figure 6: Function of flavonoids in plants.

5.3. Fertility and Reproduction. Flavonoids give color and fragrance to flowers and fruits in different plant species and attract pollinators. These pollinators help to remove and grow seeds and plants. The most important and major class of flavonoids that play a role in plant pollination is the anthocyanins present in flowers and fruits. Production of seed fruits and low level of anthocyanidin in tobacco is also achieved by blocking the pathway of rutin, a type of flavonol. Parthenocarpy is achieved by chalcone synthase gene silencing and by applying the flavonol quercetin and kaempferol these processes can be reversed. Thus, in this way, flavonoids play an essential role in the fertility and reproduction of plants [21].

5.4. Rhizosphere. Flavonoids also play an important role in the rhizosphere region of the roots of the plant by stimulating spore germination and chemoattraction of rhizobia, and they also amplify the expression of nod genes. Plant secretes flavonoids in response to infection by rhizobia such as *Azorhizobium*, *Bradyrhizobium*, *Mezorhizobium*, and *Sinorhizobium* as a result of which the plant oozes out flavonoids [22]. When these bacteria interact with the plants, they form a nodule and the bacteria remain inside the nodule as a bacteroid, this process results in the N2 fixation [22].

5.5. Extraction Processes for Flavonoids

5.5.1. Conventional Technique. There are various methods used for the extraction of flavonoids. These include percolation, maceration, hydrodistillation, Soxhlet reflux, and soaking. Among these methods, Soxhlet is the simplest, easiest to use, and most widely used method used for the extraction of flavonoids. Water infusion and marceration are the old extraction processes to adopt ethanol, acetone, and methanol usage for the extraction of flavonoids which they are still being used. Various factors are taken into account when using conventional methods. These factors are time, solvent type, mass to volume ratio, temperature, and particle size. Of the various solvents used in these extraction processes, ethanol and methanol are the most commonly used.

5.5.2. Enzyme-Assisted Technique. The enzyme-assisted method is an alternative method to the conventional method. This uses the specificity of enzymes and their regioselectivity. In this method, less solvent is used compared to

conventional methods [23]. Enzymes from various sources like fungi, bacteria, plants, fruits, and vegetables breakdown the cell wall, and this can increase the cell wall permeability and thus increases the extraction yield. Cello-bio-hydrolases, β-glucosidases, and endo- and exo-glucanases hydrolyze cellulase and increase cell permeability. Hemicellulose chains are degraded by the use of the enzymes pectin lyase, pectate lyase, and endo- and exo-polygalactouronases. Factors like treatment time, pH, and temperature and the amount of the enzyme are considered while using this method [23].

5.5.3. Extraction Using Ultrasound-Assisted Technique. 100 g of lotus leaves suspended in ethanol of concentration 40-80% and soaked in it for about 4 h in ultrasonic light for 15-40 min in ultrasound cleaner. From this, a deep brown extract was obtained, which was then filtered using the filter. This filter is then absorbed by D101 macroporous absorptive resin column at a speed of about 20 ml/min. This column is then eluted with dH2O to make this liquid colorless and then eluted with 80% ethanol. Then using a rotatory evaporator, cooled elutant was evaporated and pressure was reduced in order to obtain the flavonoids. In this extraction method, it was found that the extraction yield of flavonoids increased when ethanol was used in conc. 40-70%, but beyond 70%, the yield reduces [24].

5.5.4. Supercritical Fluid Extraction Method. Fluids that are above their thermodynamically critical temperature and pressure are known as supercritical fluids. Under these conditions, the viscosity decreases, and the diffusivity of the solvent increases. CO2, because of its flammable, nontoxic, cheap, and easy-to-use properties, is the most widely used solvent that is used in extraction methods. Low temperature which maintains the integrity of the products, high volatility of the solvent that reduces waste, and the separation of volatile from nonvolatile compounds are the advantages of using the supercritical fluid extraction method [23].

6. Therapeutic Properties of Flavonoids

Flavonoids have anti-inflammatory, antimutagenic, antiallergic, antiviral, and anticarcinogenic properties [23] (Figure 6). They also process therapeutic and cytotoxic activity. Flavonoids are inhibitors of various enzymes such as xanthine oxidase (XO), cyclooxygenase (COX), lipoxygenase, and phosphoinositol-3-kinase [25]. Flavonoids give color and aroma to the flowers; in fruits, they attract pollinators; and as a consequence, the pollinators help in seed dispersal and spore germination and seed growth. They protect the plant from biotic and abiotic stress and function as signal molecules, detoxing agents, UV filters, allopathic compounds, and phytoalexins and play an important role in drought resistance and tolerance to freezing [3].

6.1. Antioxidant Activity. Antioxidants are compounds which protect plants, animals, and humans against the effect of reacting oxygen species (ROS). Flavonoids act as an antioxidant by suppressing ROS formation either by inhibition of the enzyme or by chelating the trace elements involved in free radical generation, by scavenging ROS, or by upregulation or protection of antioxidant defenses [26]. The appearance, position, structure, and number of attached sugar units play an important role in antioxidant activity. The antioxidant activity of flavonoids is the most important activity.

6.2. Antiviral Activity. Flavonoids such as flavon-3-ol, flavones, and flavanones are effective against viral infection compared to flavones. Flavon-3-ol is more effective against HIV 1 and HIV 2 immunodeficiency [7]. According to studies, other flavonoids which show antiviral activity include quercetin, hespertin, and naringin. They possess antidengue activity [27]. These antiviral flavonoids help in the inhibition of various enzymes involved in the virus life cycle. Various studies have also focused on apigenin, vitexin, and their derivatives which were found to be active against many viruses such as hepatitis C virus, herpes simplex virus 1 (HSV-1), human hepatitis A and B and C virus, rhesus rotavirus (RRV), and influenza viruses [28].

6.3. Antimicrobial Activity. Various studies have shown that flavonoids are secreted by plants in response to bacterial infection. Flavonoids such as apigenin, galangin, flavone glycosidase, and chalone have been proved to show antibacterial properties by Cushnie and Lamb [29]. A wide variety of nonflowering medicinal plants and flowering plants show antibacterial activity by a large number flavonoid. *Asplenium nidus nidus* L. contains gliricidin 7-O-hexoside, and quercetin-7-O-rutinoside is a fern that gives protection for three pathogens, namely, *Proteus mirabilis* Hauser, *Pseudomonas aeruginosa* (Schroeter), and *Proteus vulgaris* [30]. Plants that are involved in traditional and dietary medicine also secrete various flavonoids and phenolic compounds. Nutmeg (*Myristica fragrans* Houtt.) is a flavoring agent used in India and other countries in Southeast Asia. Nutmeg activities can help to recuse a large number of species related to oxidative stress [14]. Flavonoids are also used in skin acne problems and show antibacterial activity for *P. acnes*. Chlorine-containing chloroflavin is the first flavonoid antibiotic against fungi. The peeling of oranges also contains several flavonoids that have fungistic activity against *Deuterophoma tracheiphila*. Myricetin, a flavonoid, inhibits the growth of multidrug resistance bacteria *Burkolderia cepcia* and inhibits protein synthesis by *B. cepcia* [31].

6.4. Cardioprotection. Many studies have shown the effectiveness of flavonoids in cardioprotection. Hypertension and atherosclerosis can be prevented by the usage of flavonoids. These flavonoids reduce atrial pressure, enhance the vasorelaxant process, and prevent endothelial dysfunction. Endothelial dysfunction is a leading cause of cardiovascular disease and is the major complication of atherosclerosis and arterial thrombus formation. Endothelium-dependent vasorelaxation is exerted by *anthocyanin delphinidin*. Atherosclerosis development starts with the oxidative modification of low-density lipoproteins by free radicals. Scavenger receptors take this modified LDL and lead to the formation of cells [32]. Cardioprotection is achieved by the activity of quercetin and quercetin glycosides by protecting the LDL from oxidative modification. The antioxidant activities of

polyphenolics may have significant benefits for health. This is due to the ability of polyphenols to transfer electron chelation of ferrous ions, and they also scavenge reactive oxygen species (ROS), and due to this, they are used in the protection of chronic cardioactivity [33].

6.5. Antidiabetic Activity. Flavonoids also possess antidiabetic activity by regulating carbohydrate digestion, insulin secretion, insulin signaling, glucose uptake, and adipose deposition [34]. They target multiple molecules that are involved in the regulation of several pathways such as improvement of β-cell proliferation, promoting insulin secretion, apoptosis reduction, and improving hyperglycemia by regulating glucose metabolism in the liver [35]. In US, a study was conducted where 200,000 women and men were suppelemented with dietary flavonoids. The study confirmed that a higher consumption of anthocyanins from blueberries, apples and pears, reduce the risk of diabetes. It was hypothesized that the majority of flavonoids bioactivity occurs due to their hydroxyl group, α, and β ketones [36].

6.6. Anticancer Activity. Studies have been reported that flavonoids are able to inhibit tumor cell proliferation by inhibiting formation of ROS and repression of xanthine oxidase, cyclooxygenase-2, and 5-lipoxygenase enzymes, which play important role in tumor promotion and development [37]. Flavonoids possess wide range of anticancer effects. In a study, it was reported that isorhamnetin and acacetin can inhibit human breast cancer proliferation [38]. In another study, kaempferol showed antiproliferative and apoptosis activity against breast (MCF-7) cancer, human osteosarcoma, stomach (SGC-7901), and lung (A549) carcinoma cells [39]. Another study reported that hesperidin can reduce progression of cell cycle in osteosarcoma MG-63 cells and induce apoptosis in various cancer cells like ovary, breast, prostate, and colon cancer cells [40]. Additionally, hesperidin shows antitumor and hepatoprotective effects against the development of hepatocellular carcinoma [40]. Cyanidin also showed inhibition of proliferation and induction of apoptosis in human epithelial colorectal adenocarcinoma cells [41].

7. Strategies to Enhance Flavonoid Production

Production of flavonoids can be improved via numerous techniques (Figure 7). One of the most effective tools is elicitation and feeding of precursors. In a study, the elicitation of hairy root culture by chitosan from seven species of *Psoralea* was found to enhance the production of flavonoids such as daidzein and coumestrol [42]. When the result of flavonoid production from hairy root culture and callus culture was compared, it was found that flavonoid production from hairy root culture was higher [42]. In another study, methyl jasmonate was used to improve flavonoid production in a cell suspension culture of *H. perforatum*. It was found that 100 μmol/L of methyl jasmonate treatment resulted in maximum production of flavonoid, i.e., 280 mg/L, which was 2.7 times higher than the control culture [43]. Quercetin and rutin production from hairy root culture of *F. tataricum* has been enhanced to 47.13 mg/l, which was about 3.2 times higher

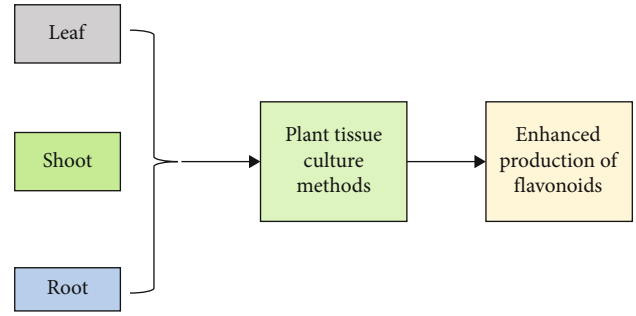

Figure 7: Strategies to enhance flavonoid production.

than that of control culture, i.e., 14.88 mg/L [44]. Shaw et al. [45] elicited an in vitro culture of *Hordeum vulgare* using CuONP and found that the leaves showed a significant enhancement of flavonol levels (~1.2 times more than the control) after 20 days of treatment. Fazal et al. [46] reported the treatment of silver and gold nanoparticles in callus cultures of *Prunella vulgaris*. They found that silver gold nanoparticles in the ration of 1 : 3 in combination with NAA enhanced flavonoid accumulation, i.e., 6.71 mg/g Dw. Genady et al. [47] used the treatment with copper sulfate nanoparticles in *O. basilicum* and found an enhanced flavonoid content. In a study, hairy root of *Dracocephalum kotschyi* was treated with iron oxide nanoparticles which showed enhanced flavonoid accumulation [48]. The cell suspension culture of *Momordica charantia* was treated with silver nanoparticles synthesized by *Bacillus licheniformis* [49], and it was found that the elicitation increased the concentration of flavonoids present in the plant, such as quercetin, kaempeferol, myricetin, catechin, naringenin, rutin, and biochanin A. A study showed that *Cucumis anguria* hairy root culture treatment with silver nanoparticles enhanced the production of flavonoids [50]. Singh et al. [51] reported the highest flavonoid production, i.e., 23.076 ± 5.128 mg QE/g extract after 20 days of CuONP treatment in case of *Withania somnifera*. Chung et al. [52] reported the treatment of *Gymnema sylvestre* cell suspension culture with copper oxide nanoparticles and found that it increased the production of total flavonoid content by two times. Nourozi et al. [53] reported the treatment of iron oxide nanoparticles in hairy root culture of *Dracocephalum kotschyi* that improved flavonoid production. Herbal products and natural phytochemicals, like flavonoids, flavonols, and other bioactive compounds, exhibit health-promoting effects. Evidence suggests that these metabolites have the potential to protect different cells from oxidative damage [54, 55]. A study reported that foliar application of jasmonic acid at a concentration of 0.25 mM resulted in improved antioxidant responses including flavonoid and anthocyanin production in *Jatropha curcas* [56]. Another study reported accumulation of flavonoids in *Isatis tinctoria* L. hairy root culture which was elicited by 179.54 μM methyl jasmonate, and it increased 11.21-folds as compared with controls [57].

8. Toxicological Evaluation of Flavonoids

Toxicological evaluation of flavonoids is also an important aspect which needs to be taken into consideration. A study

reported the evaluation of safety and toxicity of chrysin (plant flavonoid) after acute and subchronic oral administration in rats. It was found that acute oral administration of chrysin (5000 mg/kg) showed 40% mortality. In the subchronic toxicity study, daily oral administration of chrysin (1000 mg/kg) showed suggestively decreased body weight, whereas liver weight was improved significantly in male rats. Significant increase in renal and hepatic oxido-nitrosative stress was also observed, and no significant change in electrocardiographic, hemodynamic, the left ventricular function, and lung function test was found [58]. In a study, subchronic toxicity and genotoxicity of a flavonoid-rich extract from Maydis stigma was evaluated in mice. It was found that all animals survived until the planned necropsy, and no statistically significant or toxicologically relevant differences were observed in any of the treatment groups as compared to control one [59]. A study was conducted to investigate preclinical safety of four flavonoids, i.e., naringin, naringenin, hesperidin, and quercetin. Cytotoxicity was evaluated against VERO and MDCK cell lines, and it was found that quercetin was slightly more cytotoxic on cell lines than the other citroflavonoids. All flavonoids showed an LD50 value more than 2000 mg/kg, which categorizes them as low-risk substances. Similarly, predicted LD50 was LD50 more than 300 to 2000 mg/kg for all flavonoids as acute toxicity assay estimated and suggests that all these flavonoids did not show significant toxicological effects, and they were classified as low-risk, useful substances for drug development [60].

9. Conclusions and Future Prospects

Flavonoids are secondary metabolites which are secreted by plants. They provide various therapeutic applications for mankind which include cardioprotection, antidiabetic, and antiviral activity, as well as protection for plants in response to stress. They also play an important role in plant reproduction and fertility and in atmospheric nitrogen fixation. In summary, the valuable role of flavonoids for human health has generated increased consumption of these compounds. Vegetables, flowers, and seeds are rich source of flavonoids. The recognition of natural flavonoids as a good, safer source of antioxidants opens new perspectives to explore more of these compounds, focusing on new structures using new methodologies and technologies and exploiting other new natural sources. This review provides an overview of the role of flavonoids in plants, extraction procedures, and their therapeutic application for the benefit of humanity. Further production of different flavonoids using in vitro culture systems has been discussed.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Research Article

Studies of Phytochemicals, Antioxidant, and Antibacterial Activities of *Pinus gerardiana* and *Pinus roxburghii* Seed Extracts

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Pine seeds are considered as nonwood forest products (NWFP) with regularly increasing market's demand. They can be eaten in various ways such as roasted or raw. In addition, they are included in various traditional dishes like in cookies, sauces, candies, cakes, breads, and other bakery items and, moreover, for medicinal purposes. GC-MS study is performed to analyze the phytochemical compounds present in the seed extracts of *Pinus roxburghii* (Chir) and *Pinus gerardiana* (Chilgoza). In total, 25 compounds were identified each in Chir and Chilgoza. In Chir seeds, abundantly present compounds were 2,4-di-tert-butylphenol (16.6%), followed by ç-Terpinene (9.9%) and cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, (1à,3à,4á) (9.8%), whereas in Chilgoza seeds, the maximum amount of compound was 1-hexyl-1-nitrocyclohexane (17.3%), followed by phenol, 2,6-bis(1,1-dimethylethyl) (15.4%), and heptadecane, 2-methyl (8.4%). The total phenolic content of Chir seed sample was 1536 ± 4.35 (mg GAE/100 g), whereas in the Chilgoza seed extract was 642.66 ± 2.08 (mg GAE/100 g). The application of RP-HPLC-DAD system revealed that Chir and Chilgoza seeds have maximum quantity of catechin ($15.77 \pm 0.16\,\mu$g/mg and $17.49 \pm 0.32\,\mu$g/mg, respectively). Both Chir and Chilgoza seed extracts exhibited significant antioxidant (radical scavenging) potential, through H_2O_2 ($618.94 \pm 21.45\,\mu$g/mL and $575.16 \pm 19.88\,\mu$g/mL) and DPPH ($552.60 \pm 13.03\,\mu$g/mL and $429.15 \pm 3.80\,\mu$g/mL) assays, respectively. Additionally, a well-known antibacterial potential was also found in both plants' dichloromethane extracts, with 64 to 256 μg/mL of minimum inhibitory concentrations. As a whole, result shows the importance of both plants as a naturally occurring phytochemical source with significant antibacterial and antioxidant activity.

1. Introduction

Pine trees are one of the most common and widely grown species in the Himalayan region. Categorized under the *Pinus* genus and *Pinaceae* family, they are the largest conifer's families in the world and always remain evergreen woody conifer trees [1]. In the Himalayan region (HR), five species of pine are considered indigenous, distributed at different elevation such as *Pinus gerardiana* (Chilgoza), *Pinus wallichiana* (Kail), *Pinus roxburghii* (Chir), *Pinus merkusii* (Merkus), and *Pinus kesiya* syn. *Insularis* (khasi) as shown in Table 1 [2]. Among these, Chilgoza tree nuts have highly incomparable nutritious value therefore, commercialized for edible use and consumed in roasted form; these seeds are added as an ingredient in different dishes [3]. Whereas Chir trees are abundantly present and have ethno medicinal value, nuts are traditionally consumed in India and Pakistan [4, 5].

P. roxburghii, known as Chir pine trees, is generally 55 m tall and over 100 cm diameter breadth height (dbh). Tree bark is thick in dark reddish-brown while winter buds are brown, ovoid, small, and nonresinous. The tree leaves are needle-shaped in flabellate-triangular arranged in 3 per bundle and cylindrical in cross-section. Cones are pedunculate, short, ovoid, range in 10-15 × 6-9 cm, while seeds are small, about 8-12 mm in length with a long wing of 25 mm, and normally ripen in April [6].

P. gerardiana is traditionally called Chilgoza trees to a medium height (17 to 27 m) and 2-4 m in dbh. The branches are small and horizontal with glabrous bark and silver grey [7]. Leaves are needle-like shaped and dark green, arranged three per cluster. However, the male cones are longer than female ones, which are ovoid with hard woody scales. These seeds are in dark brown colored, pointed from top and cylindrical, and normally ripen in October [8].

Data available to date demonstrate that these trees are composed of different types of phenolic compounds, at varying amounts depending on the geographical origin, harvesting time and storage, with distinct biological activities being also reported [9]. In past two decades, massive studies were carried out on essential oils and extracts of several parts of pine trees. Studies generally focused on nutritional value, chemical composition, food supplements, and in drug formulation [10]. The natural bioactive compounds, including phenols, terpenes, flavonoids, alkaloids, and saponin obtained from different *Pinus* spp., have reported for their potency against several diseases, e.g., asthma, diabetes, neurodegenerative diseases, cancer, oxidative stress-related diseases, cardiovascular-related problems, liver and kidney disorders, and various pathogenic infections [2]. Among the phytochemicals present, these pine nuts usually contain inherent antioxidants that help in reducing the oxidation rate, namely, flavonoids, e.g., flavonols, and flavanones in various glycoside and aglycone form [11]. For instance various phenolic group constituents like as catechin, gallic acid and quercetin show antioxidant, antiallergic, antimicrobial, anti-inflammatory, UV protection, and anticancer activity [12, 13]. Ellagic acid has potency to inhibit the oxidation of low-density lipoprotein [14]. Vanillic acid is highly efficient to reduce oxidative stress and Aβ1-42-induced cognitive impairment, therefore very effective in Alzheimer's and other neuron-related disease [15].

Till now, studies have been carried out to assess the phytochemical composition and biological potential of Chilgoza seeds [3, 16]. In case of *P. roxburghii* except seed, the chemical composition and biological activities have been studied on its various parts, viz., needles and bark [4]. In this sense, the present study is aimed at evaluating the total phenolic content of *P. gerardiana* and *P. roxburghii* seed extracts and their bioactive compounds and at investigating the antioxidant and antibacterial roles. In addition, chemical composition and quantification of phenolic compounds were assessed by GC-MS and HPLC-DAD.

2. Materials and Methods

2.1. Chemical, Reagents, and Apparatus. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent, L-ascorbic acid, di-sodium hydrogen phosphate de-hydrate, and sodium dihydrogen phosphate dehydrate were purchased from HiMedia Laboratories Pvt. Ltd. (India). HPLC grade water, sodium carbonate, and hydrogen peroxide were procured from Loba Chemie Pvt. Ltd. (India). Gallic acid, quercetin, ellagic acid, vanillic acid, catechin, epigallocatechin gallate (EGCG), and DCM were procured from Sigma-Aldrich (USA). LC–MS grade acetonitrile, methanol, and formic acid (of 98% purity) were procured from Fisher Chemicals (Hampton, NH, USA). Colistin was procured from HiMedia Laboratories Pvt. Ltd. (India). Labeled, CHROMAFIL Xtra poly ether sulfone (PES), 25 mm, and 0.20 μm syringe filters were purchased from Macherey-Nagel (Düren, Germany). UV-vis spectrophotometer (Evolution 201) was procured from Thermo Fisher Scientific-Shanghai (China). ORBITEK shaker was purchased from Scigenics Biotech Pvt. Ltd. (India). Refrigerated centrifuge C-24 Plus was from REMI Sales and Engineering Ltd. (India). Analytical balance Aczet, CY2202 was purchased from Mettler-Toledo Pvt. Ltd. (India). Rotary vacuum evaporator-RE-52 was purchased from SONAR (India). Micropipette (20-200, 100-1000, and 0.5-10 μL) of variable volume purchased from HiMedia Laboratories Pvt. Ltd. (India).

2.2. Preparation of Seed Extracts. At room temperature, deshelled Chilgoza and Chir samples were grounded into powder with the help of commercial mixer grinder. Powdered seed samples of 50 g were added to 500 mL conical flask containing 250 mL dichloromethane (DCM) solvent each and kept in incubator cum shaker for 48 h at 37°C. Each sample was strained through Whatman no. 1 filter paper. After the extraction process, the liquid extracts were collected and then concentrated at 40°C by using a rotary vacuum evaporator. The prepared extracts were collected and stored at 4°C in refrigerator for further analysis [17].

2.3. Total Phenolic Content. The total phenolic content (TPC) of Chilgoza and Chir was determined by using Folin-Ciocalteau reagent [18]. Seed extracts (about 20 μg) were separately taken and made volume up to 1 mL by

Table 1: Geographical allocation of various pines species in HR [2].

Pine species	Habitat and distribution	Altitude (m)
P. gerardiana	Occur in drier rocky slope in some part of J&K and Kinnaur region of Himachal Pradesh	2000-3350
P. wallichiana	Found in the higher altitude of Himalayas drier region along with J&K up to Arunachal Pradesh north-eastern region of India	Till 2700
P. roxburghii Sarg.	Grows up and found in outer Himalayan valleys drier region of J&K to the Arunachal Pradesh	400-2300
P. merkusii	Found some regions in high moisture area of Indo-Myanmar border	1500
P. kesiya or *insularis*	Grows up in high moisture regions of Meghalaya north-eastern part of India	Up to 3000

adding distilled water. Then, Folin-phenol reagent (500 μL) was added into that, and 2.5 mL sodium carbonate Na_2CO_3 (20%) was also added. It was mixed properly and kept away from light for 40 min for incubation and color development. Postincubation, the absorbance was taken at 725 nm. Gallic acid calibration curve was constructed, and linearity was found in 5-25 μg/mL range. Seed extract TPC was stated in mg of gallic acid equivalent (mgGAE/100 g seed extracts) by using the standard curve.

2.4. GC-MS Study of the Seed Extracts. Each seed extract sample was diluted by adding DCM (1:10), and their fraction components were analyzed by GC-MS (TRIPLE QUAD GC-MS/MS) (Thermo Fisher, USA), equipped with an autosampler (TriPlusRSH) and DB 5 column (40 m × 0.15 mm i.d., film thickness of 0.15 μm). For analysis of two different seed extracts, the following GC-MS operating conditions were followed with slightly modification as described by Al-Owaisi et al. [19]: initial temperature was kept at 80°C and time held for a min, thereafter with 10°C/min ramping rate reached up to 180°C by holding 2 min, and finally with same ramping increased to 260°C and was held for 15 minutes. Transfer line temperature, 250°C; carrier gas, He at constant flow rate 0.7 mL/min; split ratio, 71 : 4; 1 μL of injection volume; component ionization, electron impact (70 eV) mode; EI source temperature, 230°C; m/z range, 45-450. The respected component relative concentrations were expressed as percent area on basis evolved in chromatograph. The identity of their components was done on the basis of visual interpretation and compared based on probability and literature search input as per NIST library (v. 2.2, 2014) with different types of compounds identified.

2.5. HPLC Analysis of the Seed Extracts. RP-HPLC system (Capcellpak) of Shimadzu (Kyoto, Japan) equipped well with a C-18 (2) column of phenomenex Luna (4.6 mm i.d. ×25 cm, 5 μm) and a detector of diode array (DA) (SPD-M20A, Shimadzu, Japan) were taken for the phenolic compound quantification, including gallic acid, ellagic acid, vanillic acid, epicatechin, quercetin, catechin, and EGCG isomers [3]. Solvents used to run were as follows: solvent A: aqueous formic acid of 8% and solvent B: 10 : 90, *v*/*v* (acetonitrile/methanol). The flow rate was 0.9 mL/min. Injection volume was 20 μL. The gradient was as given: 0 min, 20% B; 7 min, 35% B; 14 min, 45% B; 21 min, 65% B; 25 min, 85% B; and 32 min, 95% B. To monitor all the phenolic compounds, the DA detector wavelengths were fixed at 260, 280, or 320 nm. Standard solution preparation was done as described by Lee et al. [20]. From the stock, 0.01 mL was taken and diluted up to 1 mL diluents to make necessary dilutions and filtered through 0.22 μ PES membrane filters and injected in HPLC system.

2.6. Antioxidant Activities

2.6.1. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Scavenging Assay. The free radical scavenging potential of two seeds' extract was evaluated following the method followed by Bhatti et al. [21] with slightly modifications. Stock solutions of both seeds' extracts (1 mg) were prepared in methanol (MeOH), and further different methanolic solution concentrations (20-640 μg/mL) were prepared. From each concentration, 300 μL was added to methanolic solution 2700 μL of DPPH (4 mg/100 mL). The mixture solution was incubated in absence of light at 37°C room temperature for one hour. Free radical scavenging efficacy of extracts was based on the initial purple color disappearance. Absorbance of solution was taken at 517 nm. For positive control, ascorbic acid was used [21]. Scavenging capacity of DPPH was determined using the formula below given:

$$\text{DPPH radical scavenging activity } I\,(\%) = \frac{(\text{A blank} - \text{A sample})}{\text{A blank}} \times 100, \quad (1)$$

where the IC_{50} of DPPH radical was calculated from the line regression of the percentage of remaining DPPH radical against the sample concentration.

2.6.2. Hydrogen Peroxide Scavenging Assay (H_2O_2). The capacity of seed extract to scavenge H_2O_2 was estimated following the procedure of Bhatti et al. [21]. Briefly, 0.1 mL extract aliquots (20-640 μg/mL) were added into an Eppendorf tubes to made volume up to 0.4 mL with addition of 50 mM (pH 7.4) phosphate buffer and (2 mM) H_2O_2 solution (0.6 mL). Mixture was properly vortexed and kept for 10 min, and then, absorbance was read at 230 nm. Ascorbic acid was used as positive control [21]. The extracts' ability to scavenge the H_2O_2 was evaluated by using the following equation:

$$H_2O_2 \text{ scavenging activity percentage} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100, \quad (2)$$

where A_0 is the absorbance of blank and A_1 is the absorbance of sample.

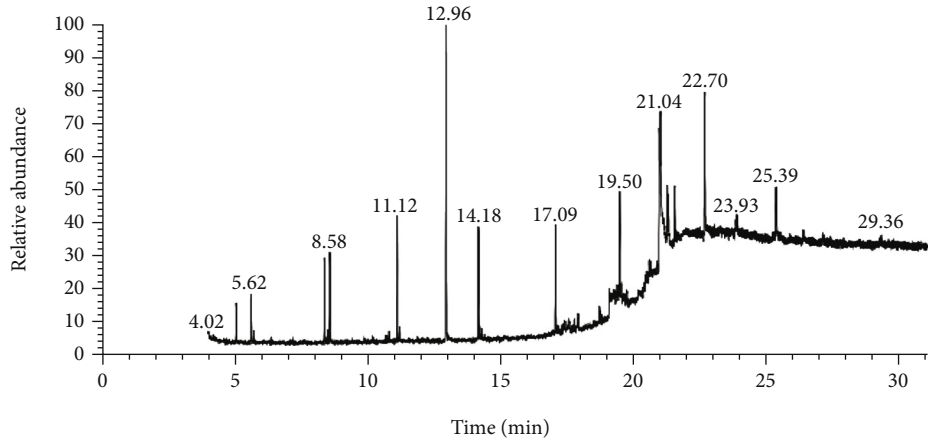


Figure 1: GC-MS profile of the DCM extract of Chilgoza with their retention time and peak assignment as in Table 2.

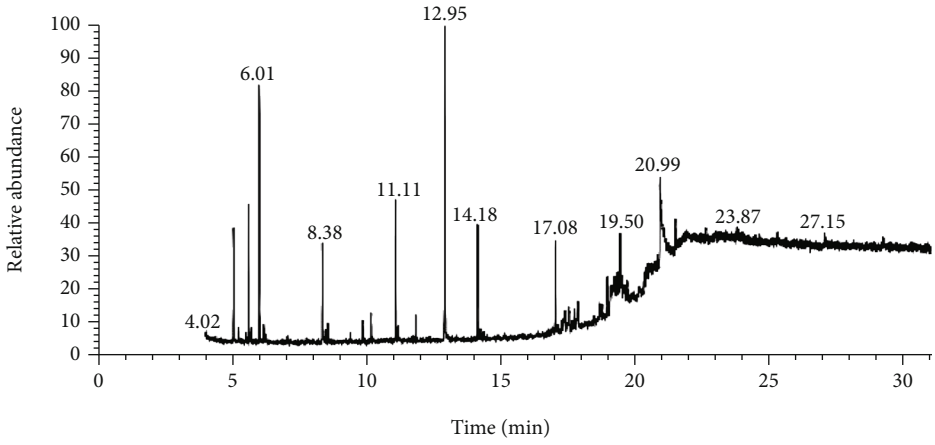


Figure 2: GC-MS profile of the DCM extract of Chir with their retention time and peak assignment as in Table 3.

2.7. Antibacterial Screening by Minimum Inhibitory Concentration (MIC). Gram-negative bacteria viz., *Salmonella typhimurium* MTCC 3224, Klebsiella pneumonia MTCC 109, and *Escherichia coli* MTCC 443 were used to study the antibacterial potential of both seeds' extracts, using the broth macrodilution technique [22]. DCM extracts were prepared in Mueller Hinton broth, and serial dilutions were obtained, ranging between 0.5 and 256 μg/mL. Bacteria ($1\text{-}2 \times 10^8$ CFU/mL) were transferred to test tubes and incubated at 37°C for 24 h. Minimum inhibitory concentrations (MIC) were determined, being considered as the lowest concentrations without visible turbidity. Colistin was used as a positive antibacterial control, while DCM was used as negative control [22].

2.8. Statistical Evaluation. Results obtained are expressed as mean value and standard deviation ($x \pm$ SD) with three times repeated trials for all experiments. The IC_{50} values were obtained by plotting inhibition-concentration curves by nonlinear regression analysis. Statistical analysis was performed using the Microsoft Excel.

3. Results and Discussion

3.1. Total Phenolic Content. The TPC of Chilgoza DCM seed extract was 642.66 ± 2.08 mg GAE/100 g, and TPC of Chir seed sample was 1536 ± 4.35 mg GAE/100 g. Hoon et al. reported that the maximum TPC in Chilgoza seeds was highest in water extract, followed by DCM, ethanol (EtOH), hexane (HEX), and ethyl acetate (EtOAc) as well as MeOH extracts [3]. Mahdhi et al. studied the high quantity of total phenols in *P. halepensis* methanolic-aqueous seed extracts 14.63 ± 0.05 mg/g gallic acid equivalent weight (GAE) [23]. Zulfqar et al. found higher TPC (77.2 ± 1.41 mg GAE/g) in methanolic extract of *P. gerardiana* dry nuts than in EtOAc extract (52.5 ± 2.9 mg GAE/g) [16]. Valero-Galván et al. found that *Pinus cembroides* grown in five states of Mexico presented different amounts of TPC in the methanol seed extract [24]. Bolling et al. report that TPC of pine nut was 68 mg GAE/100 mg; however, as per Phenol-Explorer database reports 58 mg GAE/100 mg [25]. Kadri et al. reported that Algerian pine species, viz., *Pinus pinea*, *Pinus halepensis*, *Pinus canariensis*, and *Pinus pinaster* seed extract, TPC differ within species [26]. Su et al. reported that *Pinus koraiensis*

Table 2: List of phytocompounds identified in Chilgoza by GC-MS.

Peak no.	Compound	RT	Area %	Mol. weight	Molecular formula	CAS. no.
1.	3-Carene	5.06	1.53	136	$C_{10}H_{16}$	13466-78-9
2.	2-Propyn-1-ol, acetate	5.62	2.27	98	$C_5H_6O_2$	627-09-8
3.	1-Undecanol	8.38	3.14	172	$C_{11}H_{24}O$	112-42-5
4.	Naphthalene	8.58	3.81	128	$C_{10}H_8$	91-20-3
5.	8-Heptadecene	11.12	4.75	238	$C_{17}H_{34}$	2579-04-6
6.	Phenol, 2,6-bis(1,1-dimethylethyl)	12.96	15.42	206	$C_{14}H_{22}O$	128-39-2
7.	1-Hexadecanol	14.18	5.40	242	$C_{16}H_{34}O$	36653-82-4
8.	10-Heneicosene	17.09	4.77	294	$C_{21}H_{42}$	95008-11-0
9.	(2S,4R)-p-Mentha-[1(7),8]-diene 2-hydroperoxide	17.59	1.42	168	$C_{10}H_{16}O_2$	NA
10.	2R-Acetoxymethyl-1,3,5-trimethyl-4c-(3-methyl-2-buten-1-yl)-1c-cyclohexanol	17.93	1.43	282	$C_{17}H_{30}O_3$	NA
11.	Tetradecanoic acid, 10,13-dimethyl-, methyl ester	18.75	1.31	270	$C_{17}H_{34}O_2$	267650-23-7
12.	Tetradecanoic acid, 12- methyl-, methyl ester, (S)	19.12	2.58	256	$C_{16}H_{32}O_2$	62691-05-8
13.	Acetophenone, 2-[(p-nitrophenyl)imino]	19.21	1.60	254	$C_{14}H_{10}N_2O_3$	6394-60-1
14.	2-Methyl-3-(2,2-dimethylpropyl)-butadiene	19.41	1.20	138	$C_{10}H_{18}$	90822-87-0
15.	1-Nonadecene	19.50	6.08	266	$C_{19}H_{38}$	C19H38
16.	8-Isopropyl-5-methyl-5,6,7,8-tetrahydro-2,4-quinazolinedione	20.21	1.28	222	$C_{12}H_{18}N_2O_2$	63498-93-1
17.	4-tert-Octylphenol, TMS derivative	20.62	1.20	278	$C_{17}H_{30}OSi$	8721-87-6
18.	1-Hexyl-1-nitrocyclohexane	21.03	17.30	213	$C_{12}H_{23}NO_2$	118252-09-8
19.	5,10-Pentadecadienoic acid, (E,E)-	21.29	2.34	238	$C_{15}H_{26}O_2$	64275-68-9
20.	1,1,1,3,5,5,5-Heptamethyltrisiloxane	21.34	2.31	222	$C_7H_{22}O_2Si_3$	1873-88-7
21.	1-Hexyl-2-nitrocyclohexane	21.57	3.51	213	$C_{12}H_{23}NO_2$	118252-04-3
22.	Heptadecane, 2-methyl	22.70	8.49	254	$C_{18}H_{38}$	1560-89-0
23.	Cyclotrisiloxane, hexamethyl	23.93	1.48	222	$C_6H_{18}O_3Si_3$	541-05-9
24.	4,4-Dipropylheptane	25.39	4.20	184	$C_{13}H_{28}$	17312-72-0
25.	1-propanone, 1-[5-ethyl-3-(5-nitro-2-furanyl)-1H-1,2,4-triazol-1-yl]	27.16	1.16	264	$C_{11}H_{12}N_4O_4$	35732-74-2

NA: not applicable.

seed (PKS) ethanolic extract contained higher total phenolic content of 264 mg GAE/g [27].

3.2. GC-MS Analysis of the Seed Extracts. Data obtained from the GC-MS analysis of DCM extracts of Chilgoza and Chir seeds revealed the presence of terpenoids, alcohols, alkenes, aromatic hydrocarbons, etc. (Figures 1 and 2). A total of 25 compounds were detected in Chilgoza and Chir, as shown in Tables 2 and 3. In Chilgoza seeds, the most abundant compound was 1-hexyl-1-nitrocyclohexane (17.3%), followed by phenol, 2,6-bis(1,1-dimethylethyl) (15.4%), and heptadecane, 2-methyl (8.4%), while in Chir seeds, 2,4-di-tert-butylphenol (16.6%), followed by ç-Terpinene (9.9%), cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, (1à,3à,4á) (9.8%) were the most commonly identified.

Kadri et al. reported α-pinene only in *P. pinaster*. Tables 4 and 5 display the biological activities of some compounds present in Chilgoza and Chir extracts, as reported in the various literatures [26]. However, GC-MS studies on pine nuts extracts were not updated in the literature; researchers emphasized on the essential oil analysis attained by extraction or emission from various tree parts, viz., branches, bark, cones, and needles [28].

Table 3: List of compounds identified in Chir by GC-MS.

Peak no.	Compound	RT	Area %	Mol. weight	Molecular formula	CAS no
1.	α-Pinene	5.06	4.59	136	$C_{10}H_{16}$	80-56-8
2.	3-Carene	5.62	6.22	136	$C_{10}H_{16}$	13466-78-9
3.	ç-Terpinene	6.01	9.99	136	$C_{10}H_{16}$	99-85-4
4.	1-Undecanol	8.38	4.11	172	$C_{11}H_{24}O$	112-42-5
5.	Oxirane, 2-(chloromethyl)-2-cyclopropyl	10.20	1.43	132	C_6H_9ClO	121505-35-9
6.	1-Hexadecanol	11.11	5.46	242	$C_{16}H_{34}O$	36653-82-4
7.	2,4-Di-tert-butylphenol	12.95	16.67	206	$C_{14}H_{22}O$	96-76-4
8.	10-Heneicosene	14.18	5.74	294	$C_{21}H_{42}$	95008-11-0
9.	1-Eicosanol	17.08	4.30	298	$C_{20}H_{42}O$	629-96-9
10.	5,10-Pentadecadienoic acid, (E,E)-	17.43	2.03	238	$C_{15}H_{26}O_2$	64275-68-9
11.	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-9 (phenylsulfonyl)-, (E,E)	17.58	2.82	362	$C_{21}H_{30}O_3S$	57683-67-7
12.	(2S,4R)-p-Mentha-[1(7),8]-diene 2-hydroperoxide	17.93	2.35	168	$C_{10}H_{16}O_2$	NA
13.	2,6-Dimethyl-3,5,7-octatriene-2-ol	18.75	1.77	152	$C_{10}H_{16}O$	29414-56-0
14.	10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5á-ol	19.02	1.99	220	$C_{15}H_{24}O$	19431-80-2
15.	4-tert-Octylphenol, TMS derivative	19.14	2.01	278	$C_{17}H_{30}OSi$	78721-87-6
16.	2,3-Dimethylamphetamine	19.21	2.20	163	$C_{11}H_{17}N$	75659-60-8
17.	10-Pentadecen-5-yn-1-ol	19.29	1.43	222	$C_{15}H_{26}O$	64275-59-8
18.	1,1,1,3,5,5,5-Heptamethyltrisiloxane	19.40	1.48	222	$C_7H_{22}O_2Si_3$	1873-88-7
19.	1-Hexyl-2-nitrocyclohexane	19.50	4.56	213	$C_{12}H_{23}NO_2$	118252-04-3
20.	1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl ester	19.77	1.61	238	$C_{11}H_{10}O_6$	54699-35-3
21.	3,4-Nonadien-6-yne, 5-ethyl-3-methyl	20.51	2.28	162	$C_{12}H_{18}$	61227-88-1
22.	4(3H)-Quinolinone, 3-hydroxy	20.57	1.75	161	$C_9H_7NO_2$	55759-82-5
23.	Cyclohexanol, 4-ethenyl-4-methyl-3-(1-methylethenyl)-, (1à,3à,4á)	21.00	9.86	180	$C_{12}H_{20}O$	56298-45-4
24.	1-Hexyl-1-nitrocyclohexane 2-tert-butyl-3-(tert-butylimino)-4-phenyl	21.56	2.06	213	$C_{12}H_{23}NO_2$	118252-09-8
25.	Thieno[2,3-b]pyridine, 5-ethyl-3-nitro	27.15	1.30	208	$C_9H_8N_2O_2S$	51043-51-7

NA: not applicable.

3.3. HPLC Analysis of the Seed Extracts. Six compounds were detected by HPLC-DAD system based on available standards (Supplementary Table S1). The highest quantity was of catechin ($10.49 \pm 0.32\,\mu$g/mg), followed by gallic acid ($5.39 \pm 0.39\,\mu$g/mg), ellagic acid ($5.21 \pm 0.15\,\mu$g/mg), vanillic acid ($1.6 \pm 0.12\,\mu$g/mg), quercetin ($0.84 \pm 0.04\,\mu$g/mg), and EGCG ($0.15 \pm 0.04\,\mu$g/mg) were found in Chilgoza seeds. On the other hand, Chir seeds contained catechin ($1.57 \pm 0.16\,\mu$g/mg), followed by ellagic acid ($1.47 \pm 0.06\,\mu$g/mg), gallic acid ($1.31 \pm 0.08\,\mu$g/mg), quercetin ($1.28 \pm 0.09\,\mu$g/mg), and vanillic acid ($0.27 \pm 0.02\,\mu$g/mg). EGCG was not detected in the Chir sample.

Hoon et al. also reported the highest quantity of catechin in Chilgoza DCM seed extract, but our data on EGCG contradict that obtained by this author [3]. Zulfqar et al. found that the maximum quantity of gallic acid (11.41 ppm) in methanolic extract of *P. gerardiana* dry nuts and in ethyl acetate extract quercetin was highest (165.33 ppm) [16]. Sadeghi et al. found maximum amounts of epicatechin ($10.3 \pm 0.18\,\mu$g/mg), followed by catechin ($10.1 \pm 0.18\,\mu$g/mg) in *Pinus eldarica* seeds grown in different regions of the Tehran province in Iran [39]. Mahdhi et al. compared eleven different phenolic compounds identified from *P. halepensis* methanolic-aqueous seed extract by convection-drying method and sun-drying method and reported that cirsiliol was chief flavonoid component, i.e., (0.761 and 1.916), than luteolin (0.589 and 1.760), followed by catechin (+) (0.569 and 0.888) and luteolin-7-O-glucoside (0.017 and 0.148) mg/100 g of dry weight, respectively [23].

3.4. Antioxidant Activities. The results of the antioxidant activity of Chilgoza and Chir seed extracts are shown in Table 6. Ascorbic acid showed stronger antioxidant activities in both DPPH ($326.70 \pm 9.64\,\mu$g/mL) and H_2O_2 ($375.73 \pm 11.73\,\mu$g/mL) assays as compared to both seed powder extracts tested. Comparing both extracts, the antioxidant potential of Chilgoza seed extracts in both DPPH and H_2O_2 assays was higher compared to that of Chir seed

Table 4: Biological activities of Chilgoza seed compounds.

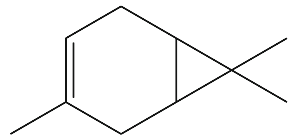
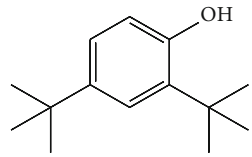
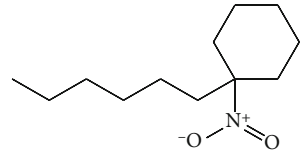
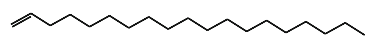
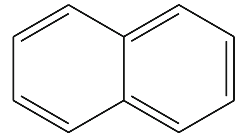
Compound name	Nature of compound	Structure	Biological activities	Ref
3-Carene	Monoterpenes		Antiacetylcholinesterase and antimicrobial	[29]
2,4-Di-tert-butylphenol or phenol, 2,6-bis(1,1-dimethylethyl)	Phenylpropanes		Antimicrobial, antioxidant, anti-inflammatory, cytotoxic, nematicidal, insecticidal, and allelopathic effect	[30]
1-Hexyl-1-nitrocyclohexane	Ketone		Antioxidant, antimicrobial, anti-inflammatory	[31]
1-Nonadecene	Alkene		Antifungal, anticancer	[32]
Naphthalene	Aromatic hydrocarbon		Anticancer, antiviral, antimicrobial, antidepressant, antineurodegenerative antidiabetic, anti-inflammatory, antitubercular, antihypertensive, antipsychotic, anticonvulsant	[33]

Table 5: Biological activities of Chir seed compounds.

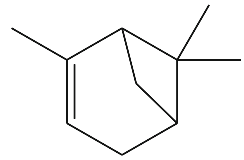
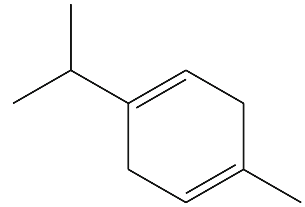
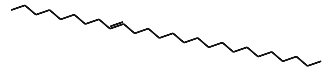
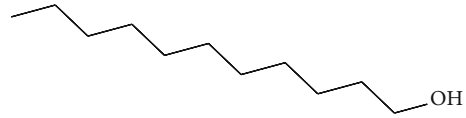
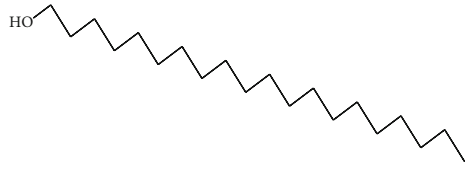
Compound name	Nature of compound	Structure	Biological activities	Ref
α-Pinene	Monoterpene		Antibiotic resistance modulation, antimicrobial, anti-*Leishmania*, antitumor, analgesic, antioxidant, anti-inflammatory, and antimalarial	[34]
ç-Terpinene	Monoterpene		Antibacterial	[35]
9-Hexacosene	Alkene		Analgesic and anti-inflammatory	[36]
1-Undecanol	Aliphatic alcohol		Bactericidal, larvicidal, and membrane-damaging activity	[37]
1-Eicosanol	Primary alcohols		Antitumor and antibacterial activity	[38]

Table 6: Antioxidant activity of Chilgoza and Chir seed extracts on DPPH and H_2O_2 assays.

Plant name	Plant part extract	Mean IC_{50} value μg/mL ± standard deviation	
		DPPH	H_2O_2
Pinus gerardiana (Chilgoza)	Seed	429.15 ± 3.80	575.16 ± 19.88
Pinus roxburghii (Chir)	Seed	552.60 ± 13.03	618.94 ± 21.45
Ascorbic acid (control)		DPPH (326.70 ± 9.64 μg/mL)	H_2O_2 (375.73 ± 11.73 μg/mL)

Table 7: Antibacterial activities of Chilgoza and Chir seed extracts.

Plant name	Plant part extract/MIC μg/mL	Bacteria
Pinus gerardiana (Chilgoza)	Seed/128	*Salmonella typhimurium* MTCC 3224
Pinus gerardiana (Chilgoza)	Seed/128	*Klebsiella pneumonia* MTCC 109
Pinus gerardiana (Chilgoza)	Seed/256	*Escherichia coli* MTCC 443
Plant name	Plant part extract/MIC μg/mL	Bacteria
Pinus roxburghii (Chir)	Seed/128	*Salmonella typhimurium* MTCC 3224
Pinus roxburghii (Chir)	Seed/64	*Klebsiella pneumonia* MTCC 109
Pinus roxburghii (Chir)	Seed/128	*Escherichia coli* MTCC 443

sample extract, which seems to be attributed to the presence of the high amount of antioxidant phytocompounds in Chilgoza samples as detected through the HPLC-DAD system, although the TPC in Chir seed samples was almost double than Chilgoza seeds.

However, earlier studies reported on pine nuts' phytochemical composition analyzes the presence of tocopherols, carotenoids, phytosterols, linoleic acids, and vitamin C, all of them revealing strong antioxidant potential, being their concentration higher than that in phenolic compounds [3, 9, 25]. The results obtained by DPPH assay to Chilgoza seeds DPPH were found accordingly with Hoon et al., who reported that an IC_{50} value of DCM seed extract was as good as compared to EtOAc, EtOH, HEX, and MeOH extracts. Moreover, Chilgoza water extract results were much better [3]. Zulfqar et al. reported that the percentage of DPPH inhibition of both MeOH and EtOAc extract of *P. gerardiana* dry nuts was 76.33 ± 2.51% and 73.67 ± 2.75% at concentration of 10 mg/mL and was statistically insignificant [16]. Valero-Galván et al. reported that methanol seed extract of *P. cembroides* grown in the five states of Mexico revealed different antioxidant activity assessed by DPPH assay [24]. *P. halepinsis* found in Palestine region displayed that methanolic extract by maceration and Soxhlet extraction method showed IC50 of 0.12 mg/mL and 0.43 mg/mL, respectively [10]. Mahdhi et al. studied maximum antioxidant activities from *P. halepensis* methanolic-aqueous seed extracts by using DPPH at concentration of 0.08 mg/mL [23]. Su et al. reported that *Pinus koraiensis* seed (PKS) ethanol extract displayed significant scavenging activity on 2,2-diphenylpicrylhydrazyl (DPPH) (EC50, 0.023 ± 0.004 mg/mL) and significant suppressive effect on lipid peroxidation in liver as well as enhance the glutathione (GSH) and superoxide dismutase (SOD) antioxidant enzyme levels and reduce malondialdehyde (MDA) content in the brain and liver of rat [27]. Stem bark hydro alcoholic extract of *P. roxburghii*, *P. wallichiana*, and *P. gerardiana* showed significant IC_{50} value (μg/mL) against DPPH at concentrations 97.54, 111.40, and 102.86, and ascorbic acid showed value at 18, respectively, and H_2O_2 (μg/mL) showed IC_{50} value at 86.90, 84.18, and 81.83 while ascorbic acid showed 16.72, respectively [40].

3.5. Antibacterial Activity. The DCM extract of both Chilgoza and Chir seeds was found effective against Gram negative bacteria, viz., *S. typhimurium* MTCC. 3224, *K. pneumonia* MTCC. 109, and *E. coli* MTCC. 443. Results of seed extracts of Chilgoza and Chir antibacterial activity are shown in Table 7. Colistin was used as a positive control (MIC value was 8 μg/mL).

Both Chilgoza and Chir seed antibacterial potentials were expected due to the occurrence of the antimicrobial compounds 3-carene, 2,4-di-tert-butylphenol, 1-hexyl-1-nitrocyclohexane, naphthalene, α-pinene, γ-terpinene, 1-undecanol, and 1-eicosanol, as reported in Tables 4 and 5. The possible target sites of phytocompounds in microbes are cell membrane, cell wall, and different enzymes. Salim et al. found that the antibacterial activities of *P. halepinsis* ethanolic seed extracts displayed good inhibition percentage against bacteria *Staphylococcus aureus*, *E. coli*, and *Shigella* at range of 0.02 g/mL [10]. Sharma et al. reported the antibacterial activity against *Pseudomonas aeruginosa*, *K. pneumonia*, and *E. coli* to the bark hydroalcoholic extract of three types of pine species, viz., *P. roxburghii* (Chir), *P. wallichiana* (kail), and *P. gerardiana* (Chilgoza) by well diffusion method, despite *P. wallichiana* displayed the most prominent antibacterial activity [40].

4. Conclusion and Future Perspectives

Secondary metabolites present in pine seed extracts are coated with excellent biological properties. In this study, the DCM seed extract of *P. gerardiana* and *P. roxburghii* revealed to be a rich source of molecules with interesting antioxidant and antimicrobial effects. The obtained results are positive and, if supported by *in vivo* studies, may be further proposed to be used for therapeutic purposes. In the

near future, deeper studies on this field should be done, and other biological effects of such trees' extracts should also be carried out in experimental trials. Other studies should also be done to a better understanding of the impact of seed collection from different regions with altitudinal variation, in addition to correlation with climate, soil, and regional geographic data in chemical composition. Equally important will be to perform a combined analysis of protein, amino acid, minerals, and lipid profiles to reach a more clear understanding on the real potentialities of these less investigated trees.

However, the Chilgoza seeds are eaten in roasted form in several countries, but still Chilgoza and Chir seeds are not utilized in functional food development. Recently, our group has developed the cookies having Chilgoza and Chir seeds used in decorated form to enhance its nutritional value [41]. Still there is a need to develop functional/nutraceutical foods using Chilgoza and Chir seeds which ultimately gives new employment horizon to the hilly area people.

The limitation of the current study is the selection of extraction solvent. We believe that DCM solvent has not much compatibility with our seed samples; that was the reason we got antioxidants and antibacterial activity at higher concentration.

Data Availability

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

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

PB and KK conceptualized and designed the project. KB performed all experiments and wrote the main manuscript text. RS analyzed the data. NC-M, MV, and NKU critically analyzed the data and drafted the final manuscript. MV and KK arranged the funds.

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Supplementary Materials

Table S1: retention time of different flavonoids. *(Supplementary Materials)*

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Research Article

Pharmacological Potential of *Avicennia alba* Leaf Extract: An Experimental Analysis Focusing on Antidiabetic, Anti-inflammatory, Analgesic, and Antidiarrheal Activity

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Avicennia alba is a mangrove plant that is extensively used to treat severe health issues. This focus of this study was to investigate the antidiabetic, anti-inflammatory, analgesic, and antidiarrheal activities of methanolic extract of *A. alba* leaves in Swiss albino mouse model. The antidiabetic, anti-inflammatory, analgesic, and antidiarrheal activities of the leaf extract were performed using alloxan-monohydrate, carrageenan-induced paw edema, acetic acid-induced writhing test and the hot plate method, and castor oil-induced method, respectively. The extract was used at doses ranging from 200 to 500 mg/kg to conduct the investigation. Leaf extract at 400 and 500 mg/kg showed potent antidiabetic activity in alloxan-induced diabetic mice. Advanced research is needed to control blood glucose levels and carrageenan paw edema-based anti-inflammatory effects. Both tests showed statistically significant result in a dose-dependent manner. The maximum dose (500 mg/kg) demonstrated potent analgesic activity in both writhing test and hot plate method. The plant extract also showed significant antidiarrheal activity at 400 and 500 mg/kg in experimental mice. However, more research is needed to explore the possible mechanisms and isolate the compounds associated with these bioactivities from the leaf extract of *A. alba*.

1. Introduction

Medicinal plants produced in mangroves are commonly used to treat many severe maladies [1–3]. *Avicennia alba* is a mangrove plant that belongs to the Acanthaceae family and referred to as "Ilva mada." It grows in tropical regions and found far from the ocean [4]. The reason behind of its name was two Latin phrases "Alba" and "putih," which indicate "white" in Latin and Malay, respectively. It is more ubiquitous on near about the rivers or on freshly formed mudbanks on the seaward side. In some ways, it is a precursor to animals that can survive in a protected environment.

A. alba's leaves and fruit morphologies can be "abnormal" if the tree is growing in the shade or suffering from low nutrition or other difficult circumstances. Normally, the flowers are little and golden, and they are usually spread out across an area. There is a long, tapering pointed end to the fruit, which is teardrop-shaped [5, 6]. Together with nutrition and fiber, the phytochemicals from *A. alba* provide a widespread defensive function against physiological complications and stress conditions. The most abundant bioactive compounds include carbohydrates, tannins, alkaloids, flavonoids, terpenoids, steroids, and phenolic compounds [7–9].

The leaves of *A. alba* were tested for antioxidant activity and appeared to be effective [10–12]. In traditional Chinese medicine, the bark and seeds of *A. alba*'s were employed as a fish poison, while the resin was used to treat birth control, ulcers, skin conditions, and even cancer [13]. *A. alba*, a mangrove, has been subjected in very few studies. Its crude extract has been shown to be effective against polymicrobial diseases [14]. *A. alba* leaves protect gastric mucosa from ethanol-induced damage [15], and the stem of the plant has been used to isolate and structurally elucidate new naphthoquinones and their analogues [1, 6]. Plant and human pathogenic bacteria, particularly oral pathogens, were shown to be inhibited by *A. alba* [13, 14]. Additionally, the plant extract has antifungal properties. Aphrodisiacs, skin disease, asthma, rheumatism, scabies, antifertility agents, paralysis, snake bites, and ulcers are only some of the conditions for which *A. alba* is employed in the Indian medical system [7, 16].

Diabetes is a common chronic disease, characterized by recurrent hyperglycemia and metabolic problems [17]. Diabetes mellitus (DM) is the eighth major cause of death, affecting 4% of world population. Its effect on various physiological systems, especially nerves and blood flow, makes it a severe medical condition in both developing and industrialized countries [18, 19]. Despite claims by trado-medical practitioners that they have a treatment for DM, there is no cure. It falls into the same category as AIDS, cancer [20, 21], and hypertension, which can only be controlled at the moment [22–24].

Inflammation is triggered due to numerous unpleasant stimuli including pathogens and physical trauma, which is used by immunological responses [25, 26]. Conversely, pain is regarded as a negative sensory and emotional perception linked to tissue injury. It is also frequently triggered by unpleasant stimulus and conveyed to the central nervous system (CNS) via specific neural networks, where it is recognized [27, 28]. It is a strategy of safeguarding the body from damage. Despite the development of sufficient medications, inflammation and pain continue to be the difficult and debilitating health issues, affecting 80% of the adult population worldwide [29]. Untreated and chronically protracted pain is the most common ailment, causing both harm and injury. Nonresolving inflammation also causes functional impairment, such as taking time off work, school, or community engagements, and can lead to the development of serious inflammatory conditions such as asthma, autoimmune disorder, systemic inflammatory, glomerulonephritis, inflammatory bowel disease, and rheumatoid arthritis [30, 31]. These debilitating diseases are the leading cause of disability and, if not appropriately monitored and maintained, can lead to death. Standard pain and inflammation medications are still the backbone for treating and managing these conditions [32]. However, they are concomitant to various health consequences and toxicity, including stomach irritation, gastric ulcer, alteration kidney function, hypertension implications, liver damage, and platelet suppression, which can lead to increased bleeding [33, 34].

Diarrhea is of major concern for morbidity and mortalities in third world nations, affecting primarily neonates and infants [35, 36]. As per UNICEF and WHO data, yearly 2.5 billion cases of diarrhea are reported worldwide; with 1.9 million children under the age of five dying from diarrhea, the majority are from developing nations. 78% of diarrheal deaths of children occur in Africa and Southeast Asia [37, 38]. Antimotility and antisecretory medications are the mainstays of diarrhea management. Opioids and their metabolites are still commonly utilized to treat diarrhea. Opioid antidiarrheals such as diphenoxylate, difenoxin, and loperamide are routinely utilized [39]. Several alternative medications that have antimotility or antisecretory properties on the bowel and can be used to treat diarrhea exist [40].

Therefore, there is a compelling necessity expand research into phytochemicals that are useful to relieve pain and inflammation. The purpose of the study is to evaluate pharmacological activity of *A. alba*, focusing on antidiabetic, anti-inflammatory, analgesic, and antidiarrheal activities in Swiss albino mouse model.

2. Materials and Methods

2.1. Chemicals and Plant Material. The standard drugs glibenclamide, indomethacin, diclofenac sodium, morphine, and loperamide were purchased from Incepta Pharmaceuticals Ltd., Dhaka, Bangladesh. Different reagents and distilled water were also purchased from British Drug House (BDH) Chemicals Ltd., Dhaka, Bangladesh. Plant material from *A. alba* was collected from Sylhet. The plant's genus and family were reported from National Herbarium, Bangladesh (DACB accession number: 40556).

2.2. Preparation of Methanol Extract. The *A. alba* leaves were harvested and cleaned with distilled water for the removal of undesirable materials. First, leaves were sun-dried before being processed into a coarse powder. Before the study began, container was used to place the powder into it and kept in a dry, cool, and dark setting. In a glass flask, 400 gm of granulated leaves of *A. alba* was steeped for 10 days in 1000 ml of 95% methanol with constant shaking and mixing. To get a clean filtrate, filtration was done for the entire mix via a fine and white cotton material and Whatman filter paper No. 1. The filtrate was kept in an open room to disperse the solvent, and the extract was found. The methanol extract of the leaves yielded 2.11% *w*/*w* yield. Extract was redissolved in 5% DMSO for oral administration.

2.3. Experimental Animals. Swiss albino mice ($n = 90$) of both sexes (20-25 g) were procured from Jahangirnagar University in Dhaka, Bangladesh, and maintained in animal cages under regular natural circumstances (22-25°C, moistness 60-70%, 12-hour light: 12-hour dull cycle). A regular pellet fed was fed to the mice. The Faculty of Allied Health Sciences Research Ethics Committee, Daffodil International University, Dhaka-1207, Bangladesh, accepted all of the procedures used in this investigation, including the use of animals (Ref: FAHSREC/DIU/2020/1006).

2.4. Phytochemical Group Screening. In the preliminary phytochemical study, the presence of certain phytochemical groups was evaluated. Colorimetric methods were used to identify flavonoids, saponins, tannins, carbohydrates, alkaloids, terpenoids, gums, phenolics, steroids, and glycosides [41–43].

2.5. Acute Toxicity Test. The extract was administered to mice in oral route ($n = 5$) at doses of 100, 200, 400, and 600 mg/kg, with % mortality measured from 24 to 7 days [44, 45].

2.6. Antidiabetic Activity

2.6.1. Experimental Design. Into five groups, the mice were separated, five mice per group. Group I was a standard control (saline, nondiabetic), group II was alloxan- (150 mg/kg-) treated control (diabetic control), group III was provided glibenclamide (10 mg/kg), and groups IV, V, and VI were taken leaf extract at doses of 200, 400, and 500 mg/kg, respectively. For 15 days, the therapeutic options were continued. The extracts and saline solution were administered orally administered.

The extract was used to evaluate the antidiabetic activity using albino mice at 200, 400, and 500 mg/kg b.w. doses in an oral glucose tolerance test in vivo. The doses were designated on the basis of their efficacy in previous studies [44].

2.6.2. Blood Glucose Determination. A fractional tail amputation process was used to detect each mouse's blood glucose level, and blood was drawn from the tail vein using a one-touch electronic glucometer with glucose test strips. The tails were rinsed in ethanol to prevent them from infection [46].

2.6.3. Body Weight (b.w.) Analysis. The experimental mice bodyweight is measured before they began the medication (day 0) and throughout the trial (days 7 and 15), as well as fluctuations in weight.

2.7. Anti-inflammatory Activity by Carrageenan-Induced Inflammatory Method. The animals were separated into 5 groups of fine mice each on the day of the experiment. They were weighted to ensure that the medication was administered correctly. Group I received 1% Tween, while groups III, IV, and V provided leaf extract at 200, 400, and 500 mg/kg b.w. doses. In group II, any anti-inflammatory medicine might be employed as a positive control. Indomethacin was utilized as the standard in this model. The animals were given 0.1 ml of 1% carrageenan in the subplanter region of the right hind paw after 30 minutes. At the level of the lateral alveolus, the paw was tagged with a permanent marker. A plethysmograph was used to take the initial reading right after the injection, as well as successive paw volumes. Paw volumes were measured at 0, 30, 60, and 120 hours. Afterward, carrageenan was injected. The volume of edema was estimated by subtracting the initial volume from the volume of the time points in consideration [47].

2.8. Analgesic Activity Test

2.8.1. Acetic Acid-Induced Abdominal Writhing Test. The experiment for analgesic activity proceeded with categorizing the mice into 5 groups. Each mice group contains 5 mice, standard (10 mg/kg diclofenac sodium in intraperitoneal route) and control (0.5% methylcellulose). Groups III, IV, and V were considered with methanol extract of *A. alba* leaves at dosages of 200, 400, and 500 mg/kg b.w., respectively. Writhing assay was followed for the assessment of analgesic activity induced by acetic acid. For the following 10 min after obtaining test data and vehicle orally 30 minutes before intraperitoneal delivery of 10 ml/kg of 0.7% acetic acid, the mice were examined for particular body contraction known as "writhing" [7]. Fifteen minutes prior to the acetic acid injection, diclofenac-sodium was given intraperitoneal injection. The percent of protective action against writhing induced by acetic acid was calculated using the formula.

$$\text{Percentage protection} = \frac{(Wc - Wt)}{Wc} \times 100, \quad (1)$$

where Wc and Wt are the mean values of the control and treated groups, respectively.

2.8.2. Hot Plate Test. This test was performed as per the instructions provided by previous researchers [48]. Methanol leaf extract (200, 400, and 500 mg/kg b.w. p.o.), morphine (5 mg/kg b.w. p.o.), and normal saline (10 ml/kg b.w.) were introduced orally to 5 mice groups ($n = 5$). Mice were kept on a hot plate (Bibby Sterilin, UK), and the latency of reaction (in seconds) for licking the jumping or hind paw was measured. The mice for the studies were those that reacted within 15 seconds and did not exhibit a lot of variance. Before and after treatment with the various medicines, recordings were collected at 0, 30, 60, 90, and 120 mins [49].

2.9. Castor Oil-Induced Antidiarrheal Activity Test. This experiment was proceeded as per the instructions of Islam et al. [50]. Before the test, the animals were separated for the diarrheal test by introducing them castor oil (0.5 ml) in oral route, and then, animals with diarrheal symptoms were selected for experimental purpose. Five groups were divided with 25 mice (five in each group). Before going through the experiment, mice were starved for 18 hours (only water was accessible). Animals in group I were given merely the vehicle (1% Tween 80, 10 ml/kg, i.p.) as a control, while those in group II were given the conventional medicine (loperamide 3 mg/kg b.w.). The extract doses for groups III, IV, and V were 200, 400, and 500 mg/kg, respectively. Castor oil (0.5 ml) was introduced to mice in oral route 30 min

afterward treatment to tempt diarrhea and were individually placed in blotting paper. The paper was replaced every hour. Diarrheal feces were counted, and the proportion of defecation inhibition was computed for each group over a 4-hour observation period.

2.10. Statistical Analysis. The SPSS (Statistical Package for the Social Sciences) statistical software, version 20.0, was used for all the data analysis. The results are demonstrated as the mean ± SEM (standard error mean) value. The statistical analysis was accomplished using one-way analysis of variance (ANOVA) pursued by Dunnett's test for all the experiments. $*P < 0.05$, $**P < 0.01$, and $*** P < 0.001$ were reported to be statistically significant.

3. Results

3.1. Phytochemical Screening. Phytochemical investigation on the methanol leaf extract of *A. alba* demonstrated the occurrence of flavonoids, saponins, tannins, alkaloids, terpenoids, gums, phenolics, carbohydrates, and steroids, as well as the absence of glycosides (Table 1).

3.2. Acute Toxicity. Normal behavior was observed in mice given the extract at doses of 100–600 mg/kg p.o. They were awake and alert, with typical grooming, contact, and pain responses. Passivity, stereotypy, or vocalization was not present. They had typical motor activity and secretory indications. The animal's alertness, grip strength, limb tone, motor activity, and gait were all normal. In mice, the extract was determined to be safe up to 600 mg/kg.

3.3. Antidiabetic Activity

3.3.1. Blood Glucose Level. The leaf extract of *A. alba* given at 200, 400, and 500 mg/kg reduced glucose levels of blood in diabetic mice dose dependently at the end of the trial, although not as much as glibenclamide-treated mice (Table 2).

3.3.2. Body Weight Changes and Fasting Blood Glucose Levels. In alloxanized diabetic mice, the leaf extract at dosages of 200, 400, and 500 significantly increased b.w. and generated a maximum drop in fasting glucose levels after 15 days of therapy (Table 3).

3.4. Anti-inflammatory Activity. The methanol extract of *A. alba* demonstrated to have anti-inflammatory activity in mice at dose levels of 200, 400, and 500 mg/kg/day b.w., respectively. Indomethacin (10 mg/kg) for leaf was found to have anti-inflammatory activity in mice at dose level of 500 mg/kg/day body weight, respectively (Table 4).

3.5. Analgesic Activity

3.5.1. Writhing Test. Methanol leaf extract of *A. alba* inhibited writhing by 39.01, 56.74, and 64.18% at dosages of 200, 400, and 500 mg/kg b.w., respectively, whereas diclofenac sodium repressed writhing by 69.86% at a dose of 10 mg/kg, and the findings were statistically significant ($**P < 0.01$, $*** P < 0.001$) (Table 5).

Table 1: Phytochemical screening of the leaf extract of *A. alba*.

Tested groups	Methanol leaf extract
Flavonoids	+
Saponins	+
Tannins	+
Carbohydrates	+
Alkaloids	+
Terpenoids	+
Gums	+
Phenolics	+
Steroids	+
Glycosides	-

(+) indicates presence; (-) indicates absence.

3.5.2. Hot Plate Test. At 90 min, the leaf extract of *A. alba* revealed maximum reaction times of 7.24, 8.40, and 9.10 seconds for dosages of 200, 400, and 500 mg/kg, respectively, whereas morphine demonstrated a maximum reaction time of 11.01 sec for dosages of 5 mg/kg at 90 min (Table 6). The findings showed that the extract considerably improved ($*P < 0.05$, $**P < 0.01$) pain threshold when comparison was done to the control group, and that the effect lasted for the full 120-minute monitoring period.

3.6. Castor Oil-Induced Antidiarrheal Activity. At dosages of 200, 400, and 500 mg/kg, the three serial dosages of *A. alba* leaf extract effectively reduced enterpooling in mice induced by castor oil. The largest dosages of leaf extract (400 and 500 mg/kg) lead to a greater diminution in both the total amount of diarrheal feces and percent inhibition of diarrhea. When compared to the negative controls, the leaves extract inhibited diarrhea by 30.91% and 46.36% at 400 and 500 mg/kg dosages, respectively. Loperamide had the highest percent inhibition of diarrhea, at 59.09% (Table 7).

4. Discussion

Many traditional medicines rely heavily on the medicinal properties of plants [51, 52]. There may be polar plant elements in *A. alba* since it grows in coastal woods. Methanol was employed in this experiment. The solvent was entirely evaporated to dryness in order to prevent any influence on the experimental animals [53]. There is a long history of therapeutic usage of the herb studied in this research, both in India and elsewhere [16]. *A. alba* leaf extract was analyzed for phytochemicals, and the results are reported in Table 1. The methanol extract of *A. alba* leaves displayed the occurrence of saponins, flavonoids, tannins, sugars, alkaloids, terpenoids, gums, phenolics, steroids, and the lack of glycosides. Leaf extract in methanol had more phytochemicals than the other extracts studied. Most secondary metabolite adaptive value remained undiscovered for many years. Metabolic wastes, it was assumed, were the only sources of these chemicals. Today, we know that a large number of secondary metabolites in plants have ecological roles. The research demonstrates that *A. alba* is an extremely

Table 2: The effect of *A. alba* leaf extract on alloxan-induced diabetic mice's blood glucose levels.

Group	Dose (mg/kg)	Blood glucose level (mg/dl)				
		Day 0	Day 4	Day 7	Day 10	Day 15
Normal saline	0.3 ml	122.60 ± 3.10	114.20 ± 1.07	108.10 ± 2.48	106.60 ± 2.70	98.8 ± 3.10
Diabetic control	0.3 ml	415 ± 4.50	423.15 ± 1.87	429.55 ± 3.10	438.10 ± 1.58	454.10 ± 3.20
Glibenclamide	10	394.20 ± 2.37	373.25 ± 2.31*	339.15 ± 2.29*	296.15 ± 3.06**	265.27 ± 2.67**
Methanol extract	200	401.30 ± 2.51	388.25 ± 2.27	372.55 ± 3.40	361.40 ± 3.24	344.25 ± 3.15*
Methanol extract	400	385.25 ± 3.27	370.35 ± 2.57	360.30 ± 3.34*	309.90 ± 3.14**	285.10 ± 2.96**
Methanol extract	500	387.55 ± 3.27	374.15 ± 3.24	366.45 ± 2.22	313.35 ± 3.27	291.25 ± 2.37

Results are expressed as mean ± SEM ($n = 5$), $^*P < 0.05$, $^{**}P < 0.01$, which considered significance compared with the control group (one-way ANOVA followed by Dunnett's test).

Table 3: The effects of *A. alba* extract on mice's body weight.

Group	Dose (mg/kg)	Body weight (g)				
		Day 0	Day 4	Day 7	Day 10	Day 15
Normal saline	0.3 ml	23.90 ± 0.27	25.14 ± 0.19	26.10 ± 0.18	27.20 ± 0.12	28.15 ± 0.21
Diabetic control	0.3 ml	27.20 ± 0.44	27.80 ± 0.17	25.55 ± 0.40	25.15 ± 0.22	24.10 ± 0.50
Glibenclamide	10	29.55 ± 0.22	30.25 ± 0.40	32.15 ± 0.25*	33.65 ± 0.12**	34.45 ± 0.10**
Methanol extract	200	26.10 ± 0.25	26.10 ± 0.21	29.95 ± 0.35	30.85 ± 0.35	32.45 ± 0.20*
Methanol extract	400	25.30 ± 0.15	26.25 ± 0.20	32.80 ± 0.14*	32.25 ± 0.10*	33.40 ± 0.45**
Methanol extract	500	26.24 ± 0.11	26.50 ± 0.36	32.95 ± 0.42*	32.85 ± 0.27*	33.95 ± 0.23**

Results are expressed as mean ± SEM ($n = 5$), $^*P < 0.05$, $^{**}P < 0.01$, which considered significance compared with the control group (one-way ANOVA followed by Dunnett's test).

Table 4: Appraisal of anti-inflammatory activity of *A. alba* leaf extract by carrageenan-induced inflammatory method.

Group	Dose (mg/kg)	Paw edema (mm)			
		0 hrs	30 hrs	60 hrs	90 hrs
Control	1% Tween	7.75	7.25	7.0	7.07
Indomethacin	10 mg/kg	4.29	4.22	3.75	3.71
Methanol extract	200	6.27	6.25	6.10	5.95
Methanol extract	400	5.88	5.77	5.60	5.50
Methanol extract	500	4.99	3.87	3.75	3.55

diversified phytochemically. Thus, these phytochemicals have a dramatic effect on plants' capacity to compete and adapt. The study of these chemicals is fascinating because they may be useful as pharmaceuticals, poisons, flavors, and industrial materials. Numerous polyphenolic chemicals found in mangrove plants, including as tannins and flavonoids, were documented to possess a variety of pharmacological properties, including antidiarrheal and analgesic properties. Plant flavonoids and pentacyclic triterpenes may act as an analgesic in rats [54]. Additionally, this research discovered that benzoquinones may block lipoxygenase pathway, which supports the usage of *A. alba* in traditional medicine to treat diarrhea. Tannins and saponins are also present and contribute to the antidiarrheal activity. Ahmed et al. [53] shown that the occurrence of alkaloids, steroids, and glycosides in the leaves of *A. alba* may result in analgesic and antidiarrheal activity.

Using a writhing model induced by acetic acid in mice, the methanol extract of *A. alba* was shown to have analgesic properties. Models of pain feeling are generated by inducing localized inflammation in response to acetic acid injections. The algesia caused by the release of endogenous chemicals by acetic acid, which is employed to produce writhing, excites the pain nerve endings [55]. It was suggested that acetic acid when injected intraperitoneally causes pain because of elevated PGF2 and PGE2 levels [56]. The extract had equivalent writhing inhibition to diclofenac sodium, the usual medication (Table 5). The analgesic properties of the extract are because of the polar molecules. This finding suggests that *A. alba*'s methanol extract may have analgesic properties. Radiating heat is also used to investigate central and peripheral effects. Thus, the findings are corroborated by the plant's ability to inhibit both peripheral and central pain pathways by the use of methanol extract. The development of heat discomfort is associated with two kinds of sensory neurons in the skin: delta and C fibers. Additionally, the skin has a high concentration of temperature-sensitive ion channels. Calcium and sodium ions can pass through the plasma membrane because it has transmembrane proteins that let them do so. In order to pinpoint the cause of pain, the brain processes action potentials generated by the ions and sends them to the spinal cord. Heat-inducing discomfort may be alleviated by analgesic herbs' ability to modulate signal conduction and action potential [57].

Table 5: Effect of leaf extract of *A. alba* using acetic acid-induced abdominal writhing test.

Group	Dose (mg/kg)	Writhing (mean ± SEM)	% of inhibition
Control	0.5% methylcellulose; 10 ml/kg	28.2 ± 0.89	—
Diclofenac sodium	10	8.5 ± 0.75	69.86***
Methanol extract	200	17.2 ± 0.40	39.01**
Methanol extract	400	12.2 ± 0.65	56.74***
Methanol extract	500	10.10 ± 9.36	64.18***

Results are demonstrated as mean ± SEM ($n = 5$), $^{**}P < 0.01$, $^{***}P < 0.001$, which considered significance compared with the control group (one-way ANOVA followed by Dunnett's test).

Table 6: Effect of leaf extract of *A. alba* using hot plate test.

Group	Dose (mg/kg)	Reaction time (sec)				
		0 min	30 min	60 min	90 min	120 min
Control	1% Tween 80, 10 ml/kg	4.07 ± 0.70	4.01 ± 0.37	4.0 ± 0.08	6.6 ± 0.40	4.15 ± 0.14
Morphine	5	4.10 ± 0.10	6.15 ± 0.16*	8.50 ± 0.12*	11.01 ± 0.18**	9.60 ± 0.20**
Methanol extract	200	4.19 ± 0.13	4.55 ± 0.22	5.55 ± 0.14	7.24 ± 0.20**	6.40 ± 0.15*
Methanol extract	400	4.44 ± 0.25	4.67 ± 0.37	6.44 ± 0.21*	8.40 ± 0.15**	6.80 ± 0.12**
Methanol extract	500	4.55 ± 0.86	5.0 ± 0.14*	6.90 ± 0.20*	9.10 ± 0.31**	7.0 ± 0.13**

Results are expressed as mean ± SEM ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, which considered significance compared with the control group (one-way ANOVA followed by Dunnett's test).

Table 7: Effects of leaf extract of *A. alba* in castor oil-induced antidiarrheal test.

Group	Dose (mg/kg)	Total number of feces (mean ± SEM)	% inhibition of defecation	Total number of diarrheal feces (mean ± SEM)	% inhibition of diarrhea
Control	1% Tween 80, 10 ml/kg	8.25 ± 0.50	—	5.50 ± 0.27	—
Loperamide	3	3.25 ± 0.23	60.61	2.25 ± 0.15	59.09
Methanol extract	200	6.10 ± 0.31	26.06	4.90 ± 0.34	10.91
Methanol extract	400	5.50 ± 0.15	33.33*	3.80 ± 0.21	30.91*
Methanol extract	500	4.90 ± 0.27	40.61**	2.95 ± 0.19	46.36**

Results are expressed as mean ± SEM ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, which considered significance compared with the control group (one-way ANOVA followed by Dunnett's test).

A diarrhea model induced by castor oil in mice was utilized to test the *A. alba* extract's antidiarrheal activity. There are several theories explaining castor oil's diarrheal impact, including the blockage of Na^+, K^+-ATPase activity, adenylate cyclase activation, or the commencement of prostaglandin (PG) production, PAF (paroxysmal atrial fibrillation), and nitric oxide (NO). When mixed with bile and pancreatic enzymes, castor oil causes diarrhea and releases ricinoleic acid from triglycerides when taken orally. The colon is the primary site of absorption and excretion of ricinoleic acid and has influence on absorption or secretion. The ricinoleate salts with K^+ and Na^+ quickly develop in the lumen of the gut as a result of the liberation of ricinoleic acid. It acts like a soap or surfactant on the mucosal surface of the digestive tract and in the intestines itself. As a general rule, ricinoleate salts induce adenyl cyclase [58] or the release of PG in intestinal epithelial cells [59]. The extract lowered the frequency of defecation and the total stool count while increasing the latent period. In addition, the *A. alba* extract contains flavonoids that have been shown to suppress the production of autacoids and prostaglandins, which means that castor oil's effects on motility and secretion may be inhibited. Antidiarrheal properties of this extract may potentially be attributable to the formation of protein tannates, which in turn strengthen the intestinal mucosa and limit secretion [60].

Antidiabetic activity as measured by body weight and fasting blood glucose level is shown in Tables 2 and 3. In disease control rats, body weight is observed to be dramatically lowered when compared to normal control rats. The therapy with a specific herbal extract resulted in a rise in body

weight. Increased body weight indicates that certain herbal extract dosages have an antihyperglycemic effect. In fasting blood glucose tests, alloxan-induced diabetic rats had an increased level of glycated hemoglobin (HbA1c) owing to an increase in blood glucose levels, which react with hemoglobin and result in the creation of glycated hemoglobin [61, 62]. The methanol extracts dramatically decreased blood glucose levels, which resulted in a drop in glycosylated hemoglobin levels. When compared to other treatment groups, the high dosage (400 and 500 mg/kg) of methanol extract *A. alba* shown superior potency. The term "fat vacuoles" or "steatosis" refers to the intracytoplasmic accumulation of triglyceride (neutral fats) in the liver of rats after alloxan injection. Accumulation of these neutral fats in the liver resulted in nonalcoholic fatty liver disease (NAFLD), which is described by hepatocyte fatty alterations, ballooning degeneration, mixed lobular inflammation, and fibrosis. Ballooning is the most significant aberration in NAFLD because it results from hepatocytes losing their usual polygonal shape and becoming bloated and spherical because of intracellular fluid buildup initiated by microtubule malfunction and decreased protein secretion [61]. Hepatic fibrosis occurs when stellate cells in the liver progressively lose their ability to produce collagen. High dosages of methanol extract from *A. alba* showed improved hepatoprotective action and preservation of the normal liver architecture, respectively [63].

It is demonstrated in this research that methanol extract of *A. alba* does not induce acute toxicity, since the LD50 value exceeds 600 mg/kg. There were no deaths or evidence of toxicity in mice treated with extracts at a level of 600 mg/kg, proving their safety in usage. Additionally, it was shown to be as safe when used in an erythrocyte hemolytic experiment [63]. For this reason, methanol *A. alba* extracts may be employed as a safe phytochemical to treat various maladies. Inflammation is caused by the breakdown of proteins, which is well-documented. Anti-inflammatory medications such as salicylic acid, phenylbutazone, and flufenamic acid, among others, have shown dose-dependent capacity to denaturate thermally generated proteins. Anti-inflammatory efficacy was tested as a means to determine the extract's potential to suppress protein cleavage. According to Mondal et al. [64], methanol extract of *A. alba* was efficient in preventing heat-induced albumin denaturation at various doses. The in vitro anti-inflammatory effect of the studied ethanol extract was also shown to be promising. When living tissues are injured, they respond by inflaming themselves. It is a multistep process that includes mediator release, enzyme activation, fluid extravasation, tissue breakdown, cell migration, and repair [64]. Carrageenan-induced acute inflammation in rats was shown to be significantly reduced by the methanol extract of *A. alba* in the current investigation [65]. A number of arachidonic acid metabolites, including prostaglandins, enhance the cardinal signs of inflammation; leukotriene B4 is a facilitator of leukocyte stimulation in the inflammatory cascade [64]. According to the findings, *A. alba* methanol extracts reduced carrageenan-induced rat paw edema at doses of 200 mg/kg, 400 mg/kg, and 500 mg/kg (Table 5). The suppression of some or all of the mediators produced within 90 hours following carrageenan injection is most likely the origin of anti-inflammatory effect against acute inflammation. NO is produced when PGs, cytokines, and iNOS are activated, indicating an inflammatory response [65]. Vasodilatation, an increase in vascular permeability, and the development of edema are all due to the action of NO. NO inhibition may be due to a mechanism that prevents iNOS activity and may also be responsible for the reduction of PG production. Finally, the data show that *A. alba* ethanol extract significantly reduces rat experimental inflammatory responses. According to past research, tannins, flavonoids, and phenolic chemicals in the plant may be responsible for this action. Therefore, further research is needed to identify the active chemical and determine if it may be used to treat chronic inflammation.

5. Conclusions

In conclusion, it was shown that the *A. alba* extract is a natural and harmless treatment with antidiabetic, anti-inflammatory, analgesic, and antidiarrheal action at doses up to 500 mg/kg. Our new results provide a scientific explanation for the plant's traditional usage. Remarkably, the methanol extract of *A. alba* displayed both central and peripheral analgesic activity, which might be attributable to the existence of such active components, since the herb has a long history of folk usage in pain and fever. Additionally, the research mentions *A. alba* leaf methanol extract's antidiabetic properties. The extract dosages (400 and 500 mg/kg) avoided excessive weight loss and maintained a favorable control of glycosylated hemoglobin. Finally, it is possible that the *A. alba* leaf has analgesic and antidiarrheal properties. These data support *A. alba*'s usage as a traditional medicine on a scientific foundation. However, further tests may be necessary to assess the plant's medicinal potential as a treatment.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Saikat Mitra and Fahadul Islam contributed equally to this work.

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Research Article

Isolation of Thymol from *Trachyspermum ammi* Fruits for Treatment of Diabetes and Diabetic Neuropathy in STZ-Induced Rats

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Terpenoids and phenols from *Trachyspermum ammi* (*T. ammi*) have reported some pharmacological actions. The objective of the work was to isolate the active constituent, its identification by spectroscopic techniques, and evaluation of the antidiabetic and neuroprotective activity from *T. ammi* on STZ Wistar rats. The dried fruits of *T ammi* were kept in a hydrodistillation apparatus to collect essential oil. The isolated fraction went through TLC, UV, FTIR, HPLC, HRMS, C^{13}, and ^{1}H NMR for characterization. Two dosage concentrations from the isolated compound were prepared as 10 and 20 mg/kg for treatment groups. The groups were tested for thermal and mechanical hyperalgesia, writhing, grip strength, spontaneous locomotor test, neuromuscular coordination tests, and histopathological and lipid profile analysis. Diabetes was induced by streptozotocin (45 mg/kg i.p.) and 12 weeks of treatment-induced diabetic neuropathy in Wistar rats. Biomarkers were evaluated to understand the neuropathic protection of thymol on STZ-treated Wistar rats. The biomarker studies (SOD, NO, LPO, $Na^{+}K^{+}$ATPase, and TNF-α) further confirmed thymol's diabetic neuropathy protective action. This study suggests that isolated compound thymol was antidiabetic and neuroprotective as it has shown controlled glucose levels defensive nerve damage in STZ Wistar rats. $P < 0.05$ level of significance was observed in the levels of endogenous biomarkers, fasting blood glucose levels, actophotometer response, and response latency in treated groups compared to the diabetic group, whereas $P < 0.001$ level of significance during lipid profile levels, thermal algesia, and neuromuscular comparison tests was noted in treated groups compared to the diabetic group.

1. Introduction

Patients suffering from diabetes mellitus (DM) observe a severe condition of peripheral nerve dysfunction called diabetic neuropathy (DN) [1, 2]. In India, the prevalence is higher (4.3%) compared to the western countries where 1%–2% population of DM faces these conditions. This could be due to the probability that Asian Indians are prone to the condition of insulin resistance [3–5]. Around 2/3rd of the diabetic population suffers from clinical or subclinical neu-

ropathic conditions, where approximately 10% of the people with diabetes face persistent pain. Diabetic neuropathy has a common feature of spontaneous pain, intractable or stimulus-induced pain [6]. There are many categories of DN where diabetic polyneuropathy (DPN) is a condition that prevails for a lifetime and is a major cause of nerve injury, foot ulceration, gait disturbance, and amputation [7–11]. The central control observed for DN is preventive management, as the check on the glycemic control helps prevent neuropathic complications [12].

The negative symptom observed in patients suffering from DN is a decrease in sensation-induced numbness, whereas the positive symptoms observed are aching, prickling, and burning sensations [13]. Mechanisms suggest that the tiny and unmyelinated nerve fibres are responsible for conveying sensations like temperature, touch, and pain, while the long white fibres get sensations of joint position and vibratory senses. Most of the patients report mild discomfort but around 25% report painful neuropathic conditions in diabetes. Pain in diabetic neuropathy often worsens at night [1, 14–16].

Diabetes has gradually become the most prominent problem in the global healthcare issue of the 21st century. The population of diabetics is predicted to double between the years 2000 to 2030 by reaching to level of 366 million people [17, 18]. This metabolic disorder shows prominence of hyperglycemia and includes defects of insulin productions or insulin secretion or both. Cases of chronic hyperglycemia in diabetes lead to dysfunctions and damages in the kidney, nerves, eyes, heart, and blood vessels. Prominent symptoms of hyperglycemia include weight loss, polydipsia, polyuria, blurred vision, and sometimes polyphagia. Susceptibility to infections and impairment of growth are chronic effects of prolonged hyperglycemia. In contrast, the acute fatal consequences of diabetes are nonketotic hyperosmolar syndrome and hyperglycemia with ketoacidosis [19, 20].

Prime causes of Type 2DM are genetics and lifestyle factors [21]. Advancing age is also a risk factor of T2DM, but the increasing incidences of obesity in childhood have resulted in prominent cases of T2DM in adolescents, children, and teenagers which is a serious concern and an emerging public health epidemic [22].

T. ammi is commonly known as "Ajwain" and is found in major parts of India, dominantly in Rajasthan and Gujarat regions. The plant belongs to the family Umbelliferae and also is called Omum, Ajowan, in Sanskrit [23, 24], Agyptischer in German, Kammun or Al-Yunan in Arabic, Hounastan in Armenian, and Xi Ye Cao Guo Qin in Dutch. This plant is native to Indian region, Pakistan, Iraq, and Afghanistan but also grows in regions near Mediterranean Sea, Egypt, and southwest Asia. The herb possesses many medicinal values and is used traditionally for curing conditions like atonic dyspepsia, abdominal tumors, lack of appetite, flatulence, bronchial problems, abdominal pains, diarrhea, piles, asthma, and amenorrhoea. Many pieces of research have proved the plant to be antinociceptive, hypolipidemic, antimicrobial, abortifacient, nematicidal, antioxidant, antifungal, antispasmodic, anti-hypertensive, antilithiasis, diuretic, antitussive, antifilarial, abortifacient, hypolipidemic, and cytotoxic and having bronchodilating actions [25].

Currently, a huge inclination to the herbal sources is noted in the case of many treatments. Since the ancient era, humans have depended on natural sources as remedies for diseased conditions. In India, Ayurvedic remedies are used as treatments for ages. Due to reported adverse reactions in synthetic medications and temporary relief, a huge population has drifted to natural and herbal sources for curing ailments. There has been a lot of reaches where herbs and their extracts have been proved useful in the treatment of diseases [26–29].

Based on the literature review, we found no study on diabetes and diabetic neuropathy of thymol isolated from dried fruits of *T. ammi*. Therefore, we planned to isolate thymol (an active constituent) from the dried fruits of *Trachyspermum ammi* and evaluate its effects on diabetes and diabetic neuropathy by experimentally inducing it on animals.

2. Material and Methods

2.1. Drugs and Chemicals. Streptozotocin was acquired from Sigma-Aldrich India, while Sanofi India Ltd. provided glibenclamide for a research work. The chemicals were of analytical grade obtained from the laboratory facility of the institute.

2.2. Instruments. UV-1700 PharmaSpec on SHIMADZU, FTIR–Spectrum Two on PerkinElmer, and HPLC on LC-2010CHT by Shimadzu were used from PSIT, Kanpur laboratory facility for performing UV and FTIR spectroscopy, while C^{13} NMR and ^{1}H NMR were performed by Indian Institute of Technology, Kanpur laboratory facility, where DMSO–D6 was used as solvent. The HRMS was done by SAIF CDRI, Lucknow, where ethanol was used as a solvent. A glucometer (Accu-chek®) was used to estimate blood glucose levels and a semiauto analyzer (Remi Industries Ltd., India) was used for biochemical analysis.

2.3. Test Animal. Adult rats weighing (180-220 g) of both genders were obtained from the animal house of Institute of Pharmacy, PSIT, Kanpur. Animals were kept in cages (polyacrylic) which were spacious and large. An ambient room temperature was maintained with 12 h light/12 h dark cycle. Purified water and standard pellet diet ad libitum were made available to experimental rats [30]. This study was approved by IAEC, and the experiment was performed in accordance to CPCSEA (Ref No. 1273/PO/Re/S/09/CPCSEA). The experimentation design chosen is mentioned in Table 1, and the process is explained in Figure 1.

2.4. Collection and Identification of Plant Material. The fruit of *T ammi* was obtained from the local market in Kanpur, Uttar Pradesh, India (Figure 2). They were collected in November 2018. The powdered fruit underwent hydrodistillation and was purified to obtain an isolated compound (Figure 3).

2.5. Identification of Compound by TLC. The TLC of the crystalline material obtained after hydrodistillation and purification showed an R_f value of 0.52. The system of TLC used had silica gel (stationary phase) and benzene : chloroform (3 : 1 V/V) as the mobile phase.

Table 1: Experimental design.

S. No.	Group number	Group type	Dosing
1	Group I	Normal control	Normal control (2% *w*/*v* acacia)
2	Group II	Diabetic control	No drug
3	Group III	Standard	0.2 mg/kg of glibenclamide
4	Group IV	Treated 1	10 mg/kg of isolated compound
5	Group V	Treated 2	20 mg/kg of isolated compound.

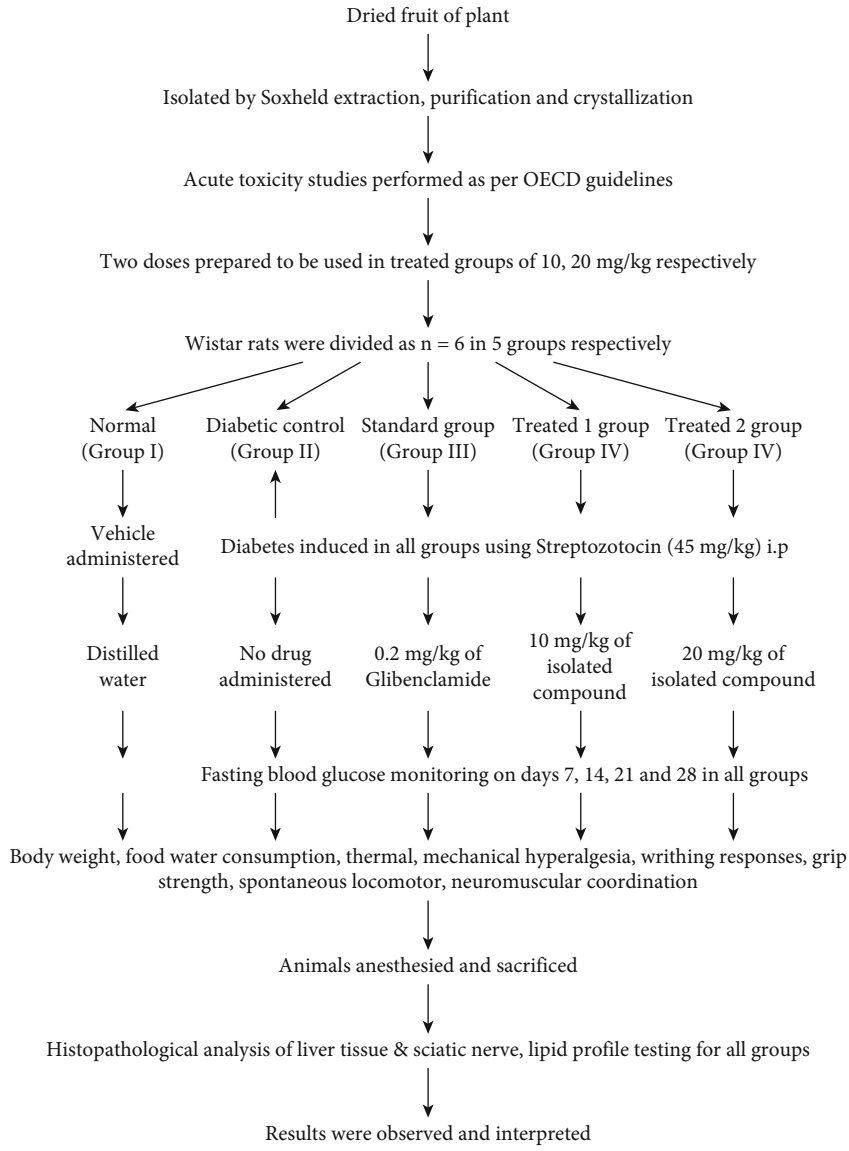


Figure 1: Experimental plan.

2.6. Identification of Compound by ^{1}H NMR, C 13 NMR, HPLC, HRMS, UV, and FTIR Analysis. The crystalline material obtained from the Crude Essential oil collected was taken for UV, HPLC, and FTIR spectroscopy studies using ethanol as a solvent. These studies were carried out in the Department Pharmacy of PSIT (Kanpur), and the results obtained are linked. The HRMS for the estimation of compound molecular weight was performed by SAIF, CDRI, Lucknow, using ethanol as a solvent. The ^{1}H NMR and C^{13} NMR were performed by the Indian Institute of Technology, Kanpur, where DMSO −D6 was used as a solvent. The melting point was registered as 50°C using a melting point apparatus. On analysing the TLC results and all spectroscopies, the interpretation of the compound was found to be thymol (Figure 4).

Figure 2: Fruit of *Trachyspermum ammi* used for isolation of active compound.

2.7. Acute Toxicity Study. The ethanolic extract of *Trachyspermum ammi* was checked up to a dose of 3200 mg/kg orally, and it was hence confirmed that the lethal dose was beyond 3200 mg/kg (b.wt) [31]. The acute toxicity experiments were done in accordance with guideline no. 425 of the OECD. The treated rats were given isolated compound doses of 5, 50, 300, and 2000 mg/kg, respectively. The dose chosen for the activity was 10 and 20 mg/kg (b. wt.) of compound isolated. Rats were then checked for their behaviour like irritability, alertness, restlessness, and fearfulness. They were also checked for neurological abnormalities like pain response, touch response, gait and spontaneous activity, and autonomic reflex-like urination as well as for defecation for 24 hours. 14 days from the administration of dose, the animals were observed for any case of mortality, and on the 14th day, they were sacrificed to isolate organs to note any visible morphological changes or signs of toxicity (Figure 5) [32–34].

2.8. Induction of Diabetes Mellitus. An intraperitoneal route was used for administration of streptozotocin (Sigma-Aldrich) at a dose of 45 mg/kg post overnight fasting of at least 12 hours to induce hyperglycemia. The hyperglycemia was confirmed post 72 hours of STZ injection and was marked by increased blood glucose levels. After the induction of diabetes, the animals were randomized as per their body weight and glucose levels thus having 4 groups according to protocol. A control group was established where diabetes was not induced. Estimation of fasting blood glucose on 7th, 14th, 21st, and 28th days was done via a glucometer from Accu-chek® [35].

2.9. Induction of Diabetic Neuropathy. Diabetes was induced, and 12 weeks after the experimentation, its effects were evaluated on the nerves to find out the possibilities of generation of diabetic neuropathy. The neuropathic development was assessed with the help of various parameters like locomotor tests and examination of the nerve tissue morphologically and microscopically on weeks 2, 4, 8, and 12 in every group.

2.9.1. Estimation of Fasting Blood Glucose. In each group, rats were fasted one day prior to testing their glucose levels on days 7, 14, 21, and 28 of experimentation. Glucose levels were obtained by collecting small amount of blood from tail and tested via a diagnostic kit [35].

2.9.2. Body Weight, Food, and Water Consumed. The body weight for each animal, consumption of food, and water were monitored during the experiment, recorded, and compared [36].

2.9.3. Estimation of Lipid Profile. Serum was separated by the help of centrifugation process by using centrifuge at a speed of 15000 rpm for 10 min—Remi Industries Ltd. (Mumbai, India). The isolated serum was analysed for TC, LDL, HDL, and triglyceride analysis using a semiautoanalyser (Span Diagnostics Ltd., India). The lipid profile estimations were performed at the end of experimentation, and the blood was collected from the retroorbital method.

2.10. Behavioural Studies

2.10.1. Thermal Hyperalgesia. Animals were kept on the analgesiometer (hot plate device) manufactured by Columbus instruments. 55 ± 1°C was set as the device temperature for the experiment. Initially, the symptoms like jumping or licking of paws were recorded and the reaction time in seconds was noted. Cut-off time for the investigation was 10 seconds to prevent any damages to rat paws. Eddy's hot plate method was used for testing the hyperalgesia on weeks 2, 4, 8, and 12 in all animals [37].

2.10.2. Writhing Responses. Assessment of neuropathic pain was done by recording writhing responses in animals. 1% *v*/*v* acetic acid (in distilled water) was administered to rats in a volume of 0.1 ml/10 g body weight to stimulate writhing responses. All episodes of stretching of back, elongation of body, and limb extensions were noted and counted in all animals [38].

2.10.3. Cold Hyperalgesia. The acetone drop test was conducted to evaluate the cold sensitivity of the rats. Animals from every group were kept in separate mesh cages for acclimatization. Fresh drops of 50 μl acetone were then applied on the midplantar surface of paws gently. The stimulation of cold was created around 2-5 sec of application, and then the responses like mild paw withdrawal, shaking, rubbing, or licking of paw were noted. These reactions were noted as nociceptive responses, no responses (antinociceptive effects), or delayed responses (delayed nociception) in terms of minutes on both paws at intervals of 5 minutes; hence, the mean response was noted [39].

2.10.4. Evaluation of Mechanical Hyperalgesia. A pinprick test was performed on rats (hind paw) where the paw was pricked with a 900 C bent gauge needle but no piercing was done. Reflexes were recorded for paw withdrawal postgentle prick, and responses were recorded in seconds. The cut-off time was 20 sec. Results were noted on weeks 2, 4, 8, and 12 of experimentation [40].

2.10.5. Grip Strength. The tests were performed to determine the neuromuscular strength of animals by dangling them on forelimbs with metal wire held tight on the poles. Duration of falling was recorded before falling on the surface hence estimating their muscle strength. Time was noted, and the interpretations were made on the basis of time of fall (as a week or damaged muscles will lead to less time of fall) [41].

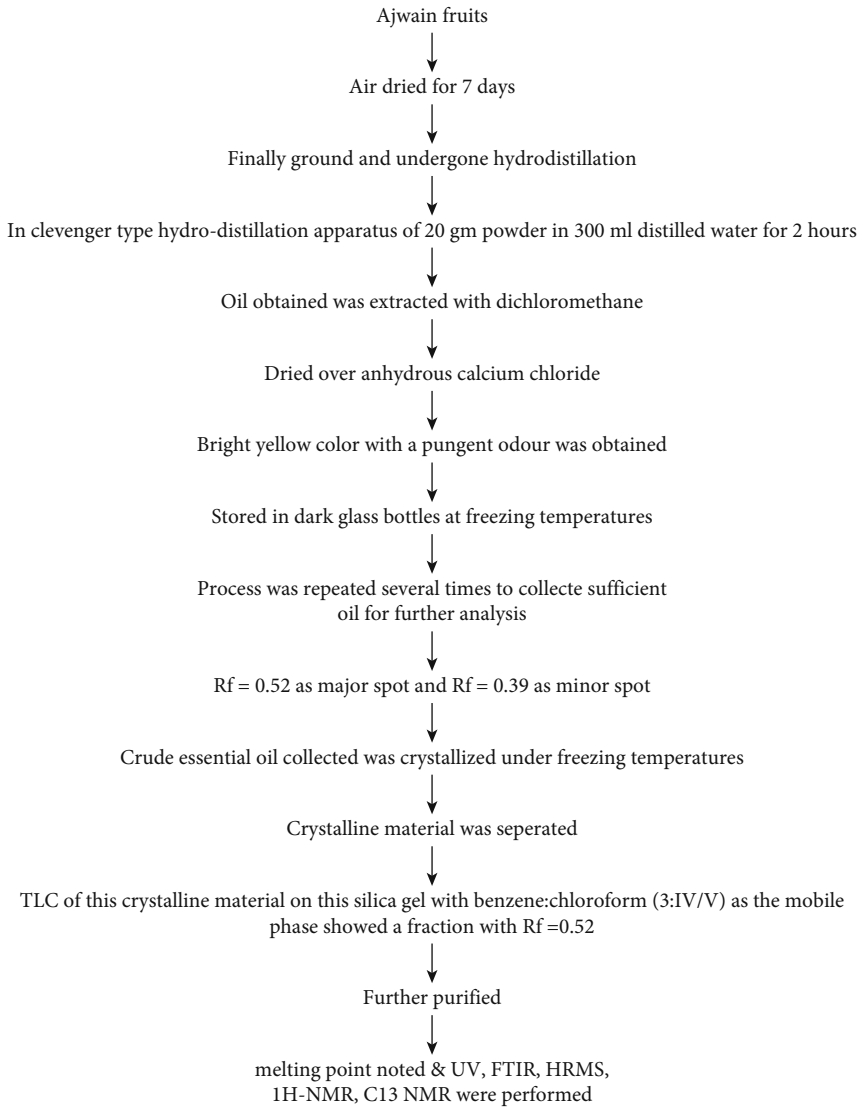


Figure 3: Isolation of thymol from the fruit of *Trachyspermum ammi*.

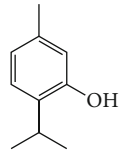


Figure 4: Structure of thymol.

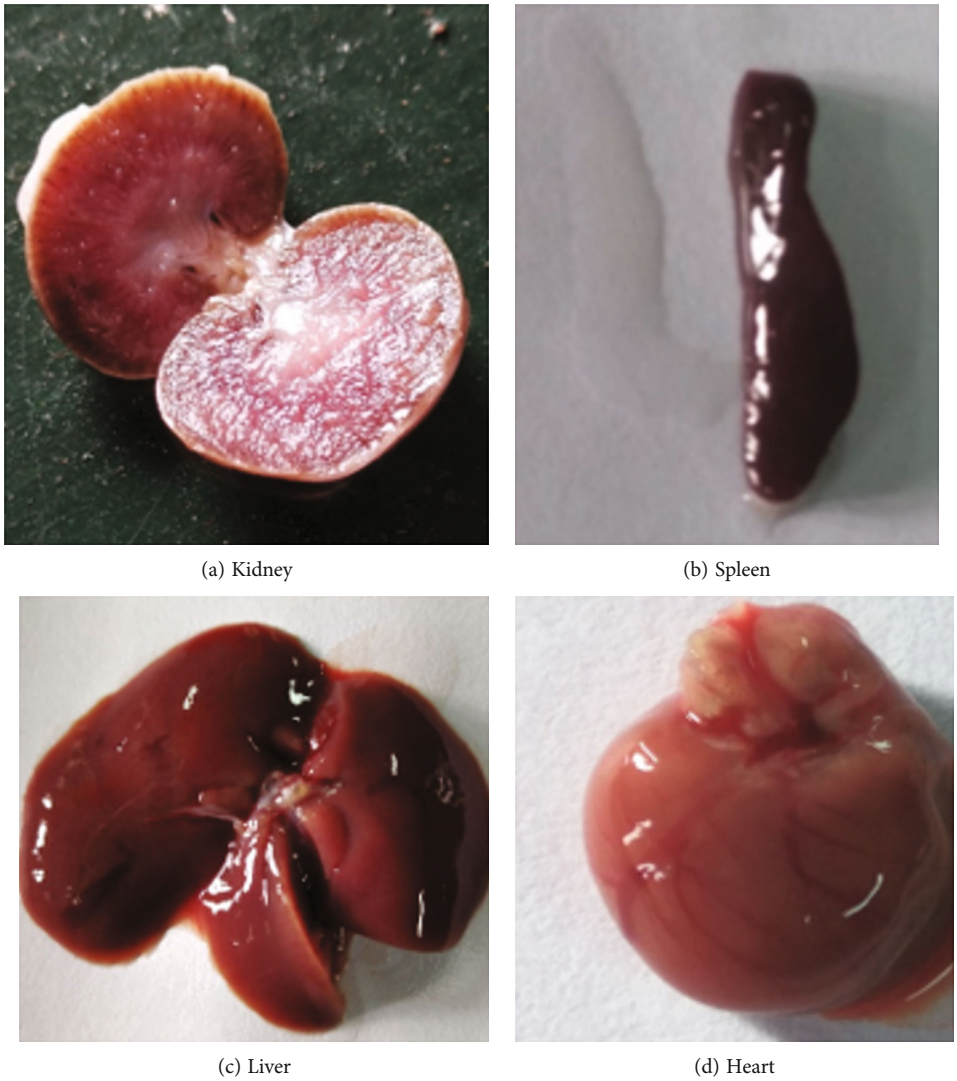
2.10.6. Spontaneous Locomotor Testing. This test was performed by introspecting the animal's behaviour and activity in an actophotometer. Animals were kept in a $30 \times 30 \times 30$ cm actophotometer with photocells and a digital counter to record the interruptions in the beam during activity recording [42].

2.10.7. Estimation of Neuromuscular Coordination. A motor coordination test was performed using a rotarod apparatus where rotarod was operating on 25 rpm speed. Cut-off time was 5 minutes, and fall-off time was recorded for all animals during the test. The low fall of time reciprocates to damaged nerve and muscle [43].

2.11. Histopathological Study. Tissues were isolated from the liver and sciatic nerves of rats at the termination of experiment. On week 12 of the study, the sciatic nerve from the lower limb's thigh region and the liver from the study animal were isolated, washed, and then stored (Figure 6). The nerve and liver were cleansed and stored in 4% formalin solution with pH 6.9. They were kept at freezing temperatures dipped in 24 hours, and then, the histopathological analysis was carried out. The samples were precisely cut and isolated and then stained with eosin and hematoxylin [44, 45].

2.12. Biochemical Estimations

2.12.1. Preparation of Homogenate of Sciatic Nerve. At the termination of the procedure, animals were exposed to a high dose of anesthesia to isolate the sciatic nerve. Homogenate of tissue was prepared using a buffer solution (0.1 M



(a) Kidney (b) Spleen

(c) Liver (d) Heart

Figure 5: Observation of visible changes on organs postacute toxicity studies.

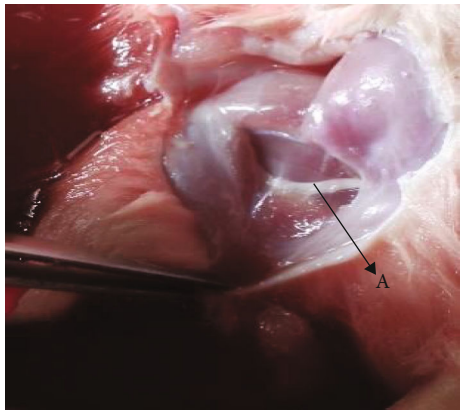


Figure 6: Isolation of sciatic nerve from the thigh region near lower limb of Wistar rat. (a) Sciatic nerve as observed in the rat was isolated from animals of all groups, freeze overnight in formalin, and sections were taken to compare the degenerations in respective groups.

Tris–HCl) with a pH of 7.4. The supernatant fluid of the homogenate was used for the estimation of membrane-bound inorganic phosphate, SOD (superoxide dismutase), lipid peroxidation (MDA content-LPO), TNF-α, and NO content (nitric oxide) [46, 47].

2.12.2. Evaluation of TNF-α. Quantification of TNF-α was done using a Thermo Scientific immunoassay kit for estimation of TNF-α levels in rat solid-phase ELISA for a duration of 4.5 h. A microplate was precoated with a monoclonal antibody, and 50 μl buffer was added to each well plate.

Samples from homogenate obtained from sciatic nerve of each group were incubated at RT for 1 hour. Immobilized antibodies will bind to TNF-α present in the well.

Washing of sample was done followed by pouring of biotinylated antibody reagents into all wells, respectively, and was then incubated at RT for 1 hour. It was further followed by washing. Then, 100 μl of streptavidin-HPR reagent was added in each well. Antibody-enzyme was then added to remove any traces of unbound antibody. 100 μl of 3,3′,5,5′

Table 2: Fasting blood glucose testing during the antidiabetic study for 28 days.

S. No.	Treatment type	Fasting blood glucose level in mg/dl			
		Day 7	Day 14	Day 21	Day 28
1	Normal control	95.4 ± 0.18	96.35 ± 1.76	97.24 ± 2.04	97.03 ± 2.19
2	Diabetic control	157.21 ± 0.32[a]	261.18 ± 2.24[a]	297.12 ± 1.37[a]	308.27 ± 0.37[a]
3	Standard	154.01 ± 0.14[*]	153.13 ± 1.91[*]	141.32 ± 1.41[*]	121.18 ± 3.23[*]
4	Treated 1	152.14 ± 1.62[*]	159.37 ± 0.37[*]	149.38 ± 3.27[*]	129.01 ± 3.45[*]
5	Treated 2	1521.53 ± 1.61[*]	152.16 ± 2.81[*]	135.14 ± 2.47[*]	117.12 ± 1.35[*]

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; followed by Tukey-Kramer's multiple comparisons test: [a]$P < 0.05$ in comparison with normal control and [*]$P < 0.05$ in comparison with the diabetic control.

Table 3: Record of the body weight postinduction of diabetes in Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Body weight (g)			
		2nd week	4th week	8th week	12th week
1	Normal control	152.3 ± 2.03	168.27 ± 2.21	190.25 ± 5.13	231.45 ± 0.26
2	Diabetic control	154.10 ± 2.40[a]	149.15 ± 1.32[a]	144.27 ± 1.53[a]	132.36 ± 3.26[a]
3	Standard	153.13 ± 1.26	150.13 ± 0.37	159.23 ± 1.32[***]	199.2 ± 1.12[***]
4	Treated 1	155.24 ± 1.32	163.4 ± 2.24[***]	168.25 ± 0.25[***]	201.34 ± 1.38[***]
5	Treated 2	159.24 ± 1.35	166.14 ± 2.14[***]	182.17 ± 1.34[***]	211.23 ± 1.42[***]

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; and [***]$P < 0.001$ in comparison with the diabetic control.

Table 4: Assessment of food and water consumption post treatment in Wistar rats.

S. No.	Treatment type	Average food & water consumption	
		Water intake (ml/day)	Food intake (g/day)
1	Normal control	13.46 ± 0.82	18.21 ± 1.34
2	Diabetic control	36.25 ± 6.34[a]	27.26 ± 0.31[a]
3	Standard	21.17 ± 2.35[***]	21.14 ± 2.62[***]
4	Treated 1	17.42 ± 1.35[***]	22.47 ± 1.37[***]
5	Treated 2	16.13 ± 0.59[***]	21.02 ± 0.15[***]

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; and [***]$P < 0.001$ in comparison with the diabetic control.

-tetramethylbenzidine (TMB)—a substrate solution—was poured which changed the color of product from blue to yellow. The color intensity was noted at 550 nm [46, 47].

2.13. Statistical Analysis. Experiments were done by grouping animals as 6 in each group. Quantitative data was tabulated via ANOVA (one-way) method, and post hoc analysis of data was done via Tukey-Kramer's test.

3. Results

3.1. Isolation. During the characterization of the isolated compound, it was analysed that UV λ max = 272.40; HPLC: retention time 9.94; HRMS-151– Molecular Ion Peak, 108 – Fragment Ion Peak; IR (KBr, cm -1): 1610 (C=C str., Aromatic ring), 1422 (O-H ben.), 1236.53 (C-O str.), 3227.02 (O-H str, Phenol), 2955.48 (C-H str). 1 H NMR (500 MHz, Chloroform) δ 4.5591 – 6.1085 (m, 3H, Ar-H), 12.32 – 3.36 (m, 4H), 7.02 – 3.36 (m, 3H), 4.0392 (s, 1H, -OH), 3.1482-3.4128 (m, 1H), 1.7083 (s, 3H), 1.7981-1.8462 (d, 6H); 13 C NMR (125 MHz, DMSO) δ 154.61, 135.53, 131.65, 125.94, 120.09, 116.01, 26.55, 22.84, 20.90. Its FTIR, C 13 NMR, 1 H NMR was compared with standards, and the compound was identified as 2-isopropyl-5-methylphenol–Thymol [48, 49].

3.2. Acute Toxicity. 14 days from the dose of administration, the animals were observed for any case of mortality. On the 14th day, they were sacrificed to isolate organs to note any visible morphological changes or signs of toxicity. The rats from all groups were checked for neurological abnormalities and autonomic reflexes. No exceptions were reported, and all results were normal. The organs isolated were observed for any morphological changes, and no changes were found. The results are recorded in Figure 5.

3.3. Fasting Blood Glucose. Glucose levels rise uncontrollably in diabetic groups which were noticed as 308.27 ± 0.37 in Group II animals (Table 2). The levels of glucose were measured in all groups where it was found that Groups IV and V have shown 129.01 ± 3.45 and 117.12 ± 1.35 mg/dl concentrations, respectively, which shows improvement in glucose levels when glucose levels were measured in all groups where it was found that Groups IV and V have shown 129.01 ± 3.45 and 117.12 ± 1.35 mg/dl concentrations, respectively,

Table 5: Effects on lipid profile of groups in Wistar rats.

S. No.	Treatment type	Lipid profile (mg/dl) posttreatment				
		TC	Triglyceride	LDL	HDL	VLDL
1	Normal control	74.29 ± 0.35	65.16 ± 0.28	36.29 ± 0.19	27.04 ± 4.15	13.28 ± 0.27
2	Diabetic control	143.29 ± 0.25[a]	157.21 ± 0.28[a]	94.19 ± 0.14[a]	15.19 ± 0.42[a]	46.38 ± 0.25[a]
3	Standard	77.23 ± 2.36***	65.48 ± 1.49***	37.16 ± 0.25***	29.8 ± 2.46***	15.12 ± 1.38***
4	Treated 1	85.02 ± 1.91***	72.26 ± 2.43***	45.26 ± 2.36***	20.35 ± 2.26***	21.13 ± 1.27***
5	Treated 2	74.59 ± 0.24***	65.15 ± 1.28***	37.24 ± 2.29***	28.03 ± 0.29***	14.01 ± 0.13***

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; and ***$P < 0.001$ in comparison with the diabetic control.

Table 6: Thermal hyperalgesia: Eddy's hot plate test on animals in all groups to assess diabetic neuropathy in Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Thermal hyperalgesia—Eddy's hot plate method—response time (s)			
		Reaction latency (2nd week)	Reaction latency (4th weeks)	Reaction latency (8th weeks)	Reaction latency (12th weeks)
1	Normal control	4.12 ± 1.43	4.19 ± 1.28	4.01 ± 1.03	4.01 ± 1.45
2	Diabetic control	6.77 ± 1.28[a]	5.19 ± 1.48[a]	8.23 ± 2.92[a]	9.85 ± 1.86[a]
3	Standard	6.25 ± 3.14	5.30 ± 4.19	5.89 ± 1.76***	5.91 ± 1.74***
4	Treated 1	6.92 ± 0.29	6.55 ± 1.32***	5.18 ± 0.21***	6.08 ± 1.41***
5	Treated 2	6.01 ± 0.48	4.41 ± 1.34	5.11 ± 1.65***	5.93 ± 0.24***

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; followed by Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; ***$P < 0.001$ in comparison with the diabetic control.

Table 7: Recoding the writhing responses to assess the neuropathic pain in all groups of Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Number of writhing responses in ten minutes (counts)			
		(2nd week)	(4th weeks)	(8th weeks)	(12th weeks)
1	Normal control	10.01 ± 0.12	11.16 ± 0.22	10.24 ± 0.22	8.13 ± 1.32
2	Diabetic control	11.14 ± 1.44[a]	10.22 ± 6.13[a]	12.58 ± 4.80[a]	13.35 ± 0.35[a]
3	Standard	12.35 ± 2.24	9.15 ± 2.34	9.64 ± 2.32***	8.94 ± 1.35***
4	Treated 1	14.94 ± 4.25	9.24 ± 1.34	9.94 ± 1.31***	8.28 ± 1.44***
5	Treated 2	13.32 ± 1.32	9.14 ± 2.11	9.61 ± 0.21***	8.24 ± 0.17***

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; and ***$P < 0.001$ in comparison with the diabetic control.

Table 8: Cold hyperalgesia: acetone drop test in all groups in Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Cold hyperalgesia (acetone drop test) in sec			
		Allodynia score in sec (2nd week)	Allodynia score in sec (4th weeks)	Allodynia score in sec (8th weeks)	Allodynia score in sec (12th weeks)
1	Normal control	4.62 ± 1.35	4.27 ± 0.25	4.07 ± 2.03	4.12 ± 1.38
2	Diabetic control	8.16 ± 2.18[a]	4.32 ± 1.91[a]	8.26 ± 0.54[a]	9.17 ± 1.34[a]
3	Standard	6.19 ± 0.37	5.19 ± 1.36	6.17 ± 1.11***	6.11 ± 2.12***
4	Treated 1	7.16 ± 1.15	5.85 ± 0.26***	6.26 ± 2.46***	5.93 ± 1.38***
5	Treated 2	6.31 ± 0.35	5.16 ± 1.82	6.31 ± 1.81***	5.15 ± 1.27***

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; followed by Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; ***$P < 0.001$ in comparison with the diabetic control.

Table 9: Mechanical hyperalgesia: pinprick test in all groups in Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Mechanical hyperalgesia (pinprick test) sec			
		Response latency in sec (2^{nd} week)	Response latency in sec (4^{th} weeks)	Response latency in sec (8^{th} weeks)	Response late response latency in sec (12^{th} weeks)
1	Normal control	2.26 ± 1.36	2.19 ± 1.48	1.97 ± 1.39	2.18 ± 1.92
2	Diabetic control	5.16 ± 1.36[a]	4.96 ± 1.04[a]	6.71 ± 0.48[a]	9.71 ± 1.04[a]
3	Standard	4.22 ± 0.23***	3.12 ± 2.05***	4.43 ± 2.82***	4.74 ± 2.72***
4	Treated 1	5.01 ± 0.11	3.15 ± 0.45***	4.21 ± 2.91***	4.89 ± 1.25***
5	Treated 2	4.14 ± 1.26***	3.38 ± 1.26	4.96 ± 2.91***	4.51 ± 0.21***

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; and ***$P < 0.001$ in comparison with the diabetic control.

Table 10: Grip strength to record the response latency in sec in all groups in Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Grip strength for each animal in a group ± SEM ($n = 6$) (sec)			
		Response latency in sec (2^{th} week)	Response latency in sec (4^{th} weeks)	Response latency in sec (8^{th} weeks)	Response latency in sec (12^{th} weeks)
1	Normal control	31.11 ± 0.13	30.97 ± 1.41	31.21 ± 1.91	34.31 ± 0.19
2	Diabetic control	13.03 ± 0.38[a]	8.93 ± 1.51[a]	6.49 ± 1.41[a]	4.01 ± 0.25[a]
3	Standard	15.14 ± 1.26*	24.31 ± 0.87*	25.56 ± 1.32*	28.21 ± 2.13*
4	Treated 1	15.77 ± 1.06*	21.11 ± 3.71*	23.27 ± 0.39*	24.35 ± 1.16*
5	Treated 2	19.03 ± 1.36*	25.25 ± 0.27*	26.18 ± 0.23*	32.16 ± 1.62*

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; followed by Tukey-Kramer's multiple comparisons test: [a]$P < 0.05$ in comparison with normal control; ***$P < 0.05$ in comparison with the diabetic control.

Table 11: Spontaneous locomotor (exploratory) test: actophotometer on all groups in Wistar rats on weeks 2, 4, 8, and 12.

S. No.	Treatment type	Actophotometer: counts per 5 minutes			
		Counts (2^{nd} week)	Counts (4^{th} weeks)	Counts (8^{th} weeks)	Counts (12^{th} weeks)
1	Normal control	121.36 ± 1.34	126.11 ± 2.52	122.52 ± 3.65	127.43 ± 2.01
2	Diabetic control	35.14 ± 3.26[a]	28.18 ± 0.14[a]	19.04 ± 1.81[a]	15.01 ± 3.24[a]
3	Standard	41.32 ± 2.42*	70.27 ± 2.44*	84.24 ± 2.22*	93.25 ± 0.42*
4	Treated 1	42.37 ± 3.25*	68.26 ± 2.32*	80.14 ± 3.62*	90.33 ± 2.66*
5	Treated 2	44.46 ± 3.23*	72.24 ± 2.72*	89.25 ± 2.43*	100.24 ± 2.55*

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; followed by Tukey-Kramer's multiple comparisons test: [a]$P < 0.05$ in comparison with normal control; ∗P <0.05 in comparison with the diabetic control.

Table 12: Neuromuscular coordination test (motor coordination): rotarod test on all groups in Wistar rats on weeks 0, 2, 4, and 6.

S. No.	Treatment type	Rotarod test (counts)			
		Counts (2^{nd} week)	Counts (4^{th} weeks)	Counts (8^{th} weeks)	Counts (12^{th} weeks)
1	Normal control	123.14 ± 2.01	125.1 ± 0.25	127.41 ± 1.02	129.37 ± 4.14
2	Diabetic control	43.11 ± 1.10[a]	21.41 ± 3.14[a]	16.24 ± 0.61[a]	10.39 ± 2.05[a]
3	Standard	50.26 ± 4.39	84.01 ± 5.23***	97.52 ± 2.19***	109.13 ± 0.28***
4	Treated 1	49.40 ± 2.91***	78.27 ± 2.71***	89.24 ± 3.27***	100.25 ± 4.82***
5	Treated 2	52.15 ± 4.16	96.13 ± 3.37***	99.24 ± 4.46***	102.18 ± 0.31***

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.001$ in comparison with normal control; ***$P < 0.001$ in comparison with the diabetic control.

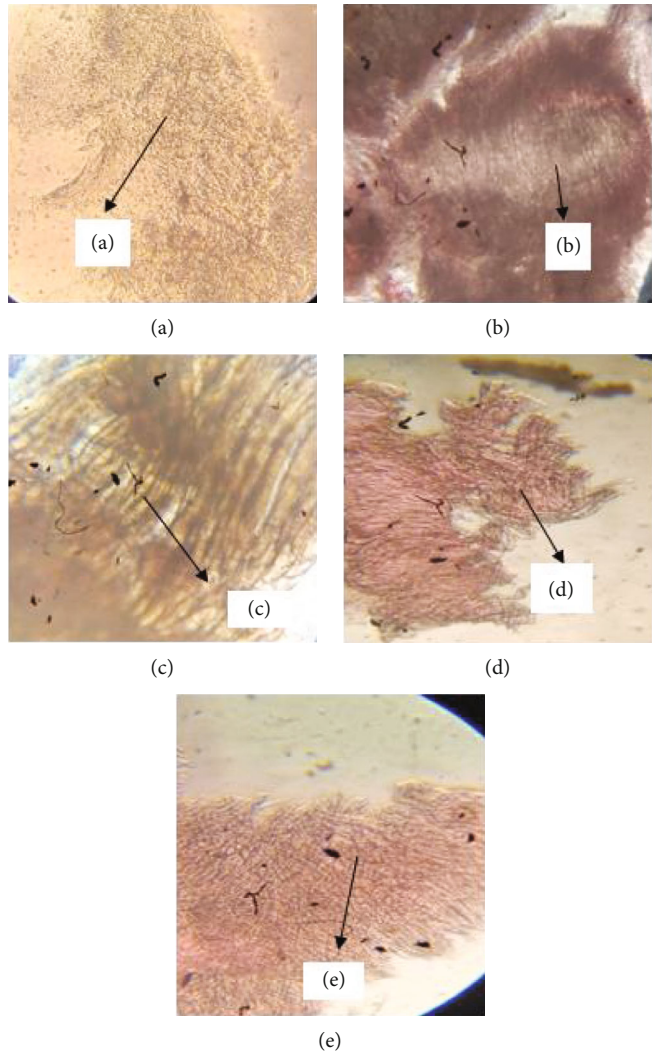


Figure 7: Analysis of longitudinal vasa nervorum in the sciatic nerves of Wistar rats on the 12th week. Samples were stained and analysed using a microscope. Group II has shown signs of swelling in the neurons. Effects of thymol isolated from fruits of Trachyspermum *ammi* were noted on the treatment groups postdosing for 12 weeks. (a) Normal nerve tissue which is not showing any signs of inflammation or nerve degeneration, damage (control group); (b) untreated groups have shown prominent signs of inflammation and damage and lipid degenerated axons showing focal peripheral axonal loss (diabetic group); (c) mild focal axonal loss shows signs of recovery from the damage induced by prolonged diabetes hence signifying the effects of standard drug on the nerves; (d) mild focal axonal loss in peripheral region showing healing action of isolated compound post regularly dosing for 12 weeks; (e) minimal axonal degeneration noted in treated group II where 20 mg/kg of isolated compound dosing was performed, hence showing the strong healing and neuroprotective action of drug on the sciatic nerve tissue.

which shows improvement in glucose levels compared with the diabetic control group on day 28 on the experiment. Group III has shown demonstrated improvement of glucose levels with 121.18 ± 3.23 mg/dl concentration on day 28.

3.4. Body Weight, Food, and Water Consumption. Bodyweight of Group IV and Group V was 201.34 ± 1.38 g and 211.23 ± 1.42 g at conclusion of the experiment, which is higher when compared against diabetic control (132.36 ± 3.26) and also efficient when compared with Group III (199.2 ± 1.12 g), while food intake was 22.47 and 21.02 g/day which is less than the diabetic control group (27.26 ± 0.31 g/day). The glibenclamide group (Group III) has shown efficient results with 21.14 ± 2.62 g/day food intake.

On recording the water intake, it was found that Group IV and Group V consumed 17.42 ± 1.35 and 16.13 ± 0.59 ml/day, which is less than the diabetes group, while Group III has shown 21.17 ± 2.35 ml/day of average water intake (Tables 3 and 4).

3.5. Lipid Profile. Groups IV and V have shown 85.02 ± 1.91 mg/dl and 74.59 ± 0.24 mg/dl levels of TC; 72.26 ± 2.43 mg/dl and 65.15 ± 1.28 mg/dl levels of triglyceride; 45.26 ± 2.36 mg/dl and 37.24 ± 2.29 mg/dl of LDL levels; 21.13 ± 1.27 mg/dl and 14.01 ± 0.13 mg/dl of VLDL levels; and 20.35 ±

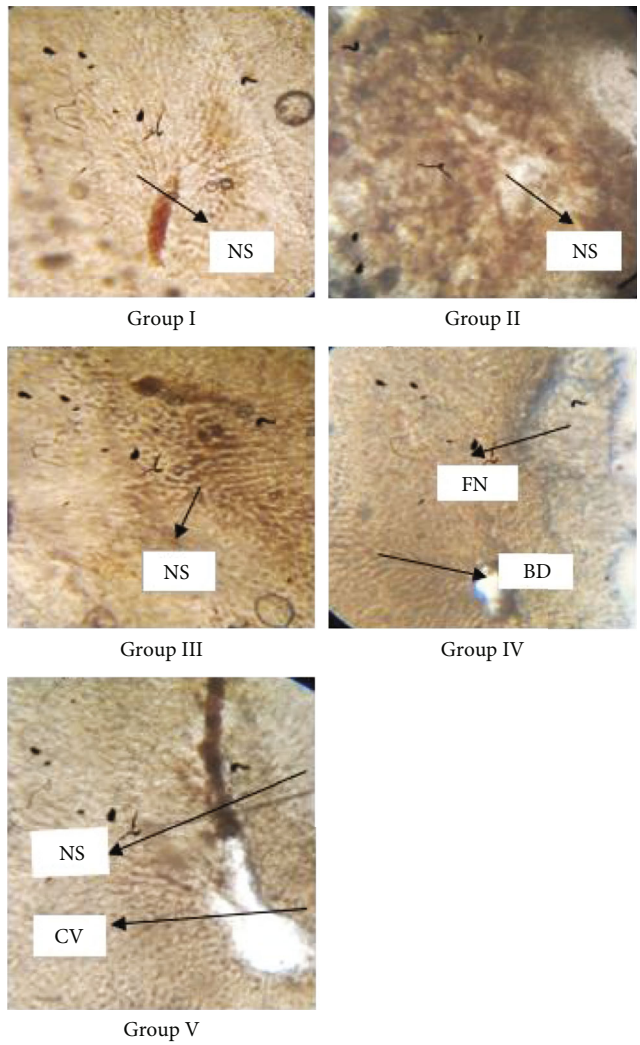
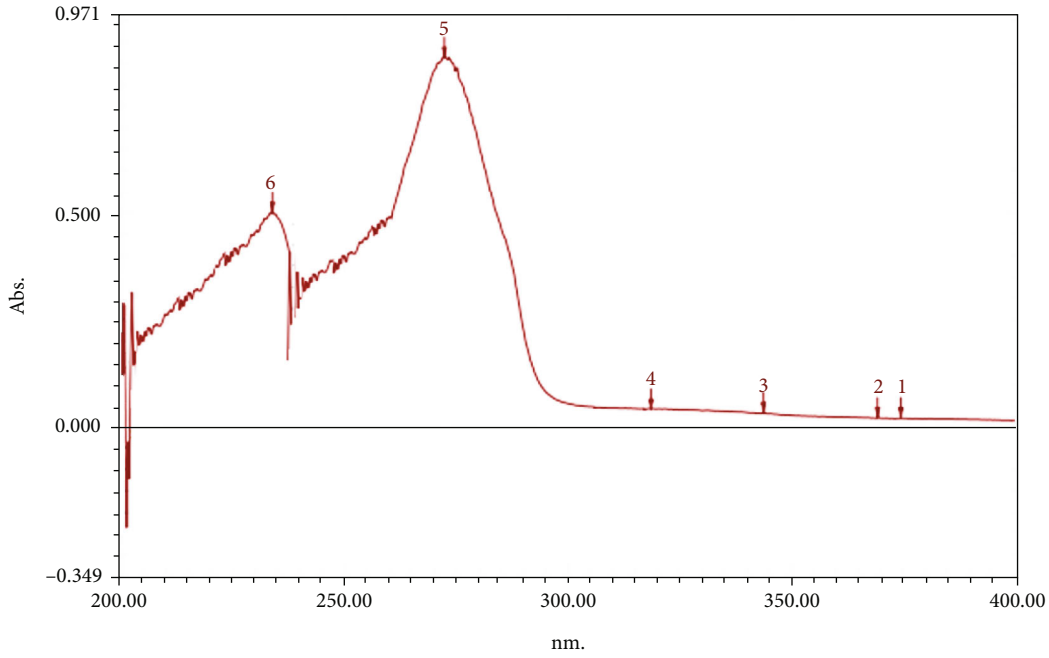


Figure 8: Histopathological analysis of the liver tissues which were obtained post 12 weeks of experiment (experimentally induced diabetic neuropathy) from all animal groups. Images represent the control group, diabetic control, standard, and treated groups. Samples were stained and show results as follows: (a) hepatic cells were observed as normal, and narrow sinusoids were clearly visible. (b) Fatty necrosis and ballooning degradation were noted in this image which represents disruptive cells in the liver. (c) Narrow sinusoids were observed in the slides, and comparatively healthy tissue was noted. (d) Normal hepatic cells with narrow sinusoids. (e) Normal hepatic cells with narrow sinusoids were observed. CV: central vein; NS: narrow sinusoids; FN: fatty necrosis; BD: ballooning degradation.

Table 13: Effect of thymol on endogenous biomarkers in rats.

S. No.	Treatment type / Group type	Endogenous biomarkers				
		SOD (U/mg of protein)	NO (μg/ml)	LPO (nM/mg of protein)	Na^+K^+ATPase (μmol/mg of protein)	TNF-α (pg/ml)
1	Normal control	24.03 ± 1.42	104.03 ± 1.36	2.34 ± 1.13	10.11 ± 1.23	51.35 ± 1.13
2	Diabetic control	5.32 ± 1.13[a]	303.11 ± 2.11[a]	9.11 ± 1.13[a]	2.34 ± 0.17[a]	160.01 ± 1.21[a]
3	Standard	16.12 ± 0.12[*]	224.32 ± 2.12[*]	6.13 ± 1.41[*]	6.21 ± 0.21[*]	119.13 ± 1.12[*]
4	Treated 1	21.11 ± 1.23[*]	158.09 ± 2.12[*]	4.13 ± 1.23[*]	7.31 ± 2.03[*]	90.21 ± 1.13[*]
5	Treated 2	23.24 ± 1.22[*]	129.22 ± 2.01[*]	2.71 ± 1.31[*]	9.34 ± 1.34[*]	67.25 ± 0.21[*]

Values are expressed as the mean ± SEM; $n = 6$. One-way ANOVA; Tukey-Kramer's multiple comparisons test: [a]$P < 0.05$ compared to normal control; [⊠]$P < 0.05$ in comparison with the diabetic control.



Measurement properties	
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Sampling interval:	0.2
Auto sampling interval:	Enabled
Scan mode:	Auto
Instrument proerties	
Instrument type:	UV-1700 Series
Measuring mode:	Absorbance
Slit width:	1.0 nm
Ligt source change wavelength:	340.8 nm
S/R Exchange:	Normal
Attachment properties	
Attachement:	None
Sample preparation properties	
Weight:	
Volume:	
Dilution:	
Path length:	10 mm
Additional information:	01/01/2007 05:42:21 AM

No.	P/V	Wavelength	Abs.	Description
1	↑	374.60	0.017	
2	↑	369.40	0.018	
3	↑	343.80	0.028	
4	↑	318.40	0.039	
5	↑	272.40	0.861	
6	↑	233.60	0.499	

Figure 9: UV spectroscopy of the isolated compound.

2.26 mg/dl and 28.03 ± 0.29 mg/dl of HDL levels which are improved values compared with the diabetic group. The standard group has shown improvements in lipid profile with TC level as 77.23 ± 2.36 mg/dl, triglyceride as 65.48 ± 1.49 mg/dl, LDL levels as 37.16 ± 0.25 mg/dl, HDL levels as 29.8 ± 2.46 mg/dl, and VLDL levels as 15.12 ± 1.38 mg/dl which have shown improvements in comparison to the diabetic group [Table 5].

3.6. Behavioural Studies

3.6.1. Estimation of Thermal Hyperalgesia. In Eddy's hot plate experiment, the response time recorded was 6.08 ± 1.41 s and 5.93 ± 0.24 s for Group IV and Group V, respectively, as recorded on the 12th week. The results justify the healing of the nerves compared with Group II, which has recorded a higher (9.85 ± 1.86 s) response time due to potential damage to the nerves due to diabetic neuropathy. Group III has shown 5.91 ± 1.74 s as response time, which improves results (Table 6).

3.6.2. Writhing Responses. Writhing responses have recorded values of 8.28 ± 1.44 and 8.24 ± 0.17 (number of reactions) on week 12, which have shown improved latency when compared against the diabetic control group (Group 2), where reported latencies on week 12 are high due to potential damage to the nerves. Group III responses have also improved (8.94 ± 1.35) [Table 7].

3.6.3. Cold Hyperalgesia. When the acetone drop test results were compared from Group IV and Group V against diabetic control on 12th week, the values obtained were 5.93 ±

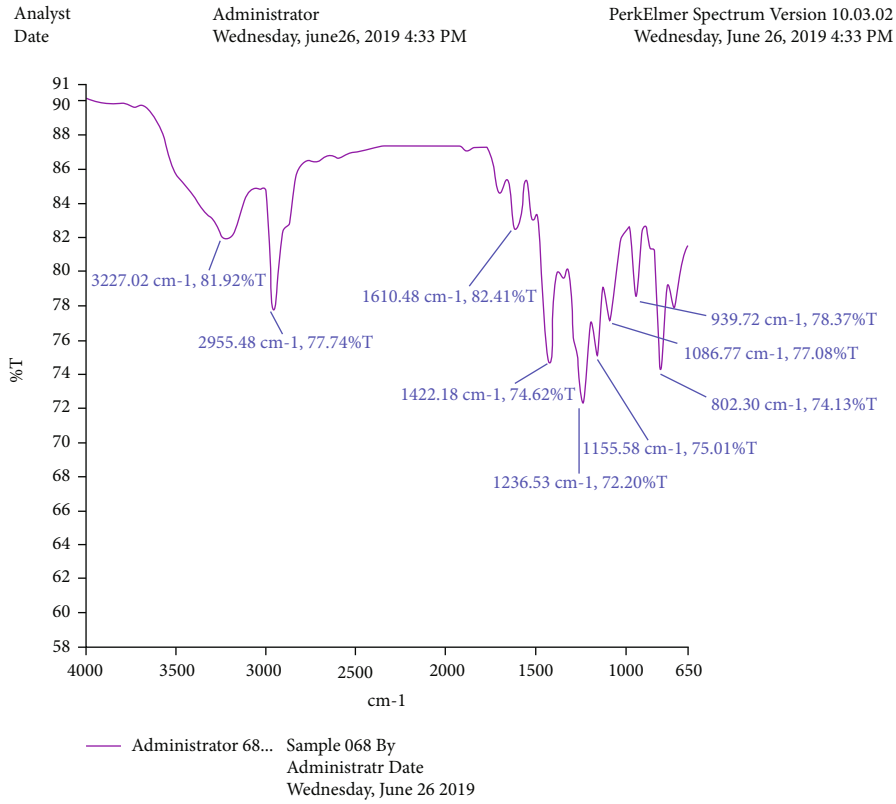

Figure 10: FTIR spectroscopy of the isolated compound.

1.38 and 5.15 ± 1.27 (sec). Here, an improvement in the latencies of the treated groups was noted compared to the diabetic group. Group III results were recorded as 6.11 ± 2.12 which had improvement compared to the diabetic control group (Table 8).

3.6.4. Mechanical Hyperalgesia: Pinprick Test. On analysing the results of Group IV and V with Group II, readings of 4.89 ± 1.25 s and 4.51 ± 0.21 s have been noted on week 12 of experimentation. Though the changes have been quite significant throughout the experiment, the results on week 12 have shown improved results in the treated groups. The standard drug group has recorded a response of 4.74 ± 2.72 s which shows improvement (Table 9).

3.6.5. Grip Strength. The animals in the diabetic group have lost their grip strength due to prolonged diabetes; hence, the diabetic group on week 12 shows poor results. In comparison, significant values of 24.35 ± 1.16 and 32.16 ± 1.62 (sec) were recorded for Groups V and V, respectively. Group III has recorded 28.21 ± 2.13 (sec) as grip strength. All treatment groups show improvement in the mechanical strength of animals (Table 10).

3.6.6. Spontaneous Locomotor (Exploratory) Test. Significant readings of 93.25 ± 0.42, 90.33 ± 2.66, and 100.24 ± 2.55 (counts) were obtained on week 12 of experimentation in Groups III, IV, and V. The readings are very similar to normal groups and significantly different from the diabetic control; hence, they show an improvement in the spontaneous locomotor activity due to healing effect of drug (Table 11).

3.6.7. Neuromuscular Coordination Testing. Motor coordination readings (rotarod test) for Groups III, IV, and V were recorded to be 109.13 ± 0.28, 100.25 ± 4.82, and 102.18 ± 0.31 which are significantly different compared to Group II on the 12th week of the experiment (Table 12).

3.7. The Histopathological Analysis. The histopathological analysis has shown normal results in treated groups, as shown in Figures 7 and 8. Little or no degradation was noted in sciatic nerve tissues similarly; no necrosis was noted in liver tissues of Groups IV and V. All results have shown significance against the diabetic control rats.

3.8. Biochemical Parameters

3.8.1. Superoxide Dismutase Effect. The results have shown significant, dose-dependent results with isolated doses of thymol. A significant reduction in the value of superoxide dismutase was found (5.32 ± 1.13 U/mg of protein) in the homogenate of the sciatic nerve obtained from diabetic rats (Group II) compared with the normal control group (Group I), i.e., 24.03 ± 1.42 U/mg of protein. Rats from treated groups have shown rise in SOD levels (21.11 ± 1.23 and 23.24 ± 1.22 U/mg of protein) as shown in Table 13.

3.8.2. The Effect of Thymol-Treated Groups on Nitrosative Stress. In the sciatic nerve, the neural nitrite levels from the diabetic groups were significantly increased to 303.11 ±

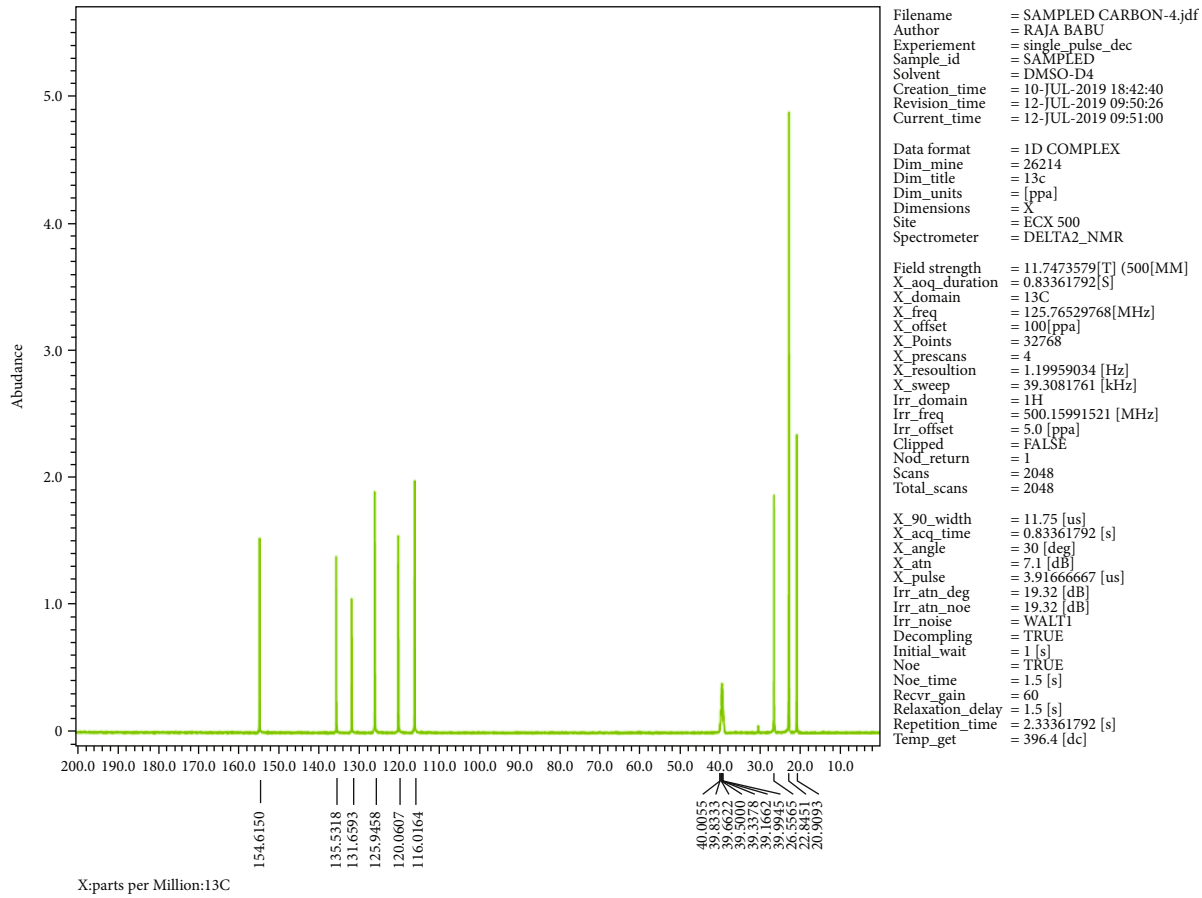

Figure 11: C^{13} NMR spectroscopy of the isolated compound.

2.11 μg/ml ($P < 0.05$) in comparison to the normal group recording values of 104.03 ± 1.36 ($P < 0.05$). Treated groups have shown values of 158.09 ± 2.12 and 129.22 ± 2.012 which are lowered significantly compared to the diabetic group rats (Table 13).

3.8.3. Lipid Peroxide Profile Evaluation. Neutral Lipid Peroxidase (LPO) levels were recorded in all groups at the end of 12 weeks of study. The diabetic group has shown a significant increase ($P < 0.05$) in LPO levels. The value recorded for Group II was 9.11 ± 1.13 nM/mg of protein compared with the levels from the normal group (2.34 ± 1.13 nM/mg of protein). The treated groups recorded values as 4.13 ± 1.23 and 2.71 ± 1.31 nM/mg of protein ($P < 0.05$) (Table 13).

3.8.4. Effect in Membrane-Bound Inorganic Phosphate. $Na^{+-}K^{+}$ATPase level (membrane-bound inorganic phosphate) after 12 weeks of study was found to be 2.34 ± 0.17 μmol/mg of protein with $P < 0.05$ in rats from the diabetic group which is low in comparison to normal group results (10.11 ± 1.23). The treated group rats have shown values of 7.31 ± 2.03 and 9.34 ± 1.34 μmol/mg of protein which is significantly different from the diabetic group (Table 13).

3.8.5. Effect in the TNF-α. A significant increase in TNF-α from sciatic nerves was noted in STZ diabetic groups (160.01 ± 1.21 pg/ml) when compared to the control group (51.35 ± 1.13 pg/ml). Treated groups have shown improved results with values of 90.21 ± 1.13 and 67.25 ± 0.21 pg/ml compared to the diseased groups (Table 13). Improvement in motor coordination was noted in treated group SOD; $Na^{+}K^{+-}$ ATPase values of treated groups receiving 10 and 20 mg/kg of thymol have shown a significant increase in the levels by $P < 0.05$ compared with those of the diabetic group. NO, LPO, and TNF-α values have a significant decrease in the levels by $P < 0.05$ compared to the diabetic group.

4. Discussion

A large part of the world's population suffers from diabetes whose major complications include hyperglycemia and diabetic neuropathy. Inflammation and hyperglycemia are the two events that alter gene expressions, thus affecting the cellular proteins, hence leading to progressive changes like diabetic complications and pathological changes. With the current growth status, diabetic neuropathy will be the prime cause of terminal stage renal diseases globally with intolerable consequences and costs for healthcare in developed countries [50, 51].

In the current treatment scenario, antidepressant drugs have been used in the treatment and regulation of neuropathic pain, which leads to the maintenance of sustained levels of neurotransmitters (norepinephrine and serotonin). As these hormones have shown a reduction in the central

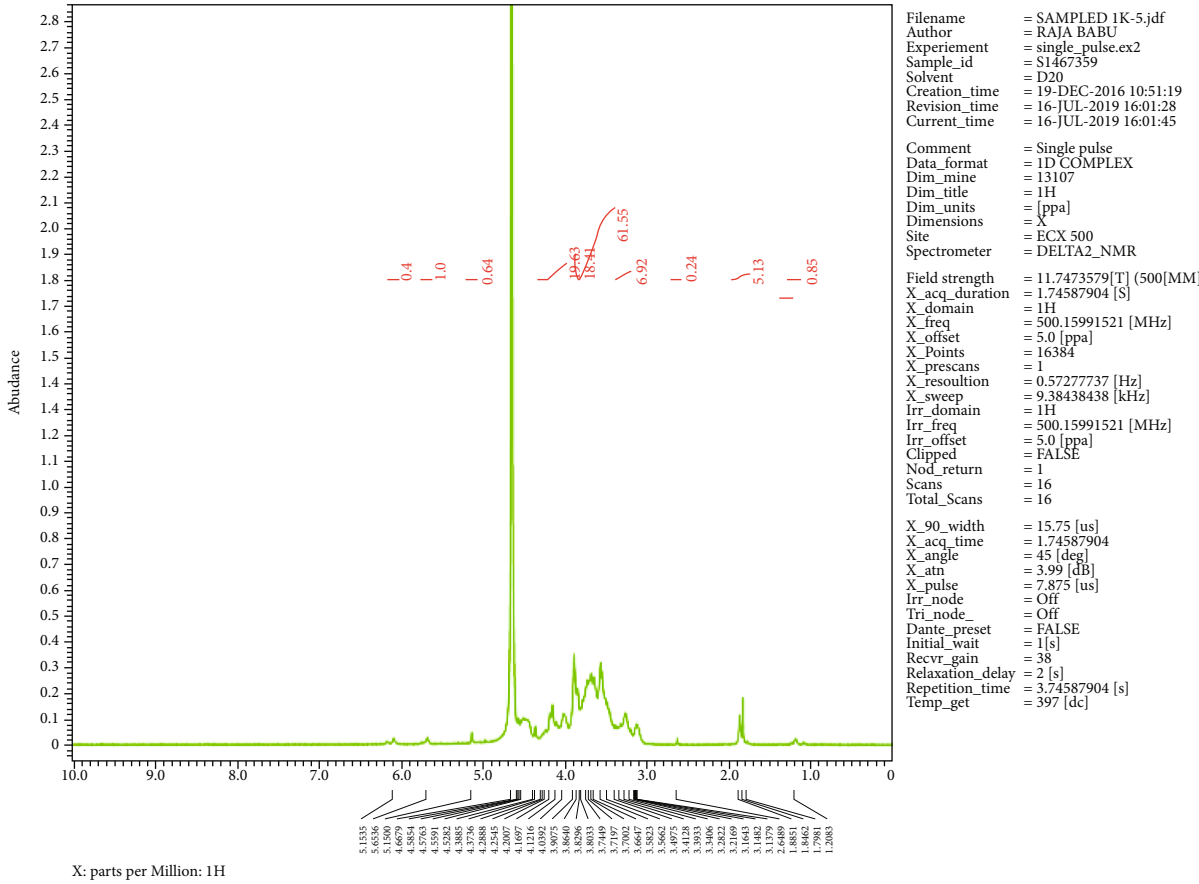

Figure 12: ^{1}H NMR spectroscopy of the isolated compound.

nervous system's pain pathways, they have shown good results in regulation and sensitivity to pain [52, 53].

Spectroscopic analysis has confirmed the presence of thymol (Figures 9–14). In the current study, the effects of thymol isolated from Trachyspermum ammi have been investigated for doses of 10 and 20 mg/kg on diabetes and diabetic neuropathy induced by streptozotocin in Wistar rats. The tests were also conducted for behaviour analysis, food, water consumption, lipid profile analysis, nociception, response latency, neuromuscular coordination, and histopathological analysis of the liver and sciatic nerve. A decrease in the blood glucose levels was noted by doses of 10 and 20 mg/kg of thymol. The reduction in blood glucose levels must be due to the stimulation of insulin secretions from beta cells that stimulate glucose metabolism and restore the remaining cells—this might be the best possible mechanism suggested by the standard drug glibenclamide [54].

This experiment has also shown protective effects of the isolated compound to treat diabetic neuropathy, which is a prominent effect of prolonged diabetes conditions. A loss of pain perception is noted in people who have diabetes due to extensive nerve damage; hence, there is the induction of peripheral neuropathy [55, 56].

The study revealed a delayed response in diabetic control group animals due to loss of perception and extensive damage to nerves, induced by prolonged diabetes. A gradual healing action was noted in treated groups which were most significant in groups receiving 20 mg/kg of thymol. In comparison, the diabetic groups have shown a trend of high sensitivity responses in nerves to delayed responses during week 12 due to extensive nerve damage. The treatment groups have also demonstrated a significant reduction in the paw withdrawal during thermal and mechanical hyperalgesia in the streptozotocin-induced Wistar rats which justifies the diabetic neuropathy protective action of thymol [57].

Loss of in nociception in diabetic animals must be due to the development of DN, which is the characterization of nerve degradation, leading to loss in the perception of pain [55, 58]. When an animal starts developing nerve damage, it tends to become lethargic and shows delayed responses due to progression of pain and development of diabetic neuropathy [59, 60]. The results obtained from the tail-flick test, thermal hyperalgesia, and cold hyperalgesia also reported a loss of perception of pain, hot or cold due to intense nerve damage in diabetic rats, and improved results in treated groups due to their nerve damage healing affect. The doses of 10 and 20 mg/kg have also shown improvement in the liver and sciatic nerve tissues, hence expressing the neuroprotection and antidiabetic action of isolated thymol.

In the analysis of vasa nervorum (longitudinal) in the sciatic nerves of Wistar rats on 12-week prominent signs of

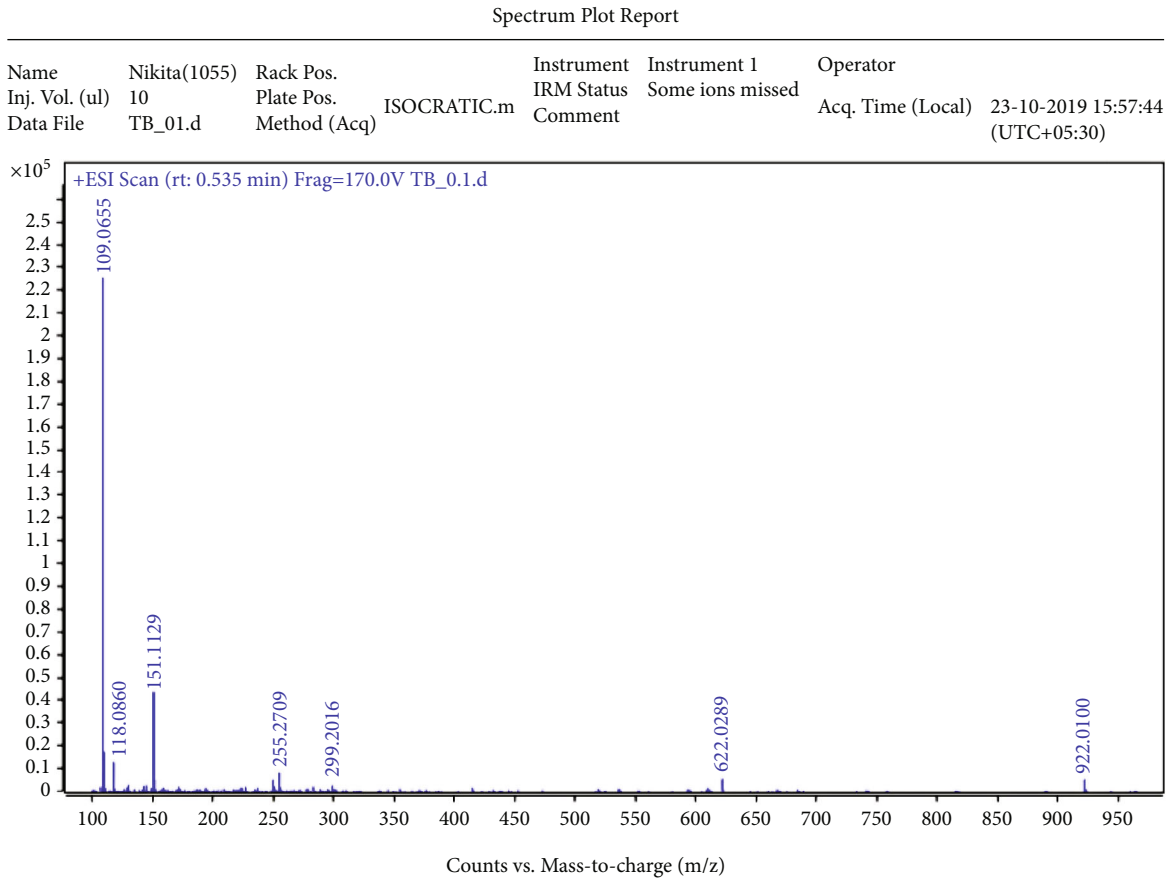

Figure 13: HRMS of the isolated compound.

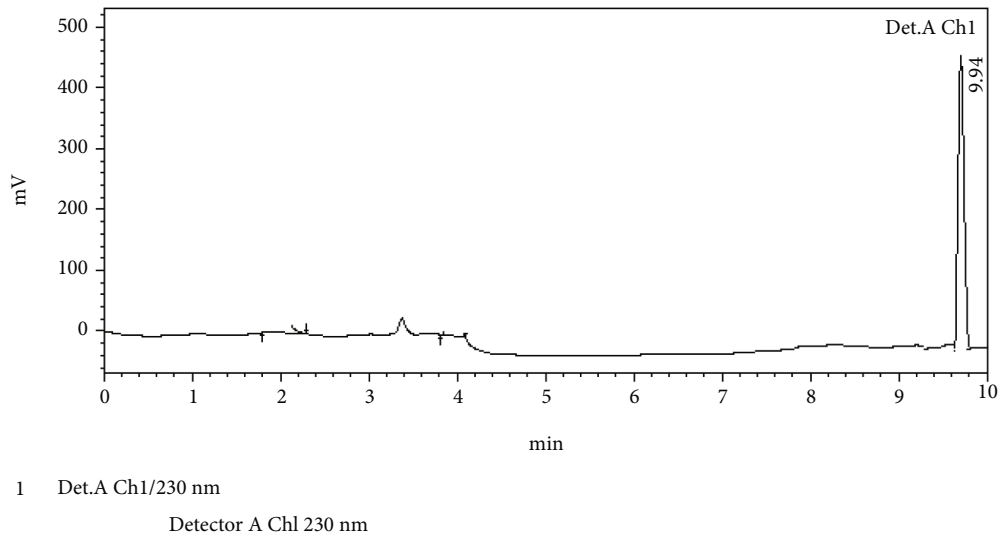

Figure 14: HPLC of the isolated compound.

inflammation and damage, lipid degenerated axons showing focal peripheral axonal loss were noted in the diabetic group while the treated groups receiving 10 and 20 mg/kg thymol have shown minimal axonal degeneration which is a sign of healing and neuroprotective action of the drug. These healing signs are the recoveries of the damage induced by prolonged diabetes conditions. Similarly, the cells in the diabetic group have shown fatty necrosis and ballooning degradation, which represents disruptive cells in the liver. At the same time, the treated groups (10 and 20 mg/kg thymol) have shown normal hepatic cells

with narrow sinusoids which are healthy liver cells. They signify the recovery of cells from diabetes. Hence, the sciatic nerve and liver cell histopathology hints on antidiabetic and neuroprotective thymol on Wistar rats.

Positive findings were recorded in thermal hyperalgesia, writhing, cold hyperalgesia responses, mechanical hyperalgesia, grip strength, spontaneous locomotor (exploratory) test, neuromuscular coordination tests, and histopathological and lipid profile analysis. The neuroprotective results were quite significant at the end of treatment that is by the end of 12 weeks. Uncontrollable lipid profiles have been the prime complications of diabetes mellitus, which is noted in 40% of the diabetes cases. Hypertriglyceridemia is a condition very commonly found in patients suffering from diabetes mellitus and is also seen in situations of insulin resistance either due to reduced catabolism or reduced production of lipoproteins [61].

Endothelial damages are caused due to the presence of superoxide and endogenous enzymes. An increase in aldose reductase and protein kinase C has shown connections with pain perceptions. Antioxidant protection is done by SOD which transforms the superoxide anions into H_2O_2. Endogenous enzymes SOD and MDA closely reciprocate to the oxidative stress. Elevation in the stress levels as depicted by MDA leads to lipid membrane disruption. This causes rearrangement of bonds in unsaturated fatty acids created by tissue damage. Increased levels of lipid peroxidase are linked to oxidative stress levels, which are decreased during the drug treatment process. Nitric oxide is an intracellular messenger, and it has a significant role in the pathological process as it combines with ROS (reactive oxygen species), an antioxidant. The process of oxidation affects cell molecules and is nonspecific [62, 63].

Superoxide anions lead to an increase in NO levels; hence, it forms peroxynitrite, which causes fast nitrosylation, DNA damage, cell death, and lipid peroxidation. These effects lead to untimely elevated pain and aggregation. Pyridyl porphyrin, the catalyst, leads to sensory neuropathy in the diabetic group. The results observed in the diabetic group are per previous studies showing worsening neuropathy [2, 64, 65].

Na^+K^+ATPase distribution must be uniform for proper generation and the conduction of bioelectricity. A reduction in chronic hyperglycemia caused a decrease in the Na^+K^+-ATPase enzyme which caused inactivation of phosphate. This further leads to activation of polyol pathways. Na^+K^+-ATPase enzyme is inhibited, but the restoration is preferred. In a recent study, thymol has shown to restore levels of Na^+-K^+ATPase hence hinting its neuroprotective action [66].

Acceleration of the production of TNF-α in neural and microvascular tissues is a prominent feature of DN. This causes damage to nerves and microangiopathy in microvascular tissues. Hence, for treatment of diabetic neuropathy, a suppression in cytokine elevation is advised. Therefore, dose-dependency of the isolated compound thymol and its function in inhibition of cytokine levels is noted [67–69].

This study noted physiological, biochemical, and histopathological deviations in thymol-administered groups. The isolated compound has also shown efficient results when compared to a standard drug for restoring the levels of biomarkers, hence more effective in treating diabetic neuropathy. The critical factors responsible for neuropathic pain, oxidative stress, cytokine release, and tumor necrosis factor-α (TNF-α) were restored by thymol dosing in rats. These were also important in restoring the membrane bound inorganic phosphate activity and apoptotic conditions; henceforth, neuropathic pain mediation was regulated. Thymol aced the significant reduction of diabetic neuropathy pain by involving various mechanisms which lead to restoration of levels of Na^+K^+ATPase, inhibition of the elevated cytokines, reduction of TNF-α, and decrease in the NO levels. Thus, the antioxidant nature and healing action of thymol isolated from the herbal extract were signified and confirmed.

5. Conclusion

In conclusion, the treatment of thymol concentrations of 10 mg/kg and 20 mg/kg has ameliorated diabetes and conditions of diabetic neuropathy in STZ rats. Hence, it is obvious that thymol's oral dosing isolated from *Trachyspermum ammi* has improved the nociceptive latency, glucose levels, and lipid profiles and has subsequently shown healing effects in the liver and sciatic nerve of the treated groups where 20 mg/kg of thymol is more potent than its lower concentration group. The future aspects of this work suggest a detailed study on the mechanism of action to be performed to get better insights into thymol's role in the treatment of neuropathy, diabetes, and other metabolic disorders.

Abbreviations

DPN:	Diabetic polyneuropathy
DN:	Diabetic neuropathy
CPCSEA:	Committee for the Purpose of Control and Supervision of Experiments on Animals
DMSO:	Dimethyl sulfoxide
OECD:	Organisation for Economic Co-operation and Development
DNA:	Deoxyribonucleic acid
DM:	Diabetes mellitus
TMB:	3,3′,5,5′-Tetramethylbenzidine
UV spectroscopy:	Ultraviolet spectroscopy
TLC:	Thin Layer Chromatography
IAEC:	Institute Animal Ethics Committee
FTIR:	Fourier-transform infrared spectroscopy
HPLC:	High-Performance Liquid Chromatography
HRMS:	High-resolution mass spectrometry
NMR:	Nuclear Magnetic Resonance
STZ:	Streptozotocin
SOD:	Superoxide dismutase
MDA:	Malondialdehyde
NO:	Nitric oxide
LPO:	Neutral Lipid Peroxidase

Na^+K^+ATPase:	Membrane-bound inorganic phosphate
TNF-α:	Tumor necrosis factor alpha
Pyridyl porphyrin:	Fe(III)tetrakis-2-(N-triethylene glycol monomethyl ether.

Data Availability

Data will be available on request to the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Daily Dose Standardization Based on Essential and Nonessential Trace Element Presence in *Berberis baluchistanica* Ahrendt Bark, Leaf, and Root

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Medicinal plants have great importance to the consumer health, as beside beneficial compounds, plants can accumulate essential and nonessential metals from soil and surrounding environments, leading to consumer health risks. Assuming this, the present study is aimed at evaluating the elemental composition and daily dose standardization based on essential and nonessential trace element presence in of bark, leaves, and roots of *Berberis baluchistanica* Ahrendt, a common medicinal plant used as a folk medicine in the region. Atomic absorption and flame emission spectroscopy were performed to analyze the presence of essential and nonessential elements manganese (Mn), copper (Cu), lead (Pb), nickel (Ni), iron (Fe), sodium (Na), and potassium (K). Among the essential elements, K was present at high concentrations in the bark ($8926.98 \pm 0.32\ \mu g/g$), leaves ($7922.77 \pm 0.42\ \mu g/g$), and roots ($6668.5 \pm 0.96\ \mu g/g$) of the plant. The estimated concentration of Na was higher in leaves ($1782.56 \pm 0.13\ \mu g/g$), followed by roots ($1089.5 \pm 0.71\ \mu g/g$) and bark ($572.8 \pm 0.62\ \mu g/g$). The Fe concentration varied in the range of $394.7 \pm 0.3\ \mu g/g$ in bark, $1298.3 \pm 0.54\ \mu g/g$ in leaves, and $1208.9 \pm 0.7\ \mu g/g$ in roots. The trace transition element Mn was highest in leaves ($42.7 \pm 0.99\ \mu g/g$), followed by roots ($33.5 \pm 0.94\ \mu g/g$) and bark ($22 \pm 1\ \mu g/g$). The Cu concentration was low, ranging from 20.1 ± 0.63 to $22.67 \pm 0.7\ \mu g/g$ in leaves, bark, and roots. The obtained concentration of nonessential element Pb was relatively lower than the permissible range (10 mgL–1) established by the World Health Organization. The elemental concentrations in all parts were within the set limits for provisional tolerable daily maximum intake (PTDMI) and provisional tolerable weekly intake (PTWI), and the hazard quotient index (HQ) was below 1 for all toxic metals. The micro and macroelemental distribution and the overall medicinal potential of any medicinal plant can be correlated for dose risk estimation, which will be useful in providing knowledge regarding the contraindication associated with folk medicines. In the present study, based on the elemental composition, it was calculated that the daily safe dose for *Berberis baluchistanica* is approximately 2-5 g/day of raw powder for an adult, which must not be exceeded to this safe range.

1. Introduction

Plants of medicinal impacts have great importance to the health of individuals and communities [1]. Medicinal plants are traditionally used for the treatment of various diseases [2]. Crude extracts of these plants comprise a mixture of different valuable bioactive components such as alkaloids, polyphenols, flavonoids, terpenoids, and minerals. These components have major antioxidant, antimicrobial, anti-inflammatory, chemo-preventative, and cytotoxic potentials. It provides safety against several diseases. Herbal medicines containing these phytochemical constituents (plant secondary metabolites) are used as alternates for synthetic and allopathic drugs and are thought to be less hazardous to humans and the environment compared to the counterpart [3]. Consumption of these medicinal plants contributes to the intake of different less important phytochemical components and other associated essential and nonessential trace elements. Along with other potential positive impacts of these plants on human health, trace elements have substantial roles in curing a variety of human diseases [4]. However, the higher concentration of these elements is also responsible for toxicity, which can even make the best drug a potent poison if the concentration of elements present in these plants exceeds the permissible specific toxicological parameters controlled by regulatory authorities. Due to their effectiveness in treating a variety of diseases, it is important to determine the concentration of these trace elements in all parts of plants along with their pharmacological properties [5].

Berberis is a recognized genus of the family *Berberidaceae*, containing approximately 550 species [6]. It is one of the most primitive angiosperms with great economic and medicinal value, as it contains berberine as a major phytochemical [7]. Different phytochemicals, such as glycosides, steroids, anthraquinones, saponins, alkaloids, phlobatanins, tannins, reducing sugars, flavonoids, and terpenoids, are also present in Berberis. The antioxidant, antidiabetic, anti-inflammatory, hepatoprotective, and hypotensive properties of berberine and berbamine found in Berberis plants have also been reported [8]. However, efficient investigations on the elemental concentrations of *Berberis baluchistanica* species are lacking.

Berberis baluchistanica Ahrendt is a wild medicinal plant locally known as Zralag in Pashto, Archin in Brahvi, and Korae in Balochi language. It is endemic to Balochistan and belongs to the family Berberidaceae. The plant is distributed in the Harboi mountain range in Kalat, Zarghun mountain in Quetta, and in Ziarat Balochistan Pakistan [9]. This medicinal plant is valued for its bark and roots and considered nontoxic and consumed in raw form, either as powder or a decoction. As it contains berberine, the plant is used for the treatment of various diseases, such as cough, fever, internal injury, eye disease, kidney stone removal, wound healing, rheumatism, and other infections of humans and livestock [10]. Previously, various secondary metabolites, such as berberisinol, berberine [11], 8-oxoberberine, oleanolic acid, palmatine [12], gallic acid, phenols, carotenoids, and vitamins [8], were isolated and found to have remarkable antioxidant, antileishmanial, antidiabetic, antibacterial, and antifungal potential [9, 10].

Traditional medicinal plants are rich in various essential and nonessential elements. These elements may be hazardous if consumed for longer periods without any restrictions. Hence, a comprehensive examination is considered significant to test the plants' essential and nonessential elements. This can be accomplished by assessing the harmful impacts of the components in light of the average upper admissible level and afterward assessing the harmfulness of elements taken from medicinal herbs. Consequently, it is necessary to quantify the essential and toxic elements along with other phytochemicals present in medicinal plants [13]. Taking into account the importance of trace elements in general well-being as well as their curative properties, medicinal plants are therefore being explored and have attracted interest regarding the effects of bioactive components, quantification of their essential and nonessential elements [14].

The present study is aimed at analyzing the elemental composition and its quantification in bark, leaves, and roots of *Berberis baluchistanica* by atomic absorption spectroscopy (AAS) and flame emission spectroscopy. Furthermore, health risks associated with nonessential elements were assessed, and the medicinal properties of all parts of the plant and their elemental distribution were correlated.

2. Materials and Methods

2.1. Plant Collection. Berberis baluchistanica plants were collected from Ziarat of Balochistan and were identified by taxonomist, Dr. Saadullah Khan Leghari Professor in Department of Botany University of Balochistan Quetta. The voucher specimens were prepared, and the plant was deposited in the Herbarium, Department of Botany, University of Balochistan Quetta, Pakistan.

The research work and the plant collection permission have been granted by the local and institutional authorities (no. UoB/Reg:/GSO/464 dated 01/06/2021).

2.2. Plant Sample Preparation. The bark, leaves, and roots of the plant were separated from each plant, washed off with distilled water to remove any kind of dust and contamination, and dried under shade for 3-4 consecutive weeks at room temperature under controlled humidity. The dried roots were separately ground into fine powder using an electrical grinder and stored in desiccators for further analysis [1].

2.3. Reagents and Solutions. All analytical grade standards of metals (Mn, Cu, Pb, Ni, Fe, Na, and K) were purchased from Merck. Standard solutions (0.1–20 mgL–1) of these metals were diluted in distilled water, and digestion was carried out with a mixture of HNO_3, H_2SO_4, and $HClO_4$ at a ratio of 5 : 2 : 1.5.

2.4. Instrument and Glassware. An atomic absorption spectrophotometer (AA 7000 Shimadzu) was used for the determination of heavy metals with hollow cathode lamps of different metals and a flame of air-acetylene. Na and K were detected using a flame emission spectrophotometer (Jenway PFP7). Various glassware including conical flasks, round bottom flasks, and different beakers were used. All

glassware were washed, dried, soaked in an HCl bath (10% *v*/*v*) for one week, and rinsed several times with deionized water before use.

2.5. Sample Digestion and Determination of Trace Elements. Digestion of the powdered plant part was accomplished according to a previously reported protocol [15]. Hence, 0.50 g powder of each sample was placed in a 50 mL round bottom flask, and a 8.5 mL mixture of acids (5 mL nitric acid, 2 mL sulfuric acid, and 1.5 mL perchloric acid) was then added. After 24 h, the flasks were heated for 30 min at 60°C, further heated at 150°C for 15 min and allowed to settle with white fumes. The digestion mixture was then transferred into a 50 mL volumetric flask, and 50 mL of distilled water was poured into the bottles, followed by filtration using filter paper (Whatman No. 1). These prepared solutions after wet digestion were studied for the detection of elements using AAS and a flame photometer. Dilutions of different concentrations were prepared from analytical grade stock standards of 1000 mgL–1 for the purpose of calibration. These dilutions were prepared immediately before running the samples. Deionized water was used throughout the investigation. For quality assurance and quality control purposes, blanks were included in each batch of samples analyzed. Atomic absorption spectrophotometry was used with hollow cathode lamps of different metals as radiation sources operated at 5 mA at 393 and 279 nm wavelengths, respectively, with optimized air acetylene flames. A flame emission spectrophotometer (Jenway PFP7) was applied for the evaluation of sodium and potassium. To obtain results within the range of the flame photometer, the samples were diluted 100-fold with deionized water. The results were expressed in (μg/g).

2.6. Hazard Quotient Assessment. Toxic metal intake causes health risks, when its consumption exceeds the set values for the upper daily intake level of a nutrient that is considered safe. For some elements, the upper daily intake level has not been established, so the health risk can be assessed by simple comparison with specific toxicological parameters controlled by regulatory authorities, i.e., the provisional maximum tolerable daily intake (PMTDI) or the provisional tolerable weekly intake (PTWI).

The health risk associated with the consumption of the toxic elements of each sampled part of the plant was evaluated by calculating the daily metal intake (DMI) and provision for weekly intake (PWI) of the metals [16]. All the calculated DMIs of metals from bark, leaves, and roots are presented in the results section.

2.7. Daily Metal Intake. The daily metal intake DMI (Eq. (1)) was calculated according to the equation [17, 18] with a slight modification

$$\mathrm{DMI} = (C) \times \frac{\mathrm{DI}}{\mathrm{BW}}, \tag{1}$$

where C represents the individual concentration of a toxic metal, DI represents the daily intake of metals per day, and BW is the bodyweight of an average individual assuming 70 kg.

The PWI was calculated by multiplying the daily metal intake by seven (Eq. (2)).

$$\mathrm{PWI} = \mathrm{DMI} \times 7, \tag{2}$$

where number 7 refers to the number of days in the week.

The obtained PWI values were compared with the PTWI of the metals as recommended by the World Health Organization (WHO) and other authorities for an average adult of 70 kg body weight.

Another way to calculate the associated intake risk from medicinal plant intake is the hazard quotient (HQ), which depends on the estimated weekly metal intake (PWI) and is inversely proportional to the oral reference dose (RfD) (Eq. (3)).

$$\mathrm{HQ} = \frac{\mathrm{PWI}}{\mathrm{RfD}}. \tag{3}$$

RfD was used to determine the safe intake for HQ, with the following values for each element: iron 800 μg/kg/day, manganese 140 μg/kg/day, and nickel 15 μg/kg/day [19]. For elements having no RfD values, we used the PTWI values to determine the HQ, lead 25 μg/kg/week, and copper 3.5 mg/kg/week or 3500 μg/kg/week.

The HQ calculus calculates the potential risk to noncarcinogenic chronic damage to human health, where HQ > 1 equals a hazard potential [20].

2.8. Statistical Analysis. All the experiments were conducted in triplicate and the results were expressed as the average ± standard deviations (SD). The magnitude of the means, standard curve, and standard deviations were calculated by using MS Excel 2010 Software. To identify significant elements, the obtained overall data were subjected to principal component analysis (PCA) using XLSTAT software.

3. Results and Discussion

The AAS is a reliable analytical technique that gives the calculated concentration of various elements. Among the essential elements, K was present at high concentrations of 8927 ± 0.3 μg g – 1 in bark, 7922.8 ± 0.4 μg g – 1 in leaves, and 6668.5 ± 0.96 μg g – 1 in roots of the plant. The estimated concentration of Na was highest in leaves (1782.6 ± 0.13 μg g – 1), followed by roots (1089.5 ± 0.7 μg g – 1) and bark (572.8 ± 0.6 μg g – 1). The Fe concentration varied in the range of 394.70 ± 0.3 μg g – 1 in bark, 1298.3 ± 0.5 μg g – 1 in leaves, and 1208.9 ± 0.7 μg g – 1 in roots. The trace transition elements Mn and Cu were found in low quantities ranging from 22 ± 1 – 42.7 ± 1 μg g – 1 and 20.1 ± 0.63 – 22.7 ± 0.7 μg g – 1. The obtained concentrations of the nonessential elements Pb were 31.5 ± 0.98 μg g – 1 in bark, 36.8 ± 1.2 μg g – 1 in leaves, and 32.1 ± 1.03 μg g – 1 in root samples (Figure 1 and Supplementary Table 1). All the elemental composition of the three parts was relatively lower than the permissible ranges established by the WHO.

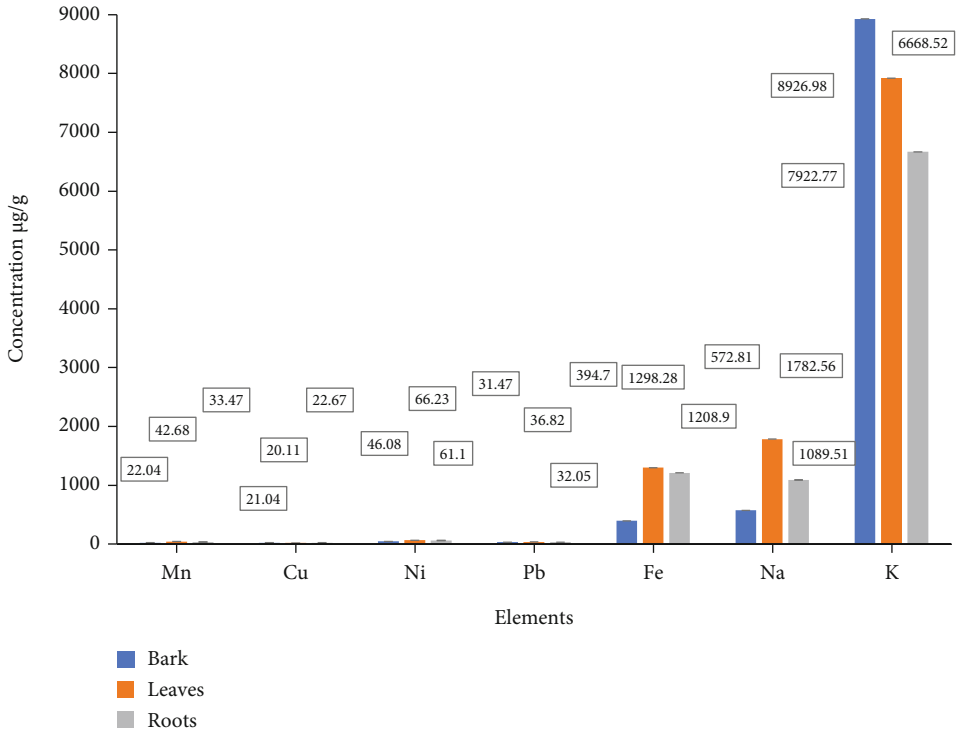

Figure 1: Comparative analysis of the concentrations of various metals in bark, leaves, and roots of *Berberis baluchistanica*.

Different parts of *Berberis baluchistanica* plants are sold in herbal medicines markets of Balochistan, because of their traditional and highly curative properties with the vernacular name Zaralg. Therefore, quality assurance of the plant has become necessary to establish the critical limits for the daily dosage uses for community. Along with the therapeutic effects, the present study is aimed at evaluating the elemental composition and daily dose standardization based on essential and nonessential trace element presence in of bark, leaves, and roots of *Berberis baluchistanica*. Among the seven detected elements, Fe, Na, K, Mn, and Cu have been classified as essential elements, while Ni and Pb are nonessential elements for the human body. All elements detected in the plant are used in the treatment of different diseases.

3.1. Daily Intake of Essential and Nonessential Metal at Nontoxic Levels. The DMI values for the evaluated metals in the present study are shown in Table 1. The results revealed that the DMI value for Mn in leaves (1707.21 μg kg$-$l) was higher than that in roots (1339.80 μg kg$-$l) and bark (881.61 μg kg$-$1) for adults weighing 70 kg. The DMI of Cu in roots (810.32 μg kg$-$l) was also relatively higher than that in bark (751.22 μg kg$-$l) and leaves (718.41 μg kg$-$l). The DMI value of Ni in bark was 16.51 μg kg$-$l, which was lower than that in leaves (23.71 μg kg$-$l) and roots (21.82 μg kg$-$l). The DMI value for Pb in leaves (218.32 μg kg$-$l) was higher than that in roots (190.24 μg kg$-$l) and bark (186.61 μg kg$-$l). The DMI of Fe in leaves (278202.43 μg kg$-$l) was higher than that in roots (259050.52 μg kg$-$l) and bark (84578.61 μg kg$-$l). The DMI value of K in bark was 446349.02 μg kg$-$l, which was higher than that in leaves (396138.50 μg kg$-$l) and roots (333426.01 μg kg$-$l). For Na the DMI value in bark was 12247.51 μg kg$-$l, which was lower than that in leaves (38197.70 μg kg$-$l) and roots (23346.64 μg kg$-$l). Overall, the concentration of toxic metal daily intake was normal and below the permissible limits in all studied parts of the plant.

3.2. Hazard Quotient and Provision for Weekly Intake. The obtained PWI values and the HQ for evaluated metals are shown in Table 2. The results revealed that the obtained PWI values for manganese in bark (6171.12 μg week$-$1), leaves (11950.41 μg week-1), and roots were 9371.62 μg week-1. The HQ values for all evaluated parts of the plant were below the toxic limit when HQ < 1. The detected (PWI) values of copper were 5259.83 μg week-1 for bark, 5027.51 μg week-1 for leaves, and 5667.54 μg week-1 for roots with HQ < 1 for bark (0.02) and leaves (0.02) and 0.023 for roots. The highest detected (PWI) value of nickel was 165.67 μg week-1 for leaves, followed by roots (152.75 μg week-1) and 115.32 μg week-1 for bark. The HQ values for bark were (0.02), leave (0.02), and roots (0.02). The quantified HQ values of lead were HQ < 1 for bark (0.75), leaves (0.87), and roots (0.76), and the obtained PWI values were 1306.20 μg week-1 in bark (1528.31 μg week-1) in leaves and 1330.12 μg week-1 in roots. As iron is not considered to have any harmful health effect, except when taken at extremely large doses, the HQ values for iron in all evaluated plant parts in the present study are in safe limits. Although it is unlikely to reach the toxic amount by taking the plant in raw form, their use, along with other iron sources, must be checked not to cause chronic intoxications.

Table 1: Daily metal intake (μg day-1) for an adult weighing 70 kg from bark, leaves, and roots of *Berberis baluchistanica*.

Heavy metals	DMI from bark (μg day-1 person-1)	DMI from leaves (μg day-1 person-1)	DMI from roots (μg day-1 person-1)
Mn	881.61	1707.21	1339.80
Cu	751.22	718.41	810.32
Ni	16.51	23.71	21.82
Pb	186.61	218.32	190.24
Fe	84578.61	278202.43	259050.52
K	446349.02	396138.50	333426.01
Na	12274.51	38197.70	23346.64

DMI: daily metal intake.

Table 2: Results of PWI (μg week-1) and health risk assessments expressed through (HQ).

Heavy metals	PWI bark (μg week-1)	HQ	PWI leaves (μg week-1)	HQ	PWI roots (μg week-1)	HQ
Mn	6171.12	0.09	11950.41	0.17	9371.62	0.14
Cu	5259.83	0.02	5027.51	0.02	5667.54	0.02
Ni	115.32	0.02	165.67	0.02	152.75	0.02
Pb	1306.20	0.74	1528.31	0.87	1330.12	0.76
Fe	592050.31	1.51	1947414.43	4.96	1813350.34	4.63

PWI: provision for weekly intake; HQ: hazard quotient.

Manganese is good antioxidant and an important element for plant and animal growth. Its deficiency causes reproductive problems in mammals, and excessive amount leads to different lungs and brain diseases [21]. The obtained concentrations of manganese in bark were $22 \pm 1\ \mu g\ g - 1$, and those in leaves ($42.7 \pm 1\ \mu g\ g - 1$) and roots were $33.5 \pm 1\ \mu g\ g - 1$. The permissible limit of Mn is 20 mg kg−1 for edible plants, the acceptable dietary intake of manganese is 2-5 mg day−1 [22], and the upper limit for manganese is 11 mg day−1. Based on the estimated PMTDI for Mn (140 μg kg−1 bw day−1), which corresponds to 980 μg kg−1 bw week−1 and 68600 μg or 68.6 mg week−1 for an adult weighing 70 kg are considered to be suitable for human adults, as shown in Table 3. The results are lower than those previously reported in the literature [23, 24] and agree with the results documented by other researchers in medicinal plants [25].

Copper is an essential element necessary for plant growth and development. It plays an important role in metabolic processes and regulates alkaloid accumulation in medicinal plants. It is used as an ailment for wound inflammation and chest and arthritis [29]. However, severe exposure to high levels of copper is associated with inflammation of brain tissues, hair loss, allergies, depression, and liver and renal damage [30]. According to Mishra et al. [4], the Cu concentration varies between 2 and 20 mgL–1, the permissible limit of copper in plants is 10 mg kg−1, and the dietary intake for an average human body is 2–3 mg day−1. The FAO/WHO has established a PTWI of 3.5 mg kg−1 body weight 3.5 mg kg−1 week−1 for an adult weighing 70 kg which corresponds to 245 mg week−1 [31]. The concentrations of copper in bark, leaves, and roots were $21 \pm 1\ \mu g\ g - 1$, $20.1 \pm 0.63\ \mu g\ g - 1$, and $22.7 \pm 0.7\ \mu g\ g - 1$, respectively, and the order of concentration was roots > bark > leaves. The obtained results are below the lower limit, as shown in Table 3. The copper concentrations in all samples are recommended for human adults. Comparing our results, Cu was found to be lower in concentration than previously reported [24]. The copper concentrations in all parts (Supplementary Table 1) were below the PTWI and HQ < 1. According to Dos Santos et al. [20], copper requirements for an adult are 900 μg day−1; therefore, the medicinal plants studied cannot be considered a source of this element.

Table 3: ADDIs (mg day−1) by a person weighing 70 kg [26, 27] and US RDA (mg day−1) [28].

Element	ADDIs (mg day−1)	US RDA (mg day−1)
Fe	15 (10–28)	10–18
Mn	2.8 (2–5)	1.0–5.0
Cu	2.5 (2–3)	1.0–3.0
Ni	0.025	0.13–0.4
Pb	0.415	***
Na	***	1500-2300
K	3500–4700	3500–4700

ADDIs: average daily dietary intake; US RDA: recommended daily dietary allowance; ***: data not available.

Nickel is an important element that controls several metabolic processes in plants. It is required for the insulin production. Its deficiency leads to cardiac function, loss of body weight, and liver problems. However, nickel is toxic at higher concentrations and shows carcinogenic side effects when consumed in high quantities [32]. High concentrations of nickel cause severe chlorosis and necrosis and anatomical changes in plants. Nickel has been reported to

cause allergic problems in humans and is adversely harmful to the lungs and nasal cavities [15]. The estimated PTWI [31] for Ni is 15 μg kg−1 day−1 for humans, which corresponds to 105 μg kg−1 bw week−1 and 7350 μg week−1 for adults weighing 70 kg. The obtained concentrations of nickel (46.08 ± 0.28 μg g − 1 in bark, 66.23 ± 0.33 μg g − 1 in leaves, and 61.1 ± 1 μg g − 1 in roots) and the PWI for bark (115.32), leaves (165.67), and roots (152.75 μg week−1) were below the provisional tolerable weekly intake and HQ < 1, as presented in Table 2.

Lead is a nonessential heavy metal. The use of Pb makes it unique regarding environmental toxicity, which is why it is very important to study heavy metals such as Pb [33]. Its deposition in the soft tissues, renal, immune, and nervous systems of the body affects its functions. The safe limit of lead for human use is 1.5 mgL−1, while in medicinal plants, the permissible limit is 10 mgL−1 [25]. It was noted that the highest Pb concentration was determined in leaves (36.8 ± 1.2 μg g − 1), followed by 32.1 ± 1.03 μg g − 1 in roots and 31.5 ± 1 μg g − 1 in bark of the plant. The presence of a relatively higher concentration of Pb in leaves may be due to airborne lead. According to the FAO/WHO, the PTWI is 25 μg lead kg−1 or 0.025 mg kg−1 body weight for humans, which is 1.5 mg week−1 [31]. There is no upper limit set for lead yet [20]; however, the obtained Pb concentrations detected in all samples were below the permissible limits set by the WHO, and HQ < 1 shows noncarcinogenic chronic damage to human health. The obtained concentration of this element was relatively lower than those found in the literature [34]. The daily dietary intake of lead for a person weighing 70 kg was approximately 0.42 mg (Table 3), and all parts had concentrations below the safe limits.

Iron is one of the most important elements known to produce red blood cells in the human body. Fe is an essential trace element (micronutrient) for living organisms. Its deficiency causes anemia, and its high amount damages tissues in the human body. Generally, iron is not considered to have harmful health effects, except when taken at extremely large doses. The acceptable limit of iron in plants is 20 mg kg−1, while it is 10 to 28 mg day−1 for human consumption [25]. The JECFA established a provisional maximum tolerable daily intake (PMTDI) of 0.8 mg/kg of body weight. The prescribed PMTDI value corresponds to 5.6 mg kg−1 bw week −1, as 392 mg week−1 for an adult weighing 70 kg. The results obtained in the present work were 394.7 ± 0.29 μg g − 1 in bark, 1298.3 ± 0.54 μg g − 1 in leaves, and 1208.9 ± 0.71 μg g − 1 in roots. The metal concentration was higher in leaves than in roots and bark. The obtained results are in agreement with previously reported data [35, 36]. The Fe concentration suggests the possible use of this medicinal plant to compensate for iron deficiency.

Sodium is an essential element that regulates fluid balance and proper functioning of muscles and nerves. Its deficiency causes mood swing, dehydration, and hair loss. According to the results, the highest concentration (1782.6 ± 0.1 μg g − 1) of Na was present in leaves, followed by 1089.5 ± 0.7 μg g − 1 in roots, and a lower concentration (572.8 ± 0.6 μg g − 1) was present in bark. The concentration of Na in *Berberis baluchistanica* was found to be in the order of leaves > roots > bark. The adequate intake range is 1500-2300 mg day−1 for an adult with 70 kg body weight [37]. The DMI value of Na in bark was 12247.51 μg kg − l, which was lower than that in leaves (38197.70 μg kg − l) and roots (23346.64 μg kg − l). The average concentration of Na present in plants is reasonably high, making this plant medicinally important. However, the obtained results showed that Na was low enough, indicating a satisfactory concentration of this element, as reported in other medicinal plants [15, 23, 38, 39].

Potassium is one of the well-known essential elements that regulate the responses of the immune system and hormones. It acts as an activator of some enzymes and works in the secretion of insulin and is involved in lowering blood pressure and kidney problems. The average concentration of K was 8927 ± 0.3 μg g − 1 in bark, 7922.8 ± 0.4 μg g − 1 in leaves, and 6668.5 ± 1 μg g − 1 in roots. The order of concentration was bark > leaves > roots, and the adequate intake range is 3500–4700 mg day−1 for an adult with 70 kg body weight. The DMI value of K in bark was 446349.02 μg kg − l, which was higher than that in leaves (396138.50 μg kg − l) and roots (333426.01 μg kg − l). Recently, Uddin et al. [9] reported that the K concentration (0.74%) from *Berberis baluchistanica* stems by energy dispersion spectroscopy was lower than the present result. The obtained results were relatively higher than those found in the literature [15, 40]. However, the results were low enough compared with those previously reported [38, 39]. There is neither any upper tolerable level set for potassium ingestion nor any reference values for weekly or monthly intake [20]. In healthy populations, excess potassium is excreted through the kidneys [41]. Therefore, the potassium amount in medicinal plants is unlikely to cause any side effects.

3.3. Principal Component Analysis. The PCA was used to determine the multidimensional overview of a multivariate data, which changes a bunch of factors, related to one another, in other uncorrelated, free, and symmetrical, principal components (PC) that are the outcome of direct blends of the first factors. The purpose is to regulate the components that clarify however much as could reasonably be expected of the information complete variety, with the smallest probable number of extents. They are designed in descending order of significance, i.e., the PC1 depicts the variation in the data, and the second, since it is symmetrical, clarifies a significant part of the remaining variation, to such an extent that the last component will be the one that gives minimal contribution to the clarification of the complete variance of the actual data [42].

The quantified elements (Mn, Cu, Pb, Ni, Fe, Na, and K) were used as variables in the PCA. By extracting the PCs with significant eigenvalues greater than 1, two principal components were produced. Analyzing the eigenvalues, it is observed that it is possible to consider only two principal components, because from this value, there are no major changes in the accumulated variance. PC1 with highest eigenvalue of 4.93 was explaining the maximum variability in the obtained set of data followed by PC2 2.07. These principal components contributed for 100% of the total variance.

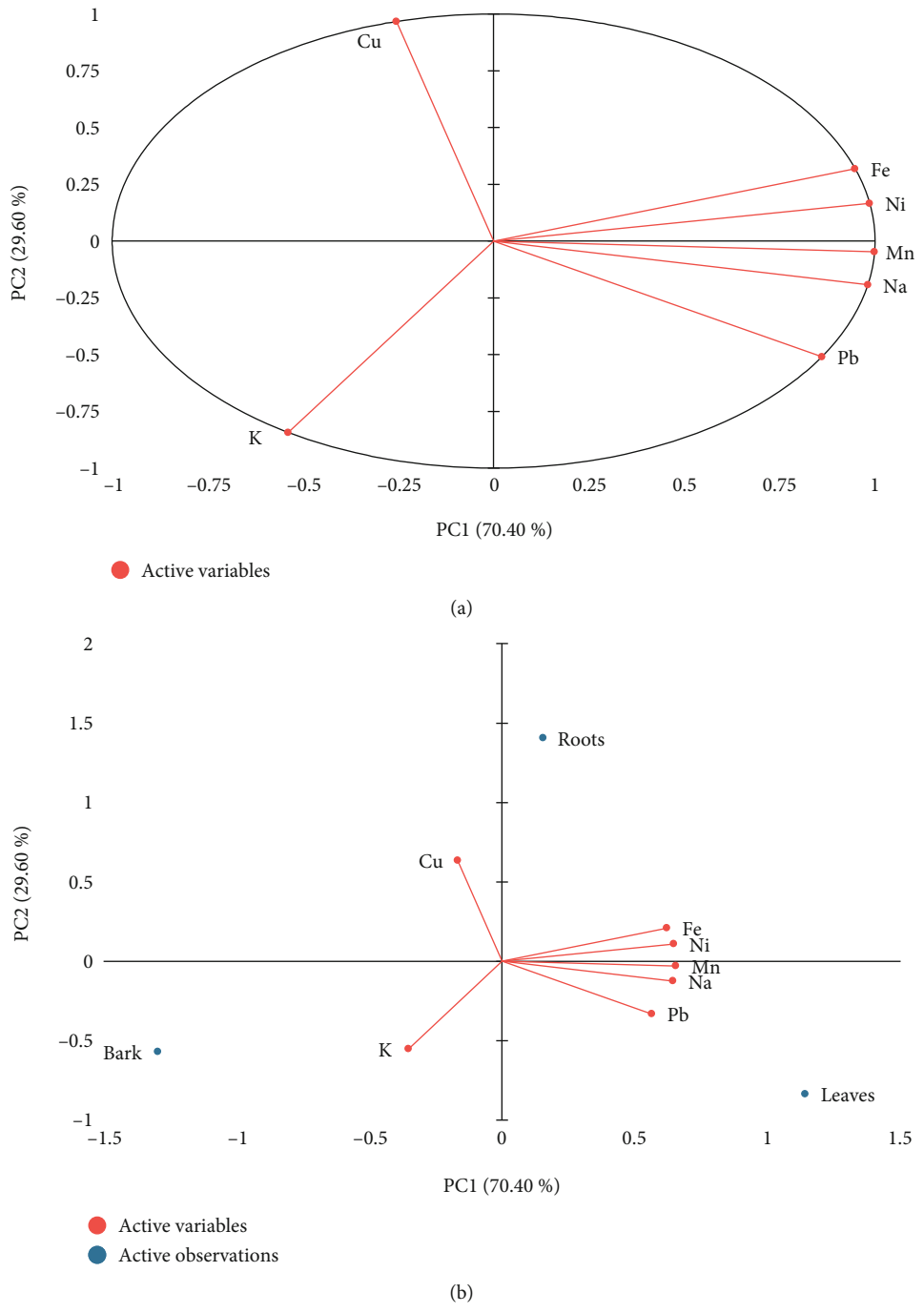

Figure 2: (a, b) Biplot diagrams of the original variables ordering and the scores in the first two principal components 1 and 2.

The first and second components showed a variance of 70.4% and 29.6%, respectively. PCA biplot in Figure 2 shows the variability between the elements, independent variables, and the investigated dependent variables. PCA variable loadings showed higher percentage contributions (Supplementary Table 2) and a positive correlation on PC1 with Mn, Pb, Ni, Fe, and Na, and negative ones with Cu and K. PC2 was positively correlated with Cu and negative ones with K which the eigenvectors gained the negative signal in PC1. The correlation between the original variables and PC1 is also negative, indicating inverse quantities acting in contrast, that is, the higher the value of Mn, Ni, Na, Fe, and Pb, the greater the contributions of these variables in the PC1, and consequently, the lower the values of the principal component from the linear combination of the original variables. From PCA biplot case score, it is evident that leaves have the highest concentration of Mn, Na, and Pb. Roots showed higher amount of Fe, Ni, and Mn. From the PC2 scores, it is evident that bark has the highest concentration of K, respectively. The overall PCA results showed that relatively higher concentration of Mn, Pb, Ni, Fe, and Na distinguished leaves from bark

and roots. PCA, further, revealed that the elemental composition of leaves is a potential outlier due to its accumulation affinity for these elements.

4. Conclusion

The concentrations of seven essential and nonessential elements were estimated in the bark, leaves, and roots of the plants, among which the concentrations of potassium and sodium were found to be higher in all three parts. The concentrations of toxic and heavy metals detected were within the permissible values established for the PMTDI and PTWI limits. All studied parts had elemental concentrations below the upper limit, with HQ values below one. Based on the presence of different elements, it has been calculated that a 2-5 g day−1 dose of raw *Berberis baluchistanica powder* is a safe dose for an adult to be used. Therefore, the use of this plant must not be exceeded to this safe range. The plant is nontoxic to use, and the medicinal qualities of the plant can be linked to their rich amounts of essential elements. The results obtained in the present study will support the safe use of all parts of the plant in traditional medicines for curing various diseases.

Data Availability

The data will be available on reasonable request.

Additional Points

Statement regarding Plant Collection. The plant collection and use were in accordance with all the relevant guidelines.

Consent

All the authors agree to publish the article in this journal.

Conflicts of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Supplementary Materials

Supplementary Table 1: concentration (μg/g) of various elements in different parts of *Berberis baluchistanica*. Supplementary Table 2: standardized eigenvectors, correlation matrix, and the percentage contribution of each original variable in the principal components. *(Supplementary Materials)*

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Research Article

The Aerial Parts of *Bupleurum Chinense* DC. Aromatic Oil Attenuate Kainic Acid-Induced Epilepsy-Like Behavior and Its Potential Mechanisms

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The aerial parts of *Bupleurum Chinense* DC. aromatic oil (BAO) were a well-known Chinese herbal medicine plant extract used to treat epilepsy. This study aimed to explore the therapeutic effect of BAO on kainic acid- (KA-) induced epileptic rats and the possible mechanism of its antiepileptic effect. The composition and content of BAO were analyzed by GC-MS, and BAO was administered orally to alleviate the epileptic behavior induced by KA brain injection. The behavior of epileptic rats was determined by Racine grading criteria. And hematoxylin-eosin staining (HE), Nissl staining, immunohistochemistry, Elisa, Western blot, and other methods were used to study the antiepileptic mechanism of BAO, and the possible mechanism was verified by the epileptic cell model of hippocampal neurons induced by the low-Mg^{2+} extracellular fluid. BAO was mainly composed of terpenoids and aliphatic compounds. And BAO could improve KA-induced epilepsy-like behavior, neuroinflammation, and neurotransmitter abnormalities in the hippocampus. Furthermore, BAO could regulate the expression of GABA, NMDAR1, Notch1, and MAP2 to improve the symptoms of epilepsy. These results were also validated at the cellular level. These results indicated that BAO could alleviate the epilepsy-like behavior through the action of the Notch/NMDAR/GABA pathway.

1. Introduction

Statistical data showed that the prevalence rate of epilepsy in China was 7.0‰. According to this estimate, there were about 9 million epilepsy patients in China, of which about 25% were refractory epilepsy, such patients could not effectively control their seizures with drugs. Among them, temporal lobe epilepsy (TLE) patients account for about 70% [1]. Although modern antiepileptic drugs (AEDs) could control about 82% of patients with seizures, there were still 1/5 patients with recurrent seizures, and because patients with epilepsy need long-term or even lifelong medication treatment, the toxic side effects of chemosynthetic drugs were particularly prominent [2]. The existing first-line AEDs almost had potentially adverse effects on the behavior and cognitive function of patients with epilepsy. Therefore, it was particularly important to explore the prevention and treatment ideas and find safer and more efficient therapies and drugs with less toxic side effects and definite curative effects. At this time, Chinese herbal medicine had attracted much attention because of its unique curative effect.

The occurrence of epilepsy was the result of the imbalance of excitation-inhibition in the brain. As the most representative inhibitory neurotransmitter in the central nervous system, the change of GABA extracellular concentration played a pivotal role in the process of neuron excitability change and abnormal discharge. However, as a rate-limiting enzyme of GABA synthesis, glutamate decarboxylase (GAD) had been

paid more and more attention to its relationship with epilepsy [3].

B. chinense, belonging to the plant family *Bupleurum* spp., was a well-known traditional Chinese medicine (TCM) that had been used for more than a thousand years, distributed mainly in Heilongjiang, Liaoning, Jilin, and Neimeng province of China [4]. It was reported that the *B. chinense* and their aerial parts had similar chemical components and pharmacological effects have a good therapeutic effect on epilepsy. In addition, some studies showed that the aerial parts of *B. chinense* had an obvious antiepileptic effect, and the curative effect was better than that of *B. chinense* [5]. Some kinds of literature have revealed that a variety of volatile oils could protect and repair GABA neurons, improve the synthesis and extracellular release of GABA transmitters in GABA neurons, reduce the reabsorption and degradation of GABA, increase the GABA content in the whole brain tissue, reduce the excitability, and reduce their excitotoxicity to nerve tissue through inhibitory postsynaptic potential, to inhibit the abnormal discharge of epilepsy and play an antiepileptic role [6].

Therefore, this study aimed to explore the effect of the aerial parts of *B. chinense* aromatic oil (BAO) on KA-induced epileptic behavior in rats through GABA regulation.

2. Materials and Methods

2.1. Chemicals. Kainic acid was obtained from Sigma-Aldrich (Shanghai, China); sodium valproate oral solution was obtained from Sanofi (Hangzhou, China); and Dian Xian Ning tablet was purchased from Kunming Chinese Medicine Factory Co. Ltd. (Kunming, China). And all other analytical reagents were purchased from Sigma-Aldrich (Shanghai, China).

2.2. Plant Extract. The aerial parts of *B. chinense* were collected in Daqing, Heilongjiang Province, China, in 2017 and identified as *Bupleurum Chinese* DC by Professor Rui-Feng Fan from Heilongjiang University of Traditional Chinese Medicine. The aerial parts were stored in the laboratory of Chinese Medicine Chemistry of the Heilongjiang University of Chinese Medicine with specimen number 20170910.

BAO was obtained by steam distillation. The BAO sample was diluted with anhydrous ether, dried with a little anhydrous sodium sulfate and identified by GC-MS (Agilent, USA). The gas chromatographic column was a quartz capillary column (30 m ×0.25 mm ×0.25 μm) with N_2 carrier gas. The column flow rate was 1 mL/min; the shunt ratio was 20: 1. The inlet temperature was 300°C, and the temperature rise rate was 60°C/min, 10°C/min to 160°C, 2°C/min to 210°C, 10°C/min to 300°C. For mass spectrometry, the carrier gas was He, ionization mode is EI, electron energy is 70 eV, ion source temperature is 20°C, scanning speed was 1 s, and scanning range is 33~550 m/z. Anhydrous ether was diluted and injected at 0.6 μL.

2.3. Animals. 70 specific pathogen-free male Sprague-Dawley rats, weighing 180~200 g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (SCXK (Liao) 2020-0001). Experimental animals were raised under standardized conditions, the temperature was 20~23°C and the humidity was 50% ~60%. All experiments were approved by the Animal Experiment Committee of Heilongjiang University of Chinese Medicine (20201230001).

2.4. Primary Cell Culture. Rat hippocampal neurons from 1 to 3 days old SD rats were cultured in neuronal culture medium containing 98% neurobasal medium (Gibco, Carlsbad, CA), 2% B27 supplement (Gibco, Carlsbad, CA) at 37°C under 5% CO_2. The low-Mg^{2+} extracellular fluid (ECF) was configured according to previous methods [7]. The neurons were cultured in the low-Mg2+ ECF (2 mL per well) at 37°C under 5% CO2. After 3 h, the neurons were rinsed twice with a neuronal culture medium and then cultured in a neuronal culture medium with VAP (1 mM) and BAO (50, 100, 200 μg/mL) at 2 mL per well. After 24 h, the cell viability was evaluated by CCK-8 kit assay [7].

2.5. Experimental Design. After 60 rats were anesthetized, the lateral ventricle was stereo-positioned, and a custom-made microinjection tube was used to lower a needle of 3.8 mm into the right lateral ventricle with a uniform injection of 3 μ of KA solution at a concentration of 0.5 μg/μL (50 rats) or 3 μ normal saline (10 rats). After the injection, the needle was retained for 5 min to establish an animal model of epilepsy caused by KA [8].

50 successful male SD rats were randomly divided into 5 groups with 10 rats in each group, model group, western medicine positive drug group (sodium valproate oral VAP, 180 mg/kg, b.w., po.), traditional Chinese medicine positive drug (Dianxianning tablets, DXN, 500 mg/kg, b.w., po.), BAO high-dose group (BAOH, 200 mg/kg, b.w., po.), and BAO low-dose group (BAOL, 50 mg/kg, b.w., po.).

2.6. Immunohistochemistry. Brain slices were immersed in 0.1 mol/L phosphate buffer (PBS) containing 0.5% Triton X-100 for 60 min and 0.6% H_2O_2 ethanol saline solution (50% ethanol and 0.9% sodium chloride) for 45 min. Normal sheep serum was incubated for 60 min, poured out without washing, and dripping with rabbit-derived glial fibrillary acidic protein (GFAP) polyclonal antibody (Abcolonal, Wuhan, China, 1 : 500), and incubated at 4°C for 36~48 h. Biotinylated secondary antibody (sheep antirabbit) was added dropwise and incubated at room temperature for 2 h. Streptavidin labeled with horseradish enzyme was added dropwise and incubated at room temperature for 2 h. Between each step, it was washed with 0.01 mol/L PBS 3 times for 10 m each time. After that, it was colored with 3,3'-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated, transparent, and sealed. The fluorescence intensity of the hippocampus was counted at 200× magnification.

2.7. Biochemical Analysis. Total protein was extracted from fresh hippocampal tissues of experimental rats in each group, and caspase3, COX2, TLR4, HMBG1, NMDAR1, GABA AR, GAD65, GAD67, GAFP, and GIRK1 expression were determined. For specific methods, please refer to the

instructions of Shanghai Enzyme-linked Biotechnology Co., Ltd. ELISA kits.

2.8. Western Blot Analysis. Total protein was extracted from fresh and interfered hippocampal tissues of rats in each group. The total protein was separated by polyacrylamide gel electrophoresis with a 20 μg sample amount and then transformed into the membrane. After sealing, the protein was incubated with primary antibody Bcl2, Bax, caspase 3, GAD65, GAD67, GIRK1, GAFP, GABA AR, Notch1, MAP2, and NMDAR1 (1 : 1000, Abcolonal, Wuhan, China) at 4°C overnight. The ECL reagent was incubated at room temperature for 1 h with an appropriate dilution concentration of corresponding HRP-labeled secondary antibody (Abcolonal, Wuhan, China). Take GADPH (1 : 1000, Abcolonal, Wuhan, China) as the internal reference. The results were analyzed with the expression level of each control group as the standard and compared with other groups.

2.9. Statistical Analysis. SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The data of the experimental results were expressed by mean ± standard error (X ± SEM), and the data were analyzed by one-way ANOVA. The homogeneity test of variance was performed first. After the test of variance, the difference between the groups was statistically significant. LSD-test was used to compare the difference, and the difference was statistically significant when $P \leq 0.05$.

3. Results

3.1. The Chemical Components of BAO. Compared with the NIST Spectral Library, the aromatic oil is isolated from the aerial parts of *B. chinense* which resulted in the identification of 63 different components, mainly containing terpenoids compounds, detailed in Table 1, and the GC chromatogram of BAO and MS of all major identified components in Supplemental Files Figures S1 and S2.

3.2. BAO Attenuated KA-Induced Epilepsy-like Behavior. Compared with the control group (CON) rats, the model group (MOD) rats developed IV-V convulsive generalized tonic-clonic seizures, forelimb spasms upright, and falls; compared with the model group, the positive drug group (VPA) could significantly improve the symptoms of epilepsy in rats and can significantly prolong the incubation period and duration of convulsions in rats. Compared with the model group, BAOH could improve the symptoms of epilepsy in rats, reduce the incidence of SE% ($P < 0.05$) (Figure 1), and prolong the incubation period and duration of convulsions in model rats. But BAOL did not improve significantly.

3.3. Observation of He Staining and Nissl Staining. Under a light microscope, it was found that BAO could reduce the histological damage caused by KA. Compared with the sham operation group, KA could significantly change the morphology of hippocampal CA3 area of brain tissue: loss of pyramidal cells and Nissl bodies, pyknosis of nuclei, hypertrophy, and massive proliferation of astrocytes and disordered and irregular arrangement of pyramidal cell layers. The pathological changes and neuron loss in the hippocampal CA3 area of brain tissue in the BAOH treatment group are significantly reduced, as shown in Figure 2.

3.4. Observation of Immunohistochemistry. In the CON group and sham group, there were fewer GFAP immunoreactive cells with thin cell bodies and protruding fibers, mainly located outside pyramidal cells and granulosa cell layers. After KA induction, edema appeared, which was manifested as hypertrophy, deep staining, deformation, and disarrangement of the cell body, and the GFAP-positive area increased significantly. However, BAOH could ameliorate the abnormal GFAP-positive area induced by KA ($P < 0.01$; Figure 3). These results suggested that BAO could inhibit hippocampal neuron loss by activating astrocytes, which was consistent with morphological changes.

3.5. Changes in Biochemical Indicators. A large number of previous studies have confirmed that the expression of caspase 3 increases after SE and caspase 3 inhibitors could reduce neuronal apoptosis after SE [9]. caspase 3 was a key protease in the caspase family. It had the characteristic of specifically cutting chromosomal DNA after aspartic acid, which ultimately led to the phasic degradation of DNA [10]. Therefore, caspase 3 was also called killer protease. These results showed a similar conclusion; KA could improve the expression of caspase 3, but treatment with BAO could reduce the caspase 3 expression.

After the seizure, neurons, astrocytes, and microglia quickly released damage-associated molecular patterns (DAMPs). At the same time, neurons and astrocytes had toll-like receptors (toll-like receptors, TLRs) activation, which was considered to be an important process of neuroinflammation caused by epileptic seizures [11]. Through Elisa's analysis, BAO could improve inflammatory protein abnormalities.

At present, neurotransmitters had been known to play an important role in the pathogenesis of epilepsy pain; that was, the excitation and inhibition process in the brain was mediated by various neurotransmitters, GABA and glutamate were the most representative transmitters involved in inhibition and excitation, and their metabolic abnormalities were closely related to the occurrence of epilepsy, while NMDAR was the ionic receptor of Glu [12, 13]. At the same time, studies have shown that there was a GAD65-GABA-GABA AR-G protein-GIRK pathway between synapses. GABA was synthesized by glutamate under the catalytic action of GAD65, released into the synaptic cleft, and bound to the N-terminal of the GABAB receptor which was fused with G protein in the postsynaptic membrane to activate the GABAB receptor [14]. After the activation of the GABAB receptor bound to the corresponding G protein, the GIRK channel opened, K^+ influx increases, and the postsynaptic membrane hyperpolarizes, resulting in postsynaptic inhibition and inhibiting the proliferation of neuronal hyperexcitability. This pathway suggested that GAD65 was related to the effect of GIRK 1 [15]. Our results showed that

Table 1: The chemical components of BAO by GC-MS.

No.	tR (min)	Name	Molecular formulae	Molecular weight g/Mol	Fixed area Proportion (%)
1	14.03	(H) Pyrrole-2-carboxaldehyde	C_5H_5NO	95.10	23.17
2	14.58	(H) Camphene	$C_{10}H_{16}$	136.23	2.36
3	15.39	(H) Bicyclol [3.1.0] hex-2-ene	$C_{10}H_{16}$	136.23	2.35
4	15.55	(H) πpinene	$C_{10}H_{16}$	136.23	13.52
5	15.87	2-pentylfuran	$C_9H_{14}O$	138.21	6.15
6	17.09	1-Methyl-2(-1-methyl)-benzene	C_9H_{11}	119.19	44.34
7	17.26	(R) D-limonene	$C_{10}H_{16}$	136.23	31.72
8	19.45	(H) Camphenol	$C_{10}H_{16}O$	152.23	6.71
9	19.54	(H) 2(10)-pinen-3-one	$C_{10}H_{14}O$	150.22	4.19
10	20.43	2-(2,2,3-Trimethylcyclopent-3-en-1-yl) acetaldehyde	$C_{10}H_{16}O$	152.23	4.89
11	20.93	Pinocarveol	$C_{10}H_{16}O$	152.23	7.65
12	22.07	(H) 3-Cyclohexen-1-ol	$C_7H_{12}O$	112.17	13.91
13	22.20	(R) Benzenemethanol	C_7H_8O	108.14	6.08
14	22.44	(R) 3-Cyclohexen-1-methanol	$C_7H_{12}O$	112.17	9.71
15	22.69	Myrtenal	$C_{10}H_{14}O$	150.22	13.81
16	23.08	3-Cyclohexen-1-ol,5-methylene-6-(1-methylethenyl)-acetate	$C_{12}H_{16}O_2$	192.25	2.53
17	24.11	Carvone	$C_{10}H_{14}O$	150.22	5.94
18	25.25	Phellandral	$C_{10}H_{16}O$	152.23	3.15
19	25.55	Isobornyl acetate	$C_{12}H_{20}O_2$	196.29	9.26
20	28.00	Neryl acetate	$C_{12}H_{20}O_2$	196.29	2.86
21	28.12	Carvyl acetate	$C_{12}H_{18}O_2$	194.27	17.77
22	28.71	Geranyl acetate	$C_{12}H_{20}O_2$	196.29	3.11
23	29.01	(R) Copaene	$C_{10}H_{16}$	136.23	5.21
24	29.35	Cyclohexane	C_6H_{12}	84.16	14.67
25	29.74	Dodecanal	$C_{10}H_{24}O$	184.32	7.93
26	31.50	Nerylacetone	$C_{13}H_{22}O$	194.31	6.16
27	33.00	1,2,4a,5,6,8a-Hexahydro-1-isopropyl-4,7-dimethylnaphthalene	$C_{15}H_{24}$	204.35	25.49
28	33.18	β-Ionone	$C_{13}H_{20}O$	192.30	3.89
29	33.58	Bicyclo [4.4.0] dec-2-ene-4-ol,2-methyl-9-(prop-1-en-3-ol-2-yl)-6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-1,2,4a,5,6,7,8,8a-octahydro-2-naphthalenol	$C_{15}H_{24}O_2$	236.35	5.16
30	33.96	1,2,4a,5,6,8a-Hexahydro-1-isopropyl-4,7-dimethylnaphthalene	$C_{15}H_{24}$	204.35	4.56
31	34.14	1-Methyl-4-(6-methylhepta-1,5-dien-2-yl) cyclohex-1-ene	$C_{15}H_{24}$	204.35	4.40
32	34.36	1-Methyl-4-(1,2,2-trimethylcyclopentyl) benzene	$C_{15}H_{22}$	202.33	10.72
33	34.65	4-Isopropenyl-4,7-dimethyl-1-oxaspiro[2.5]octane	$C_{12}H_{20}O$	180.29	3.83
34	34.96	1,6-Dimethyl-4-isopropyltetralin	$C_{15}H_{22}$	202.33	6.20
35	35.89	(H) π calacorene	$C_{15}H_{22}$	200.32	2.81
36	36.43	Caryophyllene epoxide	$C_{15}H_{24}O$	220.35	22.88
37	37.38	β-Spathulenol	$C_{15}H_{24}O$	220.35	5.27
38	37.56	Tricyclo [5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-2-methylene-6,8,8-trimethyl-tricyclo[5.2.2.0(1,6)]undecan-3-ol	$C_{15}H_{24}O$	220.35	88.77
39	37.76	Isoaromadendrene epoxide	$C_{15}H_{24}O$	220.35	17.44
40	37.88	6-methyl-3-[(2Z)-6-methylhepta-2,5-dien-2-yl]-7-oxabicyclo[4.1.0]heptane	$C_{15}H_{24}O$	220.35	100
41	38.30	Jasmoline I	$C_{21}H_{30}O_3$	330.50	18.61
42	39.03	Schembl21824483	$C_{15}H_{24}O$	220.35	24.81
43	39.58	1-Oxaspiro[2.5]octane,5,5-dimethyl-4-(3-methyl-1,3-butadienyl)-5,5-dimethyl-4-[(1E)-3-methyl-1,3-butadienyl]-1-oxaspiro[2.5] octane	$C_{14}H_{22}O$	206.32	11.21

Table 1: Continued.

No.	tR (min)	Name	Molecular formulae	Molecular weight g/Mol	Fixed area Proportion (%)
44	39.68	Patchoulane	$C_{15}H_{26}$	206.37	6.35
45	39.99	(R) Caryophyllene	$C_{15}H_{24}$	204.35	6.89
46	40.18	3,7,11-Trimethyldodeca-6,10-dien-1-yn-3-ol	$C_{15}H_{24}O$	220.35	5.31
47	40.72	Aromadendrene oxide-(2)	$C_{15}H_{24}O$	220.35	8.85
48	40.93	(H) Caryophyllene oxide	$C_{15}H_{24}O$	220.35	14.58
49	41.03	(7S,9As)-4,4,7,9a-Tetramethyl-1,2,3,6,8,9-hexahydrobenzo [7] annulen-7-ol	$C_{15}H_{26}O$	222.37	20.96
50	41.23	1,2-Bis(ethenyl)-4-propan-2-ylidenecyclohexane	$C_{13}H_{20}$	176.30	12.97
51	41.87	4a,10a-Methanophenanthren-9β-ol,11-syn-bromo-1,2,3,4,4a,9,10,10a-octahydro	$C_{18}H_{24}O_2$	272.38	6.97
52	42.16	Diepicedrene-1-oxide	$C_{15}H_{24}O$	220.35	11.10
53	42.44	7R,8R-8-Hydroxy-4-isopropylidene-7-methylbicyclo [5.3.1] undec-1-ene	$C_{15}H_{24}O$	220.35	27.61
54	43.27	(H) 10,12-Tricosadiynoic acid	$C_{23}H_{38}O_2$	364.50	9.58
55	43.46	(H) Cubenol	$C_{15}H_{26}O$	222.37	6.40
56	44.98	4,6,6-Trimethyl-2-[(1E)-3-methylbuta-1,3-dienyl]-3-oxatricyclo [5.1.0.02,4] octane	$C_{15}H_{22}O$	218.33	8.16
57	46.48	(H) Andrographolide	$C_{20}H_{30}O_5$	350.40	7.45
58	49.34	6,10,14-Trimethylpentadecan-2-one	$C_{18}H_{36}O$	268.50	29.74
59	49.45	Boronal	$C_{14}H_{22}O$	206.32	5.41
60	52.84	Farnesylacetone	$C_{18}H_{30}O_5$	262.40	6.55
61	60.97	2(3H)-Furanone, dihydro-5-undecyl	$C_{15}H_{28}O_2$	240.38	4.51
62	67.61	(R) Heptacosane	$C_{27}H_{56}$	380.70	2.98
63	70.89	1-(4-Bromobutyl)-2-piperidinone	$C_9H_{16}BrNO$	234.13	3.58

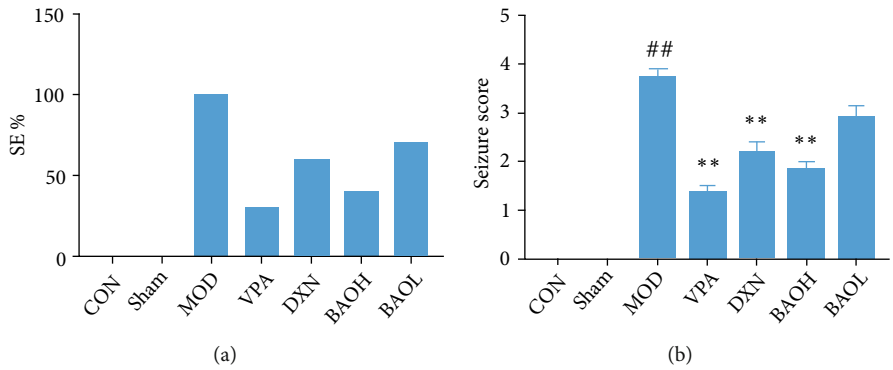

Figure 1: Effect on improving the symptom of SE in rats with epilepsy KA-induced. (a) SE% of epilepsy rats after treatment. (b) Seizure core of epilepsy rats after treatment. Compared with CON and sham groups, the difference was significant (#$P < 0.05$). Compared with the MOD group, the difference was significant ($*P < 0.05$).

BAO could improve the inhibitory GABA neurotransmission abnormalities (Figure 4).

3.6. BAO through GABA Regulation Attenuated KA-Induced Epilepsy-like Behavior. The neuronal hyperexcitability caused by the combination of KA and its receptor might be the main cause of neuronal death. KA could decrease the expression of Bcl2 and increase the expressions of Bax and caspase 3. Meanwhile, BAO could reduce neuronal cell apoptosis by improving Bcl2/Bax/caspase 3 pathway.

At present, it was known that neurotransmitters play an important role in the pathogenesis of epilepsy and pain; that was, the process of excitation and inhibition in the brain is mediated by various neurotransmitters. KA could increase the expressions of GAD65, GAD67, GIRK1, and GFAP and decrease GABA AR expression. Meanwhile, BAO could regulate the GABA signal through GAD/GIRK1 pathway.

Notch 1 knockdown increased the expression of GAD67 in the hippocampus. GAD67 was one of several forms of glutamate decarboxylase, which was responsible

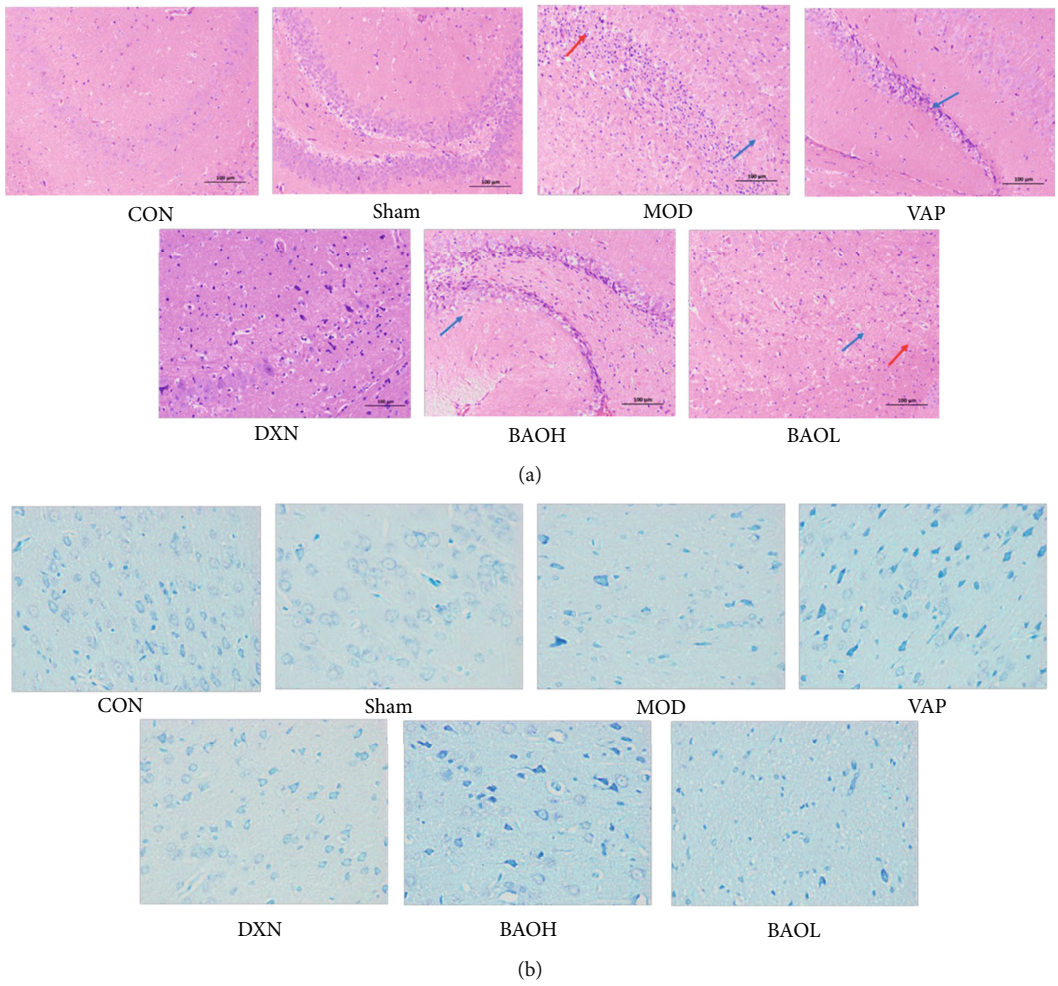

Figure 2: Effect on the pathological changes in KA-induced epilepsy rats with treatment. (a) HE staining in the hippocampus.. (b) Nissl staining in the hippocampus.

for catalyzing glutamate to produce GABA [16]. And Notch could influence NMDAR functions [17]. We could find that BAO could regulate Notch1 through MAP 2 and NMDAR 1 to attenuate the KA-induced epilepsy-like behavior.

3.7. The Effect of BAO on Primary Neuronal Cells. After the addition of low-Mg^{2+} ECF, the cell viability of hippocampal neurons was significantly decreased ($P < 0.01$), indicating the presence of neuron damage in the low-Mg^{2+} ECF-induced refractory epileptic cell model. The cell viability of neurons in BAO concentration groups is significantly increased ($P < 0.01$), and there is a dose-effect relationship, and 200 μg/mL had the most obvious effect, as shown in Figure 5. Results showed that BAO could improve neuronal apoptosis.

3.8. BAO through GABA Regulation Attenuated Hippocampal Neuron Model of Epilepsy. In refractory epilepsy, repeated and prolonged seizures tend to result in selective brain damage, including neuronal necrosis and apoptosis, mainly in the hippocampus. The loss and death of neurons were also observed in the low-Mg^{2+} ECF epileptic cell model. And BAO could regulate the abnormal expression of Bcl2, Bax, and caspase 3.

When the survival environment of neurons was changed, the neurons cultured in vitro could be induced to produce electrical seizures, which was similar to the abnormal electrical activity of neurons in human epilepsy. Mg^{2+} played a very important role in maintaining normal brain function. Studies have shown that Mg^{2+} was an important regulator of NMDAR mediated ion flow, which played a key role in excitatory nerve injury [18]. And Notch1 knockdown increased the expression of GAD67 in the hippocampus. GAD67 was one of several forms of glutamate decarboxylase, which was responsible for catalyzing glutamate to produce GABA [16, 17]. Our results showed that the expressions of Notch 1, MAP 2, NMDAR1, GAD65, GAD 67, and GIRK1 were increased; the expression of GABA AR was decreased in the Mg^{2+}-free-treated neurons, while BAO could improve the abnormal discharge of neurons by regulating Notch1/GABA/GAD/GIRK.

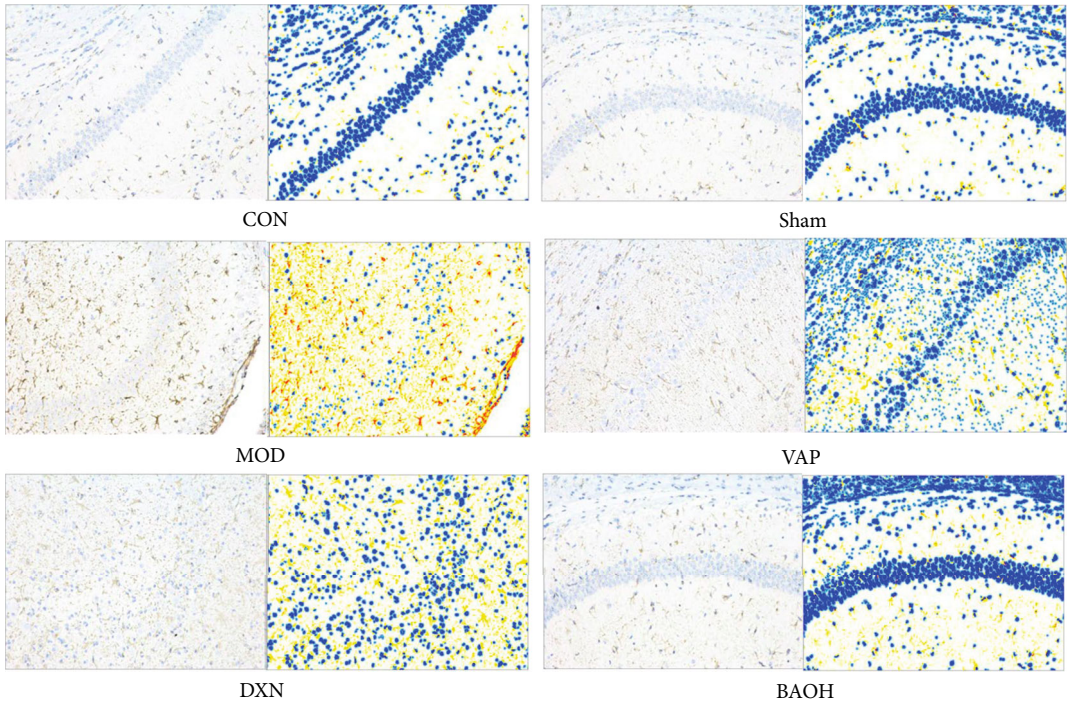

Figure 3: Effect on the expression of GFAP in KA-induced epilepsy rats with treatment.

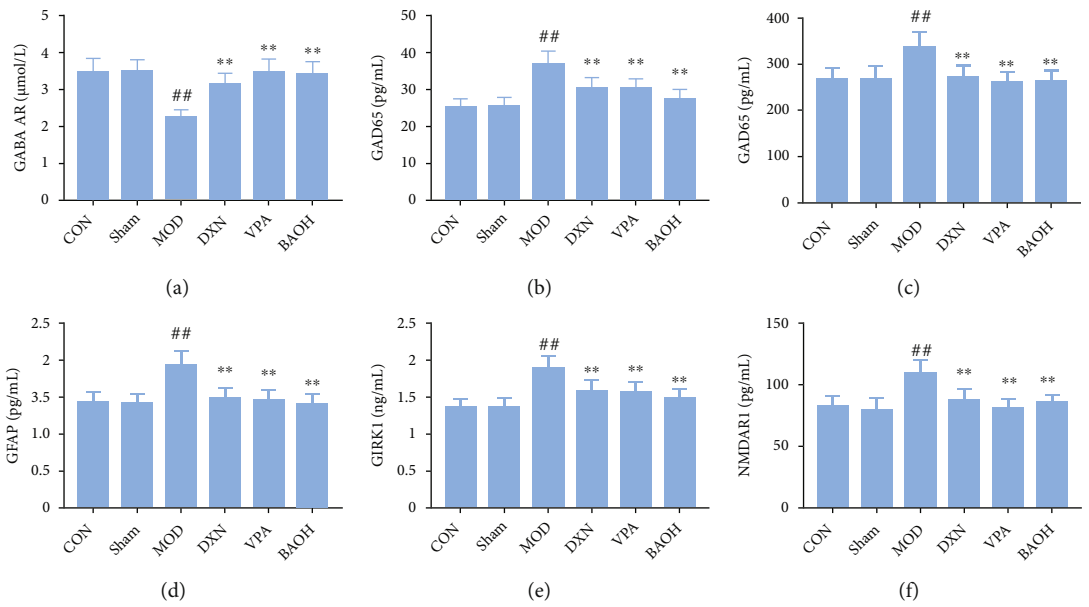

Figure 4: Effect on the changes of neurotransmitter levels in KA-induced epilepsy rats with treatment. (a) The content of GABA AR in KA-induced epilepsy rats with treatment; (b) the content of GAD65 in KA-induced epilepsy rats with treatment; (c) the content of GAD67 in KA-induced epilepsy rats with treatment; (d) the content of GFAP in KA-induced epilepsy rats with treatment; (e) the content of GIRK1 in KA-induced epilepsy rats with treatment; and (f) the content of NMDAR1 in KA-induced epilepsy rats with treatment.

4. Discussion

The aerial parts of *B. Chinense* aromatic oil (BAO) were a well-known Chinese herbal medicine plant extract used to treat epilepsy, and we found that BAO was rich in terpenoids. An increasing body of scientific literature indicates that terpenoids have multiple pharmaceutical functions, including antiepileptic activities [19, 20]. (+)-Borneol, a bicyclic monoterpene, is a common component of essential oils of medicinal herbs. This monoterpene is a positive modulator of human recombinant $\alpha 1\beta 2\gamma 2$L GABAA receptors at low concentrations of GABA [21]. Quintans-

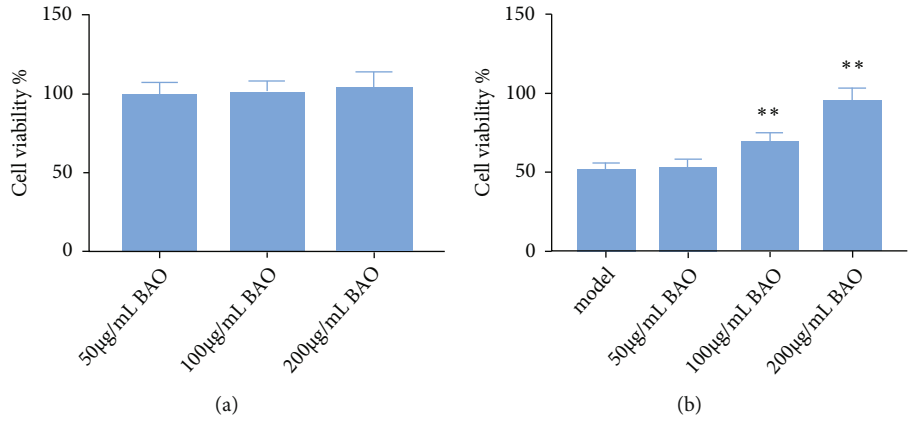

Figure 5: The effect of BAO on hippocampal neuron model of epilepsy. (a) The cell viability of different doses of BAO. (b) The effect of different doses of BAO on hippocampal neuron model of epilepsy. Compared with the model group, the difference was significant ($*P < 0.05$).

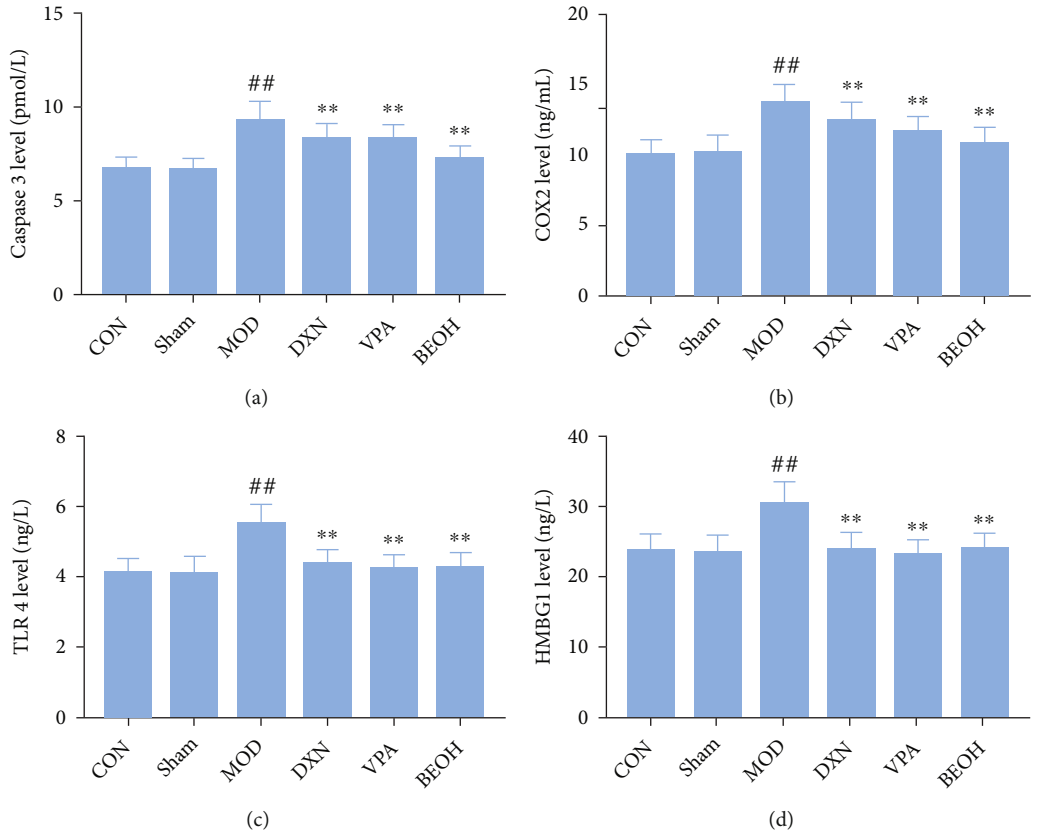

Figure 6: Effect on the expression of apoptosis and inflammatory proteins in KA-induced epilepsy rats with treatment. (a) The content of caspase 3 in KA-induced epilepsy rats with treatment; (b) the content of COX2 in KA-induced epilepsy rats with treatment; (c) the content of TLR 4 in KA-induced epilepsy rats with treatment; and (d) the content of HMBG1 in KA-induced epilepsy rats with treatment.

Júnior, Guimarães [22] revealed that pretreatment with (-)-borneol, carvacrol (phenolic monoterpene), and citral exerted a protective effect in PTZ-and MES-induced convulsions in mice. (-) Borneol and citral were more effective than carvacrol for all doses (50, 100, 200 mg/kg, i.p), resulting in increased time onset of clonic convulsions, while carvacrol was effective only at the highest dose [22]. Antagonism of PTZ-induced convulsions suggests that the terpenoids may have effects on GABAergic neurotransmission. According to NIST Spectral Library identification and analysis, BAO contains all of the above antiepileptic ingredients. Therefore, we hypothesized that BAO might ameliorate the effects of epilepsy by regulating GABA signaling pathway.

Based on BAO's ability to improve epileptic behavior and hippocampal injury, the mechanism of BAO's epileptic treatment was explored (Figures 1–4, 6). In human temporal

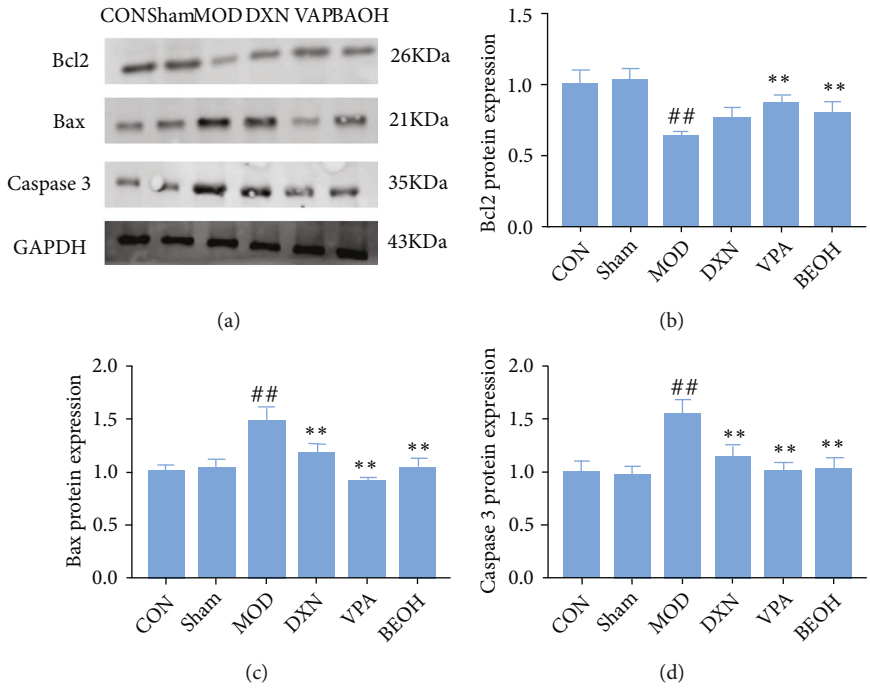

Figure 7: BAO through apoptosis pathway regulation attenuated KA-induced epilepsy-like behavior. (a) Western blotting in KA-induced epilepsy rats with treatment; (b) the expression of Bcl2 in KA-induced epilepsy rats with treatment; (c) the expression of Bax in KA-induced epilepsy rats with treatment; and (d) the expression of caspase 3 in KA-induced epilepsy rats with treatment.

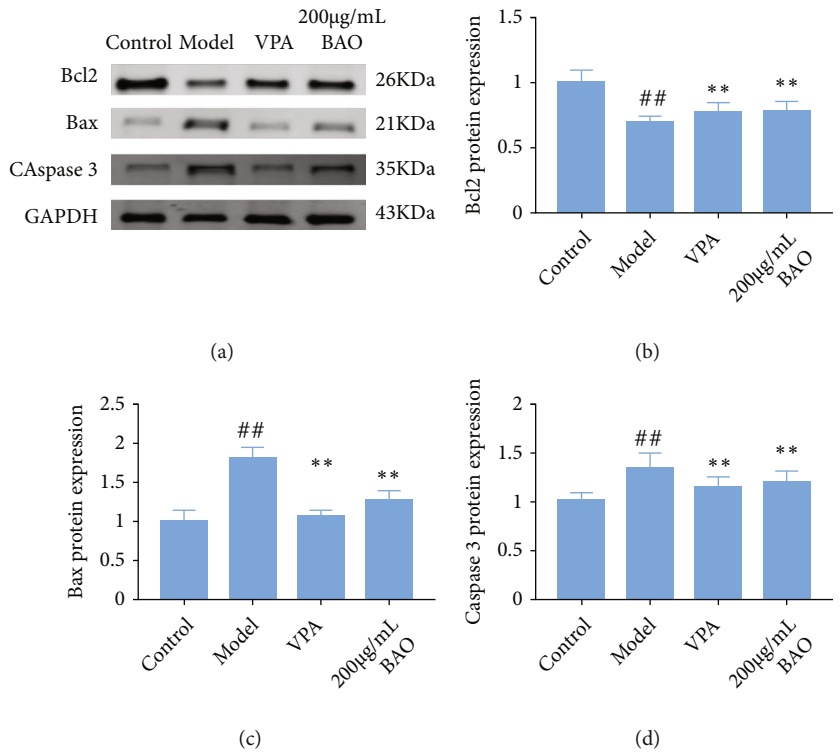

Figure 8: BAO through apoptosis pathway regulation attenuated the hippocampal neuron model of epilepsy. (a) Western blotting in hippocampal neuron model of epilepsy with treatment; (b) the expression of Bcl2 in hippocampal neuron model of epilepsy with treatment; (c) the expression of Bax in hippocampal neuron model of epilepsy with treatment; and (d) the expression of caspase 3 in hippocampal neuron model of epilepsy rats with treatment.

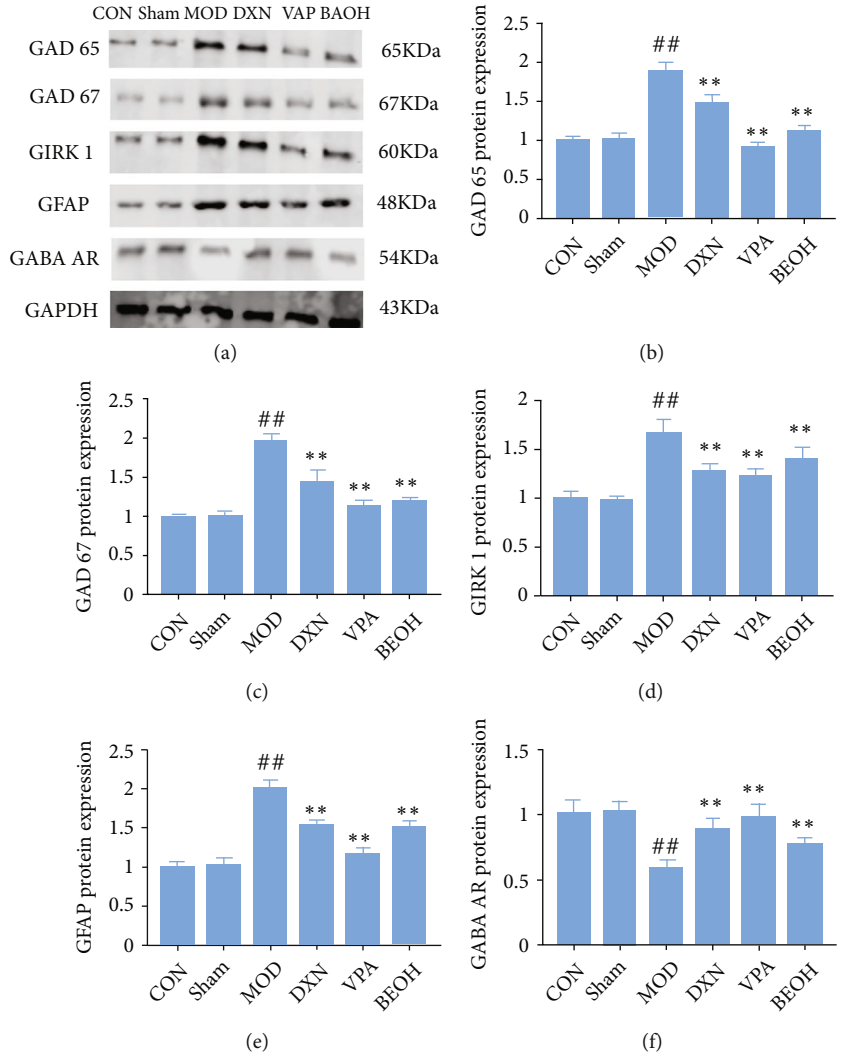

Figure 9: BAO through GABA pathway regulation attenuated KA-induced epilepsy-like behavior. (a) Western blotting in KA-induced epilepsy rats with treatment; (b) the expression ofGAD65 in KA-induced epilepsy rats with treatment; (c) the expression of GAD67 in KA-induced epilepsy rats with treatment; (d) the expression of GIRK1 in KA-induced epilepsy rats with treatment; (e) the expression of GFAP in KA-induced epilepsy rats with treatment; and (f) the expression of GABA AR in KA-induced epilepsy rats with treatment.

lobe epilepsy patients, as well as in various epileptic models such as electric or chemical lighting and local or systematic administration of epileptic agents, post-epileptic neuronal death was typically characterized by apoptosis. At the same time, the Bcl-2 gene family played an important role in the process of cell apoptosis, and caspase 3 was a key protease in mammalian cell apoptosis, located at the core of the apoptotic cascade pathway. Once caspase 3 was activated, cell apoptosis was inevitable [23, 24]. The results showed that the expression levels of caspase 3 and Bax protein in the hippocampus of KA epileptic rats were significantly higher than those of CON and sham groups, while the expression levels of Bcl-2 protein were opposite (Figures 7 and 8), while BAO could inhibit the apoptosis of epileptic neurons by interfering with the abnormal expression of caspase 3, Bax, and Bcl-2 proteins.

Astrocytes were involved in many important physiological and pathological processes in the central nervous system, including epilepsy. GFAP was specifically expressed in astrocytes. It was a characteristic marker of astrocytes. The increased expression of GFAP and the changes in astrocyte morphology suggested astrocyte activation. On the one hand, activated astrocytes protect neurons by secreting various factors; on the other hand, due to the loss of their function of regulating K^+ homeostasis, abnormal neuronal excitation and recurrent seizures would be caused by activated astrocytes. GIRK also played an important role in maintaining cell membrane resting potential and K^+ internal environment stability [25, 26]. In this study, it was found that GFAP-positive expression area and GIRK1 protein expression increased after an epileptic seizure, while GFAP and GIRK1 expression decreased significantly after BAO treatment ($P < 0.01$). These results suggested that BAO might partially inhibit the expression of GFAP, weaken the activation of astrocytes, and regulate K+ homeostasis in epileptic seizures (Figures 9 and 10).

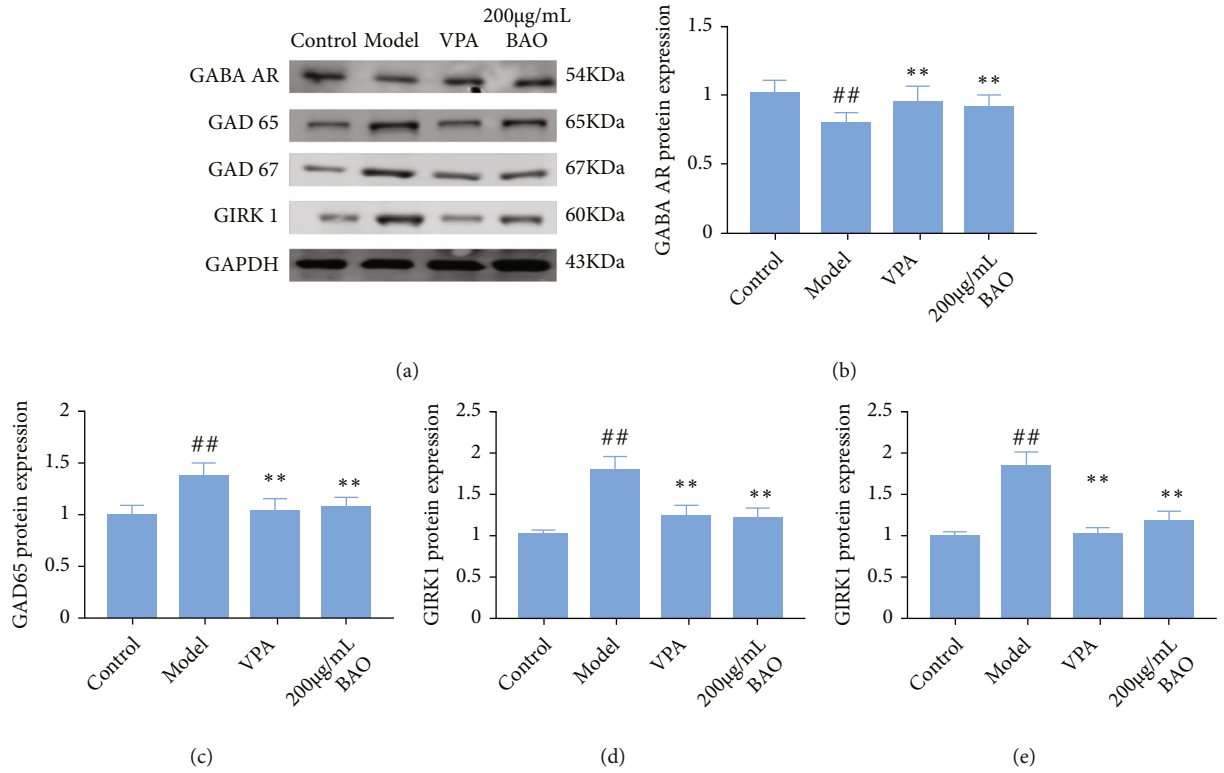

Figure 10: BAO through GABA pathway regulation attenuated the hippocampal neuron model of epilepsy. (a) Western blotting in hippocampal neuron model of epilepsy with treatment; (b) the expression of GABA AR in hippocampal neuron model of epilepsy with treatment; (c) the expression of GAD 65 in hippocampal neuron model of epilepsy with treatment; (d) the expression of GAD67 in hippocampal neuron model of epilepsy rats with treatment; and (f) the expression of GIRK1 in hippocampal neuron model of epilepsy rats with treatment.

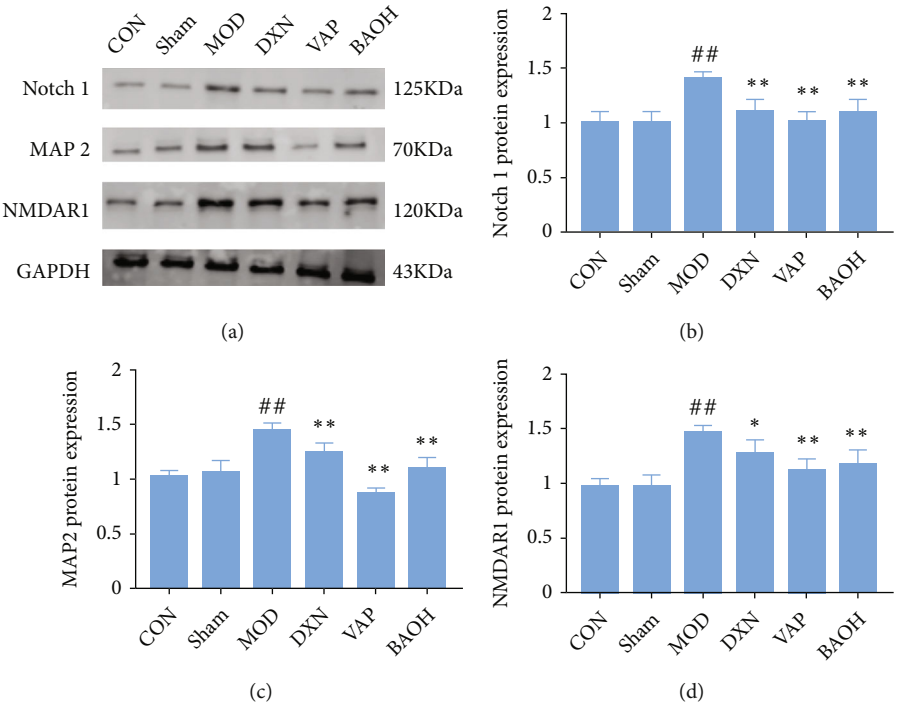

Figure 11: BAO through Notch/NMDAR pathway regulation attenuated KA-induced epilepsy-like behavior. (a) Western blotting in KA-induced epilepsy rats with treatment; (b) the expression of Notch1 in KA-induced epilepsy rats with treatment; (c) the expression of MAP2 in KA-induced epilepsy rats with treatment; and (d) the expression of NMDAR1 in KA-induced epilepsy rats with treatment.

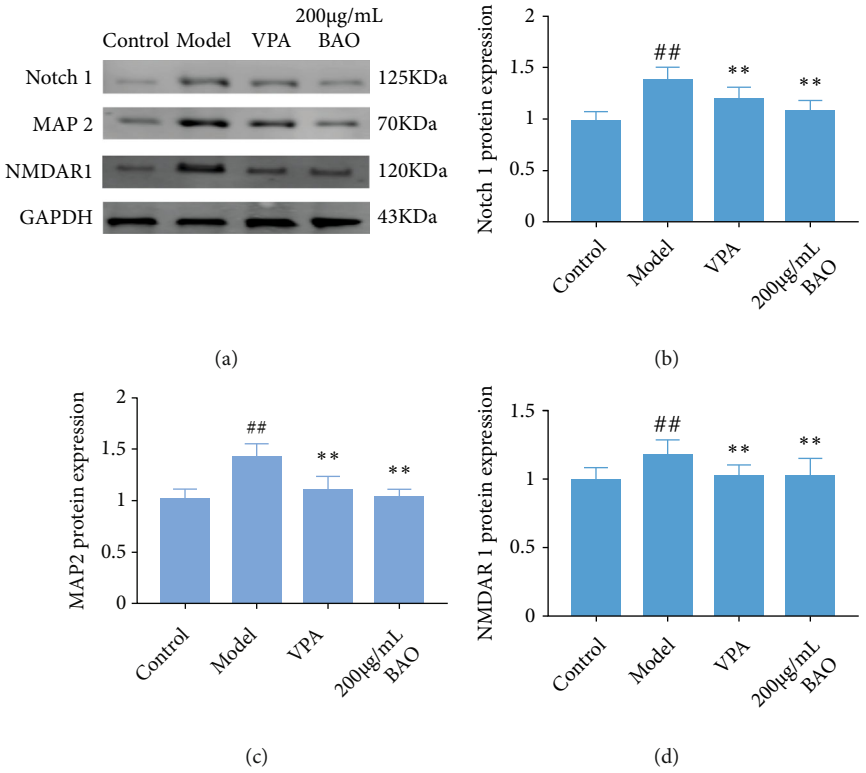

Figure 12: BAO through Notch/NMDAR pathway regulation attenuated hippocampal neuron model of epilepsy. (a) Western blotting in hippocampal neuron model of epilepsy with treatment; (b) the expression of Notch 1 in hippocampal neuron model of epilepsy with treatment; (c) the expression of MAP 2 in hippocampal neuron model of epilepsy with treatment; (d) the expression of NMDAR1 in hippocampal neuron model of epilepsy rats with treatment.

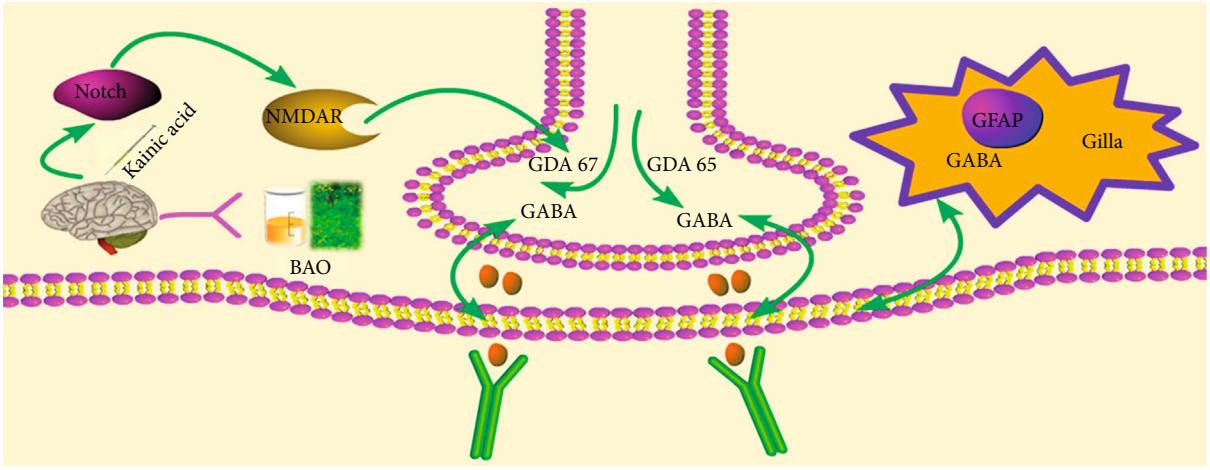

Figure 13: The mechanisms of the aerial parts of *B. chinense* aromatic oil attenuate kainic acid-induced epilepsy-like behavior.

Notch signaling played an important role in TLE neuroinflammation and neuron injury. A large number of studies have shown that Notch signaling was activated in both TLE model rats and people with TLE [27]. Consistent with previous studies, we found that Notch-1 and its downstream target gene MAP2 were significantly higher in the MOD group than in the CON and sham groups, and BAO could down-regulate Notch and MAP2 expression (Figures 11 and 12). Thus, we could speculate that Notch can affect MAP2 expression by regulating TLRs.

There was a lot of evidence that NMDAR was involved in the pathophysiology of some mental diseases, such as schizophrenia, bipolar depression, and drug addiction [28, 29]. Previous studies had found that Notch1 expression was reduced in chronic unpredictable mild stress mouse models. Interestingly, nicotine significantly increased the

expression of this protein, indicating a close relationship between NMDAR and the Notch1 receptor [30]. In addition, in the past decade, NMDAR antagonists had been used to establish schizophrenia models [31, 32], and their compounds were suitable for the treatment of bipolar depression [33]. Abnormal protein expression in neurological and psychiatric diseases was related not only to excitatory proteins but also to inhibitory proteins. For example, abnormal frontal GABA function in patients with schizophrenia was significantly associated with gamma oscillation [34]. From the perspective of neuronal oscillation, it was generally believed that the generation and modulation of theta and gamma oscillations are closely related to glutamate and GABAergic neurons [35, 36]. The knockdown of Notch1 increased the expression of GAD67 in the hippocampus, one of several forms of glutamate decarboxylase, which was responsible for catalyzing glutamate to produce GABA [16, 17]. Our results showed that BAO could regulate the Notch/NMDAR/GABA pathway to attenuate kainic acid-induced epilepsy-like behavior (Figure 13).

This study has been clear about the BAO antiepileptic active ingredients but did not reveal what compounds are significantly active ingredients in the treatment of epilepsy. The antiepileptic activities of various terpenoids from BAO will be further investigated in the following studies; researching and developing activity significantly on natural products lay the foundation for the treatment of epilepsy, at the same time provide a basis for the research of antiepileptic drugs.

5. Conclusions

In conclusion, the main active components of BAO are terpenoids, which can alleviate KA-induced epileptic behavior and play a therapeutic role in epilepsy by regulating Notch/NMDAR/GABA signaling pathway.

Abbreviations

KA: Kainic acid
GC-MS: Gas chromatography-mass spectrometry
GABA: γ-Aminobutyric acid type a
COX2: Cyclooxygenase2
TLR4: Toll-like receptor 4
HMBG1: High mobility group box 1
GAD: Glutamic acid decarboxylase
GFAP: Glial fibrillary acidic protein
GIRK: G protein gated inwardly rectifying K channels
NMDAR: N-methyl-D-aspartic acid receptor
MAP: Microtubule-associated protein
TLE: Temporal lobe epilepsy
AEDs: Antiepileptic drugs
TCM: Traditional Chinese medicine
FID: Flame ionization detection
CCK-8: Cell counting kit-8
DAB: 3,3'-Diaminobenzidine tetrahydrochloride
HE: Hematoxylin-eosin
ELISA: Enzyme-linked immunosorbent assay
BAO: The aerial parts of *B. chinense* aromatic oil.

Data Availability

The data used to support the findings of this study are included in the article. Further data or information required are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' Contributions

Xiaomao Li and Yan Liu had equal contributions. Xiaomao Li designed the experiment, completed the experiment, and wrote the first draft. Yan Liu designed the experiments and revised the manuscripts. Siyi Wang contributed to the data validation. Yikai Jiang contributed to the supervision. Adnan Mohammed Algradi polished the language of the manuscript. Yuanyuan Zhou, Juan Pan, and Wei Guan have contributed to the investigation. Haixue Kuang verified the data. Bingyou Yang provided the funding and checked the data. Xiaomao Li and Yan Liu have contributed equally to this work. Xiaomao Li and Yan Liu are the co-first authors.

Acknowledgments

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Supplementary Materials

Supplementary data associated with this article can be found in the Supporting Information. *(Supplementary Materials)*

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