Evidence-Based Complementary and Alternative Medicine

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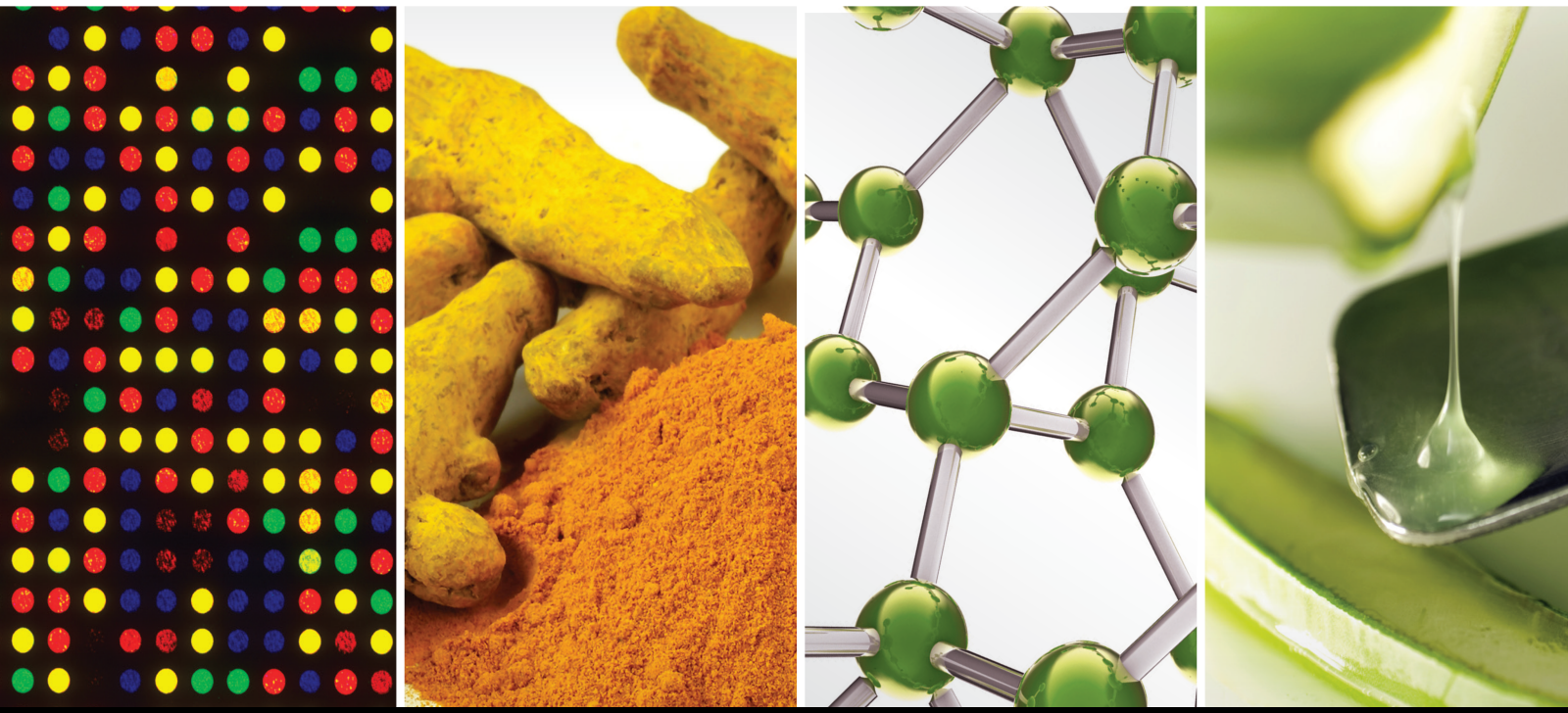
Guest Editors: Mohammad Abdollahi, Roja Rahimi, Mahnaz Khanavi, Mohammad Hosein Ayati, and Reza Baradar Jalili



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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6681540, 26 pages
https://doi.org/10.1155/2021/6681540

Review Article

Bioactive Foods and Medicinal Plants for Cardiovascular Complications of Type II Diabetes: Current Clinical Evidence and Future Perspectives

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Received 3 November 2020; Accepted 1 September 2021; Published 16 September 2021

Academic Editor: Michał Tomczyk

Cardiovascular diseases (CVDs) are the main cause of mortality in type 2 diabetes mellitus (T2DM); however, not all patients are fully satisfied with the current available treatments. Medicinal plants have been globally investigated regarding their effect in CVD, yet the field is far from getting exhausted. The current paper aims to provide an evidence-based review on the clinically evaluated medicinal plants and their main therapeutic targets for the management of CVD in T2DM. Electronic databases including PubMed, Cochrane, Embase, Scopus, and Web of Science were searched from 2000 until November 2019, and related clinical studies were included. Lipid metabolism, glycemic status, systemic inflammation, blood pressure, endothelial function, oxidative stress, and anthropometric parameters are the key points regulated by medicinal plants in T2DM. Anti-inflammatory and antioxidant properties are the two most important mechanisms since inflammation and oxidative stress are the first steps triggering a domino of molecular pathological pathways leading to T2DM and, subsequently, CVD. Polyphenols with potent antioxidant and anti-inflammatory effects, essential oil-derived compounds with vasorelaxant properties, and fibers with demonstrated effects on obesity are the main categories of phytochemicals beneficial for CVD of T2DM. Some medicinal plants such as garlic (*Allium sativum*) and milk thistle (*Silybum marianum*) have strong evidences regarding their beneficial effects; however, others have low level of evidence which reveals the need for further clinical studies with larger sample sizes and longer follow-up periods to confirm the safety and efficacy of medicinal plants for the management of CVD in T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder accompanied with reduced glucose uptake, abnormal glucose metabolism, and insulin resistance which affects 1 in 11 adults [1]. Tracing the increasing rate of T2DM during the past decades shows its incidence and prevalence to be dramatically higher than the predicted values [2, 3].

T2DM is a principal risk factor for a broad spectrum of severe conditions amongst which the most important ones are cardiovascular diseases (CVDs) [4]. More than 80% of deaths in T2DM patients occur due to cardiovascular events which caused CVD to be the leading cause of mortality in these patients [5]. Based on the Framingham study, diabetic patients are two to six times more prone to myocardial infarction and heart failure [6]. A recent meta-analysis

demonstrated higher risk of atrial fibrillation in diabetic patients [7]. Another meta-analysis revealed that diabetes significantly increases the risk of sudden cardiac death [8]. Also, hypertension is two times more prevalent in T2DM patients [9]. Diabetes is also an important risk factor for chronic heart failure [10]. Also, some cardiovascular drugs, such as statins, can increase the risk of diabetes[11]. Thus, there is a close relationship between T2DM and CVD.

Studies revealed that despite some beneficial effects of antihyperglycemic agents for the reduction of CVD risk [4], hyperglycemia is not the key concern in regard to CVD in T2DM patients [12]. In addition to glycemic control, specific management of CVD, e.g., regulation of lipid profile and HTN, is of a great importance [12]. In other words, antidiabetic agents such as metformin and glibenclamaide which only affect the blood glucose level does not have a considerable effect on CVD risk, whereas oral antidiabetics such as thiazolidinediones with insulin-sensitizing effects are more probable to reduce CVD risk [12, 13]. On the other hand, thiazolidinediones are associated with side effects such as higher risk of bone fracture due to the suppression of proosteoblastic pathways [14]. Other drug choices for the management of CVD risk in T2DM patients include antiplatelet agents and anticoagulants [4]; however, their long-term efficacy is still in doubt and more important, the adverse effects can be disturbing in long-term use, causing low patient adherence and compliance. So, scientists are globally seeking new options with better efficacy and fewer side effects for the management of CVD in T2DM.

Medicinal plants have an ancient history of use amongst people which is currently known as complementary and alternative therapies [15, 16]. Additionally, current scientific evidence supports the beneficial effects of herbal extracts and their isolated compounds in different types of CVD such as atherosclerosis [17], hypertension, and hyperlipidemia [18]. In diabetes-associated CVD, plant-derived natural supplements could play a positive role in preventing oxidative damage and inflammation [19] which are two basic mechanisms involved in the pathophysiology of T2DM-associated CVD. Thus, plants can be future candidates for the management of CVD in T2DM. The aim of the present study is to review controlled clinical trials on the cardiovascular effects of plants in T2DM patients.

2. Methods

2.1. Search Strategy. Electronic databases including Medline, Scopus, Embase, Web of Science, and Cochrane library were searched with the following search formula:

> "diabetes" [title/abstract/keyword] AND "plant" OR "extract" OR "herb" [all fields] AND "cardiovascular" OR "atherosclerosis" OR "hypertension" OR "hyperlipidemia" OR "dyslipidemia" [title/abstract/keyword]

Articles were collected from January 2000 until November 2019. Primary search results were screened by two independent investigators. No language restriction was considered.

2.2. Inclusion and Exclusion Criteria. Inclusion criteria were controlled clinical trials (using placebo or no intervention design) in which the effect of a plant in regard to a cardiovascular parameter (lipid profile, blood pressure, endothelial dysfunction, oxidative stress, or systemic inflammation) was evaluated in T2DM patients. Exclusion criteria were animal and cellular studies, human studies other than clinical trials (e.g., cohort studies and case reports), comparing the results of the herbal intervention with a standard drug (in case the standard drug was administered to both test and control groups, the study was included), choosing healthy individuals as control subjects, including type 1 diabetic patients or patients with obesity or metabolic syndrome without a diagnosis of T2DM, and administration of a mixture of herbal and nonherbal materials. Studies that assessed the effect of herbal mixtures were also excluded because the results of those studies cannot be attributed to any of the individual herbal extracts. Also, purified phytochemicals were excluded since the aim of this review is to only consider plants since they are better choices to be suggested as dietary interventions. Studies on the antidiabetic activity of plant extracts without considering a cardiovascular parameter were excluded, as well. References of the included articles were also checked to find further relevant studies.

Final included papers were screened to extract the scientific name of the plant (in case the scientific name was not mentioned, the most probable scientific name was written in the table with an asterisk sign), used part, dosage, study design, sample size (the number of patients who completed the study), duration of treatment, and outcomes. Jadad score was used to evaluate the quality of the studies [20].

3. Mechanisms of Plants Clinically Investigated to Control CVD in T2DM

Among a total of 10644 primarily obtained papers, 73 were finally included. Figure 1 shows the detailed study selection process. Final included papers are summarized in Table 1. Medicinal plants have demonstrated several benefits to control cardiovascular complications of T2DM via different mechanisms. The most important medicinal plants exerting each mechanism are discussed as follows. Figure 2 shows a schema of the mechanisms affected by bioactive foods and plants to control CVD complications of T2DM.

3.1. Glycemic Profile. Despite the numerous large-scale clinical trials designed to clarify the relationship between blood sugar and CVD in T2DM patients, the problem is not yet completely solved since intensive blood glucose control has represented conflicting results [93]. Persistent hyperglycemia in diabetic patients leads to impaired angiogenesis which is, at least in part, related to abnormal glucose flux via the hexosamine biosynthetic pathway and participates in cardiovascular mortality [94]. Also, high level of blood glucose induces the production of advanced glycemic end-products (AGEs) which accumulate during the time and cause vascular complications of T2DM [95]. Blood insulin

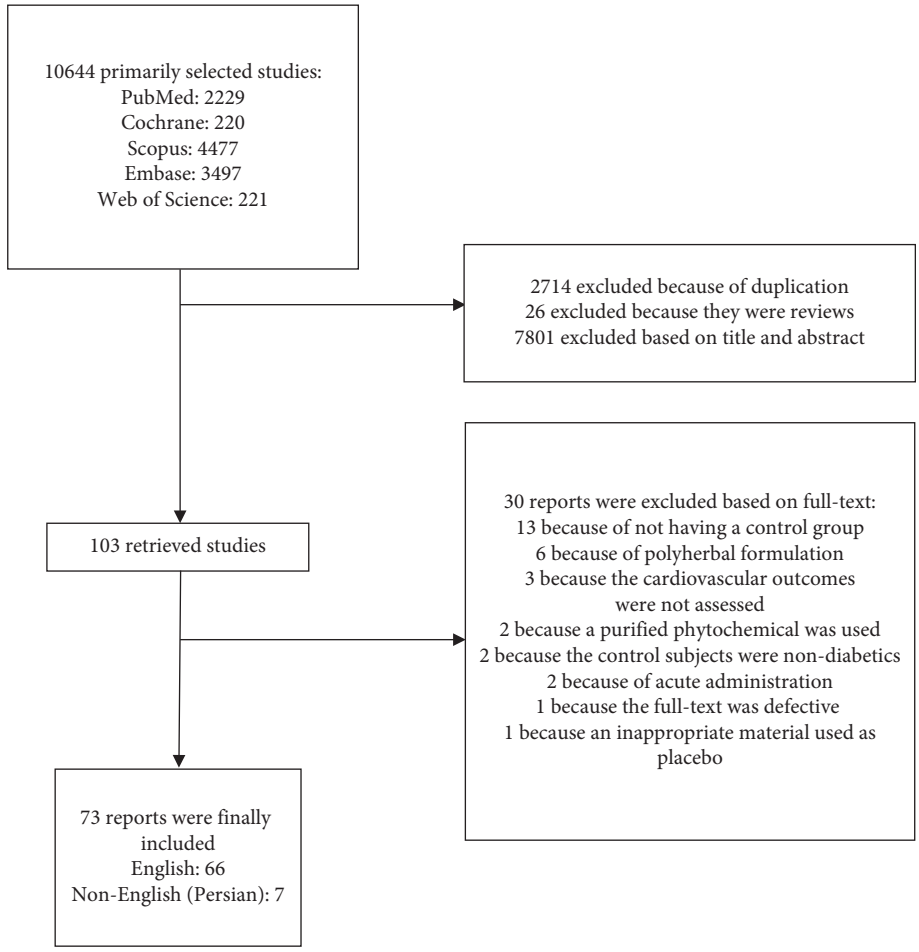

Figure 1: Study selection flow diagram.

level is another key factor launching the cardiovascular complications of T2DM. It is demonstrated that type 1 diabetic patients (in whom the main contributor is the impaired insulin level) have also high susceptibility to CVD. Thus, regardless of the other factors, abnormal blood glucose and insulin level themselves can be triggers for cardiovascular events. Insulin acts as a double-edged sword in the pathogenesis of CVD. In healthy subjects, insulin secretion leads to the dual activation of the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K). While the former activates proatherogenic factors, the latter elevates nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) which causes a relaxing effect on the vascular smooth muscles and suppresses the proatherogenic and proinflammatory mediators in plasma. By contrast, during insulin resistance, the proatherogenic cascade is well activated, whereas the antiatherogenic pathway (PI3K) is not fully responsive [93].

Several medicinal plants have shown antihyperglycemic activity in clinical studies (Table 1). Milk thistle (*Silybum marianum* (L.) Gaertn.) is a well-known medicinal plant due to its ancient use as a hepatoprotective remedy; however, significant antioxidant properties of its flavonolignans made researchers reconsider its health benefits, and thus, it is now considered as an interesting option for the treatment of several chronic diseases involved with oxidative stress [96]. Six-week administration of milk thistle supplement to T2DM patients could significantly improve glycemic parameters via reduction of FBS, FPI, and insulin resistance which was evident from the homeostatic model assessment-insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) [21]. The supplement also improved lipid profile and inflammation/oxidative damage biomarkers [22]; thus, it can be suggested as a valuable herb to manage CVD risk in T2DM [97]. *Cynara scolymus* L. or the globe artichoke is a popular vegetable widely used in different parts of the world. In a study by Nazni et al., five different food products containing artichoke extract were prepared in order to choose the most pleasant form for a clinical trial on the effect of dietary artichoke on cardiovascular parameters in T2DM patients. Wheat biscuit enriched with artichoke extract was chosen as the most acceptable form and was administered in a placebo-controlled trial to T2DM patients for a period of 90 days. The preparation could significantly improve both fasting and postprandial blood sugar, as well as the lipid profile [23]. Another trial also assessed the effect of globe artichoke in T2DM patients; however, two months of treatment resulted in no significant effect on glycemic parameters and TAG in this study and only reduced TC and LDL-C [24]. It should be

Table 1: Clinical studies on the use of medicinal plants for the management of cardiovascular complications in diabetic patients.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Acca sellowiana (O. Berg) Burret/fruit	Myrtaceae	Feijoa	150 mg	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (20) or placebo (14)	3 m	2	Oral antidiabetics and/or insulin	Vs. placebo: ↓FBS and HbA1c, ↓LDL-C, TC, and TAG, ↑HDL-C, and ↓SBP and DBP	[45]
Allium sativum L./bulb	Amaryllidaceae	Garlic	250 mg, BD	Randomized, open-label, prospective, comparative trial on obese diabetic patients treated with metformin (30) or metformin + plant (30)	3 m	1	ND	Vs. baseline and placebo: ↓FBS and GTT, ↓TC, LDL-C, and TAG, ↑HDL-C, ↓ADA and CRP, and ↓HbA1c vs. metformin alone, but not vs. baseline	[13]
Allium sativum L./bulb	Amaryllidaceae	Garlic	300 mg, BD	Placebo-controlled trial on diabetic patients treated with the plant (10) or placebo (10) as monotherapy or the plant (20) or placebo (20) concomitant with oral antidiabetics	1 m	3	None in the monotherapy groups, oral antidiabetics in the combination-therapy groups	Monotherapy: vs. baseline: ↓TAG and fructosamine; vs. placebo: ↓FBS and fructosamine, combination therapy: vs. baseline: ↓TAG and fructosamine and ↓15% in the dosage of oral antidiabetics, and vs. placebo: ↓fructosamine and no significant change in TC, LDL-C, and HDL-C	[46]
Allium sativum L./bulb	Amaryllidaceae	Garlic	300 mg, TDS	Randomized, single-blind, placebo-controlled trial on diabetic patients treated with the plant (30) or placebo (30)	6 m	2	Metformin	Vs. placebo: ↓TC, LDL-C, and TAG and ↑HDL-C	[26]
Allium sativum L./bulb	Amaryllidaceae	Garlic	300 mg, BD	Randomized, single-blind, placebo controlled in dyslipidemic diabetic patients treated with the plant (35) or placebo (35)	3 m	2	ND	Vs. placebo: ↓TC and LDL-C and ↑HDL-C	[25]
Allium sativum L./bulb	Amaryllidaceae	Garlic	300 mg, QID	Randomized, double-blind, placebo-controlled, crossover trial on 26 diabetic patients with high risk for cardiovascular diseases	1 m	3	Metformin, statins, ASA, and ACE inhibitors	Vs. placebo: no significant change in BP, lipid profile, anthropometric parameters, oxidative stress, and inflammation biomarkers	[27]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Aloe vera (L.) Burm.f./leaf gel	Xanthorrhoeaceae	Aloe	300 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic hypercholesterolemic patients treated with the plant (30) or placebo (30)	2 m	5	Metformin and glyburide	Vs. baseline and placebo: ↓FBS and HbA1c and ↓TC and LDL-C and no significant change in HDL-C and TAG	[47]
Aloe vera (L.) Burm.f./leaf gel	Xanthorrhoeaceae	Aloe	1 g daily	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (21) or placebo (22)	2 m	5	Oral antidiabetics	Vs. baseline: no significant change in glycemic parameters and lipid profile	[48]
Avena nuda L./seed	Poaceae	Naked oat	50 or 100 g/day	Randomized, single-blind, controlled trial on diabetic hyperlipidemic patients treated with usual care (63), diet intervention (61), 50 g of the plant (65), or 100 g of the plant (71)	1 m	3	Oral antidiabetics and/or insulin	Vs. control: ↓FBS, post prandial glucose, HbA1c, and HOMA-IR ↓TC, LDL-C, and TAG, ↓BW, BMI, and WC (only with high dose), and ↑HDL-C	[44]
Berberis aristata DC*/stem bark	Berberidaceae	Indian barberry	0.75 g or 1.5 g, BD	Randomized, open, controlled trial on diabetic patients treated with the low-dose of the plant (30) and high-dose of the plant (30) or left untreated (30)	9 m	2	Oral antidiabetics and antihyperlipidemics	Vs. control: ↓FBS, ↓TAG and LDL-C, ↑HDL-C, and no significant change in TC	[40]
Berberis vulgaris L./fruit	Berberidaceae	Barberry	1 g, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (21) or placebo (21)	3 m	4	Oral antidiabetics	Vs. placebo: ↓FBS and FPI, ↓TAG, TC, LDL-C, and apo-B, ↑TAOS, and no significant change in HbA1c, homocysteine, and HDL-C	[39]
Berberis sp.*/fruit	Berberidaceae	Barberry	200 ml, daily	Randomized, double-blind, controlled trial on diabetic patients treated with the plant (23) or left untreated (23)	2 m	3	Oral antidiabetics	Vs. control: ↓FBS, ↓TC, ↑PON-1, ↓SBP and DBP, ↓BW and BMI, and no significant change in HbA1c, TAG, LDL-C, HDL-C, apo A1, and apo B100	[38]
Boswellia serata Roxb. ex Colebr./gum resin	Burseraceae	Olibanum	400 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (37) or placebo (34)	3 m	4	Metformin	Vs. placebo: ↓FBS, HbA1c, and FPI, ↓TC and TAG, and no significant change in HDL-C	[49]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Brassica napus L./seed oil	Brassicaceae	Canola	31 g canola oil/2000 kcal	Randomized, controlled trial on diabetic patients treated with canola-enriched bread (55) or wheat bread (64)	3 m	3	Oral antidiabetics	Vs. control: ↓HbA1c, ↓TC, LDL-C, TAG, TC/HDL-C, and LDL-C/HDL-C, ↓Framingham CVD risk score, and no significant change in BP, HR, BW, and WC	[50]
Brassica oleracea L./sprout	Brassicaceae	Broccoli	5 or 10 g/day	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the low-dose plant (26), high-dose plant (23), or placebo (23)	1 m	5	Oral antidiabetics	Vs. baseline: ↓FBS and ↓TC and LDL-C; high-dose vs. low-dose and placebo: no significant effect on FBS, TC, LDL-C, ↓TAG, ox-LDL-C/LDL-C, AIP, and ↑HDL-C	[51]
Carica papaya L./fermented fruit	Caricaceae	Papaya	3 g, BD	Randomized, controlled trial on total of 101 diabetic patients	4 m (2 w of washout at the end)	3	—	Vs. control: ↓SBP, ↑TAG, ↑TAOS, and no significant change in glycemic parameters, TC, LDL-C, and HDL-C	[52]
Cinnamomum cassia (L.) J.Presl/bark	Lauraceae	Cinnamon	500 mg, 2, 6, or 12 caps/day	Randomized, placebo-controlled trial on diabetic patients in six groups treated with two (10), six (10), and twelve (10) plant capsules or two (10), six (10), or twelve (10) placebo capsules	40 days of treatment and 20 days of follow-up	1	Oral antidiabetics	Vs. baseline: ↓FBS, ↓TC, TAG, and LDL-C, and no significant change in HDL-C	[53]
Cinnamomum cassia (L.) J.Presl/bark	Lauraceae	Cinnamon	400 mg, TDS	Randomized, placebo-controlled trial on diabetic patients treated with the plant (29) or placebo (30)	3 m	2	Oral antidiabetics	Vs. placebo: no significant change in glycemic profile, lipid profile, and anthropometric parameters	[54]
Cinnamomum zeylanicum Blume/bark	Lauraceae	Cinnamon	1 g, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (20) or placebo (19)	2 m	4	Metformin	Vs. placebo and baseline: no significant change in glycemic profile, MDA, and TAOS	[55]
Crataegus laevigata (Poir.) DC./flower and leaf	Rosaceae	Hawthorn	400 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic dyslipidemic patients treated with the plant (24) or placebo (21)	6 m	4	ASA, statins, and antihypertensives	Vs. baseline: ↓TC, LDL-C, and non-HDL-C and no significant change in TAG and HDL-C; vs. placebo: ↓neutrophil elastase and no significant change in lipid profile, CRP, and MDA	[56]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Crataegus laevigata (Poir.) DC.*/fruit	Rosaceae	Hawthorn	600 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic hypertensive patients treated with the plant (39) or placebo (40)	4 m	5	Oral antidiabetics and antihypertensives	Vs. placebo: ↓DBP and no significant change in SBP, FBS, HbA1c, and fructosamine	[57]
Crocus sativus L./stigma	Iridaceae	Saffron	15 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (32) or placebo (32)	3 m	5	Oral antidiabetics	Vs. baseline: ↓FPG, HbA1c, TC, LDL-C, and LDL-C/HDL-C ratio; vs. placebo: ↓FPG, TC, LDL-C, and LDL-C/HDL-C ratio and no significant change in anthropometric parameters, TAG, and HDL-C	[58]
Cucurbita ficifolia Bouché/fruit	Cucurbitaceae	Pumpkin	100 g daily	Randomized, controlled trial on diabetic patients treated with the plant (20) or left untreated (20)	2 m	2	Metformin and glibenclamide	Vs. baseline: ↓FBS and HbA1c, ↓SBP and DBP, and ↓LDL-C and CRP; vs. control: ↓FBS, HbA1c and ↓SBP and DBP	[59]
Curcuma longa L./rhizome	Zingiberaceae	Turmeric	750 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (99) or placebo (100)	6 m	4	—	Vs. placebo: ↓PWV, ↓TAG, leptin, and HOMA-IR, ↑adiponectin, ↓WC, visceral fat, and total body fat, ↓BMI and LDL-C, and ↑HDL-C (numerically, but not statistically significant)	[60]
Curcuma longa L./rhizome	Zingiberaceae	Turmeric	400 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (60) or placebo (54)	3 m	5	Metformin and sulfonylureas	Vs. placebo: ↓PWV, AI, and arterial stiffness and no significant effect on BP; vs. baseline: ↓ICAM-1 and VCAM-1	[61]
Curcuma longa L./rhizome	Zingiberaceae	Turmeric	700 mg, TDS	Randomized, double-blind, placebo-controlled trial on hyperlipidemic diabetic patients treated with the plant (36) or placebo (39)	2 m	5	Oral antidiabetics and antihyperlipidemics	Vs. placebo: ↓BMI, TAG, and TC, ↓LDL-C (borderline), no significant change in glycemic profile, apolipoproteins, and other lipid markers	[62]
Cyamopsis tetragonoloba (L.) Taub./partially hydrolyzed gum	Leguminosae	Guar	5 g, BD	Randomized, controlled clinical trial on diabetic patients with MetS treated with the plant (23) or left untreated (21)	1.5 m	3	Metformin and protamine insulin	Vs. baseline: ↓HbA1c, ↓serum *trans*-fatty acid, ↓WC, and no significant change in BP, lipid profile, CRP, and ET-1	[43]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Cynara scolymus L./leaf	Compositae	Artichoke	400 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic hypercholesterolemic patients treated with the plant (36) or placebo (36)	2 m	4	Metformin and glyburide	Vs. placebo: no significant change in FBS, GTT, HbA1c, TAG, ↓TC, and LDL-C	[24]
Cynara scolymus L./petals	Compositae	Artichoke	6 g daily	Controlled trial on diabetic patients treated with the plant (15) or placebo (15)	3 m	—	—	Vs. baseline: ↓FBS and postprandial sugar, ↓TC, LDL-C, and TAG, and ↑HDL-C	[23]
Dichrostachys glomerata (Forssk.) Chiov./pod	Leguminosae	—	400 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic obese patients treated with the plant (23) or placebo (23)	2 m	2	ND	Vs. placebo: ↓FBS and HbA1c, ↓TC, LDL-C, TAG, and TC/HDL-C, ↑HDL-C, ↓SBP and DBP, and ↓BW, BMI, WC, HC, and body fat	[63]
Fragaria × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier/fruit	Rosaceae	Strawberry	25 g freeze-dried powder, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (19) or placebo (17)	1.5 m	4	Oral antidiabetics	Vs. baseline and placebo: ↓HbA1c, CRP, and MDA, ↑TAOS, and no significant change in FBS and anthropometric parameters	[64]
Ginkgo biloba L./leaf	Ginkgoaceae	Ginkgo	1 tablet (containing flavonol glycoside 19.2 mg and terpene lactone 4.8 mg), TDS	Randomized, controlled trial on diabetic patients with nephropathy treated with the plant (32) or left untreated (32)	2 m	2	Oral antidiabetics, insulin, and antihypertensives	Vs. baseline: ↑NO, ↓VWF, and no significant change in FBS and ET-1	[42]
Glycine max (L.) Merr./bean	Leguminosae	Soy	2.5 g, daily	Controlled trial on diabetic hypercholesterolemic patients treated with fenofibrate (11), plant (18), or fenofibrate + plant (7)	2 m	—	Fenofibrate	Soybean + fenofibrate vs. fenofibrate alone: ↓LDL-C and TAG and no significant change in TC and HDL-C	[65]
Glycine max (L.) Merr.*/bean (in the form of nut)	Leguminosae	Soy	60 g, daily	Randomized, controlled trial on patients treated with the plant (35) or placebo (35)	2 m	2	ND	Vs. baseline: ↓FBS, SBP, DBP, TC, LDL-C, and E-selectin and ↑TAOC and FMD; vs. control: ↓FBS, TC, LDL-C, and E-selectin, ↑TAOC, FMD, and no significant change in HDL-C and TAG	[66]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Gymnema lactiferum (L.) R.Br. ex Schult./leaf	Apocynaceae	Ceylon cow-tree	3.5 g, BD	Open-label trial on diabetic hypercholesterolemic patients treated with the plant (12) or placebo (14)	1 m	—	Oral antidiabetics	Vs. baseline: ↓FBS and HbA1c, ↓TC and LDL-C, and no significant change in HDL-C, TAG, and BW	[67]
Juglans regia L.*/nut	Juglandaceae	Walnut	56 g/ day	Randomized, controlled, single-blind, crossover trial on 24 diabetic patients	2 m	2	—	Vs. baseline: ↑FBS, ↓TC and LDL-C, ↓BP in the control group, and no significant change in anthropometric parameters, HbA1c and HOMA-IR; vs. control: ↑FMD	[68]
Juglans regia L./leaf	Juglandaceae	Walnut	100 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic hypercholesterolemic patients treated with the plant (32) or placebo (29)	3 m	5	Oral antidiabetics	Vs. baseline: ↓FBS and HbA1c, ↓TC and TAG, and no significant change in LDL-C and HDL-C; vs. placebo: ↓FBS, ↓TAG, and no significant change in FPI and c-peptide	[69]
Juglans regia L./leaf	Juglandaceae	Walnut	100 mg daily for the first week and then 100 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (20) or placebo (19)	2 m	5	Metformin and glibenclamide	Vs. baseline: ↓SBP, but not DBP, ↓BW and BMI, no significant change in lipid and glycemic profile, and ↓FBS and HbA1c in placebo	[70]
Laurus nobilis L./leaf	Lauraceae	Bay leaf	500 mg, QID	Randomized, placebo-controlled trial on diabetic patients treated with the plant (50) or placebo (15)	1 m	—	Oral antidiabetics	Vs. baseline: ↓FBS, ↓TC, LDL-C, and TAG, and ↑HDL-C	[71]
Linum usitatissimum L./seed gum	Linaceae	Flax	5 g daily (as chapatti)	Randomized, controlled trial on diabetic patients treated with the plant (60) or control chapattis (60)	3 m	2	ND	Vs. baseline: ↓FBS, ↓TC and LDL-C, and no significant change in VLDL-C, HDL-C, and TAG	[72]
Lycopersicon esculentum Mill.*/fruit	Solanaceae	Tomato	250 ml, BD	Randomized, placebo-controlled trial on diabetic patients treated with the plant (15), vitamin E (12), vitamin C (12), or placebo (13)	1 m	3	Oral antidiabetics, ACE inhibitors	Vs. baseline: ↑LDL-C resistance to oxidation and no significant change in FBS, BP, CRP, and lipid profile	[73]
Melissa officinalis L./aerial parts	Lamiaceae	Lemon balm	350 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (31) or placebo (31)	3 m	4	Oral antidiabetics	Vs. baseline: ↓ICAM-1 and TAG/HDL-C and ↑apo A-1; vs. placebo: ↓TC/HDL-C and LDL-C/HDL-C ↑apo A-1, and no significant change in ICAM-1	[74]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Morus alba L.*/leaf	Moraceae	Mulberry	1 g, TDS	Randomized, double-blind, placebo-controlled pilot trial on diabetic patients treated with the plant (12) or placebo (12)	3 m	4	Oral antidiabetics	Vs. baseline and placebo: ↓postprandial blood glucose and no significant change in FBS, BW, BP, and HbA1c	[75]
Panax ginseng C.A.Mey./root	Araliaceae	Korean ginseng	100 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (20) or placebo (20)	2 m	4	ND	Vs. baseline and placebo: ↓IL-6 and CRP and no significant change in anthropometric parameters, TNF-α, and HbA1c	[30]
Panax quinquefolius L./root	Araliaceae	American ginseng	1 g, TDS	Randomized, double-blind, placebo-controlled trial on diabetic hypertensive patients treated with the plant (30) or placebo (34)	3 m	5	Oral antidiabetics	Vs. placebo: ↓radial AI and SBP, but not DBP, and no significant change in PP and HR	[37]
Panax quinquefolius L./root	Araliaceae	American ginseng	1 g, TDS	Randomized, double-blind, placebo-controlled, crossover trial on diabetic patients treated with the plant (24) or placebo (24)	2 m	5	Oral antidiabetics, Antihyperlipidemic agents, and antihypertensive drugs	Vs. placebo: ↓FBS and HbA1c, ↓SBP, ↓TC, LDL-C, TC/LDL-C, and LDL-C/HDL-C, ↑NOx, and no significant change in DBP; vs. baseline: ↓PAI-1	[76]
Passiflora edulis Sims/fruit peel	Passifloraceae	Purple passion fruit	220 mg, daily	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (19) or placebo (21)	4 m	3	Metformin, glibenclamide, and atenolol	Vs. placebo: ↓FBS, ↓SBP, and no significant change in DBP, lipid profile, HbA1c, and BMI	[77]
Phyllanthus emblica L./fruit	Phyllanthaceae	Emblic	250 or 500 mg, BD	Randomized, double-blind, controlled trial on diabetic patients treated with low dose of the plant (20), high dose of the plant (20), atorvastatin (20), or placebo (20)	3 m	3	Oral antidiabetics	Vs. baseline and placebo: ↓HbA1c, ↓RI, ↓TC, LDL-C, and TAG, ↑HDL-C, ↓MDA and CRP, and ↑GSH and NO	[78]
Pinus maritima Mill./bark	Pinaceae	Pine	25 mg, 5 times a day	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (24) or placebo (24)	3 m	3	Oral antidiabetics and /or antihypertensives	Vs. placebo: ↓FBS and HbA1c, ↓50% in ACE inhibitor dosage, and ↓LDL-C and ET-1	[79]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Plantago ovata Forssk./husk	Plantaginaceae	Psyllium	5 g, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (21) or placebo (15)	2 m	4	Oral antidiabetics	Vs. placebo: ↓FBS and HbA1c, ↓LDL-C/HDL-C, ↑HDL-C, and no significant change in FPI, TC, TAG, and LDL-C	[80]
Portulaca oleracea L./seed	Portulacaceae	Purslane	10 g, daily	Randomized, controlled, crossover trial on diabetic patients treated with the plant + yoghurt (48) or yoghurt alone (48)	5 weeks	1	Oral antidiabetics	Vs. baseline: ↓TC and TAG, ↓SBP and DBP, ↓BW, BMI, and WC, and no significant change in FPG, FPI, HOMA-IR, LDL-C, and HDL-C; vs. placebo: ↓TAG, ↓SBP, ↓BW and BMI, and no significant change in WC, FPG, FPI, HOMA-IR, TC, LDL-C, HDL-C, and DBP	[81]
Portulaca oleracea L./herb	Portulacaceae	Purslane	60 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (23) or placebo (27)	3 m	4	Oral antidiabetics	Vs. baseline: ↓SBP and no significant change in FBS and DBP	[82]
Prunus amygdalus Batsch*/fruit	Rosaceae	Almond	56 g, daily	Randomized, crossover, controlled trial on diabetic hyperlipidemic patients treated with the plant (20) or placebo (20)	1 m	1	Oral antidiabetics	Vs. control: ↓ox-LDL-C, ↓IL-6, TNF-α, and CRP, and no significant change in MDA, ICAM-1, and VCAM-1	[83]
Prunus amygdalus Batsch*/fruit	Rosaceae	Almond	56 g, daily	Randomized, crossover, controlled trial on diabetic hyperlipidemic patients treated with the plant (20) or placebo (20)	1 m	1	Oral antidiabetics	Vs. control: ↓FBS, FPI, and HOMA-IR ↓TC, LDL-C, LDL/HDL-C, apo B, apo B/apo A-1, and nonesterified fatty acids, ↓body fat, and no significant change in BW and BMI	[84]
Punica granatum L.*/fruit	Lythraceae	Pomegranate	200 ml, daily	Randomized, single-blind, controlled trial on diabetic patients treated with the plant (30) or left untreated (30)	1.5 m	3	ND	Vs. control: ↓ox-LDL-C and anti-ox-LDL-C antibody and ↑TAOS and PON-1	[34]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Rheum ribes L./stem	Polygonaceae	Syrian rhubarb	400 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic hypercholesterolemic patients treated with the plant (18) or placebo (18)	1 m	3	Metformin and glyburide	Vs. placebo: ↓FBS, ↓TC and LDL-C, and no significant change in HDL-C and TAG	[85]
Salvia miltiorrhiza Bunge/root	Lamiaceae	Danshen	5 g, BD	Randomized, placebo-controlled trial on diabetic patients with coronary heart disease treated with the plant (31) or placebo (31)	2 m	2	Oral antidiabetics	Vs. placebo: ↓VCAM-1; vs. baseline: ↓VWF and ox-LDL-C	[41]
Salvia officinalis L./leaf	Lamiaceae	Sage	500 mg, TDS	Randomized, triple-blind, placebo-controlled trial on diabetic hyperlipidemic patients treated with the plant (40) or placebo (40)	3 m	5	Metformin and glyburide	Vs. baseline and placebo: ↓FBS and HbA1c, ↓TC, LDL-C, and TAG, and ↑HDL-C	[86]
Satureja khuzestanica Jamzad/aerial parts	Lamiaceae	—	250 mg, daily	Randomized, double-blind, placebo-controlled trial on hyperlipidemic diabetic patients treated with the plant (11) or placebo (10)	2 m	4	Oral antidiabetics and antihyperlipidemic agents	Vs. baseline: ↓TC and LDL-C, ↑HDL-C and TAOC, and no significant change in FBS, MDA, and TAG	[87]
Sesamum indicum L./seed	Pedaliaceae	Sesame	28 g, daily	Randomized, controlled trial on diabetic patients treated with the plant (20) or left untreated (16)	1.5 m	3	Oral antidiabetics	Vs. control: ↓TAG and AIP and no significant change in LDL-C, TC, and HDL-C	[88]
Silybum marianum (L.) Gaertn./seed	Compositae	Milk thistle	200 mg, TDS	Randomized, double-blind, placebo-controlled trial on hyperlipidemic diabetic patients treated with the plant (29) or placebo (25)	4 m	3	ND	Vs. baseline: ↓FBS, ↓TC, LDL-C, and TAG, and ↑HDL-C	[89]
Silybum marianum (L.) Gaertn./seed	Compositae	Milk thistle	140 mg, TDS	Randomized, triple-blind, placebo-controlled trial on diabetic patients treated with the plant (20) or placebo (20)	1.5 m	5	Oral antidiabetics	Vs. placebo: ↓MDA and CRP and ↑GSH, Gpx, SOD, and TAOS	[22]
Silybum marianum (L.) Gaertn./seed	Compositae	Milk thistle	140 mg, TDS	Randomized, triple-blind, placebo-controlled trial on diabetic patients treated with the plant (20) or placebo (20)	1.5 m	5	Oral antidiabetics	Vs. placebo: ↓FBS, FPI, and HOMA-IR, ↓QUICKI, ↓TAG and TAG/HDL-C, and ↑HDL-C; vs. baseline: ↓TC and LDL-C	[21]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Thymus kotschyanus Boiss. and Hohen./aerial parts	Lamiaceae	—	10 g, BD	Randomized, controlled trial on diabetic patients treated with the plant (32) or left untreated (32)	3 m	3	Oral antidiabetics	Vs. baseline: ↓FBS and HbA1c, ↓LDL-C, ↑HOMA-β, and no significant change in FPI, HOMA-IR, TC, and TAG; vs. control: ↑HDL-C	[90]
Urtica dioica L./aerial parts	Urticaceae	Stinging nettle	100 mg/kg	Randomized, single-blind, placebo-controlled clinical trial on diabetic patients treated with the plant (24) or placebo (21)	2 m	1	ND	Vs. baseline: ↓TNF-α; vs. placebo: ↓CRP and IL-6 and no significant change in FPI, insulin resistance, and anthropometric parameters	[32]
Urtica dioica L./aerial parts	Urticaceae	Stinging nettle	5 ml of liquid extract (containing 2.7 g/lit of dry matter), TDS	Randomized, double-blind, placebo-controlled trial on diabetic women treated with the plant (24) or placebo (23)	2 m	5	Metformin and glibenclamide	Vs. placebo: ↓FBS, TAG, and SGPT ↑HDL-C, NO, and SOD, and no significant change in other lipid profile parameters	[33]
Vaccinium macrocarpon Aiton*/fruit	Ericaceae	Cranberry	500 mg, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (15) or placebo (15)	3 m	2	ND	Vs. placebo: ↓TC, LDL-C, and TC/HDL-C, no significant change in ox-LDL-C, HDL-C, and TAG, and no significant change in BP, HbA1C, HOMA-IR, anthropometric parameters, and CRP	[91]
Vaccinium macrocarpon Aiton*/fruit	Ericaceae	Cranberry	240 ml, daily	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (29) or placebo (29)	3 m	3	Oral antidiabetics	Vs. baseline and placebo: ↓FBS, ↓apo-B, ↑apo-A-1 and PON-1 activity, and no significant change in Lp(a)	[35]
Vitis sp./seed	Vitaceae	Grape	300 mg, BD	Randomized, double-blind, crossover, placebo-controlled trial on 32 diabetic patients with high risk for cardiovascular problems	1 m	2	Oral antidiabetics or special diet control	Vs. baseline: ↓fructosamine and CRP, ↓TC, ↑GSH, and no significant change in HOMA, TAOS, and endothelial function	[36]
Vitis vinifera L./seed	Vitaceae	Grape	200 mg	Randomized, triple-blind, placebo-controlled trial on diabetic patients treated with the plant (26) or placebo (22)	2 m	3	—	↓HDL-C vs. baseline but not vs. placebo: no significant change in FBS, BP, TC, LDL-C, and TAG	[92]

Table 1: Continued.

Plant/part	Family	Common name	Dosage	Design	Duration	Jadad score	Concomitant therapy	Outcomes	Reference
Zingiber officinale Roscoe/rhizome	Zingiberaceae	Ginger	800 mg, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (33) or placebo (30)	3 m	4	—	Vs. placebo: ↓FBS, HbA1c, and HOMA-IR, ↓TC and TAG, ↑HDL-C/TC, and no significant change in HDL-C and LDL-C	[29]
Zingiber officinale Roscoe/rhizome	Zingiberaceae	Ginger	1 g, BD	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (28) or placebo (30)	2 m	4	Oral antidiabetics and antihyperlipidemics	Vs. placebo: ↓FPI, ↓LDL-C, TAG, and HOMA-IR, ↑QUICKI, and no significant change in FPG, TC, HDL-C, and HbA1c	[28]
Zingiber officinale Roscoe/rhizome	Zingiberaceae	Ginger	1 g, TDS	Randomized, double-blind, placebo-controlled trial on diabetic patients treated with the plant (22) or placebo (23)	3 m	5	Oral antidiabetics	Vs. placebo: ↓FBS, FPI, HbA1c, and HOMA-IR, ↓CRP and MDA, and ↑TAOS and PON-1	[30]

Abbreviations: FBS: fasting blood sugar, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, TAG: triacyl glycerol, ADA: adenosine deaminase, CRP: C-reactive protein, HbA1c: glycosylated hemoglobin, AIP: atherogenic index of plasma, ox-LDL-C: oxidized LDL-C, BW: body weight, ASA: aspirin, MDA: malondialdehyde, GTT: 2-hour oral glucose tolerance test, TAOS: total antioxidant status, FMD: flow-mediated dilatation, ET: endothelin, AI: augmentation index, HR: heart rate, PP: pulse pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, IL: interleukin, TNF: tumor necrosis factor, PON-1: paraoxonase-1, ACE: angiotensin-converting enzyme, RI: reflection index, GSH: glutathione, SOD: superoxide dismutase, QUICKI: quantitative insulin sensitivity check index, ICAM: intracellular adhesion molecule, VCAM: vascular adhesion molecule, PAI: plasminogen activator inhibitor (the symbol * means the scientific name is not mentioned in the original article).

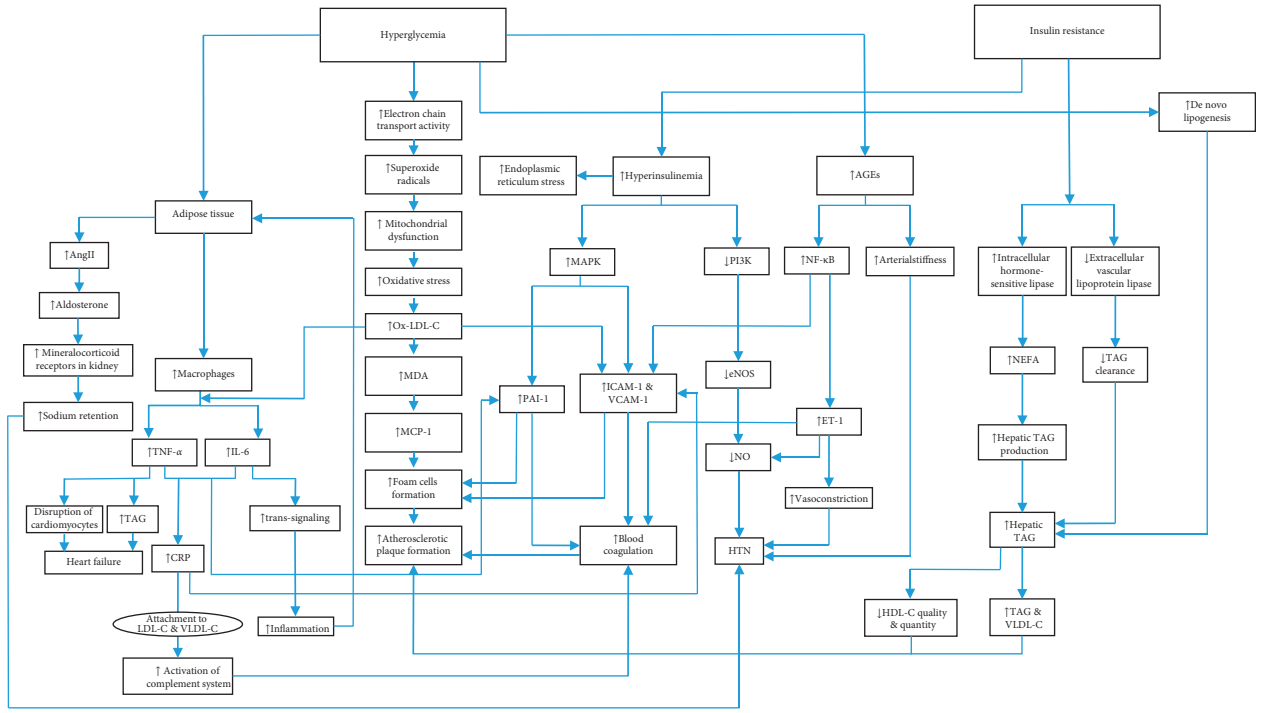

Figure 2: Therapeutic targets of bioactive foods and medicinal plants for the management of cardiovascular diseases in type 2 diabetic patients.

mentioned that, in addition to the shorter treatment period, the latter study used fiber-free extract, whereas the former study prepared the artichoke biscuits with the whole plant, containing fiber as well. This suggests the important role of dietary fibers in the clinical efficacy of artichoke.

3.2. Lipid Profile. Dyslipidemia is a common feature of T2DM with a prevalence of 37% to 56% [98]. The routine characteristics of diabetic dyslipidemia is hypertriglyceridemia in the form of elevated triacylglycerol-rich lipoproteins (TRLs), along with an increase in small dense low-density lipoprotein cholesterol (sdLDL-C) and a decrease in high-density lipoprotein cholesterol (HDL-C) [99]. TLRs are the result of fat digested from the foods as chylomicrons which contain apolipoprotein B48 (apoB48) or are released from the liver in the form of very-low-density lipoproteins (VLDL-C), containing apoB100 [100]. It seems that the reduction in CVD risk in diabetic patients via glycemic control is mostly due to the regulation of lipid profile rather than the glycemic profile itself [101]. Also, HDL-C is suggested to be an important factor which independently determines the risk of CVD in diabetic patients [102] since its quality and quantity are dysregulated even before the clinical diagnosis of diabetes [103]. In diabetic subjects, the level of intracellular hormone-sensitive lipase is augmented, while the extracellular vascular lipoprotein lipase is reduced due to insulin resistance or deficiency. The former results in the higher release of nonesterified fatty acids from adipose tissues which consequently increases hepatic production of triacylglycerol (TAG), whereas the latter causes reduced clearance of TAG from plasma; both finally result in the increased blood TAG level [103]. Increased blood sugar in T2DM elevates de novo lipogenesis from glucose [104]. Also, abnormal increase in insulin level along with insulin resistance causes disturbance in the production of hepatic sterols, endoplasmic reticulum stress, reduced apoB100 catabolism, and stimulation of VLDL-C production (especially $VLDL_1$ which is larger and contains higher amount of TAG) that results in elevated blood VLDL-C level [99]. On the other hand, abnormal structure of HDL-C, as well as a decrease in its production, causes the antiatherogenic activity of these particles to be reduced compared with that of normal subjects [99].

Considering the abovementioned importance of lipid levels in T2DM, it is not far-fetched that most studies assessing the effect of medicinal plants in T2DM evaluate the effect of the treatments on the lipid profile of patients. Garlic (*Allium sativum* L.) is amongst the most evident medicinal plants to regulate the lipid profile. The bulb contains sulfated compounds such as S-allyl cysteine, allicin, and alliin. When chopped, alliinase in garlic turns alliin to allicin [105]. Garlic is suggested to have an antihypertrophic effect on the heart via the elevation of hydrogen sulfide (H_2S) and NO [106]. Alliin has shown an inhibitory effect on 3-hydroxy 3-methyl glutaryl coenzyme A (HMG-CoA) reductase activity which is the same mechanism as statins [106]. Garlic also showed antihypertensive activity [107] as well as an inhibitory effect on adenosine deaminase (ADA) activity, an enzyme possibly involved in insulin resistance [108]. Some studies reported significant effect of garlic supplement with a dose of 500 mg/day [108] or 300 mg, twice daily [25, 26] in the regulation of lipid profile; whereas some others such as the study of Atkin et al. reported no such effect even with a higher administered

dose [27]. This might be due to the different design of the two studies as the patients in the latter study received conventional antidiabetics, as well. Also, the latter study assessed the effect of garlic for a shorter period of time which shows the need for longer treatment to achieve the therapeutic effect. Overall, garlic can be considered as an important dietary intervention to manage a series of cardiovascular complications such as HTN, hyperlipidemia, and atherosclerosis [109] which is also reported in T2DM patients; however, future studies are needed to determine the optimum dose and dosage form to achieve a satisfying therapeutic outcome. Ginger rhizome (*Zingiber officinale* Roscoe) is another medicinal plant which is also used as a popular spice in cooking. Three months of supplementation with ginger in T2DM patients could significantly decrease LDL-C and TAG and improve HDL-C. Ginger could also significantly reduce fasting plasma insulin and HOMA-IR, showing an overall improvement in lipid profile, as well as insulin resistance [28]. These results regarding the insulin resistance and TAG were also confirmed in another study by Arablou et al.; however, the reported data on the LDL-C, HDL-C, and FBS were somehow controversial [29]. While the former study reported a nonsignificant effect on blood glucose, HDL-C, and HbA1c, the latter study showed a significant decrease in FBS and HbA1c, as well as an increase in HDL-C which might be due to the longer period of supplementation. Ginger could also improve the biomarkers of oxidative damage and inflammation [30], suggesting the plant as a multipotential choice to manage CVD in T2DM. Gingerol and shogaol are two main active ingredients of the plant responsible for several pharmacological activities such as anti-inflammatory and vasorelaxant effects via the inhibition of prostaglandin and leukotriene synthesis. Also, ginger has demonstrated antiplatelet activity in animal studies which is another beneficial mechanism in CVD [110]; however, it should be noted that, in patients taking conventional antiplatelet agents, high doses of ginger may show a synergistic pharmacodynamic herb-drug interaction, thus, should be taken with caution.

3.3. Systemic Inflammation. The presence of inflammation in T2DM has been demonstrated years ago through cohort studies that revealed higher incidence of the disease in patients with higher level of acute-phase proteins such as C-reactive protein (CRP) and proinflammatory cytokines such as interleukin-6 (IL-6) in comparison to the subjects with normal values of these markers [111]. The increased level of inflammatory markers in T2DM is suggested to be mostly due to a general inflammatory status involving the whole body's immune system rather than a local inflammation of pancreas. Adipose tissue, abnormally hosting a large number of macrophages in obese subjects which produce tumor necrosis factor (TNF), and the liver, in which TNF-α- and IL-6-induced CRP is produced, seem to be the main participants in this process [112]. Inflammation is a common characteristic of T2DM and CVD. Overproduction of TNF-α and proinflammatory ILs results in cardiovascular events such as atherosclerosis. The importance of CRP in the prediction of CVD risk in T2DM is as high as LDL-C and HDL-C. On one hand, CRP attaches to LDL-C and VLDL-C particles and triggers blood coagulation via the activation of the complement system. On the other hand, it stimulates the production of soluble adhesion molecules, facilitating the formation of foam cells, and makes the endothelium of blood vessels prone to atherosclerotic plaque formation [113]. The deep involvement of CRP in cardiovascular events, along with its long half-life suggests this marker as an independent factor predicting CVD risk in T2DM [114]. Elevated level of TNF-α is also associated with increased risk of heart failure via the elevation of TAG, possibly due to stimulating VLDL-C production and disruption of cardiomyocytes [113]. In contrast, IL-6 seems to have a pleiotropic role in CVD which in not yet completely clarified; however, the mechanisms are somehow explained after the discovery of its two underlying signaling pathways, i.e., classic signaling and trans-signaling. While the activation of the former pathway results in anti-inflammatory effects and regulates the metabolism, the latter exacerbates inflammation during pathological conditions. The ratio of IL-6, soluble IL-6 receptor, and glycoprotein 130 (gp 130, a common signal transducer in the IL-6 family) determines the final results of IL-6 activation [115]. It is also demonstrated that IL-6 and TNF-α interaction plays an important role in endothelial dysfunction in the animal model of diabetes [116].

Some medicinal plants have been reported to be effective on the serum level of inflammatory markers in T2DM patients. *Panax ginseng* C.A. Mey. (Chinese ginseng or Korean ginseng) is a globally well-known medicinal plant with a wide spectrum of indications in traditional Chinese medicine (TCM). Administration of ginseng supplement to diabetic patients over a period of eight weeks significantly reduced the serum level of IL-6 and CRP in comparison to both baseline values and the placebo group [31]. One of the main classes of active ingredients of ginseng are triterpene structures called ginsenoside, several of which have demonstrated anti-inflammatory properties via the modulation of IL-6 production [117], and their beneficial effects in CVD are demonstrated in preclinical studies [118]. Another medicinal plant effective on the level of inflammatory markers is stinging nettle (*Urtica dioica* L.) which decreased IL-6 and CRP vs. placebo and TNF-α compared with baseline levels [32]. Another trial also demonstrated a higher level of NO in patients treated with nettle extract in comparison to placebo [33]. Based on the preclinical investigations, the antidiabetic effect of stinging nettle is attributed to its phenolic compounds and seems to be due to cytoprotective effects on the pancreas, α-glucosidase and α-amylase inhibition, and modulation of glucose transporter type 4 [119]; however, the exact subcellular mechanism of the plant in the modulation of inflammatory mediators needs to be further investigated.

3.4. Oxidative Stress. Oxidative stress is an inseparable part of T2DM and is closely related to the cardiovascular complications of this disease [120, 121]. High blood sugar

causes oxidative stress via the elevation of AGEs, glucose auto-oxidation, and hexosamine and polyol pathways induction, as well as mitochondrial damage due to overactivation of the electron chain transport system, resulting in the overproduction of superoxide radicals which are naturally deactivated by superoxide dismutase (SOD) enzyme [95, 122]; however, the excessively produced radicals directly destruct mitochondrial DNA. This damage causes a series of events leading to mitochondrial dysfunction and abnormal cellular energy expenditure, further inducing oxidative stress [95]. Oxidative stress in diabetic patients causes LDL-C particles turn into an oxidized form (Ox-LDL-C), evident from the serum level of malondialdehyde (MDA, a byproduct of lipid peroxidation) that consequently stimulates the production of monocyte chemoattractant protein-1 (MCP-1), a trigger for the formation of foam cells [121]. This oxidation process is partially prevented by paraoxonase-1 (PON-1), an esterase linked with apo A-1 of HDL-C particles, thus, plays a protective role against the development of atherosclerotic plaque formation [123]. PON-1 is also suggested to be a reliable marker for the prediction of atherosclerosis risk in T2DM [124]. Glutahtoine (GSH) is another part of the endogenous antioxidant defense mechanism which is altered in T2DM. It is demonstrated that the GSH level of erythrocytes and its absolute synthesis rate in diabetic patients are significantly lower than those of normal subjects, possibly due to nonglycemic mechanisms [125].

Punica granatum L. (pomegranate) is native to the Mediterranean region, especially Iran. The fruit juice is a rich source of two polyphenol subcategories, namely, anthocyanins and ellagitannins, with potent antioxidant and anti-inflammatory properties, as well as previously demonstrated beneficial effects in cardiovascular problems [126]. In a randomized, single-blind clinical trial, T2DM patients received a daily amount of 200 ml pomegranate juice for 6 weeks. Compared to the untreated group, pomegranate could significantly improve antioxidant status via the elevation of PON-1 and decrease in Ox-LDL-C, as well as its specific antibodies [34]. Shidfar et al. reported the significant effect of cranberry, another anthocyanin-rich fruit, toward the prevention of oxidative damage in T2DM via the improvement of PON-1 [35]. Grape seed extract, rich in proanthocyanidins, has demonstrated antioxidant properties in T2DM which was evident from the increased level of GSH [36]. It seems that anthocyanin-rich fruits are a reliable source of antioxidant components, especially in regard to improvement in PON-1 activity , thus, are clinically valuable to manage chronic oxidative damage in T2DM [127].

3.5. Blood Pressure. Hypertension (HTN) in T2DM is linked with a dramatic raise in the incidence of CVD so that a 10 mmHg decrease in systolic blood pressure (SBP) is accompanied with 11% and 13% lower risk of myocardial infarction and microvascular complications, respectively [128]. Also, T2DM patients with diastolic blood pressure (DBP) lower than 80 mmHg showed a lower risk of stroke and mortality compared with those with a DBP of 90 mmHg [129]. Chronic inflammation in T2DM causes endothelial dysfunction, evident from the reduction of flow-mediated dilatation (FMD), makeing an imbalance between endogenous vasodilators such as NO and vasoconstrictors such as endothelin-1 (ET-1). Also, inflammation and oxidative stress stimulate the production of antgiotensin II in adipose tissue which results in increased aldosterone, the activation of mineralocorticoid receptors in the kidney, sodium retention, and consequently, HTN [130, 131]. Furthermore, the production of AGEs due to high blood sugar causes stiffness of the arterial wall, further exacerbating HTN [9].

American ginseng (*Panax quinquefolius* L.) is grown in the North American continent. In a clinical trial in T2DM patients with well-controlled essential HTN, American ginseng ethanolic extract, standardized based on 10% of total ginsenosides, was administered to evaluate the vascular effects. The results showed a significantly lower SBP in the active group compared with placebo. Additionally, the augmentation index which is a noninvasive method for the evaluation of arterial stiffness was significantly reduced in patients treated with ginseng [37]. Preclinical studies suggest the inhibitory effect on glucotoxicity and endothelial dysfunction [132] and vascular smooth muscle cell proliferation [133] to be the possible vasculoprotective mechanisms of American ginseng. Barberry is another plant that demonstrated beneficial effects on both SBP and DBP in T2DM patients compared with the no intervention group [38]. This effect may be partly mediated by berberine, an alkaloid in barberry fruit, with previously demonstrated antihypertensive and vasodilatory properties in preclinical studies [134, 135]. Barberry fruit was also effective in reducing blood glucose and insulin, as well as lipid profile, which is also attributed to berberine [39]. Barberry fruit contains anthocyanins, a group of polyphenols, which may also contribute to the health benefits of the fruit; however, a clinical trial on the barberry stems containing 2.23% berberine as the major alkaloid [40] (but a possibly lower anthocyanin content) in T2DM patients further confirms the significant role of this alkaloid in glycemic and cardiovascular effects of barberry.

3.6. Endothelial Function. Endothelial dysfunction is suggested as an independent predictor of T2DM development risk. Adhesion molecules including intracellular adhesion molecule (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) are produced as a result of endothelial dysfunction. They are demonstrated to get increased years before the diagnosis of T2DM, and thus, they are not only the markers of vascular damage but also predict the occurrence of T2DM [136, 137]. High production of AGEs in T2DM activates nuclear factor-κB (NF-κB) which consequently stimulates gene expression of several factors, including ICAM-1, VCAM-1, and ET-1 [138]. Enhanced activity of ET-1 negatively affects the cardiovascular status of diabetic patients due to vasoconstrictor and procoagulant activities of ET-1, as well as a reduction in NO-mediated

vasodilation [139]. On the other hand, proinflammatory cytokines such as TNF-α and IL-6 trigger the coagulation process via the elevation of von Willebrand Factor (VWF) and plasminogen activator inhibitor-1 (PAI-1), resulting in a procoagulant state in the impaired endothelium. Furthermore, increased ox-LDL-C induces the production of proinflammatory cytokines, MCP-1, and adhesion molecules, all of which facilitate the process of atherosclerosis [138].

In a pilot-controlled trial, *Salvia miltiorrhiza* Bunge. was assessed in T2DM patients with coronary heart disease. The plant is commonly known as danshen and is a popular natural remedy for CVD in TCM. Two-month supplementation with danshen extract significantly decreased the level of VCAM-1 and VWF, demonstrating an improvement in endothelial function [41]. Salvianolic acids and diterpene structures called tanshinones are two major categories of danshen phytochemicals, both of which participate in beneficial effects of the plant in CVD [140]. Another study evaluated the vasculoprotective effect of ginkgo (*Ginkgo biloba* L.) on T2DM patients with nephropathy. Ginkgo, known as a living fossil, contains a mixture of flavonoids (ginkgoflavone glycosides) and diterpenes (ginkgolides and bilobalide) as the main active ingredients [141]. The plant has shown an inhibitory effect on ox-LDL-C-induced endothelial dysfunction in human umbilical vein endothelial cells [141]. Supplementation with ginkgo leaf extract could significantly attenuate the abnormally increased VWF and elevate NO level. Also, there were no significant differences in FBS, showing that the plant can be coadministered along with conventional antidiabetics without increasing the risk for hypoglycemia [42].

3.7. Anthropometric Parameters. The relationship between obesity and T2DM has been demonstrated in several studies. Recently published results of the ACCORD (Action to Control Cardiovascular Risk in Diabetes) study showed that T2DM patients with a body mass index (BMI) higher than 40 have the highest risk of nonfatal myocardial infarction and cardiac death [142]. Adipocytes secrete nonesterified fatty acids and hormones such as adiponectin and leptin which affect metabolism and insulin sensitivity. The imbalance between the release of these agents results in β-cell dysfunction and insulin resistance [143]. Molecular assessments also revealed that the gene expression of AGE ligands, NF-κB, and PI3K is dysregulated in obesity and T2DM [144]. Although obesity, in general, is known as a risk factor for T2DM, the distribution of body fat is a more important indicator of insulin resistance. It is suggested that waist circumference is the determinant of abdominal obesity and can be used to predict the risk of T2DM and CVD [145, 146]. Aside direct exacerbating effects of T2DM on CVD, the coexistence of obesity in these patients further worsens the condition since an accumulating body of evidence introduces obesity as one of the prime suspects of CVD [147].

Guar gum is a product of *Cyamopsis tetragonoloba* (L.) Taub. seeds and is a water-soluble polymer of galactose and mannose usually used in a partially hydrolyzed form due to the unpleasantly high viscosity of the original form [148]. Six-week supplementation with daily 10 g dose of guar gum could significantly reduce HbA1c and serum *trans*-fatty acids compared with baseline values. The plant could also significantly reduce waist circumference; however, no such effect was observed for body weight (BW) [43]. Previous studies have shown beneficial effects of guar gum in weight loss due to the gel-forming properties and decreasing gastric emptying speed, as well as antiappetite activity [148]; thus, lack of the slimming effect in this trial might be due to the short study period or low administered dose. Another plant with high content of water-soluble fiber which is assessed in regard to CVD risk factors in T2DM is *Avena nuda* L., commonly known as naked oat. In this dietary intervention, patients were instructed to replace one of the main foods of their regimen with a product providing 50 or 100 g of naked oat. One month of naked oat intake decreased BW, BMI, and waist circumference in comparison to the control group in a dose-dependent manner. Both lipid and glycemic profile were also improved during this intervention [44]. It can be inferred from the two studies that water-soluble fibers are one of the important categories of plant-based products able to help weight loss and glycemic/lipid profile regulation in T2DM patients; however, since they are mostly effective in high doses, they are suggested to be administered as dietary interventions.

4. Safety

Most of the studies included in this review reported no significant difference between the frequency of adverse effects in the active and passive groups, especially in case of dietary interventions, showing that the preparations were well tolerated. Most of the reported adverse effects were limited to transient low-grade gastrointestinal complications which disappeared after a while or by dose reduction. Also, some studies assessed the safety by measuring the biomarkers of hepatic and renal toxicity including aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP), as well as blood urea and creatinine, respectively. Most studies reported no significant change in the evaluated biomarkers except within the normal range. Taken together, herbal interventions are generally safe in regard to the adverse effects; however, some other considerations such as the possibility of herb-drug interactions via pharmacokinetic or pharmacodynamic interactions with the conventional antihyperglycemic and cardiovascular drugs are possible which should be taken into account [149, 150]. As an example, ginseng and garlic can increase prothrombin time, and if being administered to patients under treatment with aspirin or warfarin, the synergistic effect can result in abnormal bleeding [151]. Also, mucilaginous herbal materials, such as different gums, as well as resinous compounds, can delay/decrease the oral absorption of concomitantly used conventional drugs and consequently affect their pharmacokinetics; thus, it is recommended to

take such herbal supplements with a proper time interval with conventional drugs [152].

5. Discussion

The current paper reviewed recent advances regarding the effectiveness of plants as dietary interventions for the management of cardiovascular complications in T2DM. It is well understood that any individual plant can act via several mechanisms which suggest it as a multifaceted approach to target different pathways involved in the pathogenesis of CVD in T2DM.

Two of the most important mechanisms by which several medicinal plants could improve cardiovascular outcomes of diabetic patients are antioxidant and anti-inflammatory properties. Oxidative damage and inflammation can trigger each other due to a negative feedback loop so that the free radicals can induce inflammation, and inflammatory mediators increase oxidative stress in a chronic pathological condition such as T2DM. Discovering detailed cellular pathways which participate in the pathogenesis of cardiovascular damage is the Rosetta stone for decoding the fundamental therapeutic targets in CVD of T2DM patients. Plants are rich sources of secondary metabolites preventing the free radicals produced due to the oxidative damage in different tissues which is now demonstrated in clinical studies (Table 1). Although most of the included trials have focused on the lipid and glycemic profile which are classic outcomes to be assessed in CVD, recent studies have tried to also measure oxidative damage and inflammation biomarkers due to the growing evidence supporting the involvement of these two mechanisms in the primary stages of T2DM.

The most important category of plant-derived secondary metabolites with antioxidant activities are polyphenols (Table 2). Polyphenols comprise several subcategories including flavonoids, anthocyanins, lignans, and phenolic acid, each of which have numerous number of studies supporting their antioxidant and anti-inflammatory activities [17]. Curcumin, resveratrol, quercetin, and epigallocatechin gallate (EGCG) which are today widely used as antioxidant supplements all belong to different classes of polyphenols.

Another important category of phytochemicals are essential oil-derived compounds such as small terpenes and terpene alcohols. These compounds have demonstrated relaxing effects on the muscle cells, thus, can improve HTN via vasorelaxant effects on the smooth muscles of blood vessels [153, 154].

Fiber-containing plants are also significantly effective in controlling CVD risk factors in T2DM patients by slowing the absorption of dietary fats and improvement of anthropometric parameters. One of the positive points regarding this category of phytochemicals is that they are usually tasteless compounds, so they can be prepared as different enriched food products without affecting the original taste, providing higher patient compliance.

In addition to fibers, other herbal materials can also be provided as enriched foods, e.g., beverages, breads, biscuits, corn flakes, or other types of usually taken snakes. Plants with active ingredients resistant to heat can be added to baked products such as the preparation used in the study of Nazni et al. [23]. Anthocyanin-rich fruits such as barberry, pomegranate, and cranberry can be prepared as cold beverages with natural bright colors which can be easily taken as a daily routine. Such preparations with medicinal properties without having the appearance of a typical medicine are more welcomed by patients since diabetic patients usually receive lots of conventional medicines and thus the addition of a pack of healthy biscuits or a glass of natural juice would be more pleasant than another series of capsules/tablets.

Some of the abovementioned medicinal plants have strong evidence to support their beneficial effects in CVD of T2DM; however, some others have only limited data regarding their safety and efficacy. Also, some medicinal plants have controversial data obtained in different clinical trials. One of the obvious reasons explaining these controversial results is the difference between study duration and sample size. Some biomarkers need a specific minimal time to be changed as the results of an intervention which is not considered in some trials. For instance, HbA1c, as the gold standard of glycemic control, needs a 1.5 to 2 months of time to show the results of the intervention, whereas some clinical trials are designed for a shorter period of time, resulting in nonsignificant inter/intragroup difference which may be wrongly considered as a negative result [155].

The dosage and formulations are other important factors affecting the result of a study. Some phytochemicals such as curcuminoids in turmeric have highly lipophilic structures, causing a poor oral bioavailability; thus, bioavailability-enhanced formulations provide higher serum concentrations of the active ingredients and consequently better clinical outcomes. Another important reason which usually remains undiscussed is the different baseline characteristics of the selected patients. Several factors including the onset of T2DM, duration of previous pharmacotherapy to control the disease, and patient adherence to the prescribed medicines, as well as genetic factors such as the race and family history of T2DM, can cause different treatment responses to the same therapeutic interventions and should be carefully considered when comparing the outcomes of different trials on the same plant. Thus, negative results obtained in patients with a long history of T2DM, usually evident from the level of HbA1c, do not necessarily mean that the intervention cannot be effective in newly diagnosed patients [155].

The amount of main active ingredients in the herbal preparation is another factor which may affect the final results. As it has been discussed in several previous literature [156, 157], the amount of phytochemicals depends on several factors such as the time of harvest, storage condition, and extraction method. Lack of optimization of the production procedure results in nonuniform preparations which can affect the clinical outcome. The best way to solve this problem is to consider an optimum standardization procedure based on the major components of the plant. This can be based on a precise technique such as high-performance liquid chromatography (HPLC) or an easier but faster method such as colorimetric spectroscopic techniques.

Table 2: Major possible mechanisms attributed to different phytochemical categories as protective agents in cardiovascular complications of type 2 diabetes mellitus.

Phytochemical categories	Possible mechanisms
Flavonoids, anthocyanins, and other polyphenols	Antioxidant, anti-inflammatory, and cytoprotective properties
Volatile terpenes and terpenoids	Regulation of high blood pressure via vasorelaxant effects
Nonvolatile terpenes and terpenoids	Anti-inflammatory, antihyperglycemic, antihypertensive, and vasculoprotective properties
Fibers	Slowing the dietary fat and sugar absorption and improvement of anthropometric parameters
Sulfated compounds	Cytoprotective effects on cardiomyocytes, antihypertensive and antihyperlipidemic effects, and anticoagulant activities
Alkaloids	Antihypertensive and vasorelaxant properties

Announcing the level of main active compounds of the herbal preparations in the clinical trials can help comparing the quality of preparations in different studies and gives a more reliable judgement regarding the obtained results.

One of the limitations of our study is that the types of interventions were diverse; thus, the results cannot be subjected to statistical analysis since they do not fulfill the essential criteria for systematic reviews and meta-analysis. However, the collected information is valuable as a comprehensive interpretation of the current clinical evidence on the management of CVD in T2DM by medicinal plants. Another limitation is that only placebo-controlled trials were included in this paper. These criteria were considered in order to include fewer studies with more homogeneity so that different interventions can be compared with each other. There are several uncontrolled studies or studies on the comparison of an herbal intervention with a positive control which can also provide valuable data and may be the subject of future review articles on the same topic.

6. Conclusions

Overall, several plants are capable of improving the cardiovascular complications of T2DM and can be suggested as complementary therapies or dietary interventions along with conventional medicines. Active components of the clinically effective medicinal plants can also be used as new backbones to develop semisynthetic structures with higher potency and controlled profile of adverse effects for CVD. Further preclinical studies to clarify the exact cellular and subcellular mechanisms of these natural products, as well as well-designed clinical studies, are necessary to confirm the safety and efficacy of plant-based therapies for the management of CVD in T2DM.

Conflicts of Interest

Authors declare no conflicts of interest.

Authors' Contributions

RB performed the search, edited the tables, and wrote the main body of the manuscript. MF and RR designed the study, prepared the table, and edited the final manuscript. MR and SN prepared the tables and edited the final manuscript.

Acknowledgments

This study was partially supported by Tehran University of Medical Sciences (TUMS), Grant no. 96-04-86-37025.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6641031, 16 pages
https://doi.org/10.1155/2021/6641031

Review Article

Targeting miRNA by Natural Products: A Novel Therapeutic Approach for Nonalcoholic Fatty Liver

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Received 16 November 2020; Accepted 2 August 2021; Published 14 August 2021

Academic Editor: Mozaniel Oliveira

The increasing prevalence of nonalcoholic fatty liver disease (NAFLD) as multifactorial chronic liver disease and the lack of a specific treatment have begun a new era in its treatment using gene expression changes and microRNAs. This study aimed to investigate the potential therapeutic effects of natural compounds in NAFLD by regulating miRNA expression. MicroRNAs play essential roles in regulating the cell's biological processes, such as apoptosis, migration, lipid metabolism, insulin resistance, and adipocyte differentiation, by controlling the posttranscriptional gene expression level. The impact of current NAFLD pharmacological management, including drug and biological therapies, is uncertain. In this context, various dietary fruits or medicinal herbal sources have received worldwide attention versus NAFLD development. Natural ingredients such as berberine, lychee pulp, grape seed, and rosemary possess protective and therapeutic effects against NAFLD by modifying the gene's expression and noncoding RNAs, especially miRNAs.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the chronic liver diseases nowadays which threatens human health leading to liver dysfunction. NAFLD is caused by the abnormal accumulation of fat (more than 5% of liver weight), especially triglycerides, in the liver of people who are not addicted to alcohol [1, 2]. As this disease's incidence has a close association with lifestyle, it is more common in developed countries. In the absence of proper treatment, this disease can progress, and nonalcoholic steatohepatitis (NASH) could be created [3]. NASH also can increase the risk of cirrhosis and hepatocellular carcinoma (HCC) due to environmental conditions and genetic factors [4].

NAFLD is a multifactorial disease that initiates and develops due to the interactions between various risk factors. In addition to diet, hyperlipidemia, diabetes mellitus, environmental factors, obesity, insulin resistance (IR), and genetic factors such as gene expression or single-nucleotide polymorphism (SNP) have a crucial role in the formation of NAFLD [1, 5]. The environmental risk factors include air pollution, water or food pollution, and chemical materials. The most significant air pollution is particles less than 2.5 micrometres that are harmful to human health and are involved in the pathogenesis of NAFLD through nuclear

factor-κB (*NF-κB*), c-Jun *N*-terminal kinase- (*JNK*-) activator protein 1 (*AP1*), and toll-like receptor 4 (*TLR4*) activation [5, 6]. Furthermore, the principal food or water pollution and chemical risk factors that include aflatoxin, trichloroethylene, pesticides, heavy metals, and trihalomethanes also have a significant impact [5, 7].

Moreover, many genetic and epigenetic risk factors performed crucial roles in the susceptibility and progression of NAFLD. Recent studies have shown that a wide variety of modifications include copy number variation, structural variation, genes, long noncoding RNAs (lncRNAs), or microRNA expression changes in NAFLD [8]. Some of these genes (lipid biosynthetic regulating transcription factors, nuclear receptors, fibrogenesis, and inflammatory response factors) are involved in NAFLD genetic susceptibility [9], for instance, the NLR family pyrin domain-containing 6 (*NLRP6*), NLR family pyrin domain-containing 3 (*NLRP3*), and interleukin-18 (*IL-18*) genes' expression or SNPs in patatin-like phospholipase domain-containing protein 3 (*PNPLA3*-rs738409 and rs6006460) [2, 10]. Transmembrane 6 superfamily member 2 (TM6SF2-rs58542926) and membrane-bound O-acyltransferase domain-containing 7 (*MBOAT7*-rs641738) genes play essential roles in the initiation or progression of this disease [11]. Noncoding RNAs (ncRNAs) such as microRNAs (miRNAs) and LncRNA could not encode any protein and transcribe from the cell genome. MicroRNAs play essential roles in regulating the cell's biological processes, such as apoptosis, migration, lipid metabolism, insulin resistance, and adipocyte differentiation by downregulating its targeting genes [12, 13]. According to this, gene structure changes (epigenetic modifications such as methylation and acetylation) or gene expression changes are among the NAFLD causes. Therefore, miRNAs' role in the biogenesis, pathogenesis, development, and progression of this disease is not unusual [14, 15].

Natural products (NPs) are chemical agents derived from living organisms such as bacteria, fungi, plants, and animals. Research has shown that some of these substances have therapeutic effects and could be used as pharmacological agents to treat various diseases [16]. Nowadays, more than 80% of medicines are made of natural compounds [17]. Subsequently, with the development of molecular techniques, it has been found that one of the beneficial effects of these natural products on the disease is through modifying the expression of the gene and noncoding RNAs, especially miRNAs. These substances produce epigenetic changes in the cell by modification of histones and DNA. As a result, an increase or decrease in the expression of some genes or miRNAs occurs [18]. Thus, using natural products could adjust miRNA profiles, inhibit metastasis, increase drug susceptibility, inhibit cancer progression, and treat or prevent many diseases such as NAFLD [19]. As identified, some of these compounds, such as ellagitannin, resveratrol, curcumin, genistein, and epigallocatechin-3-gallate, could inhibit proliferation, induce apoptosis, and modify cell behaviour through the effect on miRNAs' expression profiles [20].

The aim of this study is to investigate the usefulness of natural agents (as an essential factor in creating epigenetic changes) in the regulation of miRNA for the treatment of nonalcoholic fatty liver disease. We expect that this would provide new plans in NAFLD therapies by using natural products.

1.1. A Brief Overview of miRNAs. Noncoding RNAs (ncRNAs) are groups of RNAs that do not synthesize any proteins. In general, according to their length, ncRNAs are divided into two groups. Small ncRNAs are less than 200 nucleotides, and long ncRNAs consist of more than 200 nucleotides. Small ncRNAs consist of small nuclear RNAs (snRNAs), PIWI-interacting RNAs (piRNAs), transfer RNAs (tRNAs), and miRNAs. Among ncRNAs, microRNAs (miRNAs) are the most studied molecules to date [21]. miRNAs are highly conserved small noncoding RNAs involved in regulating the expression of approximately 60% of mammalian genes at the posttranslation level by multiple mechanisms [22]. These molecules are located on the intergenic, exonic, or intronic regions of all human chromosomes, except for the Y chromosome [20].

Lin-4 was the first miRNA discovered by Victor Ambros in 1993 that targeted the 3′ UTR of lin-14 mRNA from *Caenorhabditis elegans*. After many studies and the advancement of human knowledge, the number of known molecules has increased dramatically. Today, over 48860 mature microRNAs and 38589 hairpin precursors from 271 organisms are included in the miRBase database [23, 24]. In vitro and bioinformatics studies have shown that many miRNAs can target three prime untranslated regions (3′ UTR) of a single gene. In contrast, miRNA solely can bind to the 3′ UTR of several genes and regulate their expression [25].

Moreover, based on their function, miRNAs are divided into two groups. The first group is oncomiR, which is overexpressed in diseases or cancers and inhibits genes that are essential for human health. The second group is tumour suppressor miRNAs that are downregulated or silenced in cancers and illnesses. As a result, their inhibitory trait is removed from the oncogenic signalling pathways [18]. miRNAs are affected by genetic and epigenetic modifications occurring in the cell genome. For instance, any agent that leads to DNA methylation of the loci of miRNAs could reduce miRNA expression; in contrast, the region's demethylation increases miRNA expression [26]. Therefore, by modifying the methylation or demethylation of genes responsible for the generation or improvement of NAFLD through various materials or methods, such as natural products, we can catch a basic level to manage this illness [24].

miRNAs could bind to mRNA 3′ UTR through their seed region. The seed position is in the 5′ untranslated region (5′ UTR) of miRNAs between nucleotides 2 and 8. Since this region is not more than a few nucleotides in length, its complementary sequence may be present in several different mRNAs so that miRNA may target several other genes [27]. It has also been shown that miRNA may have more than one complementary site on the 3′ UTR of its target gene. Therefore, miRNA's effect on the gene is more significant than miRNA by only one complementary binding site [28].

Although these single-strand RNAs (18–28 nucleotides) (miRNAs) have an essential role in regulating and controlling the biological activities of healthy cells, they are also involved in the development and pathogenesis of various diseases, especially cancer. To date, there have been numerous studies showing that miRNAs are involved in proliferation, differentiation, apoptosis, and cell migration [19, 29].

The miRNAs' biogenesis is initiated at the nucleus by RNA polymerase II. First, miRNAs are transcribed as primary miRNA (pri-miRNA) by several thousand nucleotides. Then, pri-miRNA is cleaved by Drosha and DGCR8 to produce pre-miRNA (70–100 nt) [30]. miRNA is then transported from the nucleus to the cytoplasm by RanGTP and exportin 5. Dicer binds to it in the cytoplasm, performs secondary cleavage, and produces double-stranded RNA (mature miRNA) [31]. After mature miRNA is inserted into the RNA-induced silencing complex (RISC), it could target 3′ UTR mRNAs of the target genes [32].

Nevertheless, some reports indicated that miRNAs could bind to 5′ UTR of the target gene in addition to the 3′ UTR [15]. For example, hsa-miR-24-3p, miR-34a, miR-US25-1, and miR-103a-3p could target 5′ UTR of the c-Jun activation domain-binding protein 1 (Jab1), axis inhibition protein 2 (AXIN2), human cytomegalovirus (HCMV), and GPRC5A, respectively [33, 34]. miRNAs can increase the stability or expression of target genes by binding to 5′ UTR of theirs. For example, miR-10a or miR-122 increases the expression and strength of ribosomal protein mRNAs and RNA of the hepatitis C virus, respectively [35, 36].

miRNAs could regulate gene expression in both direct and indirect methods. Instantly, they destroy or inhibit the translation of target mRNA. Nevertheless, indirectly, they could inhibit upstream genes that are the inhibitor, activator, or transcription factor for the target gene [37, 38]. Alterations of miRNAs have been reported in a wide range of diseases, including human pathological liver conditions. Previous studies showed that these molecules have an essential role in NAFLD's pathogenesis and could apply as a potential therapeutic target [4].

1.2. MicroRNAs as a Therapeutic Target for NAFLD. Liver biopsy is the standard method for the diagnosis of NAFLD. However, this method is an invasive procedure that can cause many problems and discomfort for patients, especially children and the elderly. Therefore, attaining a new and noninvasive method such as serum biomarkers (may be miRNAs) and ultrasound and imaging techniques is essential to detect it before clinical symptoms occur [39]. By regulating the expression or activity of many genes, miRNAs play a crucial role in regulating lipid metabolism in the liver [40]. There is a close relationship between NAFLD and the expression profile of miRNAs. It has been reported frequently that these molecules' expression changes in animal models and NAFLD/NASH patients.

Furthermore, miRNAs can simultaneously affect several genes from different molecular and signalling pathways (glucose metabolism, lipid metabolism, inflammation, and oxidative stress). This finding shows the significance of miRNAs as therapeutic target biomarkers [24, 41, 42]. In addition to body tissues, miRNAs are significantly present in body fluids such as serum, saliva, plasma, and urine to be used for early detection, prognosis, or treatment monitoring of diseases [43].

Circulating miRNAs or exosomal miRNAs are resistant to RNAse degradation due to their complexation with lipids, proteins, or lipoproteins. These molecules are found in the body fluids due to cellular damage or cell secretion and are detectable by available methods [44]. Exosomal miRNAs or exomiRs play fundamental functions in cellular communications by the entrance to the intercellular space. They are sometimes tissue-specific, and their expression changes in pathological conditions. Therefore, they have this potential to be employed as a molecular marker for NAFLD [45, 46].

Many studies have been performed to determine the relationship of circulating miRNAs with NAFLD as a novel potential biomarker. In this regard, miRNAs which are most important include miR-99a-3p, miR144-3p, miR-200b-5p, miR-200-3p [47], miR-34a, miR-122, miR-16 [48], miR-21, and miR-451 [49]. Some of these miRNAs, such as miR-34a-5p, miR-375, and miR-301a-3p, could also indicate severity from NAFLD [50].

Moreover, in one study, 84 circulating miRNAs were analyzed to determine which of them are associated with NAFLD. The results showed that some miRNAs (miR-192, miR-122, miR-19a/b, miR-375, and miR-125b) are overexpressed in the steatosis sample. It further supposed a strange relationship between miRNA-192, miR-122, and miR-375 and nonalcoholic steatohepatitis (NASH) [51]. It has been shown that improper nutrition can lead to obesity by altering 6% miRNAs [52]. Here, we discuss if particular miRNAs are correlated by the progression of NAFLD in humans.

1.3. miR-34a. One of the miRNAs that increased in high-fat-fed mice is miR-34a. Human studies have likewise shown that this miRNA is closely associated with metabolic syndrome and NASH severity. Moreover, its expression in the tissue and serum of NAFLD/NASH patients increased [4, 53]. The role of miR-34a in NAFLD's pathogenesis is due to the development of lipid accumulation in hepatocytes and decreasing fatty acid B-oxidation through inhibition of the Sirtuin 1 (*SIRT1*) gene [54]. miR-34a could indirectly reduce peroxisome proliferator-activated receptor alpha (*PPAR-α*) gene activity and also could enhance sterol regulatory element-binding transcription factor 1c (*SREBP-1c*), peroxisome proliferator-activated receptor-*γ* coactivator-1*α* (*PGC-1α*), and farnesoid *X* receptor (*FXR*) gene activity. Thus, by impairing metabolic sensors, NAFLD-associated lipids' metabolism will be deregulated [55, 56]. Additionally, restraint of the *SIRT1* gene expression could activate proapoptotic genes such as *P53* and Src homology 2 domain-containing- (SHC-) transforming protein 1 isoform p66Shc variant (*P66SCH*), thereby increasing the susceptibility of hepatocyte cells to apoptosis and oxidative stress [57, 58]. Therefore, downregulation of miR-34a leads to the rational

expression of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*), *PPAR-α*, and *SIRT1* genes and improves the steatosis [56].

1.4. miR-33a/b. Another miRNA that plays an essential role in fatty liver disease by influencing lipid metabolism is miR-33. In humans, this miRNA has two members (miR-33a and miR-33b) that are located in the intron region of the sterol regulatory element-binding transcription factor 2 (*SREBP2*) and sterol regulatory element-binding transcription factor 1 (*SREBP1*) genes, respectively. These two genes play fundamental roles in controlling cholesterol and lipid synthesis [59, 60]. Moreover, these miRNAs play a vital role in regulating insulin signalling, de novo lipogenesis, triglyceride accumulation, and fatty acid oxidation. Consequently, inhibition of their expression or function could increase fatty acid oxidation, insulin sensitivity, elevated serum HDL, and decreased serum VLDL [61, 62]. Sirtuin 6 (*SIRT6*) and insulin receptor substrate 2 (*IRS-2*) are the target genes of miR-33, which play an essential role in controlling glucose metabolism. In vivo and in vitro studies suggest that overexpression of miR-33 plays a critical role in NAFLD development by affecting lipids and carbohydrate metabolism [41, 60, 61].

1.5. miR-155. One of the miRNAs that was downregulated in the serum and liver tissue of NAFLD patients is miR-155. Overexpression of miR-155 by any instruments led to a decrease in the expression of lipid metabolism-related genes such as *SREBP1*, liver *X* receptor (*LXR*), and fetal alcohol syndrome (*FAS*), thereby reducing intracellular lipid accumulation [63, 64]. Overall, the downregulation of the miR-155 expression level, with its crucial role in expanding adipose tissue mass, could facilitate NAFLD and obesity.

1.6. miR-451. Many studies have shown that the expression level of miR-451 decreased in high-fat-fed mice (HFD), hepatocyte- (HepG2-) treated cells with palmitic acid, and patients with NASH symptoms. As miR-451 is a negative regulator for proinflammatory cytokines (such as tumour necrosis factor-*α* (*TNF-α*), interleukin-8 (*IL-8*), and *NF-κB p65* subunit), its downregulation enhances the excretion of these cytokines through the AMP-activated protein kinase (AMPK)/AKT pathway [65]. Therefore, another proper objective for NAFLD treatment is the upregulation of miR-451 by natural products. The downregulation of the miR-451 level could facilitate NAFLD and obesity due to its significant role in expanding adipose tissue mass.

1.7. miR-375. Another miRNA that is considered for molecular targeting in the treatment of NAFLD is miR-375. This miRNA expression increased in the liver and serum of NAFLD patients and high-fat-fed mice, respectively, compared with steatosis samples and the control group. MiR-375 is a crucial regulator of glucose homeostasis, so its downregulation leads to a reduction in the amount of adiponectin receptor 2 (*AdipoR2*), interleukin-6 (*IL-6*), leptin, and *TNF-α*, ultimately reducing the lipid accumulation [51, 66].

1.8. miR-192. The miR-192 expression is altered in fatty liver disease. This miRNA has a profibrogenic power and decreases NASH. Nevertheless, its serum expression increased due to hepatocytes' secretion during the NASH's pathophysiological stages [67, 68].

1.9. miR-27a/b. miR-27a/b is one of the molecules that plays an essential role in abiogenesis. Its overexpression inhibits *SREBP1* and *FAS* genes and increases lipolysis, secretion of free fatty acids, and glycerol from the cell [69, 70].

1.10. miR-24. Another miRNA that increased in animal or human models of NAFLD is miR-24. As previously shown, miR-24 overexpressed in the HepG2 cell line was treated by fatty acids and in the liver of high-fat-fed mice. This miRNA could increase lipid accumulation in the hepatocytes by targeting the insulin-induced gene 1 (*Insig1*) gene that is an inhibitor of lipogenesis [53, 71]. Therefore, miR-24 inhibitors could play a crucial role in improving NAFLD.

1.11. miR-149. Studies have shown that there is a close relationship between NAFLD and miR-149 expression. For example, the expression of this miRNA in NAFLD mice and fatty acid-treated HepG2 cell lines increased. miR-149 leads to increased lipid accumulation and lipogenesis by inhibiting the fibroblast growth factor 21 (*FGF-21*) gene. Consequently, the downregulation of miR-149 and upregulation of *FGF-21* by pharmacological methods can enhance lipid metabolism and could improve NAFLD [72, 73].

1.12. miR-21. The expression of miR-21 in NAFLD patients' serum and liver tissue is different from that of healthy controls. This miRNA expression is increased in patients' hepatocytes, whereas serum expression is lower than in the control group [49, 67]. Nutrition plays a vital role in miRNA expression; previous studies have shown that miR-21 increased in the liver of high-fat mice and fatty acid-treated HepG2 cells. miR-21 affects lipogenesis, NAFLD, and cancer induction by its inhibitory effect on human polybromo-1 (*HPB1*) and *PPAR-α* [74, 75].

1.13. miR-122. miR-122 is the most abundant hepatic miRNA. It considers for approximately 70% of miRNAs expressed in that tissue and plays a fundamental role in the maturation, differentiation, and proliferation of hepatocytes [76, 77]. Serum level of miR-122 is associated with liver fibrosis in NAFLD patients, and its accuracy in describing NAFLD severity is higher than liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [67]. Another notable feature of this biomarker is that its expression in NASH samples is approximately 7.2 and 3.1 fold, respectively, compared to healthy and steatosis samples [51]. However, there is a difference between serum and liver

expression of miR-122 in NAFLD individuals. In other words, miR-122 tissue expression decreased in NAFLD/NASH patients compared to controls, but in contrast, its serum expression was upregulated in NAFLD/NASH patients [4, 78]. The increased serum level is that hepatocytes' destruction releases it in the fluid between the cells and blood [79]. Therefore, it can be used as a biomarker for noninvasive diagnosis of fibrosis development and liver injury. Studies revealed that mice lacking expression of this miRNA showed an increase in lipogenesis, lower serum cholesterol/triglyceride levels, fibrosis enhancement, NASH, and hepatocellular carcinoma, in addition to increased expression of TNF-α, IL-6, and C-C motif chemokine ligand 2 (CCL2) genes [80, 81]. Further investigations showed that miR-122 increased the expression of acetyl-CoA carboxylase (*ACC1*), diacylglycerol O-acyltransferase 2 (*DGAT2*), *FAS*, and *SREBP1* genes that play essential roles in de novo lipogenesis [40, 76, 82].

1.14. miR-185. miR-185 is another miRNA involved in regulating the insulin signalling pathway, cholesterol homeostasis, and lipid metabolism that is downregulated in fatty acid-treated HepG2 cells (palmitic acid). Furthermore, overexpression of miR-185 in the C57BL/6 mouse model of NAFLD (high-fat diet mice) has been shown to decrease liver steatosis and increase insulin sensitivity. Molecular studies have also demonstrated that miR-185 performs its function by inhibiting the expression of lipid metabolism-related genes, including the *SREBP1c*, *HMGCR*, *FAS*, and *SREBP2* genes, and induces insulin sensitivity by promoting the phosphatidylinositol-3-OH kinase (PI3K)/AKT2 pathway via enhancing IRS-2 gene expression [83]. Therefore, it is one of the potential therapeutic targets in the NAFLD and could be overexpressed using dendrosomal curcumin [25].

Due to miRNAs' function in regulating different biological pathways, these molecules are considered therapeutic targets nowadays. Since these miRNAs could regulate the gene expression, up-/downregulation is a beneficial mechanism for preventing or treating various diseases [84]. The activity and function of miRNAs can be modified using competing endogenous RNAs (ceRNAs) such as lncRNAs and circular RNAs (circRNAs). Numerous studies have shown that one or more miRNAs' expression can be altered using natural products [20].

1.15. Is Natural Product Clinically Useful in NAFLD? NAFLD's current pharmacological management, including drug and biological therapies, is expensive, possesses temporary relief, and has some adverse effects. Numerous clinical studies confirmed the ability of natural products in the management of NAFLD. In this context, various dietary fruits or medicinal herbal sources have received worldwide attention versus NAFLD development. This section represents the advantages of natural products in the management of NAFLD in human studies.

In a randomized, single-blind clinical trial, Shidfar et al. investigated the effects of extra virgin olive oil on the severity of steatosis in NAFLD patients on a weight loss diet. Fifty patients (19 women and 31 men) with nonalcoholic fatty liver were randomized to receive the hypocaloric diet enriched with olive oil (olive oil group) or the hypocaloric diet with normal fat (control group) for 12 weeks. It was found that ALT and AST levels significantly decreased in the olive oil group than in the control group. The result showed that consuming a diet containing olive oil enhances weight loss's beneficial effects and improves liver enzymes' level [85].

Because curcumin significantly improves lipid-modifying hepatic steatosis, a clinical study was reviewed to evaluate its efficacy in patients with NAFLD. In a randomized, double-blind, placebo-controlled trial, Rahmani et al. studied the effect of curcumin on the liver fat content on forty patients in the curcumin group (70 mg) and forty patients in the placebo group for eight weeks. Curcumin showed a significant decrease in serum levels of total cholesterol, liver fat content, triglycerides, body mass index, aspartate aminotransferase, and alanine aminotransferase [86]. In another work, Panahi et al. investigated the effects of curcumin on the metabolic profile in 87 patients with grades 1–3 of NAFLD. After eight weeks, the result confirmed that 1000 mg/day curcumin supplementation could decrease uric acid ($p < 0.001$) and serum lipids ($p < 0.001$) compared to the placebo group [87].

Coenzyme Q10 (CoQ10) is a natural compound that could positively affect the inflammatory status, the grade of hepatic steatosis, and liver enzymes' activity in patients with NAFLD. In a randomized, double-blind, placebo-controlled trial, 41 patients with NAFLD were divided into two groups and treated daily with 100 mg of CoQ10 capsule (intervention group) or placebo (placebo group) for three months. Results showed that the intervention group, compared to the control group, had a significant decrease in the grade of hepatic steatosis and serum levels of high-sensitivity C-reactive protein (*hs-CRP*), *AST*, gamma-glutamyl transpeptidase (*GGT*), and *TNF-α* ($p < 0.05$) [88].

In another study, Abidov et al. considered 151 volunteers (113 patients with NAFLD and 38 volunteers with normal liver fat). They treated them three times a day for 16 weeks with Xanthigen (300 mg brown seaweed extract containing 2.4 mg fucoxanthin + 300 mg pomegranate seed oil). The results showed that Xanthigen could significantly reduce body weight and body fat content in both groups and decrease waist circumference, liver fat content, and liver enzymes only in the NAFLD group. Hence, this natural drug has a promising function in obesity management [89].

Resveratrol is a natural compound whose therapeutic effect on NAFLD has been evaluated in many clinical trials. A previous study with forty patients showed that using this supplement (500 mg per day) for 12 weeks with adequate physical activity could significantly reduce steatosis grade, mass index, weight, waist circumference, and liver enzymes compared to the placebo group (medium-chain triglyceride) ($p < 0.05$). Resveratrol supplementation also significantly reduced inflammatory markers such as *IL-6*, *hs-CRP*, and *NF-κB* and hepatocellular apoptosis compared with the placebo group ($p < 0.05$). Furthermore, resveratrol

supplementation with lifestyle modification was more effective than lifestyle modification alone [90].

Soy milk is another substance that affects the metabolic characteristics of NAFLD patients. The results of its eight-week consumption in 66 NAFLD patients showed that soy milk consumption (240 ml per day) could significantly diminish the level of serum insulin ($p = 0.04$), homeostasis model assessment of insulin resistance ($p = 0.03$), and blood pressure ($p = 0.04$). It can also increase the quantitative insulin sensitivity check index (*QUICKI*) ($p = 0.04$) compared to the control group [91]. The results of a randomized clinical trial conducted by Eslami et al. showed that 8-week administration of soy milk with a low-calorie diet in 70 patients could significantly reduce serum ALT and *hs-CRP* compared with the control group, which followed only a low-calorie diet ($p < 0.05$) [92]. Kani et al. conducted a parallel randomized clinical trial on 45 patients with grades 1 and 2 of NAFLD. They evaluated the efficacy of soy nut consumption on the serum leptin and inflammation level. Low-calorie diet, low-calorie low-carbohydrate diet, and low-calorie low-carbohydrate soy-containing diet (30 grams of soy nuts instead of 30 grams of red meat) were three groups, in which patients were randomized. After eight weeks, the results demonstrated a significant difference in reducing systolic and diastolic blood pressure, glycemic indices, fasting blood sugar (*FBS*), *hs-CRP*, and serum insulin level in the low-calorie low-carbohydrate soy-containing diet group compared to low-calorie or low-calorie low-carbohydrate diets. It has been found that these diets can move patients in grade 2 to grade 1, and the disease of some patients improved completely [93].

In a randomized controlled clinical trial, Gheflati et al. assessed the efficacy of purslane seeds in 54 individuals with NAFLD. Eight-week consumption of the purslane seeds (10 g/day) along with a low-calorie diet significantly reduced serum concentrations of low-density lipoprotein cholesterol (*LDL*), *FBS*, *QUICKI*, and total cholesterol, compared with the control group (only the low-calorie diet) ($p < 0.05$) [94].

Zataria multiflora (ZM) is a thyme-like plant, a member of Lamiaceae family. A clinical trial evaluated the effect of *Zataria multiflora* in 85 patients with NAFLD. Patients were randomized to receive 700 mg plant powder ($n = 45$) or placebo ($n = 40$) twice daily for 12 weeks. Results revealed that *Zataria multiflora* could significantly reduce insulin resistance, insulin serum level, and blood pressure compared to the placebo group. However, there was no significant difference between *hs-CRP*, *TNF-α*, the grade of the fatty liver in ultrasonography, ALT, and other outcomes in the two groups [95].

Evaluation of the potential therapeutic effect of silymarin and vitamin E on liver tissue improvement in 36 NAFLD patients showed that daily intake of two tablets of silymarin (540.3 mg) and vitamin E (36 mg) with a hypocaloric diet for three months improved the noninvasive NAFLD index [96].

Previous studies demonstrated that ancient *Triticum turgidum* ssp. *turanicum* (Khorasan wheat), which is commercially known as Kamut, has a beneficial effect on human health. The results of comparing the treatment of Kamut to the control group (wheat products) in NAFLD patients with moderate liver steatosis showed a significant reduction of ALT, AST, alkaline phosphatase (ALP), and cholesterol in the Khorasan group ($p < 0.05$). Finally, Kamut could significantly improve the liver steatosis grade by reducing circulating proinflammatory *TNF-α*, *IL-8*, interferon-gamma (*IFNγ*), and the interleukin-1 receptor antagonist (*IL-1RA*) [97].

2. Modulation of miRNA Levels by Natural Products

Nowadays, plant secondary metabolites as multiple target compounds are widely used in disease treatment with complex pathogenesis. These compounds exert their therapeutic and pharmacologic effects by regulating the gene expression of critical signalling pathways [98, 99]. Another prominent mechanism of secondary metabolism is up- and downregulation of miRNA [100]. Various studies showed that the anticancer, anti-inflammatory, antihypercholesterolemic, antidiabetic, cardioprotective, and neuroprotective effects of alkaloids, flavonoids, coumarins, terpenes, iridoids, cardiac glycosides, and isothiocyanates are related to the regulation of miRNAs' expression [101–106]. For example, several investigations showed that berberine, an isoquinoline alkaloid isolated from different Berberidaceae and Ranunculaceae family species such as *Berberis vulgaris* and *Coptis chinensis*, regulated different miRNAs' expression [107–109]. Lu et al. reported that berberine suppressed microRNA-21 expression in the human colorectal cancer cell line (*HCT116*) at 100 μM [110]. Also, osthole (20 mg/kg, twice a day, for six weeks) as prenylated coumarin showed its anti-Alzheimer's effects due to the increased miRNA-101a-3p expression in the cortex and hippocampus of mice [111]. Polyphenols are other phytochemicals that have regulation activity on miRNAs' expression [112]. Apigenin (4′,5,7-trihydroxyflavone) is a flavonoid, isolated from several genera including *Artemisia, Matricaria, Teucrium, Petroselinum, Apium,* and *Achillea* [113, 114], that has been shown to inhibit miRNA-103 expression at 40 mg/kg dose for 14 days intraperitoneally (i.p) in transgenic mice. The suppression of miRNA-103 ameliorated insulin sensitivity and glucose tolerance [115]. Downregulation of miRNA-29a has critical roles in various tumour and inflammatory diseases such as atherosclerosis, cholestasis, pediatric liver disease, and thoracic aneurysms [116]. Gracillin is a steroidal saponin that is mainly separated from *Dioscorea* spp. (Dioscoreaceae) [117]. This compound (10 mg/kg, i.p, 7 days) showed cardioprotective and anti-inflammatory effects due to an increase in the expression of miRNA-29a that had been suppressed by lipopolysaccharide (LPS, 10 mg/kg, i.p) in mice cardiomyocytes [118]. Antrocin [119], benzyl isothiocyanate [120], capsaicin [121], curcumin [122], quercetin [123], genistein [124], ginsenoside [125], emodin [126], oleuropein [127], resveratrol [128], and other secondary metabolites that could affect the regulation of miRNAs' expression and their natural sources are shown in Table 1.

Table 1: Some secondary metabolites which show the pharmacological effects through the regulation of miRNAs.

Category structure	Secondary metabolite	miRNAs	Pharmacological results	Natural source*	Ref.
Alkaloids	Berberine	↑miRNA-101	Blocking endometrial tumour growth and migration, *in vitro*, human endometrial cancer cell lines (AN3CA and HEC1A), 10, 30, and 100 μM, 24 hours (h)	*Coptis chinensis* = *Coptidis Rhizoma* (Ranunculaceae), *Berberis vulgaris* (Berberidaceae)	[108]
		↓miRNA-122	Improving lipid hemostasis and hepatic gluconeogenesis, *in vivo*, diabetic mice, 40 mg/kg, 160 mg/kg, oral administration, 4 weeks	*Coptis chinensis* (Ranunculaceae), *Berberis vulgaris* (Berberidaceae)	[129]
		↑miRNA-203	Increasing the chemotherapy response of two cisplatin-resistant gastric cell lines 7901/DDP and BGC-823/DDP, *in vitro*, 10 μM, 48 h	*Coptis chinensis* (Ranunculaceae), *Berberis vulgaris* (Berberidaceae)	[130]
	Rhynchophylline	↑miRNA-331-5p	Reducing ketamine dependence, *in vivo*, 60 mg/kg, i.p, 3 days	*Uncaria rhynchophylla* (Rubiaceae)	[131]
	Topsentin	↓miRNA-4485	Photoprotective effects, *in vitro*, human keratinocyte cell line (HaCaT), 2.5, 5, and 10 μM, 6 h	*Spongosorites genitrix* (Halichondriidae)	[132]
	Tetrandrine	↓miRNA-155	Antidiabetic effects, *in vivo,* 100 mg/kg, i.p, mice, 48 h	*Stephania tetrandra* (Menispermaceae)	[133]
	Nicotine	↓miRNA-99b, ↓miRNA-192	Carcinogenicity, *in vitro*, non-small-cell lung cancer cell lines NCI-H460 and A549, 100 μM, 48 h	*Nicotiana* spp. (Solanaceae)	[134]
	Camptothecin	↓miRNA-125b	Anticancer activity, *in vitro*, human cervical cancer (HeLa) and human immortalized myelogenous leukemia (K562) cell lines, 10 μM, 48 h	*Camptotheca acuminata* (Nyssaceae)	[135]
	Palmatine	↑miRNA-200c	Suppressing breast cancer, *in vitro*, human breast cancer cell line (MCF-7), 10 μM, 2 days	*Coptis chinensis* (Ranunculaceae)	[136]
	Vincristine	↓miRNA-34a	Anticancer activity against human retinoblastoma cell lines: HCT116 (CCL-247), WERI-Rb1 (HTB-169), *in vitro*, and Y79 (HTB-18), 2.5 nM, 48 h	*Catharanthus roseus* (Apocynaceae)	[137]
Coumarins	Osthole	↑miRNA-9	Anti-Alzheimer's activity, *in vitro*, neurons (from the cortex of neonatal mice), and human neuroblastoma cell line (SH-SY5Y), 50 μM, 24 h	*Cnidium monnieri* (Apiaceae)	[138]
	Esculetin	↓miRNA-19b, ↑miRNA-30c	Cardioprotective activity, *in vitro*, human aortic endothelial cells (HAECs), 2.5 μM, 2 h	*Artemisia capillaris,* (Asteraceae), *Citrus limonia* (Rutaceae)	[139]
Flavonoids	Apigenin	↓miRNA-122	Antihepatitis C virus, *in vitro*, human hepatoma cell line has an HCV replicon reporter construct (Huh7-Feo), 5 μM, 5 days	*Matricaria chamomilla* (Asteraceae), *Apium graveolens* (Apiaceae)	[140]
	Chrysin	↑miRNA-9	Anticancer activity, *in vitro*, human gastric cell line (AGS), 35, 55, and 70 μM, 24 h	*Passiflora* spp. (Passifloraceae)	[141]
	Genistein	↑miRNA-574-3p	Inhibiting proliferation on human prostate cancer cell line (PC3 and DU145), *in vitro*, 25 and 50 μM, 24 h	*Glycine max* (Fabaceae)	[142]
	Resveratrol	↓miRNA-31	Treatment effects on ulcerative colitis, *in vivo*, 100 mg/kg, oral administration, 5 days	*Vitis* spp. (Vitaceae)	[128]
	Luteolin	↓miRNA-301-3p	Inhibiting proliferation, *in vitro*, human pancreatic cancer cell line, 25 and 50 μM, 48 h	*Achillea millefolium* (Asteraceae)	[143]

Table 1: Continued.

Category structure	Secondary metabolite	miRNAs	Pharmacological results	Natural source*	Ref.
Iridoids	Geniposide	↑miRNA-124a	Antirheumatoid arthritis activity, *in vitro*, human rheumatoid fibroblast-like synoviocyte line (MH7A), 50 μM, 24 h	*Gardenia jasminoides* (Rubiaceae)	[144]
	Oleuropein	↓miRNA-519d	Increasing radiotherapy sensitivity, *in vitro*, human nasopharyngeal carcinoma cell lines (HNE1 and HONE1), 200 μM, 24 h, *in vivo,* 1% w/v added to the mice drinking water, 7 days	*Olea europaea* (Oleaceae)	[127]
	Catalpol	↑miRNA-200	Anticancer activity, *in vitro* human ovarian cancer, (OVCAR-3), 50 and 100 μg/ml, 48 h	*Rehmannia glutinosa* (Scrophulariaceae)	[145]
Isothiocyanates	Benzyl isothiocyanate	↑miRNA-99a	Anticancer activity, *in vitro,* human bladder cancer cell lines (5637 and T24), 10 and 20 μM, 24 h	*Brassica oleracea* (Brassicaceae)	[120]
	Phenethyl isothiocyanate	↑miRNA-194	Anticancer activity, *in vitro,* human prostatic adenocarcinoma cell line (LNCaP and PC3), 2.5 μM, 24 h	*Raphanus sativus* (brassicaceae)	[146]
	Allyl isothiocyanate	↓miRNA-155	Anti-inflammatory activity, mouse macrophage line (RAW264.7), 10 μM, 24 h	*Brassica* spp. (Brassicaceae)	[147]
Quinones	Emodin	↑miRNA-34a	Suppressing liver tumour, *in vitro*, human liver cancer cell line (HepG2), 10 and 100 nM, 24 h, *in vivo*, hypodermic injection 1 and 10 mg/kg, 30 days	*Rheum palmatum, Polygonum cuspidatum*, *Polygonum multiflorum* (Polygonaceae)	[126]
	Shikonin	↑miRNA-140-5p	Reducing lung injury induced by sepsis, *in vitro*, mouse lung epithelial cells (MLE-12), 50 μg/mL, 24 h, *in vivo*, specific pathogen-free (SPF) rat, 50.0 mg/kg, lingual vein injection, 6 h	*Lithospermum erythrorhizon* (Boraginaceae)	[148]
Saponins	Dioscin	↑let-7i	Nephroprotective activity, *in vitro,* standard rat kidney cell line (NRK-49F) and human kidney proximal tubular epithelial cell line (HK-2), 50, 100, and 200 ng/ml, 12 h, *in vivo,* Sprague Dawley (SD) rats (20, 40, and 80 mg/kg, i.p) and C57BL/6J mice (15, 30, and 60 mg/kg, i.p), 7 days	*Dioscorea* spp. (Dioscoreaceae)	[149]
	Ginsenoside Rg6	↑miRNA-146a	Reducing lung injury induced by sepsis, *in vitro*, bone-marrow-derived macrophage (BMDM) cell line, 20 μM, 1 h, *in vivo,* mice, 20 mg/kg, i.p, (pretreatment for 2 h)	*Panax ginseng* (Araliaceae)	[125]
	Timosaponin A-III	↑miRNA-200c, ↑miRNA-141	Anticancer activity, *in vitro,* human breast adenocarcinoma cell lines (MDA-MB-231 and MCF7), 2, 4 μM, 48 h	*Anemarrhena asphodeloides* (Asparagaceae)	[150]
Simple phenols	Capsaicin	↑miRNA-449a	Anticancer activity, androgen-sensitive human prostate adenocarcinoma cells (C4-2 and LNCaP), 100 μM, 48 h	*Capsicum annuum* (Solanaceae)	[121]
	Curcumin	↑miRNA-34a	Anticancer activity, *in vitro,* human breast adenocarcinoma cell lines (MDA-MB-231 and MCF7), 30 μM, 48 h	*Curcuma longa* (Zingiberaceae)	[122]
	Ferulic acid	↓miRNA-590	Improving spinal cord repair after injury, *in vitro*, neural stem cell (NSC), 10 μM, 24 h	*Bambusa* spp. (Poaceae)	[151]

Table 1: Continued.

Category structure	Secondary metabolite	miRNAs	Pharmacological results	Natural source*	Ref.
Terpenes	Paeoniflorin	↑miRNA-124	Anticancer activity, *in vitro*, human gastric cancer cell line (MGC-803), 20 μM, 48 h	*Paeonia lactiflora* (Paeoniaceae)	[151]
	Triptolide	↑miRNA-137	Nephroprotective effects on diabetic rats, *in vitro*, human renal mesangial cell line (HRMC), 10 ng/ml, *in vivo*, 100 μg/kg, oral administration, 12 weeks	*Tripterygium wilfordii* (Celastraceae)	[152]
	Betulinic acid	↑miRNA-27a	Anticancer activity, *in vitro*, human breast adenocarcinoma cell lines (MDA-MB-231), 2.5, 5, and 10 μM, 24 h, *in vivo,* female athymic BALB/c nude mice, 20 mg/kg, oral administration, 25 days	*Betula* spp. (Betulaceae)	[153]
	Antrocin	↑let-7c	Anticancer activity, *in vitro*, non-small-cell lung cancer cell lines (H441), 5 μM, 12 h	*Antrodia camphorata* (Polyporaceae)	[119]

*The most important natural sources containing secondary metabolisms.

2.1. Role of miRNA Signalling in Preventive and Therapeutic Potentials of Natural Products in NAFLD. Escalating evidence showed that natural products (the substances produced naturally by living organisms) possess protective or therapeutic effects against NAFLD (Figure 1) by regulating various microRNAs' expression. In a study, Adi et al. evaluated the identification of susceptibility genes and examined their diet behaviour. For this purpose, they studied the protective roles of a high-protein fish oil (HPO) diet on type 2 diabetes (T2D) and NAFLD in NONcNZO10 (NZ10) mice. Twelve mice were randomized to receive a control diet (CD). The other 12 mice were randomized to receive an HPO diet for 19 weeks, and microRNA expression profile changes and hepatic gene, steatosis, and blood chemistry were analyzed. The results confirmed that dietary protein and fish oil have protective effects against the development of T2D and NAFLD by downregulating miR-411 (>8-fold decrease by high glucose treatment of endothelial cells), miR-155 and miR-335 (>2-fold decrease by suppressing inflammation), miR-21 (>2-fold decrease as a marker of NAFLD by targeting HMGCR expression), miR-143 (>2-fold decrease by targeting the oxysterol-binding protein-related protein 8 (ORP8)), and miR-29a,b,c (>2-fold decrease by targeting FOXA2) [154]. Also, Wang et al. reported that the therapeutic potential of fish oil supplementation on cholesterol metabolic disorder and hepatic triglyceride is mediated through regulating the particular miRNAs' expression (rno-miR-33-5p and rno-miR-34a-5p) in Western-style diet-induced NAFLD rats [155].

Since recent studies have emphasized the miR-34a expression association with apoptosis in NAFLD, Shan et al. investigated the antiapoptotic effect of carnosic acid (CA), a phenolic compound extracted from the leaf of *Rosmarinus officinalis* (Lamiaceae), in fifty experimental rats that were randomly divided to receive CA or the high-fat diet (HFD) for ten weeks. The protective effect of CA against NAFLD was proved through the activation of SIRT1/p66shc by inhibiting miR-34a [58]. In another work, Yang et al. reported that the therapeutic potential of berberine on NAFLD is mediated through reducing liver uncoupling protein-2 (UCP2) mRNA expression and the regulation of lipid metabolism. For this purpose, NAFLD rats were divided into the standard control group (regular diet with distilled water), the model control group (high-fat diet with distilled water), and the berberine group (high-fat diet with berberine solution). After 12 weeks, they found that, unlike the regular group that was devoid of protein expression, there was a significant increase in the model group.

Moreover, berberine significantly decreased the expression of UCP2 mRNA in comparison to the model group ($p < 0.01$) [156]. A phenolic-rich extract of lychee pulp (LPP) is useful for improving lipid metabolism in the liver by suppressing miR-33 and miR-122. This extract's hypolipidemic effects were studied by dividing mice into the HFD group or HFD combined with the LPP group. The result showed that the daily administration of the LPP for ten weeks could decrease the triglyceride, total serum cholesterol, fatty acid synthase mRNA, and corresponding protein expression levels [157]. Gracia et al. investigated the effect of resveratrol on miRNA-103-3p, miRNA-107-3p, and miRNA-122-5p expression in 16 rats, which were fed an obesogenic diet to induce liver steatosis. After 12 weeks of treatment, the results showed that resveratrol has protective effects against the development of liver fat by downregulating miR-103 (2.49-fold decrease), miR-107 (2.08-fold decrease by reducing carnitine palmitoyltransferase 1A (CPT1A) protein), and miR-122 (2.59-fold decrease by reducing FAS protein expression) [158]. Joven et al. demonstrated that the administration of plant-derived polyphenols for ten weeks was effective in hepatic metabolism, decreased liver steatosis, insulin resistance, and the expression of miR-103 and miR-107 in mice fed with the high-fat diet-induced fatty liver compared to the chow diet.

Moreover, polyphenols attenuated the expression of miR-122, which had not been altered with a fat-rich diet [159]. Grape seed proanthocyanidin extract (GSPE)

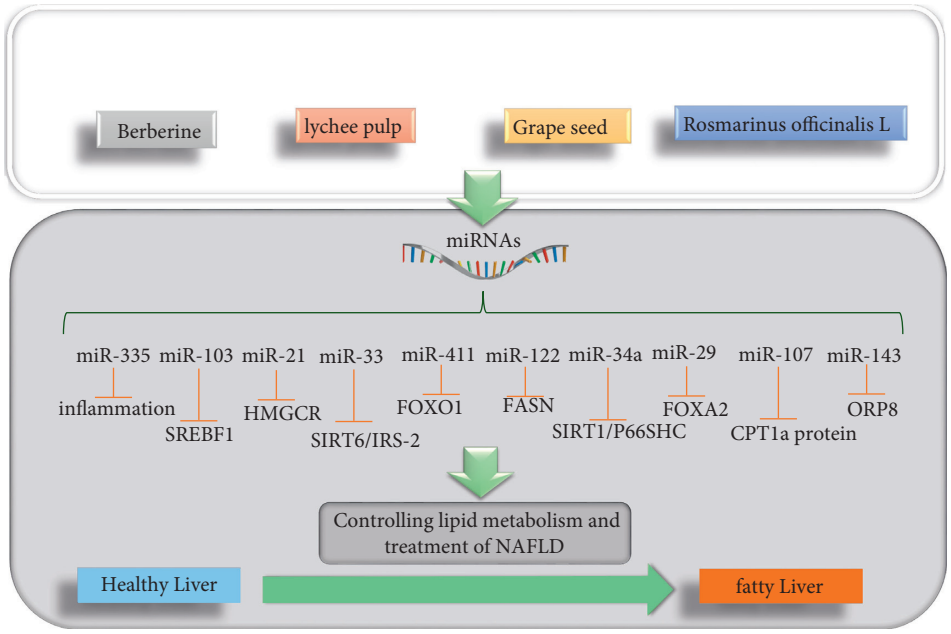

Figure 1: Natural products with preventive/therapeutic effects on NAFLD, acting via microRNAs.

effectively controls lipid metabolism by reducing liver fat regulators such as miR-33 and miR-122. The results showed that proanthocyanidin treatment decreased lipogenesis by repressing miR-12. Besides, it increased hepatic cholesterol efflux to procreate new HDL particles by suppressing miR-33 [160]. Baselga-Escudero and his colleagues studied the association of these miRNAs with lipemia. For this purpose, they analyzed these miRNAs in the livers of dyslipidemic cafeteria diet-fed rats and cafeteria diet-fed rats supplemented with proanthocyanidins and/or v-3 PUFAs. The results showed that, unlike the cafeteria diet, which showed an increasing effect, GSPE suppressed miR-33 and miR-122. However, SREBP2, the host gene of miR-33a, was significantly repressed by v-3 PUFAs but not by proanthocyanidins [161].

3. Conclusions

NAFLD is one of the most common metabolic diseases, which, in addition to its complications, is directly related to many other diseases such as cardiovascular problems, cancer, and kidney failure. Therefore, it is necessary to discover effective drugs due to widespread and critical fatty liver complications. Although various medications, such as thiazolidinediones, polyunsaturated fatty acids, and statins, have been proposed as invalid guidelines for NAFLD treatment, their effect on fatty liver treatment is not exact. On the contrary, identifying cellular mechanisms associated with NAFLD's occurrence and development, such as expression changes of related microRNA, including miR-34a, miR-155, miR-451, and miR-21, can be beneficial in finding effective treatments for this disease. The use of natural compounds for the treatment of NAFLD has long been considered. Various studies showed that the anticancer, anti-inflammatory, antihypercholesterolemic, antidiabetic, cardioprotective, and neuroprotective effects of alkaloids, flavonoids, coumarins, terpenes, iridoids, cardiac glycosides, and isothiocyanates are related to the regulation of miRNAs' expression. Various dietary fruits or medicinal herbal sources have received worldwide attention versus NAFLD development. So, natural compounds can cure various diseases, including NAFLD, by affecting the expression of microRNA. This study found that natural compounds such as polyphenols can play an essential role in improving and treating NAFLD by altering the expression of various microRNAs and NAFLD-related genes. However, extensive clinical studies are needed for their therapeutic approach in NAFLD patients.

Conflicts of Interest

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

Acknowledgments

INSF is acknowledged for their general support.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6628911, 6 pages
https://doi.org/10.1155/2021/6628911

Research Article

The Efficacy of *Rosa damascena* on Liver Enzymes in Nonalcoholic Fatty Liver Disease: A Randomized Double-Blind Clinical Trial

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Received 1 January 2021; Revised 18 April 2021; Accepted 2 August 2021; Published 13 August 2021

Academic Editor: Reza B. Jalili

Objective. This study aimed to appraise the potential effects of *Rosa damascena* preparation on nonalcoholic fatty liver disease (NAFLD). *Design*. In the randomized, double-blind placebo-controlled clinical trials, seventy-four patients with NAFLD were prescribed either 1 g *Rosa damascena* powder or placebo three times in a day for 12 weeks. All patients were provided the lifestyle modification instructions and recommended following them precisely. ALT, AST, FBS, and lipid profiles were measured at the baseline after 12 weeks of studying. The Mann–Whitney *U* test was correctly used to compare the changes of variables among the groups. *Results*. Seventy-two patients completed the study in two groups. Sixty-seven patients were men, and the mean ± standard deviation of age was 40.11 ± 9.05 years. The *Rosa damascena* group showed a considerable decrease in the serum ALT (23.83 ± 24.82 vs. 16.19 ± 27.41, $p = 0.042$), waist circumference (99.73 ± 10.01 vs. 101.52 ± 8.84, $p = 0.003$), triglyceride (TG) (186.29 ± 76.75 vs. 184.47 ± 73.05, $p = 0.001$), cholesterol (167.47 ± 34.48 vs. 184.11 ± 33.54, $p = 0.001$), low-density lipoprotein (LDL) (99.17 ± 28.66 vs. 107.52 ± 25.42, $p = 0.001$), and elevation in serum high-density lipoprotein (HDL) (41.85 ± 6.56 vs. 39.20 ± 5.00, $p < 0.007$) compared to the control group. Improving fatty liver degree due to liver ultrasound was higher in the *Rosa damascena* group than the control group ($p = 0.001$). *Conclusion*. *Rosa damascena* meaningfully improves liver function in NAFLD. Hence, it can be recommended along with lifestyle modification for these patients. Further studies are recommended with a larger sample size.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) remains the universal cause of chronic hepatic disorders [1]. Fatty droplets typically occupy the liver in the notable absence of other causes of hepatic steatosis [2]. However, its pathophysiology is not clearly known, but the multiple-hit hypothesis properly including steatosis, active inflammation, insulin resistance, active hormones promptly released from adipose tissue, specific nutrients, gut microbiota, genetic, and epigenetic factors were respectfully suggested [3]. There is a relationship between metabolic syndrome and NAFLD [4]. It is a silent disease, but without adequately treating it undoubtedly delivers two severe outcomes: (1) intrahepatic: steatohepatitis, cirrhosis, and hepatocellular carcinoma. (2) Extrahepatic: diabetes mellitus type 2, hyperlipidemia, hypertension, ischemic heart disease, and extrahepatic cancers, especially colorectal carcinoma [5]. Its most favorable

treatment is lifestyle modification and drug prescription [6]. Antioxidant and immunomodulating drugs can treat this disease [7].

Persian medicine (PM) represents an ancient medical school which recommended some lifestyle modification methods (*Seteye-Zaroorieh*), herbal drugs, and hand practice or *a'amal-e-yadavee* such as massage and blood sampling methods (phlebotomy or fasd, cupping or hijamat, and leech therapy) for the treatment of disease [8]. Herbal preparation in PM was recommended for liver disease [9]. *Rosa damascena* is one of these cultivated plants that is planted and traditionally used in many countries such as Iran. The hepatoprotective effect of *Rosa damascena* is in terms of its components such as phenolic acid and flavonoid components [10]. Its petals have antioxidant and anti-inflammatory properties, and in animal studies, its effectiveness to clean the liver from accumulated triglycerides and prevent the inflammation of liver was shown [11]. Regarding PM principles, *Rosa damascena* has a pleasant odor; each aromatic herb reinforcing main organs such as the liver [12]. Since there is no clinical trial for the efficiency of *Rosa damascena* on NAFLD, this study was designated to show the *Rosa damascena* beneficial effect on liver enzymes and ultrasound grade in the patients with NAFLD.

2. Methods and Materials

2.1. Trial Design. This randomized, double-blind, placebo-controlled clinical trial was accomplished on two parallel groups of patients with NAFLD. Patients were appropriately allocated into two groups: intervention and placebo, by the block randomization method. The study protocol was approved by Medical Ethics Committee of Iran University of Medical Sciences (IR. IUMS REC.1398.406) and registered in Iran Clinical Trials Registry (ID: https://clinicaltrials.gov/ct2/show/IRCT20191006044994N1}).

2.2. Participants. The patients who met the inclusion criteria were enrolled in Behest Clinic affiliated with Iran University of Medical Sciences in Tehran, Iran. The study began on 1st March 2020 and ended on November 2020.

2.2.1. Inclusion Criteria

The patients (18–80 y) with nonalcoholic fatty liver disease

Liver enzymes rising (AST > 38, ALT > 40)

Ultrasound report grade 1 or 2 or three of fatty liver

2.2.2. Exclusion Criteria. Acute or chronic liver disease, malignancy, pregnancy, uncontrolled diabetes mellitus, alcohol consumption, thyroid disease, psychological disorders, lactation, consumption of hepatotoxic drugs within the past 6 months, consumption of drugs which can affect the assessed biochemical tests of study within the previous 3 months (e.g., metformin, vitamin E, oral contraceptive pills, statins, and glucocorticoids), heart and adrenal insufficiency, and that did not want to willingly participate.

2.3. Intervention. After signing a written informed consent and taking a complete history of patients at the start of the study, they were randomly divided into two groups as case and control. Patients in both groups were given dietary recommendations due to classical medicine and recommended walking at an average speed for 40 minutes daily. The patients in the intervention group and who were in the placebo group received two capsules, each containing 500 mg of petal powder of *Rosa damascena* or toast powder, three times in a day, 30 minutes before a meal or 2 hours after the meal. Both groups were carefully followed for 12 weeks. *Rosa damascena* and placebo capsules were packaged in similar containers and labeled accordingly. Consumption of less than 80% of the drug during the trial was considered as drug intolerance, and the patient was excluded from the trial.

For concealment, sealed envelopes were used, so that the distinct number was recorded on similar envelopes. The drug and placebo were coded in similar capsules and identical packages with the same color and aroma. The patient and researcher were unaware of how the drug or placebo was coded.

This study aimed to appraise the potential effects of *Rosa damascena* preparation on liver enzymes in nonalcoholic fatty liver disease (NAFLD), especially ALT enzyme, at the beginning and the end of the 12th week of study.

Upon entering the study and completing it, all participants were evaluated in terms of blood pressure, waist circumference, height, and weight. A standard mercury calibrated sphygmomanometer was used to measure the blood pressure. Participants rested for 15 minutes, and in a sitting position, blood pressure was taken from their right hand. A rubber meter was properly used to accurately measure the waist circumference in the upright position. A standard meter and an accurate scale were properly used to accurately measure the height and weight. BMI was calculated due to standard formula: BMI = weight (kg)/height (m^2).

All these measurements were performed by a trained nurse. Blood samples were collected from the right hand of all participants after 12 hours of fasting. Complete blood count (CBC), biochemical (FBS, BUN, Cr, total cholesterol, TG, HDL, LDL, AST, and ALT) tests, and U/A (S. G) were precisely measured at weeks 0 and 12. For a significant amount of liver enzymes, photometric assay, and accurate measurement of blood glucose and other biochemical tests, an enzymatic colorimetric method using the standard kits (Pars Azmoun Company, Iran) was properly used. An experienced radiologist satisfactorily performed a liver ultrasound at the marked beginning and the end of controlled study.

2.3.1. Fatty Liver Score according to Ultrasound Grades

(1) Mild increased hepatic echogenicity with normal visualization of diaphragm and intrahepatic vessels

(2) Moderate increased hepatic echogenicity with mildly impaired visualization of diaphragm and intrahepatic vessels

(3) Severe increased hepatic echogenicity and poor or nonvisualization of diaphragm and intrahepatic vessels.

2.4. Drug and Placebo Preparation. *Rosa damascena* was collected from Marand city located in East Azerbaijan province and authenticated by a botanist from School of Pharmacy, Tehran University of Medical Science, Tehran, Iran (voucher no: pmp 584). The rose petals were carefully separated from the sepals and, after drying and crushing, were placed in 500 mg gelatin capsules. *Rosa damascena* capsules were used for better acceptance in patients. Placebo powder (toasted flour) was also filled in 500 mg capsules similar to *Rosa damascena* capsules.

2.5. Sample Size. Sufficient sample size was carefully calculated by proper utilization of pilot study information and the standard formula for accurately comparing two means and correctly observing 20 units of essential ALT difference between two specific groups with an alpha error of 0.05, power 80%, and with the expectation of 20% potential loss to proper follow-up. Ultimately, 74 patients (37 persons in each group) were enrolled.

2.6. Statistical Analysis. The patients with medication compliance as 80% or more entered the analysis. Data were carefully analyzed by SPSS software (version 17). The specific Kolmogorov–Smirnov test promptly confirmed the standard distribution of variables. The effective mean ± standard deviation or number and frequency percentage were properly used to typically describe the variables. The chi-square or Fisher exact test and independent *t*-test or Mann–Whitney *U* test were typically used to accurately compare the independent variables between two groups. Within-group analyses were typically performed using the Wilcoxon signed ranks test. *P* values less than 0.05 were considered statistically significant.

2.7. Safety Measures and Adverse Events. At the beginning of the study, a form was provided to patients in each group to record possible drug side effects such as gastrointestinal symptoms, constipation, diarrhea, and the effect on libido. CBC, BUN, and serum creatinine levels at weeks 0 and 12 were normal, and there were no significant changes in both groups. There was no report of severe adverse events in any patients.

3. Results

234 patients were enrolled, and 160 subjects who had not met the inclusion criteria were excluded. Seventy-four patients who met the inclusion criteria and agreed to participate in the study were divided into two groups. Thirty-seven patients were assigned to the intervention group and 37 patients to the control group. From each group, one individual was excluded in terms of COVID-19. In total, seventy-two cases completed the study. Thirty-six patients were in the *Rosa damascena* group and 36 patients in the placebo group (Figure 1).

The baseline characteristics of studying groups are summarized in Table 1. At the baseline, the frequency of all the primary and secondary outcome variables was statistically identical among groups except systolic blood pressure. To control the confounding effect of baseline systolic blood pressure, percent changes of systolic blood pressure were compared among groups. Regarding demographic characteristics, the mean age of participants was 41.5 ± 9.85 and 38.7 ± 8.07 years in the intervention and control groups, respectively. There were no significant differences in baseline demographic data and age, gender, and BMI between the two groups (Table 1).

Regarding laboratory indices, there were no significant differences in FBS, grade of NAFLD in ultrasound, ALT and AST, lipid profiles, and urine-specific gravity levels between two groups at the beginning of study (Tables 1 and 2).

Significant differences were observed due to baseline systolic blood pressure (p value = 0.004) (Table 1).

Following the patient in both groups, compliance with lifestyle did not indicate any significant differences among the groups. We followed the patients in both groups in terms of compliance with lifestyle modification every week and recalled them to do the diet and exercise instructions. Weight loss among 3–5% during 12 weeks was considered to assess the compliance of life and exercise modification between two groups; at the end of study, the weight change in the participants of two blocs was about 3–5% (Table 1).

3.1. Primary Outcome. Analyzing the results after 12 weeks reflected that there was a substantial reduction in the serum ALT levels in both groups. The percent decrease of ALT was significantly higher in the *Rosa damascena* group than in the control group ($p = 0.042$) (Table 1).

3.2. Secondary Outcomes. Except HDL, lipid profiles significantly decreased in the intervention group (TG, Chol, and LDL, $p > 0.001$), but HDL significantly increased in the intervention group ($p < 0.007$) (Table 1).

In both groups, the anthropometric factors such as weight, BMI, and WC reduced after 12 weeks, but WC was significantly decreased in the intervention group ($p < 0.003$) (Table 1).

Diastolic pressure significantly decreased in the intervention group ($p = 0.001$). Platelet count and SG were significantly decreased in the intervention group (platelet, $p > 0.002$ and SG, $p > 0.001$) (Table 1).

Moreover, there was a significant reduction in NAFLD grade in ultrasound in the *Rosa damascena* group compared to the placebo over the studying period (p value = 0.001) (Table 2).

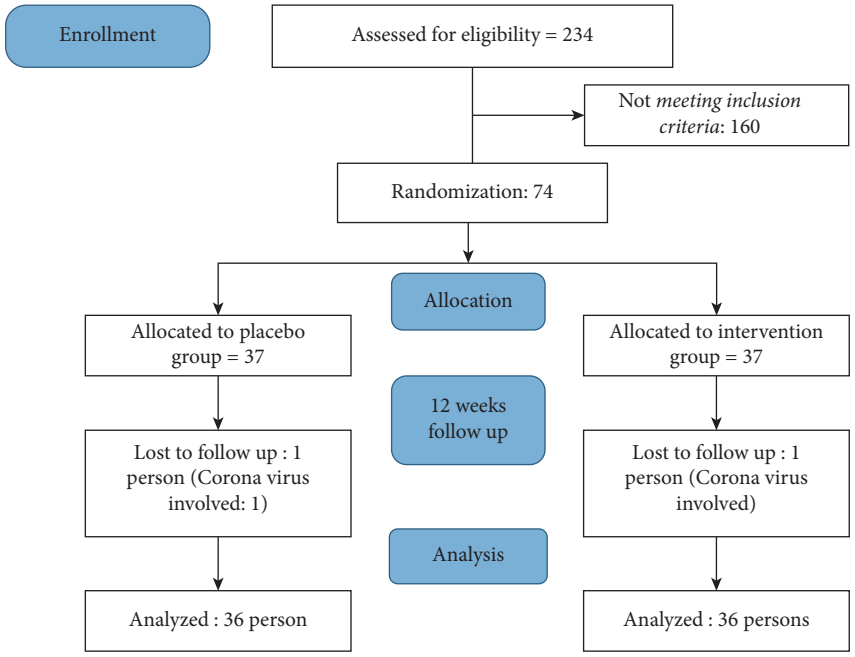

Figure 1: Participant flow.

Table 1: Amount variables at different times.

Variables	Group	Baseline mean ± SD	*P* value	After 12 weeks mean ± SD	*P* value	Percent change mean ± SD	*P* value
BMI (kg/m^2)	Rd	29.68 ± 3.66	0.064	28.46 ± 3.62	0.640	4.08 ± 3.96	0.102
	Placebo	30.05 ± 3.88		29.22 ± 3.85		2.71 ± 3.18	
WC (cm)	Rd	103.69 ± 10.64	0.967	99.73 ± 10.01	0.424	3.75 ± 2.76	0.003
	Placebo	103.79 ± 8.89		101.52 ± 8.84		2.14 ± 2.71	
ALT (IU/L)	Rd	69.05 ± 25.38	0.364	45.22 ± 19.45	0.262	31.52 ± 27.3	0.042
	Placebo	66.41 ± 25.24		50.22 ± 20.60		18.66 ± 29.62	
AST (IU/L)	Rd	1.82 ± 38.38	0.937	30.19 ± 9.83	0.787	13.40 ± 33.86	0.166
	Placebo	37.44 ± 16.03		31.83 ± 12.60		11.33 ± 20.59	
Systolic BP (mmHg)	Rd	125.47 ± 13.47	0.004	118.52 ± 11.33	0.18	5.33 ± 4.18	0.001
	Placebo	113.08 ± 11.70		113.47 ± 11.62		−0.395 ± 3.00	
Diastolic BP (mmHg)	Rd	82.47 ± 12.17	0.97	82.47 ± 11.21	0.717	3.90 ± 7.78	0.001
	Placebo	82.60 ± 7.20		82.60 ± 7.51		0.22 ± 3.85	
FBS (mg/dl)	Rd	108.85 ± 26.31	0.251	103.11 ± 26.77	0.713	3.12 ± 16.14	0.103
	Placebo	100.08 ± 25.12		103.94 ± 40.7		−3.42 ± 15.89	
TG (mg/dl)	Rd	225.44 ± 121.16	0.112	186.29 ± 76.75	0.764	9.61 ± 35.11	>0.001
	Placebo	190.70 ± 100.29		184.47 ± 73.05		−3.67 ± 25.55	
Cholesterol (mg/dl)	Rd	199.23 ± 30.81	0.214	167.47 ± 34.48	0.048	14.66 ± 18.95	>0.001
	Placebo	189.14 ± 35.29		184.11 ± 33.54		2.13 ± 8.01	
HDL (mg/dl)	Rd	36.82 ± 7.22	0.961	41.85 ± 6.56	0.044	−14.94 ± 13.03	0.007
	Placebo	35.85 ± 4.06		39.20 ± 5.00		−9.8312.65	
LDL (mg/dl)	Rd	112.91 ± 28.12	0.112	99.17 ± 28.66	0.208	19.97 ± 20.83	>0.001
	Placebo	112.08 ± 27.26		107.52 ± 12.60		3.28 ± 9.44	
Platelet (mm^3 × 10^3)	Rd	257.86 ± 43.69	0.333	237.36 ± 43.99	0.768	7.53 ± 9.11	>0.002
	Placebo	240.40 ± 78.53		238.25 ± 54.21		0.05 ± 17.88	
Urine SG	Rd	**1019.61 ± 7.04**	0.276	1025.79 ± 5.82	0.027	−0.60 ± .54	>0.001
	Placebo	1021.47 ± 8.86		1022.02 ± .75		−0.05 ± 5.17	

BMI, body mass index; WC, waist circumference; ALT, alanine aminotransferase AST, aspartate aminotransferase; FBS, fasting blood sugar; TG, triglyceride, HDL, high-density lipoprotein, LDL, low-density lipoprotein, SG, specific gravity, minus means to increase; Mann–Whitney *U* test.

4. Discussion

This study is the first randomized clinical trial investigating the effect of *Rosa damascena* petals on NAFLD. In this double-blind clinical trial, ordinary consumption of 3 g (two capsules, three times in a day) of *Rosa damascena* petal capsules for 12 weeks were compared to the placebo. There was no considerable gap in BMI among two groups. Various investigations indicated that 5–10% weight loss is considered for dietary consumption and exercise efficiency to improve

Table 2: The ultrasound changes at different times.

Ultrasound grade	Baseline		P value	After 12 weeks		P value
	Rd = 36	Placebo = 36		Rd = 35	Placebo = 36	
Grade 1	7	8		25	9	
Grade 2	27	24	0.635**	10	22	0.001**
Grade 3	2	4		0	5	

**Person chi-square test.

the NAFLD [13]. Weight loss percent was less than 5% in both groups (Table 1).

The results showed that *Rosa damascena* could reduce liver enzymes (especially ALT), lipid profile, and blood pressure, meaningfully improve metabolic syndrome components, and increase urine SG.

Considering multihit hypothesis, steatosis persists as the first event in the pathogenesis of NAFLD. Davoodi et al. showed that *Rosa damascena* could significantly reduce the final liver fat accumulation, TG, TC, and LDL-C serum concentrations, and hepatic enzymes in an experimental study [11]. Active inflammation is the second notable event in NAFLD pathogenesis [3]. *Rosa damascena* organic compositions such as flavonoids and phenols naturally have anti-inflammatory outcomes and severely inhibit destructive effects of active inflammation on the liver. Flavonoids components inhibit de novo lipogenesis and modulate intestinal microbiota imbalance [10].

Previous studies indicated that NAFLD increases hypertension, following cardiovascular diseases. Proper treatment of NAFLD can reduce cardiovascular disease incidence [14]. *Rosa damascena* naturally has cyanidin-3-O-B glucosides which efficiently remain as an antihypertensive agent in terms of inhibiting angiotensin-converting enzyme [15]. Many patients reported less depression and better sleep at the end of the study. These effects may be due to strengthening the vital organs, such as the brain, heart, and liver [16].

This study showed that urine-specific gravity increases after *Rosa damascena* consumption. Specific gravity represents an expression of urine concentration in terms of density. Fluid density critically depends on the sufficient number of solute particles present and their relative mass. Specific gravity measurement is used to reasonably assess the measurable quantity of solutes present in urine. It accurately reflects the ability of functioning kidneys to naturally produce the effective urine [17].

Another outcome was a reduction in the platelet count. Flavonoids contain many components, such 4-methylcatechol that is normally formed by human microflora. It poses a strong antiplatelet effect which can typically decrease the elevated incidence of cardiovascular diseases. This information can help explain the antiplatelet potential of orally given flavonoids containing formulas such as *Rosa damascena* [18].

In brief, after a favorable review of properties as mentioned above of *Rosa damascena*, it is typically recommended that before doing liver-destroying drugs such as halothane, advised patient should employ *Rosa damascena* for a certain period and typical dose along with lifestyle modification. This effect is due to synchronous properties of antioxidant activity and liver protector of *Rosa damascena*.

The safe dose of powder of *Rosa damascena* is up to 12 g [12]. Winther et al. found that the effective dose of *Rosa damascena* when used as the dried powder is 5 g per day [19]. We use 3 g or two 500 mg capsules three times a day. No significant reduction in the additional variables such as FBS might were due to the low doses of preparing in this study, and higher dosage or other types of preparation or more prolonged treatment time might be needed.

There were some limitations such as the short duration of the study and using *Rosa damascena* as a capsule for better acceptance in the patients (in the PM, its decoction form is recommended). The effect of *Rosa damascena* on liver histology (liver biopsy) was unstudied because it was quite invasive. The prevalence of the COVID-19 pandemic prevents the patients from participating in the study or causes them to leave the study.

5. Conclusion

This study showed that *Rosa damascena* petals powder consumption with a daily dose of 3 g for 12 weeks improved ALT enzyme, reduced fatty liver grade in ultrasound (Table 2), and better metabolic syndrome components in informed patients with NAFLD. Regarding the cost-effectiveness and availability of *Rosa damascena* and the lack of any reports for serious complications of this plant using, *Rosa damascena* can be recommended in patients with NAFLD. Further research with larger sample size and long-term follow-ups of patients, further research with a larger sample size can provide a stronger document about the usage of *Rosa damascena* as complementary medicine to treat the NAFLD.

Data Availability

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

Disclosure

This study was an active part of a Ph.D. thesis of Dr. Sayed Ali-Hahi Moravej.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The study was supported by Iran University of Medical Sciences. The authors sincerely thank all patients and the

team that typically worked on this study, especially Mrs. Abdollahi, Dr. Behrooz Moghbelalhosein, and Dr. Amir Mohammadi Dehdezi.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 9932291, 17 pages
https://doi.org/10.1155/2021/9932291

Research Article

Mineral Content, Chemical Analysis, *In Vitro* Antidiabetic and Antioxidant Activities, and Antibacterial Power of Aqueous and Organic Extracts of Moroccan *Leopoldia comosa* (L.) Parl. Bulbs

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Received 18 March 2021; Revised 20 May 2021; Accepted 30 June 2021; Published 23 July 2021

Academic Editor: Mahnaz Khanavi

Medicinal plants are a rich source of bioactive phytochemicals or bionutrients. Studies carried out during the past few decades have shown that these phytochemicals play an important role in preventing metabolic diseases such as cancer and diabetes. The present study was dedicated to the analysis of mineral and chemical composition and evaluation of antidiabetic, antioxidant, and antibacterial properties of aqueous and organic extracts of *Leopoldia comosa*, a plant with a long history of therapeutic and food use. Mineral content was determined using inductively coupled plasma atomic emission spectroscopy. Chemical composition was carried out by extraction of essential oils, preparation of aqueous and organic extracts, and qualitative and quantitative analysis. The biological study consisted of the evaluation of antidiabetic activity by inhibition of three enzymes, antioxidant activity by five tests, and antibacterial activity by the disc diffusion method. The correlation between chemical composition and antidiabetic and antioxidant properties was explored by PCA. The results showed that *L. comosa* contains high levels of Fe, K, P, Na, Cu, Mg, and Ca with values, respectively, in the order of 33552, 1843.14, 756.36, 439.65, 303.9, 272.37, and 20.55 mg/kg. Quantitative analysis showed that the diethyl ether extract had the highest content of polyphenols ($129.75 \pm 0.29\,\mu$g GAE/mg E), flavonoids ($988.26 \pm 0.18\,\mu$g QE/mg E), and tannins ($30.22 \pm 0.15\,\mu$g CE/mg E). All extracts of *L. comosa* possess inhibitory activity of alpha-amylase, alpha-glucosidase, and beta-galactosidase enzymes, mainly the decocted and the acetone extract. The antioxidant results showed that organic extracts are more active than aqueous extracts especially diethyl ether extract which was similarly found to have an antibacterial effect on *Listeria innocua* and *Proteus mirabilis*. PCA allowed us to deduce that phenolic compounds, flavonoids, and tannins are strongly correlated with antioxidant and antidiabetic activity. *L. comosa* may have potential remedy in the prevention of metabolic disease.

1. Introduction

Biological systems are continuously exposed to oxidants, either generated endogenously by metabolic reactions or exogenously, such as air pollutants. Reactive oxygen species such as the superoxide anion (O_2°) and the hydroxyl radical (OH), are very unstable species with unpaired electrons, capable of initiating the oxidation of proteins, lipids, and nucleic acids leading to alterations in cell structures and mutagenesis [1, 2]. Experimental studies have reported that the overproduction of free radicals with a deficiency of antioxidants is involved in the development of diabetes [3–5].

Parallel to oxidative stress, the evolution of our lifestyles, especially the modification of eating habits, with the overconsumption of fatty and sugary foods, coupled with a low intake of fruits and vegetables, plays a major role in the onset of diabetes. These dietary imbalances, combined with a lack of physical activity, lead to calorie intake over needs and energy storage in the adipose tissue. However, not all excess energy is stored in the form of fat, as some of the

macronutrients are oxidized in the mitochondria, which promotes the production of free radicals [6].

Type 2 diabetes mellitus or non-insulin-dependent diabetes (NIDDM) is a multifactorial disease characterized by severe deregulation of glucose homeostasis. The World Health Organization (WHO) has predicted that between 2014 and 2045, the number of diabetics will double from 422 million to approximately 629 million people [7]. The incidence rate of NIDDM is higher in economically developed countries, particularly the US, where 9.1% of the population (29 million) has diabetes. In Morocco, between 2011 and 2015, the number of diabetics has increased from 1.5 million to more than 2 million, i.e., 25% more in 5 years. 80% of diabetes cases are type 2 [8].

In addition to the problem of oxidants, the rapid development of resistance in microbial agents and drug-induced side effects constitute a major public health problem, even in the most developed countries [9]. Indeed, the main determinant of the appearance of this resistance is probably the pressure of antibiotic selection to which microbial populations are subjected. Thus, for all these reasons, research is now focusing on new therapeutic alternatives such as medicinal plants that have been used for centuries in the treatment of many diseases. Active components responsible for antioxidant, hypoglycemic, and antibacterial activity may include polysaccharides, ascorbic acid (vitamin C), carotenoids, triterpenoids, alkaloids, flavonoids, coumarins, phenolic substances, and peptides [6].

Within the framework of the investigations of the phytochemical properties and valorization of the pharmacological activities of the natural substances of the Taza region, carried out by our laboratory: natural Substances, Pharmacology, Environment, Modelling, Health and Quality of Life (SNAMOPEQ) [10–15], we have selected for the present study a medicinal plant named *Leopoldia comosa* (L.) commonly called "Bssilla" which despite its use in traditional Moroccan medicine for its therapeutic properties [16] and its exploitation for its economic value has not been investigated either to study its chemical composition or to evaluate its pharmacological properties either in Morocco or in Southern Mediterranean countries. Indeed, in our previous preliminary work related to the ethnomedicinal and socioeconomic value that we have conducted from March 2018 to May 2019 in the province of Taza, Morocco has shown that this plant is used in the treatment of dermatological and digestive disorders. It also represents an important source of income for the population and farmers of the plant in the region of Taza, especially as Morocco is involved in the export of this plant abroad, particularly to Italy as this plant is used by Italians in food and the treatment of many diseases [16].

Hence, the interest of this study was to evaluate for the first time the mineral composition of the bulb of Moroccan *L. comosa* and to conduct phytochemical studies by a qualitative and quantitative analysis of secondary metabolites (alkaloids, polyphenols, flavonoids, tannins, anthraquinones, anthracenosides, quinones, saponins, and sterols) and pharmacological studies by the evaluation of antidiabetic, antioxidant, and antibacterial biological activities. The antidiabetic activity was studied by three assays using the enzymes responsible for inhibiting polysaccharide degradation; alpha-amylase, alpha-glucosidase, and beta-galactosidase; antioxidant activity by five different methods (H_2O_2, ABTS, DPPH, FRAP, and RP); and antibacterial activity by the disc diffusion method. A principal component analysis (PCA) was also performed to investigate the correlation between the contents of phenolic compounds and the results of the *in vitro* tests for antidiabetic and antioxidant activities.

2. Materials and Methods

2.1. Plant Material. Leopoldia comosa (L.) bulbs were harvested in the province of Taza, Morocco (geographic coordinates: N 34°13.605′ W 004°01.711′, altitude: 469 m) during the spring, March 2018, and the plant was identified by Dr. Abdelmajid Khabbach, the botanist of the Laboratory of Natural Substances, Pharmacology, Environment, Modelling, Health and Quality of Life (SNAMOPEQ), Polydisciplinary Faculty of Taza (FPT), Sidi Mohamed Ben Abdellah University of Fez, Morocco. A voucher specimen was deposited in the herbarium under the code SA 2018/05. The bulb was cleaned, peeled, and left to dry in the shade and at room temperature. The full name of the plant was taken as described on the website (https://www.theplantlist.org; *Leopoldia comosa* (L.) Parl.).

2.2. Mineral Content of Leopoldia comosa (L.). The analysis of the mineral composition of *L. comosa* bulbs (potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), phosphorus (P), copper (Cu), iron (Fe), selenium (Se), strontium (Sr), and zinc (Zn) was determined using inductively coupled plasma atomic emission spectroscopy ((ICP-AES) HORIBA JOBIN YVON) as previously described [17]. Thus, 0.5 mg of *L. comosa* bulbs was digested with nitric acid and perchloric acid (25%: 75%) solution, before being incinerated at 110°C, and then brought back dry until the mineralization was discolored on a sand bath. The residue was dissolved in 10 mL HCL (5%), and the contents were filtered through 0.45 μm porosity filters until a clear solution was obtained. The sample solution was made up to a final volume of 25 mL with distilled water and analyzed by atomic absorption spectrophotometry.

2.3. Preparation of Extracts

2.3.1. Aqueous Extraction. It is a method of preparation using distilled water in three modalities that vary according to temperature and extraction time. The aqueous extracts were prepared according to the methodology described previously in our work [10, 13–15]:

(1) *Decoction.* 10 g of the plant material was mixed with 100 mL of distilled water in a ground glass flask, topped with a condenser, and left to boil at a stable boiling temperature for 20 minutes.

(2) *Infusion.* 100 mL of boiling distilled water was poured onto 10 g of the plant material in a beaker for 30 minutes.

(3) *Maceration.* 10 g of the plant material was mixed with 100 mL of cold distilled water in a beaker for 24 hours.

After filtration, the 3 prepared aqueous extracts were frozen at (−80°C) for 24 hours, lyophilized using a (Heto PowerDry LL3000).

2.3.2. Organic Extraction. The organic extracts were prepared by two different methods; the first technique is realized under hot conditions using a Soxhlet apparatus, where 100 g of plant material was introduced into a cartridge of cellulose attached to a ball and surmounted by a refrigerant and 1000 mL of three solvents of increasing polarity (diethyl ether, acetone, and ethanol) used separately was vaporized and then condensed while remaining in contact with the plant material. The extraction is ended when the solvent of extraction becomes clearer, six hours for our experimental conditions. The second technique was cold maceration by pouring 1000 ml of the solvents described previously separately on 100 g of the plant material for 48 hours.

Organic extracts were evaporated on a rotary evaporator (Büchi AG CH-9230) under vacuum at 40–50°C and stored with the aqueous extracts at 4°C for uses in phytochemical and pharmacological studies.

2.3.3. Extraction of Essential Oils (EOs) by Hydrodistillation. The essential oils (EOs) were extracted by hydrodistillation using a Clevenger apparatus. The extraction was done twice, with fresh bulbs of *L. comosa* and the second time after drying the bulbs at room temperature. The extraction consisted of immersing 100 g of the bulbs (fresh or dried) in a flask filled with one liter of distilled water, which was then brought to a boil.

2.4. Phytochemical Analysis

2.4.1. Qualitative Analysis of Phytochemicals. Phytochemical screening tests allow us to characterize the presence or absence of secondary metabolites through a qualitative analysis based on coloration and/or precipitation reactions. In this study, the search for different secondary metabolites, such as alkaloids, tannins, saponins, anthracenosides, anthraquinones, quinones, flavonoids, and sterols, was carried out on the plant bulb and the nine aqueous and organic extracts prepared from *L. comosa* as described in the previous work of our laboratory [10, 13–15].

2.4.2. Quantitative Analysis of Phytochemicals. The dosage of secondary metabolites in the *L. comosa* bulbs was conducted according to the results of the phytochemical screening tests. Thus, the content of polyphenols, flavonoids, and tannins was determined. The assay was carried out as described in previous work in our laboratory [12–15].

(1) Determination of Polyphenol Content. The Folin–Cio calteu method [18] was used to determine the total phenolic content of our extracts. A volume of 0.5 mL of each of our nine aqueous and organic extracts or gallic acid was introduced into test tubes, 2.5 mL of Folin–Ciocalteu reagent was added, and then 4 mL of 7.5% (m/v) sodium carbonate was added. The different solutions were kept in a water bath for 30 minutes. Absorbance was measured at 765 nm using a SPECUVIS2 UV/Vis Spectrophotometer, No: HF1309003. The polyphenol content in the extracts was expressed in microgram (μg) gallic acid equivalent per milligram (mg) of extract (μg GAE/mg E).

(2) Determination of Flavonoid Content. The quantification of flavonoids in aqueous and organic extracts of *L. comosa* was carried out by the colorimetric method of aluminum chloride $AlCl_3$, based on the protocol described by Dewanto et al. [19] and as presented in our previous work [13]. The flavonoid content was expressed as μg quercetin equivalent/mg of extract (μg QE/mg E).

(3) Determination of Tannin Content. The content of tannins in *L. comosa* extracts was determined by the vanillin method according to the protocol of [20] and as presented in our previous work [13]. A volume of 50 μL of each sample of our nine aqueous and organic extracts or catechin was added to 1500 μL of the vanillin/methanol solution (4%, w/v) and then mixed using a vortex. Then, 750 μL of concentrated hydrochloric acid (HCl) was added and allowed to react at room temperature for 20 minutes. The absorbance was measured at 500 nm, and the concentration of tannins was expressed in microgram (μg) catechin equivalents per milligram (mg) of extract (μg CE/mg E).

2.5. Biological and Pharmacological Assays

2.5.1. Study of Antidiabetic Activity

(1) Alpha-Amylase Inhibitory Assay. The alpha-amylase inhibition assay was performed using the 3, 5-dinitrosalicylic acid (DNSA) method [21]. Different concentrations of extracts from the bulb of *L. comosa* were prepared in saline phosphate buffer (Na_2HPO_4/NaH_2PO_4 (0.02 M) at pH 6.9). A volume of 200 μL of alpha-amylase solution (2 units/mL) was mixed with 200 μL of the extract and was incubated for 10 min at 30°C. Thereafter, 200 μL of the starch solution (1% in water (w/v)) was added to each tube and incubated for 3 min at 30°C. The reaction was terminated by the addition of 200 μL DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3, 5-dinitrosalicylic acid solution) and was boiled for 10 min in a water bath at 85–90°C. The mixture was cooled to ambient temperature and was diluted with 5 mL of distilled water, and the absorbance was measured at 540 nm using a SPECUVIS2 UV/Vis spectrophotometer, no: HF1309003. Acarbose was used as a positive control.

The α-amylase inhibitory activity was expressed as percent inhibition and was calculated using the following equation:

$$\text{inhibition}\,(\%) = 100 \times \left[\frac{(\text{Ac} - \text{Acb}) - (\text{As} - \text{Asb})}{(\text{Ac} - \text{Acb})}\right], \quad (1)$$

where Ac refers to the absorbance of the control (enzyme and buffer), Acb refers to the absorbance of control blank (buffer without enzyme), As refers to the absorbance of the sample (enzyme and extract), and Asb is the absorbance of sample blank (extract without enzyme). The concentration of extract providing 50% inhibition (IC50) was calculated from the calibration curve.

(2) Alpha-Glucosidase Inhibitory Assay. The inhibitory potency of aqueous and organic extracts of *L. comosa* against alpha-glucosidase enzyme was evaluated by measuring the formation of 4-nitrophenol by alpha-glucosidase after reaction with 4-p-nitrophényl-α-D-glucopyranoside (pNPG) according to the method of Lordan et al. [22]. To perform this test, a reaction mixture containing 150 μL of extracts prepared in sodium phosphate buffer (0.1 M/pH = 6.7) at various concentrations and 100 μL of α-glucosidase solution (0.1 U/mL) was preincubated at 37°C for 10 min. Subsequently, 200 μL of 1 mM of p-nitrophényl-α-D-glucopyranoside (pNPG) solution in sodium phosphate buffer (0.1 M/pH = 6.7) was added and incubated at 37°C for 30 min. The reaction was terminated by adding 1 mL of sodium carbonate solution (Na_2CO_3/0.1 M) and the absorbance was measured at 405 nm. Acarbose was included as a positive control, and the percentage inhibition was determined as described in the alpha-amylase assay, and the IC50 values were determined.

(3) Beta-Galactosidase Inhibitory Assay. The *in vitro* assessment of antidiabetic activity by inhibition of beta-galactosidase is a test based on the arrest of beta galactoside degradation by inhibition of intestinal β-galactosidase activity. Indeed, the beta-galactosidase or lactase is an enzyme capable of hydrolyzing lactose by transforming it into glucose and galactose [10]. For this purpose, a mixture of 150 μL of different concentrations of the extracts and 100 μL of sodium phosphate buffer (0.1 M at pH = 7.6) containing the enzyme solution beta-galactosidase (1 U/mL) was incubated at 37°C for 10 min. Then, 200 μL of the substrate 2-nitrophenyl β-D-galactopyranoside (1 mM) solubilized in sodium phosphate buffer was added. The reaction mixtures were incubated at 37°C for 30 min. After incubation, 1 mL Na_2CO_3 was added to stop the reaction and the absorbance was recorded at 410 nm using a spectrophotometer. Quercetin was used as a positive control and the percentage inhibition was determined as described in the alpha-amylase assay, and the IC50 values were determined.

2.5.2. Study of Antioxidant Activity. Various methods were adopted to assess the antioxidant activity *in vitro* of *L. comosa* extracts, namely, hydrogen peroxide scavenging assay (H_2O_2), ABTS or TEAC (equivalent antioxidant capacity of Trolox), DPPH (2, 2-diphenyl-1-picrylhydrazyl), ferric reducing antioxidant power assay (FRAP), and reducing power (RP).

(1) Hydrogen Peroxide Scavenging Assay (H_2O_2). The ability of *L. comosa* aqueous and organic extracts to scavenge H_2O_2 was determined using the method of Ruch et al. [23]. A hydrogen peroxide solution (40 mM) was prepared in a solution of phosphate saline buffer (PBS, pH 7.4). The concentration of hydrogen peroxide was determined after 10 minutes by absorption at 230 nm using a spectrophotometer.

The percentage scavenging of H_2O_2 by our extracts and ascorbic acid was determined according to the equation:

$$(\%) = \left[\frac{((\text{Ac})(\text{As} - \text{Asb}))}{\text{Ac}}\right] \times 100, \quad (2)$$

where Ac is the absorbance of the control (H_2O_2 + phosphate-buffered saline), As is the absorbance of the sample (H_2O_2 in phosphate-buffered saline + extract), and Asb is the absorbance of the blank (extract + phosphate-buffered saline) [13].

(2) Trolox Equivalent Antioxidant Capacity Using ABTS (TEAC). The antioxidant activity of the nine prepared extracts was determined according to the protocol of Re et al. [24] and as presented in our previous work [13]. The stock solution was prepared by mixing an ABTS solution of (7 mM) with potassium persulfate (2.45 mM), and the mixture was left in the dark at room temperature for 12–16 hours before use. 30 μL of our extracts were reacted with 3 mL of the $ABTS^+$ solution, and the absorbance was measured at 734 nm after 1 min using a spectrophotometer. Trolox was used as the reference standard, and results were expressed in μg Trolox equivalent per milligram of extract (μg TE/mg E).

(3) DPPH (2, 2-Diphenyl-1-picrylhydrazil) Free Radical Scavenging Activity. The chemical compound 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was one of the first free radicals used to study the relationship between the structure and antiradical activity of phenolic compounds. It has an unpaired electron on an atom of the nitrogen bridge [25]. To make this test, 3 mL of different concentrations of our extracts were added to 1 mL of the DPPH solution (200 μM) and the mixture was left in the dark for 30 min at 30°C, and the absorbance was measured at 517 nm using a spectrophotometer. Trolox, BHT, and ascorbic acid were used as the reference standard.

(4) Ferric Reducing-Antioxidant Power Assay (FRAP). The antioxidant power of iron reduction (FRAP) was used to measure the ability of extracts to reduce the TPTZ-Fe (III) complex to TPTZ-Fe (II) measured at wavelength 593 nm [26]. 100 μL of the *L. comosa* extracts was reacted with 3000 μL of the FRAP solution (25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL $FeCl_3 \cdot 6H_20$) for 30 minutes in the dark. The results were expressed in μg Trolox equivalent per milligram of extract (μg TE/mg E) [13].

(5) Reducing Power Assay (RP). The RP assay was developed to measure the ability of the extracts tested to reduce ferric iron (Fe^{3+}) present in the potassium ferricyanide complex

K_3Fe (CN) 6 to ferrous iron (Fe^{2+}). Based on the protocol developed by Oyaizu in 1986 [27] and as described in our publication [13], results were expressed in μg ascorbic acid equivalent per milligram of extract (μg AAE/mg E).

2.5.3. Study of Antibacterial Activity

(1) Bacterial Strains. The antibacterial activity of organic extracts of *L. comosa* was tested against six reference bacterial strains; these are pathogenic bacteria frequently involved in infectious diseases. Three bacteria are Gram-positive, *Staphylococcus aureus* (CECT976), *Bacillus subtilis* (DSM6633), and *Listeria innocua* (CECT 4030), and three Gram-negative bacteria, *Escherichia coli* (K12), *Proteus mirabilis*, and *Pseudomonas aeruginosa* (CECT118).

(2) Inoculum Preparation. The inoculum suspension was obtained by taking colonies from 24 hours' cultures. The colonies were suspended in a sterile aqueous solution of NaCl (0.9%) and shacked for 20 seconds. The density was adjusted to the turbidity of a 0.5 McFarland Standard (10^8 CFU/mL, colonies forming a unit per mL).

(3) Agar Disc Diffusion Assay. Antibacterial activity of organic extracts of *L. comosa* was determined by the agar disc diffusion assay according to the method described by Sharififar et al. [28]. A suspension of microorganisms from an inoculum of 10^8 CFU/mL was inoculated on the surface of agar plates containing 20 mL of Mueller Hinton Agar using a sterile swab. Sterile discs (6 mm in diameter) soaked in different concentrations of the extracts prepared from *L. comosa* bulbs (40, 80, and 100 mg/mL) solubilized in DMSO (10%) were placed on the surface of the agar plate. Then, the plates were closed and incubated at 37°C for 20 hours. The antibacterial effect of our extracts was evaluated by measuring the zone of inhibition formed around the discs and expressed in mm against the six bacterial strains tested. Negative control was produced by DMSO (10%) while the positive control is represented by the two antibiotics tetracycline and amikacin. All tests were repeated three times, and the results were calculated as follows: mean ± standard deviation.

2.6. Statistical Analysis. The results were expressed as the mean ± standard error. Nonlinear regression analysis was adopted to determine the IC50 values of the four assays (alpha-amylase, alpha-glucosidase, beta-galactosidase, and DPPH assays). The data were analyzed by one-way analysis of variance (one-way ANOVA), Turkey: compare all pairs of column procedure for the significance of the difference. A difference in the mean values of $P < 0.05$ was considered to be statistically significant. The analysis was performed with GraphPad Prism® 5.0 software. Principal component analysis (PCA) was performed by the XLSTAT software.

3. Results

3.1. Mineral Composition of Leopoldia comosa (L.) Bulbs. According to our bibliographic research, we did not find any studies relating to the evaluation of the mineral composition and nutritional value of *L. comosa*. The results of the analysis of the mineral composition of *L. comosa* in our study represent the first investigation conducted in this plant. This shows that the *L. comosa* bulb has high levels of Fe (33552), K (1843.14), P (756.36), Na (439.65), Cu (303.9), Mg (272.37), and Ca (20.55) mg/kg of plant material. However, lower values were noted for the 3 elements Se, Sr, and Zn (<0.01 mg/L).

3.2. Phytochemical Study of Leopoldia comosa (L.) Bulbs

3.2.1. Yields of Aqueous and Organic Extractions of Leopoldia comosa (L.) Bulbs. The extraction yield is the ratio between the weight of the compounds or substances that can be extracted depending on the nature of the solvent used, the extraction method, and the nature of the plant material used, whether dry or fresh. The yield is expressed as a percentage and is calculated by the following formula:

$$R = \frac{\text{PA}}{\text{PB}} \times 100, \tag{3}$$

where A is the extraction yield in (%), PA is the weight of compound in g, and PB is the weight of dry plant material in g.

The yields obtained were highly variable, ranging from 0.2 to 8%. The highest yield was obtained for the most polar solvent, water, with the decoction modality with a percentage of 8%, followed by the infused extract and the aqueous macerate with values of about 7% and 5.2%, respectively, whereas organic solvents have low values, whose highest yield was obtained with the most polar solvents, namely, ethanol, acetone, and diethyl ether, and with cold extraction by maceration with values of about 1.3%, 0.8 %, and 0.5%, respectively. Ethanolic, acetone, and diethyl ether extracts prepared by Soxhlet showed values of about 1%, 0.6%, and 0.2%, respectively.

3.2.2. Extraction Yields of Essential Oils (EOs) from Leopoldia comosa (L.) Bulbs. The results of the investigations of the extraction of essential oils from *L. comosa* bulb whether fresh or dried allowed us to note an absence of essential oils in this part of the plant.

3.2.3. Phytochemical Screening. Phytochemical screening was carried out on *L. comosa* in two parts: first on the plant bulbs and the second on the aqueous and organic extracts prepared. The results obtained revealed the presence of flavonoids, catechin tannins, and quinones in the case of the plant bulbs and the 9 aqueous and organic extracts prepared. The families of anthracenosides and anthraquinones are present in the bulb and organic extracts while they are absent in the aqueous extracts.

3.2.4. Polyphenol, Flavonoid, and Tannin Contents. Phenolic compounds are the most diverse compounds of secondary metabolites found in plant organs, which can be used as therapeutic agents, preservatives, additives, and food supplements [29]. Table 1 summarizes the results obtained for the polyphenol, flavonoid, and tannin contents of the aqueous and organic extracts of the *L. comosa* bulbs.

According to Table 1, we noticed that the contents of phenolic compounds, flavonoids, and tannins vary according to the aqueous or organic extraction method and the hot or cold extraction modality.

Aqueous extracts showed lower contents of phenolic compounds than the organic extracts with a significant difference ($P < 0.05$). For aqueous extracts, the difference was nonsignificant ($P < 0.05$) between decocted, infused, and macerated and showed values of 4.28 ± 0.02, 4.44 ± 0.02, and 4.69 ± 0.01 μg GAE/mg E, respectively. For organic extracts, the difference is significant between the six prepared extracts ($P < 0.05$). The diethyl ether extract prepared by Soxhlet with the least polar solvent showed the highest value which is of the order of 129.75 ± 0.29 μg GAE/mg E, followed by the macerated diethyl ether extract, acetone extract, macerated acetone extract, ethanolic extract, and lastly the ethanolic extract prepared by maceration with values of the order of 115.81 ± 0.24, 69.96 ± 0.01, 61.43 ± 0.04, 20.49 ± 0.08, and 18.20 ± 0.04 μg GAE/mg E, respectively.

For the flavonoid content, the aqueous extract prepared by the infusion mode was found to be richer than the decocted and macerated with values on the order of 90.82 ± 0.59, 82.15 ± 0.26, and 78.63 ± 0.21 μg QE/mg E, respectively, with a significant difference ($P < 0.05$) between decocted and infused, and infused and macerated, and the no significant difference between decocted and macerated. For organic extracts, always hot extraction makes it possible to extract more flavonoids than cold extraction used by the same solvent, in particular for diethyl ether extract followed by acetone and ethanolic extracts with values of the order of 988.26 ± 0.18, 330.15 ± 1.45, and 147.63 ± 0.57 μg QE/mg E, respectively.

In the case of tannins, we obtained the highest content with the diethyl ether extract prepared by Soxhlet (30.22 ± 0.15 μg CE/mg E), and in the case of aqueous extracts, the macerate gives the best yield (18.68 ± 0.11 μg CE/mg E).

3.3. Biological and Pharmacological Assays

3.3.1. Antidiabetic Activity

(1) Alpha-Amylase Inhibitory Assay. The results of the evaluation of the alpha-amylase inhibitory activity of aqueous and organic extracts from the bulb of *L. comosa* are shown in Table 2. IC50 values were calculated for all aqueous and organic extracts and the reference standard, and a lower IC50 value indicates a higher inhibitory activity. The results obtained showed that the aqueous extracts and particularly the decocted extract have a high alpha-amylase inhibitory capacity with an IC50 of 1200.66 ± 13.79 μg/mL, which is twice better than the macerated extract with an IC50 of 2752.33 ± 8.11 μg/mL. Similarly, for organic extracts, we have recorded that hot extraction by Soxhlet gives interesting results compared to cold extraction by maceration, of which ethanolic extract, acetone extract, and diethyl ether extract had IC50s, respectively, of 2264 ± 22.86 μg/mL, 2219.33 ± 3.31 μg/mL, and 2512.33 ± 5.98 μg/mL versus IC50 values of 2384 ± 7.40 μg/mL, 2289.66 ± 7.45 μg/mL, and 2897.66 ± 4.76 μg/mL, respectively, for the same extracts prepared by cold maceration. A highly significant difference was observed for all extracts and the reference standard, acarbose, which had an IC50 of 616.33 ± 6.58 μg/mL.

(2) Alpha-Glucosidase Inhibitory Assay. To explore the antidiabetic activity of aqueous and organic extracts of *L. comosa*, the alpha-glucosidase inhibition assay was performed and the results are shown in Table 2. According to this table, all the extracts tested showed an interesting hypoglycemic property with IC50s ranging from 85.41 ± 3.86 μg/mL to 268.23 ± 2.85 μg/mL. The acetone extract was most active with an IC50 of 85.41 ± 3.86 μg/mL which is significantly lower than the reference standard, acarbose (IC50 = 195 ± 5 μg/mL). For the aqueous extracts, the decocted was the active extract with an IC50 of 238.53 ± 2.35 μg/mL against an IC50 of 268.23 ± 2.85 μg/mL for the cold prepared macerated extract. In this test, hot extraction seems to be the best method to obtain the great hypoglycemic power of aqueous and organic extracts of *L. comosa* bulb.

(3) Beta-Galactosidase Inhibitory Assay. Based on our literature search, this study presents for the first time the results of beta-galactosidase inhibition. According to Table 2, we note that organic extracts are more active than aqueous extracts, with a better activity obtained by the acetone extract with an IC50 of the order of 163.5 ± 2.51 μg/mL. For the aqueous extracts, the decocted recorded an IC50 value of 205.43 ± 2.22 μg/mL against 245.5 ± 9.26 μg/mL for the macerated extract. These results are in line with those of the alpha-amylase and alpha-glucosidase inhibition assay, for which we found that hot extraction is the best method for preparing the extracts responsible for inhibiting the enzymes of the antidiabetic activity.

Several previous studies have reported that diabetes is associated with oxidative stress [3–5], and this through the accumulation of free radicals that can lead to changes in the genetic material of the cell and thus modify its metabolic functioning. Similarly, the chronic hyperglycemic state of diabetes mellitus leads to oxidative stress including several mechanisms such as the auto-oxidation of glucose leading to the formation of the superoxide anion radical and activation of the hexosamine pathway. For these reasons, we continued our study and tested the antioxidant activity of aqueous and organic extracts of *L. comosa* to determine their ability to scavenge free radicals using five different and complementary tests with different mechanisms (H_2O_2, ABTS, DPPH, FRAP, and RP).

3.3.2. Antioxidant Activity. In this study, the antioxidant potential of the 9 aqueous and organic extracts from the bulb of *L. comosa* was determined by five methods, and the results

Table 1: Polyphenol, flavonoid, and tannin contents of aqueous and organic extracts of *Leopoldia comosa* (L.) bulbs.

Extracts of *Leopoldia comosa* bulbs		Polyphenols (μg GAE/mg E)[x]	Flavonoids (μg QE/mg E)[y]	Tannins (μg CE/mg E)[z]
Aqueous	Decocted	**4.28 ± 0.02**[a]	82.15 ± 0.26[a]	17.06 ± 0.11[a]
	Infused	**4.44 ± 0.02**[a]	**90.82 ± 0.59**[b]	16.62 ± 0.04[b,a]
	Macerated	**4.69 ± 0.01**[a]	78.63 ± 0.21[c, a]	**18.68 ± 0.11**[c]
Organic	Ethanolic	20.49 ± 0.08[b]	147.63 ± 0.57[d]	12.28 ± 0.17[d]
	Macerated ethanolic	18.20 ± 0.04[c]	128.00 ± 0.23[e]	9.8 ± 0.18[e]
	Acetone	69.96 ± 0.01[d]	330.15 ± 1.45[f]	16.2 ± 0.23[f, a, b]
	Macerated acetone	61.43 ± 0.04[e]	308.45 ± 0.6[g]	15.06 ± 0.15[g]
	Diethyl ether	**129.75 ± 0.29**[f]	**988.26 ± 0.18**[h]	**30.22 ± 0.15**[h]
	Macerated diethyl ether	115.81 ± 0.24[g]	793.67 ± 1.49[i]	23.24 ± 0.09[i]

Data are expressed as mean ± standard deviation ($n = 3$). Different letters in the same column indicate a significant difference ($P < 0.05$). [x]μg of gallic acid equivalent per mg of dry plant extract. [y]μg of quercetin equivalent per mg of dry plant extract. [z]μg of catechin equivalent per mg of dry plant extract.

Table 2: IC50 (μg/mL) of aqueous and organic extracts of *Leopoldia comosa* (L.) bulbs for the inhibition of alpha-amylase, alpha-glucosidase, and beta-galactosidase assays.

Extracts of *Leopoldia comosa* bulbs		Alpha-amylase (IC50 μg/mL)[x]	Alpha-glucosidase (IC50 μg/mL)[x]	Beta-galactosidase (IC50 μg/mL)[x]
Aqueous	Decocted	**1200.66 ± 13.79**[a]	**238.53 ± 2.35**[a]	**216.9 ± 8.67**[a]
	Infused	2880 ± 8.05[b]	258.93 ± 1.38[b]	**205.43 ± 2.22**[b]
	Macerated	2752.33 ± 8.11[c]	268.23 ± 2.85[c, b]	245.5 ± 9.26[c]
Organic	Ethanolic	2264 ± 22.86[d]	257.96 ± 2.72[d,b,c]	182.23 ± 7.88[d]
	Macerated ethanolic	2384 ± 7.40[e]	162.7 ± 2.79[e]	196.2 ± 4.42[e]
	Acetone	**2219.33 ± 3.31**[f,d]	**85.41 ± 3.86**[f]	**163.5 ± 2.51**[f]
	Macerated acetone	2289.66 ± 7.45[g,d]	**85.95 ± 1.92**[g,f]	200.43 ± 12.15[g]
	Diethyl ether	2512.33 ± 5.98[h]	136.03 ± 0.95[h]	240.23 ± 13.45[h]
	Macerated diethyl ether	2897.66 ± 4.76[i, b]	130.80 ± 1.39[i, h]	291.83 ± 10.83[i]
Reference standard	Acarbose	616.33 ± 6.58[j]	**195 ± 5**[j]	—
	Quercetin	—	—	171.16 ± 2.90[j]

Data are expressed as mean ± standard deviation ($n = 3$). Different letters in the same column indicate a significant difference ($P < 0.05$). [x]Concentration that inhibits 50% of the activity in micrograms per milliliter.

of the H_2O_2, ABTS, DPPH, FRAP, and RP tests are presented in Table 3 and show that all the extracts prepared from *L. comosa* bulbs possess significant antioxidant properties.

(1) Hydrogen Peroxide Scavenging Assay (H_2O_2). Table 3 presents for the first time the results of hydrogen peroxide scavenging of *L. comosa* bulb extracts and those of ascorbic acid used as the reference standard. Aqueous extracts showed great activity by this test with a high percentage of scavenging of H_2O_2, respectively, of the order of 62.12 ± 0.2%, 61.89 ± 0.3%, and 61.72 ± 0.1% for decocted, infused, and macerated with a no significant difference ($P < 0.05$) between these three aqueous extracts. Organic extracts also showed better H_2O_2 scavenging activity, and the hot diethyl ether extract prepared by Soxhlet was the most active with a percentage of 62.67 ± 0.06%, followed by the two hot prepared ethanolic and acetone extracts with a percentage of 61.3 ± 0.16% and 61.24 ± 0.2%. The hot-prepared diethyl ether extract was the only one that showed a no significant difference ($P < 0.05$) with the reference standard (ascorbic acid) which showed a percentage of 63.63 ± 0.47%.

(2) Trolox Equivalent Antioxidant Capacity Using ABTS (TEAC). Trolox equivalent antioxidant capacity is based on the inhibition of $ABTS^+$ radical solution absorbance when it is exposed to an antioxidant. It should be noted that the higher the TEAC value, the more active the molecule is. The results of the ABTS test are expressed in μg Trolox equivalent per milligram of extract (μg TE/mg E) (Table 3). Antioxidant capacity is classified in the following order: diethyl ether > acetone > macerated diethyl ether > macerated acetone > ethanolic > macerated
ethanolic > decocted > infused > and aqueous macerate last.

(3) 2, 2-Diphenyl-1-picrylhydrazil Free Radical Scavenging Activity (DPPH). To study the antiradical activity of our 9 aqueous and organic extracts, we evaluated their ability to scavenge the free radical $DPPH^\circ$. $DPPH^\circ$ is a free radical that accepts an electron or hydrogen radical to become a stable molecule, and its purple color shows a characteristic absorption at 517 nm. The results of this test are expressed in IC50 illustrated in Table 3. The scavenging effect of the DPPH radical showed an activity that is dependent on the nature of the solvent employed, the extraction modality, and the concentration tested.

The results of this test showed that for the aqueous extracts, the decocted and the infused have IC50 values of 1011.33 ± 4.37 and 1089.33 ± 0.92 μg/mL, respectively, with a no significant difference and that they remain more active than that of the aqueous macerate (1140 ± 20.64 μg/mL). The

Table 3: Antiradical and antioxidant activity of aqueous and organic extracts of *Leopoldia comosa* (L.) bulbs via the five tests H_2O_2, ABTS, DPPH, FRAP, and RP.

Extracts of *Leopoldia comosa* bulbs		H_2O_2 (%)[a]	ABTS (μg TE/mg E)[b]	DPPH (IC50 μg/mL)[c]	FRAP (μg TE/mg E)[b]	RP (μg AAE/mg E)[d]
Aqueous	Decocted	**$62.12 \pm 0.2^{a,\ d}$**	**27.46 ± 0.69^{a}**	**1011.33 ± 4.37^{a}**	12.9 ± 0.1^{a}	**8.36 ± 0.06^{a}**
	Infused	$61.89 \pm 0.3^{a,\ d,\ e}$	17.18 ± 0.17^{b}	1089.33 ± 0.92^{b}	$11.16 \pm 0.52^{b,\ a}$	**7.91 ± 0.14^{a}**
	Macerated	$61.72 \pm 0.1^{a,\ d,\ e}$	6.63 ± 0.31^{c}	1140 ± 20.64^{c}	**15.27 ± 0.1^{c}**	**10.68 ± 0.13^{a}**
Organic	Ethanolic	$61.3 \pm 0.16^{b,\ a}$	225.86 ± 1.04^{d}	139.4 ± 6.93^{d}	131.55 ± 0.26^{d}	59.40 ± 0.21^{b}
	Macerated ethanolic	$61.09 \pm 0.05^{c,\ b}$	89.47 ± 0.68^{e}	220.5 ± 2.91^{e}	49.24 ± 0.13^{e}	18.86 ± 0.05^{c}
	Acetone	$61.24 \pm 0.2^{d,\ b,\ c}$	364.96 ± 0.28^{f}	$99.76 \pm 0.04^{f,\ d}$	277.74 ± 0.67^{f}	147.39 ± 1.07^{d}
	Macerated acetone	$60.94 \pm 0.2^{e,\ b,\ c}$	343.02 ± 1.44^{g}	$100 \pm 0.03^{g,\ f,\ d}$	225.77 ± 0.15^{g}	133.32 ± 0.8^{e}
	Diethyl ether	**62.67 ± 0.06^{f}**	**381.63 ± 0.63^{h}**	**10.08 ± 0.01^{h}**	**394.77 ± 0.74^{h}**	**356.7 ± 0.92^{f}**
	Macerated diethyl ether	$61.91 \pm 0.1^{g,\ a}$	$360.93 \pm 0.25^{i,\ f}$	$10.15 \pm 0.04^{i,\ h}$	358.77 ± 0.74^{i}	283.95 ± 0.59^{g}
Reference standards	Trolox	—	—	1.75 ± 0.09j	—	—
	Ascrobic acid	**63.63 ± 0.47^{f}**	—	0.17 ± 0.02k	—	—
	*B*HT	—	—	0.17 ± 0.02k	—	—

Data are expressed as mean ± standard deviation ($n = 3$). Different letters in the same column indicate a significant difference ($p < 0.05$). [a]H_2O_2 scavenging activity (%) of *L. comosa* extracts at the concentration of 100 μg/mL. [b]μg of Trolox equivalent per mg of dry plant extract. [c]Concentration that inhibits 50% of the activity in micrograms per milliliter. [d]μg of ascorbic acid equivalent per mg of dry plant extract.

organic extracts showed higher activity compared to the aqueous extracts with an IC50 of 10.08 μg/mL for diethyl ether extract, followed by macerated diethyl ether extract, acetone extract, macerated acetone, ethanolic extract, and macerated ethanol with IC50s, respectively, of the order of 10.15 ± 0.04, 99.76 ± 0.04, 100 ± 0.03, 139.4 ± 6.93, and 220.5 ± 2.91 μg/mL, with a significant difference between ethanolic extract, macerated ethanolic extract, acetone extract, and a no significant difference between the diethyl ether extract prepared by hot and cold modalities; likewise, for hot and cold acetone extract, the difference is no significant.

(4) Ferric Reducing-Antioxidant Power Assay (FRAP). The antioxidant power of *L. comosa* extracts was estimated from their ability to reduce the TPTZ-Fe (III) complex to TPTZ-Fe (II) measured at wavelength 593 nm. The results are expressed in μg Trolox equivalent per milligram of extract (μg TE/mg E).

The results obtained are presented in Table 3, and we noticed that all the tested extracts have a strong capacity for reducing iron with a significant difference ($P < 0.05$) between the aqueous and organic extracts and that the organic extracts are more active than aqueous extracts whose difference between decocted and infused is not significant, represented by values of 12.9 ± 0.1 and 11.16 ± 0.52 μg TE/mg E, respectively. For organic extracts, the diethyl ether extract is the most active with a value of 394.77 ± 0.74 μg TE/mg E, followed by the macerated diethyl ether, acetone, macerated acetone, ethanolic, and macerated ethanolic extract with values, respectively, of the order of 358.77 ± 0.74, 277.74 ± 067, 225.77 ± 0.15, 131.55 ± 0.26, and 49.24 ± 0.13 μg TE/mg E. The organic extracts showed a highly significant difference ($P < 0.05$) between them.

(5) Reducing Power Assay (RP). In this test, the yellow color of the test solution changes to different shades of green and blue, depending on the reducing power of each compound. A higher absorbance at 700 nm indicates a higher reducing power of the extract. The results are expressed in μg ascorbic acid equivalent per milligram of extract (μg AAE/mg E). The reducing power of our 9 prepared extracts is shown in Table 3. A highly significant difference between the aqueous and organic extracts was observed, and that the organic extracts have high reducing power, especially the diethyl ether extract with an iron reduction value of 356.7 ± 0.92 μg AAE/mg E.

3.3.3. Antibacterial Activity. According to our bibliographic research, this is the first study dedicated to the evaluation of the antibacterial activity carried out on the *L. comosa* bulbs. The results of the antibacterial activity were expressed from the measurement of the diameter of the inhibition halos. According to Table 4, extracts of *L. comosa* showed activity against *Proteus mirabilis* with an inhibition diameter ranging from 8.5 mm for the ethanolic extract to 10.5 mm for diethyl ether extract at the concentration of 100 mg/mL, while for *Listeria innocua* only the hot and cold prepared diethyl ether extracts showed an inhibition zone ranging from 9 mm to 10 mm.

3.4. Principal Component Analysis (PCA). In our study, the PCA was performed on individuals represented by the different extracts prepared from *L. comosa* bulbs and the variables are the measurements concerning the dosage of polyphenols, flavonoids, and tannins, the five tests of antioxidant activity (H_2O_2, ABTS, DPPH, FRAP, and RP), and the three tests of antidiabetic activity**:** alpha-amylase, alpha-glucosidase, and beta-galactosidase inhibition.

3.4.1. Correlation Matrix (Pearson (n)). The correlation matrix between the methods of antioxidant activity and antidiabetic activity and the results of the determination of

Table 4: Diameters of the inhibition zone (in mm) of different organic extracts from *Leopoldia comosa* (L.) bulbs against six pathogenic bacterial strains.

Strains	Ethanolic extract			Macerated ethanolic extract			Acetone extract			Macerated acetone extract			Diethyl ether extract			Macerated diethyl ether extract			Standard (+)		Standard (−)
	40 mg/ml	80 mg/ml	100 mg/ml	40 mg/ml	80 mg/ml	100 mg/ml	40 mg/ml	80 mg/ml	100 mg/ml	40 mg/ml	80 mg/ml	100 mg/ml	40 mg/ml	80 mg/ml	100 mg/ml	40 mg/ml	80 mg/ml	100 mg/ml	Tetra 20 μg/ml	AK 30 μg/ml	DMSO (10%)
											B.G-										
Ecoli	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12	0	0
Prote	7	7	**8.5**	8	7	**7.5**	9	7.5	**8**	10	7	**9.5**	11	8	**10.5**	10	10	**10.5**	23	0	0
Psdm	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0	21	0
											B.G+										
Staph	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13	0	0
Bacil	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0	24.5	0
Lister	—	—	—	—	—	—	—	—	—	—	—	—	—	9	**9**	—	10	**10**	0	26.5	0

B.G-: Gram-negative bacteria; *Ecoli*: *Escherichia coli* K12; *Prote*: *Proteus mirabilis*; *Psdm*: *Pseudomonas aeruginosa* CECT118; B.G+: Gram-positive bacteria; *Staph*: *Staphylococcus aureus* CECT976; *Bacil*: *Bacillus subtilis* DSM6633; *Lister*: *Listeria innocua* CECT4030; Tetra: tetracycline; Ak: amikacin; DMSO: dimethylsulfoxide.

polyphenols, flavonoids, and tannins are shown in Table 5. According to the latter, we noticed a better correlation between polyphenols and flavonoids with a correlation coefficient r which is equal to 0.9705 and between flavonoids and tannins with $r = 0.8396$. For the antioxidant activity tests, we found that there is a difference in correlation between the five tests, with the best correlations found for the DPPH test with the FRAP test with a correlation coefficient ($r = 0.9439$), DPPH, and RP ($r = 0.9549$) and between DPPH and ABTS ($r = 0.8982$). The H_2O_2 test showed a low correlation with the ABTS test ($r = 0.4240$) and with the DPPH test ($r = 0.5792$). A positive correlation was observed for the content of polyphenols and flavonoids, with the ABTS, RP, and FRAP tests. The H_2O_2 test showed a positive correlation with the tannin content of the extracts ($r = 0.8354$). For the antidiabetic activity tests, we noticed that there is a weak correlation between the three tests alpha-amylase, alpha-glucosidase, and beta-galactosidase.

3.4.2. Graphical Representation of the Principal Component Analysis (PCA). The results of the PCA are presented according to the two axes $F1$ and $F2$ because their cumulative percentage explains 84.54% of the information retained (Figure 1). The first principal component ($F1$) explains 63.54% of the total information, and the second one ($F2$) shows 21.01%.

According to Figure 1, the F1 axis is mainly constructed by the positive correlation between the ABTS, DPPH, RP, and FRAP tests and the contents of polyphenols, flavonoids, and tannins. The F2 axis is formed by the H_2O_2, alpha-glucosidase, and beta-galactosidase inhibition assays (Figure 1).

4. Discussion

4.1. Mineral Composition of Leopoldia comosa (L.) Bulbs. According to our bibliographical research, the present study represents the first investigation carried out on the mineral composition of *L. comosa* bulbs. The results obtained showed that *L. comosa* is an important source of the mineral elements: Fe, K, P, Na, Cu, Mg, and Ca, which are involved in the defense mechanism against oxidative stress and thus protect the body from cancer and cardiovascular disease [30]. Among the best-known antioxidant minerals such as zinc and selenium, zinc plays a global antioxidant role, as it is directly involved in the constitution of an anti-free radical enzyme: superoxide dismutase. This mineral is involved in the activity of more than 200 enzymes, particularly those involved in protection against free radicals and those involved in protein synthesis. Hence, it is important in the phenomena of cell renewal, cicatrization, and immunity. Selenium also participates in the fight against free radicals, being an essential component of certain antioxidant enzymes. It also has a stimulating effect on immunity and therefore contributes in general to the body's defense reactions.

The bulb of *L. comosa* could be considered as a good dietary complement because of its high content of Fe, K, P, Cu, and Mg, and because of its low Na/K ratio, it could also be used as a protective agent against cardiovascular diseases.

The food use of the bulb of *L. comosa* has a long history in Mediterranean countries. Indeed, the shape and taste of the *L. comosa* bulb are very similar to garlic, onion, and leek. In the past, peasants, during their work, picked and ate the bulbs with bread. Nowadays, the bulbs are peeled, cut, and fried in olive oil, sometimes mixed with cheese and eggs. In some places, for example, in the region of Salerno, Italy, they are boiled and served with a sweet and sour sauce [31]. *L. comosa* bulbs contain mucilages, sugars, latex, waxes, and traces of volatile oil. Thanks to these substances, the plant is widely used in food [31].

4.2. Yields of Aqueous and Organic Extractions of Leopoldia comosa (L.) Bulbs. The solvents used for *L. comosa* extraction showed significantly different extraction capacities between aqueous and organic extracts. From the results, we note that the extraction yield depends on the choice of solvent, extraction time, temperature, and extraction modality. Other studies have reported that the yield also varies according to the chemical nature of the sample [32]. In our study, the more polar the solvent, the higher and more important the yield is, which is consistent with the results of other work carried out by our laboratory on plants of the Taza region [10, 13–15]. Also, hot extraction by decoction modality seems to be the best method to obtain a better aqueous extraction yield, which agrees with the results of our laboratory by Bouabid et al. [10], who stated that the decoction of *Atractylis gummifera* (L.) gives a yield of 35% against 24% for maceration. A similar result was obtained in our work on another plant *Juglans regia* (L.) of the family Juglandaceae [13]. Indeed, the decoction of *Juglans regia* (L.) gives a yield of 14% compared to 8.75% for the aqueous macerate. The results of our study concur with those of previous studies in our laboratory which have shown that the extraction yield depends on the choice of solvent and extraction method [10, 13–15] and that for a better yield. The use of polar solvents is recommended.

In comparison with other work carried out on *L. comosa* bulbs, the study carried out by Larocca et al. in Italy reported a yield of $7.61 \pm 0.10\%$ for the extract prepared by the hydroalcoholic solvent (water/methanol 70%) by maceration using centrifugation of 80 rpm (rotation per minute) for 24 hours at 30°C [33]. Another study conducted by Loizzo et al. [34] in Italy on *L. comosa* bulbs showed that the yield was 3.16% for the ethanolic extract prepared by maceration for 48 hours compared to 1.3% for the macerated ethanolic extract of Moroccan *L. comosa* bulbs. So, the yield is almost twice what we got in our study. This can be explained by the geographical place of the plant's harvest, as well as the application of centrifugation in the maceration process.

The results of the hydrodistillation method from the bulbs of *L. comosa* showed us an absence of essential oils in this plant part, as according to our bibliographical research, no previous report has described the presence of essential oils in *L. comosa* bulbs. Other methods can be used to confirm the absence of essential oils in *L. comosa* bulbs, such as steam extraction, organic solvent extraction, or ultrasonic extraction.

Table 5: Correlation coefficient between chemical composition and tests for antidiabetic and antioxidant activities of aqueous and organic extracts of *Leopoldia comosa* (L.) bulbs.

Variables	Polyphenols	Flavonoids	Tannins	H_2O_2	ABTS	RP	FRAP	DPPH	Alpha-amylase	Alpha-glucosidase	Beta-galactosidase
Polyphenols	**1**	**0.9705**	0.7381	0.3407	**0.8873**	**0.9905**	**0.9807**	**0.9545**	−0.3081	0.6769	−0.3813
Flavonoids		**1**	**0.8396**	0.5272	0.7708	**0.9890**	**0.9188**	**0.9233**	−0.3112	0.4969	−0.5158
Tannins			**1**	**0.8354**	0.4240	0.8007	0.6492	0.5792	−0.2023	0.1585	−0.6514
H2O2				**1**	−0.0375	0.4389	0.2218	0.2049	0.0719	−0.3094	−0.5861
ABTS					**1**	0.8515	**0.9549**	**0.8982**	−0.2220	0.8135	−0.0031
RP						**1**	**0.9646**	**0.9439**	−0.3006	0.5834	−0.4245
FRAP							**1**	**0.9549**	−0.2872	0.7234	−0.2399
DPPH								**1**	−0.3231	0.6222	−0.3180
Alpha-amylase									**1**	−0.1038	0.3942
Alpha-glucosidase										**1**	0.1751
Beta-galactosidase											**1**

H_2O_2: hydrogen peroxide scavenging assay; ABTS: Trolox equivalent antioxidant capacity (TEAC) method/ABTS radical cation decolorization assay; FRAP: ferric reducing-antioxidant power assay; RP: reducing power method; DPPH: DPPH scavenging activity.

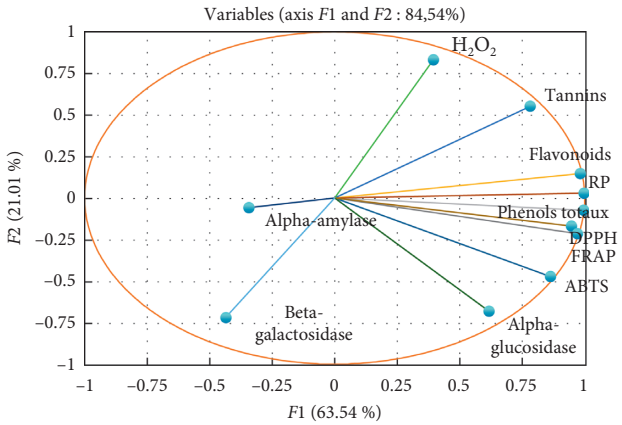

Figure 1: Graphical representation of the principal component analysis (PCA) of the different variables of the chemical composition and tests for antioxidant and antidiabetic activities. H_2O_2: hydrogen peroxide scavenging assay; ABTS: Trolox equivalent antioxidant capacity (TEAC) method/ABTS radical cation decolorization assay; FRAP: ferric reducing-antioxidant power assay; RP: reducing power method; DPPH: DPPH scavenging activity.

4.3. Phytochemical Screening. The results of the phytochemical screening revealed the presence of flavonoids, catechin tannins, and quinones in the plant bulb and the 9 aqueous and organic extracts prepared. For the families of anthracenosides and anthraquinones, we can deduce that distilled water does not allow for their extraction and that only organic extracts allow for the extraction of these two families. Tests for alkaloids, saponins, and sterols were negative on the bulb and all prepared extracts.

In addition to the presence of phenolic compounds in *L. comosa* bulbs, previous studies have reported the presence of other chemical families. Indeed, Parrilli et al. [35] reported that the bulb of *L. comosa* is an important source of triterpene and glycoside, of which eucosterol 4a, a terpene of the nor-27 lanostane family, is the major compound. The same team was able to characterize the structure of glycosides from *L. comosa* [36]. In 1984, Adinolfi et al. were able to determine the structure of new triterpenes from *L. comosa* bulbs harvested in Italy [37, 38]. These authors continued their research and were able to identify two new 3-benzyl-chromanones, named 7-O-methyl-3.9-dihydropunctatin 1 and 8-O-demethyl-7-O-methyl-3.9-dihydropunctatin 2 [39], and in 1985, they were able to isolate, from the bulb of *L. comosa* harvested in Italy, three new homoisoflavanones, and their structures were elucidated: muscomosin, comosin, and 8-odemethyl-8-O-acetyl-7-O-methyl-3.9-dihydropunctatin [40].

A comparison with other plants from the Taza region studied under the same experimental conditions in our laboratory (SNAMOPEQ) allows us to say that the presence or absence of different secondary metabolites varies according to the botanical family, the species, and the geographical place of the plant's harvest as well as the different solvents used for extraction. For example, the study carried out by Senhaji et al. [15] showed that *Anabasis aretioïdes* harvested in the Figuig region is characterized by the presence of saponins, catechin tannins, and sterols, whereas the qualitative study carried out by Bentabet et al. on the same plant harvested in Algeria shows the presence of alkaloids, tannins, saponins, reducing sugars, and coumarins [41]. Another study was carried out by Senhaji et al. [14] on *Ajuga iva* Subsp. *Pseudoiva* showed the presence of 4 chemical families, flavonoids, sterols, saponins, and catechin tannins, while another study review reported the presence of other families for the plant *Ajuga iva* [42]. The study conducted by Bouabid et al. [10] on *Atractylis gummifera* (L.) which belongs to the Asteraceae family revealed that the plant contains flavonoids, tannins, saponins, quinones, and sterols. The families of Asteraceae (*Atractylis gummifera*) and Lamiaceae (*Ajuga iva* Subsp. *Pseudoiva*) are among the most exploited families in traditional Moroccan medicine [43, 44].

4.4. Polyphenol, Flavonoid, and Tannin Contents of Leopoldia comosa Bulbs. The contents of polyphenols, flavonoids, and tannins in an extract are parameters that depend strongly on

the operating conditions of the extraction, and in particular on the nature and polarity of the solvent [12–15]. As Table 1 shows, the solvents used in extraction affected significantly ($P < 0.05$) the content of phenolic compounds in *L. comosa*.

The solvents diethyl ether followed by acetone and ethanol were found to be more effective in extracting phenolic compounds than water. This result indicates that *L. comosa* bulbs contain many fewer polar compounds.

Although water extraction gives us a high extraction yield, it is not the right solvent for the extraction of phenolic compounds. This could be explained by the fact that water extracts only water-soluble bioactive compounds; besides, many other residual substances and impurities are present in aqueous extracts. Organic extracts have higher levels of phenolic compounds than aqueous extracts.

The results obtained are in agreement with many results previously reported by our laboratory indicating that phenolic compounds are generally more soluble in polar organic solvents than in water [12, 13, 15].

In comparison with the work carried out by Casacchia et al. on the bulb *L. comosa* from Italy [45], the contents of phenolic compounds were of the order of 39.53 ± 0.027 and 49.80 ± 0.012 mg CAE/g MF (chlorogenic acid equivalent (CAE) per g fresh material (MF)), respectively, for the decocted and the steamed bulb extract cooked for 15 min; these results are expressed by another reference standard (chlorogenic acid) and not the gallic acid which we used and which is the most used. For the flavonoid content, the decocted has a value of 0.64 ± 0.026 and the steamed bulb extract of 1.63 ± 0.010 mg QE/g MF (mg equivalent of quercetin per gram of fresh material). Similarly, the study carried out by Larocca et al. in Italy [33] reported the content of phenolic compounds and flavonoids for the hydroalcoholic extract (water/methanol 70%) which was around 57.67 ± 0.72 mg GAE/g E and 18.79 ± 0.36 mg QE/g E, respectively.

According to our bibliographic research, the tannin content of *L. comosa* bulbs has not been reported in any previous reports, and our study is the first one that has investigated and dosed the tannins.

Our study has highlighted the presence and content of tannins for the first time for the bulb of *L. comosa*, so we can say that Moroccan *L. comosa* has high contents of polyphenols, flavonoids, and in particular tannins extracted by the less polar solvent diethyl ether. Also, we can deduce that the choice of the solvents to be used is essential for the extraction and determination of the secondary metabolites of a plant and its pharmacological valorization by the studies of the biological properties.

4.5. Antidiabetic Activity. In several epidemiological studies, postprandial glycemia is a major independent risk factor for cardiovascular disease in both glucose intolerant and type 2 diabetic patients. People with low glucose tolerance or diabetes often have high postprandial blood glucose levels for long periods [46, 47]. Alpha-amylase and alpha-glucosidase are two enzymes responsible for the degradation of carbohydrates. This degradation allows the absorption of glucose and increases blood glucose levels [10, 48]. As a result, inhibition of these two enzymes limits the increase in postprandial glycemia and may therefore be an important strategy for reducing blood glucose levels in type 2 diabetics.

The results of the present study reveal that the aqueous and organic extracts of *L. comosa* bulbs have a high alpha-amylase inhibition activity, with a high inhibitory power of IC50 of 2752.33 ± 8.11 μg/mL for the decocted and an IC50 of 2264 ± 22.86 μg/mL for the ethanolic extract. In comparison with other studies on the bulb of *L. comosa*, Casacchia et al. in Italy reported IC50s of 730 ± 0.13 μg/mL and 690 ± 0.02 μg/mL for the extract of the bulb steamed for 15 min and decocted, respectively [45]. In another study carried out in Italy on *L. comosa* bulbs, Larocca et al. found an IC50 of 75.17 ± 0.52 μg/mL for the hydroalcoholic extract (water/methanol 70%) [33]. Similarly, the study by Loizzo et al. in the same country found IC50s of 81.3 ± 2.77 and 166.9 ± 3.4 μg/mL for ethanolic and n-hexane extracts prepared from the bulb of *L. comosa*, respectively [34]. Therefore, we can conclude that the results of the alpha-amylase inhibition activity of *L. comosa* bulb extracts are different even for bulbs harvested in the same country, which can be explained by several parameters, including the choice of solvent, extraction ratio, extraction method, treatment or not of the plant before use, and place and season of harvest of the plant.

The results of alpha-glucosidase inhibition by aqueous and organic extracts of *L. comosa* bulbs are promising, mainly the acetone extract ($IC50 = 85.41 \pm 3.86$ μg/mL) which proved to be 3 times more active than the reference standard, acarbose ($IC50 = 247.23 \pm 2.85$ μg/mL). Our results are consistent with the work carried out by Larocca et al. in Italy who reported an IC50 of 85.33 ± 0.38 μg/mL for the hydroalcoholic extract (water/methanol 70%) of *L. comosa* bulbs [33]. However, our results are better than those obtained by Loizzo et al. who reported IC50s of 112.8 ± 3.3 and 166.9 ± 3.4 μg/mL, respectively, for the ethanolic and n-hexane extract of *L. comosa* bulbs [34].

The results of inhibition of aqueous and organic extracts of *L. comosa* bulbs by the beta-galactosidase inhibition test are presented for the first time in our study. All the extracts tested showed a high hypoglycemic power with a better activity presented by the aqueous extracts, especially the decocted ($IC50 = 205.43 \pm 2.22$ μg/mL), and for the organic extracts, the acetone extract was the most active with an IC50 value of 163.5 ± 2.51 μg/mL.

4.6. Antiradical and Antioxidant Activity

4.6.1. Hydrogen Peroxide Scavenging Assay (H_2O_2). The study of the antioxidant activity of *L. comosa* bulbs by the H_2O_2 test represents the first study carried out by this test. The importance of this test is shown by the ability of the extracts tested to scavenge the H_2O_2 radical, as the latter is an oxidant and can directly inactivate some enzymes, generally by oxidation of essential thiol groups (-SH). Hydrogen peroxide can rapidly cross the cell membrane and once inside the cell, H_2O_2 can probably react with Fe^{2+} and

eventually Cu^{2+} and form a hydroxyl radical, and this may be the cause of its many toxic effects. Therefore, the removal of hydrogen peroxide is very important. The aqueous and organic extracts of *L. comosa* were tested for their antioxidant capacity by the hydrogen peroxide scavenging method, and as shown in Table 3, all extracts, at the concentration of 100 μg/mL, showed scavenging capacity against H_2O_2. In our study, diethyl ether extract represents the highest percentage of H_2O_2 scavenging ($62.67 \pm 0.06\%$), which is significantly higher than that obtained by aqueous and other organic extracts. These results are higher than those obtained in our laboratory [12, 14, 15] who found that the macerated methanolic extract of *Atractylis gummifera* (L.), *Ajuga iva* Subsp. *Pseudoiva*, and *Anabasis aretioïdes* gives a percentage of scavenging, respectively, of the order of $19.24 \pm 1.10\%$, $22.17 \pm 0.30\%$, and $5.32 \pm 0.23\%$.

4.6.2. Trolox Equivalent Antioxidant Capacity Using ABTS (TEAC). The results of the ABTS test represent the first study conducted using this test because according to our literature search, no studies have been done using this test for the bulb of *L. comosa*. In this test, the antioxidant reduces the ABTS•+ cation radical generated by ammonium persulfate. Our results show that aqueous extracts have lower TEAC contents than organic extracts and that diethyl ether extract gives the highest TEAC value (381.63 ± 0.63 μg TE/mg E) which is highly correlated with the content of polyphenols, flavonoids, and tannins in this extract. Extraction by organic solvents using Soxhlet in our study seems to be the most efficient method to extract phenolic compounds and therefore allows obtaining a very good antioxidant activity. These results disagree with previous work carried out by our laboratory which indicates that organic extraction by maceration is the best method to obtain good antioxidant activity [12–15] which can be explained by the qualitative and quantitative difference in the chemical composition of our plant subject of this study.

4.6.3. 2, 2-Diphenyl-1-picrylhydrazil Free Radical Scavenging Activity (DPPH). The DPPH radical scavenging test showed an activity that is dependent on the nature of the solvent used, the extraction modality, and the concentration tested. Thus, the diethyl ether extract of *L. comosa* bulbs at 5 μg/mL showed a scavenging effect of 31.10% which increases to 96.43% at 100 μg/mL.

IC50 values were calculated for all aqueous and organic extracts and reference standards (ascorbic acid, Trolox, and BHT); a lower IC50 value indicates higher antiradical activity. For the aqueous extracts, we note that the decocted presents the best activity with an IC50 of 1011.33 ± 4.37 μg/mL. Organic extracts showed higher activity compared to aqueous extracts with an IC50 of 10.08 ± 0.01 μg/mL for the diethyl ether extract. Therefore, the polar solvent diethyl ether is the most suitable solvent for *L. comosa* to extract its bioactive molecules. Our results are better in comparison with previous work carried out in Italy by Loizzo et al. [34] on the macerated ethanolic extract and the hexane extract of *L. comosa* which obtained IC50 values of the order of 40.9 ± 1.8 and 46.6 ± 1.5 μg/mL, respectively. Another study carried out by Larocca et al. in Italy found an IC50 of 36.73 ± 0.49 μg/mL for the hydroalcoholic extract (70% water and methanol) [33]. Similarly, for the study carried out by Casacchia et al. in Italy, the decoction recorded an IC50 value of 9630 μg/ml [45]. These results can be explained by the choice of extraction solvents and by the hot and cold extraction modality adopted and also by the chemotype.

4.6.4. Ferric Reducing-Antioxidant Power Assay (FRAP). The reduction and oxidation of a chemical are defined as a gain or loss of electrons, respectively. A reducing agent is a substance that gives electrons and, therefore, causes the reduction of another reagent. The FRAP test is widely used in the evaluation of the antioxidant power of food polyphenols. The antioxidant potency of *L. comosa* extracts was estimated based on its ability to reduce the TPTZ-Fe (III) complex to TPTZ-Fe (II) measured at wavelength 593 nm. It appears that organic extracts show a higher antioxidant capacity by the FRAP test than aqueous extracts. The reducing capacity of the diethyl ether extract was the most powerful among the nine extracts tested (394.77 ± 0.74 μg TE/mg E). These results correlate with work carried out in our laboratory [13–15] which found that organic extracts show great antioxidant power compared to aqueous extracts and that hot extraction gives better results than cold extraction by this test.

4.6.5. Reducing Power Assay (RP). The reducing power test is based on the reduction of the $Fe^{3+/}$ferricyanide complex to ferrous ion (Fe^{2+}) in the presence of reducing agents (antioxidants) measured at wavelength 700 nm. In this test, the classification of the extracts is the same as that obtained with the FRAP test, and this means that the molecules responsible for the reduction of the complex are the same as those of the reducing power. The diethyl ether extract proved to be the most active with an iron reduction value of 356.7 ± 0.92 μg AAE/mg E which is due to its high content of phenolic compounds and therefore its ability to transfer electrons. The study conducted by Loizzo et al. in Italy [34] reported that the ethanolic extract prepared by cold maceration for 48 hours and the n-hexane extract had IC50s of 78.8 ± 2.8 and 113.6 ± 3.7 μg/mL, respectively.

4.7. Antibacterial Activity. On this plant and to the best of our knowledge, this study is the first to provide data on antibacterial activity. The results show that among all the extracts tested, the diethyl ether extract has a moderate antibacterial effect on two strains: one Gram- (*Listeria innocua*) and the other Gram+ (*Proteus mirabilis*). The promising effect of the diethyl ether extract could be attributed to its phenolic content with values, respectively, of the order of 129.75 ± 0.29 μg GAE/mg E, 988.26 ± 0.18 μg QE/mg E, and 30.22 ± 0.15 μg CE/mg E for polyphenols, flavonoids, and tannins. Indeed, phenolic compounds have been shown to have strong antibacterial activity [49].

Using the same bacterial strains, the work carried out in our laboratory (SNAMOPEQ) with organic extracts (methanol, macerated methanol, chloroform, ethyl acetate, and petroleum ether) prepared from the aerial part of *Anabasis aretioïdes* showed that the inhibition diameter varies from 7 to 13.5 mm depending on the bacterial strain used and the choice of solvent for extraction. Of the five extracts tested, the ethyl acetate extract at a concentration of 100 mg/mL showed moderate antibacterial activity against *Staphylococcus aureus* CECT976, *Proteus mirabilis*, *Bacillus subtilis* DSM6633, *Escherichia coli* K12, and *Pseudomonas aeruginosa* CECT118 with an inhibition diameter of 13.5, 12.5, 11.5, 10.5, and 8 mm, respectively. However, the less polar extract prepared by petroleum ether did not affect the bacterial strains [15]. These results are different from our study which revealed that the diethyl ether extract whose polarity is close to that of the petroleum ether was found the most active. Therefore, the use of different solvents for extraction is important to select the one that gives the best result.

Besides, the antibacterial activity of phenolic compounds has been extensively studied against a wide range of microorganisms and has demonstrated potent activity and interesting synergistic properties with antibiotics [49, 50]. However, the absence of activity for some extracts of *L. comosa* does not mean the total absence of phenolic compounds but could be due to the low amount of these compounds or their antagonistic action by the presence of other compounds. The compounds responsible for the antibacterial activity of the *L. comosa* bulb are characterized by their solubility in less polar solvents.

4.8. Principal Component Analysis (PCA). PCA was performed with $n = 9$ extracts prepared from *L. comosa* bulbs. The PCA is associated with a diagonal Pearson correlation matrix between the nine extracts and the principal component factors. The results of the present study allowed us to determine the different correlations between polyphenols, flavonoids, and tannins; the five methods of antioxidant activity; and the three tests of antidiabetic activity. According to Figure 1, we found a positive correlation between the ABTS, DPPH, RP, and FRAP tests and the contents of polyphenols, flavonoids, and tannins. These results are in agreement with the literature which indicates that phenolic compounds play an important role in the scavenging of free radicals [13, 51, 52]. For tests of antidiabetic activity, we noticed a weak correlation between the three tests alpha-amylase, alpha-glucosidase, and beta-galactosidase and the contents of polyphenols, flavonoids, and tannins, which is in disagreement with the literature of which several studies have reported that polyphenols can have considerable hypoglycemic properties [12, 46]. This can be explained by the antagonistic action between the families present in *L. comosa* bulbs or the presence of other chemical families in the bulbs of *L. comosa* that we have not dosed and which have a greater action in the inhibition of the enzymes responsible for the antidiabetic activity.

5. Conclusions

This study, undertaken for the first time in Morocco, allowed us to describe and determine the mineral, chemical composition, and pharmacological properties of the aqueous and organic extracts of the bulb of *Leopoldia comosa* (L.), a spontaneous plant from the region of Taza, Morocco, which is characterized by a high production of this plant.

L. comosa is an important source of the mineral elements in particular: Fe (33552), K (1843.14), P (756.36), Na (439.65), Cu (303.9), Mg (272.37), and Ca (20.55) mg/kg plant matter.

Phytochemical screening carried out both on the bulb and the aqueous and organic extracts prepared from it shows that *L. comosa* is rich in polyphenol, flavonoid, tannin, quinone, anthraquinone, and anthracenoside compounds, mainly the diethyl ether extract. The results of the *in vitro* antidiabetic activity showed that *L. comosa* extracts possess inhibitory activity of the enzymes alpha-amylase, alpha-glucosidase, and beta-galactosidase, in particular, the aqueous extract prepared by decoction and the acetone extract, which were found to be the most active in all three tests. The results of the antioxidant activity show that all the extracts prepared from *L. comosa* have high antioxidant power, especially the diethyl ether extract prepared by Soxhlet, which presents significant values via the five tests of antioxidant activity (H_2O_2, ABTS, DPPH, FRAP, and RP). The results of the antibacterial activity show that among all the extracts tested, the diethyl ether extract has a moderate antibacterial effect on two strains: one Gram- (*Listeria innocua*) and the other Gram+ (*Proteus mirabilis*). The results of the principal component analysis (PCA) allowed us to conclude that there is a positive correlation between the ABTS, DPPH, RP, and FRAP tests and the contents of polyphenols, flavonoids, and tannins. For antidiabetic activity, a weak correlation was obtained between the three assays alpha-amylase, alpha-glucosidase, and beta-galactosidase and phenolic compounds, flavonoids, and tannins.

In addition to the economic and ethnomedicinal values represented by wild Moroccan *L. comosa* because it represents an important source of income for the population in the region of Taza, Morocco, and is used in traditional Moroccan medicine [16], the present study underlines the importance of *L. comosa* as a medicinal and food plant by highlighting its richness in mineral elements and chemical compounds at the origin of the important biological antidiabetic, antioxidant, and antibacterial activities. Our results of the mineralogical and chemical analyses and the *in vitro* evaluation of the plant's antidiabetic and antioxidant activities are promising and encourage us to continue the *in vivo* study of the antidiabetic and antioxidant activities mainly for the aqueous decocted extract and the organic acetone extract.

Abbreviations

L. comosa: *Leopoldia comosa*
ROS: Reactive oxygen species
H_2O_2: Hydrogen peroxide scavenging assay

TEAC or ABTS:	Trolox equivalent antioxidant capacity method/ABTS radical cation decolorization assay
FRAP:	Ferric reducing-antioxidant power assay
RP:	Reducing power method
DPPH:	1.1-Diphenyl-2-picrylhydrazyl scavenging activity
PCA:	Principal component analysis
ANOVA:	Analysis of variance
IC50:	Concentration that causes 50% inhibition
EO:	Essential oils.

Data Availability

The experimental data used to support the findings of this study are incorporated into the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

MB performed experimental studies, statistical analysis, and manuscript preparation. FL designed the experiments, offered consistent guidance, analyzed the data, manuscript preparation, and review, and edited the final version and submitted it for publication. SS, NL, and KB participated in the experimental studies. HT designed the experiments, provided consistent guidance, and carried out manuscript preparation and review.

Acknowledgments

The authors thank the Centre CURI (Regional University Interface Center), Université Sidi Mohamed Ben Abdellah (USMBA) of Fez, Morocco, for the determination of the mineral composition of the plant, Dr. Abdelmajid Khabach, for the botanical identification of the plant, and Mourad Stitou, a Ph.D. student of SNAMOPEQ Laboratory, for his help in the XLSTAT software. This study was conducted with the support of the Sidi Mohamed Ben Abdellah University (USMBA).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 9970678, 8 pages
https://doi.org/10.1155/2021/9970678

Research Article

The Effect of Cinnamaldehyde on iNOS Activity and NO-Induced Islet Insulin Secretion in High-Fat-Diet Rats

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Received 19 March 2021; Revised 7 June 2021; Accepted 3 July 2021; Published 13 July 2021

Academic Editor: Reza B. Jalili

Introduction. Obesity and insulin resistance are associated with alterations in nitric oxide level and insulin secretion. Previous studies demonstrated that cinnamaldehyde (CNMA) improved islet insulin secretion and restored nitric oxide (NO) level, but its underlying mechanisms have not been investigated. This study aimed to investigate the effect of CNMA on inducible nitric oxide synthase (iNOS) activity and NO-induced islet insulin secretion in high-fat-diet (HFD) treated rats. *Materials and Methods*. Forty male Wistar rats (12 weeks old) were randomly divided into four equal groups, namely, control, CNMA, HFD, and HFD + CNMA. Control and CNMA groups were treated with standard laboratory animals' diet, while HFD and HDF + CNMA groups were fed with an HFD diet enriched with 25% *W*/*W* tail fat for 16 weeks. CNMA was administrated orally (20 mg/kg body weight, daily) during the study period. Islet insulin secretion and the inducible NOS activity in the presence or absence of L-NAME (NO synthase inhibitor, 5 mmol/L) were evaluated. *Results*. L-NAME-suppressed insulin secretion in control, HFD, and HFD + CNMA groups; however, in the CNMA group, it could not exhibit such effect ($P < 0.01$). Islets of HFD-treated animals showed significantly higher iNOS activity than controls. CNMA treatment significantly suppressed iNOS activities in CNMA and HFD + CNMA groups compared with control and HFD, respectively. *Conclusion*. These results suggest that the beneficial effect of CNMA on insulin secretion might be due to its inhibitory effect on iNOS activity.

1. Introduction

Metabolic syndrome (MS) is defined as a collection of interrelated risk factors that can predict type 2 diabetes mellitus, stroke, cardiovascular disease, and other health threats. These risk factors include abdominal obesity, hyperglycemia, dyslipidemia, and high blood pressure with a core component of insulin resistance (IR). The pathophysiology of MS is not well understood. However, it is well known that nitric oxide (NO) plays a crucial role in the pathogeneses of metabolic syndrome [1] and is closely linked with insulin signaling [2]. Also, apparent data suggest that NO is involved in the development of IR and type 2 diabetes [3, 4]. On the other hand, NO has long been used as a vasodilator, and nitrate compounds are frequently used for angina, hypertension, and heart failure.

To study the pathophysiology of MS, numerous animal models of dietary approaches to imitate the disease condition in humans have been established. In this approach, single-type diets (high fructose, high sucrose, or high fat) or combinations of diets (high fructose/fat or high sucrose/fat) are frequently used [5]. Several high-fat diets (HFD) with different fat sources (plant- or animal-derived) as well as fat contents (vary between 20 and 60% of total energy) have been extensively used [5–7].

NO is a highly diffusible gaseous molecule with a short half-life in blood (a few seconds). NO plays essential roles such as cell signaling and vasodilator molecules in several biological processes. Therefore, its production is strictly controlled in almost every cell type [8]. The NO synthesis within cells is regulated by different enzyme isoforms and

nonenzymatic pathways [9]. The enzymatic pathway is one of the most important ways in which NO production is regulated. In this pathway, NO formation is catalyzed by NO synthase (NOS) enzyme through a series of redox reactions in which NOS utilizes tetrahydrobiopterin, NADPH, and molecular oxygen to convert l-arginine to l-citrulline and NO. There are three isoforms of NOS in mammals: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) that play important roles as dichotomous effects in human biology and diseases [8, 9].

In pathophysiological conditions such as diabetes, obesity, and insulin resistance, NOS activity is altered [10]. However, the NO production could be increased in hyperglycemia, but its bioavailability decreases due to degradation in reaction with free oxygen radicals [10]. Recent evidence demonstrated that alteration in iNOS expression is involved in the pathogenesis of IR, obesity, and diabetes [11, 12]. iNOS stimulation could produce a higher amount of NO than other isoforms of NOS (eNOS and nNOS) [11]. Despite the protective effects of NO in physiological concentration, its overproduction in pathophysiological conditions such as IR and obesity can lead to irreversible tissue damage [10–12]. Also, iNOS-derived NO primarily causes disturbance of carbohydrate metabolism through β-cell dysfunction and impaired insulin secretion [12]. NO also participates in insulin production and secretion. However, its role in insulin secretion is disputable [13]. It is revealed that high levels of NO inhibit the activity of cytochrome *c* oxidase and produce ATP, leading to impaired glucose-stimulated insulin secretion [12].

Owing to its short half-life, measuring nitric oxide is difficult. Therefore, assessments of plasma nitrate and nitrite (NO_X) are being used as markers for the activities of NOS and the production of NO. There is a strong correlation between serum NO_X levels and NO production so that the determination of NO_X in the blood has been reported to be the most useful method of quantitation of NO production in the body [14].

Cinnamaldehyde (CNMA) can be isolated from several plant sources such as *Cinnamomum cassia* and *Cinnamomum burmannii*. The CNMA has been widely studied for its several pharmacological activities [15]. CNMA has many medicinal activities including antioxidant, antibacterial, anticancer, immunomodulatory, and antidiabetic activities [6, 16–18]. A part of the therapeutic effects of CNMA was accomplished by modulation in NO production [19]. As mentioned above, NO produced in islets of the pancreas participates in the synthesis and secretion of insulin. Moreover, there is evidence showing that CNMA could improve NO balance in cell lines [20] and islet insulin release in rats with metabolic syndrome [6]. However, the role of NO in the beneficial effects of CNMA on insulin secretion has not been studied directly. To illuminate this uncharted area, in the present study, we examined the NO pathway. Accordingly, the aim of this study was to investigate the impact of CNMA on iNOS activity and NO level in HFD-treated rats.

2. Materials and Methods

2.1. Animals and Sample Preparation. All procedures involving animals were performed according to the guides and rules in care and use of Laboratory Animals in Scientific Affairs with the Iranian Ministry of Health and Medical Education following the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The animal experiments were approved by the Ethics Committee of Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences (permit code: 91/08/168).

Male Wistar rats (12 weeks old, 220–250 g) were purchased from Pasteur Institute, Tehran, Iran. Animals were allowed to acclimatize for 14 days to the laboratory environment (12 h light/dark cycle, 30–35% humidity, and 20–24°C temperature) before the experiment. Afterwards, they were randomly divided into four groups of 10 rats each. Group 1 received standard diet (control); group 2 was treated with a semi-purified HFD (HFD); group 3 received standard diet plus 20 mg/kg (6) CNMA by daily gavage for 16 weeks (control + CNMA); and group 4 was treated with a semipurified HFD plus 20 mg/kg CNMA by daily gavage for 16 weeks (HFD + CNMA).

CNMA was purchased from Sigma Company (W228613, China) and was diluted with corn oil 2% (*v*/*v*). It was provided weekly and stored at room temperature and in a cold-dark place.

Semipurified HFD contained 25% (*w*/*w*) of fat (1% soya bean oil and 24% tail fat). The HFD was prepared monthly and conserved in a cold room at 4°C. Both regular and HFD were provided by Javaneh Khorasan animal food company (Mashhad, Iran). All animals had free access to their relative diets during the study period. At the final 16th week of the study, overnight-fasted animals were anesthetized by sodium pentobarbital (60 mg/kg, intra-peritoneally) [21]. Blood samples were collected from the heart; plasma was obtained by centrifugation (5,000 rpm for 20 min at 4°C) for the evaluation of lipid profile, NO_X, glucose, and insulin. Finally, the pancreas of animals was dissected out for islet isolation. The animals and their food weighed weekly. The food intake was calculated as food consumption (g/16 weeks) × diet energy (kcal/g).

2.2. Islet Isolation Protocol. The isolation of islets of Langerhans was performed as described previously [6]. Immediately after blood collection, the common bile duct was cannulated at the proximal end, and its opening to the duodenum was ligated, and then collagenase (Roche, Cat. Number 1213, Germany) solution (0.5 mg/ml in ice-cold Hanks' balanced salt solution, HBSS; pH 7.4) was injected into the common bile duct. Then, the pancreas was resected and incubated at 37°C for 10 min. Islets were hand-collected under a stereomicroscope (Kyowa Optical, SDZTR-PL model, Japan). Each incubation well contained five islets in 1.0 ml of Krebs–Ringer buffer (pH = 7.4) supplemented with

0.5 g/dl bovine serum albumin (BSA; Fluka, USA) and 16.7 mM glucose. They also were gassed with 95% O_2 and 5% CO_2 for 5 min to obtain constant pH and oxygenation. To investigate the role of NO, the incubation mediums of six wells of each group were supplied by different NOS inhibitors: aminoguanidine (AG) or L-NG-nitro arginine methyl ester (L-NAME, Sigma). All incubations were performed at 37°C for 60 min. Then, aliquots of the supernatant were removed and stored at −20°C for insulin measurement.

2.3. iNOS Activity Assay. To evaluate iNOS activity, 200 islets were collected and stored in 200 μl lysis buffer containing ice-cold HEPES (20.0 mM; pH 7.4; Sigma), L-dithiothreitol (1.0 mM, Sigma), and protease inhibitor cocktail (1 tablet dissolved in 10 ml buffer; Cat. No. 11836153001, Roche Co., Germany) with EDTA supplement. The samples were stored at −80°C. On assay day, the islet aliquots were sonicated using 40% intensity for 10 seconds. After centrifugation at 35,000g for 10 min at 4°C, supernatants were used for protein and enzyme assays. Protein concentration was determined by the Bradford method. The islet samples were kept on ice at all times. For iNOS activity assay, 100 μl of the supernatant was added to reaction solution composed of 20.0 mmol/L HEPES (pH 7.4), 0.2 mM L-arginine (Sigma), and 2 mM NADPH (Sigma) in a total volume of 0.5 ml and then incubated under constant air bubbling (1 ml/min) at 37°C for 120 min. The blank/control samples contained all reaction components except NADPH. Aliquots of the incubated islet samples (100 ml) were then passed through an Agilent 1100 series high-performance liquid chromatography (HPLC) system with a fluorescence detector for analysis of the L-citrulline formed. Because L-citrulline is produced in equimolar to NO concentrations and more stable than NO, it is considered as NO production level. iNOS activity was calculated from the different L-citrulline AUC (area under the curve) between samples and their respective blanks/controls. L-citrulline was applied as standard. The L-citrulline HPLC methods were precisely described in the previous publication [21].

2.4. Measurement of Plasma NO_X Concentration. For indirect measurement of NOS activity, nitric oxide metabolite (nitrite + nitrate) concentration was measured using the Griess protocol.

First, 50 μL of zinc sulfate (15 mg/mL) was added to each sample (1 ml) and was shaken vigorously. After that, 100 μL of vanadium III chloride (8 mg/mL) in 1 M HCl was added to each well. Eventually, 100 μL of Griess reagent containing 50 μL sulfanilamide (2%) and 50 μL N-(1-Naphthyl)ethylenediamine dihydrochloride (0.1%) was added to each well. The plate was incubated for 20 min at 37°C, and absorbance was read at 540 nm. Sodium nitrate (0–150 μM) was used as a standard [22]. The intra-assay coefficient of variation for plasma NO_X was 3.12%.

2.5. Insulin and the Biochemical Plasma Assay. Islet and plasma insulin were assayed using an ELISA kit (insulin; Mercodia, Sweden). Fasting plasma glucose (FPG) was measured by the glucose oxidase method (Zistchem Co., Iran). The triglyceride (TG), total cholesterol (TC), HDL, and LDL cholesterol were measured by commercially available kits (Zistchem Co., Iran). The intra-assay coefficients of variation for insulin, glucose, TG, and TC were 7.34, 1.12, 1.97, and 1.22, respectively.

2.6. Statistics. All analyses were validated by D'Agostino and Pearson (omnibus K2 test performed with Prism version 5) normality test. Bartlett's test was used for evaluating the homogeneity of variances between groups. Data on weight were analyzed by two-way repeated measure analysis of variance (RM-ANOVA). Parameters including HOMA-IR, adiposity index, plasma glucose, TG, TC, HDL, and LDL were analyzed by two-way ANOVA with the factors being HDF and CNMA. The Bonferroni test was used for post hoc comparison. Islet insulin secretion of each group was analyzed by one-way ANOVA. The statistical analysis was performed using the GraphPad Prism software (version 5). Results were expressed as means ± SD of triplicate experiments. A value of $P < 0.05$ was considered statistically significant. The sample size was precisely chosen based on previous HFD studies [6, 16, 21, 23, 24].

3. Results

3.1. Serum NO_X Levels. The results of plasma NO_X levels are presented in Figure 1. Plasma NO_X levels were varied according to diet ($P = 0.003$) and CNMA treatment ($P = 0.01$), with an interaction between diet VNMA ($P < 0.001$). NO_X level in the HFD group was significantly higher than control and other studied groups ($P < 0.001$). CNMA administration could effectively reduce ($P < 0.01$) as well as normalize plasma NO_X level in HFD-treated rats, which was similar to that of the control group. However, CNMA gavage in standard diet-treated animals did not exhibit a significant effect on plasma NO_X level.

3.2. Effects on Insulin Secretion. To investigate the effects of the two chemically different NOS inhibitors L-NAME and AG on islet insulin secretion, we assessed the insulin release from isolated islets incubated at 16.7 mM glucose in the presence/absence of these inhibitors. L-NAME supplementation (5 mmol/L) [2] significantly decreased insulin secretion from isolated islets belong to both HFD and control rats in the presence of 16.7 mmol/L glucose (Figure 2). Overall, at this high concentration of glucose (16.7 mmol/L), the insulin secretion was lower in islets of control (−50%) and HFD (−42%) groups. This effect was much more pronounced in the presence of L-NAME. The diet and CNMA consumption significantly reduced NO-induced islet insulin secretion (diet effect: $p = 0.01$; CNMA effect: $P = 0.001$). The addition of AG (10 mmol/L, 60 min incubation) [2] also markedly decreased insulin secretion from islets of both control and HFD groups (Figure 2). The results of two-way ANOVA revealed that

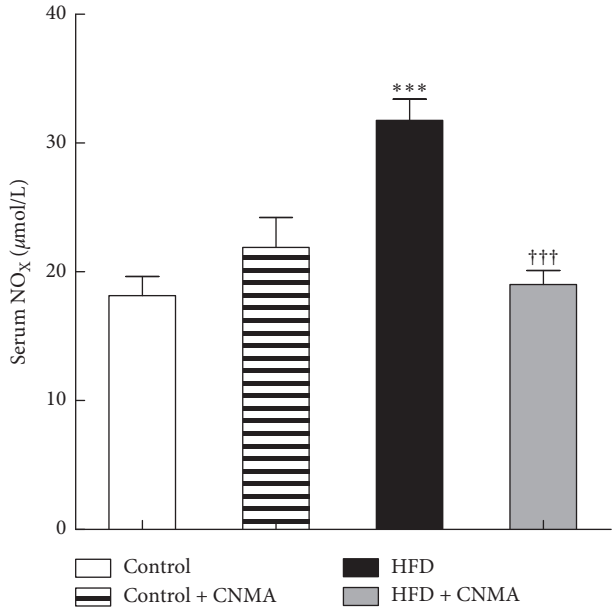

Figure 1: Serum NO_X (nitrite + nitrate) concentration in different experimental groups; NO_X was measured in serum of 10 rats in each group. Bartlett's test showed that variances are equal ($P > 0.05$) for all measures. Data were analyzed by one-way ANOVA and followed by the Bonferroni posttest. Results are presented as mean ± SEM. *** Significant difference ($P < 0.001$) vs. controls and †† significant difference ($P < 0.001$) vs. HFD. All significant value was reported after calculation of Bonferroni corrections.

CNMA significantly decreased islet insulin secretion in HFD and/or control groups (diet effect: $P = 0.105$; CNMA effect: $P < 0.0001$). Similarly, the islet insulin release was lower at the presence of AG in the control (−53%) and HFD (−47%) groups (Figure 2). The results revealed that there was no difference in islet insulin secretion of the control + CNMA group in the presence or absence of either L-NAME or AG (Figure 2). Only AG supplementation significantly inhibited insulin secretion in the HFD + CNMA group.

The reduction ratio of insulin secretion was also calculated. Accordingly, the lowest reduction ratio of the islet insulin secretion was observed in CNMA-treated groups (Figure 2). In other words, these findings showing that CNMA consumption attenuated the role of NO in insulin secretion.

3.3. Results of iNOS Activity. iNOS activity was determined as NO formation measured in the absence of Ca^{2+} and calmodulin (Figure 3). The iNOS activity varied according to diet ($P < 0.001$) and CNMA ($P < 0.001$); however, no statistically significant interaction was found between diet and CNMA on iNOS activity ($P = 0.52$).

In comparison with control animals, HFD treatment during 16 weeks increased iNOS activity ($P < 0.001$). CNMA treatment markedly inhibited iNOS activity in islet of HFD-treated animals ($P < 0.01$) compared with the HFD-untreated group. However, the iNOS activity in HFD + CNMA group was still higher than the control group ($P < 0.01$; Figure 3).

4. Discussion

The present study aimed to investigate the effects of HFD and dietary CNMA supplementation on NO-induced islet insulin secretion and plasma NO_X. The most important finding of this study was that CNMA supplementation reduced NO-induced islet insulin secretion and acted as an iNOS activity inhibitor irrespective of the diet. As shown in the present study, rats that consumed a high-fat diet developed metabolic syndrome criteria including insulin resistance, hyperglycemia, and obesity associated with increased body fat stores. Consistent with previous studies, our findings demonstrated that CNMA supplementation prevented insulin resistance and decreased plasma glucose level and adiposity in HFD rats [6, 16, 25, 26].

This study was performed to investigate the role of NO in antidiabetic activity of CNMA through assessments of plasma NO_X and islet iNOS activity as well as insulin secretion in normal and HFD-treated rats.

In all studied groups, insulin secretion decreased after NO synthesis inhibited with L-NAME; however, the reduction was only significant in untreated animals. NO has physiological importance in the biphasic secretion of insulin [27]. It is the main glucose regulator of islet insulin secretion [28]. The stimulatory effect of NO on insulin release is consistent with what has been found in previous reports [29–34], indicating that NO synthesis inhibition has diminished glucose-stimulated insulin secretion [32, 34]. Panagiotidis et al. have also reported that glucose-stimulated

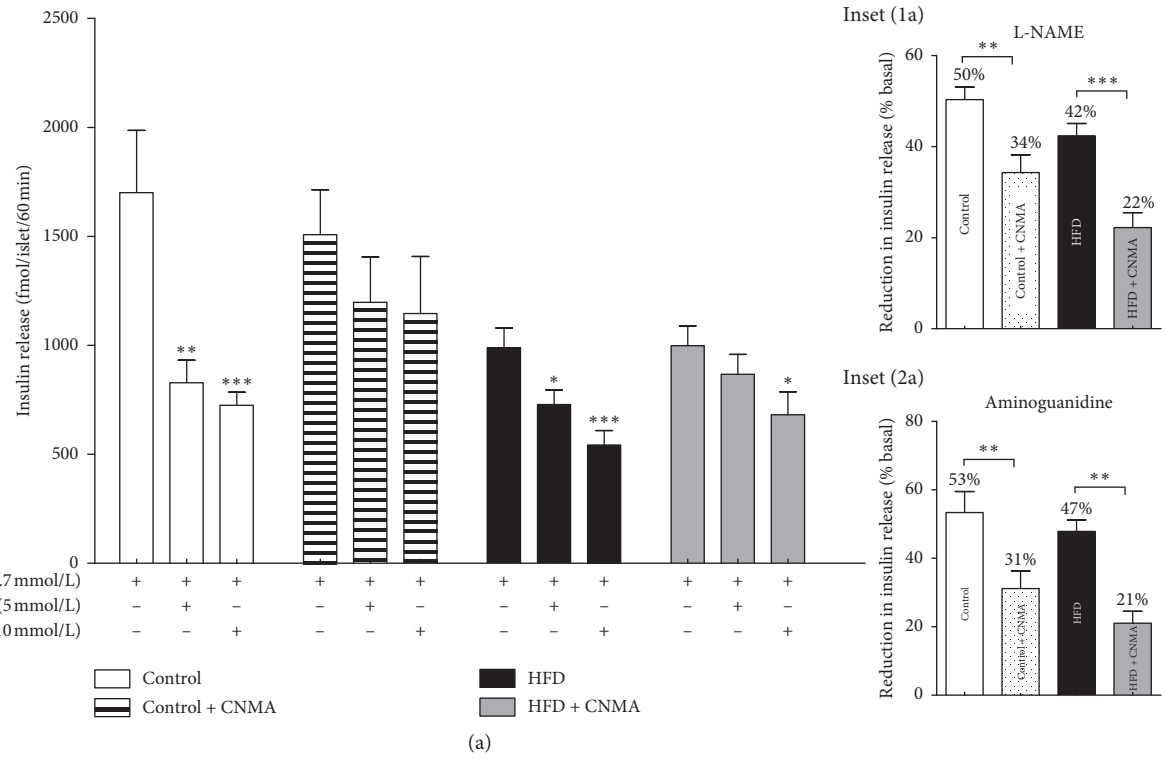

FIGURE 2: Effect of nitric oxide (NO) on insulin secretion of isolated islets from experimental groups. Insulin release was measured during 1 hour from groups of five islets at 16.8 mmol/L glucose alone and the presence of aminoguanidine (AG) and L-NAME after overnight fasting. Values are presented as mean ± SEM. for eight cups. Bartlett's test showed that variances are equal ($P > 0.05$) for all measures. Data were analyzed by one-way ANOVA and then the Bonferroni posttest. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with glucose 16.7 mmol/L in each group versus control in insets after the Bonferroni correction.

insulin secretion enhanced the intra-cellular concentration of Ca^{++}, which in turn intrinsically increased the islet NOS activity. On the other hand, other studies have theorized that NO has an inhibitory effect on glucose-stimulated insulin secretion so that inhibition of NO synthesis stimulates insulin secretion [35, 36]. They have theorized many mechanisms, including the reduction in the activity of carbohydrates metabolism enzymes such as phosphofructokinase [36] and neutralization of the thiol/sulfhydryl groups of enzymes involved in insulin secretion. It has been emphasized that these effects are independent of cGMP [35, 37]. It has been demonstrated that NO increased the insulin gene expression in the short term (24 hours) and enhanced the β-cell function by PI 3-kinase signaling [38]. NO influences the activity of calcium channels and insulin release in a concentration-dependent manner. At low concentrations, NO facilitates insulin secretion through increasing the cGMP; however, at high concentrations, it has an inhibitory effect on insulin secretion [39, 40]. A previous electrophysiological study performed using NO donors on the beta cell line and primary beta cells has shown that NO also had a dual effect on the ATP-dependent potassium channel (K_{ATP}) activity, that is, it increased K_{ATP} current and inhibited insulin secretion with its indirect effect. This effect was mediated by a decrease in the metabolism, the ATP/ADP ratio reduction, and then hyperpolarization of the beta cell membrane. However, NO, through directly affecting the K_{ATP}, inhibited K_{ATP} current, depolarized the beta cell membrane, and consequently, increased insulin secretion [41]. NO has been interfered with the glycolysis [42] and citric acid cycle enzymes activity [43]. It has been revealed that NO is connected to the SH group (sulfhydryl group) of serum proteins and plasma membrane proteins [44]. It has also been proven that the factors connecting to the SH group directly inhibit the K_{ATP} currents of beta cells and insulin secretion [45]. Given the contradictory roles of NO on insulin secretion as well as its complicated mechanisms on the insulin vesicles release, we can conclude that in this study, NO had an insulin stimulation effect, and its importance remained strong following the HFD treatment.

We previously reported that the HFD decreased islet insulin secretion and CNMA significantly reduced insulin secretion and content in isolated islets of a high-fat diet [6]. The role of NO in insulin secretion seems to be the same in HFD and control islets. In the present study, the insulinotropic effect of NO in animals receiving CNMA was much less than control and HFD animals so that the rate of insulin secretion decreased in these islets after inhibition of NO. Accordingly, two hypotheses can be suggested: (1) the physiological role of CNMA on insulin secretion is similar to NO mimetic, and in consequence, the NO production due to enzyme activity was decreased and (2) CNMA acted as an NOS inhibitor. According to

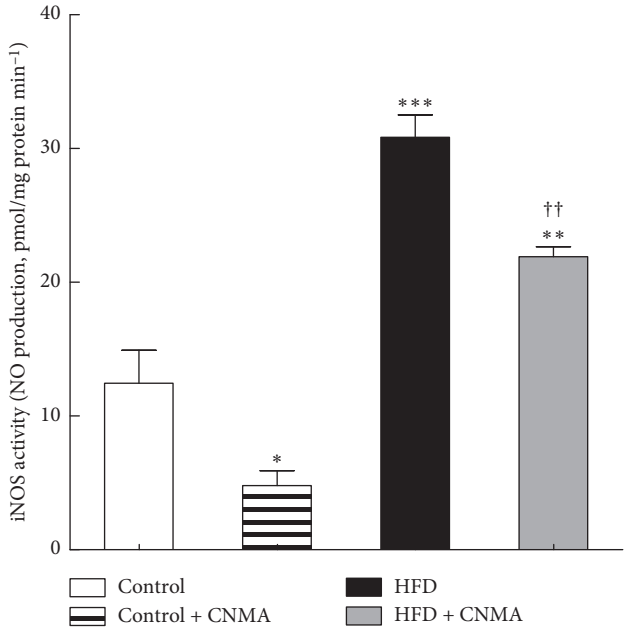

Figure 3: Comparison of iNOS (inducible nitric oxide synthase) activity in different experimental groups; iNOS activity was measured in six samples. The results are presented as mean ± SEM. Bartlett's test showed that variances are equal ($P > 0.05$) for all measures. Data were analyzed by one-way ANOVA and then the Bonferroni posttest. *, **, and ***Significant differences ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively) compared with the control group, and ††significant difference ($P < 0.01$) compared with the HFD group. The values remain significant even after Bonferroni corrections.

the results of the current study, the activity of the iNOS enzyme in animals consumed CNMA was lower than that of the control and HFD groups. Furthermore, plasma NO_X level in the HFD group confirms these results, and it seems to CNMA acted as an inhibitor of the iNOS enzyme. Other studies on the macrophage cell line (RAW 264/7) have shown that CNMA reduced the NO level and iNOS activity [20, 46, 47]. It also has been imposed that CNMA through anti-inflammatory effects (NF-κB inhibition) could inhibit the NO production [47]. On the other hand, another study has investigated the vasorelaxant effect of CNMA on the mesenteric vascular bed and found that CNMA, similar to NO, could activate the NO/sGC/PKG signaling pathway as well as its downstream potassium channels [48].

In conclusion, HFD increased iNOS activity in islets. CNMA restored the stimulatory effect of NO on insulin secretion by normalized iNOS and serum NO_X. Therefore, the part of beneficial effects of CNMA on carbohydrate metabolism was developed from the NO pathway by reducing islet iNOS activity.

This study has some limitations that should take into consideration for interpreting the data. First, we did not assess possible adverse effects of CNMA in other systems. Second, based on the experimental design, the reactive nitrogen species (RNS such as peroxynitrite (OONO−)) as well as its oxidative products including S-nitrosothiols and nitrotyrosine were not evaluated. Further experimental researches are required to reveal the exact NO pathway by which CNMA modulates islet insulin secretion.

Data Availability

Data are available from the corresponding author upon request through the data access committee or institutional review board.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Zomorrod Ataie and Mohammad Dastjerdi contributed equally.

Acknowledgments

The authors acknowledge Mr. Mehran Hosseini for his critical editing of the manuscript.

Supplementary Materials

The composition of HFD was precisely described in Table 1 of supplementary data [6]. After 16 weeks of HFD ingestion, the metabolic syndrome criterion was clearly induced (obesity, hyperglycemia, and insulin resistance without any alteration in serum lipid profile). The data are reported in the supplementary file. Cinnamaldehyde gavage improved insulin resistance accompanied by restoration plasma insulin and glucose level as well as reduced weight gain due to HFD. (*Supplementary Materials*)

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 5583691, 7 pages
https://doi.org/10.1155/2021/5583691

Research Article

The Effects of *Berberis integerrima* Fruit Extract on Glycemic Control Parameters in Patients with Type 2 Diabetes Mellitus: A Randomized Controlled Clinical Trial

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Received 26 January 2021; Revised 29 April 2021; Accepted 16 June 2021; Published 5 July 2021

Academic Editor: Mohammad Hossein Ayati

Background. *Berberis integerrima* Bunge fruits have been utilized in traditional medicine to control diabetes mellitus (DM). However, no clinical survey has been done in this regard. This study was conducted to clinically evaluate the effects of fruit extract of this plant in improving glycemic control indices in patients with type 2 DM (T2DM). *Methods*. In a randomized controlled clinical trial, patients with T2DM who met the inclusion criteria were randomly divided into two groups of drug (*Berberis*) and control to receive the extract solution 5 ml twice daily (equivalent to 1000 mg of dry extract) with standard treatment (metformin) or only standard treatment, respectively, for 8 weeks. Before and after the intervention, fasting blood sugar (FBS), serum glycosylated hemoglobin (HbA1c), serum insulin, the homeostasis assessment model for insulin resistance (HOMA-IR), body mass index (BMI), and systolic and diastolic blood pressure were determined and compared between the two groups. *Results*. During the study, 30 and 35 patients in the drug and control groups, respectively, completed the study. Although no significant changes occurred in any parameter within each group, postintervention FBS (117.5 [107–128.8] versus 134 [120–142], $P = 0.001$) and HbA1c (7 [6.4–7.7] versus 7.5 [6.8–7.9], $P = 0.045$) were significantly lower in the drug group than in the control one. In terms of other parameters, there were no significant differences between the groups. *Conclusion*. Consumption of *B. integerrima* Bunge fruit extract at a dosage of 1000 mg daily decreases FBS and HbA1c but does not affect HOMA-IR in patients with type 2 diabetes mellitus.

1. Introduction

Diabetes mellitus (DM), including type 2 DM (T2DM), is one of the most common endocrine disorders that, if left untreated, can lead to neurological, cardiovascular, and renal complications as well as foot ulcers and subsequent infection [1].

Proper treatment for glycemic control is imperative to slow the devastating complications of DM [2]. Drugs used to treat T2DM have several adverse effects including

hypoglycemia, obesity, allergic reactions, gastrointestinal disorders (anorexia, nausea, and vomiting), lactic acidosis, and anemia [3]. On the other hand, many patients remain hyperglycemic despite pharmacotherapy with combination of drugs [4]; treatment-resistant T2DM is now a common challenge in clinical practice. Therefore, finding new drugs or supplements for adjunctive therapy is mandatory to reduce the required dose of antidiabetic drugs and consequent adverse effects and to improve glycemic control in difficult-to-treat cases.

The use of herbal medicines has increased due to the public idea of fewer side effects and lower cost [5]. So far, the positive effects of many herbs in lowering blood glucose or reducing the side effects of hyperglycemia have been known [6]. The antidiabetic effects of plants are attributed to their phytochemicals including phenolic compounds, flavonoids, terpenoids, and alkaloids that have shown hypoglycemic effects [7].

Berberis plants (commonly known as barberry), including *Berberis integerrima* Bunge, from the Berberidaceae family [8] are used in the traditional medicine of some countries, including Iran, China, Pakistan, and India, to control blood sugar [9]. Barberry fruit has antioxidant [10], anti-inflammatory [11], antihypertensive [12], hypoglycemic [13], and lipid-lowering [14] effects. *B. integerrima* fruits are rich in compounds such as berberine, berbamine, protoberberine, polyphenols, anthocyanins, and pectin [15]. Berberine has been suggested to lower blood glucose by several mechanisms including the increase in insulin sensitivity, stimulating glycolysis in peripheral tissues, inhibiting gluconeogenesis in the liver, and inhibiting the gastrointestinal absorption of carbohydrates [16, 17]. Furthermore, the anthocyanins in barberry fruit may also have a role in its hypoglycemic effect [18].

Some animal studies have shown the antidiabetic effect of *B. integerrima* fruits [18, 19]. However, to the best of our knowledge, no clinical study has been done in this regard. Therefore, the present study was performed to clinically evaluate the effects of fruit extract of this plant in improving glycemic control indices in patients with type 2 diabetes.

2. Materials and Methods

2.1. Extraction. Fresh fruits of *B. integerrima* were purchased from the nearby market in the southern Khorasan province and confirmed by a taxonomist in Ferdowsi University of Mashhad with voucher number SAM-1890 deposited in Samsam-Shariat Herbarium, Pharmacognosy Department, Isfahan University of Medical Sciences (IUMS).

Plant material (15 kg) was cut into modest pieces twice by a crushing machine. Extraction was done with ethanol : water (6 : 4) in maceration tank for four days with three repetitions. The extract was then concentrated with a rotary evaporator (Heidolph, Germany) and stored in a refrigerator. To determine the percentage of the dry weight of the extract, a crucible was placed in an oven at 110°C for 20 minutes and weighed after cooling in a desiccator. Then, 1 g of the weighed plant extract was poured into the crucible and placed in the oven again. After one hour, the crucible was cooled in the desiccator and weighed (by three times). Using the following formulas, the extraction yield was calculated as 46.7% (w/w).

$$\text{Dry weight \%} = \left(\frac{\text{dry weight}}{\text{extract weight}}\right) \times 100;$$
$$\text{Extraction yield} = \left(\frac{\text{dry weight percentage} \times \text{total amount of concentrated extract}}{\text{fruit weight}}\right) \times 100. \quad (1)$$

2.2. Extract Standardization by Total Phenolic and Total Flavonoid Content. For standardization of the extract, the content of polyphenols was determined by Folin-Ciocalteu method [20]. For this purpose, 1 g of the extract was transferred to a volumetric flask and reached the volume of 10 ml with methanol. Then, to 20 μl of the sample, 1.58 ml of water, 100 μl of the Folin-Ciocalteu reagent, and 300 μl of 20% sodium carbonate solution were added and shaken well to be completely mixed. The solution was kept at 20°C for 2 hours in a dark environment, and then the absorption was read at 765 nm against blank. The gallic acid solution was used as the standard for polyphenolic compounds. After preparing different concentrations of gallic acid solution, absorption reading at 765 nm, and drawing the standard curve($y = 0.0551x + 0.0303$), total phenol content (TPC) of the extract was calculated as 86.88 ± 0.246 mg gallic acid equivalent per gram of dry extract.

The total flavonoid content (TFC) of dry extract was calculated using spectrometry method of AlCl3-flavonoid complex as reported before [20]. Briefly, a 10% diluted solution of dry extract (100 μL) was added to the test tubes containing 100 μL of 20% AlCl3 and 20 μL of glacial acetic acid, and methanol was added to 3 mL. After 30 min of incubation, the absorbance was read at 415 nm. Based on the quercetin calibration formula, $y = 0.046x - 0.0086$, TFC of the extract was calculated as $3.88 \pm 0.0.17$ mg quercetin equivalent per gram of dry extract.

2.3. Preparation of Oral Solution (Syrup) from the Extract. To prepare barberry syrup, a mixture of 8.5 g of concentrated extract with several solvents and excipients including water, 70% ethanol, polyethylene glycol (PEG), tween, pectin, and poloxamer was tested in different ratios to obtain a clear and

stable solution. Finally, the clearest and the most stable solution with the following ingredients and ratios (w/v) was selected for administration to the patients: carboxymethylcellulose (CMC) 0.5%, aspartame 1.5%, poloxamer 2.5%, polyethylene glycol 12.5%, citric acid 2%, and distilled water 100 ml. Furthermore, the orange extract was used as the flavoring agent.

2.4. Study Design. This study was a randomized controlled clinical trial conducted from September 2018 to April 2020 in the Faculty of Pharmacy and Isfahan Endocrine & Metabolism Research Center (IEMRC), both affiliated to IUMS, Isfahan, Iran. The study was approved by the ethics committee of IUMS with the ethics code IR.MUI.RESEARCH.1398.104 and registered at Iranian Registry of Clinical Trials with the code IRCT20150721023282N5.

2.5. Patients and Interventions. Participants were selected from diabetic patients referred to the diabetes clinic of IEMRC. All patients were met prior to participation to become utterly familiar with the study, and, if agreed, their consent was obtained.

The study inclusion criteria were (1) age between 18 and 75 years; (2) being diagnosed with type 2 diabetes (according to the diagnostic criteria of the American Diabetes Association) for at least 2 years; (3) treatment with metformin; (4) glycosylated hemoglobin (HbA1c) between 6.5 and 9%; (5) not consuming alcohol and other abused substances; (6) no liver or kidney disease; (7) not taking oral antidiabetic drugs such as sulfonylureas and glinides and insulin products; (8) no pregnancy and lactation.

Patients were excluded from the study if they did not follow the medication instructions for more than three days, developed allergic reaction to barberry extract during the study, or changed their treatment for diabetes (change in dose, type, or the number of medications) during the study.

By convenience sampling, all subjects who met the inclusion criteria were consecutively chosen and randomly divided into two groups of drug (barberry extract) and control. Block randomization method was utilized for randomization. In this way, all possible blocks of 4 were numbered and after that, using random number table, the specified blocks were chosen and the individuals of each group were selected based on the arrangements within the blocks.

Demographic characteristics of patients including age, sex, height, weight, BMI, and systolic (SBP) and diastolic (DBP) blood pressure were recorded. Appropriate diet and maintenance of physical activity were suggested to both groups during the study. Dietary instructions were given by a qualified dietician. Before starting treatment, 10 ml of venous blood sample was taken from each patient in the two groups in the fasting state (8 to 12 hours overnight) centrifuged within a maximum of 30 to 45 minutes of sampling, and serum glucose (FBS), HbA1c, and insulin levels were determined (due to the experiments of all patients in one step, the centrifuged samples were stored at freezer −70°C until assays). Moreover, using the following formula, the homeostasis assessment model for insulin resistance (HOMA-IR) was calculated and recorded as an indicator of insulin resistance:

$$\text{HOMA} - \text{IR} = \frac{[\text{fasting insulin}\,(\mu\text{U/mL}) \times \text{fasting glucose}\,(\text{mmol/L})]}{22.5}. \tag{2}$$

For patients of the drug group, in addition to the standard treatment according to the physician's opinion, barberry extract syrup was administered at a dose of 5 ml twice a day (equivalent to 1000 mg of prepared dry extract) for eight weeks. The control group received only standard treatment. At the end of the 8th week of treatment, by taking blood samples from the two groups, the mentioned indicators were measured and recorded again. Finally, the recorded results of the two groups were compared with appropriate statistical tests.

Patients were asked to report any side effects from the syrup. Furthermore, in order to evaluate the patients' compliance for interventions including diet and physical activity issues, they were contacted by telephone every other day and visited in the third week of intervention, when, for participants of drug group, the previous syrup bottle was taken and evaluated in terms of the amount of consumed content, and a new bottle was given to them.

2.6. Outcome Measures. The primary outcome measures were the changes in FBS, HbA1c, serum insulin levels, and HOMA-IR, while the secondary outcome measures included the changes in BMI, SBP, and DBP at the end of intervention compared to the baseline values.

2.7. Sample Size Calculation. Considering that the main variables of this study were continuous quantitative type, the following formula was used for sample size calculation:

$$n = \frac{\left(Z_{\alpha/2} + Z_{\beta}\right)^2 \times 2\text{SD}^2}{d^2}, \tag{3}$$

where n is the required sample size in each group, d is the clinically important difference between the groups, SD is the standard deviation, $Z_{\alpha/2}$ is the standard normal z-value for a significance level $\alpha = 0.05$, which is 1.196, and Z_{β} is the standard normal z-value for the power of 80%, which is 0.84.

According to HbA1c values in a previous study [21], the SD and d quantities were considered 0.6 and 0.5, respectively. Therefore, a calculated sample size of at least 22 patients in each group was obtained.

2.8. Statistical Analysis. SPSS software version 24 was used to perform statistical analysis. Quantitative data were reported as mean ± SD or median (IQR) and qualitative data as number and percent. Due to nonnormal distribution of data, Wilcoxon signed-rank test was used to compare the preintervention and postintervention values within each group, while the Mann–Whitney U test was applied for comparison of values in each time point between the groups. Analysis of covariance (ANCOVA) was used to compare the changes in the parameters between the two groups. The significance level was considered to be $P < 0.05$.

3. Results

3.1. Patients. During the study, out of 250 patients surveyed, 150 patients met the inclusion criteria, of whom 70 were willing to cooperate and were randomized to the groups. During the study, 5 people from the drug group were excluded from the study due to either side effects of the syrup after the first doses (2 patients) or reluctance to continue the participation (3 patients). Finally, 30 and 35 patients in drug and control groups, respectively, completed the study (Figure 1). The baseline characteristics of patients are shown in Table 1. As seen, there was no significant difference between the groups regarding age, sex distribution, and consumed antidiabetic drug (other than metformin).

3.2. Efficacy Evaluation. Table 2presents the changes of the evaluated parameters in each group along with the comparison of their values between the two groups. As can be seen, even though no significant change occurred in any parameter within each group, a statistically significant difference was observed between the two groups regarding postintervention FBS ($P = 0.001$) and HbA1c ($P = 0.045$); both parameters were significantly lower in the drug group than in control as confirmed by ANCOVA with the control of baseline values. In terms of other parameters, there were no significant differences between the groups.

3.3. Side Effects. During the study, only two patients in the drug group complained of heartburn and weakness (one patient in each case) who were excluded from the study. The other patients who completed the study did not report any side effects.

4. Discussion

In this study, in the group receiving barberry syrup, at the end of the eighth week, there was a significant decrease in FBS and HbA1c compared to the control group as well as a slight increase in fasting insulin and HOMA-IR which was not significant compared to the control group with a higher increase of these parameters. In addition, consumption of

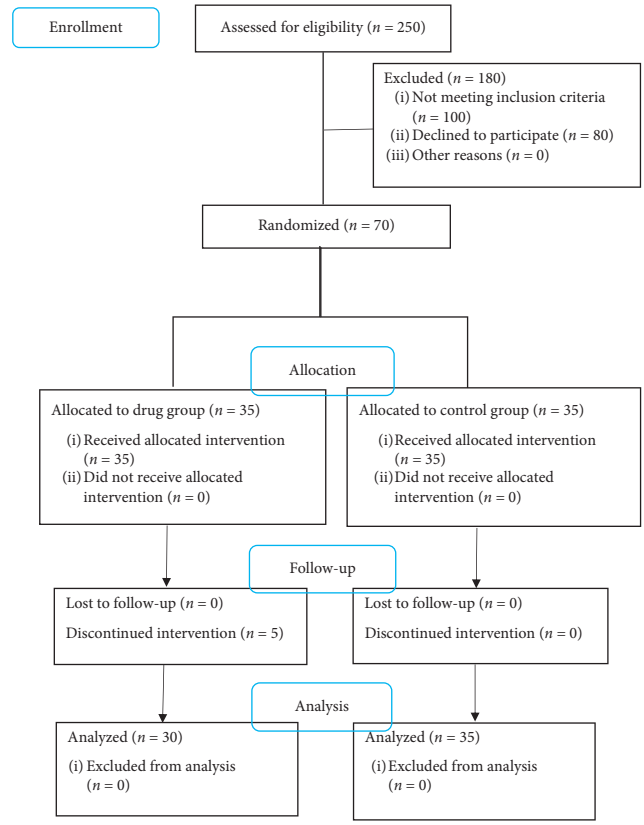

Figure 1: The CONSORT flowchart of the study.

this extract did not affect BMI and blood pressure. According to our research, this is the first human study of *B. integerrima* fruits in diabetic patients, and other studies have been animal type with different results.

In an animal study performed on diabetic rats, the effects of *B. integerrima* fruit extract (1000 mg/kg) on blood sugar and serum insulin levels were investigated. Based on the results, at the end of the eighth week, weight gain, insulin and blood glucose levels, and HOMA-IR decreased significantly [18]. In a study on streptozotocin-induced diabetic rats, the anthocyanin fraction extracted from *B. integerrima* fruit reduced blood glucose levels and body weight compared with untreated diabetic rats [18]. Therefore, it seems that barberry fruit anthocyanins play a major role in its hypoglycemic effects.

Despite the lack of a clinical study on the effect of *B. integerrima* fruits on type 2 diabetes mellitus, in a recent survey by Sanjari et al., the use of *B. integerrima* root extract (480 mg/day) by T2DM patients resulted in a significant reduction of FBS, HbA1c, and 2-hour postprandial glucose (2-hPG) equivalent to metformin [22]. Consistent with our results, this study shows the beneficial effects of this species of *Berberis* on T2DM. In addition, in a recently published clinical study, the effects of *B. integerrima* fruit extract (at a dose of 1500 mg per day for 3 months) on FBS and several nonglycemic indices were evaluated in patients with rheumatoid arthritis treated with glucocorticoids. According to the results, the consumption of this extract was associated with a significant reduction in FBS [23]. This is in agreement

Table 1: Baseline characteristics of study patients.

Parameter	Drug group (*Berberis*) ($n = 30$)	Control group ($n = 35$)	
Age (years; mean ± SD)	56.10 ± 7.20	57.60 ± 7.70	0.416
Sex (n)			
Male	5	6	0.959
Female	25	29	
Antidiabetic drug (n)*			
Acarbose	1	1	
Sitagliptin	8	8	0.837
Linagliptin	1	1	
Pioglitazone	7	5	

*Antidiabetic drug other than metformin.

Table 2: Preintervention and postintervention values of parameters and their comparison between the two groups.

Parameter	Group	Time		P value**	OR (95% CI)$^{\delta}$
		Baseline	End (week 8)		
BMI (kg/m^2)	Control	29.5 ± 4.4	29.6 ± 4.3	0.180	1.375 (0.619–3.058)
	Berberis	4.4 ± 29.7	4.4 ± 29.7	0.970	
	*P*value*	0.856	0.935	0.492***	
FBS (mg/dl)	Control	128 (118–141)	134 (120–142)	0.110	0.979 (0.956–1.002)
	Berberis	125 (114.8–134.5)	117.5 (107–128.8)	0.110	
	*P*value*	0.498	0.001	0.026***	
Serum insulin (U/ml)	Control	4.8 (3.1–10.1)	7.5 (3.8–9.4)	0.296	0.975 (0.881–1.079)
	Berberis	5 (2.9–8.6)	6 (3.6–9.1)	0.698	
	*P*value*	0.963	0.400	0.792***	
HbA1c (g/dl)	Control	7.2 (6.7–8.4)	7.5 (6.8–7.9)	0.872	1.413 (0.860–2.321)
	Berberis	7.4 (6.8–7.7)	7 (6.4–7.7)	0.430	
	*P*value*	0.879	0.045	0.045***	
HOMA-IR	Control	26.8 (14.9–54.5)	43.5 (23.3–55.1)	0.368	1.003 (0.987–1.020)
	Berberis	28.8 (15.4–46.1)	30.7 (17.9–46.4)	0.910	
	*P*value*	0.803	0.124	0.517***	
SBP (mm Hg)	Control	13 (12–14)	13 (12–14)	0.936	0.884 (0.592–1.319)
	Berberis	12 (11–14)	12 (11–14)	0.315	
	*P*value*	0.450	0.234	0.246***	
DBP (mm Hg)	Control	8 (7–9)	8 (7-8)	0.415	1.308 (0.754–2.268)
	Berberis	8 (7-8)	8 (7–9)	0.565	
	*P*value*	0.521	0.994	0.298***	

The values are mean ± SD for BMI and median (IQR) for other parameters. BMI, body mass index; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis assessment model for insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure. *Comparison of preintervention and postintervention values between the two groups (Mann–Whitney U test). **Comparison of preintervention and postintervention values within each group (Wilcoxon signed-rank test). ***Comparison between the two groups with control of baseline values (ANCOVA test). $^{\delta}$Odds ratio and 95% confidence interval for comparison of mean differences between the groups.

with the results of our study. However, since the patients were not diabetic in the mentioned study, the mean FBS values were lower than ours. Therefore, it seems that the hypoglycemic effects of barberry could occur even in nondiabetic patients.

There are several studies on the hypoglycemic effects of *Berberis vulgaris*, another species of barberry. However, as the total phenolic content and antioxidant activity of *B. integerrima* are higher than those of *B. vulgaris* [24], the first plant could be a reasonable choice for this study. In the study of Shidfar et al., the effect of *B. vulgaris* fruit extract (at a dose of 3 g per day for 3 months) on the levels of insulin, blood glucose, and HbA1c in type 2 diabetic patients was investigated [25]. According to the results, consumption of the extract led to a significant reduction in FBS, which is consistent with our results. However, contrary to our results, there was no significant change in HbA1c levels, while insulin levels and HOMA-IR decreased significantly. Therefore, it seems that the blood glucose-lowering effect is decisive for barberry species. However, the ideal dose and dose-response relationship for this effect should be determined in further studies. On the other hand, the effect of barberry extract on insulin level and insulin resistance index (HOMA-IR) has been different between the studies. In our study, insulin levels increased slightly, which could indicate that part of the hypoglycemic effect was due to increased

insulin secretion. Since, in the study of Shidfar et al., contrary to our research, insulin levels and, consequently, HOMA-IR decreased, a similar effect might be observed with *B. integerrima* extract if the dose and duration of intervention increased. Of note, our evaluated dose was one-third of the dose prescribed in the above-mentioned study, and the duration of our study was one month less than that. In fact, hyperinsulinemia in type 2 diabetic patients is a compensatory response to insulin resistance-induced hyperglycemia resolving overtime after proper glucose control. Therefore, the lack of effect on insulin resistance index (HOMA-IR) in our study may be due to an insufficient effect on hyperglycemia or short intervention duration or both.

The hypoglycemic effect of barberry could be due to various mechanisms. In an animal study on diabetic rats, *B. vulgaris* resulted in increased insulin secretion and pancreatic beta cells and subsequent blood sugar reduction [26]. Because oxidative degradation of the pancreas plays a role in the T2DM development, antioxidant compounds such as barberry fruit extract [10] may be effective in reducing this damage and improving glycemic control [27]. Phenolic compounds in barberry play an essential role in its antioxidative effects [28]. Notably, as mentioned previously, the amount of these compounds in *B. integerrima* fruit is more than that in *B. vulgaris* [24]. Moreover, increased insulin secretion, as also seen in our study, can be a reason for lowering blood glucose by barberry [26].

One of the substances in *B. integerrima* is the alkaloid berberine [15]. The antidiabetic effects of berberine have been demonstrated in numerous studies [16]. Increased insulin sensitivity, activation of the AMPK (adenosine monophosphate-activated protein kinase) pathway, induction of glucagon-like protein-1 secretion from the intestine, stimulation of glycolysis in peripheral tissues, inhibition of gluconeogenesis in the liver, increase of glucose transporters in the cells, decrease in the activity of α-glucosidase, and consequent inhibition of carbohydrates absorption are some of the suggested mechanisms for this effect [16, 17].

The anthocyanins in barberry fruit may also have a role in its antidiabetic effect. In the study of Sabahi et al., anthocyanins of *B. integerrima* fruit decreased glucose levels, increased liver glycogen, and resulted in weight loss in the treated rats [18]. In the clinical study of Yang et al. on prediabetic and early untreated diabetic patients, use of purified anthocyanins (320 mg/day) significantly reduced HbA1c (-0.14%) [29]. The antioxidant properties of anthocyanins protect pancreatic beta cells against oxidative stress. Anthocyanins also release insulin from the pancreas and increase AMPK phosphorylation and activation of this enzyme, leading to increased glucose transport to the muscle cells and decreased serum glucose levels [18].

Although in the present study *B. integerrima* did not have a significant effect on body weight, BMI, and blood pressure in diabetic patients, the weight loss and hypotensive effects of *Berberis* plants have been shown previously [30, 31]. Furthermore, barberry is used in the traditional medicine to treat hypertension. Berberine has been suggested to have antiobesity effect by inhibiting adipogenesis [32]. Since the impacts of barberry extract on these parameters were not primary objectives of this study, more controlled studies are needed to evaluate such effects.

The main limitations of our study were relatively low sample size, lack of placebo and blinding (due to technical problems), and no precise control for patients' diet and calorie intake. However, this is the first pilot clinical study showing the beneficial effects of *B. integerrima* fruit extract on glycemic control of T2DM patients. Of note, more clinical studies with larger sample size and longer duration are necessary to confirm these effects.

5. Conclusion

Consumption of *B. integerrima* Bunge fruit extract at a dosage of 1000 mg daily decreases FBS and HbA1c but does not affect HOMA-IR in patients with type 2 diabetes mellitus. Therefore, this extract can be considered as a dietary supplement in the treatment of type 2 diabetes mellitus.

Data Availability

Ethically, the obtained data are confidential as this work is a clinical trial. However, in necessary situations, the data will be sent to the reader via e-mail upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to acknowledge the laboratory staff of Isfahan Endocrine and Metabolism Research Center (IEMRC) for its assistance. This work was supported by the Vice-Chancellery for Research and Technology at Isfahan University of Medical Sciences (Grant no. 398036).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 5587938, 23 pages
https://doi.org/10.1155/2021/5587938

Review Article

A Review on Antidiabetic Activity of *Centaurea* spp.: A New Approach for Developing Herbal Remedies

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Received 18 February 2021; Revised 19 May 2021; Accepted 14 June 2021; Published 5 July 2021

Academic Editor: Francesca Mancianti

Objective. Diabetes mellitus (DM) is a long-life metabolic disorder, characterized by high blood glucose levels. The hyperglycemic condition generally leads to irreversible nerve injury and vascular damage. Among different types of diabetes, type 2 is more common and has spread all over the world. Although various therapeutic approaches have been developed to control type 2 DM, regulating blood glucose levels has still remained a controversial challenge for patients. Also, most prescription drugs cause different side effects, such as gastrointestinal disorders. Thus, developing novel and efficient antidiabetic agents possessing fewer adverse effects is in high demand. *Method*. The literature was comprehensively surveyed *via* search engines such as Google Scholar, PubMed, and Scopus using appropriate keywords. *Results*. Medicinal plants, both extracts and isolated active components, have played a significant role in controlling the blood glucose levels. Good-to-excellent results documented in the literature have made them a precious origin for developing and designing drugs and supplements against DM. *Centaurea* spp. have been traditionally used for controlling high blood glucose levels. Also, the antidiabetic properties of different species of *Centaurea* have been confirmed in recent studies through *in vitro* assays as well as *in vivo* experiments. *Conclusion*. Potent results encouraged us to review their efficacy to open a new horizon for development of herbal antidiabetic agents.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease which is described by hyperglycemia and high blood sugar levels in postprandial and fasting state. It is characterized by defects in insulin secretion, insulin action, or both of them [1]. The total number of diabetic patients in the world has been anticipated to rise from 171 million in 2000 to 366 million in 2030 [2]. Considering the long-term side effects of DM, it has become one of the major causes of morbidity in the world [3]. There are different types of diabetes based on its pathogenesis, including insulin-dependent (type I), noninsulin-dependent (type II), and gestational. Type 2 DM is more common than the other types in which the body's insulin receptors become resistant to the normal insulin effects. Then, β cells of the pancreas respond to the high blood glucose levels by producing more insulin to manage the situation. However, the insulin overproduction makes β cells wear themselves out [4, 5].

Patients with DM may experience some complications such as retinopathy, neuropathy, nephropathy, cataracts, peripheral vascular insufficiencies, and damaged nerves resulting from chronic hyperglycemia [5–7]. High blood glucose levels in type 2 DM can be controlled by using

insulin or oral antidiabetic drugs [8]. Different pathways and mechanisms are considered for preventing the progression of the disease. They may include inhibition of intestinal α-glucosidase and α-amylase, inhibition of aldose reductase, insulin synthesis and secretion, inhibition of lens aldose reductase, oxidative stress protection, inhibition of formation of advanced glycation end products, lowering plasma glucose levels, altering enzyme activity of hexokinases and glucose-6-phosphate, inhibition of postprandial hyperglycemia, stimulation of GLUT-4, decreasing activity of G6P, and reducing the level of skeletal hexokinases [5].

One of the most popular approaches to the management of blood glucose levels is the inhibition of key enzymes [9]. α-Glucosidase and α-amylase are two carbohydrate digestive enzymes which can cause elevated postprandial hyperglycemia (PPHG); thus, their inhibition plays a significant role in controlling PPHG in patients with type 2 DM. Inhibition of α-glucosidase leads to the reduction of disaccharide hydrolysis, and inhibition of α-amylase disrupts the breakdown of starch to simple sugars. Some of these compounds are clinically used, and the results have shown significant reduction of blood glucose levels in patients [10, 11]. The most important side effect related to the approved Food and Drug Administration (FDA) antitype 2 DM drugs, including voglibose, acarbose, miglitol, sulphonylureas, and thiazolidine, is gastrointestinal problems such as swelling, abdominal distraction, diarrhea, and meteorism, which need more attention. Thus, investigation of different therapeutic agents with lower side effects is in high demand. Accordingly, herbal remedies have absorbed lots of attention [12–14] and different medicinal plants such as *Abelmoschus moschatus*, *Alangium salvifolium*, *Azadirachta indica*, *Bidens pilosa*, *Boerhaavia diffusa*, *Capsicum frutescens*, *Cassia alata*, *Eclipta alba*, *Embellica officinalis*, *Ficus carica*, *Gentiana olivier*, *Glycyrrhiza glabra*, *Gymnema sylvestre*, *Hordeum vulgare*, *Ipomoea aquatic*, *Juniperus communis*, *Mangifera indica*, *Momordica charantia*, *Ocimum sanctum*, *Punica granatum*, and *Zingiber officinale* have demonstrated enzyme inhibitory activity possessing desirable effects on diabetes and hyperglycemia [15–33]. Furthermore, various phytochemicals such as alkaloids, sesquiterpene and saponins, polysaccharides, flavonoids, dietary fibers, ferulic acid, tannins, limonene, and oleuropeoside have been studied for their inhibitory activity toward enzymes involved in the one set and progression of type 2 DM, which deserve to be considered for the development and production of herbal anti-DM supplements [5, 24, 34–43].

The genus *Centaurea* (family Asteraceae, tribe Cardueae, subtribe Centaureinae) compromises approximately 600 species worldwide, from Asia, Europe, and tropical Africa to North America [44]. *Centaurea* spp. have long been used in traditional medicine to cure various ailments such as diabetes, diarrhea, rheumatism, malaria, hemorrhoids, and neurological disorders. They have also been used in the treatment of inflammation, common cold, fever, cough, and ophthalmic disorders and their liver strengthening, wound healing, and anti-itching effects have been important [45–50]. A wide range of secondary metabolites, including sesquiterpene lactones (SLs) [44, 51–53], flavonoids [45, 46, 54, 55], lignans, and alkaloids [44, 45, 55], have been isolated from different *Centaurea* spp. The genus *Centaurea* is known for possessing sesquiterpene lactones (SLs) [56, 57] and phenolic compounds [58]. Herein, focusing on the hypoglycemic activity of various species of *Centaurea* in both folk and modern medicine [59–66], we reviewed different reports on their antidiabetic potency to develop herbal drugs and supplements for controlling blood sugar.

2. Methods

The literature was completely searched *via* search engines such as Google Scholar, Pub Med, and Scopus using keywords, including DM, *Centaurea*, hyperglycemia, medicinal plants, antidiabetic plants, α-glucosidase, α-amylase, high blood glucose levels, enzyme inhibition, plant-based diets, folk medicine, and treatment. All results were extracted and analyzed in a comprehensive manner.

3. Results

Antidiabetic activity of *Centaurea* spp. (Figure 1) has been usually investigated through the *in vitro* inhibition of α-glucosidase and α-amylase as well as *in vivo* studies on rats and mice (Table 1). However, no clinical trials have been conducted. α-Glucosidase and α-amylase are clinically responsible for glucose disorders in patients with type 2 DM. Reported results have been summarized in Table 1.

3.1. In Vitro Assays

3.1.1. Centaurea bornmuelleri. *In vitro* α-amylase and α-glucosidase, as well as antioxidant activities of *Centaurea bornmuelleri*, have been reported in the literature. Among methanolic, aqueous, and ethyl acetate extracts of aerial parts of *C. bornmuelleri*, the ethyl acetate extract was found to be more potent than the others toward α-amylase and α-glucosidase [67] (Table 1). Other studies confirmed the antibacterial and antioxidant activity of the methanolic extract of the plant [80]. Also, it could inhibit the growth of colon cancer cells under *in vitro* conditions [81].

3.1.2. Centaurea calcitrapa. *Centaurea calcitrapa* has been used in folk medicine for the treatment of ophthalmic and skin diseases, common fever, jaundice, and digestive disorders [82–84]. In an *in vitro* study, the antidiabetic activity of methanolic extract of aerial parts of the plant was investigated. It could inhibit α-glucosidase with IC_{50} value of 4.38 ± 0.31 mg/ml comparing with acarbose ($IC_{50} = 1.41 \pm 0.07$ mg/ml) [68] (Table 1). It is worth mentioning that the extract has also shown antibacterial activity against *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Salmonella*, *Enterobacter*, *Enterococcus,* *Acinetobacter*, and *Escherichia* genera [85–87]. Furthermore, *C. calcitrapa* has depicted significant antioxidant activity through β-carotene/linoleic acid bleaching assay. *In vivo* antioxidant assay in mice at the doses of 50 and 100 mg/kg/day within 21 days afforded a protective effect against erythrocytes hemolysis [88].

(a)

(b)

(c)

Figure 1: Some *Centaurea* species deposited in the herbarium of the Faculty Of Pharmacy, Tehran University of Medical Sciences. (a) *Centaurea bruguierana*. (b) *Centaurea patula*. (c) *Centaurea depressa*.

Table 1: Antidiabetic activity of *Centaurea* spp.

Entry		*Centaurea* spp.	Action	Part	Extract	Activity[a]	Reference
1	*In vitro* studies	*C. bornmuelleri*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	33.12 ± 0.32 (mg ACAE[b]/g extract)	[67]
2		*C. bornmuelleri*	α-Glucosidase inhibition	Aerial parts	MeOH	10.17 ± 0.91 (mg ACAE/g extract)	[67]
3		*C. bornmuelleri*	α-Glucosidase inhibition	Aerial parts	Decoction	1.95 ± 0.07 (mg ACAE/g extract)	[67]
4		*C. bornmuelleri*	α-Glucosidase inhibition	Aerial parts	Infusion	2.36 ± 0.25 (mg ACAE/g extract)	[67]
5		*C. bornmuelleri*	α-Amylase inhibition	Aerial parts	Ethyl acetate	19.90 ± 0.89 (mg ACAE/g extract)	[67]
6		*C. bornmuelleri*	α-Amylase inhibition	Aerial parts	MeOH	16.73 ± 0.34 (mg ACAE/g extract)	[67]
7		*C. bornmuelleri*	α-Amylase inhibition	Aerial parts	Decoction	3.98 ± 0.22 (mg ACAE/g extract)	[67]
8		*C. bornmuelleri*	α-Amylase inhibition	Aerial parts	Infusion	3.54 ± 0.66 (mg ACAE/g extract)	[67]
9		*C. calcitrapa*	α-Glucosidase inhibition	Aerial parts	MeOH	4.38 ± 0.31 (mg/ml)	[68]
10		*C. centaurium*	α-Amylase inhibition	Roots	MeOH	32.51 ± 0.34%	[69]
11		*C. centaurium*	α-Amylase inhibition	Roots	Aqueous	—	[69]
12		*C. centaurium*	α-Amylase inhibition	Roots	Polyphenol	—	[69]
13		*C. centaurium*	α-Amylase inhibition	Roots	*n*-Hexane	158 (μg/ml)	[69]
14		*C. depressa*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	46.11 ± 0.97%	[70]
15		*C. depressa*	α-Glucosidase inhibition	Aerial parts	Chloroform	53.45 ± 1.98%	[70]
16		*C. depressa*	α-Amylase inhibition	Aerial parts	Ethyl acetate	36.93 ± 0.97%	[70]
17		*C. depressa*	α-Amylase inhibition	Aerial parts	Chloroform	43.97 ± 0.92%	[70]
18		*C. drabifolia* subsp. *detonsa*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	43.10 ± 2.41%	[70]
19		*C. drabifolia* subsp. *detonsa*	α-Glucosidase inhibition	Aerial parts	Chloroform	36.03 ± 0.24%	[70]
20		*C. drabifolia* subsp. *detonsa*	α-Amylase inhibition	Aerial parts	Ethyl acetate	25.58 ± 0.38%	[70]
21		*C. drabifolia* subsp. *detonsa*	α-Amylase inhibition	Aerial parts	Chloroform	25.28 ± 0.38%	[70]
22		*C. fenzlii*	α-Glucosidase inhibition	Aerial parts	MeOH	0.331 (mmol ACAE/g dry weight)	[71]
23		*C. fenzlii*	α-Amylase inhibition	Aerial parts	MeOH	0.354 (mmol ACAE/g dry weight)	[71]
24		*C. hypoleuca*	α-Glucosidase inhibition	Flowers	EtOH	10.33 ± 0.04 (mmol ACAE/g extract)	[72]
25		*C. hypoleuca*	α-Glucosidase inhibition	Flowers	MeOH	12.77 ± 0.61 (mmol ACAE/g extract)	[72]
26		*C. hypoleuca*	α-Glucosidase inhibition	Flowers	Ethyl acetate	19.61 ± 0.05 (mmol ACAE/g extract)	[72]
27		*C. hypoleuca*	α-Glucosidase inhibition	Stems	EtOH	9.10 ± 0.06 (mmol ACAE/g extract)	[72]
28		*C. hypoleuca*	α-Glucosidase inhibition	Stems	MeOH	8.66 ± 0.08 (mmol ACAE/g extract)	[72]

Table 1: Continued.

Entry	*Centaurea* spp.	Action	Part	Extract	Activity[a]	Reference
29	*C. hypoleuca*	α-Glucosidase inhibition	Stems	Ethyl acetate	12.62 ± 0.21 (mmol ACAE/g extract)	[72]
30	*C. hypoleuca*	α-Amylase inhibition	Flowers	EtOH	82.65 ± 1.31 (mmol ACAE/g extract)	[72]
31	*C. hypoleuca*	α-Amylase inhibition	Flowers	MeOH	102.41 ± 1.18 (mmol ACAE/g extract)	[72]
32	*C. hypoleuca*	α-Amylase inhibition	Flowers	Ethyl acetate	106.72 ± 1.10 (mmol ACAE/g extract)	[72]
33	*C. hypoleuca*	α-Amylase inhibition	Stems	EtOH	63.64 ± 1.05 (mmol ACAE/g extract)	[72]
34	*C. hypoleuca*	α-Amylase inhibition	Stems	MeOH	66.66 ± 0.67 (mmol ACAE/g extract)	[72]
35	*C. hypoleuca*	α-Amylase inhibition	Stems	Ethyl acetate	72.41 ± 0.61 (mmol ACAE/g extract)	[72]
36	*C. karduchorum*	α-Glucosidase inhibition	Roots	Hydrophilic (80%EtOH, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	5.35 ± 0.08 (mg/ml)	[73]
37	*C. karduchorum*	α-Glucosidase inhibition	Stems	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	1.42 ± 0.10 (mg/ml)	[73]
38	*C. karduchorum*	α-Glucosidase inhibition	Leaves	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	0.63 ± 0.00 (mg/ml)	[73]
39	*C. karduchorum*	α-Glucosidase inhibition	Flowers	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	1.51 ± 0.22 (mg/ml)	[73]
40	*C. karduchorum*	α-Amylase inhibition	Roots	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	Not active	[73]
41	*C. karduchorum*	α-Amylase inhibition	Stems	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	Not active	[73]
42	*C. karduchorum*	α-Amylase inhibition	Leaves	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	14.63 ± 0.67 (mg/ml)	[73]
43	*C. karduchorum*	α-Amylase inhibition	Flowers	Hydrophilic (80% ethanol, 19% H_2O, and 1% of 0.1% trifluoroacetic acid, v/v/v)	Not active	[73]
44	*C. kotschyi* var. *persica*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	42.35 ± 2.22%	[70]
45	*C. kotschyi* var. *persica*	α-Glucosidase inhibition	Aerial parts	Chloroform	49.42 ± 0.92%	[70]
46	*C. kotschyi* var. *persica*	α-Amylase inhibition	Aerial parts	Ethyl acetate	36.16 ± 0.13%	[70]
47	*C. kotschyi* var. *persica*	α-Amylase inhibition	Aerial parts	Chloroform	42.72 ± 0.17%	[70]
48	*C. papposa*	α-Glucosidase inhibition	Aerial parts	Dichloromethane	227.6 ± 4.4 (μg/ml)	[8]
49	*C. papposa*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	791.9 ± 1.8 (μg/ml)	[8]
50	*C. papposa*	α-Glucosidase inhibition	Aerial parts	*n*-Butanol	Not active	[8]

Table 1: Continued.

Entry	*Centaurea* spp.	Action	Part	Extract	Activity[a]	Reference
51	*C. patula*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	54.88 ± 1.11%	[70]
52	*C. patula*	α-Glucosidase inhibition	Aerial parts	Chloroform	56.11 ± 0.24%	[70]
53	*C. patula*	α-Amylase inhibition	Aerial parts	Ethyl acetate	31.70 ± 0.04%	[70]
54	*C. patula*	α-Amylase inhibition	Aerial parts	Chloroform	33.30 ± 0.04%	[70]
55	*C. pulchella*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	35.59 ± 0.58%	[70]
56	*C. pulchella*	α-Glucosidase inhibition	Aerial parts	Chloroform	60.31 ± 2.13%	[70]
57	*C. pulchella*	α-Amylase inhibition	Aerial parts	Ethyl acetate	21.54 ± 0.04%	[70]
58	*C. pulchella*	α-Amylase inhibition	Aerial parts	Chloroform	59.54 ± 0.59%	[70]
59	*C. saligna*	α-Glucosidase inhibition	Leaves	Ethyl acetate	23.80 ± 0.06 (mmol ACAE/g extract)	[74]
60	*C. saligna*	α-Glucosidase inhibition	Leaves	MeOH	12.57 ± 1.97 (mmol ACAE/g extract)	[74]
61	*C. saligna*	α-Glucosidase inhibition	Leaves	Aqueous	3.32 ± 0.40 (mmol ACAE/g extract)	[74]
62	*C. saligna*	α-Amylase inhibition	Leaves	Ethyl acetate	0.80 ± 0.01 (mmol ACAE/g extract)	[74]
63	*C. saligna*	α-Amylase inhibition	Leaves	MeOH	0.59 ± 0.01 (mmol ACAE/g extract)	[74]
64	*C. saligna*	α-Amylase inhibition	Leaves	Aqueous	0.16 ± 0.01 (mmol ACAE/g extract)	[74]
65	*C. tchihacheffii*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	58.23 ± 0.53%	[70]
66	*C. tchihacheffii*	α-Glucosidase inhibition	Aerial parts	Chloroform	53.45 ± 1.40%	[70]
67	*C. tchihacheffii*	α-Amylase inhibition	Aerial parts	Ethyl acetate	29.89 ± 1.01%	[70]
68	*C. tchihacheffii*	α-Amylase inhibition	Aerial parts	Chloroform	40.26 ± 0.29%	[70]
69	*C. triumfettii*	α-Glucosidase inhibition	Aerial parts	Ethyl acetate	69.88 ± 1.16%	[70]
70	*C. triumfettii*	α-Glucosidase inhibition	Aerial parts	Chloroform	41.12 ± 0.77%	[70]
71	*C. triumfettii*	α-Amylase inhibition	Aerial parts	Ethyl acetate	42.84 ± 0.34%	[70]
72	*C. triumfettii*	α-Amylase inhibition	Aerial parts	Chloroform	22.40 ± 0.17%	[70]
73	*C. triumfettii*	α-Glucosidase inhibition	Stems	EtOH	3.74 ± 0.05 (mmol ACAE/g extract)	[14]
74	*C. triumfettii*	α-Glucosidase inhibition	Stems	MeOH	3.77 ± 0.05 (mmol ACAE/g extract)	[14]
75	*C. triumfettii*	α-Glucosidase inhibition	Stems	Ethyl acetate	4.13 ± 0.04 (mmol ACAE/g extract)	[14]

Table 1: Continued.

Entry		*Centaurea* spp.	Action	Part	Extract	Activity[a]	Reference
76		*C. triumfettii*	*α*-Glucosidase inhibition	Flowers	EtOH	2.27 ± 0.01 (mmol ACAE/g extract)	[14]
77		*C. triumfettii*	*α*-Glucosidase inhibition	Flowers	MeOH	2.09 ± 0.03 (mmol ACAE/g extract)	[14]
78		*C. triumfettii*	*α*-Glucosidase inhibition	Flowers	Ethyl acetate	1.42 ± 0.05 (mmol ACAE/g extract)	[14]
79		*C. triumfettii*	*α*-Amylase inhibition	Stems	EtOH	137.39 ± 0.76 (mmol ACAE/g extract)	[14]
80		*C. triumfettii*	*α*-Amylase inhibition	Stems	MeOH	127.57 ± 0.72 (mmol ACAE/g extract)	[14]
81		*C. triumfettii*	*α*-Amylase inhibition	Stems	Ethyl acetate	165.47 ± 0.72 (mmol ACAE/g extract)	[14]
82		*C. triumfettii*	*α*-Amylase inhibition	Flowers	EtOH	137.42 ± 0.75 (mmol ACAE/g extract)	[14]
83		*C. triumfettii*	*α*-Amylase inhibition	Flowers	MeOH	114.06 ± 0.50 (mmol ACAE/g extract)	[14]
84		*C. triumfettii*	*α*-Amylase inhibition	Flowers	Ethyl acetate	116.85 ± 0.85 (mmol ACAE/g extract)	[14]
85		*C. urvillei* subsp. *hayekiana*	*α*-Glucosidase inhibition	Aerial parts	Ethyl acetate	67.66 ± 0.05%	[70]
86		*C. urvillei* subsp. *hayekiana*	*α*-Glucosidase inhibition	Aerial parts	Chloroform	43.65 ± 0.39%	[70]
87		*C. urvillei* subsp. *hayekiana*	*α*-Amylase inhibition	Aerial parts	Ethyl acetate	43.20 ± 0.59%	[70]
88		*C. urvillei* subsp. *hayekiana*	*α*-Amylase inhibition	Aerial parts	Chloroform	17.53 ± 0.08%	[70]
89		*C. alexanderina*	Reduction of blood glucose level	Leaves	MeOH		[75]
90	*In vivo* studies	*C. aspera*	It exhibited an important hypoglycemic effect by oral route and chronic administration in diabetic rats; the extract obtained by exhaustion with hot water showed an acute hypoglycemic activity in normal animals	Flowers	Aqueous	—	[76]
91		*C. bruguierana*	The ethyl acetate extract resulted in the best reduction of blood glucose The aqueous extract resulted in the best reduction of PEPCK activity and increment in hepatic GP activity	Aerial fruiting parts	Aqueous, dichloromethane, ethyl acetate, and methanol	—	[77]

Table 1: Continued.

Entry	*Centaurea* spp.	Action	Part	Extract	Activity[a]	Reference
92	*C. corubionensis*	Consumption of aqueous extracts of leaves and flowers at the dose of 5 g/kg led to the reduction of blood glucose levels; aqueous extract of flowers (50 mg/ml) could increase insulin release from isolated islets of Langerhans	Leaves and flowers	Aqueous and EtOH	—	[78]
93	*C. horrida*	Reduction in blood glucose level in chronic and acute condition Using the extract significantly improved peripheral nerve function of diabetic mice *via* hot plate and tail flick tests	Herb and roots	MeOH	—	[79]

[a]IC_{50} values reported as mg/ml, μg/ml, mmol ACAE/g extract, or inhibition percent (%). [b]ACAE = acarbose equivalent.

3.1.3. Centaurea centaurium. *In vitro* α-amylase inhibitory activity of methanolic, aqueous, polyphenol, and *n*-hexane extracts of *Centaurea centaurium* was assayed by Conforti et al. [69]. The *n*-hexane extract was the most potent extract with an IC_{50}value of 158 μg/ml. However, aqueous and polyphenol extracts were inactive, and the methanolic extract was found to be weak with an inhibition percent of $32.51 \pm 0.34\%$ at the concentration of 1000 μg/ml.

3.1.4. Centaurea depressa, Centaurea drabifolia, Centaurea kotschyi, Centaurea patula, Centaurea pulchella, Centaurea tchihacheffii, Centaurea triumfettii, and Centaurea urvillei. The chloroform and ethyl acetate extracts of aerial parts of eight *Centaurea* spp. including *C. depressa*, *C. drabifolia*, *C. kotschyi*, *C. patula*, *C. pulchella*, *C. tchihacheffii*, *C. triumfettii*, and *C. urvillei* were investigated for their α-glucosidase and α-amylase inhibitory activity by Zengin et al. All *Centaurea* spp. extracts were able to inhibit both enzymes at the concentration of 2 mg/mL (Table 1) and compared with acarbose, inducing inhibitory activity toward α-amylase and α-glucosidase with inhibition percent of 50.51% and 44.16% at 1 mg/ml. The chloroform extract of *C. pulchella* and *C. depressa* and the ethyl acetate extract of *C. urvillei* showed the most potent α-amylase inhibitory effects with inhibition percent of 59.54%, 43.97%, and 43.20%, respectively. The antiglucosidase effect was reported in the following order: ethyl acetate extract of *C. triumfettii* (69.88%) > ethyl acetate extract of *C. urvillei* (67.66%) > chloroform extract of *C. pulchella* (60.31%) [70].

It should be mentioned that antioxidant, antibacterial, antinociceptive, antipyretic, and anticholinesterase activities of these species were also proven [14, 70, 89–93].

3.1.5. Centaurea fenzlii. The methanolic extract of *Centaurea fenzlii* has shown α-glucosidase and α-amylase inhibitory activity as 0.331 mmol ACAE/g dry weight and 0.354 mmol ACAE/g dry weight, respectively [71]. The plant has also shown antioxidant, antityrosinase, and anticholinesterase activities, as well as cytotoxicity against colon and MCF-7 breast cancer cell lines [71, 94, 95].

3.1.6. Centaurea hypoleuca. Ethanolic, methanolic, and ethyl acetate extracts of aerial parts (flower and stem) of *Centaurea hypoleuca* have depicted *in vitro* inhibitory activity toward α-glucosidase and α-amylase. It should be noted that the ethyl acetate extract of the plant flowers resulted in higher activity than that of the stem as well as other extracts (Table 1) [72]. Also, all extracts demonstrated moderate-to-good antioxidant, antimicrobial, and anticholinesterase activities [72].

3.1.7. Centaurea karduchorum. The dried powder of *Centaurea karduchorum* has been traditionally used for wound healing [96]. Also, tea prepared from aerial parts of the plant was found to be helpful for the treatment of diabetes, which was investigated and proven in recent studies. Among ethanolic extracts obtained from roots, stems, leaves, and flowers of the plant (Table 1), the leaves extract showed the best inhibitory activity against α-glucosidase ($IC_{50} = 0.63 \pm 0.00$ mg/ml); however, it could not efficiently inhibit the α-amylase ($IC_{50} = 14.63 \pm 0.67$ mg/ml) [73].

Comparing α-glucosidase inhibitory activity of *C. karduchorum* with that of cinnamon which is known for its antidiabetic activity revealed potent efficacy of *C. karduchorum* since the activity of various extracts of cinnamon was calculated in the range of $IC_{50} = 0.42$–4.0 mg/ml [73, 97].

Table 2: Chemical compounds isolated from *Centaurea* spp.

Entry	*Centaurea* spp.	Phytochemical constituents	References
1	*C. alexanderina*	Sesquiterpene lactones and flavonoids (kaempferol 3-*O*-rutinoside, rutin, apigenin 7-*O*-galacturonic acid methyl ester, apigenin 7-*O*-β-D-glucoside, astragalin, centaurein, vicenin, vitexin, isovitexin, kaempferol, apigenin, quercetin, jaceosidin, and nepetin)	[75, 104, 115, 116]
2	*C. aspera*	Sesquiterpene lactones (dehydromelitensin, melitensin, isomelitensin, eudesmanolides, and dihydrostenophyllolide) and flavonoids (6-methoxyluteolin (nepetin), 6-methoxyacacetin (pectolinarigenin), 6-methoxyapigenin (hispidulin), and 6-methoxychrisoeriol (jaceosidin)).	[52, 116–118]
3	*C. bornmuelleri*	Flavonoids (afzelin, astragalin, isorhamnetin, apigenin, quercetin, luteolin, and kaempferol), phenolic acids (caffeoylquinic acids and chlorogenic acid), sterol (stigmast-4-en-3gamma-ol), and lignans (arctiin, arctigenin, matairesinol, and matairesinoside)	[67, 92, 119]
4	*C. bruguierana*	Sesquiterpene lactones (cnicin and dehydromelitensin-8-acetate) and flavonoids (kaempferol, rutin, quercetin, cirsimaritin, cirsilinelol, and eupatilin)	[77, 104, 112, 113, 120–123]
5	C. calcitrapa	Sterols, sesquiterpene lactones and their closely related group of triterpenoids, lignans, flavonoids (apigenin, luteolin, scutellarein, chrysoeriol, nepetin, jaceosidin, eupatorin, kaempferol, kaempferide, jaceidin, and centaureidin), alkaloids (stizolphine and choline), and phenolic acids (derivatives of hydroxycinnamic acids: *p*-coumaric, ferulic, caffeic, and chlorogenic acid; derivatives of hydroxybenzoic acids: *p*-hydroxybenzoic, protocatechuic, gallic, and gentisic acid)	[124–132]
6	*C. centaurium*	Fatty acids (11, 14-eicosadienoic acid methyl ester, 9-octadecenoic acid methyl ester, and 9-octadecenoic acid) and terpenes (cypirene, α-zingiberene, β-farnesene, β-santalene, β-bisabolene, β-himachalene, and azulene)	[69]
7	*C. corubionensis*	Has not been fully characterized	
8	*C. depressa*	Phenolic compounds, condensed tannins, flavonoids (luteolin, kaempferol, scutellarein 7-β-D-glucuronoside, scutellarein 5-β-D-glucuronoside, quercetin, isoquercitrin, quereimeritrin, and apigenin), monoterpenoid (piperitone), sesquiterpenoid (elemol), and sesquiterpene lactones (solstitialin A and acetyl solstitialin)	[70, 90, 92, 133–137]
9	*C. drabifolia*	Flavonoids, sesquiterpene lactones (belonging to the guaiane class; centaurea lactone, cynaropicrin, aguerin B, 8α-isovaleryloxyzaluzanin C, 8α-acetoxyzaluzanin C, and 4β,15-dihydro-3-dehydrosolstitialin A), and phenolic compounds (protocatechuic acid, 5-caffeoylquinic acid, 5-feruloylquinic acid, orientin, vitexin, quercetin, quercetin-3-*O*-glucoside, patuletin-*O*-hexoside, luteolin, luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucoside, isovitexin, apigenin, and hispidulin)	[138–142]
10	*C. fenzlii*	Flavonoids (cirsiliol, isorhamnetin, hispidulin, and cirsimaritin)	[95]
11	*C. horrida*	Flavonoids (horridin, apigenin, rutin, apigenin-3-O-glucuronide, kaempferol-3-*O*-glucuronide, apigenin-8-C-α-L-arabinoside, apigenin-6-C-α-L-arabinoside, apigenin-7-O-β-D-glucoside, apigenin6,8-di-C-β-D-glucoside, scutelarein-7-*O*-β-D-glucoside, kaempferol-3-*O*-β-D-glucoside, kaempferol-3-O-α-L-rhamnoside, vitexin, isovitexin, orientin, schaftoside, hispidulin, fisetin, quercetin, quercetin-3-*O*-α-L-rhamnoside, and quercetin-3-*O*-β-D-galactoside), lactones, phenolic acids, pentacyclic triterpenes, sterol glucoside, and Q acid derivatives	[104, 143–146]
12	*C. hypoleuca*	Sesquiterpene lactones (centaurepensin, acroptillin, cynaropicrin, janerin, linichlorin, and repin) and phenolic compound (catechin and chlorogenic acid)	[72, 126, 147–150]
13	*C. karduchorum*	Phenolic compounds (chlorogenic acid, apigenin, and luteolin glycosides)	[73, 150, 151]
14	*C. kotschyi*	Sesquiterpene lactones (germacrene D, β-caryophyllene, β-cedrene, β-bisabolene, and bicyclogermacrene), phenolic compounds, and flavonoid (patuletin-7-O-glucoside)	[70, 116, 152, 153]
15	*C. papposa*	Phenolic acids (quinic acid, malic acid, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, salicylic acid, vanillic acid, coumarin, syringic acid, apigenin, and apigetrin), flavonoids, and terpenes	[8, 154, 155]
16	*C. patula*	Phenolic compounds (protocatechuic acid, caffeic acid, 5-feruloylquinic acid, orientin, vitexin, patuletin-*O*-hexoside, luteolin-7-*O*-glucoside, isovitexin, quercetin, apigenin, hispidulin, and luteolin), sesquiterpenes (spathulenol), and diterpene alcohol (phytol)	[141, 156]
17	*C. pulchella*	Phenolics content, condensed tannins, and fatty acid (linoleic acid, α-linoleic acid, and palmitic acid)	[70]

Table 2: Continued.

Entry	*Centaurea* spp.	Phytochemical constituents	References
18	*C. saligna*	Flavonoids (rutin, hesperidin, quercetin, luteolin, kaempferol, and apigenin) and phenolic compounds (rosmarinic acid and *p*-coumaric acid)	[74]
19	*C. tchihacheffii*	Phenolic compounds	[70]
20	*C. triumfettii*	Phenolic compounds (chlorogenic acid, ferulic acid, p-coumaric acid, and caffeic acid)	[14, 150]
21	*C. urvillei*	Flavonoids (naringenin-7-*O*-β-D glucuronopyranoside, 6-hydroxykaempferol-7-*O*-β-D glucuronopyranoside, hispidulin-7-*O*-β-D-glucuronopyranoside, apigenin-7-*O*-β-D-methylglucuronopyranoside, hispidulin-7-*O*-β-D-methylglucuronopyranoside, hispidulin-7-*O*-β-D-glucopyranoside, apigenin-7-*O*-β-D-glucopyranoside, kaempferol, apigenin, luteolin, eriodictyol-7-*O*-β-D-glucuronopyranoside, arbutin, salidroside, and 3,5-dihydroxyphenethyl alcohol-3-*O*-β-D-glucopyranoside)	[70, 92, 157]

3.1.8. Centaurea papposa. In vitro α-glucosidase inhibitory activity of *n*-butanol, dichloromethane, and ethyl acetate extracts of *Centaurea papposa* was studied by Mawahib et al. Among them, dichloromethane extract displayed a greater inhibitory activity ($IC_{50} = 227.6 \pm 4.4\,\mu$g/ml) comparing with acarbose ($275.4 \pm 1.6\,\mu$g/ml). The ethyl acetate extract exhibited weak anti-α-glucosidase activity ($IC_{50} = 791.9 \pm 1.8\,\mu$g/mL), and the *n*-butanol extract, however, was inactive [8].

3.1.9. Centaurea saligna. Centaurea saligna has been traditionally used as a wound healing agent, astringent, and tonic. Moreover, its choleretic, diuretic, antibacterial, antirheumatic, and antipyretic activities have been reported [49, 74, 98]. The plant also has demonstrated anticholinesterase, antityrosinase, antiradical, antimicrobial, and antiproliferative properties on LNCaP, HCT-116, and MCF-7 cancer cell lines [74, 99, 100].

Methanolic, aqueous, and ethyl acetate extracts of *C. saligna* leaves were studied against α-glucosidase (3.32–23.80 mmol ACAE/g extract) and α-amylase (0.16–0.80 mmol ACAE/g extract) by Zengin et al. Among them, the ethyl acetate extract showed the most potent anti-α-glucosidase activity (23.80 mmol ACAE/g extract). It is clear that those extracts exhibited weak inhibitory activity toward α-amylase [74].

3.1.10. Centaurea triumfettii. Leaves of *Centaurea triumfettii* have been traditionally used as foodstuff [92, 101]. Biological activities of methanolic, ethanolic, and ethyl acetate extracts of stems and flowers of *Centaurea triumfettii* have been reported by Acet [14]. The ethyl acetate extract of the stems showed potent inhibitory effects on α-amylase (165.47 ± 0.72 mmol ACAE/g extract) and α-glucosidase (4.13 ± 0.04 mmol ACAE/g extract). The plant has also shown the antioxidant capacity and antibacterial activity [14, 91, 102].

3.2. In Vivo Assay

3.2.1. Centaurea alexanderina. Centaurea alexanderina has shown different biological activities such as anti-inflammatory, analgesic, hepatoprotective, and antibacterial (against *Pseudomonas aeruginosa*) effects and cytotoxicity on A-495 lung cancer cells [75, 103].

Antidiabetic properties of the 80% methanolic extract of leaves of *C. alexanderina* at the doses of 300 and 600 mg/kg have been studied under *in vivo* conditions in normoglycemic as well as streptozotocin- (STZ-) induced diabetic rats. Those results were compared with glibenclamide (50 mg/kg) as the standard drug. Administration of the extract at the dose of 600 mg/kg led to a remarkable reduction of the elevated blood glucose by 9.4% and 10.5% after 1 and 2 h, respectively. However, using the dose of 300 mg/kg decreased the related item to 2.8% after 2.5 h. Using 300 and 600 mg/kg of extracts daily within two months in the STZ-induced diabetic model led to the reduction of plasma glucose levels by 2.7% and 4.9%, respectively. However, the reduction of test days to 30 days affected the efficacy of extract, and the corresponding levels reduced to 1.1% and 3.8%, respectively [75].

3.2.2. Centaurea aspera. Aqueous extracts of *Centaurea aspera* flowers were investigated for their hypoglycemic activity in normal and alloxan-diabetic rats. It exhibited an important hypoglycemic effect by oral route and chronic administration in diabetic rats comparing with glibenclamide. It should be mentioned that the extract obtained by exhaustion with hot water showed an acute hypoglycemic activity in normal animals [76].

3.2.3. Centaurea bruguierana. Hypoglycemic activity of different extracts of *Centaurea bruguierana* and the mechanism of action was investigated in STZ-alloxan-diabetic rats by Khanavi et al. The aqueous and dichloromethane extracts at the dose of 400 mg/kg and the ethyl acetate and methanol extracts at the dose of 200 mg/kg, obtained from aerial fruiting parts of the plant, were investigated. The ethyl acetate extract afforded the best activity to reduce the blood glucose levels up to 50.0%, while methanol, dichloromethane, and aqueous extracts reduced that up to 45.7%, 41.7%, and 29.5%, respectively. Glibenclamide showed a 34.5% reduction. The best result from reduction of phosphoenolpyruvate carboxykinase (PEPCK) activity (84.0%) and increasing hepatic glycogen phosphorylase (GP) activity

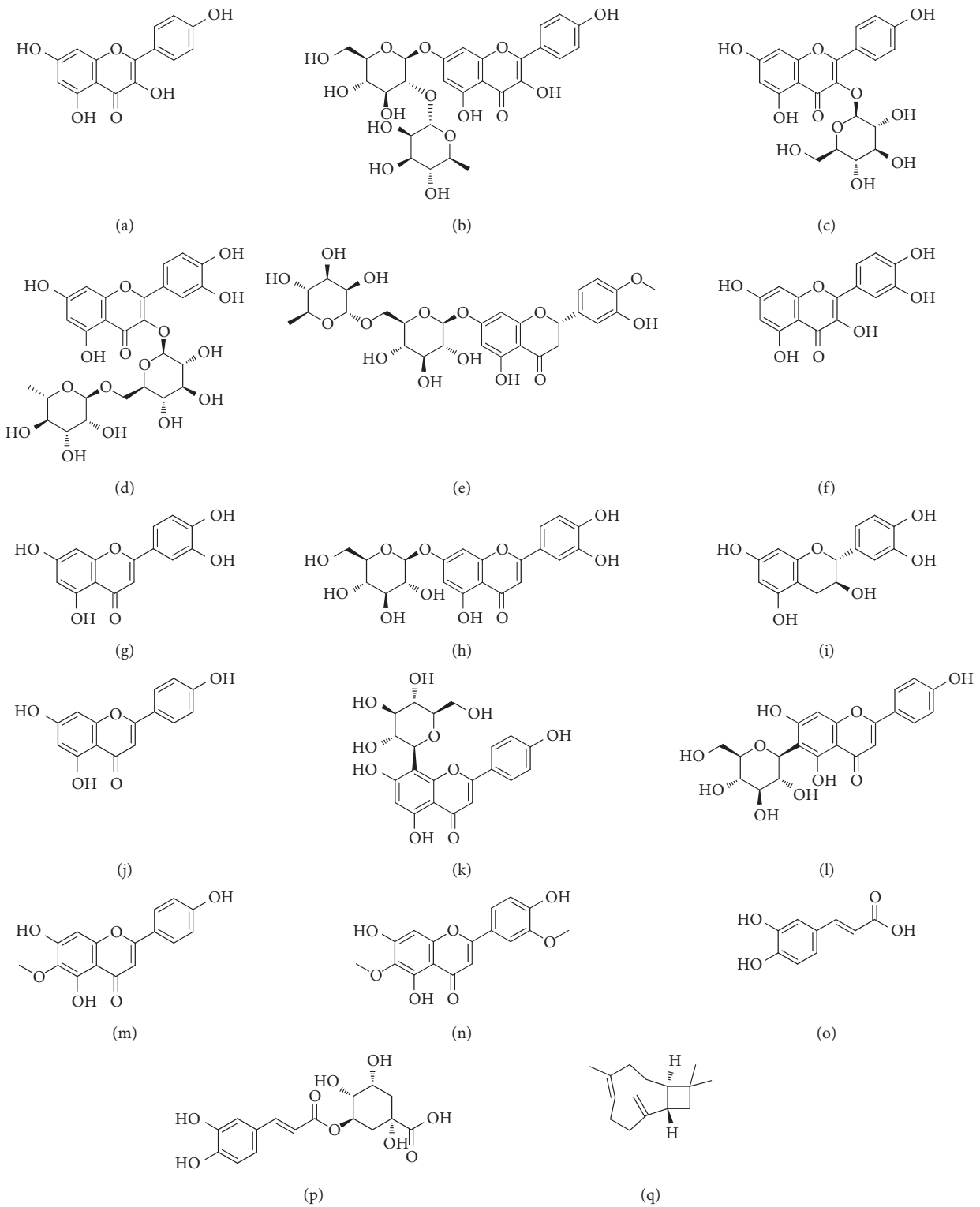

Figure 2: The chemical structure of constituents isolated from *Centaurea* spp., responsible for antidiabetic activity. (a) Kaempferol. (b) Kaempferol 3-*O*-rutinoside. (c) Astragalin (kaempferol-3-glucoside). (d) Rutin. (e) Hesperidin. (f) Quercetin. (g) Luteolin. (h) Cynaroside (luteolin-7-*O*-glucoside). (i) Catechin. (j) Apigenin. (k) Vitexin. (l) Isovitexin. (m) Hispidulin. (n) Jaceosidin. (o) Caffeic acid. (p) Cholorogenic acid. (q) β-Caryophyllene.

(134.5%) points of view was related to the aqueous extract comparing with those of glibenclamide (62.5% and 133.0%), respectively. *C. bruguierana* depicted no effect on blood insulin, but it was able to reduce blood glucose by stimulation of hepatic glycogenolysis and inhibition of gluconeogenesis [77, 104].

3.2.4. Centaurea corubionensis. Chuclá et al. studied the effect of aqueous and ethanolic extracts of leaves and flowers of *Centaurea corubionensis* on normoglycemic rats, circulating insulin levels in anesthetized rats, glucose-induced hyperglycemic rats, and alloxan-diabetic rats at different doses of 2.5, 5, and 10 g/kg [78]. Consumption of aqueous extracts of leaves and flowers at the dose of 5 g/kg led to the reduction of blood glucose levels by 19 and 16%, respectively. Also, 6 h after administration of aqueous extract of leaves (5 g/kg), the serum glucose and insulin levels were reported to be 97.2 (mg%) and 10.2 (μU/ml) comparing with tolbutamide (75 mg/kg) with those values of 84.4 (mg%) and 9.2 (μU/ml), respectively. Moreover, aqueous extract of flowers (50 mg/ml) could increase insulin release from isolated islets of Langerhans to 36 μU/ml. However, no effect was observed on alloxan-diabetic animals, and it may be associated with severe damage of the pancreas by the alloxan. Hypoglycemic properties of *C. corubionensis* can be achieved by the undamaged pancreas *via* raising serum circulating insulin.

3.2.5. Centaurea horrida. Raafat et al. investigated the antidiabetic effect of the methanolic extract of *Centaurea horrida* herb and roots in alloxan-induced diabetic mice comparing with glibenclamide. All results were generally obtained more significantly than those of glibenclamide. The plant has been traditionally used to lower blood glucose levels [79]. It was found that administration of the extract at dose of 100 mg/kg led to the reduction of blood glucose levels from 219.33 to 106.56 mg/dL. Investigation of the subacute effect of the extract exhibited the reduction of blood glucose levels from 121.84 mg/dL on 1th day to 105.42 mg/dL on the 8th day at the same dose. The subacute effect of the extract on body weight in alloxan-induced diabetic mice also revealed that using the extract at different doses of 5, 25, 50, and 100 mg/kg did not lead to a significant overweight in mice which was comparable to the positive control. *In vivo* assessment of the antioxidant activity of the extract demonstrated that treated mice with doses of 25, 50, and 100 mg/kg had no remarkable increase in serum catalase activity. However, it was clear that long-term treatment of diabetes with all doses, particularly with a high dose of extract, induced a reversed effect on catalase activity, which may be associated with reduced oxidative stress. It is worth mentioning that using the extract significantly improved peripheral nerves function of diabetic mice *via* hot plate and tail flick tests. This is an important result as uncontrolled high blood glucose levels can damage peripheral nerves causing diabetic neuropathy [79, 105, 106]. It has been suggested that hypoglycemic effect of the plant is achieved by the inhibition of the endogenous glucose production or inhibition of intestinal glucose absorption and controlling dietary glucose uptake in the small intestinal tract. It is believed that the mechanism is independent of insulin secretion [79].

The elastase and tyrosinase inhibitory effects of *C. horrida* have also been reported [107].

4. Discussion

Herbal medicine has occupied a particular position in healing purposes, and their use has grown significantly over recent years. In this respect, there are a wide range of reports on the antidiabetic activity of medicinal plants [108], which can be fully considered for the development of efficient drugs and supplements.

4.1. Toxicity. It should not be forgotten that all natural remedies are not essentially safe, and all herbal medicine users should be aware of the risks that they carry [93, 109]. To reach this goal, the toxicity of plants should be investigated for better knowing the range of safety. According to the literature, there are no enough data on the toxicity of reported *Centaurea* spp. in this paper, and most plant toxicity tests should be conducted.

Orally administration of 80% methanolic extract of *C. alexanderina* by different groups of mice ($n = 10$) in the dose range of 50–3000 mg/kg resulted in no fatality and the LD_{50} value was assumed to be greater than 3000 mg/kg [75].

LD_{50} value for the methanolic extract of *C. urvillei* was calculated as 115.5×10^{-2} using the brine shrimp lethality bioassay [110]; likewise, the LC_{50} values for methanolic and diethyl ether extracts of *C. triumfettii* were obtained as 266.5 and 166.6 μg/ml, respectively [111].

Cytotoxicity of petroleum ether, chloroform, ethyl acetate, *n*-butanol, and remaining methanolic fractions of the methanolic extract of *C. bruguierana* depicted that petroleum ether and remaining methanolic fractions were nontoxic toward NIH-3T3 cells (Swiss embryo fibroblast) [112]. However, in a study reported by Nasr et al. [113], chloroform, ethyl acetate, *n*-butanol, and methanol fractions of the plant showed toxicity on HUVEC cells (a noncancerous cell line).

As reported by Erol-Dayi et al. [114], evaluation of cytotoxicity of methanolic and aqueous extracts of *C. calcitrapa*, *C. ptosimopappa*, and *C. spicata* indicated the lack of toxicity of aqueous extract of *C. ptosimopappa* and *C. spicata* on Hela (human cervix adenocarcinoma) and Vero (normal African green monkey kidney) cells ($IC_{50} > 1000$ μg/ml). Those methanolic extracts were found to be more toxic ($IC_{50} > 200$ μg/ml) on the same cells. The aqueous extract of *C. calcitrapa* showed moderate toxicity on both cells ($IC_{50} > 400$ μg/ml), whereas the methanolic extract demonstrated an inhibitory effect with $IC_{50} < 100$ μg/ml on Hela and Vero cells (92.5 and 91.7 μg/mL, respectively). It

indicated that the methanolic extract of *calcitrapa* needs more attention from the toxicity point of view.

According to the results reported by Conforti et al. [69], based on the brine-shrimp toxicity test on the roots of *C. centaurium*, the LC_{50}value was calculated as 44.05 mg/ml for the methanolic extract, while LC_{50}values for the polyphenolic, lipophilic, and water fractions were found to be 157.44, 25.98, and 152.81 mg/ml, respectively.

4.2. Constituents Isolated from Centaurea spp. and Their Antidiabetic Activity Mechanism of Action (MOA). The antidiabetic activity of *Centaurea* spp. is definitely indebted to the presence of phytochemicals. Isolated constituents from discussed plants are listed in Table 2. In this respect, sesquiterpenes, flavonoids, and phenolic compounds have been generally reported in the literature (Figure 2).

4.2.1. Sesquiterpene Lactones. Sesquiterpenoids have shown potent antidiabetic activity *via* various mechanisms such as inhibition of enzymes involved in hyperglycemia, protecting β-pancreatic cells, preventing oxidative and inflammatory damages associated with the disease, and improving insulin secretion. They can improve insulin sensitivity by regulating glucose transport and key proteins of the insulin signaling pathway. They have also exhibited lipid-lowering actions [158].

Sesquiterpene lactones have exhibited hypoglycemic effects in STZ-induced diabetic mice by improving the function of pancreatic islets, increasing glycolysis, and decreasing gluconeogenesis as well as antioxidant and hypolipidemic activities, which have been assessed by using *in vitro* assays. The mechanism of antidiabetic activity may involve an antioxidant effect, improving insulin sensitivity, and stimulation of pancreatic β-cells to secret insulin [159]. Sesquiterpene lactones have also shown *in vitro* inhibitory effects on α-glucosidase and α-amylase [160]. They can be used for the treatment of diabetes through the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathway [158, 161]. They have also reduced the production of chemokines, such as MCP-1, TGF-β1, and FN, activate NF-κB, and inhibited sugar-induced degradation of IκBα, confirming the efficacy of sesquiterpene lactones as drug candidates for the treatment of diabetic nephropathy [158, 162].

β-Caryophyllene, as a sesquiterpene lactone derivative, has shown antihyperglycemic activity in STZ-induced diabetic rats. Oral administration of β-caryophyllene significantly decreased glucose and increased insulin levels. Moreover, reversing the glycoprotein levels in plasma and tissues of diabetic rats to near normal and decreasing proinflammatory cytokines detected using histological and immunohistochemical studies demonstrated the antioxidant capacity of this compound [163, 164]. It should be noted that chronic use of β-caryophyllene has also depicted good results in the prevention or reduction of diabetes-related neuropathy and depressive-like behavior in mice (assessed by marbles test) [165].

4.2.2. Flavonoids. Flavonoids are one of the major components of *Centaurea* spp. Four flavonoids including scutellarein, nepetin, apigenin, and hispidulin were evaluated for their α-glucosidase inhibitory effects comparing with acarbose and the order of the activity was obtained as scutellarein > nepetin > apigenin > hispidulin > acarbose.
Also, the synergistic effects from the combination of each flavonoid with acarbose at different concentrations were observed. It was perceived that the best synergistic effect was related to the combined apigenin-acarbose which acted as a noncompetitive inhibitor [166].

The antihyperglycemic effect of apigenin may be related to the inhibition of α-glucosidase, preventing oxidative stress conditions, decreasing insulin resistance, decreasing hepatic gluconeogenic enzymes activity, and increasing serum insulin levels [167–169]. Apigenin can enhance the metabolism of glucose *via* suppression of the activities of gluconeogenic enzymes and aldose reductase. It also prevents diabetic complications such as cataracts, retinopathy, and neuropathy due to the intracellular sorbitol accumulation. Glucose is converted to sorbitol in the polyol pathway, catalyzed by aldose reductase [170].

Vitexin and isovitexin are two apigenin isomers, and their α-amylase inhibitory effects and antioxidant potentials have been investigated *via in vitro* assays. Vitexin and isovitexin exhibited significant anti-α-amylase activity with IC_{50}values of 4.6 and 13.8 μM, respectively. Also, antioxidant activity was assayed through DPPH free radical scavenging assay, which showed IC_{50} values of 92.5 and 115.4 μM, respectively [171]. Vitexin also depicted inhibitory effect on α-glucosidase ($IC_{50} = 52.805\,\mu$M) which was comparable with that of acarbose ($IC_{50} = 375\,\mu$M) [172]. In addition, computer-aided studies of vitexin-amylase, isovitexin-amylase, and vitexin-glucosidase complexes in the active site of related enzymes confirmed the construction of desired interactions with amino acid residues [171, 172]. Another *in vitro* study using cell culture revealed that vitexin protected pancreatic β-cells from high-glucose-induced damage, inhibited islet β-cell apoptosis, and improved insulin release and sensitivity. The underlying mechanism may increase the expression of transcription factor Nrf2, resulting in increased intracellular antioxidant molecules, and suppress the inflammatory signaling pathway. Besides, vitexin enhances insulin production by activating insulin signaling *via* the activation of phosphorylation of IR, IRS-1, and IRS-2 [173].

Hispidulin is another important flavonoid compound inducing antidiabetic activity. Oral administration of hispidulin to STZ-induced hyperglycemia mice effectively mitigated postprandial and fasting hyperglycemia and glucose tolerance, which was associated with a dual mechanism, promoting β-cell function and suppressing hepatic glucose production [174].

Kaempferol has also depicted remarkable α-glucosidase and α-amylase inhibitory activity [175, 176]. Oral administration of kaempferol significantly improved blood glucose control in obese mice, which was associated with suppressing hepatic gluconeogenesis and improving insulin sensitivity and secretion [177, 178]. It was found that kaempferol-3-*O*-rutinoside was also a potent

inhibitor of α-glucosidase, being over 8 times more active than the reference drug, acarbose, under *in vitro* conditions [179].

Astragalin has shown hypoglycemic activity on Wistar rats (10 mg/kg) and improved insulin secretion in the glucose tolerance test. Investigation of isolated pancreatic cells treated with astragalin (100 μM) led to Ca^{2+} influx stimulation *via* a mechanism involving ATP-dependent potassium channels, L-type voltage-dependent calcium channels, the sarco/endoplasmic reticulum calcium transport ATPase (SERCA), and PKC and PKA (protein kinase) [180].

Rutin is also an important flavonoid possessing antihyperglycemic effects *via* various mechanisms, including decrease of carbohydrates absorption from the small intestine, inhibition of tissue gluconeogenesis, increase of tissue glucose uptake, stimulation of insulin secretion from β-cells, and protecting Langerhans islet against degeneration. Rutin also decreases the formation of sorbitol, reactive oxygen species, advanced glycation end-product precursors, and inflammatory cytokines [181].

Luteolin and luteolin 7-*O*-glucoside have shown good α-glucosidase inhibitory activity. However, luteolin was found to be more potent than acarbose by the inhibition of 36% at the concentration of 0.5 mg/ml. Although luteolin could inhibit α-amylase effectively (IC_{50} in the range of 50 to 500 μg/ml), it was less potent than acarbose [182].

Jaceosidin is another flavonoid compound, and its antihyperglycemic capacity has been assessed through various *in vivo* studies. The results showed that jaceosidin supplementation significantly lowered fasting blood glucose levels and reduced insulin resistance. As it was also found that jaceosidin supplementation increased antioxidant capacity by enhancement of catalase and GSH-px activities, a relevant relationship between antioxidant and antihyperglycemic effects of jaceosidin can be concluded. Jaceosidin could improve endoplasmic reticulum stress and attenuate insulin resistance *via* SERCA2b (sarco/endoplasmic reticulum Ca^{2+}-ATPase 2b) upregulation in mice skeletal muscles [183, 184].

Hesperidin has shown antidiabetic activity. It has inhibited obesity, hyperglycemia, and hyperlipidemia, and decreased insulin resistance. These effects might be closely related to the activation of AMPK, which regulate the insulin signaling pathway and lipid metabolism [185]. Hesperidin ameliorates pancreatic β-cell dysfunction and apoptosis in a streptozotocin-induced diabetic rat model [186].

The antidiabetic activity of quercetin is also important. It has reduced fasting and postprandial hyperglycemia in an animal model of DM [187]. An *in vivo* study revealed the hypoglycemic effects of quercetin, but no changes were observed in the activity of lipogenic enzymes and lipoprotein lipase. It can be concluded that the antidiabetic activity of quercetin is comparable with that of antiobesity activity [188]. There are different reports on the α-glucosidase inhibitory effect of quercetin, which describe its multilateral antidiabetic activity [187, 189, 190].

Oral administration of catechin to STZ-induced diabetic rats resulted in a potential agonist characteristic that is capable of activating the insulin receptors and producing a glucose tolerance pattern. The hypoglycemic effect of catechin is associated with its insulin mimetic activity [191]. It has been indicated that catechin significantly decreased the different lipid parameters, hepatic, and renal function enzyme levels along with HbA1c levels in diabetic rats while remarkably increased the high-density lipoprotein (HDL) levels with values comparable with the glibenclamide. Also, α-glucosidase and α-amylase inhibitory activity of catechin have been reported with inhibition percent of 80% and 79%, respectively [192].

4.2.3. Phenolic Compounds. Phenolic compounds have shown versatile and attractive antidiabetic activity. Caffeic acid, a known phenolic acid compound, could protect mice pancreatic islets from oxidative stress induced by multiwalled carbon nanotubes (MWCNTs) [193]. Investigation of the effect of caffeic acid and cinnamic acid on glucose uptake in TNF-R-induced insulin-resistant hepatocytes showed that they may eliminate insulin resistance by improving insulin signaling and enhancing glucose uptake in insulin-resistant cells, which described their antihyperglycemic potential [194]. In another report, glucose uptake into the isolated adipocytes was raised by caffeic acid. The increase of glucose utilization by caffeic acid seems to be responsible for lowering plasma glucose [195].

Chlorogenic acid could also reduce fasting blood glucose levels [196–198]. It has shown an inhibitory effect on α-amylase as potent as acarbose; however, its α-glucosidase inhibitory activity was far weaker than that of acarbose [199, 200].

The effect of phenolic compounds, particularly in the management of type 2 diabetes, has attracted lots of attention [201]. They are characterized by the presence of hydroxyl group(s) on the aryl moiety and endorsed by their antioxidant activity due to high potency of hydroxyl groups as hydrogen donors [202]. As it has been accepted that the formation of reactive oxygen species (ROS) is associated with hyperglycemia [203], using antioxidants is preferred to treat and reduce the complications of DM. Also, it has been proven that consuming a diet low in fat and rich in antioxidants may reduce the risk of obesity and insulin resistance [204–207].

Phenolic compounds comprise a wide range of phenolic acids and flavonoids. Flavonoids in turn contain anthocyanin pigments, flavonols, flavones, flavanols, and isoflavones. Polymerization of flavanols leads to the formation of tannins in which the esterification of phenolic groups affords cyclic chromenones such as ellagic acid. However, condensed tannins known as proanthocyanidins, for example, catechin, epicatechin, and gallocatechin, are obtained from the condensation of flavanols [208].

Centaurea spp. have been frequently reported to possess anthocyanins [207, 209–211] and their biological activities such as antioxidant, antiallergic, anti-inflammatory, antiviral, antiproliferative, antimutagenic, antimicrobial, and anticarcinogenic activities. Also, different properties such as improvement of microcirculation, protection from cardiovascular damage and allergy, prevention of peripheral capillary fragility,

prevention of diabetes, and vision improvement are fully considered in the literature [207, 212–222]. Also, the role of anthocyanins is well described for their effect on the prevention of diabetic cataracts [207, 218, 223]. The presence of apigenin in *Centaurea* spp. [224] has been confirmed, and its activity against thyroid neoplasms as well as anxiolytic, anti-inflammatory, and antinociceptive properties has been reported [225–227]. The presence of flavonoids in *C. bornmuelleri* is significant and might be responsible for the desired activity [67]. The phytochemical analysis of *C. calcitrapa* proved the presence of sterols, sesquiterpene lactones, and their closely related group of triterpenoids, bisabolenes, lignans, and flavonoids as the main secondary metabolites [124–130]. *C. hypoleuca* contains higher amounts of catechin and chlorogenic acid than the other phenolic compounds, which are known to be responsible for various biological activities such as antioxidant, neuroprotective, antidiabetic, hepatoprotective, and antiarthritic properties [72, 147–149]. High levels of apigenin (2472 μg/g extract), known as a common dietary flavonoid, has absorbed attention in *C. saligna*. *In silico* study has confirmed the construction of H-bonding and pi-pi stacking interactions between apigenin and the α-glucosidase active site [74]. Chlorogenic acid has been identified as the main phenolic compound in *C. triumfettii* [14]. *C. karduchorum* is known to possess abundant amounts of phenolic compounds, mainly luteolin glycosides (glucoside and glucuronide) and chlorogenic acid [73]. Some studies confirmed the activity of luteolin and/or its glycosides against diabetes and neurodegenerative diseases through the reduction of glucose uptake, oxidative stress, and inflammation [151]. Chlorogenic acid has chemopreventive and hypoglycemic effects [150], and it is the main component of medicinal plants characterized by their antioxidant, anti-inflammatory, and enzyme inhibitory activities [150, 189, 228]. *C. bruguierana* possessed sesquiterpene lactones and flavonoids (kaempferol, rutin, and quercetin) [77, 104, 120]. Also, the plant has been documented for its antiplasmodial and antipeptic ulcer effects [77, 229, 230]. The antidiabetic property of *C. karduchorum* as a herbal tea is directly dependent on the high levels of bioactive phenolic derivatives profiting from synergistic interactions of those compounds [73]. The presence of terpenes has been confirmed through qualitative analysis in *C. papposa*, which may explain the favorite activity toward α-glucosidase [154]. High total phenolic and flavonoid contents of *C. pulchella* and *C. urvillei*, respectively, may explain their antidiabetic activity [70]. Phytochemical examination of aerial parts of *C. horrida* indicated the presence of pentacyclic triterpenes, sterol glucoside, quinic acid derivatives, phenolic acid derivatives, and flavonoids as well as horridin [143, 144].

As mentioned above, discussed species of *Centaurea* are known to possess a high content of phenolic compounds, which explains their antitype 2 DM activity.

Inhibition of α-glucosidase and α-amylase has been found to be a versatile tool for the treatment of type 2 diabetes [231, 232]. Apart from synthetic compounds [233–237], a wide spectrum of medicinal plants have been introduced to possess those enzymes inhibitory activity [238], and flavonoids have been well described in this field [239]. Amphiphilic property of phenolic moiety provides favorite interactions with enzymes *via* the construction of H-bonding and hydrophobic interactions with the polar groups of enzymes and hydrophobic amino acid residues, respectively.

An important point comes back to side effects related to α-amylase inhibitors. They include abdominal distention, flatulence, meteorism, and possibly diarrhea which are consequence of high activity of the enzyme. It seems that extreme inhibition of pancreatic α-amylase results in the abnormal bacterial fermentation of undigested carbohydrates in the colon [240–242]. In this respect, dual inhibitors such as *C. saligna* and *C. karduchorum* possessing weak inhibition of α-amylase and high inhibition of α-glucosidase are desirable for the treatment of type 2 DM.

Finally, the efficacy of *Centaurea* spp. under *in vivo* conditions has followed various mechanisms such as lowering blood glucose levels, stimulation of hepatic glycogenolysis, inhibition of gluconeogenesis, and insulin secretion and circulation.

5. Conclusion

In conclusion, the antidiabetic activity of some *Centaurea* spp., which has been studied for controlling hyperglycemia, was reviewed. The results obtained from *in vitro* and *in vivo* studies confirmed the efficacy of *Centaurea* spp. for the treatment of type 2 DM. *In vitro* assays generally focused on the α-glucosidase and α-amylase inhibitory activity, and the effectiveness of *C. bornmuelleri*, *C. calcitrapa*, *C. centaurium*, *C. drabifolia*, *C. depressa*, *C. fenzlii*, *C. hypoleuca*, *C. karduchorum*, *C. kotschyi*, *C. papposa*, *C. patula*, *C. pulchella*, *C. saligna*, *C. tchihacheffii*, *C. triumfettii*, and *C. urvillei* has been investigated. Among them, dichloromethane extract of *C. papposa* was found to be the most potent inhibitor of α-glucosidase, and the *n*-hexane extract of roots of *C. centaurium* showed the highest activity toward α-amylase (Table 1). *In vivo* studies of *C. alexanderina*, *C. aspera*, *C. bruguierana*, *C. corubionensis*, and *C. horrida* revealed that *C. horrida* and *C. bruguierana* were found to be more potent than glibenclamide and *C. corubionensis* was comparable with tolbutamide. These results demonstrated that *Centaurea* spp. deserve to be widely studied through clinical trials to prove their antidiabetic effects. Also, data related to the acute and chronic toxicity are in high demand to develop safe *Centaurea* spp.-based supplements and drugs against type 2 DM.

Data Availability

The data supporting this review are from the previously reported studies and data sets which have been cited. The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Samaneh Fattaheian-Dehkordi and Reza Hojjatifard contributed to the literature review and writing the manuscript. Mina Saeedi and Mahnaz Khanavi carried out the supervision, methodology, writing, reviewing, and editing.

Acknowledgments

The authors would like to thank the partial support from Tehran University of Medical Sciences.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 5585650, 14 pages
https://doi.org/10.1155/2021/5585650

Research Article

Cafeteria Diet-Induced Metabolic and Cardiovascular Changes in Rats: The Role of *Piper nigrum* Leaf Extract

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Received 2 March 2021; Revised 30 April 2021; Accepted 24 May 2021; Published 2 June 2021

Academic Editor: Roja Rahimi

Background. Cafeteria diet is known to induce excessive body fat accumulation (obesity) that could cause metabolic and cardiovascular changes and even death. The increase in prevalence over time and the failure in treatment options make obesity a real public health problem. The present study assessed the preventive effect of the hydro-ethanolic extract of the *Piper nigrum* leaf on the development of metabolic and cardiovascular changes in cafeteria diet fed Wistar rats. *Methods*. Thirty-six male rats were divided into 5 groups of 6 rats each: a normal control group (Nor.), a negative control group (Neg.), two groups administered different doses of extract in mg/kg (E250 and E500), and a group administered atorvastatin 10 mg/kg (Ator., reference drug). The animals were fed with experimental diets (standard and cafeteria) for a period of 5 weeks. Food and water intake were assessed daily, and the body weight assessed weekly. At the end of the feeding, plasma lipid profile and markers of hepatic and renal function were assessed. Furthermore, the relative weights of the adipose tissue and the organs were assessed. The liver, kidneys, and heart homogenates were assessed for markers of oxidative stress while the aorta was histopathologically examined. *Results*. Cafeteria diet-induced weight gain of 30% and increased triglyceride, total cholesterol, and low-density lipoprotein cholesterol level of more than 50%. Equally, an increase in the relative weight of accumulated adipose tissues of more than 90%, oxidative stress, and alteration in the organ structure were visible in cafeteria diet fed rats (Neg). Treatment with *P. nigrum* extract significantly prevented weight gain, dyslipidemia, oxidative stress, and alteration in the architecture of the aorta. The effect of *P. nigrum* extract was comparable to that of the reference drug. *Conclusion*. *Piper nigrum* leaf may prevent weight gain and possess cardioprotective activity with a strong antioxidant activity.

1. Background

Abnormal accumulation of body fat with health risks defines obesity which is becoming a real public health problem as people are adopting cafeteria diets feeding habits. Obesity is a global epidemic with more than 650 million cases globally between 1975 and 2016 representing approximately 13% of the world's adult population [1]. In Africa, obesity has reached alarming proportions, especially among children where the number of cases increased by almost 50% since the 2000s [1]. Behavioural changes towards high fat diet and decrease in physical exercise contribute to imbalance of energy between excess calories intake and energy spent [2, 3]. Hence, obesity occurs when energy intake surpasses energy expenditure resulting to large energy stores as body fat particularly in the adipose tissues. Accumulation of body fat induces accumulation of adipocytes (hyperplasia) and their size (hypertrophy) bringing about structural and functional changes, activation of inflammatory signalling pathways [4, 5], and discomfort (depression and rejection)

[6]. Obesity is a major risk factor for several chronic diseases, including diabetes, cardiovascular diseases, and some types of cancers [7] which not only cause a decline in quality of life but also cause serious complications and premature death [8]. Notwithstanding the multifactorial aetiology of obesity, the prevalence is on the rise with environmental and behavioural factors (including dietary habits) being the major contributors rather than genetic variation [9].

Obesity has been related to induction of oxidative stress with resulting complications like endothelial dysfunction, non-alcoholic fatty liver disease, microvascular complications, and nephropathy [10, 11] and cardiovascular diseases [12]. Obese subjects generally possess low antioxidant defense with enhanced levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [13]. More so, in the case of central obesity, the antioxidant mechanism decreases further in an inversely proportional fashion. Obesity related hepatic inflammation is associated with tumour formation in dietary induced obesity in mice [14]. Ulla et al. [13] reported an increase in oxidative stress in experimental animals fed cafeteria diet characterized by an increase in lipid peroxidation and a collapse in antioxidant defense enzymes (catalase, superoxide dismutase, and glutathione peroxidase). Meanwhile, Udomkasemsab et al. [12] reported the implication of cafeteria diet in inducing inflammatory and oxidative stress markers. For this reason, plants that possess antioxidant and anti-inflammatory properties are thought to possess anti-obesity activities as shown in the studies of Ulla et al. [13] and Udomkasemsab et al. [12]. Such medicinal plants contain a wide variety of bioactive components that have beneficial effects on body metabolism and fat oxidation [15].

Currently, many strategies such as increase in physical activities, healthy dietary habits, anti-obesity drugs, and in extreme cases surgery have been put in place for the management of obesity. However, in most cases, these measures do not help because of the complexity of the pathology, cost, and adverse effects of certain pharmacological protocols. Sometimes only 5 to 10% of obese subjects maintain normal weight with a healthy lifestyle [15]. Hence, management of obesity in an efficient way remains a real challenge for the pharmacological industries and the health services. This has challenged many researchers to study medicinal plants and medicinal foods that can limit weight gain in order to prevent obesity. Animal models for obesity were developed by feeding rodents a semi-purified diet for several weeks with a fat content of more than 40% energy based on animal fats and the rodents developed obesity, hyperglycaemia, hypertriglyceridemia, and hyperleptinemia mimicking the pathophysiology of human obesity and metabolic syndrome [9, 16, 17]. Hence, diet-induced obesity models have become one of the most frequently used in studying obesity with high fat and cafeteria diets often employed in rats which have exhibited evidence of the role of over nutrition in energy homeostasis, body weight regulation, and adiposity [18–21]. The cafeteria diet consists of highly energetic and highly palatable human foods along with chow diet to trigger diet-induced obesity in laboratory rodents [22]. In the absence of a purified high diet, the cafeteria diet is an alternative to induce obesity in that it prevents the use of very high intakes of a particular type of fat while inducing continuous hyperphagia and increased energy intake [23, 24] with a substantial amount of sugar and salt, which increases the appetite of rodents [25]. Buyukdere et al. [26] concluded that cafeteria diet is more suitable in inducing obesity and adiposity in young rats as compared to high fat diet.

Piper nigrum belongs to the Piperaceae family and is widely used in the food industry because of its culinary applications as well as health benefits, as spices and preservatives, and in the herbal medicine and cosmetic industry [27]. Both the berries and leaf of *P. nigrum* are served as hot spices and in some traditional clinics they are used in stimulating appetite in patients [28]. These *Piper* species are also used in folk medicine for the treatment of coughs, intestinal diseases, bronchitis, venereal diseases, colds, and rheumatism [28]. In some local communities in Africa, these spices are referred to as bush pepper in order to distinguish them from the common domesticated peppers [28]. In earlier studies, *Piper nigrum* demonstrated lipid-lowering effects and antioxidant capacity by prolonging the lag time for LDL + VLDL oxidation in the present of copper (II) ion [29–31]. Oral administration of aqueous extract of *P nigrum* leaf presented antioxidant defense and anti-atherogenic activity in hamsters fed atherogenic diet [31, 32]. In high-fat diet fed rats, the ethyl acetate and aqueous extracts of *Piper nigrum* seeds regulated body weight, percentage fat, and fat-free mass [33]. Phytochemical studies revealed the major bioactive molecules in *Piper nigrum* to include polyphenols, alkaloids, terpenoids, tannins, and oils while the major isolated compounds reported are piperine, piperidine, pellitorine, cepharadione, piperolactum, paprazine, and sylvamide [34]. Of these compounds, piperine has been widely studied to act on pharmacological systems [35]. Accumulated evidence from in vitro and in vivo studies has shown that piperine possesses anticancer [36, 37] and neuroendocrine modulator effects [38]. Some other studies have demonstrated the expectorant, antiflatulent, and cholesterol-lowering properties of *Piper nigrum* is due to its piperine [39, 40].

Most of the data generated on *Piper nigrum* are based on the berries and its chemical constituents with very little attention on the leaf that are often and commonly consumed as vegetables or spices. Secondly, there is no study that considered the effect of *Piper nigrum* leaf extract on cafeteria diet which mimics human feeding habit. Hence, the present study tests the hypothesis that hydro-ethanolic extract of the leaf of *Piper nigrum* (Piperaceae) possess antioxidant activity that prevents obesity and atherosclerosis development in cafeteria diet fed rats. The aim of this study was to evaluate the anti-obese, antioxidant, and cardioprotective effect of *Piper nigrum* (Piperaceae) leaf hydro-ethanolic extract on cafeteria diet fed rats. In order to verify the protective effect of *Piper nigrum* extract against obesity, and cardiovascular disease, we induce obesity by cafeteria diet as earlier described [41]. Hence, the experimental animals will be characterized by physiological changes which are similar to humans. Our findings would be informative on the health

benefits of spices and/or medicinal food in oxidative stress and obesity related to the feeding habits.

2. Methods

2.1. Plant Collection and Preparation. Fresh leaves of *Piper nigrum* were collected in the locality of Djombé-Penja in the Littoral region, Cameroon in August 2017. No permission was needed for plant collection. The identification of the plant was done at the national herbarium of Yaoundé, Cameroon where the voucher specimen is kept under the number 2528 SRFK. 4 kg of fresh leaf of *Piper nigrum* was cleaned and dried at room temperature away from sunlight and then crushed to obtain a fine powder. The powder (1.615 kg) was macerated with 6.5 L of hydro-ethanolic solvent (70/30 ethanol and distilled water v/v) for 72 hours in a percolator. The mixture was filtered and the solvent evaporated with a rotavapor (Buchi R110) and then oven-dried at 40°C. The hydro-ethanolic extract (273.1 g) obtained gave an extraction yield of 6.82% which was then stored at −20°C and used for the study.

2.2. Phytochemical Screening. The extract was screened qualitatively for the identification of the different secondary metabolites in the hydro-ethanolic extract. The method described by Harbone [41] was used for the test of anthraquinones, saponins, sterols, terpenoids, steroids, flavonoids, coumarins, phenols, tannins, and glucosides. And for alkaloids, and anthocyanins, the Odebeyi and Sofowora method [42] was applied.

2.3. Composition of the Cafeteria Diet. Obesity was induced by a cafeteria diet composed according to the protocol of Darimont et al. [43]. The composition of the standard laboratory chow and the cafeteria (experimental) diet are presented in Table 1. These were the two types of diets used in this study: the normal and cafeteria diet.

2.4. Experimental Animals and Treatment. This study was carried out in line with the practice and principles of the institution on the use of experimental animals respecting the 2011 Guide for the Care and Use of Laboratory Animals, 8th edition, and the Animal Welfare Act. Thirty male Wistar Albino rats 6–7 weeks old and weighing between 123 g and 130 g obtained from the animal house of the Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé, Cameroon, were used for this study. The rats were housed in wire meshed (3 per cage) cages and maintained at 24 ± 2°C temperature and a cycle of 12 : 12 hours light/dark with free access to food and water at all time. After adaptation into the laboratory conditions for one week, the rats were randomly divided into 5 groups of 6 rats each (still maintained in 3 per cage) and fed for five weeks as follows:

(i) Group 1: Normal control (Nor.), administered standard diet

(ii) Group 2: Negative control (Neg.), administered cafeteria diet

(iii) Group 3: (Ator.): cafeteria diet + atorvastatin 10 mg/kg

(iv) Group 4: (E250): cafeteria diet + Hydro-ethanolic extract 250 mg/kg

(v) Group 5: (E500): cafeteria diet + Hydro-ethanolic extract 500 mg/kg

All animals were fed with cafeteria diet except the animals of group 1 which was fed with standard diet and the feeding lasted five weeks. Alongside feeding, the animals were administered distilled water (Nor. and Neg.), atorvastatin (Ator.), and plant extracts (E250 and E500) in a volume of 100 ml/kg by oral intubation.

2.5. Sacrifice of Rats and Samples Collection. At the end of the feeding period (5 weeks), the experimental animals were fasted for 16 hours then taken into a separate room where they were weighed and sacrifice under pentobarbital sodium anesthetized (humane pharmaceutical grade, 78 mg/kg of body weight administered intraperitonially) and blood collected by cardiac puncture in to heparinize tubes. In brief, when the animal was asleep after anaesthesia as determined by no withdrawal of limb with pinching and by no response to a penlight shone in the eye, the animal was fixed on a dissecting board and incised mid-ventrally. Then, the chest part was opened by cutting the diaphragm and 5 ml of blood removed from the left ventricle (close to the surface of the chest) by cardiac puncture with a 5 ml syringe (21 *G* needle). In cases where the animal's heart was still beating after cardiac puncture, extra anaesthesia was administered to sacrifice the animal. The blood collected was centrifuged at 3000 rpm for 15 min at 4°C to separate the plasma which was stored in aliquots at −20°C for further biochemical analysis. The liver, kidney, heart, aorta, visceral fat, subcutaneous fat, and epididymal fat were carefully collected, rinsed with 0.9% NaCl, dried, and immediately weighed. Then a portion of liver, kidney, and heart was excised, and 10% w/v homogenate was prepared in ice-cold Tris-HCl 50 mM solution, respectively, and centrifuged at 3000 rpm for 25 min at 4°C. The supernatant obtained was used for the estimation of lipid peroxidation (malondialdehyde) and antioxidant activity (catalase, superoxide dismutase, and glutathione).

2.6. Determination of Body Weight, Lee Index, and Metabolic Efficiency Index. During the period of treatment, body weight of each animal was measured once per week in order to follow the development of over weight of the animals. The Lee index was calculated using the final body weight and nasal-anal length as in the equation below (equation (1)) [44]. Rats with Lee index (LI) higher or equal to 300 (Li ≥ 300) were considered obese.

$$\mathrm{LI} = \left(\frac{\sqrt[3]{\text{body weight}}}{\text{nasal} - \text{anal length (cm)}} \right) \times 1000. \quad (1)$$

The metabolic efficiency (ME) is the measure of how the body utilizes fat as an energy source. That is the body's ability to conserve its fat reserves or to mobilize them in the form of

Table 1: Composition of different diets (g/g of ingredient).

Ingredients	Standard diet (g)	Energetic value (kcal)	Cafeteria diet (g)	Energetic value (kcal)
Corn flour	530	1852.35	278	971.61
Wheat flour	128	463.36	55	199.10
Cheese	—	—	110	249.70
Biscuit	—	—	110	498.30
Soy	310	130.51	160	67.36
Pork liver pate	—	—	110	262.72
Banana chips	—	—	50	265.40
Peanuts	—		50	294
Chocolate	—		50	275
Vitamin complex (multi vitamin plus)	20	—	20	—
Bone powder	10	—	5	—
Salt	2	—	2	—
Total		2446.22		3083.19

energy. This index was calculated as follows (equation (2)) [45]:

$$\mathrm{ME} = \frac{\text{body weight gain}}{\text{food intake}}. \quad (2)$$

2.7. Determination of Food and Water Consumption. The evaluation of the average of food and water intake per rat was recorded daily by subtracting the quantity of remaining food every day from the initial quantity provided the previous day.

2.8. Biochemical Parameters Analysis. Plasma concentration of transaminases, glucose, urea, creatinine, bilirubin, protein, triglycerides (TG), total cholesterol (TC), and HDL cholesterol (HDL-C) were all estimated through colorimetric methods with commercially available test kits according to the manufacturer's recommendations. The level of LDL-cholesterol (LDL-C) was calculated by subtracting the HDL cholesterol levels from the total cholesterol levels.

The atherogenic index (AI) (equation (3)) was calculated by using the method of Bais et al. [46].

$$\mathrm{AI} = \frac{[\mathrm{CT}] - [\mathrm{HDL}]}{[\mathrm{HDL}]}. \quad (3)$$

2.9. Antioxidant Parameters Analysis. Antioxidant analysis was performed on the homogenate of different organs: liver, kidneys, and heart. The supernatant was used for the estimation of malondialdehyde (MDA) according to the protocol described by Wilbur et al. [47]. Glutathione (GSH) was assessed using Ellman's method [48], superoxide dismutase (SOD) activity was determined using the method described by Misra and Fridovich [49], and catalase activity was assessed using the method describe by Sinha [50].

2.10. Histological Examination. The aorta collected from all animals was rinsed and fixed in 10% buffered formalin, embedded in paraffin, and cut into 5 μm sections that were stained with hematoxylin and eosin. These were observed under a microscope at 100x magnification. The histomorphometry of the aorta was evaluated with Image J software version 1.49.

2.11. Statistical Analysis. The data was analysed statistically using the GraphPad Prism 7.00 software. The results were expressed as mean ± SEM of 6 animals. The values were compared using the One-Way Analysis of Variances (ANOVA) test followed by the Tukey multiple comparison test. The differences were considered significant at $p < 0.05$.

3. Results

3.1. Phytochemical Screening of Hydro-Ethanolic Extract Piper nigrum Leaf. The results of the phytochemical screening of the hydro-ethanolic extract of the *Piper nigrum* leaf are summarized in Table 2. A plethora of secondary metabolites were identified, including gallic tannins, alkaloids, bound anthraquinones, phenolic compounds, flavonoids, and coumarins. The extract tested negative for terpenoids, anthocyanins, saponins, and glucosides.

3.2. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on Food and Water Consumption. Figure 1 presents the effects of the hydro-ethanolic extract of *Piper nigrum* leaf (E250 and E500 mg/kg) on food and water consumption of experimental animals. A significant decrease in food intake ($p < 0.001$) in the negative control group (untreated group) was observed compared to normal control group (Figure 1(a)). All groups of animals had an increase of water intake in the first week which slightly dropped in the second week. The pick of water consumption was attained at the third week for the normal and negative control animals and the atorvastatin (Ator.) administered group (Figure 1(b)). Amongst the plant extract treated groups, it was the E250 that had the lowest water consumption.

Each point represents the mean ± SEM, $n = 6$. $a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ significant differences from the normal control; $1 = p < 0.05$, $2 = p < 0.01$, $3 = p < 0.001$ significant differences from the negative control; Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E250 and E500: animals fed with

Table 2: Phytochemical screening of *Piper nigrum* hydro-ethanolic extract.

Compounds	Results
Gallic tannins	+
Alkaloids	+
Anthraquinones (bounds)	+
Phenolic compounds	+
Flavonoids	+
Coumarins	+
Terpenoids	-
Anthocyanins	-
Saponins	-
Glucosides	-

(+): present, (-): absent.

cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg.

3.3. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on Lee Index, Metabolic Efficiency Index, and Atherogenic Index. With respect to the different indexes, no significant difference was found between the normal and the negative control groups (Table 3). However, a significant decrease in the atherogenic index (AI) of 57.06% ($p < 0.01$) was observed in the E500 treated group when compared to the negative control. No significant difference in Lee's index was observed across groups. Animals in the negative control group had significantly high metabolic efficiency (ME) of 73.02% ($p < 0.001$) compared to the normal control group. This increase was significantly inhibited to 30.44% ($p < 0.01$) and 44.70 ($p < 0.001$) in groups treated with atorvastatin and E500, respectively.

Each value represents mean ± SEM, $n = 6$. $a = p < 0.05$, $c = p < 0.001$ significant differences from the normal control; $2 = p < 0.01$; $3 = p < 0.001$ significant differences from the negative control. Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg. LI : Lee index, ME : metabolic efficiency, AI : atherogenic index.

3.4. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on Body Weight Gain in Cafeteria Diet Fed Rats. Figure 2 shows the effects of the hydro-ethanolic extract of *Piper nigrum* leaf on the weight change of rats fed with a cafeteria diet during an experimental period of five weeks. Feeding with cafeteria diet induced a significant increase in body weight in the negative control group of up to 30% ($p < 0.05$) compared to the normal control group in the fourth week of feeding. No significant difference was noted between groups at week one. In the second week of treatment, E250, E500, and atorvastatin significantly inhibited the increase in body weight of 41.81% ($p < 0.001$), 34.84% ($p < 0.01$), and 29.86% ($p < 0$) 01), respectively. At the third and fourth week of treatment, E250 significantly ($p < 0.05$) inhibited the effect of cafeteria diet in body weight by 38.46% and 36.41% and E500 inhibited by 35.67% ($p < 0.05$) and 34.52%, respectively. At the 5th week of treatment, while the body weight of the different groups continues to increase at different rates, the E500 group showed a significant inhibition of 42% ($p < 0.001$) compared to the negative control. Overall, the 250 mg/kg and 500 mg/kg extract resulted in a greater inhibition in weight gain in rats throughout the experiment.

Each point represents the mean ± SEM, $n = 6$. $a = p < 0.05$, $c = p < 0.001$; significant differences from the normal control; $1 = p < 0.05$, $2 = p < 0.01$, $3 = p < 0.001$ significant differences from the negative control; Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E.250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg.

3.5. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on the Relative Weight of Fats and Organs. Figure 3(a) and 3(b) show the effects of the hydro-ethanolic extract of *Piper nigrum* leaf on the relative weight of fats and organs of experimental animals, respectively. A significant increase in the relative weight of subcutaneous (129.36%, $p < 0.001$), visceral (177.08%, $p < 0.001$), and epididymal adipose tissues (96.65%, $p < 0.01$) was observed in cafeteria diet fed rats (negative control). A significant decrease in the relative weight of visceral adipose tissue (VAT, 67.66%) and epididymal adipose tissue (EAT, 44.26%) was observed in the E250 group compared to the negative control. The E500 equally decreased the percentage of subcutaneous, VAT and EAT by 52.59% ($p < 0.01$), 61.15% ($p < 0.001$), and 45.90% ($p < 0.01$) compared to the negative control. Overall, the relative weight of adipose tissues of the animals treated with the doses of 250 and 500 mg/kg of the extract was comparable to those of the normal group.

Feeding cafeteria diet significantly increased the relative weight of the liver of 55.80% ($p < 0.001$) as indicated in the negative control group compared to the normal control group. *P nigrum* extract significantly decreased the relative weight of the liver of the order 20.43% ($p < 0.05$) and 39.18% ($p < 0.001$) in the groups treated with 250 mg/kg and 500 mg/kg, respectively. Similarly, a significant increase in the relative weight of the aorta in the negative control group (126.28%) was observed compared to the normal group. However, administered extracts significantly inhibited the increase in aorta weight to 54.29% ($p < 0.001$) and 55.80% ($p < 0.001$), respectively, in the group receiving the doses of 250 mg/kg and 500 mg/kg. No significant difference was found in the relative weights of kidney and heart of different groups of animals.

Each bar represents the mean ± SEM, $n = 6$. $a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ significant differences from the normal control; $1 = p < 0.05$, $2 = p < 0.01$, $3 = p < 0.001$ significant differences from the negative control; Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E.250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg. SCAT: subcutaneous adipose tissue; VAT: visceral adipose tissue; EAT: epididymal adipose tissue.

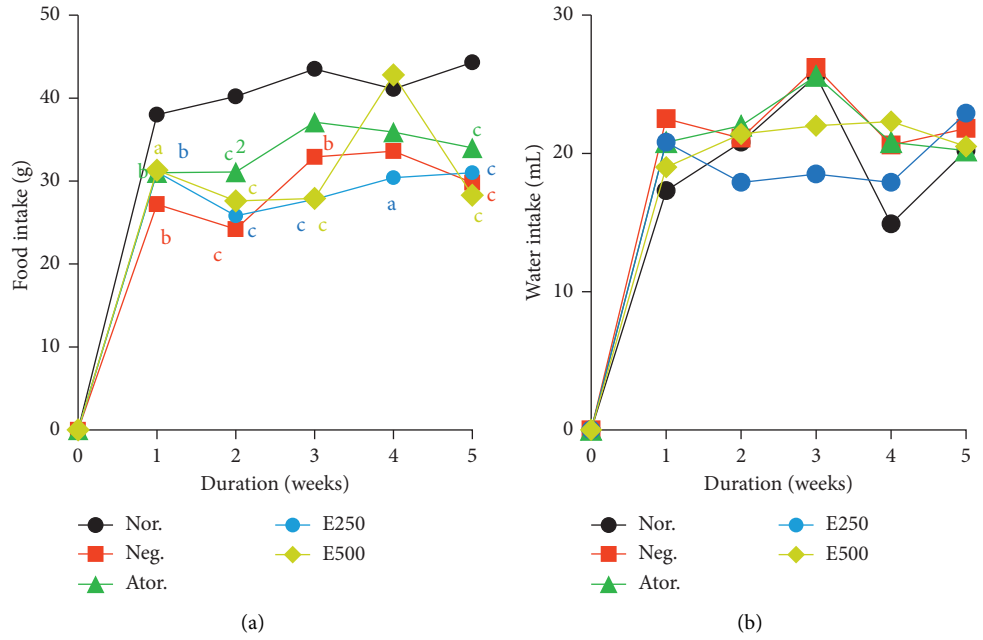

Figure 1: Effects of hydro-ethanolic extract of *Piper nigrum* leaf on food (a) and water (b) consumption.

Table 3: Effects of *Piper nigrum* on Lee index, metabolic efficiency index, and atherogenic index.

	Nor.	Neg.	Ator.	E250	E500
LI	0.32 ± 0.01	0.31 ± 0.007	0.31 ± 0.009	0.31 ± 0.008	0.30 ± 0.004
ME	0.786 ± 0.02	1.36 ± 0.04c	0.946 ± 0.012	1.09 ± 0.05	0.752 ± 0.033
AI	1.76 ± 0.12	1.90 ± 0.18	1.45 ± 0.26	1.56 ± 0.22	0.82 ± 0.01a2

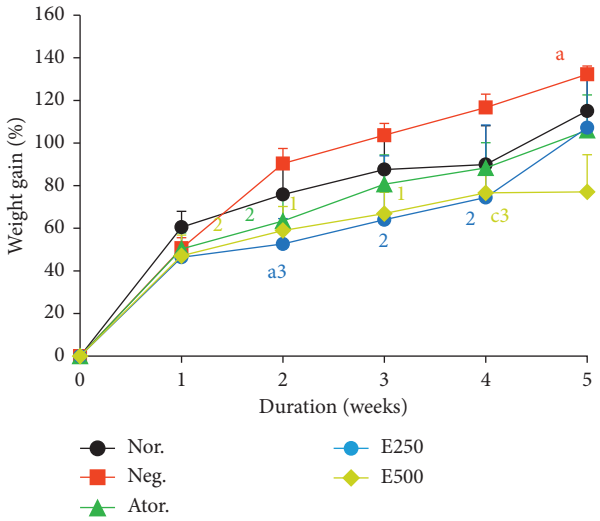

Figure 2: Effects of hydro-ethanolic extract of *Piper nigrum* leaf on body weight variation.

3.6. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on Plasma Lipid Profile. Results of the effects of hydro-ethanolic extract of *Piper nigrum* leaf on lipid profile parameters in cafeteria diet fed rats are presented in Table 4. The cafeteria diet significantly increased the triglyceride, total cholesterol, LDL-C, and HDL-C concentrations in the order of 50.44% ($p < 0.001$); 66.90% ($p < 0.001$), 111.39%, and 41.86% ($p < 0.05$), respectively. Treatments with Ator, E250, and E500 significantly decreased the triglyceride levels of 39.41% ($p < 0.001$), 35.29% ($p < 0.001$), and 47.52% ($p < 0.001$), respectively. Only the E250 and E500 significantly decreased the total cholesterol level by 15.12% ($p < 0.05$) and 33.61% ($p < 0.001$), respectively, compared to the negative control. A significant decrease in LDL-C of 63.01% ($p < 0.01$) was noted in the E500 group compared to the negative control.

Each value represents mean ± SEM, $n = 6$. $a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ significant differences from the normal control; $1 = p < 0.05$, $2 = p < 0.01$, $3 = p < 0.001$ significant differences from the negative control. Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E.250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg.

3.7. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on Plasma Blood Glucose Levels and of Hepatic and Renal Function. Table 5 presents the effects of hydro-ethanolic extract of *Piper nigrum* leaf on plasma blood glucose levels and some markers of hepatic (ALAT and ASAT) and renal (bilirubin, creatinine, and urea) function in cafeteria diet fed rats. Cafeteria diet significantly increased the transaminase activity (ALAT and ASAT), glucose, total protein, and bilirubin contents, respectively, of 64.51% ($p < 0.001$), 84.81%

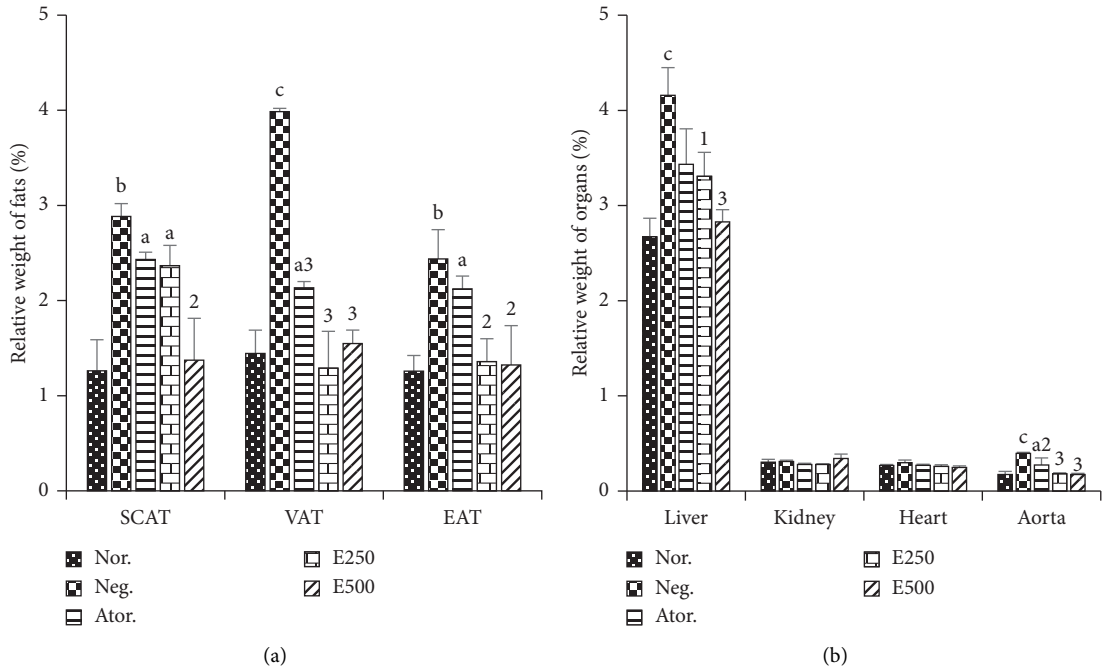

Figure 3: Effects of hydro-ethanolic extract of *Piper nigrum* leaf on adipose tissues and organs.

($p < 0.001$), 83.76% ($p < 0.001$), 97.35% ($p < 0.001$), and 20.68% ($p < 0.05$) with respect to the normal control.

Each value represents the mean SEM, $n = 6$. $a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ significant differences from the normal control; $1 = p < 0.05$, $2 = p < 0.01$, $3 = p < 0.001$ significant differences from the negative control. Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E.250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg.

Also, an increase in creatinine and urea concentration of 88.8% and 2.64% was observed in negative control group compared to the normal group. However, E250 significantly decreased ALAT and ASAT activity by 38.23% ($p < 0.001$) and 50.68% ($p < 0.05$), respectively, while E500 significantly decreased by 57.10% ($p < 0.001$) and 49.31% ($p < 0.001$). The E250 animals induced a significant inhibition in blood glucose levels of 16.74% ($p < 0.001$) compared to the negative control. The significant decreases in total protein levels of 57.86% ($p < 0.001$), 50.67% ($p < 0.001$), and 66.89% ($p < 0.001$) were observed in the E250 and E500 groups, respectively. Only the E500 animals showed a significant decrease in the level of bilirubin. Cafeteria diet significantly ($p < 0.001$) increased the concentration of creatinine as presented by the negative control. The effect of cafeteria diet on creatinine was significantly ($p < 0.001$) inhibited by the administration of *Piper* extract with E500 having the best effect. The effect of E500 was comparable to atorvastatin and the normal control.

3.8. Effects of Hydro-Ethanolic Extract of Piper nigrum Leaf on Lipid Peroxidation and Antioxidant Enzymes in Organs. Figure 4(a) reveals a significant decrease in catalase activity in the heart tissue of cafeteria diet fed rats (40.14%, $p < 0.001$) compared to the normal control group. When *Piper* extracts were administered, they effectively prevented the collapse in catalase activity in the heart tissue, thus boosting the antioxidant activity. However, no significant difference between groups was observed in the liver and kidney catalase activity.

Cafeteria diet induced a significant decrease in SOD activity of 60.67% ($p < 0.01$) in the kidney of the negative control group but did not have any effect on the liver and heart SOD (Figure 4(b)). The increase in SOD activity in the kidney was inhibited significantly by atorvastatin (159.74%, $p < 0.01$) and the E250 and E500. In the heart homogenate, a significant increase in SOD activity of 85.61% ($p < 0.05$) and 114.15% ($p < 0.01$) was observed in atorvastatin and E250 groups compared to the negative control. No significant differences were found in the liver of the negative control and the other treated groups.

With respect to GSH, a significant decrease of 85.94% ($p < 0.001$), 44% ($p < 0.01$), and 70.15% ($p < 0.001$) was noted, respectively, in the liver, kidney, and the heart of the negative control group compared to the normal control group (Figure 4(c)). Compared to the negative control group, a significant increase in GSH levels of 347.69% ($p < 0.001$) and 381.17% ($p < 0.001$) was noted, respectively, in the liver of atorvastatin and E250. Only the E500 group showed a significant increase in the level of GSH (163.67%, $p < 0.001$) in the kidney compared to the negative control. In the heart, the E250 and E500 groups showed an increase in GSH level of 112.28% ($p < 0.001$) and 111.69%. ($p < 0.001$).

Measurement of lipid peroxidation in animal organs was quantified by the level of MDA in homogenates. Figure 4(d) indicates a significant increase in MDA level in heart homogenates of 188.17% ($p < 0.001$) in the negative control

Table 4: Effects of hydro-ethanolic extract of *Piper nigrum* leaf on lipid profile.

	Nor.	Neg.	Ator.	E250	E500
TG (mg/dL)	113 ± 0.54	$170 \pm 6{,}02^{c}$	103 ± 0.76^{3}	110 ± 2.37^{3}	89.2 ± 2.9^{c3}
Cholesterol (mg/dL)	71.3 ± 4.79	106 ± 6.67^{c}	91.5 ± 4.46^{b}	101 ± 4.41^{b}	79 ± 3.42^{2}
HDL-C (mg/dL)	25.8 ± 1.09	36.6 ± 1.73^{a}	40.5 ± 3.42^{c}	40 ± 2.39^{b}	43.2 ± 2.07^{c}
LDL-C (mg/dL)	22.9 ± 4.07	35.4 ± 6.44	30.4 ± 6.89	39 ± 3.44	17.9 ± 1.9

Table 5: Effects of hydro-ethanolic extract of *Piper nigrum* leaf on markers of hepatic and renal function.

	Nor.	Neg.	Ator.	E250	E500
ALAT (U/I)	12.4 ± 0.49	20.4 ± 1.54^{c}	12.6 ± 0.70^{3}	12.5 ± 0.69^{3}	8.75 ± 1.47^{3}
ASAT (U/I)	23.7 ± 1.7	43.8 ± 3.09^{c}	30.3 ± 1.57^{1}	21.6 ± 2.63^{3}	22.2 ± 2.94^{3}
Glucose (mg/dl)	117 ± 1.18	215 ± 4.27^{c}	164 ± 2.49^{c3}	179 ± 6.30^{c3}	203 ± 5.71^{c}
Urea (mg/dl)	38.9 ± 0.60	39.8 ± 0.76	37.3 ± 0.40	36.8 ± 0.76	37.8 ± 0.76
Total protein (g/dl)	8.31 ± 0.20	16.4 ± 1.26^{c}	7.01 ± 0.75^{3}	6.91 ± 0.39^{3}	8.09 ± 0.50^{3}
Creatinine (mg/dl)	2.25 ± 0.47	4.25 ± 0.75^{c}	2.5 ± 0.28^{3}	3.0 ± 0.40^{b1}	2.67 ± 0.66^{3}
Bilirubin	14.5 ± 0.27	17.5 ± 1.46^{a}	17.5 ± 0.45^{a}	16.0 ± 0.46	13.6 ± 0.41^{2}

group compared to the normal control group. On the other hand, no significant difference was found in the homogenates of liver and kidneys of these two groups. Treatment with E500 induced a decrease in the level of MDA in the liver of 52.29% ($p < 0.01$) and in the kidney of 39.70% ($p < 0.01$) compared to the negative control group. In heart homogenates, a significant decrease in the MDA level of 62.73% ($p < 0.001$) and 74.87% ($p < 0.001$) was noted, respectively, in the groups treated with atorvastatin and with E250 and E500. The E250 extract had a better effect on restoring catalase and SOD activities than E500 in the heart tissues.

Each bar represents the mean ± SEM, $n = 6$. $a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ significant differences from the normal control; $1 = p < 0.05$, $2 = p < 0.01$, $3 = p < 0.001$ significant differences from the negative control. Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E.250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg.

3.9. Effects of the Hydro-Ethanolic Extract of Piper nigrum Leaf on the Architecture of the Aorta. Figure 5 shows the effects of the hydro-ethanolic extract of *Piper nigrum* leaf on the architecture of the aorta. The consumption of a cafeteria diet induced a significant thickening of the media and the structural disorganization of the intima in negative control group compared to normal control group (Figure 5(a)). The E250 and E500 groups showed a significant decrease in the thickness of the media. All groups exhibited a structural rearrangement of the architecture of the aorta which was comparable to the normal control group. The histomorphometry data of the aorta under Image J software presented in histograms (Figure 5(b)) are the extrapolated thickness of the aorta. The aorta of the negative control rats had the highest accumulation of fats measuring up to $46.78 \pm 4.84\,\mu$m compared to normal ($27.37 \pm 2.98\,\mu$m). Atorvastatin reduced the thickening of the aorta to $35.38 \pm 2.67\,\mu$m while the *Piper* extracts reduced the thickening to $33.66 \pm 4.70\,\mu$m (E250) and $22.36 \pm 1.14\,\mu$m (E500). The effect of the plant extract was dose-related and comparable to that of atorvastatin.

Each bar represents the mean ± SEM, $n = 6$. $a = p < 0.05$, $b = p < 0.01$, $c = p < 0.001$ significant differences from the normal control; $1 = p < 0.05$, $3 = p < 0.001$ significant differences from the negative control. Nor.: normal control; Neg.: negative control; Ator.: atorvastatin (10 mg/kg); E.250 and E500: animals fed with cafeteria diet and treated with hydro-ethanolic extract of *Piper nigrum* leaf at doses of 250 and 500 mg/kg.

4. Discussion

The present study reported cafeteria diet-induced weight gain leading to obesity in negative control group compared to normal control group but with a reduction in food intake similar to earlier report by Bais et al. [46]. This justifies that weight gain does not necessarily depend on the amount of food consumed but more on the caloric intake as provided in the cafeteria diet whose consumption led to an increase in energy reserves and an increased weight gain [25, 43]. With cafeteria diet life style being embraced in modern times where people do not have time for a regular and/or organic food, this study provides a possible outcome in its consumption, and the importance of spices such as *P. nigrum* if added to a meal.

The overall increased relative weight of adipose tissue and organs, and the concentrations of total cholesterol, LDL-C, HDL-C, and triglyceride in cafeteria diet fed rats corroborated the results of Cabot et al. [51] and Meryem et al. [52]. This may be as a result of an excessive accumulation of fats in the tissues, linked to a very high bioavailability of lipids and the need for the body to store them in one way or another, without taking into account the lipotoxic effects generated by this excessive storage [51]. The dyslipidemia observed was directly related to the accumulation of fat in adipose tissue including visceral adipose tissue. According to Grundy et al. [53], the accumulation of fat promotes the acceleration of the flow of free fatty acids from the portal vein to the liver, which contributes to the hypersecretion of

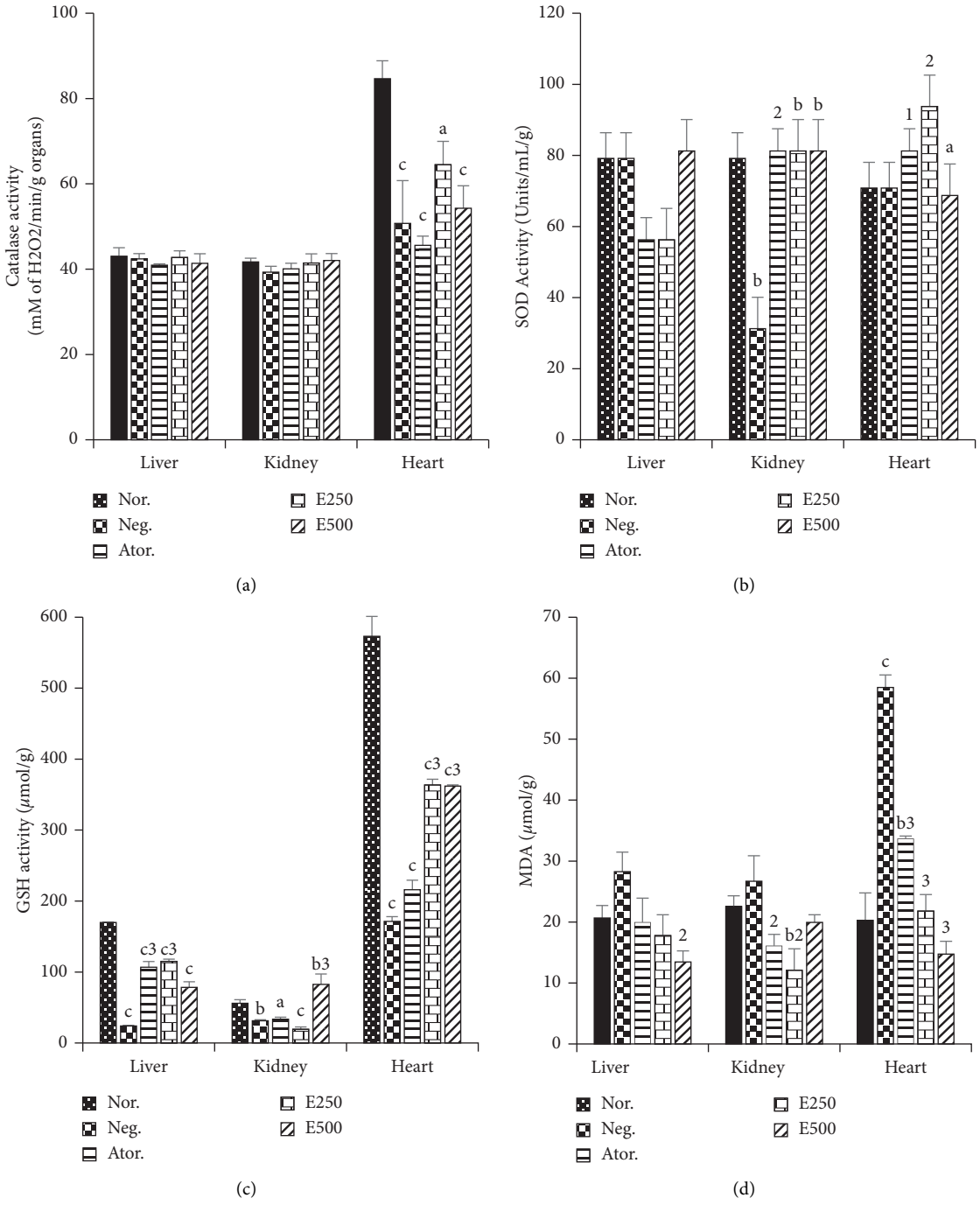

Figure 4: Effects of hydro-ethanolic extract of *Piper nigrum* leaf on some parameters of oxidative stress.

VLDL particles (very-low-density lipoproteins) accounting for the high plasma triglycerides concentrations observed in this study. The elevation of plasma triglyceride levels is generally accompanied by numerous disturbances in lipid metabolism. This is the case of the increased expression of the hydroxy-methyl-glutaryl Coenzyme-A (HMG-CoA) enzyme, increased LDL-cholesterol particle size, decreased HDL-C, increased atherogenic risk, and inhibited lipoprotein lipase activity [52, 54, 55]. The increase of HDL-C in negative control in the present study could be explained by the presence of phytoestrogens contained in soy (included in the diet) that promote the synthesis of HDL-C in the liver [56]. This effect of phytoestrogens varies from subject to subject and would be greater in individuals with excess cholesterol in the body [57].

Rats treated the with extract of *Piper nigrum* and atorvastatin presented an overall protective effect in weight gain and amelioration of lipid profile in cafeteria diet intoxication. The similarity in dietary intake between the negative control group and the groups treated with different doses of plant extract suggests that the extract had no anorectic effects that could justify the low rate of weight gain

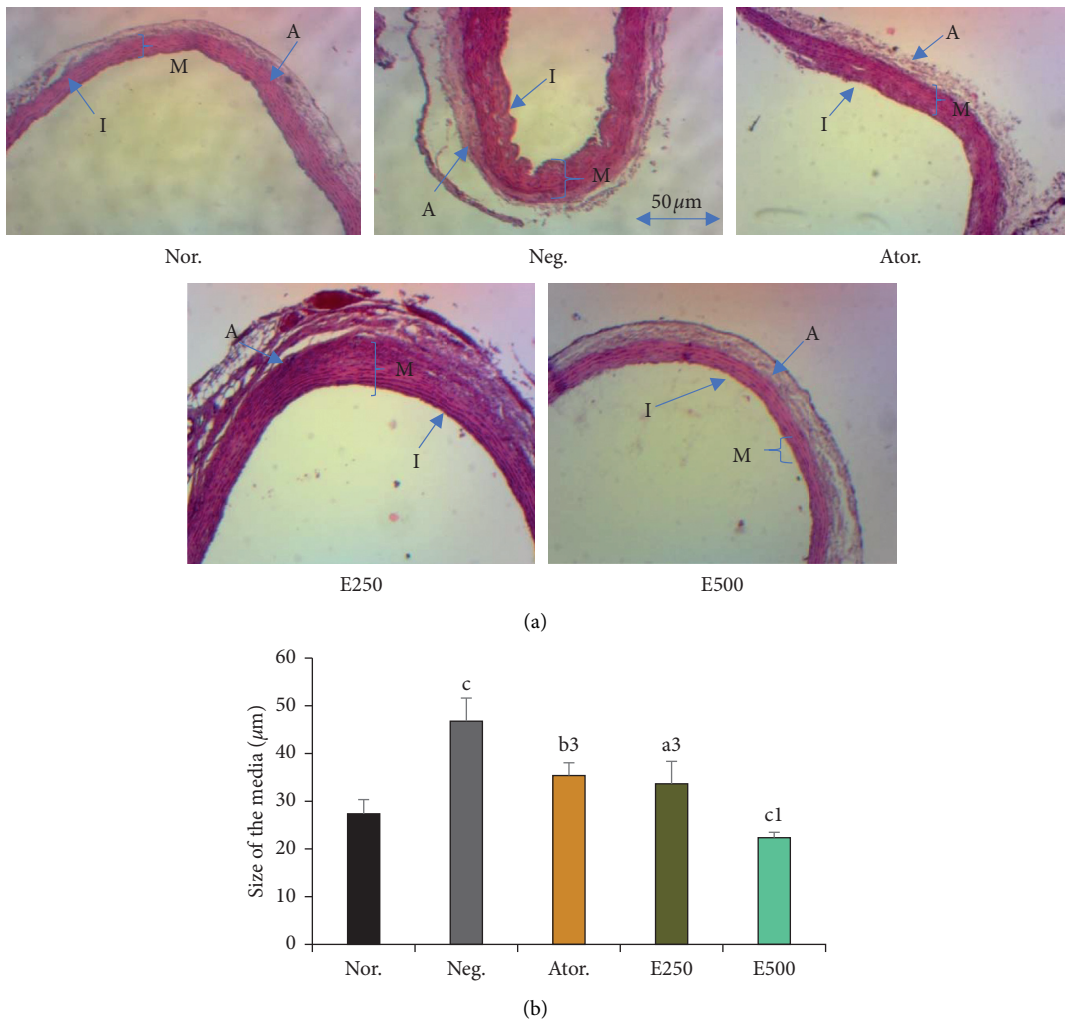

Figure 5: Effects of hydro-ethanolic extract of *P. nigrum* leaf on the architecture of aorta. (a) Hematoxylin-eosin 40X. (b) Histomorphometry data of the aorta under Image J software version 1.49.

observed. The inhibition of weight gain may be attributed to the thermogenic effects of the plant and its action on the metabolism of fats. Indeed, *Piper nigrum* contains a plethora of secondary metabolites involved in many biochemical processes. This is the case of piperine, a potent alkaloid that inhibits lipid accumulation by stimulating metabolic enzymes such as lipoprotein lipase and adenosine monophosphate activated protein kinase (AMPK) that promote the use of lipids by peripheral tissues [30, 58]. It also contains phenolic compounds and flavonoids which would be involved in thermogenesis and decrease the expression of genes involved in weight gain and differentiation of preadipocytes into adipocytes [59]. In an earlier study, Ibrahim et al. [60] reported a higher total flavonoid, total phenolic, gallic acid, and rutin content in the leaves of *P. nigrum* compared to the seeds which may be responsible for the biological effect of the plant. Hence, consumption of the *Piper nigrum* leaf may be more beneficial to health than the seeds.

With regard to the different indexes, an increase in the atherogenic index and the metabolic efficiency was noted in the negative control group compared to the normal control group related to the development of obesity in these animals. According to Lossa et al. [61] and Bais et al. [46], this increase is linked to the consumption of the cafeteria diet similar to most high-calorie diets linked to an increase in metabolic efficiency and atherogenic risk. The animals treated with the different doses of the extract and atorvastatin showed a decrease in these different indexes compared to the negative control. The decrease in metabolic efficiency and the atherogenic index could be justified by an increase in energy expenditure [45, 62] and by the cardioprotective effects of the plant [63].

The blood glucose concentration and markers of renal and hepatic functions presented an increase in transaminases, glucose, urea, creatinine, bilirubin, and total proteins in cafeteria fed animals (negative control) compared to normal control. Earlier researchers have equally reported the effect of cafeteria diet on hyperglycaemia associated with insulin resistance or glucose intolerance [64], liver damage indicative of increased transaminases and bilirubin levels [46]. Equally, renal damage that results in increased levels of

urea and creatinine and inflammatory phenomena has been reported in cafeteria diet intoxicated experimental animals [65, 66]. *Piper nigrum* extract and atorvastatin were able to prevent modification of all these parameters and maintained them towards normal. Hence, properties which are indicative of the hepatoprotective and nephroprotective activity of the plant related to bioactive compounds that possess antioxidant properties [67, 68]. Mir et al. [59] also linked the decrease in glucose concentration to flavonoids which possess strong antioxidant activities. However, the decrease in glucose concentration was not dose dependent as the E250 presented a better protective effect than E500. The high blood glucose concentration in E500 compared to E250 might have resulted from the effect of a by-product that interfered with its antihyperglycemic activity. The effect of *P nigrum* on glucose metabolism may be an important tool in the management of hyperglycaemia resulting from overweight and obesity.

Superoxide dismutase protects the tissues against reactive oxygen species by scavenging superoxide radical and producing hydrogen peroxide which is the substrate of catalase. Catalase then reduces hydrogen peroxide to oxygen and water. Reduced glutathione acts as a substrate of glutathione peroxidase and Glutathione-S-Transferases and yields water molecule and other organic hydroperoxides. Obesity have been reported to induce a collapse of the antioxidant defense mechanism characterized by diminishing activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) resulting in an accumulation of lipid peroxides and reactive oxygen species. [13]. Cafeteria diet consumption has been associated with obesity and oxidative stress in experimental animal by alteration in the catalase, SOD, glutathione, and MDA levels [30, 67]. Also, obesity has been reported to induce systemic oxidative stress, resulting in a collapse in the antioxidant defense system characterized by decreased activity of catalase, superoxide dismutase, reduced glutathione levels, and an increase in the MDA marker of lipid peroxidation [69]. In the present study, though the cafeteria diet significantly altered the antioxidant defense mechanism, it did not have a significant effect on liver and kidney CAT activity. However, the plant extract improved the antioxidant defense system by an increase in the antioxidant enzymes activity and a decrease in MDA accumulation at E250 and E500 dose levels. However, the E250 had a better effect of catalase and SOD activities in the heart tissue. The non-significant changes in CAT activity in liver and kidney tissues of obese rat may be attributed to increase in oxygen consumption by the adipose tissues and the change of CAT enzyme activity is dependent on oxygen consumption. Meanwhile, the heart tissue uses a large amount of fatty acids and glucose as energetic substrate and the final oxidation of these fuels occurs in the mitochondria by aerobic mechanisms, which justify the heart tissue susceptibility to oxidative stress. In a similar way, sixteen weeks fed high fat diet had no significant effect on liver and heart CAT activity in experimental rats [70]. The unexpected high SOD activity in the liver and the heart tissue may present false positive results considering the effect of cafeteria diet on the atorvastatin and the extract treated groups.

Histopathological analysis of the architecture of the aortic revealed a thickening of the media and structural disorganization of the intima in negative control animals following consumption of a cafeteria diet. Indeed, the hypercaloric diet induces dyslipidemia which later caused an accumulation of lipids mainly by the action of LDL-C in the arteries. These factors in turn lead to structural alteration of the vessel and ultimately to the formation of atheromatous plaques [71]. The extract at different doses prevented the thickening of the media and induced a structural rearrangement of the intima. It could be justified by the antioxidant properties of the plant that would limit the oxidation of LDL-cholesterol and the progression of atherosclerosis closely related to cardiovascular complications [32, 72].

5. Conclusion

This study was designed to evaluate the preventive effect of hydro-ethanol extract of *Piper nigrum* leaf on cafeteria diet-induced obesity in rats. The cafeteria diet consumed by animals during the experimental period led to an increase in body weight and relative weight gain of the organs, dyslipidemia with an increased risk of atherogenesis, liver and kidney damage, and oxidative stress. Hence, such dietary lifestyle may be detrimental to human health. Co-administration with *Piper nigrum* leaf extract inhibited the effect of the cafeteria diet which justifies the health benefits of *Piper nigrum*. This may be important in the human physiology when overwhelmed with oxidative stress.

Abbreviations

AI:	Atherogenic index
ALAT:	Alanine aminotransferase
AMPK:	Adenosine monophosphate activated protein kinase
ANOVA:	Analysis of variance
ASAT:	Aspartate aminotransferase
Ator.:	Atorvastatin
EAT:	Epididymal adipose tissue
Eq:	Equation
g:	Gram
GSH:	Glutathione
HCl:	Hydrochloric acid
HDL:	High-density lipoprotein
HMG-CoA:	3-hydroxy-3-methylglutaryl-Co-enzyme A
IMPM:	Institute of Medical Research and Medicinal Plants Studies
kg:	Kilogram
LDL:	Low-density lipoprotein
LI:	Lee index
MDA:	Malondialdehyde
ME:	Metabolic efficiency index
mg/dL:	Milligrams per decilitre
mg/kg:	Milligrams per kilogram

NaCl: Sodium chloride
Neg.: Negative control
Nor.: Normal control
P. nigrum: *Piper nigrum*
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
SCAT: Subcutaneous adipose tissue
SEM: Standard error of mean
SOD: Superoxide dismutase
TC: Total cholesterol
TG: Triglyceride
VAT: Visceral adipose tissue
VLDL: Very-low-density lipoprotein
WHO: World Health Organization.

Data Availability

The datasets used and analysed during the study are included in the manuscript without any restriction.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

DLEM followed up the animal feeding and participated in laboratory analysis, present at every step of the project. YSAF participated in the laboratory analysis, prepared the first draft of the manuscript, and participated in the revision and formatting of the final version. ADT participated in laboratory analysis and discussed laboratory data. JRM was in charge of the animal house and followed up the feeding protocol and animal welfare. LRYT participated in the laboratory analysis. ElMB carried out phytochemical screening. ATT analysed and discussed all data derived from extraction and phytochemical screening. DD participated in setting the experimental designs and supervised the histopathological examinations. GAA conceived and designed the project, participated in data analysis and discussion, and revised the final form of the manuscript. All authors read and approved the final version.

Acknowledgments

This study was supported by The World Academy of Science (TWAS) Grant number: RGA No. 16-493 RG/CHE/AF/AC_G – FR3240293297 dated 12 December 2016. The funding provided students support, equipment, and reagents used for this study.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 5531367, 10 pages
https://doi.org/10.1155/2021/5531367

Research Article

The Immunomodulating Effect of Baicalin on Inflammation and Insulin Resistance in High-Fat-Diet-Induced Obese Mice

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Received 15 February 2021; Revised 20 April 2021; Accepted 20 May 2021; Published 27 May 2021

Academic Editor: Reza B. Jalili

Background. Obesity is a chronic low-grade systemic inflammation state, which causes insulin resistance, diabetes, and other metabolic diseases. Baicalin is known to have anti-inflammatory and antiobesity effects. In this study, we investigated the cellular and molecular immunological effects of baicalin on obesity-induced inflammation. *Methods*. Male C57BL/6 mice were assigned to four groups: the normal chow, high-fat diet (HFD), BC2 (HFD + baicalin 200 mg/kg), and BC4 (HFD + baicalin 400 mg/kg) group; the three groups except normal chow were fed with a high-fat diet for 8 weeks to induce obesity followed by baicalin treatment with two doses for 8 weeks. The body weight, epididymal fat weight, liver weight, food intake, oral glucose tolerance test (OGTT), oral fat tolerance test (OFTT), and serum lipids were measured. We evaluated insulin resistance by measuring the serum insulin level and homeostatic model assessment of insulin resistance (HOMA-IR). Also, the major obesity-associated immune cells including monocytes, macrophages, T lymphocytes, and dendritic cells in the blood, fat, and liver and the inflammatory and insulin signaling-related gene expressions in the fat and liver were evaluated. *Results*. Baicalin significantly reduced the body weight and liver weight and improved serum fasting glucose, insulin, HOMA-IR, free fatty acid, HDL cholesterol, and the levels of glucose and triglyceride at each time point in the OGTT and OFTT. In the analysis of immune cells, baicalin significantly decreased inflammatory Ly6C^{hi} monocytes, M1 adipose tissue macrophages (ATMs), and M1 Kupffer cells. On the contrary, baicalin increased anti-inflammatory M2 ATMs and liver CD4+ T cells and CD4/CD8 ratio. In the analysis of inflammatory and insulin signaling molecules, baicalin significantly downregulated the gene expression of tumor necrosis factor-α, F4/80, and C-C motif chemokine 2 while upregulated the insulin receptor mRNA expression. *Conclusion*. From these results, baicalin can be a promising treatment option for obesity and its related metabolic diseases based on its anti-inflammatory property.

1. Introduction

It is now generally accepted that obesity causes chronic low-grade inflammation throughout the body [1]. Local inflammation, especially in the excessive fat-deposited liver and adipose tissues, mediates systemic low-grade inflammation and results in obesity-induced insulin resistance (IR) [2, 3]. Almost immune cells including monocytes, macrophages, lymphocytes, and dendritic cells (DCs) are involved in obesity-induced inflammation, among which the number and activation level of macrophages is particularly increased [4]. In an obese state, adipose tissue macrophages (ATMs) and Kupffer cells (KCs) highly infiltrate into tissues and switch from M2 to M1 polarization [3, 5]. In addition, the imbalance between subtypes of T cells occurs, and it is involved in the recruitment and differentiation of macrophages [6, 7]. Also, the proinflammatory cytokines such as tumor necrosis factor (TNF)-α and C-C motif chemokine 2 (Ccl2) accelerate inflammatory response and damage insulin-signaling networks [3, 5]. Therefore, modifying the immune cell response may ameliorate obesity-induced inflammation and insulin resistance [8].

Baicalin is a bioactive flavonoid from *Scutellaria baicalensis*, which has beneficial effects on hyperglycemia and hyperlipidemia and is also linked to anti-inflammatory, antidiabetic, and antiobesity effects *in vivo* and *in vitro* [9–12]. In terms of the anti-inflammatory effects, baicalin has been reported to limit M1 polarization and promote M2

polarization *in vitro*, but most previous studies were related to inflammation caused by infection or limited to specific cytokines [13–15]. As the effects on obesity-induced sterile inflammation in a systemic manner have not been studied, it is difficult to gain the overall insight of how baicalin affects the immune system of the obese state [10, 16–18].

In this study, we examined the metabolic effects of baicalin on glucose and lipid metabolism and investigated its immunological mechanism in the blood, fat, and liver at the cellular and molecular levels.

2. Materials and Methods

2.1. Animal Models and Experimental Design. Six-week-old male C57BL/6 mice (Central Experiment Animals, Korea) weighing 19–21 g were used. Mice were kept in an animal room under the standard 12-hour light and dark cycle with 40–70% humidity and allowed *ad libitum* access to food and water. After adaptation for a week, the mice were divided into four groups of six mice per group: the normal group (NC group), HFD group, BC2 group, and BC4 group. Body weight of each mouse was recorded weekly before the morning feeding from baseline to the last sampling using an electronic scale (CAS 2.5D, Korea). Before and after the morning feeding, food was weighed to record the amount of food consumed. To compare the obese with the lean, the mice were assigned to two groups and fed the normal chow or high-fat diet containing 60% fat (HFD; Research Diets) for 8 weeks. In the following 8 weeks, the HFD-fed mice except normal chow were divided into 3 groups and additionally fed normal saline, 200 mg/kg/day or 400 mg/kg/day baicalin daily using Zonde. Baicalin was purchased from abcr GmbH (Karlsruhe, Germany). Baicalin with a purity higher than 98% was used after analysis by nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), and mass spectrometry (MS). All animal procedures were approved by the Kyung Hee Medical Animal Research Ethics Committee (KHMC-IACUC 19-009).

2.2. Oral Glucose Tolerance Test (OGTT) and Oral Fat Tolerance Test (OFTT). Fasting blood glucose (FBG) was measured from the tail veins of mice using a strip-operated blood glucose sensor (ACCU-CHEK Performa, Australia). For the OGTT, blood glucose was measured in the same manner at 30, 60, and 120 minutes after oral administration of glucose (2 g/kg body weight). After overnight fasting, triglyceride (TG) concentration was measured from a blood sample from the tail vein and the mice were gavaged with olive oil (Sigma, USA) by 2 mL/kg body weight. For the OFTT, TG measurements were taken at 120, 240, and 360 minutes using the Accutrend Plus (Roche, USA) point-of-care device and Accutrend triglyceride strips by using the Triglyceride Colorimetric Assay Kit (Cayman, USA) [19].

2.3. Assessment of Insulin Resistance by Measuring Insulin Level and HOMA-IR. We extracted blood from the tail vein of mice and collected them in BD Microtainer serum separator tubes. The blood samples were centrifuged at 2,000 G for 20 minutes to obtain serum. The serum insulin level was measured by using the ultrasensitive mouse insulin ELISA kit (Crystal Chem Inc., USA). The samples and insulin standards were plated into a 96-well antibody-coated microplate by 5 μL each and incubated for 2 hours at 4°C. After 5 times of washing, anti-insulin enzyme conjugate was combined into each well for 30 minutes at room temperature. After 7 times of washing, enzyme substrate solution was added and incubated for 40 minutes. Then, we added reaction stop solution to each well and waited for 10 minutes. Finally, we analyzed the microplate by using the ELISA reader at 450 nm. HOMA-IR was calculated by the following formula: fasting blood glucose (mg/dℓ) × fasting blood insulin (ng/mL) × 0.0717225161669606 [19].

2.4. Lipid Profile and Hepatic and Renal Function Safety Test. The mice were sacrificed under anesthesia with ether, and the blood samples were collected from the heart before the sacrifice. From the blood samples collected from the heart, nonesterified fatty acid (NEFA), TG, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), phospholipid, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and creatinine levels were estimated. After centrifuging the blood at 3,000 rpm for 20 minutes, the supernatant was stored at −40°C. The serum lipids and other biochemical parameters were analyzed by using the ELISA kit (MyBioSource, USA).

2.5. Isolation of Stromal Vascular Cells (SVCs) and Liver Immune Cells. The epididymal fat pads were placed in a solution of phosphate-buffered saline (PBS; Gibco, USA) and 2% bovine serum albumin (BSA; Gibco, USA) and cut into discs with a diameter of 1–2 mm size using a pair of round scissors. Tissues were mixed with collagenase (Sigma, USA) and DNase I (Roche, USA) and shaken for 20–25 minutes at 37°C. Nondigested adipose tissue was removed by filtering the mixture through a 100 μm filter (BD Biosciences, USA) after mixing with 2% BSA/PBS and 5 mM EDTA. After centrifuging the sample at 1,000 rpm for 3 minutes, the supernatant was discarded and the pellet was mixed with PBS and 2% fetal bovine serum (FBS; Sigma, USA). SVCs were obtained by refiltering the mixture through a 100 μm cell strainer followed by centrifugation at 200 rpm for 10 minutes to pellet the SVCs. Liver tissue was prepared after PBS (pH 7.0) perfusion by needle insertion into the portal vein. Samples without gall bladder tissue were mixed with RPMI 1640 medium containing 100 mL/L fetal calf serum (FCS), squashed in a 60 mm Petri dish, and filtered through 200G stainless mesh. After adding 8 mL Percoll (final 36.3%), 9 mL PBS, and 200 μL heparin, samples were centrifuged at 2,000 rpm for 20 minutes. After pouring off the supernatant containing parenchymal cells and washing with PBS, the samples were incubated in 1X ACK lysis buffer (Lonza) at room temperature for 10 minutes to dissolve red blood cells. After centrifugation at 1,500 rpm for 5 minutes with PBS, unnecessary tissue was removed using a

100 μm cell strainer. Finally, nonparenchymal cells, including immune cells, were pelletized by recentrifugation at 1,500 rpm for 5 minutes [19].

2.6. Fluorescence-Activated Cell Sorting (FACS) Analysis of ATMs, KCs, T Lymphocytes, DCs, and Monocytes. The cell number in each EDTA sample was adjusted to 105 after counting using a Cellometer (Nexcelom Bioscience LLC, USA). After FcBlock (BD Pharmingen, USA) was added at a ratio of 1 : 100, samples were incubated for 10 minutes. Then, samples were mixed and incubated with fluorophore-conjugated antibodies for 20 minutes in the dark. To analyze ATMs, CD45-APC Cy7 (BioLegend, USA), CD68-APC (BioLegend, USA), CD11c-phycoerythrin (CD11b-PE; BioLegend, USA), and CD206-FITC (BioLegend, USA) were used. To analyze KCs, CD45-FITC, F4/80-APC, CD11c-phycoerythrin (CD11b-PE; BioLegend, USA), and CD206-FITC (BioLegend, USA) were used. To analyze T cell population, CD45-FITC (BioLegend, USA), CD3-APC (BioLegend, USA), CD4-PerCp CY5.5 (BioLegend, USA), and CD8-phycoerythrin (BioLegend, USA) were used. Finally, to analyze monocytes, CD45-FITC, CD11b-PerCp CY5.5, and Ly6C-APC (BioLegend, USA) were used. After washing and centrifugation at 1,500 rpm, cells were analyzed using an FACSCalibur (BD Bioscience, USA) instrument and the percentages of the various cell types were determined using the FlowJo program (Tree Star, Inc., USA).

2.7. RNA Extraction and Real-Time PCR. The epididymal fat pads and liver were dissected from the mice. Samples were wrapped in aluminum foil, placed in liquid nitrogen, and defrosted at −70°C. They were pulverized with 300 μL of ZR RNA buffer and then centrifuged at 1,000 rpm. The supernatant was transferred to Zymo-Spin III columns that were placed in 2 mL collection tubes, and two washes with 350 μL of RNA wash were performed. After samples were centrifuged at 1,000 rpm with elution buffer, the eluted RNA was stored at −70°C until analysis. Gene expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). cDNA was synthesized from 1 mg RNA using the Advantage RT-for-PCR Kit (Clontech, USA) with 10 nM dNTPs, recombinant RNase inhibitor, MMLV reverse transcriptase, and 5x reaction buffer. After reaction at 42°C for 60 minutes and 94°C for 5 minutes, qRT-PCR was performed in a reaction mixture comprising 2x SYBR reaction buffer, primers, and distilled H_2O using a 7900HT Fast Real-Time PCR System (Applied Biosystems®, Waltham, MA, USA) [19]. Sequences of the primers used for amplification are as follows: TNF-α, 5′-TTCTG TCTAC TGAAC TTCGG GGTGA TCGGT CC-3′ and 5′-GTATG AGATA GCAAA TCGGC TGACG GTGTG GG-3′; F4/80, 5′-CTTTGGCTATGGGCTTCCAGTC-3′ and 5′-GCAAG-GAGGACAGAGTTTATCGTG-3′; Ccl2, 5′-AGGTCCCT GTCATGCTTCTGG-3′ and 5′-CTGCTGCTGGTGATCCT CTTG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene), 5′-AGTCCATGCCAT-CACTGCCACC-3′ and 5′-CCAGTGAGCTTCCCGTT-CAGC-3′. The threshold cycle for each gene was converted to a relative quantitation measure based on the expression of EF-1α using SDS Software 2.4 (Applied Biosystems®, Waltham, MA, USA). Fold-change values were standardized based on the expression in the NC group as 1.

2.8. Statistical Analysis. Statistical analyses were performed with GraphPad PRISM 5 (GraphPad Software Inc., San Diego, USA). Groups were analyzed by one-way analysis of variance (ANOVA) and Tukey's post hoc test. Results are denoted by means ± standard error of the mean (SEM); two-tailed p values of <0.05 were considered significant. Significant differences from the HFD group are represented by asterisks: * for $p < 0.05$; ** for $p < 0.01$; and *** for $p < 0.001$.

3. Results

3.1. Effects of Baicalin on Body Weight, Epididymal Fat Pads, and Liver Tissue. Mice in the HFD group consumed more daily food intake than those in the NC group (15.42 ± 2.17 g vs. 9.49 ± 0.86 g, $p < 0.01$) and became obese by HFD for 8 weeks (Figures 1(a) and 1(b)). However, comparing the BC2 group with the HFD group, the mean body weight was significantly decreased by baicalin (39.34 ± 2.59 g vs. 45.53 ± 1.26 g, $p < 0.05$) (Figure 1(a)). The epididymal fat weights of the BC2 and BC4 groups were decreased compared with that of the HFD group (Figure 1(c)). The liver weights of the BC2 and BC4 groups were significantly decreased by baicalin compared with the HFD group (1.05 ± 0.10 g in the BC2 group vs. 1.44 ± 0.09 g in the HFD group, $p < 0.01$; 1.12 ± 0.10 g in the BC4 group vs. 1.44 ± 0.09 g in the HFD group, $p < 0.05$) (Figure 1(d)).

3.2. Effects of Baicalin on Glucose and Lipid Profiles. The BC2 group showed a significantly lowered FBG level compared with the HFD group (150.6 ± 8.44 mg/dL vs. 177.3 ± 9.12 mg/dL, $p < 0.05$) (Figure 1(e)). The FBC level of the BC4 group was not significantly different from that of the HFD group. To demonstrate the effects of baicalin in glucose metabolism, we performed the OGTT. Comparing BC2 and BC4 groups with the HFD group, the glucose levels after 30 min and 60 min were significantly decreased by baicalin (30 min: 301 ± 10.50 mg/dL in the BC2 group vs. 351 ± 17.34 mg/dL in the HFD group, $p < 0.05$; 309.6 ± 7.74 mg/dL in the BC4 group vs. 351 ± 17.34 mg/dL in the HFD group, $p < 0.05$; 60 min: 234.8 ± 26.96 mg/dL in the BC2 group vs. 277 ± 7.69 mg/dL in the HFD group, $p < 0.05$; 195 ± 11.60 mg/dL in the BC4 group vs. 277 ± 7.69 mg/dL in the HFD group, $p < 0.001$) (Figure 1(e)). The BC4 group had significantly decreased the area under curve (AUC) compared with the HFD group (839.0 ± 25.28 vs. 1004.5 ± 40.05, $p < 0.001$) (Figure 1(f)).

To show the effect of baicalin on lipid metabolism, we analyzed NEFA, TG, TC, LDL-C, HDL-C, and phospholipid levels, which increased due to obesity. Mice in the BC2 group had significantly lower levels of NEFA, TG, and HDL-C compared with the HFD group (NEFA: 2.59 ± 0.11 mEq/L vs. 3.05 ± 0.17 mEq/L, respectively, $p < 0.05$; TG: 35.2 ± 4.03 mg/dL

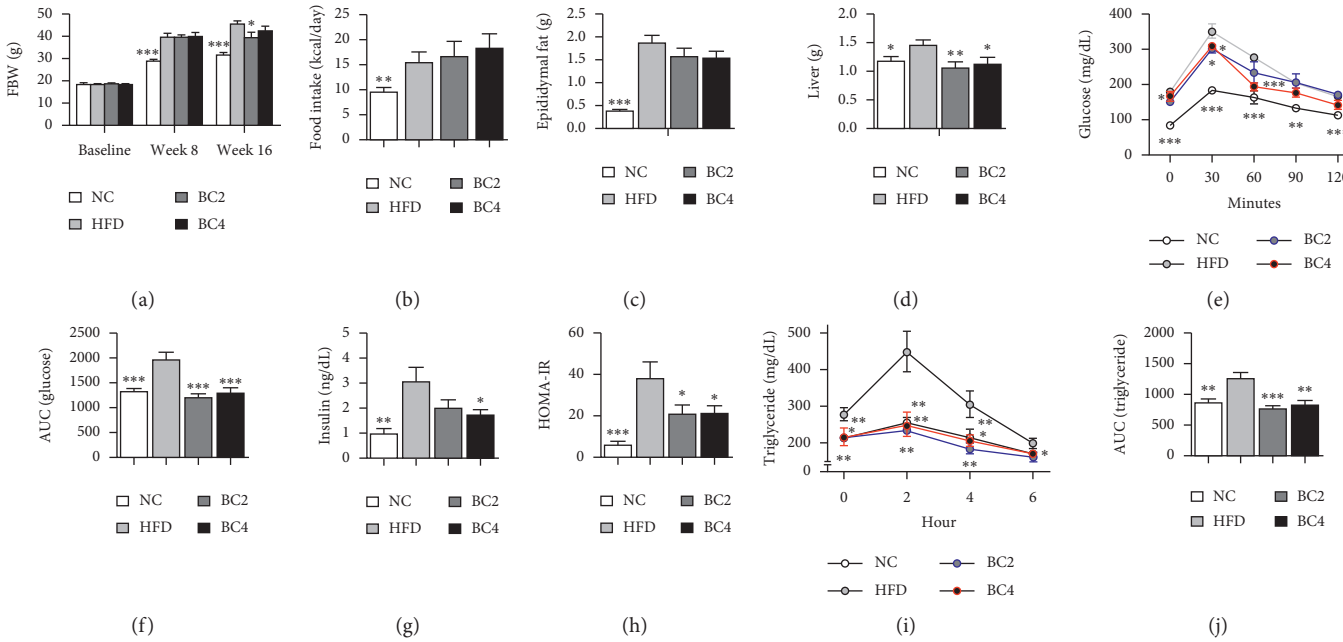

Figure 1: Effects of baicalin on (a) body weight, (b) food intake, (c) epididymal fat weight, (d) liver weight, (e) oral glucose tolerance test (OGTT) results, (f) area under the curve (AUC) of OGTT, (g) serum insulin level, (h) HOMA-IR, (i) oral fat tolerance test (OFTT) results, and (j) AUC of OFTT. Data shown are means ± standard errors of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the HFD group. $n = 6$ mice per group.

vs. 50.89 ± 6.31 mg/dL, respectively, $p < 0.05$; HDL-C: 198.6 ± 12.56 mg/dL vs. 279.2 ± 16.96 mg/dL, respectively, $p < 0.01$) (Figures 2(b), 2(d), and 2(f)). BC2 also lowered the other serum lipid levels, but it was not significant. Mice in the BC4 group had lower serum lipid levels compared with the HFD group (Figures 2(a), 2(c), and 2(e)). In the OFTT, the HFD group had higher TG levels than the NC group at all time points except for the 6 hr time point. Mice in the BC2 group showed significantly decreased TG levels at all time points compared with the HFD group (0 hr, $p < 0.01$; 2 hr, $p < 0.01$; 4 hr, $p < 0.01$; 6 hr, $p < 0.05$) (Figure 1(i)), which were even lower than those in the NC group. BC4 also significantly lowered TG levels at 0, 2, and 4 hr compared with the HFD group (0 hr, $p < 0.05$; 2 hr, $p < 0.01$; 4 hr, $p < 0.05$) (Figure 1(g)). Mice in the HFD group had higher AUCs compared with the NC group. The AUCs of both BC2 and BC4 groups were significantly reduced compared with that of the HFD group (1216.8 ± 66.5 and 1306.4 ± 105.1 vs. 1982.2 ± 130.1, respectively, $p < 0.001$) (Figure 1(j)).

3.3. Effects of Baicalin on Serum Insulin Level and HOMA-IR. To evaluate the effects on insulin resistance, we measured the fasting insulin level and HOMA-IR. The insulin level was remarkably higher in the HFD group compared with the NC group (3.03 ± 0.62 ng/mL vs. 0.98 ± 0.20 ng/mL, $p < 0.01$). We observed decrease in both BC2 and BC4 groups, but only the BC4 group showed significant difference compared with the HFD group (1.76 ± 0.20 ng/mL vs. 3.03 ± 0.62 ng/mL, $p < 0.05$) (Figure 1(g)). Similar pattern was observed in HOMA-IR. HOMA-IR of the HFD group was significantly higher than that of the NC group (38.28 ± 7.78 vs. 6.15 ± 1.60, $p < 0.001$). Both BC2 and BC4 groups showed a significant decrease in HOMA-IR compared with the HFD group (21.02 ± 4.45 and 21.71 ± 3.05 vs. 38.28 ± 7.78, $p < 0.05$) (Figure 1(h)).

3.4. Effects of Baicalin on Hepatic and Renal Function. For examining the safety of baicalin treatment, serum AST and ALT levels were estimated after 16 weeks. Mice in the HFD group had a higher level of ALT compared with the NC group. BC2 lowered the ALT level as half significantly compared with the HFD group (34.4 ± 10.25 U/L vs. 73.78 ± 11.11 U/L, $p < 0.05$), but it was not dose-dependent (Figure 2(h)). The levels of AST and creatinine were not different between all groups (Figure 2(g)).

3.5. Effects of Baicalin on Monocytes. Mice in the HFD group had higher $Ly6C^{hi}$ monocyte and lower $Ly6C^{low}$ monocyte fraction compared with the NC group. BC2 and BC4 significantly lowered $Ly6C^{hi}$ monocyte fraction compared with the HFD group (36.63 ± 1.21% and 37.68 ± 3.73% vs. 48.02 ± 2.51%, respectively, $p < 0.05$) (Figure 3(a)). BC2 and BC4 lowered $Ly6C^{low}$ monocyte fraction compared with the HFD group without statistical significance.

3.6. Effects of Baicalin on ATMs and KCs. Mice in the HFD group had a significantly higher percentage of ATMs compared with the NC group. BC2 and BC4 significantly lowered the percentage of ATMs compared with the HFD group (48.38 ± 6.01% in BC2 group vs. 60.24 ± 2.76% in the HFD group, $p < 0.05$; 46.44 ± 4.89% in BC4 group vs.

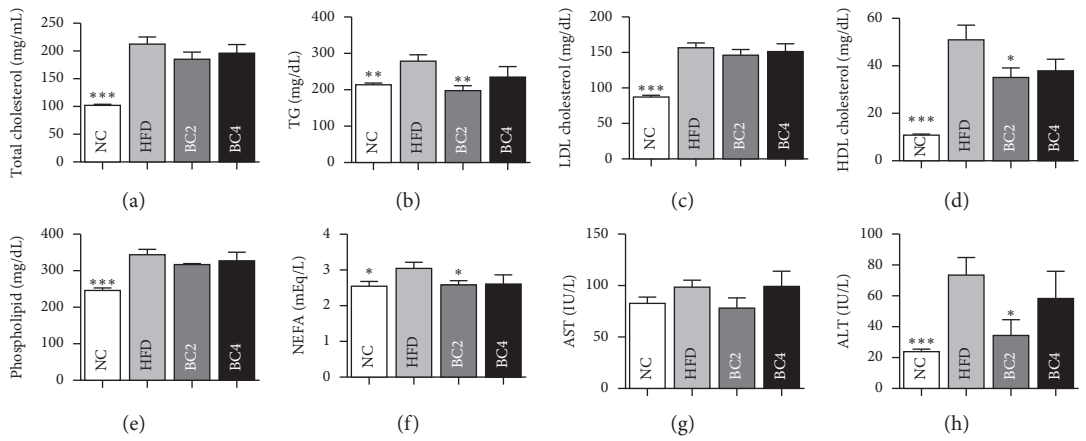

Figure 2: Effects of baicalin on (a) total cholesterol, (b) triglyceride, (c) LDL cholesterol, (d) HDL cholesterol, (e) phospholipid, (f) nonesterified fatty acid (NEFA), (g) aspartate aminotransferase (AST), and (h) alanine aminotransferase (ALT) levels. Data shown are means ± standard errors of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the HFD group. $n = 6$ mice per group.

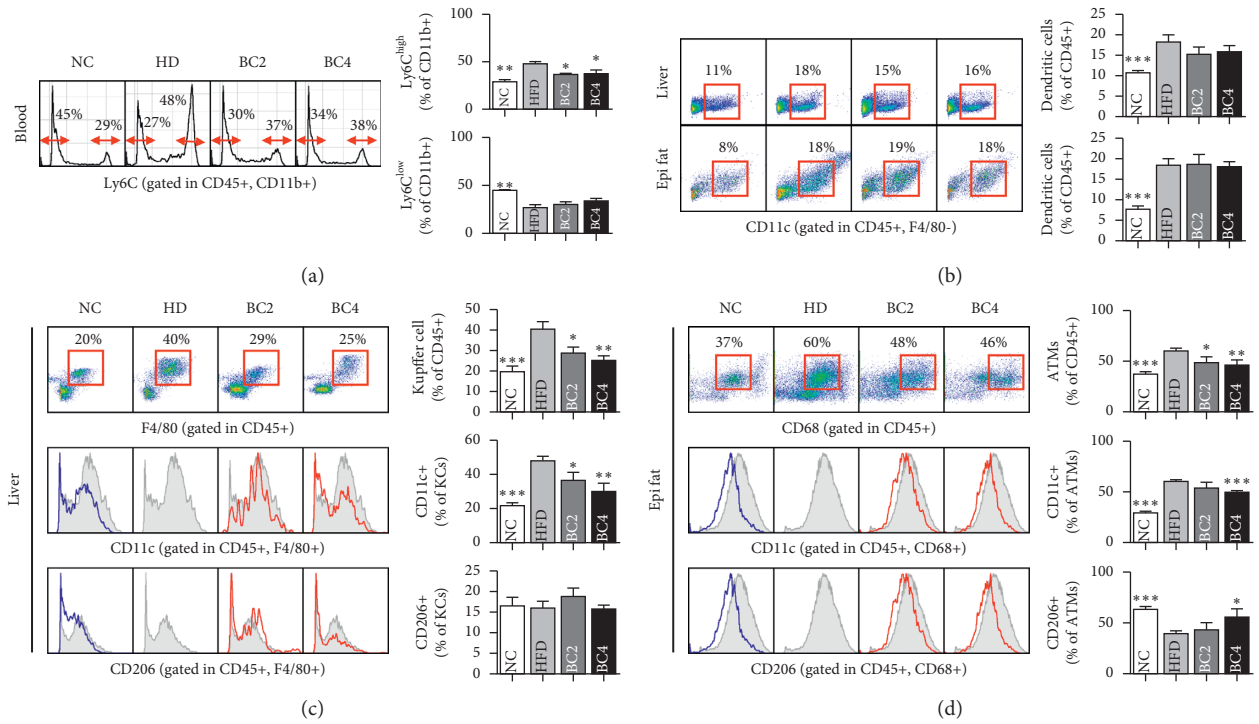

Figure 3: Effects of baicalin on (a) Ly6C monocytes in the CD11b+ population, (b) dendritic cells in liver and epididymal fat tissues, (c) liver Kupffer cells, CD11c+ Kupffer cells, and CD206+ Kupffer cells, and (d) adipose tissue macrophages, CD11c+ adipose tissue macrophages, and CD206+ adipose tissue macrophages. Data shown are means ± standard errors of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the HFD group. $n = 6$ mice per group.

60.24 ± 2.76% in the HFD group, $p < 0.01$) (Figure 3(d)). Mice in the HFD group had more CD11c+ ATMs and fewer CD206+ ATMs compared with the NC group ($p < 0.001$). However, BC4 decreased CD11c+ ATMs and increased CD206+ ATMs significantly compared with the HFD group (50.16 ± 1.07% vs. 60.24 ± 1.70%, $p < 0.001$; 55.99 ± 7.97% vs. 39.12 ± 3.04%, $p < 0.05$, respectively) (Figure 3(d)). In the liver tissue, the mice in the HFD group had higher percentages of KCs compared with the NC group. However, BC2 and BC4 lowered the percentage of KCs significantly compared with the HFD group (28.55 ± 3.15% in the BC2 group vs. 40.37 ± 3.61% in the HFD group, $p < 0.05$; 25.17 ± 2.26% in the BC4 group vs. 40.37 ± 3.61% in the HFD group, $p < 0.01$) (Figure 3(c)). Mice in the HFD group had a higher percentage of CD11c+ KCs and a lower percentage of CD206+ KCs compared with the NC group. However, BC2 and BC4 lowered the percentage of CD11c+ KCs compared with the HFD group significantly in a dose-dependent

manner ($36.26 \pm 4.90\%$ in the BC2 group vs. $47.72 \pm 2.70\%$ in the HFD group, $p < 0.05$; $29.95 \pm 4.92\%$ in the BC4 group vs. $47.72 \pm 2.70\%$ in the HFD group, $p < 0.01$) (Figure 3(c)). The percentage of CD206+ KCs was not significantly different among the HFD, BC2, and BC4 groups.

3.7. Effects of Baicalin on T Cells and DCs in Blood, Adipose Tissue, and Liver. Mice in the HFD group had a significantly higher proportion of CD4+ T cells, lower proportion of CD8+ T cells, and lower CD4/CD8 ratio compared with the NC group. In adipose tissue, BC2 and BC4 enlarged the proportion of CD4+ T cells significantly compared with the HFD group ($15.23 \pm 1.64\%$ in the BC2 group vs. $9.84 \pm 0.82\%$ in the HFD group, $p < 0.01$; $12.86 \pm 1.52\%$ in the BC4 group vs. $9.84 \pm 0.82\%$ in the HFD group, $p < 0.05$) (Figure 4(a)). Regarding CD8+ T cells, the proportions in both BC2 and BC4 groups were not significantly different from those of the HFD group (Figure 4(b)). The CD4/CD8 ratio of BC2 and BC4 groups were significantly higher than that of the HFD group (2.36 ± 0.36 in the BC2 group vs. $1.38 \pm 0.15\%$ in the HFD group, $p < 0.01$; 1.90 ± 0.27 in the BC4 group vs. $1.38 \pm 0.15\%$ in the HFD group, $p < 0.05$).

In the liver, BC4 enlarged the percentage of CD4+ T cells significantly compared with the HFD group ($22.64 \pm 0.85\%$ vs. $18.73 \pm 1.50\%$, $p < 0.05$) (Figure 4(a)). Regarding CD8+ T cells, there was no change by baicalin treatment (Figure 4(b)). The CD4/CD8 ratio of BC2 and BC4 groups were significantly increased compared with that of the HFD group (1.20 ± 0.02 in the BC2 group vs. 0.97 ± 0.03 in the HFD group, $p < 0.001$; 1.19 ± 0.06 in the BC4 group vs. 0.97 ± 0.03 in the HFD group, $p < 0.001$). In blood, the populations of CD4+ and CD8+ T cells were not significantly different among all groups (Figures 4(a) and 4(b)). The number of $CD45^+$ $F4/80^-$ $CD11c^+$ DCs in adipose and liver tissues was significantly increased by HFD, but there was no change by baicalin treatment (Figure 3(b)).

3.8. Effects of Baicalin on the Expression of Inflammatory and Insulin Signaling-Related Genes. In the adipose tissue, the transcript levels of TNF-α, F4/80, and Ccl2 were analyzed. Mice in the HFD group showed higher levels of TNF-α, F4/80, and Ccl2 mRNA compared with the NC group. However, BC2 and BC4 lowered TNF-α mRNA levels compared with the HFD group significantly (4.84 ± 0.66 in the BC2 group vs. 9.24 ± 1.31 in the HFD group, $p < 0.001$; 4.33 ± 0.38 in the BC4 group vs. 9.24 ± 1.31 in the HFD group, $p < 0.001$) (Figure 5(a)). Both BC2 and BC4 groups had significantly lower F4/80 and Ccl2 mRNA levels than the HFD group (F4/80: 2.78 ± 0.45 in the BC2 group vs. 5.81 ± 0.93 in the HFD group, $p < 0.001$; 3.67 ± 0.61 in the BC4 group vs. 5.81 ± 0.93 in the HFD group, $p < 0.05$; Ccl2: 2.40 ± 0.34 in the BC2 group vs. 4.29 ± 0.76 in the HFD group, $p < 0.05$; 2.08 ± 0.41 in the BC4 group vs. 4.29 ± 0.76 in the HFD group, $p < 0.05$) (Figures 5(b) and 5(c)).

In the liver tissue, transcript levels of insulin receptor (IR), insulin receptor substrate-1 (IRS-1), and IRS2 were analyzed to investigate the effects of baicalin on hepatic insulin signaling pathway. Mice in the HFD group had significantly decreased IR and IRS-2 mRNA levels and increased IRS-1 mRNA level compared with the NC group ($p < 0.001$). BC2 and BC4 groups had significantly higher IR mRNA levels than the HFD group (0.65 ± 0.11 in the BC2 group vs. 0.44 ± 0.06 in the HFD group, $p < 0.05$; 0.81 ± 0.10 in the BC4 group vs. 0.44 ± 0.06 in the HFD group, $p < 0.01$) (Figure 5(d)). Transcript levels of IRS-1 and IRS-2 in BC2 and BC4 groups were not significantly different from those in the HFD group (Figures 5(e) and 5(f)).

4. Discussion

This is the first study to investigate the metabolic effects and immunological mechanism of baicalin on obesity-induced inflammation in cellular and genetic levels. We discovered the cellular mechanisms by modulating $Ly6C^{hi}$ monocytes, M1 ATMs, M1 KCs, and CD4/CD8 T cells ratio and the genetic mechanism by decreasing mRNA expressions of inflammatory genes and increasing insulin signaling-related genes.

Obesity increases the risk of metabolic diseases including dyslipidemia, T2DM, nonalcoholic fatty liver disease (NAFLD), cardiovascular disease, and cancer by causing a chronic state of low-grade systemic inflammation [2, 3, 20]. Local inflammation in adipose tissue and the liver is the main contributor to systemic inflammation. The primary characteristic of obesity-induced inflammation is the extensive infiltration of macrophages into adipose tissue. Proinflammatory cytokines from ATMs damage insulin-signaling networks, aggravate infiltration, and induce proinflammatory differentiation of macrophages [3, 5]. At the same time, lipolysis in adipose tissue increases free fatty acid flux and fat oxidation in the liver, resulting in poor glucose oxidation and fatty liver. As gluconeogenesis increases, obesity-induced IR prior to beta-cell dysfunction plays a major role in the initial stage of T2DM [1, 20]. Therefore, anti-inflammatory modulation in metabolic organs is a promising treatment target of obesity and its associated metabolic diseases. In this study, we assessed the metabolic features, the major immune cells especially macrophages, and inflammatory and insulin-signaling genes of obese mice to gain insights into antiobesity and antidiabetic effects of baicalin.

Baicalin treatment significantly decreased body weight without any change in the weight of epididymal fat pads. This indicated that the anti-inflammatory properties of baicalin as reflected by improvements in glucose and lipid metabolism were not due to a loss in adipocyte volume but rather to changes in the immune cell population in the blood and adipose and liver tissues. It represented that baicalin treatment is a systemic immunomodulating treatment for obesity-associated metabolic diseases, especially T2DM and NAFLD. Mice treated with baicalin exhibited significantly lower glucose levels at 0, 30, and 60 min in OGTTs. Given that the first-phase insulin response is the principal predictor of T2DM development, these results suggest that baicalin improves glucose metabolism [21]. We also noticed that baicalin ameliorated insulin resistance by observing the decrease in the insulin level and HOMA-IR. NEFA release

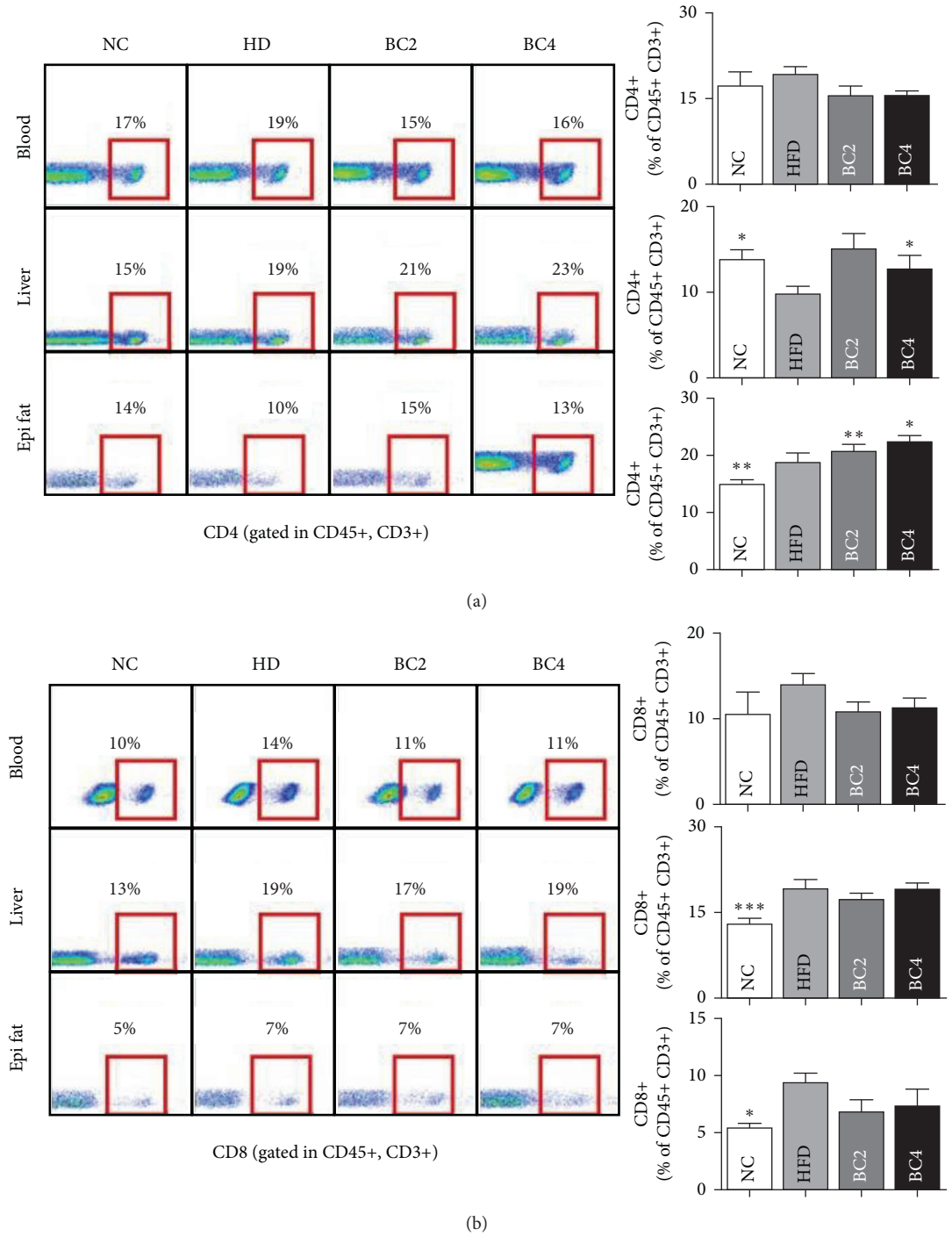

Figure 4: Effects of baicalin on (a) CD4+ T cells in the blood, liver, and epididymal fat pads and (b) CD8+ T cells in the blood, liver, and epididymal fat pads. Data shown are means ± standard errors of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the HFD group. $n = 6$ mice per group.

due to lipolysis of adipocytes inhibits insulin-mediated glucose uptake [22, 23]. Baicalin was observed to significantly reduce TG levels at all time points studied in OFTTs and the levels of NEFA and HDL-C. The observed changes in NEFA and TG but not TC are consistent with the findings of Xu et al. [24] but differ from those of Xi and colleagues. Xi et al. [17] reported that administration of baicalin for 14 weeks to obese mice increased HDL-C and reduced TC and LDL-C. Considering that hepatic lipoprotein metabolism proceeds in the order of NEFA uptake, TG synthesis, and VLDL-C hydrolysis, the results after 8 weeks are expected to be the same as Xi et al.'s result after 14 weeks. This study has a potential limitation that we had not established the positive control group. We aimed to assess the effects of baicalin on

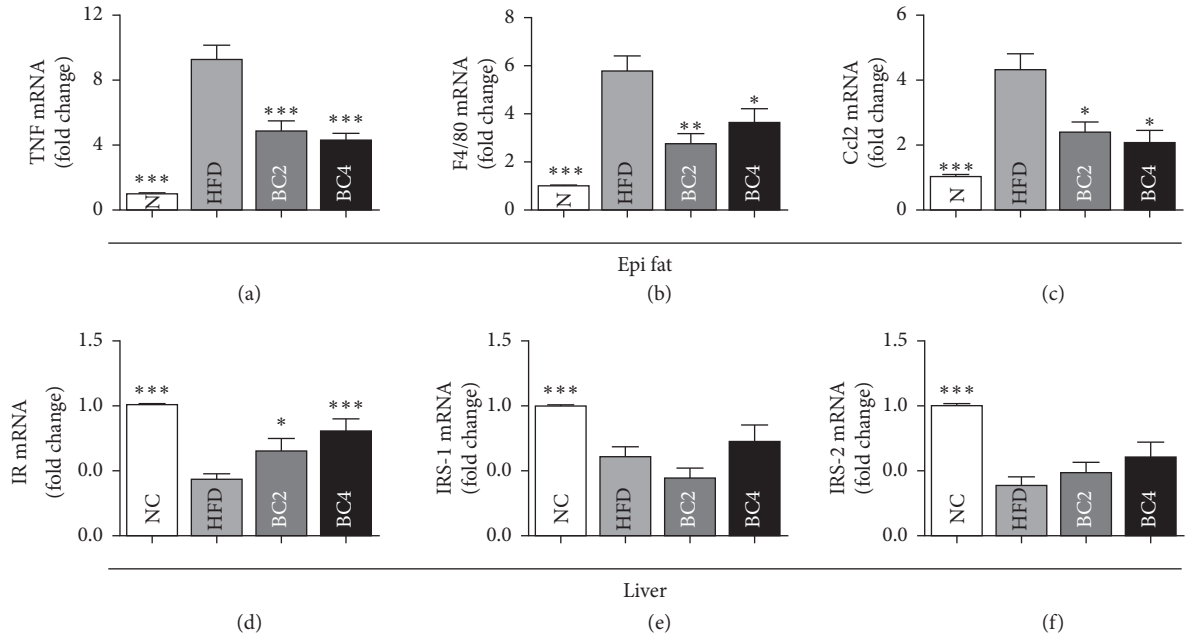

Figure 5: Effects of baicalin on the gene expression of (a) TNF-α, (b) F4/80, and (c) Ccl2 in epididymal fat pads and (d) IR, (e) IRS-1, and (f) IRS-2 in the liver. Data shown are means ± standard errors of the mean (SEM). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the HFD group. $n = 6$ mice per group.

various metabolic and immunological markers for the first time and observed its beneficial actions. Further studies are required comparing baicalin with conventional medicine such as metformin or statins to obtain more robust evidence on the treatment effect of baicalin on obesity and its associated diseases.

In this study, we demonstrated that baicalin modulated the proportion of ATMs and KCs based on a decrease in Ly6C^{hi} monocytes. Ly6C^{hi} monocytes respond to lipopolysaccharides and worsen inflammation, while Ly6C^{low} monocytes remodel the inflammation sites. Ly6C^{hi} and Ly6C^{low} monocytes are more likely to differentiate into M1 and M2 macrophages, respectively, when infiltrating into local tissues. Classically activated CD11c+ macrophages (M1) produce proinflammatory cytokines such as TNF-α as well as a high quantity of reactive oxygen species (ROS), while alternatively activated CD206+ macrophages (M2) produce anti-inflammatory cytokines such as IL-4 and IL-10. KCs are hepatic macrophages, and the expansion of M1 KCs results in hepatic inflammation, leading independently to nonalcoholic steatohepatitis (NASH) and hepatic IR [5]. In the obese state, HFD-induced hypercholesterolemia resulted in selective expansion of Ly6C^{hi} monocytes and inhibited the conversion from Ly6C^{hi} to Ly6C^{low} monocytes [25, 26], and a shift to M1 ATMs strongly mediated the development of IR [8, 27]. However, baicalin significantly modulated the monocyte phenotype with fewer Ly6C^{hi} monocytes and more Ly6C^{low} monocytes. This change from Ly6C^{hi} to Ly6C^{low} monocytes in the blood contributed to an M2-dominant shift in ATMs and KCs. Also, baicalin significantly restricted M1 KC activation with no change in M2 KCs, suppressing the hepatic inflammation. Taken together, we discovered that baicalin modulated macrophage differentiation along with Ly6C^{hi} monocytes' decrement, leading to anti-inflammation in the adipose tissue and liver.

The distribution of T cells also affects obesity-induced inflammation and hepatic steatosis. CD4+ regulatory T (Treg) cells are known to suppress inflammation by checking ATMs, while CD8+ T cells affect M1 polarization by secreting IFN-γ [1, 6]. As CD4+ T cells have 4 subtypes, CD4+ Th1 cells and an imbalance between T17 and Treg cells stimulate M1 expansion. Furthermore, CD4+ Th2 cells induce M2 polarization. In this study, baicalin significantly enhanced only the CD4+ T cell population in adipose tissue with no change in the CD8+ T cell population. However, in the liver, baicalin increased the number of CD4+ T cells and inhibited CD8+ T cells significantly. Further study is needed to determine how baicalin affects subtypes of CD4+ T cells including Th1, Th2, Treg, and Th17 cells [28, 29]. DCs differentiated from monocytes are potent antigen-presenting cells that activate CD4+ T cells, especially Th1 cells, leading to M1 ATM expansion in adipose tissue. Hepatic DCs are also known to play key roles in the pathogenesis and resolution of NASH [30, 31]. In this study, baicalin did not affect the number of adipose DCs but decreased hepatic DCs slightly. This indicates that the anti-inflammatory effects are primarily due to its effects on macrophages. However, natural killer cells such as IFN-γ-secreting immune cells that can affect M1 macrophages should also be investigated in future studies.

Inflammatory proteins, especially Ccl2 (encoding MCP-1), recruit macrophages into obese adipose tissue and TNF-α further activates the inflammatory cascade, maintaining obesity-induced systemic inflammation [32]. Consistent with previous results, we demonstrated that baicalin significantly decreased the mRNA expression of Ccl2 and

TNF-α. F4/80 is a specific marker to murine macrophages. We observed a significant decrease in F4/80 expression but no change in adipose DCs after baicalin treatment, indicating that the effects of baicalin are restricted to the ATM population, not the adipose DC population, and are mediated by the downregulation of Ccl2 and TNF-α. Insulin binds to the insulin receptor and activates the tyrosine kinases IRS1 and IRS2. In obesity, IR is demonstrated by the downregulation of IRS2 and persistent expression of IRS1, and inflammatory cytokines including TNF-α downregulate IRS2 mRNA expression [33, 34]. In this study, we observed a lower expression of IRS1 mRNA in the HFD group than that of the NC group, but no significant change in IRS1 mRNA levels after baicalin treatment. However, baicalin significantly upregulated IR mRNA expression.

Our findings suggest that oral administration of baicalin to obese mice has favorable effects on the systemic inflammatory state by reducing Ly6C^{hi} monocytes in the blood, proinflammatory macrophages, and T cells in the liver and adipose tissues, leading to improvement of obesity-induced IR, diabetes, dyslipidemia, and metabolic diseases.

5. Conclusion

This new finding suggests that baicalin is a promising treatment for obesity-related metabolic diseases by improving systemic inflammatory conditions, and further study including clinical trial should be required to confirm these results.

Data Availability

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Disclosure

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

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this manuscript.

Acknowledgments

This research was supported by a grant from the Korea Health Technology R&D Project through Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (Grant no. HF20C0022).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6631963, 9 pages
https://doi.org/10.1155/2021/6631963

Research Article

The Effect of a Persian Herbal Medicine Compound on the Lipid Profiles of Patients with Dyslipidemia: A Randomized Double-Blind Placebo-Controlled Clinical Trial

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Received 9 December 2020; Revised 9 March 2021; Accepted 21 April 2021; Published 20 May 2021

Academic Editor: Mohammad Hossein Ayati

Introduction. It has been well established in the world that lipid disorders promote the development of atherosclerosis and its clinical consequences. This study aimed to assess the impacts of a Persian medicinal (PM) compound on lipid profile. *Materials and Methods*. From June 21 to October 21, 2020, a randomized double-blind controlled clinical trial was conducted with 74 dyslipidemic patients, who were randomly divided into two equally populated groups: one prescribed with a Persian medicinal herbal compound ($n = 37$) and a placebo group ($n = 37$). A Persian herbal medicine including fenugreek, sumac, and purslane is introduced. Biochemical parameters including 12-hour fasting serum levels of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), and triglyceride (TG) were measured before the initiation and after the completion of study protocol. *Results*. Percent changes of biochemical parameters include the following: intervention group = cholesterol: 35.22, Tg: 45.91, LDL: 24.81, HDL: 2.05, VLDL: 8.94 and placebo group = cholesterol: 6.94, Tg: −7.3, LDL: 7.37, HDL: 2.88, VLDL: −0.14. The serum levels of total cholesterol ($p = 0.01$) and LDL ($p = 0.01$) significantly decreased and no increase was recorded in HDL ($p = 0.03$) levels over time in the intervention group. Furthermore, between-group analysis showed a statistically significant difference between the intervention and placebo groups in this regard. VLDL ($p = 0.2$) and TG ($p = 0.2$) levels also decreased, however not significantly. *Conclusion*. This study showed that a Persian medicinal herbal compound could be safe and beneficial to decrease the levels of serum cholesterol and LDL in dyslipidemic patients. However, larger long-term studies are recommended to clarify this effect.

1. Introduction

Dyslipidemia is an abnormal metabolic condition often recognized by disorders in lipid profile-containing serum cholesterol, TG, HDL, LDL, and VLDL [1, 2]. Cardiovascular diseases, of which dyslipidemia is a critical yet controllable risk factor, cause many deaths in the world [3]. Approximately half the popular risk of myocardial infarction and one-quarter of ischemic stroke risk are estimated by elevated LDL and cholesterol levels [4]. In addition to cardiovascular effects, dyslipidemia causes complications in other organs [5–7]. The prevalence of hypertriglyceridemia is also likely to increase in patients with diabetes, metabolic syndrome, and/or obesity [8]. About 80% of cardiovascular disease reports come from low- and middle-income countries. However, the majority of trials which were conducted in North America or Europe involve mainly white persons [9], even though dyslipidemia is generally various among different races or

ethnic groups [10], especially Asian population who is considered to be at higher risk for the adverse effects of statin use than white population [11, 12]. Statins are the most common drugs used to prevent and treat dyslipidemia [13, 14]. Regarding the fact that most patients should use statins for a long time, it is critical to consider that statin therapy's potentially harmful effects on muscles and the liver were known for some time. Besides, new concerns have emerged regarding the risk of new-onset diabetes mellitus, cognitive impairment, and hemorrhagic stroke [12, 15]. Combination therapy is more effective in many cases of dyslipidemia [16, 17]. Various studies in herbal and complementary medicine have proven medicinal plants to be effective in treating dyslipidemia [18–22]. Cooperative use of certain foods or herbal medications may increase or decrease the therapeutic effects of statins. However, there is little information about it [23]. In Persian medicine, which is several thousand years old, medicinal plants are widely used in the treatment of diseases. In this study, based on the principles of Persian medicine, three medicinal plants have been selected, combined, and prescribed to the patients without making a change in their nutritional habits and macronutrients.

The main goal was to investigate the therapeutic effects of this medicinal compound on the lipid profiles of dyslipidemic patients for the first time. The mentioned compound contains *Trigonella foenum-graecum* (fenugreek, in Persian: "Shanbalileh"), *Portulaca oleracea* (purslane, in Persian: "Khorfeh"), and *Rhus corindria* (sumac, in Persian: "Somagh"), all of which have individually various medical effects on lipid profile [24–31].

2. Materials and Methods

2.1. Study Design. This double-blind randomized clinical trial was designed to perform on 74 patients, including 31 men and 43 women. The patients, who were randomly recruited for sampling, all had disordered lipid profiles and were diagnosed with dyslipidemia based on Adult Treatment Panel (ATP) III guidelines after they visited specific clinics in Arak City [32]. Participants were individually randomized to one of two parallel groups.

A recommendation for reporting the randomization clinical trial was conducted based on the definition made by the statement of consolidated standards of reporting randomized clinical trials (CONSORT).

2.2. Participants. The inclusion criteria are indicated as follows:

Having filled out the questionnaire of consent required for participation in the study.

Total serum cholesterol more than 200 mg/dL, LDL more than 100, and TG between 200 and 400 mg/dl HDL less than 29 in men and less than 35 in women.

No medical history of recent serious heart, kidney, liver, and/or brain disease. No pregnancy in women. Age from 18 to 75.

The exclusion criteria are as follows:

Discontinuance of cooperation and/or approval from the patient during the process of treatment. The appearance of drug reaction and/or serious complications. Using any herbal medication other than the one prescribed during research process. Medical history of diabetes and/or uncontrolled hypothyroidism.

The questionnaire used for data collection included demographic variables that registered age, sex, residence area, height, weight, exercise, and food habits or regimen.

2.3. Intervention. The patients were divided into two groups of 37, categorized as intervention and placebo. The two were kept as similar as possible (especially due to physical activity and dietary habits) and were both prescribed with capsules identical in weight, shape, and packaging. Based on the results of the study of Asghari et al. [33] and in the pilot study, which took place before the main research and was performed on 10 patients, the period of treatment was 6 weeks.

The capsules given to each group were identical in color and all had covers produced from a common type of gelatin. The contents of capsules were first prepared with a specific formula, adapted from the most valid resources of PM [31, 34–36], and then inserted into the capsules and packed. The intervention group's capsules contained 600 mg of active herbal pharmaceutical ingredients, including Fenugreek seeds, Sumac, and Purslane [37, 38]. The plants were prepared dry and no extract was prepared from them. These capsules were prepared in the Pharmacy faculty of Shahid Beheshti University of Medical Sciences. The placebo group received capsules containing the same weight in wheat starch.

Each patient was asked to orally take one capsule after breakfast, lunch, and dinner (3 capsules/day) for six weeks. Moreover, each patient was given 126 capsules. They were each frequently contacted and periodically examined, while being permitted to continue their classic routine of treatment. Thus, all patients took one Atorvastatin 20 mg tablet daily before and during the process of treatment with herbal medicine. The patients were evaluated after 6 weeks of treatment and then 4 weeks after the end of treatment period. They were contacted for a while afterward to make sure there were no probable drug complications.

2.4. Sample Size and Randomization. The patients were randomly placed into two groups of 37, keeping the two as similar as possible. These groups were recognized as intervention and placebo, both prescribed with capsules identical in weight, shape, and packaging.

Permuted balanced block randomization with a block size of 4 and 6 was used to generate the random sequence, which was kept by an epidemiologist. In addition to the random sequence list, unique codes were assigned to each patient which the bottles were labeled with. In addition to the use of random blocks with different patterns, due to the

use of unique codes for each drug package, it was not possible to predict the assignment of individual to groups. This method of randomization guarantees group balance and concealment.

Using Stata software, considering alpha 5%, power 90%, and based on Gheflati et al. [39] studying that the mean of LDL was 120.8 standard deviation (SD = 24.8) in the intervention group (purslane seeds) and 148.4 (SD = 35.8) in the control group, the required sample size in each group was estimated to be 27 cases; considering the probability of attrition, 37 cases were calculated for each group. By expanding this sample size to the number of groups, 74 cases were entered into the study.

Count (percent) and mean SD were, respectively, used to describe categorical and continuous variables. To check the normality assumption of continues variables, the Shapiro–Wilk test was used. To compare the categorical variables among groups, likelihood ratio Chi-square test was used. Two independent *t*-tests and Mann–Whitney test were used to compare the continuous variables among intervention and control groups. All analyses were done by Stata statistical software version 13 (Stata Corp, College Station, TX, USA) at a significant level less than 0.05.

2.5. Measurements and Outcomes. Levels of serum cholesterol, TG, HDL, LDL, and VLDL were measured at the beginning and the end of study, which was 42 days later.

To measure the biochemical parameters, 4 ccs (cubic centimeters) of venous blood was extracted from the patients, who had not consumed anything in the past 12 hours, in a sitting position in the early morning. The routine was then performed using BT 300 Alfaclassic Auto Analyzer with the sampling of 0.1 μL (microliter), Iranian-made Parsiazmoon kits, and the lab-sized high-speed refrigerator-equipped Universal centrifuge D-7200 German-made.

To determine patients' BMIs (body mass index) before and after the study, their heights and weights were carefully measured and registered using GLAMOR digital scale and Chinese-made MOMERT wall stadiometer (without shoes or hats). No significant changes were observed in the patients' BMIs before and after the treatment course.

3. Results

From June 21 to October 21, 2020, 74 (58.1% female and 41.9% male) patients with baseline abnormal lipid profiles were included and randomized to receive herbal capsules (caps) or placebo. The mean ± SD of age was 48.7 ± 10.3 years and the baseline characteristics were well-balanced among the randomized arms, as indicated in Table 1. All patients received concomitant lipid-lowering therapy with statins. Their regular dietary habits and routines of physical activity were not changed; therefore, no significant changes were observed in the patients' BMIs after the study.

During the study period, three patients (two in the intervention and one in the placebo arm) discontinued their medications due to personal reasons. All other participants were able to take their assigned medication through treatment course without reporting issues. After the follow-up at the sixth week, the blood sample was available and the outcomes were verified in 71 (95.9%) of patients.

3.1. Lipid Profile. Herbal compound effect compared to placebo on the lipid profile is summarized in Table 2 which is indicated in Figure 1. The level of serum cholesterol and LDL was significantly reduced in the intervention group as compared to placebo ($p = 0.01$). Moreover, TG and VLDL levels also decreased of using the herbal caps ($p = 0.2$) but could not significantly increase HDL levels significantly (Figures 2 and 3).

4. Discussion

Dyslipidemia is a major risk factor for atherosclerosis and diabetes. For many decades, medicinal plants were used to treat various diseases due to their beneficial effects and few side effects [9, 18]. In the present study, the effects of an herbal capsule on cholesterol, triglyceride, HDL, LDL, and VLDL levels during 6 weeks were investigated. It was discovered that this capsule had significantly improved serum cholesterol and LDL levels in dyslipidemic patients. Although it could reduce the levels of triglyceride and VLDL in the intervention group compared to the control group, it was not significant.

The difference between statistical significance and clinical importance should always be borne in mind.

Fenugreek contains a volatile oil, alkaloids (including trigonelline), saponins (based on diosgenin), flavonoids, mucilage, protein, fixed oil, vitamins A, B1, C, and minerals.

The hypolipidemic effects of fenugreek seeds were ascribed to the presence of saponins, sapogenin, and partially, 4-hydroxy isoleucine [40]. Conversely, clinical studies in patients with lipid-related problems suggest that saponin deprived fenugreek seed powder can significantly reduce serum TG as well as total cholesterol [41].

In a study performed by Rao et al. on forty overweight and diabetic subjects, the following factors were decreased after the prescription of fenugreek seeds for 12 weeks: fasting blood glucose, total cholesterol, non-high-density lipoprotein (non-HDL) cholesterol, VLDL, and TG.

The study mentioned above was performed under specific limitations. Particularly, it did not have a concurrent control group [42].

Double-blind study of Geberemeskel et al. on 114 new diabetic patients with no complications indicated that using *Trigonella foenum-graecum* seed powder solution had resulted in the decrease of serum TG and LDL levels and the increase of HDL levels [24]. From Anacardiaceae family, while *Rhus coriaria* and *Rhus glabra* are considered safe for most people, some species, such as *Rhus radicans*, *Rhus diversiloba*, and *Rhus vermix*, contain the allergen urushiol and can cause severe skin and mucus membrane irritation. *Rhus coriaria* is found in Syria, Iran, and the Mediterranean.

As previously proved by several basic pieces of research, various parts of sumac contain a high variety of medicinally

Table 1: Baseline characteristics.

	Total ($n = 74$)	Intervention arm ($n = 37$)	Control arm ($n = 37$)
Female sex	43 (58.1%)	20 (54.1%)	23 (62.2%)
Male sex	31 (41.9%)	17 (45.9%)	14 (37.8%)
Age, years	48.7 ± 10.3	48.8 ± 10.7	48.6 ± 10
Marital status			
Single	6 (8.1%)	3 (8.1%)	3 (8.1%)
Married	64 (86.5%)	30 (81.1%)	34 (91.9%)
Widowed/divorced	4 (5.4%)	4 (10.8%)	0 (0%)
Occupation			
Unemployed	1 (1.4%)	1 (2.7%)	0 (0%)
Self-employed	9 (12.2%)	3 (8.1%)	6 (16.2%)
White collar worker	25 (33.8%)	12 (32.4%)	13 (35.1%)
Blue collar worker	3 (4.1%)	2 (5.4%)	1 (2.7%)
Housewife/retired	36 (48.6%)	19 (51.4%)	17 (45.9%)
Height (cm)	166.7 ± 8.6	167.7 ± 9.2	165.7 ± 7.9
Weight (kg)	77.8 ± 13.4	77.3 ± 12.9	78.3 ± 14.1
BMI (kg/m^2)	27.9 ± 3.5	27.4 ± 3.3	28.3 ± 3.8
Smoking	12 (16.2%)	7 (18.9%)	5 (13.5%)
Exercise	7 (9.5%)	3 (8.1%)	4 (10.8%)
Alcohol consumption	1 (1.4%)	1 (2.7%)	0 (0%)

Data are represented as number (%) or mean ± standard deviation. BMI, body mass index; cm: centimeter; kg: kilogram; kg/m^2: kilogram per square meter.

Table 2: Comparison and changes in the lipid profile between intervention and placebo groups after 6 weeks (mean ± SD).

Parameters	Intervention group ($n = 35$)	Baseline placebo group ($n = 36$)	p value	Intervention group ($n = 35$)	After 6 weeks placebo group ($n = 36$)	p value
Serum cholesterol (mg/dL)	234.56 ± 7.35	227 ± 6.85	0.46	198.65 ± 6.24	222.08 ± 6.38	0.01
HDL (mg/dL)	44.24 ± 1.58	41.45 ± 1.37	0.18	42.2 ± 1.33	38.58 ± 1.03	0.03
LDL (mg/dL)	136.56 ± 5.85	138.31 ± 7.24	0.85	110.02 ± 6.01	133.61 ± 6.69	0.01
VLDL (mg/dL)	53.28 ± 3.05	50.44 ± 2.66	0.48	45.35 ± 2.64	49.87 ± 2.62	0.2
Serum TG (mg/dL)	272.48 ± 15.53	252.29 ± 13.23	0.32	231.88 ± 14.08	256.02 ± 13.4	0.2

SD: standard deviation; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very-low-density lipoprotein; TG: triglyceride; mg/dl: milligram per deciliter.

significant phytochemical components [43–45]. Due to their high resin binding capacity, polyphenols can effectively reduce the lipid absorbance from the gastrointestinal tract. Moreover, an evident anti-oxidant property can be achieved from relatively high amounts of water-soluble tannins in sumac fruits [45, 46].

The consumption of sumac may be effective to decrease cardiovascular risk factors in patients with mild-to-moderate dyslipidemia [47].

To illustrate, thirty adults with dyslipidemia were randomly assigned to a sumac or placebo group in a clinical trial [47]. The difference between the two, the placebo group and the sumac group, indicated that BMI and total cholesterol levels were significantly decreased. However, plasma levels of TG did not change significantly across the treatment. LDL particles require oxidation to be the initiator of atherosclerosis process within vessel cell walls [48]. Other considerable effects of sumac on dyslipidemia include antioxidant properties and activities of free radical cleansing against lipid peroxidation as the initial stage of atherosclerosis [47–50].

Purslane contains several therapeutic values, nearly all of which are associated with the presence of many biologically active compounds including flavonoids, alkaloids, coumarins, and high contents of omega-3 fatty acids. These properties utterly provide considerable benefits to prevent the heart attacks and strengthening the immune system. Besides, they have favorable effects on cholesterol and triglyceride levels [51–55].

In Sabzghabaee's study [29] on 74 persons, the clinical effects of *Portulaca oleracea* seeds on dyslipidemia in obese adolescents, who were evaluated in two groups (placebo and drug intervention), were assessed. Total cholesterol, LDL, and TG levels showed statistically significant changes over time (one month) in the P. oleracea group ($p < 0.05$).

Moreover, due to PM sources, the process of food digestion is divided into four stages: 1st: gastric, 2nd: hepatic, 3rd: vascular, and 4th: tissue metabolism. Fenugreek, sumac, and purslane exert their therapeutic effects by improving the first and second stages and the physiological activity of liver and gastrointestinal tract, controlling dyslipidemia as a result [31, 34, 35, 56–58].

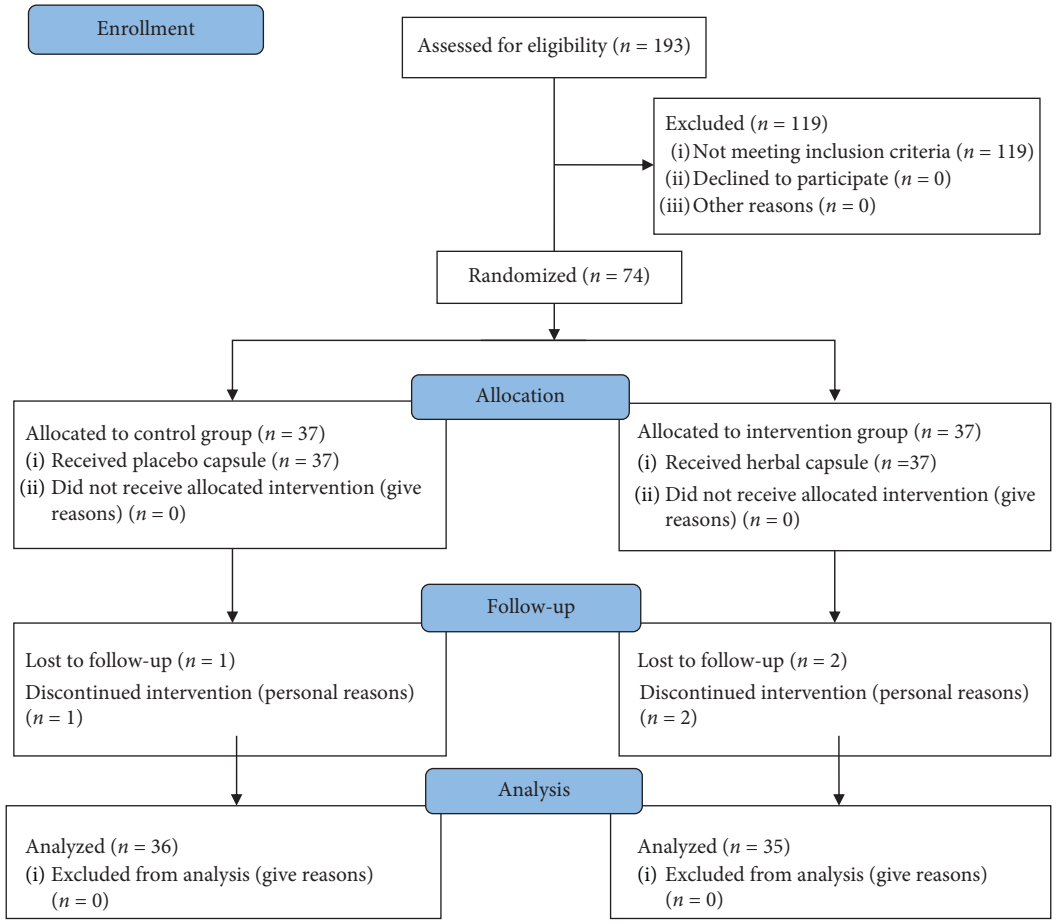
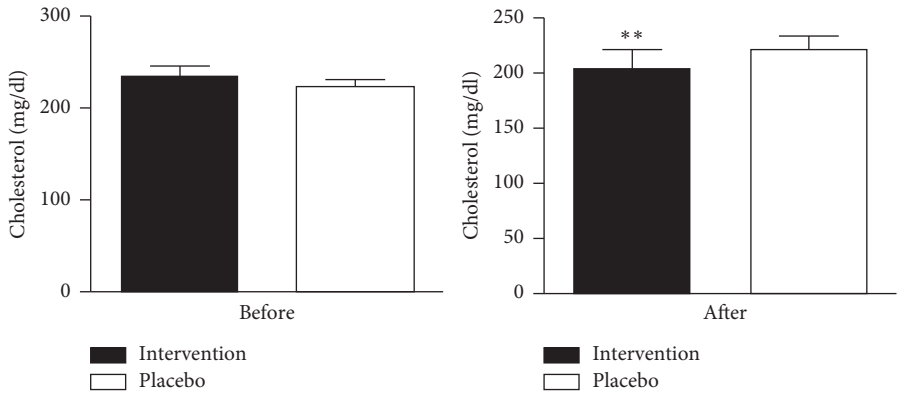


Figure 1: CONSORT flow diagram of the study.



(a)

Figure 2: Continued.

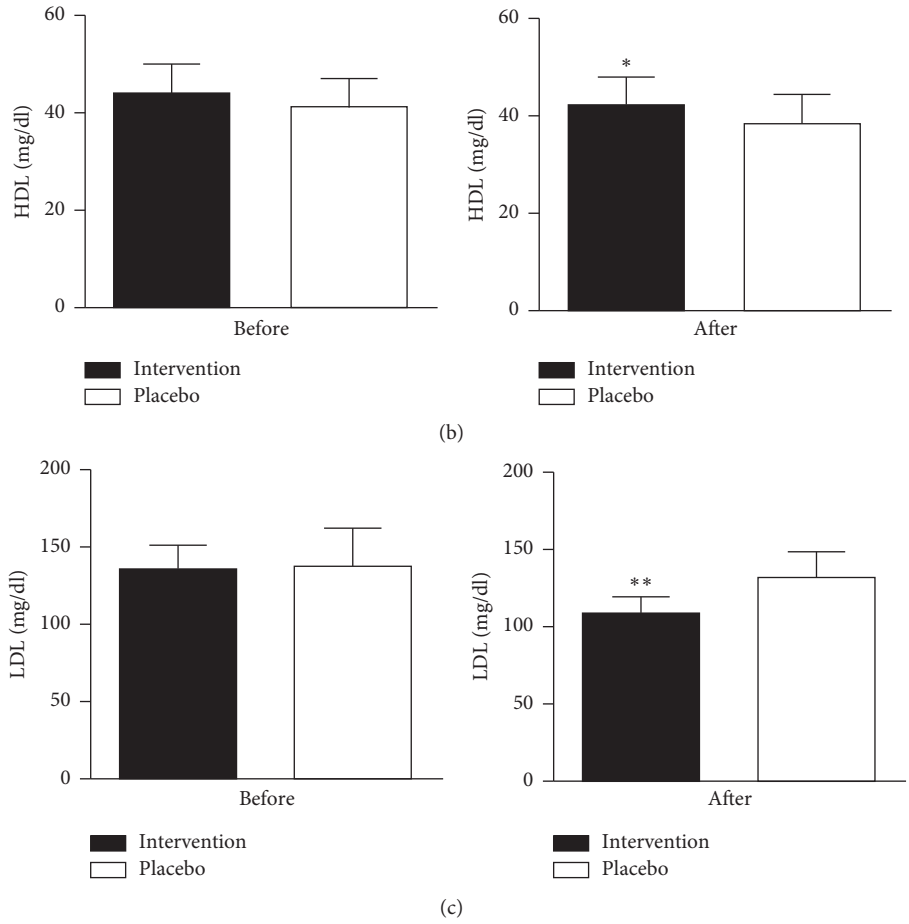


Figure 2: Comparison of cholesterol levels (a), HDL level (b), and LDL level (c) between the intervention and placebo groups before and after the trial. Data are expressed as mean ± standard error of mean (SE). p value< 0.05 was considered statistically significant. *Significantly different compared to the placebo group ($p < 0.05$). *Significantly different compared to the placebo group ($p < 0.01$). Independent t-test analysis was applied to evaluate the data.

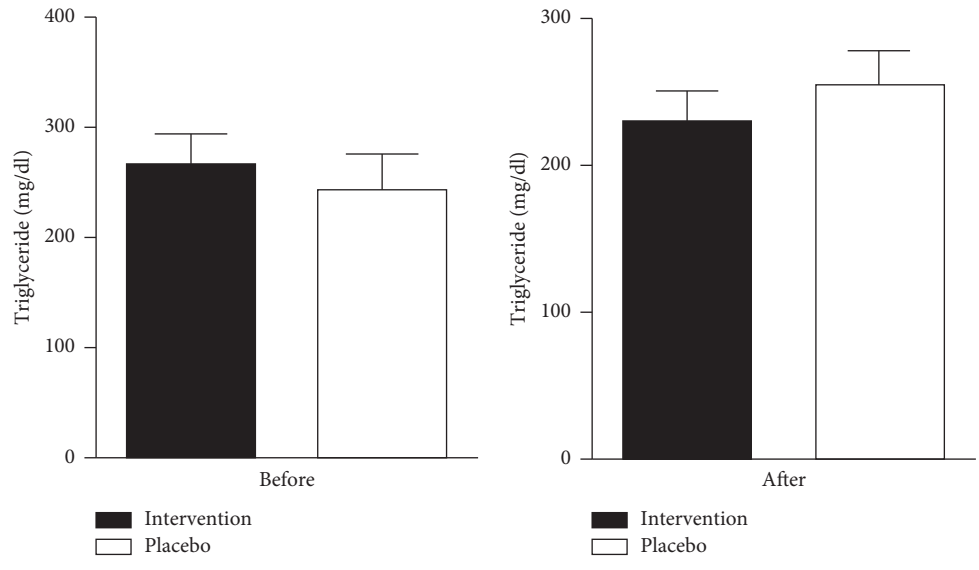
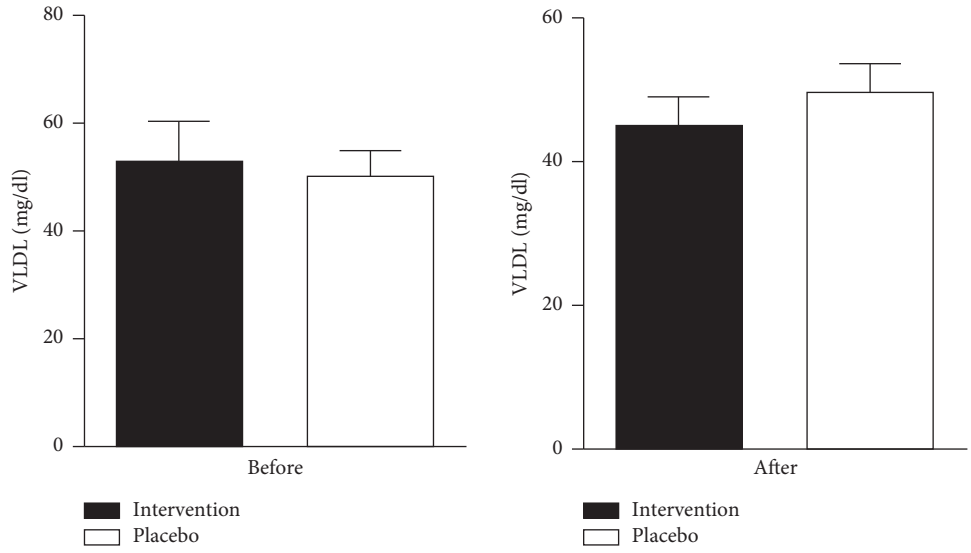


Figure 3: Continued.



(b)

Figure 3: Comparison of triglyceride levels (a) and VLDL level (b) between the intervention and placebo groups before and after the trial. Data are expressed as mean ± SE. p value< 0.05 was considered statistically significant. Independent t-test analysis was applied to evaluate the data.

4.1. Study Limitations

(1) This study was performed in a short period. Thus, an increase in time and the number of patients would help conduct more widespread research.

(2) Future studies can be improved if the analysis of the effects of this medicine on dyslipidemic patients is complemented with other metabolic disorders such as hypothyroidism or diabetes mellitus.

(3) By running this herbal compound through animal tests, consequently followed by human tests, not only can higher doses be analyzed but its independent effect can also be compared to statins.

(4) Paraclinical tests and liver ultrasonography also provide useful information through the analysis, measurement, and comparison of other biomarkers such as liver enzymes, hormonal tests, anti-oxidant, and anti-inflammatory markers.

Our findings suggest the possibility of discovering effective and safe natural polypills derived from the knowledge of PM. Polypills in nature has the advantage of being available, safe, and inexpensive. Besides, there are years of experience with such herbal remedies in traditional practice and its adoption will be easier for patients.

5. Conclusion

In this clinical trial, the effects of an herbal compound containing fenugreek, sumac, and purslane on dyslipidemic patients were assessed. The results indicated that this compound is effective in reducing cholesterol and LDL levels but cannot increase HDL levels. However, its effects on TG and VLDL levels were not statistically satisfactory. Moreover, it seems that this medication can effectively manage dyslipidemia as a complementary therapeutic used alongside currently available treatment methods. Moreover, it can be referred to as a setting for future studies.

Data Availability

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

Ethical Approval

The study was licensed by the ethics committee of the Arak University of Medical Sciences, based on the guidelines of the Declaration of Helsinki (Hong Kong revision, 1983) and good clinical practice. This study was registered under No. IRCT20141027019705N4 at the Iranian registry of the clinical trial center.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6646702, 16 pages
https://doi.org/10.1155/2021/6646702

Research Article

Preparation of Polyurethane/Pluronic F127 Nanofibers Containing Peppermint Extract Loaded Gelatin Nanoparticles for Diabetic Wounds Healing: Characterization, In Vitro, and In Vivo Studies

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Received 12 December 2020; Revised 2 April 2021; Accepted 30 April 2021; Published 15 May 2021

Academic Editor: Francesca Mancianti

Diabetic ulcer is regarded as one of the most prevalent chronic diseases. The healing of these ulcers enhances with the use of herbal extracts containing wound dressings with high antibacterial property and creating a nano-sized controlled release system. In this study, new peppermint extract was incorporated in the polyurethane- (PU-) based nanofibers for diabetic wound healing. The peppermint extract was used as an herbal antimicrobial and anti-inflammatory agent. The absorption ability of the wound dressing was enhanced by addition of F127 pluronic into the polymer matrix. The release of the extract was optimized by crosslinking the extract with gelatin nanoparticles (CGN) and their eventual incorporation into the nanofibers. The release of the extract was also controlled through direct addition of the extract into the PU matrix. The results showed that the release of extract from nanofibers was continued during 144 hours. The prepared wound dressing had a maximum absorption of 410.65% and an antibacterial property of 99.9% against *Staphylococcus aureus* and *Escherichia coli* bacteria. An in vivo study indicated on significant improving in wound healing after the use of the extract as an effective compound. On day 14, the average healing rate for samples covered by conventional gauze bandage, PU/F127, PU/F/15 (contained extract), and PU/F/15/10 (contained extract and CGN) prepared with different nanoparticle concentrations of 5 and 10 was 47.1 ± 0.2, 56.4 ± 0.4, 65.14 ± 0.2, and 90.55 ± 0.15%, respectively. Histopathological studies indicated that the wound treated with the extract containing nanofibers showed a considerable inflammation reduction at day 14. Additionally, this group showed more resemblance to normal skin with a thin epidermis presence of normal rete ridges and rejuvenation of skin appendages. Neovascularization and collagen deposition were higher in wounds treated with the extract containing nanofibrous wound dressing compared to the other groups.

1. Introduction

Recently, nano-sized drug delivery systems of herbal drugs have become an attractive topic for researchers due to their potential for enhancing activity as well as overcoming problems related to herbal medicines [1]. Polymeric nanoparticles, nanofibers, liposomes, and nano-emulsion, regarded as the novel drug delivery systems, have been widely used due to their unique advantages including bio-availability, high solubility, high pharmacological activity,

nontoxicity, distribution, sustained delivery, and protection from physical and chemical degradation [2]. The novel drug delivery system is the method that an optimum amount of drug is administered to the patient which helps to minimize drug degradation and loss. However, it is important that the drug can be delivered to the patient continuously and in appropriate time. The efficacy of a drug is depended on the amount of drug delivered to the patient. For some drugs, an optimum concentration range exists and concentrations above or below this range produce no therapeutic effect. Also, the rate of delivering a drug to the patient is important. Very slow or very fast delivery progress influences on the treatment of severe diseases. In this regard, the control release of drugs is highly suggested [3].

To create controlled release systems, two strategies can be employed: (i) mixing the target drug with scaffold precursors during fabrication [4] and (ii) incorporating drugs into micro/nanocarriers [5]. These carriers should have superior properties, such as high encapsulation efficiency, keeping the drug preserved during storage, easy administration to the target site, and presentation of a controlled release rate [6]. Biodegradable polymers such as poly (lactic acid), poly (lactic acid-co-glycolic acid), poly (ε-caprolactone), and gelatin have been used as the carrier for producing micro- and nanospheres [7]. One method for creating a controlled release system is production of crosslinked structures with high swelling ability. Tannic acid, extracted from Chinese gall, is the gallic ester of d-glucose. Due to its multiple phenolic groups that can interact with biological macromolecules, tannic acid can play an excellent cross linker role [8]. Chitosan-gelatin sponge for absorbing surgical hemostatic materials was prepared by the use of tannic acid as the crosslinking agent [9].

Gelatin is a protein obtained through the partial hydrolysis of animal collagens, and it is known as a biocompatible and biodegradable polymer which has been widely used in pharmaceuticals, cosmetics, and food products [10]. Gelatin can also be easily crosslinked because of its intrinsic protein structure with the high number of different functional groups and used as a targeted drug delivery vehicle [11]. Crosslinked gelatin/nanoparticles composite coating on microarc oxidation film for corrosion and drug release are produced [12]. Also, gelatin nanoparticles as a swelling controlled delivery system for chloroquine phosphate are produced [13].

Diabetes mellitus is one of the most prevalent chronic diseases. The healing of these kinds of ulcers is too long due to poor perfusion, inflammation, and the presence of necrotic tissue [14]. The presence of moist, warm, and nutritious in wounds environment makes it an ideal place for the growth of microorganisms. In this regard, preparation of wound dressing with high antibacterial property is needed. Delivery of antimicrobial agents and a platelet-derived growth factor via biodegradable nanofibers for repair of diabetic infectious wounds was investigated [15].

The use of herbal extracts with several therapeutic effects and few side effects is highly suggested compared to chemical and synthetic drugs [16]. Many of these extracts accelerate wound healing due to their antimicrobial, anti-inflammatory, analgesic, and tissue regeneration properties. Curcumin-loaded poly (ε-caprolactone) nanofibers are prepared, and their diabetic wound healing is investigated [17]. In addition, polyurethane/carboxymethylcellulose nanofibers containing *Malva sylvestris* extract for healing diabetic wounds is prepared by our research team previously [14]. *Mentha piperita*, known as peppermint, has been widely used in folk remedies, food, cosmetics, and pharmaceutical industries as an antimicrobial agent. This plant also showed chemopreventive, antioxidant, and antimutagenic potential, renal actions, and antiallergenic effects [18]. It is used as an analgesic to treat headache. It has also antinematodal, antiviral, and antifungal properties [19]. Previously, peppermint essential oil was loaded into the polycaprolactone (PCL) electrospun fiber mats for wound healing [20].

The novelty of the present study can be summarized as follows: (i) the peppermint extract was not crosslinked with gelatin nanoparticles and its release behavior was not investigated, (ii) PU/F127 pluronic blend nanofibers was not prepared before, (iii) the peppermint extract was not loaded into a polymeric nanofibrous structure and its release behavior was not investigated, and (iv) the effect of peppermint extract on diabetic wound healing was not evaluated. In this study, peppermint extract was loaded in the crosslinked gelatin nanoparticles (CGN), and then, the prepared nanoparticles, beside to the extract, were incorporated on electrospun polyurethane PU/F127 nanofibers. The electrospinning method was used due to their attractive properties such as low cost, flexibility, integrability, relatively simple, and high efficacy [21].

2. Materials and Methods

2.1. Materials. Dried *Mentha piperita* L. (peppermint) was purchased from a local herbal drug market (Tehran, Iran) and was identified and deposited at the Herbarium of Pharmacy Faculty, Tehran University of Medical Sciences, Iran (PMP-1301). Gelatin, dimethylformamide (DMF), ethanol, and tannic acid were obtained from Merck. Pluronic F127 and polyurethane PU (M_w = 110,000) were purchased from Sigma-Aldrich and Cardio Tech., Japan, respectively.

2.2. Extract Preparation. Dried peppermint leaves (1000 g) were powdered and extracted by ethanol/distilled water (80/20) at ambient temperature using the maceration method in a constant time of 72 hours for three times by 6 liters solvent. The obtained extracts were combined, filtered, and dried under vacuum at 40°C.

2.3. Preparation of Crosslinked Gelatin Extract Nanoparticle. 5 g gelatin powder was dissolved in 20 mL distilled water. 1 g extract was dissolved separately in 5 mL ethanol. The solutions were mixed together and stirred for 1 hour. Different amounts (0.3, 0.6, 0.9, and 1.2 g) of tannic acid were dissolved in 5 mL distilled water, and the solution was added dropwise to the mixture of gelatin and extract during 10

minutes. After that, the mixtures were heated to evaporate solvents. The obtained product was powdered using a dry ball mill. The powder was named as CGN (crosslinked gelatin nanoparticle).

2.4. Preparation of Nanofibers. PU solution was prepared by dissolving 2.1 g PU in 30 mL DMF. 0.21 g F127 was added to the solution to increase the hydrophilicity of final nanofibers. 15% w/w extract and different amounts of 5, 10, and 20% w/w CGN were added to the PU/F127 solution, and they were stirred for 30 minutes. The prepared solutions were electrospun under a high voltage range of 16–20 kV, and the distance between tip of needle and collector is 16 cm and feeding rate of 0.6 mL/h. The electrospinning apparatus was from Fanavaran Nano-Meghyas Co. (Iran). The electrospinning was performed at ambient pressure and temperature (25°C) and relative humidity of 40%. The obtained nanofibers were placed in a vacuum oven at room temperature for 48 hours to ensure the residual solvent is removed. The abbreviations of the names used in this study are presented in Table 1.

2.5. Characterization

2.5.1. Physical and Chemical Characterization. The surface morphology of nanofibers was investigated using a field-emission scanning electron microscope (FE–SEM, Sigma, Zeiss Germany). Particle size and particle size distribution were determined by image analysis using an optical microscope (using the free Scion software) on a computer equipped with the Global Lab Image software, in the optical microscope, Jenaval–Carl Zeiss (Germany). The contact angle between nanofiber mats and the liquid phase was measured using the static digital method described in the standard D5725-97 (ASTM, 2003). To do this, an optical microscope (Olympus SZ-STU2) equipped with a digital camera (Olympus Camedia C-3040) was used. The FTIR spectrum of samples was examined by the FTIR spectroscopy (ThermoNicolet NEXUS 870 FTIR from Nicolet Instrument Corp., USA). The thermal degradation analysis (TGA) of the samples was performed on a TGA-PL thermoanalyzer from UK. In each case, a 5 mg sample was examined under N_2 at a heating rate of 5°C/min from room temperature to 650°C.

2.5.2. Assessment of Swelling Ratio. The swelling ratio (fluid absorption) of nanofibers was calculated as the following equation:

$$\text{Swelling ratio}\,(\%) = \frac{M - M_d}{M_d} * 100, \tag{1}$$

where M and M_d are the weights of the swollen samples and the dried samples in an oven at 40°C. Swollen sample was prepared by immersing 0.1 g of nanofibers in 50 mL of phosphate buffered saline (PBS, pH 7.4) at 35 ± 0.1°C for 24 hours.

2.5.3. Assessment of Total Phenolic and Flavonoid Contents. In order to determine the total phenolic content, the Folin–Ciocalteu reagent was used [22]. To do this, 1 mL of the sample was mixed with 7.5 mL of distilled water and 0.3 mL of Folin–Ciocalteu's phenol (diluted 1 : 10). After 3 min, 1 mL of Na_2CO_3 solution (20%, w/v) was added to the mixture. The mixture was kept in a dark place for 1 hour, and then, its absorbance was measured at 760 nm using a single beam UV spectrophotometer. Results were expressed as gallic acid equivalents (mg of gallic acid (GAE)/mg dry weight extract).

The colorimetric method was used to evaluate the total flavonoid content [23]. In this method, different concentrations of a standard solution of catechin were added to 1 mL distilled water. Then, 75 ml of $NaNO_2$ solution (5%) was added to the mixture. After 5 min, 75 mL of $AlCl_3$ (10%) was added, and 6 min later, 500 mL of NaOH (1 N) was mixed to the solution. The mixture was diluted by adding 2.5 mL of distilled water, and its absorbance at the wavelength of 510 nm was determined using a spectrophotometer. Total flavonoid content was expressed as mg catechin equivalents (CE/g dry weight).

2.5.4. Assessment of Release Behavior. The release behavior of effective compounds from nanofibers was investigated by immersing the nanofibers (0.1 g) in 40 mL of phosphate buffered saline (PBS, pH 7.4 at 37°C) in the time range of 0–160 h. The Folin method was used to investigate the amount of released compounds.

2.5.5. Assessment of Water Vapor Transmission Rate. ASTM E96-00 standard was used to determine the water vapor transmission rate (WVTR). To do this, nanofibers were sealed over the circular opening of glass tubes filled with 10 ml distilled water. The diameter of tube was 13 mm. Then, it was placed in a chamber containing a saturated solution of ammonium sulfate at the constant temperature and humidity of 37°C and of 85%, respectively. The WVTR ($g/m^2 \cdot day$) was determined as

$$WVTR = \frac{W_i - W_t}{A \times t}, \tag{2}$$

where A, T, W_i, and W_t designated the area of tube mouth (m^2), the trial time, and the weights of trial before and after, respectively.

2.5.6. Assessment of Antibacterial Activity. The colony counting method was used to evaluate the antibacterial activity of nanofibers against both Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC 6538) and Gram-negative *Escherichia coli* (*E. coli*, ATCC 8739) (33 m1). In this method, 60 mg of nanofibers was placed into 5 mL sterilized Luria–Bertani (LB) broth solution with the concentration of 1.5×10^5 colony-forming units (CFU) of bacteria. The mixtures were cultured at 37°C in a shaking incubator for 12 hours. One hundred microliters of each of these cell solutions was seeded onto LB agar using a surface spread plate

Table 1: The abbreviations of the names.

Name	Polyurethane	Polyurethane/ Pluronic F127	Polyurethane/Pluronic F127/ Dried peppermint extract (containing 15% w/w extract respect to PU)	Crosslinked gelatin nanoparticles	Polyurethane/Pluronic F127/Dried peppermint extract (15% w/w)/ Crosslinked gelatin nanoparticles (containing 10% w/w CGN) containing 0.9 g tannic acid and 1 g peppermint extract
Abbreviation	PU	PU/F	PU/F/15	CGN	PU/F/15/10

technique. The plates were incubated at 37°C for 24 hours. The numbers of bacterial colonies (CFU) were counted. Pure phosphate-buffered saline (PBS) was also tested as blank control.

$$\text{Antibacterial activity}\,(\%) = \left(\frac{B-T}{T}\right) * 100, \tag{3}$$

where T = cfu*/ml of the test sample; cfu, concentration of colony of bacteria; B = blank sample, and cfu*, colony-forming unit. The MICs of the extracts were determined by the agar dilution method.

2.5.7. MTT Assay. The cell culture investigation was conducted by the protocol reported previously [24]. The human umbilical cord matrix (hUCM) cells were seeded at a seeding density of 1×10^6 per well/1 mL medium in 96-well flat-bottom plates. After 24 h, the cultured cells were treated with different samples and free peppermint extract (dissolved in DMSO, the final DMSO concentration < 0.1%). Medium with 0.1% DMSO and control nanofibers were used as controls. Cytocompatibility of the wound dressings were characterized by using 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT). Detecting of the formazan solution was conducted by a single beam UV spectrophotometer (CE-CIL CE2021) at 570 nm.

2.6. Preparation of Diabetic Rats and the Wound Healing Test. Male Wister weighing 150–200 g were used for diabetic experiments which were conducted according to Tehran University of Medical Sciences ethical guidelines for the care and use of laboratory animals (the ethical permission code is IR.TUMS.VCR.REC.1397.545). A single intraperitoneal injection of 70 mg/kg bodyweight of sterile streptozotocin (STZ) (Sigma, St Louis, MO, USA) in sodium citrate (0.1 mol/L, pH 4.5) was performed for inducing diabetes in rats. Animals were anesthetized by intraperitoneal injection of xylazine (13 mg/kg) and ketamine (66.7 mg/kg). The dorsal hair of rats was shaved, and 1.5 cm diameter full thickness wounds were created with a biopsy punch. The rats were randomly divided into four groups—each with five rats. The animal wounds covered by PU/F/15 and PU/F/15/10 nanofibers were classified as group 1 and group 2, respectively. The controls rats in groups 3 and 4 were treated with PU/F nanofibers and conventional gauze bandage, respectively. Wound dressings were changed every 4 days.

The healing rate was calculated with the following equation:

$$\text{Healing rate}\,(100\%) = \frac{\text{primitive area} - \text{nonhealing area}}{\text{primitive area}} * 100. \tag{4}$$

Wound observations were performed on rats that were sacrificed 3 days ($n = 5$), 7 days ($n = 5$), 14 days ($n = 5$), and 21 days ($n = 5$) after treatment. The wound dressings were changed every 4 days.

2.7. Histopathological Analysis. The animals from each group were euthanized 7, 14, and 21 days posttreatment, and the skin tissues were harvested and immediately fixed in the 10% neutral buffered formalin (PH = 7.26) for 48 h. The fixed tissue samples were then processed, embedded in paraffin, and sectioned to 5 mm thickness. Finally, the sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). The histological slides were evaluated by the independent reviewer, using light microscopy (Olympus BX51; Olympus, Tokyo, Japan). Epithelialization, inflammatory cell infiltration, fibroplasia, and granulation tissue formation are assessed in different groups, comparatively.

2.8. Histomorphometry Analysis. On day 21, epithelialization was evaluated by a semiquantitative method. To do this, a 5-point scale, 0 (without new epithelialization), 1 (25%), 2 (50%), 3 (75%), and 4 (100%), was used. Evaluations were also scored in respect to angiogenesis according to the number of new vessels within the scar tissue, using a 5-point scale as follows: 0 (none), 1 (few), 2 (moderate), 3 (many), or 4 (considerably). Histomorphometric analysis was used to investigate neovascularization and collagen density. All experiments were analyzed using a computer software Image-Pro Plus® V.6 (Media Cybernetics, Inc., Silver Spring, USA).

2.9. Statistical Analysis. All results were compared using Kruskal–Wallis analysis. Results with P values of less than 0.05 were considered statistically significant. Statistical analyses were performed using the SPSS software, version 20.0 (SPSS, Inc., Chicago, USA).

3. Result and Discussion

3.1. Microscopic Analysis. The FE-SEM images of CGN, PU, PU/F, PU/F/15, PU/F/15/5, and PU/F/15/10 at different magnifications are shown in Figure 1. As can be seen, the shape of CGN was mostly spherical, and they had smooth surface. Particle size distribution curve of nanoparticles

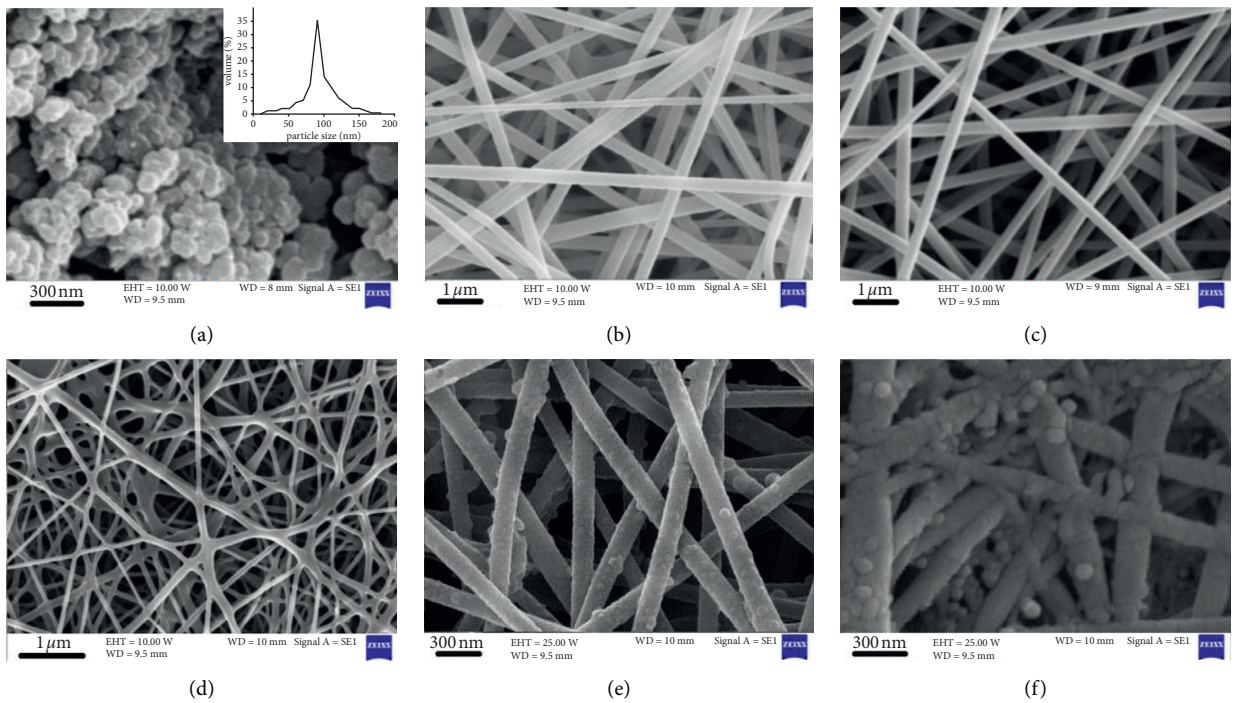


Figure 1: FE-SEM images of (a) CGN (inset image: pore size distribution curve of CGN), (b) PU, (c) PU/F, (d) PU/F/15, (e) PU/F/15/5, and (f) PU/F/15/10.

(inset of Figure 1(a)) showed a relatively narrow structure centered at 91 nm. PU nanofibers had smooth surface, and the average nanofibers diameter was 170.7 nm. After the addition of F127 into the PU matrix, the average nanofibers diameter increased to 176 nm. This can be due to the interaction of F127 with PU chains that resulted in increasing the spinning solution viscosity. The changes of the viscosity as a function of the shear rate for different spinning solutions at 25°C are shown in Figure 2. It was clear that the viscosity of solutions was increased after the addition of F127, extract, and CGN into the PU matrix. The increase of solution viscosity was more pronounced after the addition of CGN. This can be related to the stronger interaction of CGN with PU polymer due to higher number of functional groups in the gelatin structure. The shear thinning effect was observed for all samples. At low shear rates, the polymer chains absorb most of the applied energy, and they resist orienting in the flow direction resulting in high viscosity values. With increasing the shear rates, the structure of the polymer chains starts to break down and flows finally. The viscosity of PU, PU/F, PU/F/15, PU/F/15/5, PU/F/15/10, and PU/F/15/20 solutions at the shear rate of 10° was 1.56, 1.62, 1.8, 2.08, 2.2, and 2.5 Pa.s, respectively. According to Figure 1, for PU/F/15, some conglutinations especially at the touching points of nanofibers were seen. The presence of some nanoparticles on the surface of PU/F/15/5 and PU/F/15/10 nanofibers was detected. Nanofibers were not obtained when the CGN concentration was 20% w/w. This was related to the high viscosity value of spinning solution that resulted in unbalance condition of electrostatic repulsion, surface tension, and viscoelastic forces [25]. The number of nanoparticles on the nanofiber surface was increased when the CGN

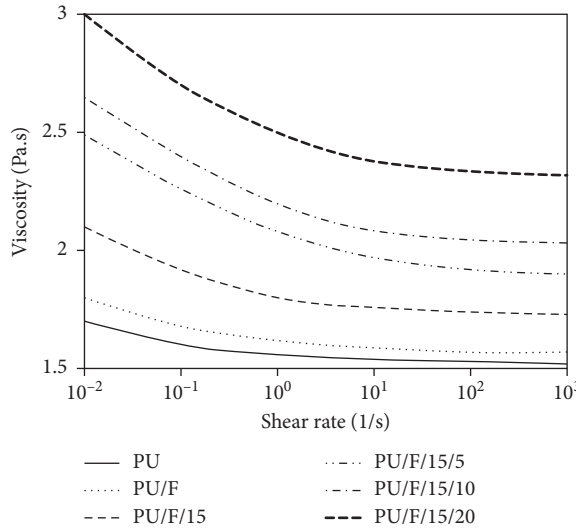


Figure 2: The changes of the viscosity as a function of the shear rate for different spinning solutions at 25°C.

concentration in the spinning solution increased from 5% to 10% w/w. The average diameter of PU/F/15/5 nanofibers was 253.4 nm.

TEM images of nanofibers are shown in Figure 3. It was clear that the F127 was dispersed into the PU matrix (Figure 3(a)). For PU/F/15 sample, a homogenous structure with some conglutinations was observed. It was found that the addition of extract in polymer matrix enhanced the interactions between PU and F127. Also, the presence of CGN in whole parts of PU/F/15/10 nanofibers was observed, indicating a good dispersion ability of CGN which can be

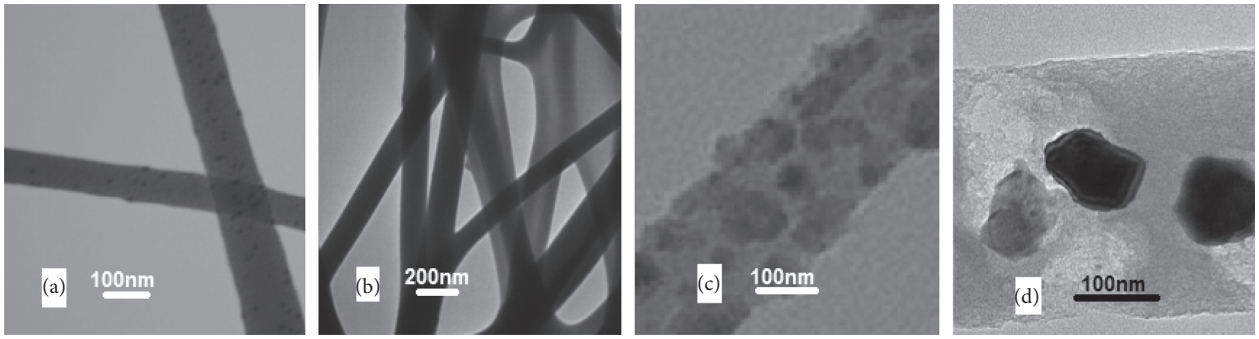


Figure 3: TEM images of (a) PU/F, (b) PU/F/15, and (c-d) PU/F/15/10.

related to the high functional group density of gelatin. The diameter of PU/F/15 and PU/F/15/10 obtained by TEM analysis was 210.5 and 278 nm, respectively.

3.2. FTIR Analysis. The FTIR spectra of gelatin, CGN, PU/F, and PU/F/15/10 are presented in Figure 4. For gelatin, bands appeared at 1655 cm^{-1} and 1548 cm^{-1} were an indication of stretching vibration of the C=O bond (amid I) and coupling of bending vibration of the N-H bond and stretching vibrations of C-N bonds (amid II), respectively [26]. The band at 1655 cm^{-1}, related to vibrations of amide I was split due to both random coil and α-helix conformation of gelatin [27]. The peak at 3384 cm^{-1} was related to the overlap of stretching vibrations of OH and NH groups. The asymmetric and symmetric stretching vibrations of methylene groups in the glycine and proline were reflected at 2968 cm^{-1} and 2904 cm^{-1}, respectively [28]. The band at 1255 cm^{-1} was assigned to the overlapped stretching vibrations of C-N and N-H deformation from amide linkages. The peak observed at 1240 cm^{-1} was related to the asymmetric COC stretching vibrations [29].

After the crosslinking process, the bands at 3384 and 1255 cm^{-1} were shifted to the 3314 cm^{-1} and 1247 cm^{-1}, respectively, which can be an indication of hydrogen bonding and protein conformation [30]. The peak appeared at 810 cm^{-1} attributed to the bending vibrations of C-H groups of phenyl rings. The peak at 615 cm^{-1} is assigned to CH out of plane bending vibrations of ethylene systems [31]. The appeared band at 1663 cm^{-1} was related to the stretching vibration of C=C in aromatic rings of phenolic compounds. The appeared band at 1490 cm^{-1} was attributed to the stretching vibration of C-C in aromatic rings of tannic acid [32].

For PU/F, the bands at 3338 cm^{-1} were related to the overlapped stretching vibration of OH and NH groups. The bands at 2977, 1715, and 1260 cm^{-1} were assigned to the stretching vibrations of C-H, C=O, and C-O in PU, respectively. The peak at 1220 cm^{-1} and 1555 cm^{-1} were attributed to the stretching vibration of C-O-C and bending vibration of the NH group, respectively.

For PU/F/15/10, the peaks appeared at 811 cm^{-1} and 612 cm^{-1} were related to the bending vibrations of C-H groups of phenyl rings and out of plane bending vibrations of CH groups of ethylene systems, respectively [31]. The appeared band at 1662 cm^{-1} was related to the stretching vibration of C=C in aromatic rings of phenolic compounds. Bands appeared at 1647 cm^{-1} and 1538 cm^{-1} were assigned to presence of CGN in the polymer matrix.

For this sample, the bands at 1224 cm^{-1} and 1268 cm^{-1} were intensified which indicated that the incorporated extract might have interacted with the ether groups of polymers through hydrogen bonds [33]. Also, the band, related to the vibrations of OH and NH groups, was shifted to lower wavenumbers (3297 cm^{-1}). Shifting of bands towards lower wavenumbers was also detected for the bands at 1647 cm^{-1} and 1538 cm^{-1}. Furthermore, the intensity of bands at 3297 cm^{-1} and 1628 cm^{-1} (related to the bending vibration of OH groups) was decrease compared to PU/F curve. These observations indicated on taking place interactions between extract ingredients' functional groups with hydroxyl and amino groups of polymers. Also, some interactions that occurred between CGN and PU resulted in increasing the solution viscosity. The presence of extract was confirmed by observing the peak at 3052 cm^{-1} which can be related to the stretching vibration of CH in aromatics. It was suggested that the extract and CGN play a crosslinking role in PU matrix.

3.3. TGA Analysis. The results of TG and DTG analyses, performed on different nanofibers, are shown in Figure 5. As can be seen, all curves had a similar 3-step structure including initial, main, and char decomposition. At the initial step (25.5–150°C), the weight loss for PU/F, PU/F/15, PU/F/15/5, and PU/F/15/10 was 14.34%, 12.7%, 15.78%, and 18.11%, respectively. The weight loss in this step was due to some physical damages, occurred in polymer chains with amorphous phases and the removal of the physically adsorbed water molecules [34]. The second step was the main thermal region in which the temperature ranged from 150°C to 400°C. In this step, the weight loss was related to the removal of chemisorbed water and degradation of polymer chains. It was clear that the thermal resistance was enhanced after the addition of CGN into the polymer matrix. This can be due to higher amounts of adsorbed water molecule (due to higher absorption capability of CGN containing nanofibers) and interaction of CGN, extract, and polymer together. Previously, it is reported that the presence of herbal extract in polymer matrix increases the hydrogen bonds of

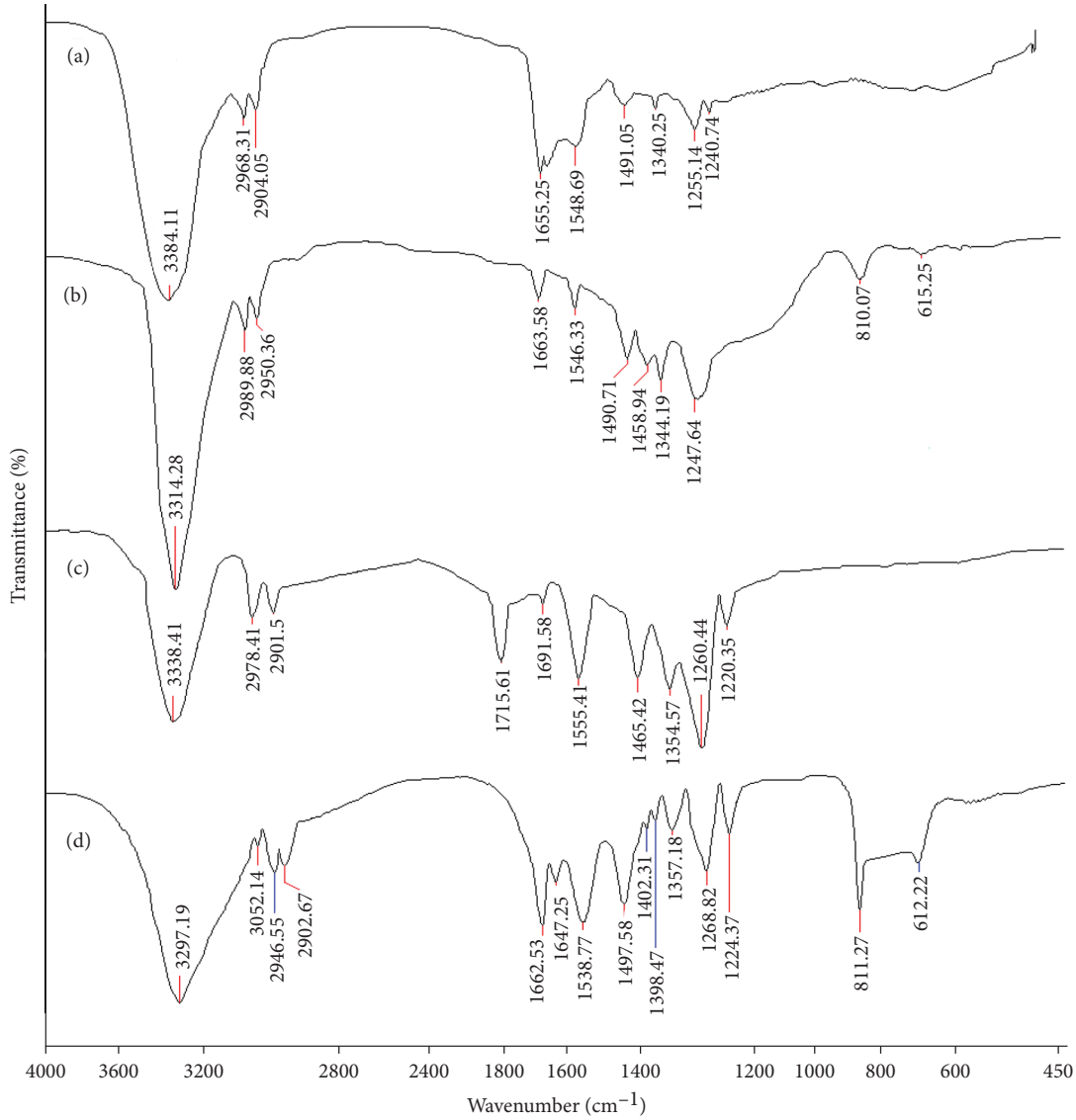


Figure 4: FTIR spectra of (a) gelatin, (b) CGN, (c) PU/F, and (d) PU/F/15/10.

the components [35]. As mentioned in the FTIR section, the extract and CGN played a crosslinking role in the carrier polymer. Production of char occurred at the third step where the temperature was higher than 400°C. In this step, the release of chemisorbed water was continued and also carbon dioxide molecules were produced.

3.4. Mechanical Properties. The stress strain curves of PU/F, PU/F/15, and PU/F15/10 samples are presented in Figure 6. It was found that the tensile strength and the elongation at break values of nanofibers enhanced due to the adhesive property and the plasticizer effect of extract [14, 36]. For PU/F/15/10 nanofibers, the tensile strength value was greater than the value for PU/F/15 sample, while the elongation at break value decreased compared to PU/F/15 nanofibers. As mentioned in the FTIR section, the crosslinked gelatin nanoparticles interacted with PU and herbal extract. Increasing the crosslinking degree resulted in decreasing the elongation property of nanofibers. Poor mechanical properties may generate some damage in the regenerated tissues at the change of wound dressing [37].

3.5. Antibacterial Properties. Since chronic wounds remain open for long periods, they are highly exposed to the risk of bacterial infection. For these wounds, microorganisms can colonize and form a biofilm in the wound bed which develops high resistance against the antimicrobial agents and immune system [38]. The presence of a biofilm in the majority of chronic wounds is reported by researchers [39]. In this study, the results of the antibacterial test (Figure 7) showed that the PU/F nanofibers had no antibacterial property, while samples containing herbal extract had 99.9% antibacterial activity against both Gram-positive and Gram-negative bacteria. It is stated that the peppermint essential oil is made up of menthol, menthone, menthyl esters, and further monoterpene derivatives (pulegone, piperitone, and

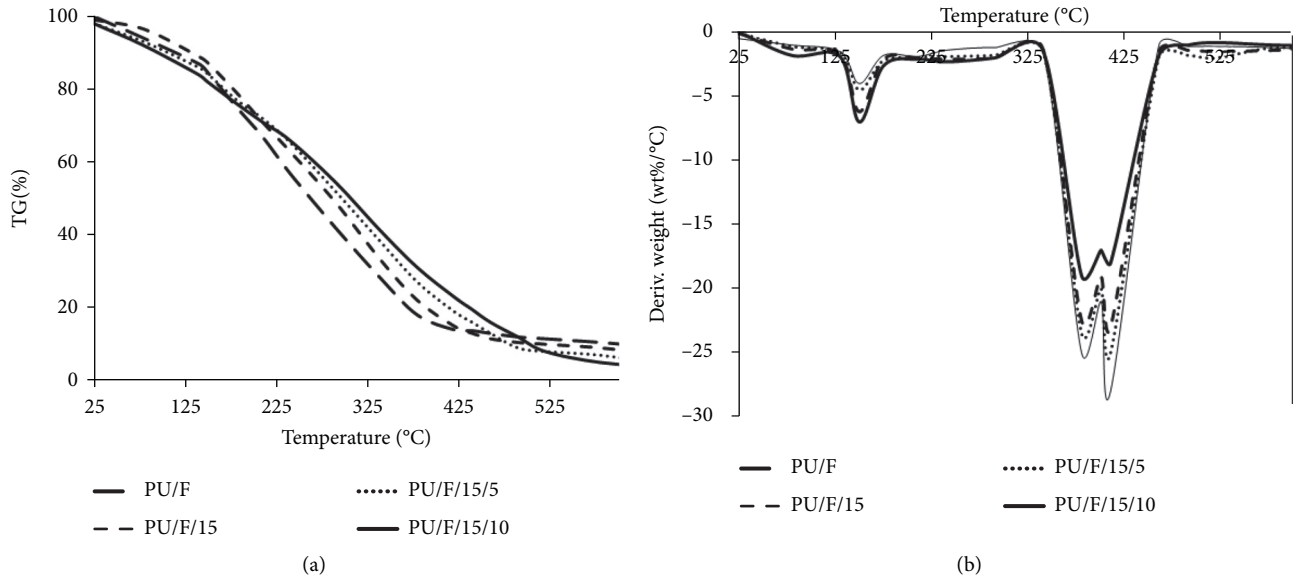


Figure 5: (a) TG and (b) DTG curves of different nanofibers.

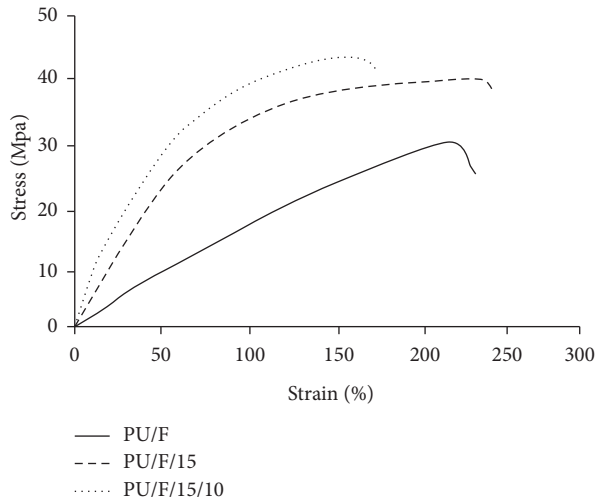


Figure 6: The stress strain curves of different nanofibers.

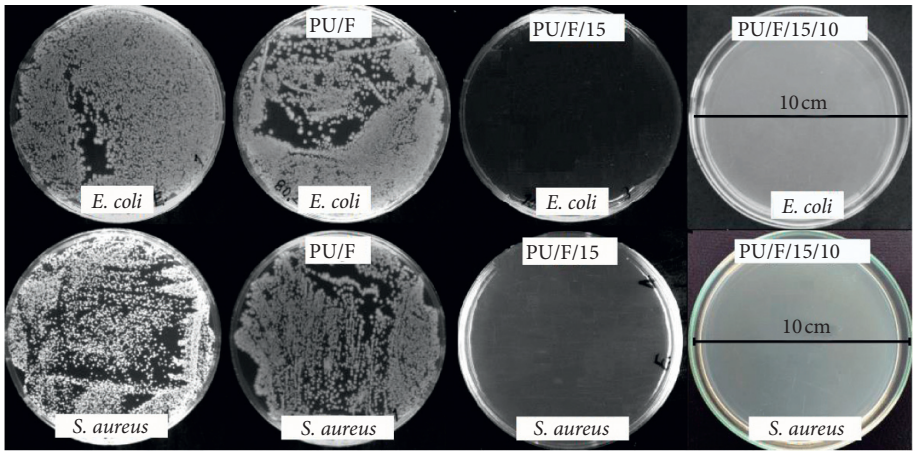


Figure 7: Antibacterial activity of different nanofibers.

menthofuran) [40, 41]. Phenolic compounds that significantly contributed to the antibacterial activity of the herbal extract through the enzyme inhibition resulted from the reaction of phenolic compounds with sulfhydryl compounds or interactions with the protein [42]. Total phenolic content of the extract, determined by the Folin–Ciocalteu reagent, was 224.8 mg GAE/g. Total phenolic content is highly dependent on the solvent, used for extraction, the water content, and the time of extraction [43]. Phenolic constituents of peppermint extract were investigated previously, and quantity of compounds including caffeic acid, eriocitrin, luteolin-7-O-glucoside, naringenin-7-O-glucoside, hesperidin, isorhoifolin, rosmarinic acid, eriodictyol, and luteolin was determined [44]. Also, flavonoids synthesized by plants are effective against bacterial infections due to their ability to form complex with extracellular soluble proteins and with bacterial cell walls [45]. Total flavonoid content for peppermint extract was 200.47 mg CE/g. The MICs value against *S. aureus* and *E. coli* bacteria was 0.26 ± 0.01 and 0.29 ± 0.01 mg/ml, respectively. It was found that *S. aureus* was more sensitive to herbal extract than *E. coli* which can be related to variation in the structure of the cell wall [46].

3.6. Fluid Absorption and WVTR Tests. Ideal wound dressings should have superior properties including (i) absorbing excessive exudates, (ii) protecting the wound from bacterial infiltration, (iii) permeability of gaseous and fluid, (iv) removal ability without trauma, and (v) being nontoxic and nonallergenic [47]. Exudates facilitate the wound healing process; however, excessive levels of exudates result in tissue maceration and infections [48]. In this regard, regulation of moist wound is very important. The fluid absorption (%) values for PU, PU/F, PU/F/15, PU/F/15/5, and PU/F/15/10 were 2.77, 328.41, 316.73, 388.71, and 410.65%, respectively. Conventional PU wound dressings are nonabsorbent due to nonporous structure and hydrophobic nature of PU [49]. In this study, low absorption value of PU nanofibers can be attributed to the porous structure of nanofibers which enables the fluid molecules to penetrate into the voids existing among the nanofibers. It was clear that the addition of F127 into the PU matrix increased significantly the absorption value due to hydrophilic nature of F127. It was also discovered that the addition of extract into the nanofibers only slightly decreased the absorption percentage. This was related to the increased average nanofibers diameter. The presence of CNG in nanofibers enhanced the absorption percentage due to high absorption capacity of CGNs. The absorption value for CNGs prepared with 0.9% tannic acid was 494.26%. The hydrophilic nature of different nanofibers was investigated by the contact angle test. The water contact angle for PU, PU/F, PU/F/15, PU/F/15/5, and PU/F/15/10 nanofibers was 98.5, 25, 25.4, 20, and 19.2°, respectively. The results indicated that the addition of F127 significantly increased the hydrophilicity of nanofibers. Also, the presence of CGN on the nanofiber surface decreased the water contact angle values due to hydrophilic nature of CGN. Decreasing the water contact angle and enhancing the hydrophilicity property of PU nanofibers after the addition of herbal extract were reported by researchers [50].

The average WVTR values for PU, PU/F, PU/F/15, PU/F/15/5, and PU/F/15/10 were 357.24, 1880.63, 1854.05, 2095.24, and 2255.47 $g/m^2 \cdot$day, respectively. Low WVTR value of PU nanofibers was related to the hydrophobic nature of polymer and low porosity of nanofibers. The porosity of PU, PU/F, PU/F/15, PU/F/15/5, and PU/F/15/10 samples was 59, 63.55, 69.4, 70.9, and 72.11%, respectively. Finally, it was found that the hydrophilicity of nanofibers played a more significant role than porosity in enhancing the WVTR values.

3.7. Release Behavior of Samples. The release behavior of samples was investigated, and the results are shown in Figures 8(a) and 8(b). In this study, beside to CGNs, the extract was mixed with polymer matrix, and its release behavior was investigated. In such systems, the pore size, crosslinking density of scaffold, and additives along with scaffold degradation could influence on the drug release rate. In this regard, at first, the release behavior of CGN was investigated, and the results are shown in Figure 8(a). As can be seen, CGN showed a gradual release up to 81.2% of the total amount of loaded extract during 72 h, and the apparent loss was related to covalent binding of herbal compounds to the polymer matrix. Such a result is reported by researchers [51]. From the figure, it was obvious that the released amount of extract was dependent on the tannic acid concentration in crosslinking procedure. With increasing the content of tannic acid from 0.3% to 1.2%, the amount of released extract from nanoparticle decreased from 97.8% to 81.21%. The solubility (%) of CGN prepared with different amounts of 0.3, 0.6, 0.9, and 1.2% tannic acid was determined as 21.14, 10.75, 0.1, and 0.09%, respectively. It was concluded that the gelatin structure was fully crosslinked when the amount of tannic acid was higher than 0.9%. The result showed that the increase of CGN crosslinking degree resulted in increasing the releasing time up to 72 h. The equilibrium time point of CGN prepared with 0.3, 0.6, 0.9, and 1.2% tannic acid was 28, 52, 72, and 72 h, respectively. In this regard, the amount of 0.9% tannic acid was selected as the optimum. Figure 8(b) shows the release behavior of different nanofibers. It was obvious that the release profile of PU/F/15 presented a three-step structure. At the first step (0–36 h), a quick release of extract was seen, and the amount of released extract was 56.02%. The entrapped extract on the surface of nanofibers is responsible for the intense slop of the release profile during the first 36 h. The incorporation of extract on the nanofiber surface is attributed to the limited physical interactions between the extract and the polymer matrix in the electrospinning process [22]. However, the presence of F127 with the large number of ethylene oxide groups increased the hydrophilic property of nanofibers. This was resulted in increasing the extract diffusion in the polymer matrix, and the release of extract was continued in longer times. The amount of released extract in the second step was 17.15%, and the equilibrium point was 62 h. For PU/F/15/10 sample, the amount of released extract showed a

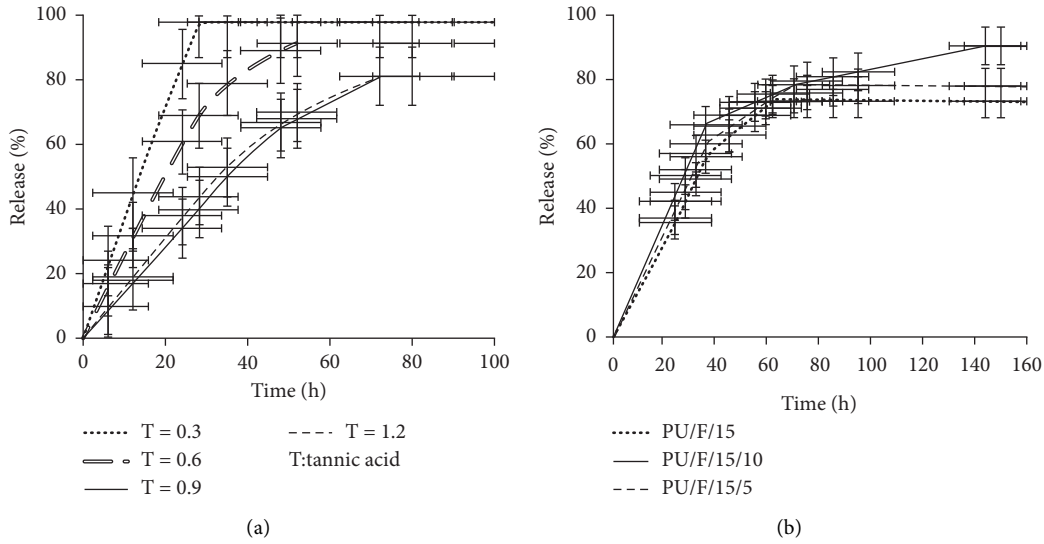
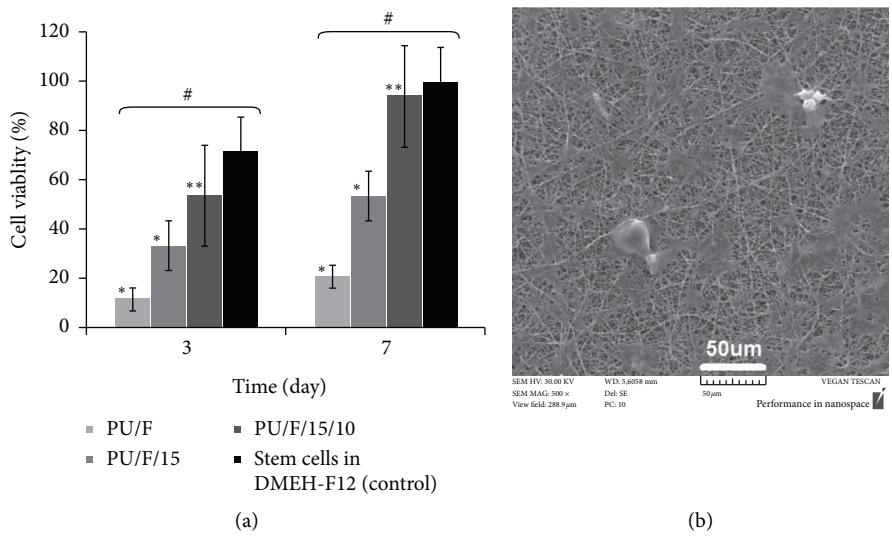


Figure 8: Release behavior of herbal compounds from (a) CGN and (b) different nanofibers.

17.8% increase at the time of 36 h. This increase was attributed to the released herbal compound from nanoparticles distributed on the surface of nanofibers. After 62 h, the releasing of herbal compounds was continued for the next 82 h, and the equilibrium point was achieved at 144 h. At the initial hours, the detected extract in the solution was attributed to the mixed extract with polymer blend. After that, the swelling of nanofibers caused penetration of water molecules into the inner layers of polymer and nanoparticles. Thus, the swelling of nanoparticle was begun at longer times that resulted in gradual release of extract during 62–144 h. The total release amount of peppermint extract for PU/F/15/10 was 17.42% higher than PU/F/15. It is reported that the drug release from gelatin nanoparticles can be occurred according to three mechanisms including desorption, diffusion, and biodegradation of nanoparticles [52]. It was suggested that desorption of surface-bound/adsorbed and diffusion through the carrier matrix were the main mechanisms in releasing herbal compounds from nanoparticles. Fully crosslinking of gelatin nanoparticles caused to emerge a controlled release behavior of nanofibers. Similar behavior was observed for PU/F/15/5. The amount of released extract at the time of 36 h was 60.1%, and the equilibrium time point was 95 h.

3.8. MTT Assay. The result of the MTT test for PU/F and PU/F/15/10 at days 3 and 7 is shown in Figure 9. It was found that the absorbance values at the day 7 showed an increase of 160% and 176.6% for PU/F/15 and PU/F/15/10 compared to the absorbance values of day 3, respectively. The number of grown cells on PU/F/15/10 nanofibers was much higher than PU/F nanofibers. Also, the absorption values of PU/F/15 and PU/F/15/10 samples were 75% and 94.64% of absorption values of control samples, respectively, indicating on the nontoxic nature of the prepared wound dressing. This can be due to natural and organic nature of herbal extract. As mentioned in contact angle and WVTR analyses, the hydrophilicity of nanofibers increased after the addition of extract. Increasing the moist and porosity of the scaffold resulted in enhancing the cell compatibility and grown cells number. Furthermore, the surface of extract containing samples showed high antibacterial property, which makes these nanofibers a suitable place for cell growing. The presence of hUCM cells on the wound dressings after 7 days was investigated by microscopic analysis, and the result is shown in Figure 9(b). High accumulation of stem cells on nanofibers was an indication of nontoxic nature of nanofibers and the effectivity of this scaffold in cell proliferation.

3.9. Wound Healing and Histological Examination. Wound healing mainly involves three phases including inflammation, proliferation, and wound closure and remodeling. In diabetic patients, impaired wound healing resulted from abnormal inflammatory response and insufficient fibroblast proliferation. A representative wound on an animal in each group and the wound healing percentages of diabetic rats on days 0, 3, 7, 14, and 21 after treatment are shown in Figures 10 and 11, respectively. As can be seen, there were no significant changes in wound area for sample covered with gauze bandage on day 3. This was related to the lack of moisture on the surface of the wound caused to generate some damage in the regenerated tissues at the change of gauze bandage. For the sample covered with PU/F nanofibers, better healing (higher wound closure percentage) was observed due to the higher absorption ability of PU/F wound dressing in comparison with gauze bandage that resulted in taking up wound exudates and providing a moist wound healing environment [53]. Also, relatively hindering property of the prepared scaffold from the penetration of bacteria into the wound surface can be another reason for higher efficiency of PU/F compared to gauze bandage. Delaying of the wound healing rate in wounds covered with gauze bandage and PU/F compared to the other groups was attributed to the existence of bacteria in wounds or because of their histopathological



(a) (b)

FIGURE 9: (a) The viability of wound dressings after 3 and 7 days, Data are expressed as mean ± SD from three individual experiments. *, $p < 0.05$, **, $p < 0.01$, and $^{\#}p < 0.001$. (b) SEM images of hUCM cells after 7 days seeded on PU/F/15/10 nanofibers.

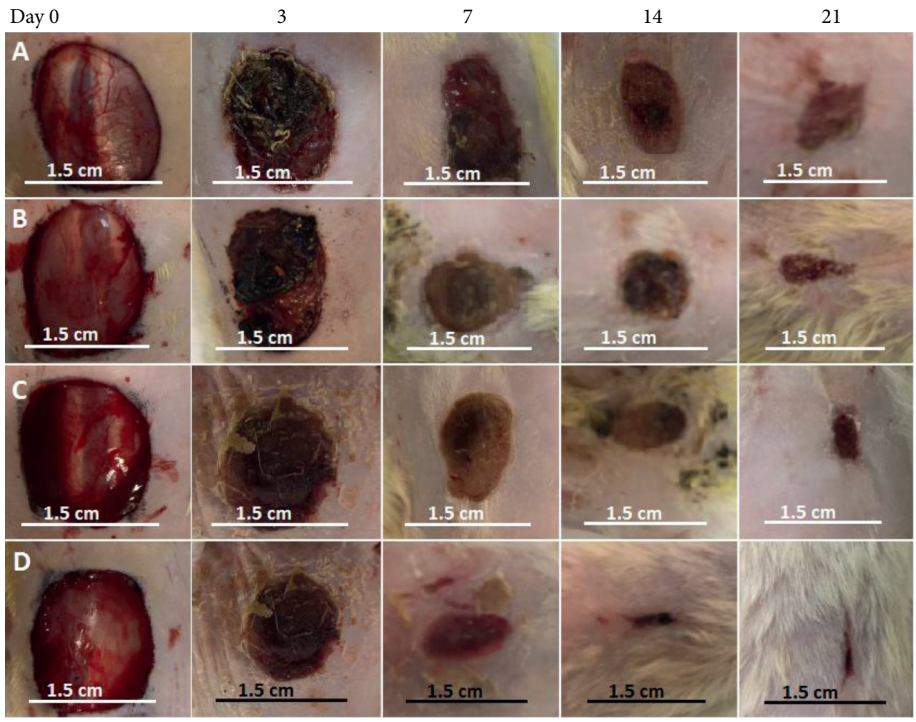


FIGURE 10: Representative wounds on an animal on days 0, 3, 7, 14, and 21 after treatment for (a) conventional gauze bandage, (b) PU/F, (c) PU/F/15, and (d) PU/F/15/10.

lesions [54]. The wound healing percentages of diabetic rats on day 3, treated with gauze bandage, PU/F, PU/F/15, and PU/F/15/10, were 2.55 ± 0.1, 5.84 ± 0.2, 10.11 ± 0.1, and $12.75 \pm 0.25\%$, respectively. The results showed that the wound contraction process occurred rapidly for wound treated with extract containing wound dressings compared to the other dressings. This can be related to reducing the inflammation phase time and acceleration in entry into the proliferation phase [55]. The wounds treated with PU/F/15 and PU/F/15/10 showed a significant closure on day 7 compared to other wounds. The wound closure for these samples was 40.22 ± 0.5 and $59.47 \pm 0.2\%$, respectively. As can be seen, the healing percentage for PU/F/15/10 treated wound was much higher than PU/F/15. The results of wound healing were in agreement with the results of the release test. The release equilibrium point of PU/F/15 was 59 h, and after that, the amount of released compound was close to zero. Since the wound dressings were changed after 4 days, the possibility of growing bacteria on the wound surface increased and the effective herbal drugs was not in contact with wound.

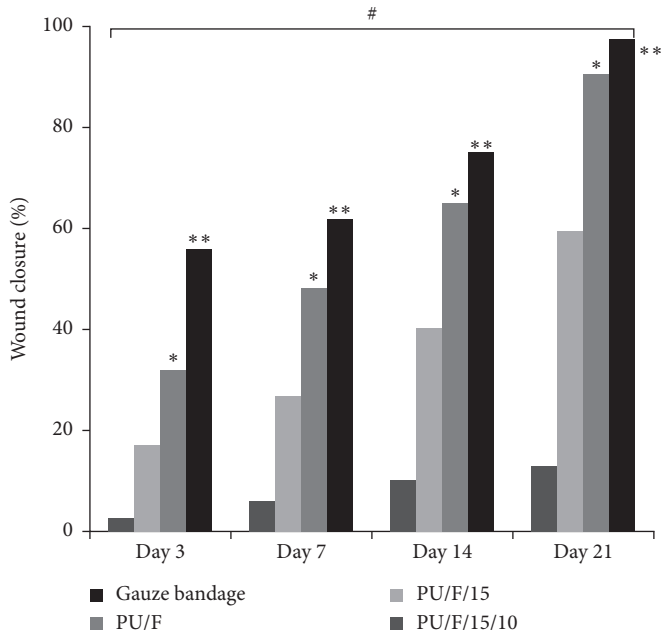


Figure 11: The effect of different wound dressings on healing of diabetic wounds. $*$, $p < 0.05$, $**$, $p < 0.01$, and $^{\#}p < 0.001$.

The wound healing percentages for samples covered with PU/F/15 and PU/F/15/10 were 65.14 ± 0.2 and $90.55 \pm 0.15\%$ on day 14, respectively. These values enhanced to 75 ± 0.22 and $97.5 \pm 0.1\%$ on day 21, respectively. It was clear that the wound healing for PU/F/15/10 treated wound proceeded faster than PU/F/15 treated wound. This can be attributed to the controlled release of herbal extract that resulted in contact of extract continually to wound surface and promote wound healing. Also, antioxidant and antibacterial properties of peppermint can facilitate the wound contraction and wound healing [49].

Histopathological images on days, 7, 14, and 21 are shown in Figure 12. For PU/F/15 and PU/F/15/10 covered samples, large number of inflammation cells was observed on day 7, while it was too low for PU/F-treated wound. Inflammation occurs at the initial step of the wound healing process, and uncontrollable inflammatory response is associated with unhealed diabetic ulcer [56]. Also, the presence of necrosis was detected for all samples. For sample covered with PU/F/15/10 nanofibers, regenerative responses and tissue granulation were observed on day 7. The granulation tissue is an important indicator for evaluating wound healing [57]. For PU/F/15 treated wound, the crusty scab covered the wound area, and the inflammation was also evident in the defect on day 14. At day 14, formation of fibroblast cell, collagen fibers, and connective fibrils were observed for PU/F/15/10-treated wound. It was reported that most of herbal drugs have anti-inflammatory property which accelerates collagen fiber development and epithelium regeneration [58]. Higher production of the collagen matrix correlates with increased scar formation. As can be seen, for PU/F/15/10 treated wound, the inflammation was significantly alleviated, and regeneration of the skin was more pronounced on day 14 compared to the other samples. For this sample, the collagen turnover was higher than PU/F/15 which was attributed to the higher released amounts of extract. The higher collagen turnover results in the higher granulation rate of tissue components [59]. A thick layer of reepithelialization and small blood vessels intermingled with fibroblastic cells were seen for PU/F/15 and PU/F/15/10-treated wounds on day 21. For PU/F/15/10, the thickness of reepithelialized layer and the number of blood vessels were higher than PU/F/15. The presence of menthol with various biological properties, such as antimicrobial, anticancer, and anti-inflammatory agent, could reduce the inflammation and facilitate of nerve regeneration [60, 61].

3.10. Histomorphometric Analysis. The histomorphometric analysis for PU/F/15 (group 1), PU/F/15/10 (group 2), and PU/F (Control, C) was performed at 7, 14, and 21 days after skin injury, and the results are presented in Tables 2 and 3. As can be seen, reepithelialization in the group treated with extract containing nanofibers (groups 1 and 2) was better than other treatment groups at day 14. The wound healing process is heavily dependent on collagen synthesis. Therefore, to further investigate the effect of different treatments on wound healing, sections of animal skin tissues were stained with MT staining (Figure 11). This staining was used to recognize the progress of collagen synthesis during GT formation and matrix remodeling. Collagen fibers were stained blue-green in the MT staining method, in which the intensity of this color corresponds to the relative amount of deposited total collagen and reflects the advancement of collagen synthesis and remodeling. The results indicated that the groups 1 and 2 had the greatest collagen synthesis. In contrast, the rate of collagen fiber synthesis and deposition in the wound were the lowest in group C. Reducing the inflammation phase time, accelerating in entering to the proliferation phase, tissue granulation, and wound contraction and higher collagen production were assigned to healing mechanism.

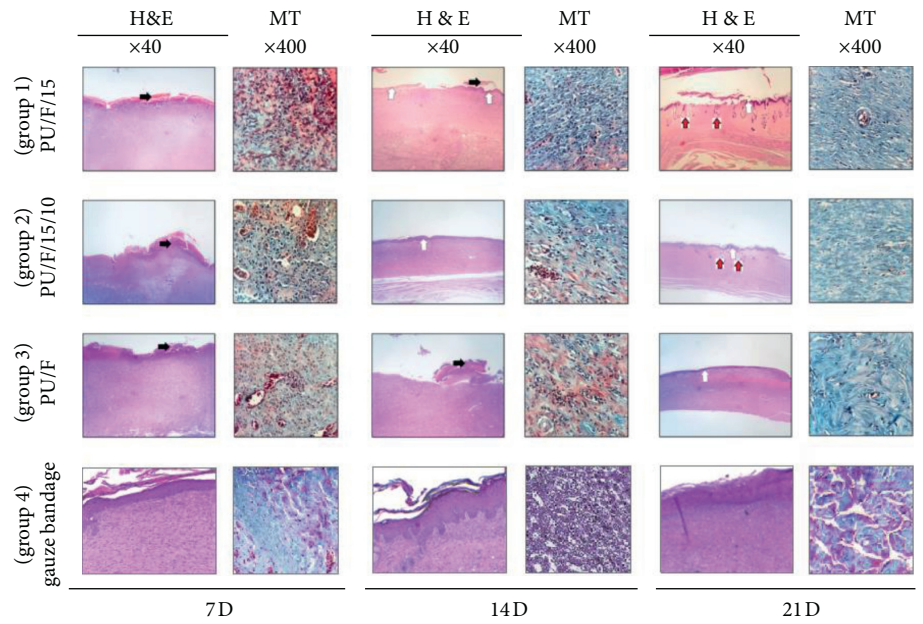


Figure 12: H&E and MT stained microscopic sections of healed incisions in treatment groups, Black thick arrows, crusty scab; white thick arrow, epidermal layer; red thick arrows, rejuvenation of skin appendages.

Table 2: Histomorphometric analysis of different experimental groups.

Group	Angiogenesis	Epitheliogenesis (score)
1 (PU/F/15)	1 (7 d) 1 (14 d) 2 (21 d)	0 (7 d) 1 (14 d) 4 (21 d)
2 (PU/F/15/10)	1 (7 d) 2 (14 d) 3 (21 d)	1 (7 d) 3 (14 d) 4 (21 d)
3 (PU/F)	1 (7 d) 1 (14 d) 2 (21 d)	0 (7 d) 0 (14 d) 3 (21 d)
4 (gauze bandage)	0 (7 d) 1 (14 d) 2 (21 d)	0 (7 d) 0 (14 d) 2 (21 d)

Table 3: Histomorphometric analysis of different inflammatory cells and collagen deposition (%).

Group	Inflammatory cells/3HPF	Collagen deposition (%)
1 (PU/F/15)	158 (7 d) 101 (14 d) 42 (21 d)	34.3 ± 3.0 (7 d)$*$ 55 ± 5.5 (14 d)$**$ 80.3 ± 3.5 (21 d)
2 (PU/F/15/10)	132 (7 d) 83 (14 d) 23 (21 d)	39.6 ± 7.5 (7 d)$**$ 64.6 ± 5.03 (14 d)$**$ 88.6 ± 3.7 (21 d)
3 (PU/F)	175 (7 d) 136 (14 d) 79 (21 d)	21.0 ± 5.0 (7 d) 31 ± 6.2 (14 d) 50.0 ± 4.3 (21 d)
4 (gauze bandage)	179 (7 d) 142 (14 d) 80 (21 d)	17.2 ± 1.5 (7 d) 25.5 ± 4.1 (14 d) 40.8 ± 2.8 (21 d)

$*$, $**$ values indicate the treatment group versus group C. $*$, $P < 0.05$, $**$, $P < 0.01$.

4. Conclusion

In this study, hydrophilic polyurethane-based wound dressings containing peppermint extract were prepared by electrospinning technique. The extract release was controlled by the addition of crosslinked extract-gelatin nanoparticles with addition to direct use of extract in the polymer matrix. The equilibrium releasing time and the maximum buffer absorption were 144 h and 410.65%, respectively. FTIR results indicated on generating of hydrogen bonds between the extract, CGN, and carrier polymers. Antibacterial and control release properties of the extract containing wound dressings were the reason of the fast and acceptable wound healing process. The wound closure percentage for PU/F/15 and PU/F/15/10 samples was 65.14 ± 0.2 and 90.55 ± 0.15%, respectively, on day 14. The result of MTT analysis indicated the nontoxic nature of the wound dressing. Histopathological analysis revealed that the use of extract containing wound dressings reduced the inflammation phase time and caused to accelerate entry into the proliferation phase. Also, regenerative responses, tissue granulation, and wound contraction proceeded faster than control. Formation of fibroblast cell, collagen fibers, and connective fibrils were more observable in extract containing scaffold treated wound. Higher production of the collagen matrix correlates with increased scar formation. The higher collagen turnover results in the higher granulation rate of tissue components. A thick layer of reepithelialization and small blood vessels intermingled with fibroblastic cells were seen. Further studies related to advanced investigations such as cell migration, hemolysis, monitoring blood serum cytokine levels, and human cytokine synthesis inhibitory factor can be evaluated to understand the mechanism of wound healing in a better and accurate way. In conclusion, the prepared nanofibers showed potent wound healing activity for diabetic ulcers. Also, the prepared scaffolds have potential for applications in antibiotic-free bacterial infection treatment as wound healing materials.

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.

Acknowledgments

This project was supported by Iran National Science Foundation, and it was performed at Tehran University of Medical Sciences (TUMS, grant no. 39119) and was a part of Dr. Almasian Post Doc. Research (grant no. 37827).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 5521562, 12 pages
https://doi.org/10.1155/2021/5521562

Research Article

Crataegus pinnatifida Bunge Inhibits RANKL-Induced Osteoclast Differentiation in RAW 264.7 Cells and Prevents Bone Loss in an Ovariectomized Rat Model

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Received 27 January 2021; Revised 21 February 2021; Accepted 8 March 2021; Published 30 March 2021

Academic Editor: Reza B. Jalili

Osteoporosis is characterized by a decrease in bone microarchitecture with an increased risk of fracture. Long-term use of primary treatments, such as bisphosphonates and selective estrogen receptor modulators, results in various side effects. Therefore, it is necessary to develop alternative therapeutics derived from natural products. *Crataegus pinnatifida* Bunge (CPB) is a dried fruit used to treat diet-induced indigestion, loss of appetite, and diarrhea. However, research into the effects of CPB on osteoclast differentiation and osteoporosis is still limited. *In vitro* experiments were conducted to examine the effects of CPB on RANKL-induced osteoclast differentiation in RAW 264.7 cells. Moreover, we investigated the effects of CPB on bone loss in the femoral head in an ovariectomized rat model using microcomputed tomography. *In vitro*, tartrate-resistant acid phosphatase (TRAP) staining results showed the number of TRAP-positive cells, and TRAP activity significantly decreased following CPB treatment. CPB also significantly decreased pit formation. Furthermore, CPB inhibited osteoclast differentiation by suppressing NFATc1, and c-Fos expression. Moreover, CPB treatment inhibited osteoclast-related genes, such as *Nfatc1, Ca2, Acp5, mmp9, CtsK, Oscar*, and *Atp6v0d2*. *In vivo*, bone mineral density and structure model index were improved by administration of CPB. In conclusion, CPB prevented osteoclast differentiation *in vitro* and prevented bone loss *in vivo*. Therefore, CPB could be a potential alternative medicine for bone diseases, such as osteoporosis.

1. Introduction

Osteoporosis is characterized by a decrease in bone microarchitecture and an increased risk of fracture [1]. Bone remodeling is balanced between bone formation by osteoblasts and bone resorption by osteoclasts [2]. However, the excessive activity of osteoclasts induces osteoporosis, rheumatoid arthritis, and periodontitis. Thus, the inhibition of the osteoclast differentiation and its activity plays a role in the treatment strategy of osteoporosis.

Osteoclasts are *giant*, multinucleated cells derived from hematopoietic stem cells. Receptor activator of nuclear factor kappa-β ligand (RANKL) is essential for osteoclast differentiation [3, 4]. The binding of RANKL to RANK stimulates tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), activating mitogen-activated protein kinase (MAPKs) and nuclear factor kappa-β (NF-κB). As a result, it induces the expression of NFATc1 and c-Fos, known as essential transcription factors for osteoclast differentiation. These transcription factors induce the expression of osteoclast-related genes such as tartrate-resistant acid phosphatase (TRAP), carbonic anhydrase II (CA2), matrix metallopeptidase 9 (MMP-9), ATPase H+ transporting lysosomal 38 kDa V0 subunit d2 (ATP6v0d2), osteoclast associated receptor (OSCAR), and cathepsin K (CTK) [5, 6].

Bisphosphonate and selective estrogen receptor modulators (SERMs) are frequently used as treatments. However, long-term treatment of these agents causes side effects such as Paget's disease of bone, breast cancer, prostate cancer, hot flashes, and night sweats [7–10]. Therefore, there is a need

for integrating complementary and alternative medicines for osteoporosis based on natural products with few side effects. Consequently, the importance of developing an alternative treatment for osteoporosis has increased currently.

Crataegus pinnatifida Bunge (CPB) is the dried fruit of *Crataegus pinnatifida* Bung, called "Sansa" in Korea [11]. Previous studies have shown that CPB has antioxidant and anti-inflammatory effects [12, 13]. Chlorogenic acid is the major component of *Crataegus pinnatifida* Bunge and has an inhibitory effect on osteoclast differentiation induced by RANKL [14]. It has also been linked to anti-inflammatory and antioxidant effects [15, 16]. Osteoporosis is caused by endocrine, metabolic, and mechanical factors. Furthermore, recent studies have shown that the risk of developing osteoporosis is increased in inflammatory conditions [17, 18]. Therefore, we hypothesize that CPB may have a positive effect on bone metabolism.

In this study, we investigated the *in vitro* effects of CPB on RANKL-induced osteoclast differentiation. In addition, we also investigated the *in vivo* effects of CPB on bone loss in an ovariectomized (OVX) model.

2. Materials and Methods

2.1. Chemicals and Reagents. RANKL was purchased from PeproTech (London, UK). Dulbecco's modified eagle medium (DMEM) was purchased from Welgene (Daejeon, Korea). Minimum Essential Medium Eagle alpha-modification (α-MEM), penicillin/streptomycin (P/S), Dulbecco's phosphate buffered saline (DPBS), and normal serum were purchased from Gibco (Gaithersburg, MD). Fetal bovine serum (FBS) was supplied by Atlas Biologicals (Fort Collins, CO). TRAP staining kit, bicinchoninic acid (BCA) solution, and 17β-estradiol (E_2) were obtained from Sigma Aldrich (St. Louis, MI, USA). Cell Titer 96® AQueous nonradioactive cell proliferation assay (MTS) was obtained from Promega (Madison, WI, USA). Osteo assay surface multiple well plate was obtained from Corning Inc. (Corning, NY, USA). Anti-β-actin (cat. no. sc-8432) and anti-c-Fos (cat. no. sc-447) were supplied Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NFATc1 (cat. no. 556602) was supplied by BD Pharmingen (San Diego, CA, USA). Secondary antibodies (cat. no. 111-035-045, 115-035-062) were supplied by Jackson ImmunoResearch (West Grove, PA, USA). Polymerase chain reaction (PCR) primers were obtained from Genotech (Daejeon, Korea). SuperScript II Reverse transcription kit and SYBR green were obtained from Invitrogen (Carlsbad, CA, USA). Taq polymerase was obtained from Kapa Biosystems (Woburn, MA, USA). Avidin-biotin complex (ABC) kit and 3,3′-diaminobenzidine (DAB) were obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). All reagents used in the experiments were of analytical grade.

2.2. Preparation of CPB Extract. CPB was obtained from Omni Herb Inc. (Seoul, Korea). The sample extract was prepared by decocting 600 g dried herb with 6 L boiling distilled water (dH_2O) for 2 h. Next, the filtrate was evaporated using a vacuum evaporator and freeze-dried into powder. The yield from the dried herbs was 39.6% (freeze-dried powder: 237.8 g), and the powder was subsequently stored at −20°C.

2.3. High-Performance Liquid Chromatography Analysis. Quantitative analysis of main components in CPB was performed using an A Waters 2695 system equipped with a Waters 2487 Dual λ absorbance detector and X-bridge C18 Column (250 mm × 4.6 mm, 5 μm). CPB dissolved in dH_2O. CPB was passed through a 0.2-μm membrane filter and 10 μL volume of the filtrate was injected into the HPLC column. The mobile phases are composed of solvent A (acetonitrile) and solvent B (H_2O (1% acetic acid)). The detection time was 0–30 min. The flow rate was 1.0 mL/min.

2.4. Cell Culture and Cell Viability. The RAW 264.7 cells were purchased from Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cells were cultured in DMEM supplemented with 1% P/S and 10% FBS. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 (Thermo Fisher, Waltham, MA, USA). The MTS assay was performed to examine the toxicity of CPB on RAW 264.7 cells. RAW 264.7 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. The CPB was administered at 125, 250, 500, and 1000 μg/mL for 24 h. Afterwards, 20 μL MTS solution was added to the wells for 2 h. The absorbance (490 nm) was measured by an enzyme-linked immunosorbent assay (ELISA) reader. Results were indicated as a percentage of the control. Cytotoxicity was considered as cell viability less than 90% of the control.

2.5. TRAP Staining and Pit Assay. RAW 264.7 cells were seeded at a density of 5×10^3 cells/well in 96-well plate. After 24 h, RAW 264.7 cells were differentiated with α-MEM supplemented with 1% P/S, 10% FBS, and RANKL (100 ng/mL). The media was changed every 2 days. After 5 days, the osteoclast cells were fixed with 10% formalin for 10 min and then stained using a TRAP staining kit, according to the manufacturer's instructions. Afterwards, cells were rinsed with dH_2O and dried at room temperature. Multinucleated osteoclasts were considered as TRAP-positive cells with three or more nuclei (red color). To measure TRAP activity, differentiation medium was transferred to new 96-well plates and TRAP solution (4.93 mg pNPP + 850 μL 0.5 M acetate solution + 150 μL tartrate solution) was added to 96-well plate at 37°C for 1 h. TRAP activity was measured at 405 nm by an ELISA reader. To examine pit formation, RAW 264.7 cells were seeded at a density of 5×10^3 cell/well in a multiple well osteo assay surface plate and incubated for 5 days. Thereafter, the cells were removed using NaClO. The pit area was measured by ImageJ version 1.46 (National Institutes of Health, Bethesda, MD, USA).

2.6. Western Blotting. RAW 264.7 cells were incubated with RANKL and various concentrations of CPB extract (125, 250, 500, and 1000 μg/mL) for 24 h. Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-

Cl, 150 mM NaCl, 1% NP–40, 0.5% sodium deoxycholate, and 0.1% SDS) consisting of proteinase inhibitors (Sigma Aldrich; Merck KGaA, Darmstadt, Germany) to obtain the protein. Thereafter, total protein quantification was done using BCA assay according to the manufacturer's instructions. The protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes were blocked (5% skim milk) for 1 h and incubated overnight at 4°C with primary antibodies for β-actin (1 : 1,000), NFATc1 (1 : 1,000), and c-Fos (1 : 1,000). After 24 h, the membranes were incubated with secondary antibodies (1 : 10,000) for 1 h at room temperature. The protein was visualized by enhanced chemiluminescence (ECL) (Whatman plc; GE Healthcare) and protein band densitometry was measured by ImageJ version 1.46. All data were normalized to the β-actin density.

2.7. Reverse Transcription-Quantitative PCR (RT-qPCR). RAW 264.7 cells were incubated with CPB (125, 250, 500, and 1000 μg/mL) for 4 days and the RANKL (100 ng/mL). Total RNA was extracted from RAW 264.7 cells using TRIzol reagent, according to the manufacturer's instructions. Then, cDNA was synthesized using the reverse transcription kit (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed with a C1000 Touch™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) and Taq polymerase. The PCR cycling conditions were initial denaturation cycle at 95°C for 5 min, followed by 30–40 cycles of amplification at 94°C for 30 sec, annealing at 53–58°C for 30 sec, and extension at 72°C for 30 sec. Primers for osteoclast-related genes are described in Table 1. The reaction was electrophoresed on 1–1.2% agarose gels stained with SYBR. The agarose gel was visualized using NαB$^{\text{I}}$™ (Neoscience, Suwon, Korea). The expression level of mRNA in the analyzed gene was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using ImageJ version 1.46.

2.8. Animal Experiments and Induction of OVX Rats. Animal experiments were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals approved by the Committee on Animal Experimentation of Kyung Hee University (KHUASP (SE)-15-101). Female Sprague–Dawley (SD) rats (12 weeks of age) were purchased from Nara Biotech (Seoul, Korea). SD-rats were housed at 22 ± 2°C, with a relative humidity of 53–55% in a 12 h light-dark cycle. In this study, all animals had *ad libitum* access to water and food. The SD-rats were acclimatized for one week before surgery. To establish an OVX model, the rats were anesthetized with 100% oxygen and 5% isoflurane to remove the ovaries. The sham group did not have their ovaries removed but received the same stress. The rats were divided into five groups ($n = 8$ per group) as follows: (1) sham group; sham-operation, treated with dH_2O, (2) OVX group; OVX-induced, treated with dH_2O, (3) E_2 group; OVX-induced, treated with 100 μg/kg 17β-estradiol, (4) CPB-L group; OVX-induced, treated with 13.2 mg/kg CPB, and (5) CPB-H group; OVX-induced, treated with 132 mg/kg CPB. The dose of CPB was calculated as follows: In Korean medicine, the recommended single dose for an adult is 8 g/60 kg, effectively equating to 3.168 g (yield, 39.6%) CPB powder of 8 g dried herbs. Therefore, the CPB-L group was administered 13.2 mg/kg CPB. Since the metabolism of rodents is faster than that of humans, the high-dose group was administered 10 times the concentration of the low-dose group [19]. Thus, CPB-H group was administered 132 mg/kg CPB. To prevent infection at the surgical site, all rats received injections with 4 mg/kg gentamicin for 3 days after surgery. E_2 and CPB were dissolved in dH_2O and administered orally once per day for 8 weeks. Body weights were measured once a week. During the experiments, all animals showed no side effects and exhibited no abnormal behavior. After 8 weeks, the experimental animals were anesthetized with 100% oxygen and 5% isoflurane and sacrificed by lethal cardiac puncture and cervical vertebrae dislocation.

2.9. Microcomputed Tomography Analysis. After sacrifice, the femur samples were fixed in 10% neutral buffered formalin (NBF) at room temperature for 24 h. The femoral head was analyzed using microcomputed tomography (micro-CT) (SkyScan1176; Bruker Corporation, Kontich, Belgium). Bone microstructure parameters such as bone mineral density (BMD), bone volume/total volume (BV/TV), and structure model index (SMI) were analyzed using NRecon software (SkyScan version 1.6.10.1; Bruker Corporation, Billerica, MA, USA).

2.10. Hematoxylin and Eosin (H&E) Staining. The fixed femur samples were decalcified in ethylenediaminetetraacetic acid (EDTA) for 4 weeks at room temperature. Afterwards, femur samples were dehydrated and embedded in paraffin. Femur samples were sectioned using a rotary microtome (5 μm-thick, ZEISS, Oberkochen, Germany), then dried and stained with H&E. Changes in tissue parameters, such as femoral head area, were observed using an inverted light microscope (magnification, 40x and 100x; Olympus Corporation, Tokyo, Japan). The trabecular area was measured by ImageJ version 1.46.

2.11. Immunohistochemistry Staining. Sectioned tissues were paraffinized and rehydrated to prepare for immunohistochemistry (IHC). Femur tissue slides were treated with 0.3% hydrogen peroxide-methanol to inhibit endogenous peroxidase. Subsequently, nonspecific reactions were blocked with normal serum for 1 h at room temperature. After washing thrice with PBS, sections were incubated with primary antibody at 4°C overnight and then incubated with secondary antibodies for 1 h at room temperature. The tissues were incubated with an ABC kit for 30 min at room temperature, followed by staining with DAB solution and counterstaining with hematoxylin. Histological changes were analyzed using a light microscope (magnification, 100x and 200x).

Table 1: Primer sequences for RT-qPCR.

Gene name	Primer sequence (5′-3′)	Tm (°C)	cycle	Accession no
MMP-9 (*Mmp9*)	F: CGA CTT TTG TGG TCT TCC CC R: TGA AGG TTT GGA ATC GAC CC	58	30	NM_013599.4
CTK (*Ctsk*)	F: AGG CGG CTA TAT GAC CAC TG R: CCG AGC CAA GAG AGC ATA TC	58	26	NM_007802.4
TRAP (*Acp5*)	F: ACT TCC CCA GCC CTT ACT ACC G R: TCA GCA CAT AGC CCA CAC CG	58	30	NM_007388.3
NFATc1 (*Nfatc1*)	F: TGC TCC TCC TCC TGC TGC TC R: CGT CTT CCA CCT CCA CGT CG	58	32	NM_198429.2
OSCAR (*Oscar*)	F: CTG CTG GTA ACG GAT CAG CTC CCC AGA R: CCA AGG AGC CAG AAC CTT CGA AAC T	53	35	NM_001290377.1
CA2 (*Ca2*)	F: CTC TCA GGA CAA TGC AGT GCT GA R: ATC CAG GTC ACA CAT TCC AGC A	58	32	NM_001357334.1
ATP6v0d2 (*Atp6v0d2*)	F: ATG GGG CCT TGC AAA AGA AAT CTG R: CGA CAG CGT CAA ACA AAG GCT TGT A	58	30	NM_175406.3
GAPDH (*Gapdh*)	F: ACT TTG TCA AGC TCA TTT CC R: TGC AGC GAA CTT TAT TGA TG	58	30	NM_008084.3

2.12. Statistical Analysis. Data are presented as mean ± standard error (SEM) of the mean for three replicates. Differences between the control and CPB treatment groups were analyzed using one-way ANOVA followed by a Dunnett's post hoc in GraphPad PRISM version 5.01 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was determined at $p < 0.05$.

3. Results

3.1. Quantitative Analysis of the CPB Extract. HPLC was used to confirm the main component of CPB [20]. As shown in Figure 1, the retention times of CPB are identical to the retention times of the chlorogenic acid standards.

3.2. Effect of CPB on RANKL-Induced TRAP Activity and Pit Formation. To determine the cytotoxic effect of CPB, RAW 264.7 cells and osteoclast were treated with CPB concentrations from 125, 250, 500, and 1000 μg/mL. In this study, none of the CPB concentrations affected cell viability in either RAW 264.7 cells or osteoclasts (Figures 2(a) and 2(b)). To investigate the effect of CPB on RANKL-induced osteoclast differentiation and pit formation, TRAP staining and pit assay was used. RANKL increased the number of TRAP-positive cells and TRAP activity compared with the untreated control group, confirming osteoclast differentiation. CPB treatment of differentiated osteoclasts decreased the number of TRAP-positive cells and TRAP activity in a dose-dependent manner. In addition, the pit area was increased with RANKL treatment, compared to untreated controls, and decreased by CPB treatment in a dose-dependent manner (Figures 2(c)–2(f)).

3.3. Effect of CPB on RANKL-Induced Expression of NFATc1 and c-Fos. To examine the expression of NFATc1 and c-Fos, we performed western blotting (Figures 3(a) and 3(b)). NFATc1 and c-Fos expression were significantly increased in the RANKL-induced cells compared to the nonstimulated control group. Therefore, the expressions of NFATc1 and c-Fos were suppressed by CPB in a dose-dependent manner.

3.4. Effect of CPB on RANKL-Induced of Osteoclast-Related Genes. To investigate the effect of CPB on osteoclast-related genes in RANKL-induced RAW 264.7 cells, RT-qPCR was performed. Treatment with RANKL increased the mRNA levels of *Nfatc1*, *Ca2*, *Acp5*, *mmp9*, *CtsK*, *Oscar*, and *Atp6v0d2*. In contrast, CPB reduced these mRNA levels in a dose-dependent manner, the most effective dose in all instances (Figures 4(a) and 4(b)).

3.5. Effect of CPB on OVX-Induced Models. To analyze the effect of CPB on OVX-induced postmenstrual osteoporosis, we orally administered E_2, CPB-L, and CPB-H to the OVX-induced rats daily for 8 weeks. As shown in Figure 5(a), the body weight of both treated and untreated OVX-induced rats significantly increased after 3 weeks as compared to that of the sham group. However, there was no significant difference in body weight between the OVX group and E_2, CPB-L, and CPB-H, respectively. The uterus weight decreased in the OVX group as compared with that in the sham group (Figure 5(b)). Furthermore, the uterus weight increased in the E_2 group as compared with that in the OVX group, with no effect observed in CPB-L and CPB-H, femur weights significantly decreased in the OVX group compared to that in the sham group (Figure 5(c)). However, there was no difference in femur weight between the E_2, CPB-L, and CPB-H groups compared to the OVX group. Tibia weight and ash were decreased in the OVX group compared with the sham group (Figures 5(d) and 5(e)), though there were no significant differences between E_2, CPB-L, and CPB-H, compared to the OVX group.

3.6. Effect of CPB on Bone Loss in OVX-Induced Models. In the micro-CT image, the bone density in the femoral head of OVX group was decreased compared with the sham group (Figure 6(a)). Furthermore, E_2 and CPB-H significantly increased bone density in the femoral head compared with the OVX group. From the results of the bone microstructure

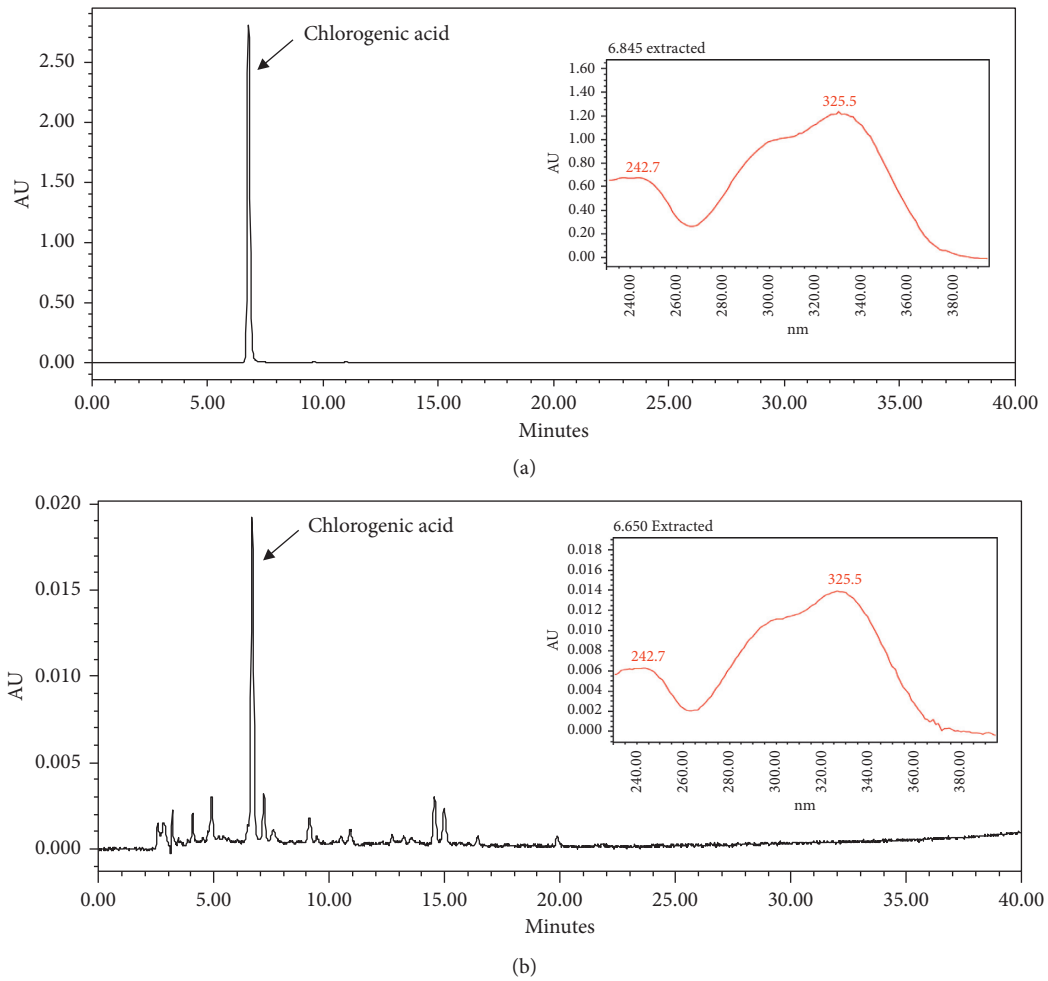

Figure 1: Quantitative HPLC of (a) chlorogenic acid standard and (b) CPB. The HPLC-analysis for standards and sample solutions. (a) Chlorogenic acid standard solution; (b) CPB samples were detected at 330 nm.

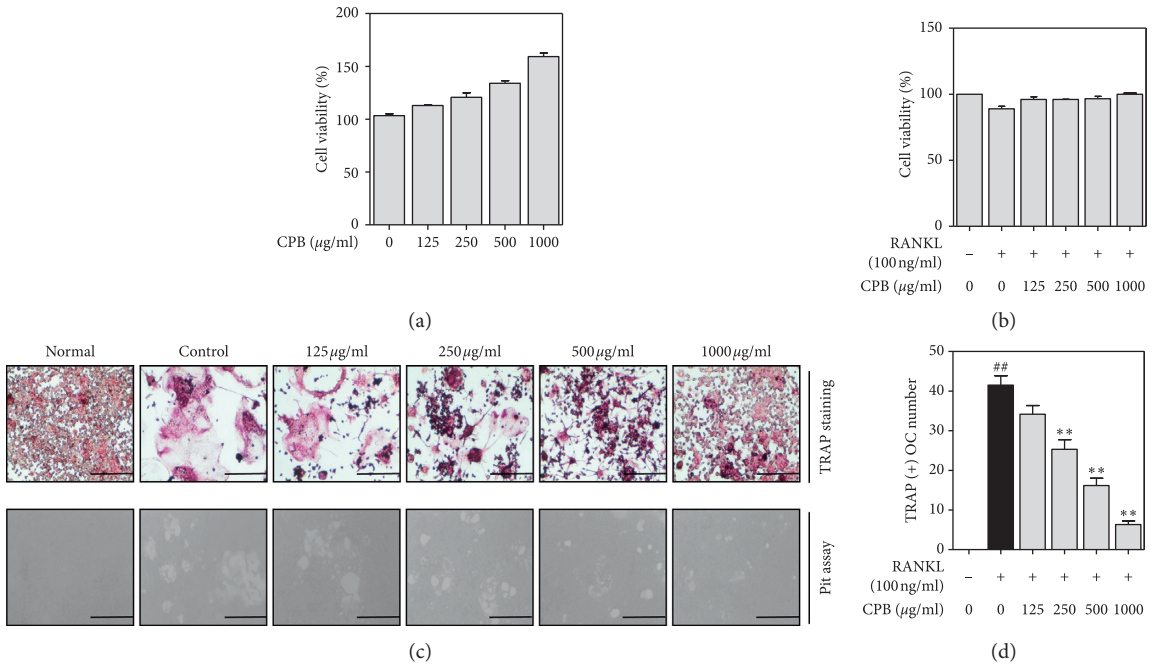

Figure 2: Continued.

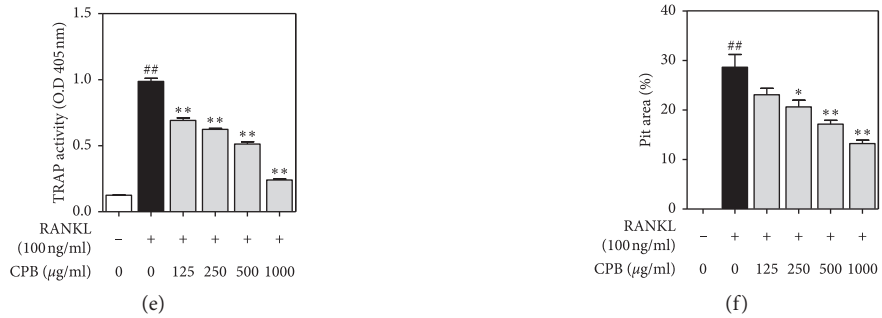

Figure 2: Effect of CPB on cell viability, osteoclast differentiation, and bone formation. (a) RAW 264.7 cells were measured by MTS assay of CPB treatment for 24 h. (b) After differentiation into osteoclasts for 5 days, cytotoxicity was measured using MTS. (c) TRAP-positive cells and pit area were captured using an inverted microscope (100x, Scale bars: 200 μm). (d) TRAP-positive cells were counted with an inverted microscope. (e) TRAP activity was measured with an ELISA reader (405 nm). (f) The pit area was measured with ImageJ version 1.46 (100x, Scale bars: 200 μm). The results are presented as the mean ± SEM ($n = 3$). $^{\#\#}p < 0.01$ compared to the normal group (untreated cells), and $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group (only-RANKL treated cells).

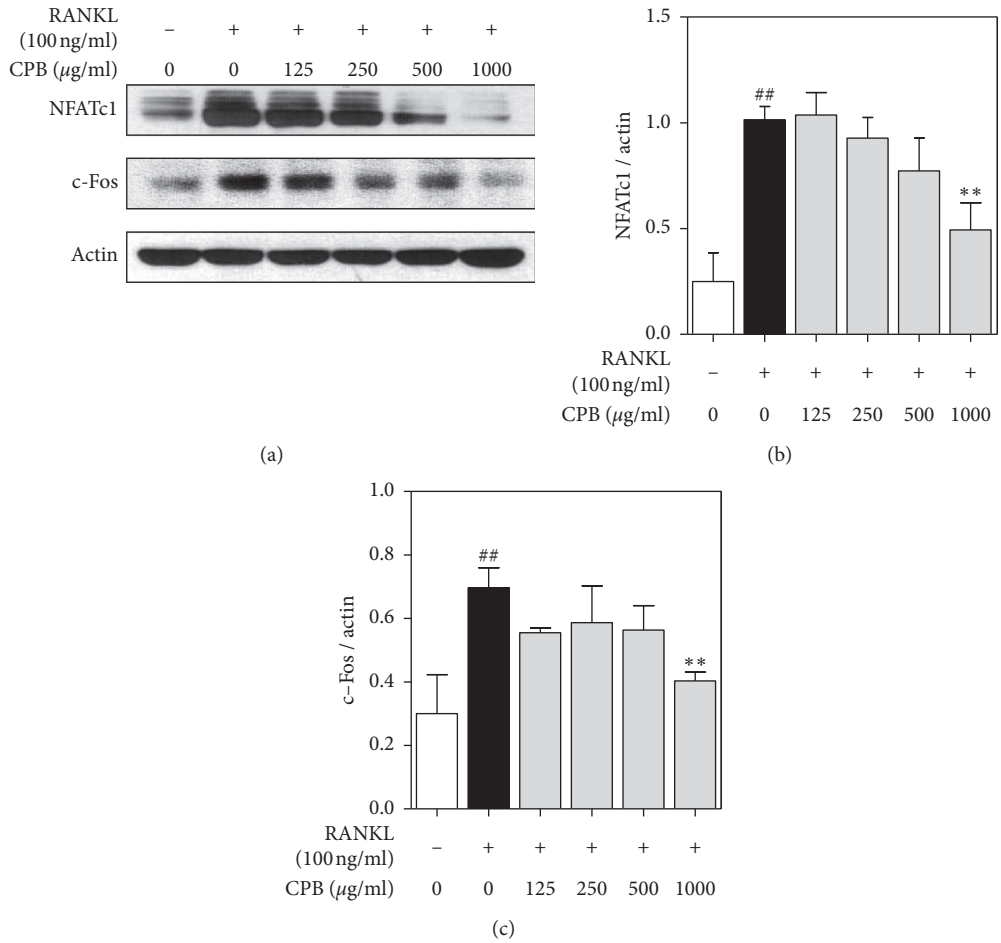

Figure 3: Effect of CPB extract on transcription factor such as NFATc1 and c-Fos. (a) RAW 264.7 cells were treated with RANKL (100 ng/mL) and CPB treatment for 24 h. The expressions of NFATc1 and c-Fos were determined by western blotting. (b) NFATc1 and c-Fos were normalized to Actin with ImageJ version 1.46. The results are presented as the mean ± SEM ($n = 3$). $^{\#\#}p < 0.01$ compared to the normal group (untreated cells), and $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group (only-RANKL treated cells).

analysis, BMD was significantly decreased in the OVX group compared to sham (Figure 6(b)), while in E_2 and CPB-H groups, BMD increased compared with the OVX group. As shown in Figure 6(c), BV/TV was decreased in the OVX group compared with the sham group. E_2 significantly increased BV/TV compared to the OVX group. CPB-L and

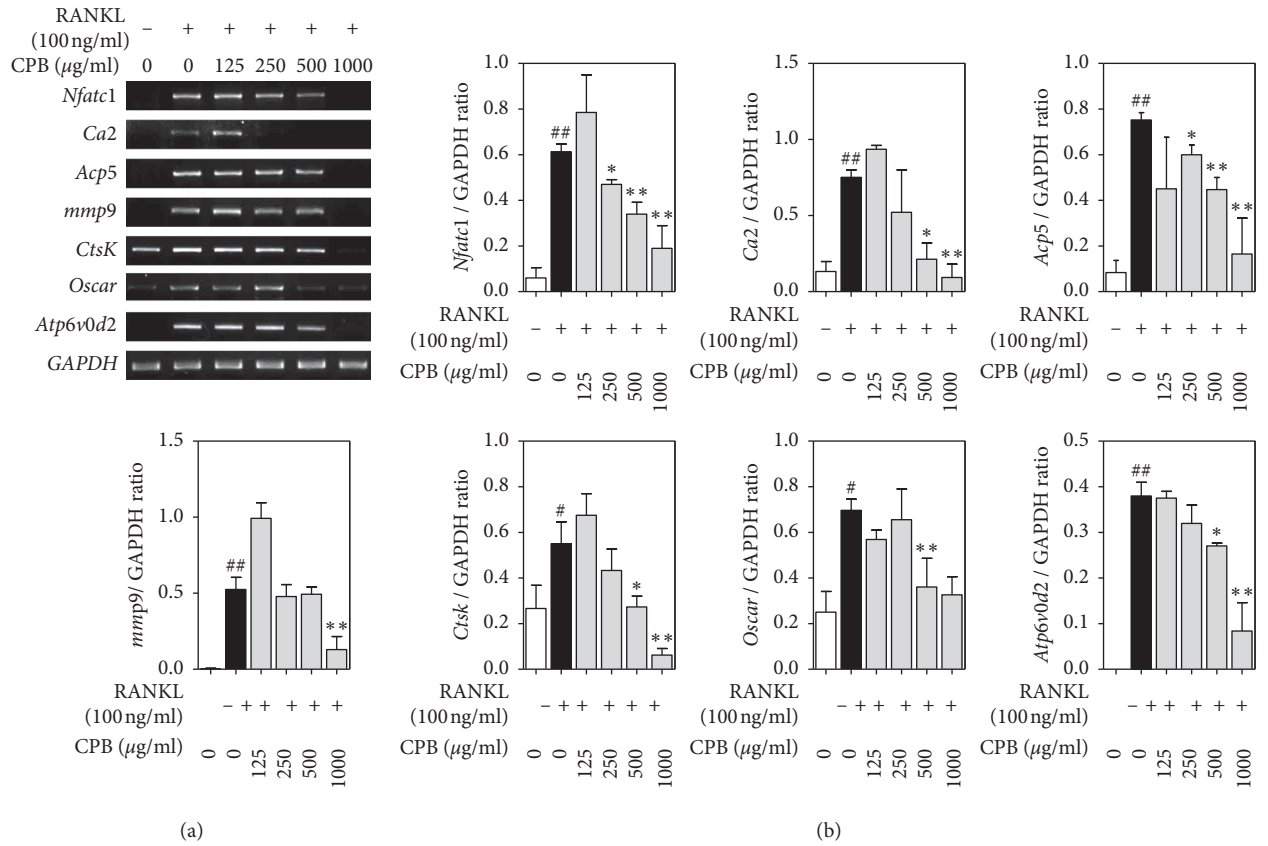

Figure 4: Effect of CPB extract on osteoclast-related genes. (a) RAW 264.7 cells were treated with RANKL (100 ng/mL) and CPB treatment for 4 days. RT-qPCR was used to determine the mRNA levels of osteoclast-related genes. (b) The levels of mRNA were normalized to GAPDH. The results are presented as the mean ± SEM ($n = 3$). $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to the normal group (untreated cells), and $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group (only-RANKL treated cells).

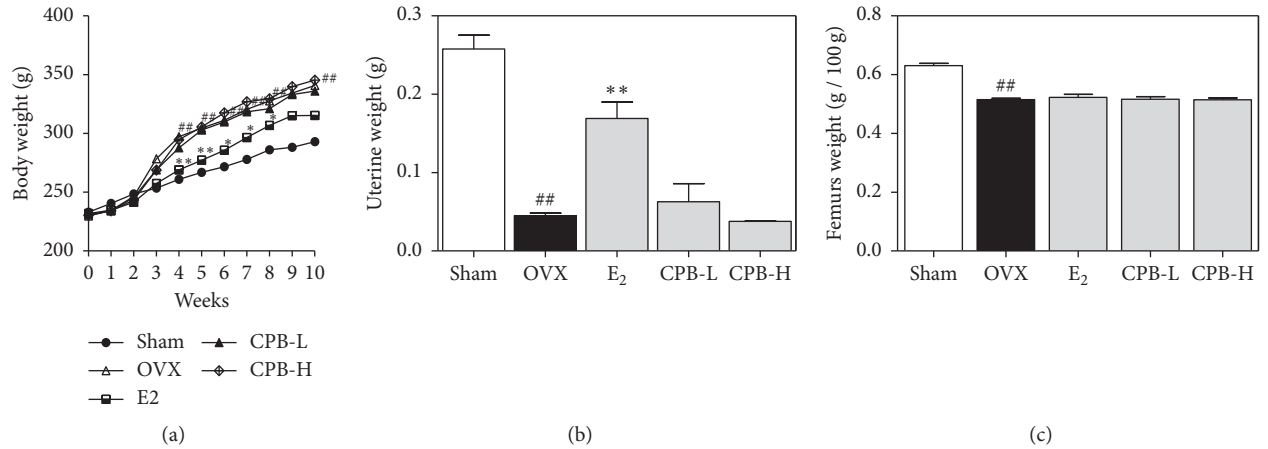

Figure 5: Continued.

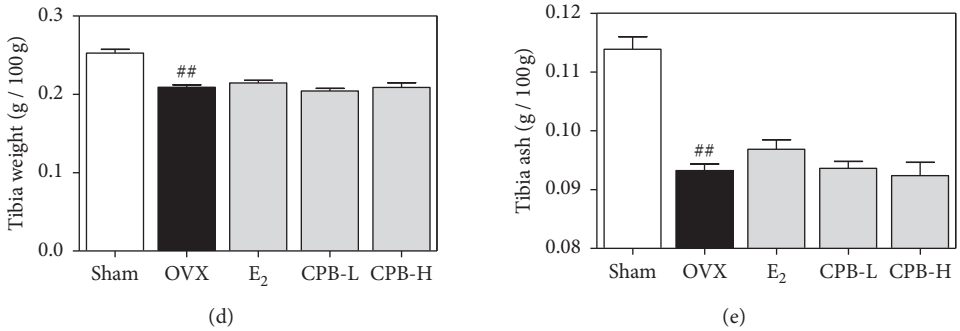

Figure 5: Effect of CPB on OVX-induced model. (a) The body weight was measured once a week. (b) Uterus weight, (c) femurs weight, (d) tibia weight, and (e) tibia ash was measured after sacrifice. The results are presented as the mean ± SEM of each experimental group ($n = 8$). $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to the normal group (sham group), and $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group (OVX group).

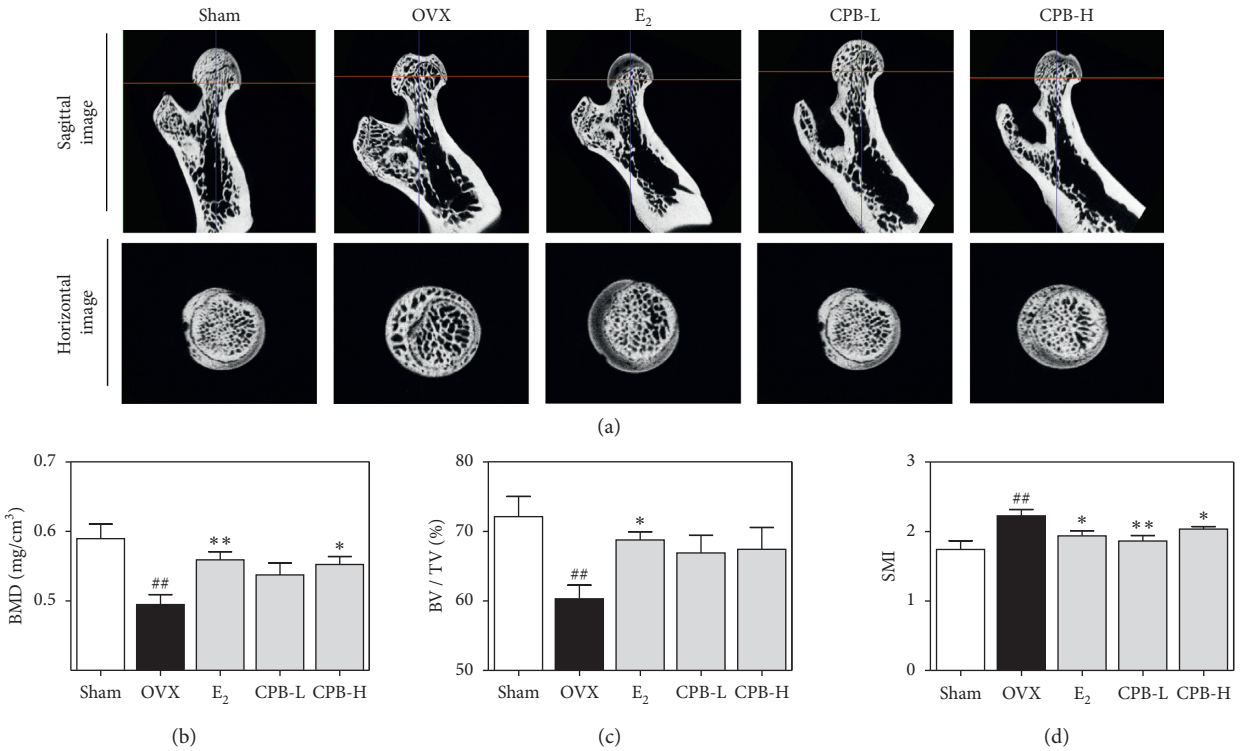

Figure 6: Effect of CPB on an osteoporosis rat model. (a) Analysis of micro-CT in the femoral head. The bone microstructure parameters, such as (b) BMD, (c) BV/TV, and (d) SMI, were measured by micro-CT. The results are presented as the mean ± SEM for each experimental group ($n = 8$). $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to the normal group (sham group), and $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group (OVX group).

CPB-H groups had increased BV/TV but not significantly. In addition, SMI was increased in the OVX group compared to the sham group (Figure 6(d)). In contrast, SMI was reduced in all three groups: E_2, CPB-L, and CPB-H, compared to the OVX group.

3.7. Effect of CPB on Trabecular Area and Expression of CTK in the Femoral Head. To measure the trabecular area, bone tissues were stained with H&E (Figure 7(a)). To determine the effect of CPB treatments on the CTK in OVX-induced rats, we perform the IHC staining (Figure 7(b)). The trabecular area was decreased in the OVX group when compared with that of the sham group. Treatments with E_2, CPB-L, and CPB-H inhibited the loss of the trabecular area compared with that of the OVX group (Figure 7(c)). Furthermore, OVX groups significantly increased CTK compared to the sham group. Concurrently, E_2, CPB-L, and CPB-H groups reduced CTK compared to the OVX group (Figure 7(d)).

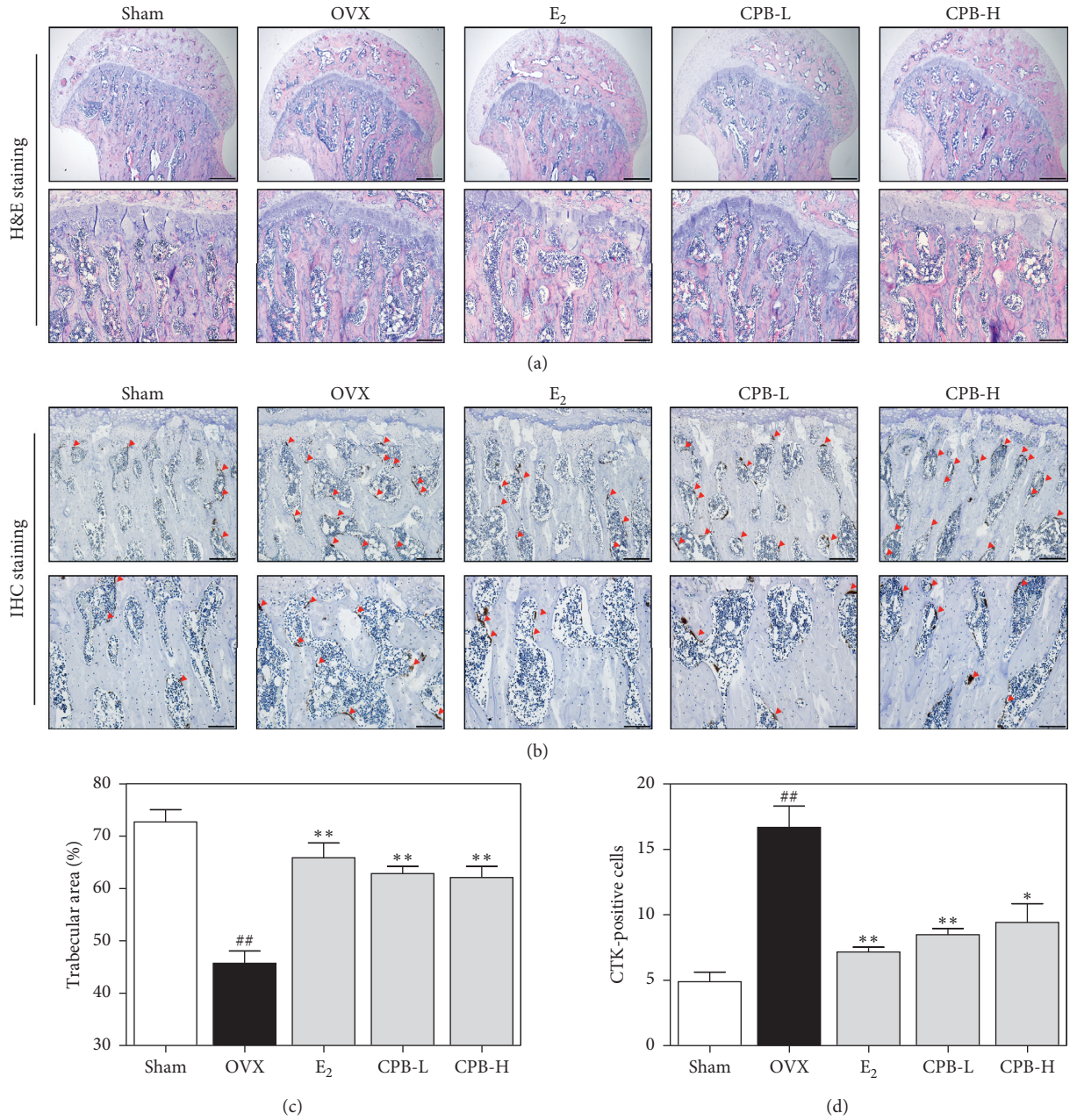

Figure 7: Effect of CPB on OVX-induced bone loss model. (a) The histology of the bone tissues was examined using H&E staining, and (b) IHC staining. (c) The trabecular area was measured using ImageJ version 1.46. (d) CTK-positive cells were counted using ImageJ version 1.46. CTK-positive cells are indicated by red arrows. The results are presented as the mean ± SEM for each experimental group ($n = 8$). $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared to the normal group (sham group), and $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group (OVX group).

4. Discussion

According to recent studies, various side effects have been reported with the administration of bisphosphonate and SERM, which are currently used for the treatment of osteoporosis. This has prompted many researchers to search for safer alternative medicinal agents with fewer side effects for osteoporosis treatment [7–10]. In this study, we examined the osteoclastogenesis and anti-osteoporosis effects of CPB on RAW 264.7 cells. *In vitro*, CPB demonstrated an inhibitory effect on osteoclast differentiation by inhibiting transcription factors and osteoclast-related genes. *In vivo*, CPB also prevented bone loss in OVX-induced rat models.

TRAP is a known osteoclast phenotype marker, and TRAP staining is a standard method used to determine osteoclast expression and activation [21, 22]. In the present study, TRAP staining results showed a significant decrease in TRAP-positive cells and TRAP activity following CPB treatment. Pit formation is commonly used to measure the osteoclasts' differentiation and bone resorption ability [23, 24]. As a result of the experiment, CPB significantly

suppressed the pit area. It is unclear whether CPB reduces pit formation by inhibiting the ability of osteoclasts to bone resorption, or it controls pit formation by inhibiting osteoclast differentiation, but the TRAP staining and pit assay results, CPB, seem to regulate bone resorption by inhibiting osteoclast differentiation.

Transcription factors, such as NFATc1 and c-Fos, are essential in osteoclast differentiation [25, 26]. In a previous study, c-Fos-deficient cells were not able to differentiate into osteoclasts [11]. In contrast, excessive expression of c-Fos causes osteosarcoma and chondrosarcoma [27]. Furthermore, NFATc1-deficient mice develop osteopetrosis due to blocked osteoclast differentiation [28]. It has also been reported that embryonic stem cells deficient in NFATc1 cannot differentiate into osteoclasts upon RANKL stimulation [29]. Therefore, c-Fos and NFATc1 are important factors for osteoclast differentiation [25, 26]. The present study showed that CPB significantly decreased the expression of c-Fos and NFATc1 and subsequent osteoclast differentiation.

c-Fos regulates bone resorption markers, such as CA2, which acidifies the bone surface during bone resorption [30–32]. Furthermore, NFATc1 regulates the expression of osteoclast-specific genes such as TRAP, MMP-9, CTK, ATP6v0d2, and OSCAR [25]. MMP-9 and CTK are involved in the process of osteoclast differentiation and play an important role in osteoclast precursors and bone resorption [6, 33, 34]. MMP-9 has a negative correlation with BMD, and overexpression of MMP-9 attenuates osteoclast formation [35, 36]. CTK is a cysteine proteinase mainly expressed in osteoclasts. CTK is known to play an important role in breaking down the organic phases of bone during bone resorption [37]. According to previous studies, a deficiency of CTK indicates an osteoporosis phenotype [38]. Therefore, it was found that the deficiency of CTK is associated with the inhibition of osteoclast activity, and CTK is an effective target in the treatment of osteoporosis. ATP6v0d2 is an essential factor required for cell-cell fusion. Previous studies found ATP6v0d2-deficient mice present with an osteopetrosis phenotype due to abnormal osteoclast maturation [39, 40]. OSCAR regulates osteoclast differentiation and cell maturation and is a costimulatory receptor for osteoclast differentiation through activation of NFATc1. It is known that OSCAR may contribute to the etiology and severity of osteoporosis and rheumatoid arthritis [41, 42]. The present study showed that CPB significantly decreased the expression of osteoclast-related genes (*Nfatc1*, *Ca2*, *Acp5*, *mmp9*, *CtsK*, *Oscar*, and *Atp6v0d2*) in RANKL-induced osteoclast differentiation in RAW 264.7 cellsvia regulation of c-Fos and NFATc1 signaling.

OVX-induction is widely used in postmenopausal osteoporosis research. According to a previous study, OVX-induced rats share similar symptoms to human osteoporosis, such as the increase in body weight [43]. In addition, loss of uterus weight demonstrates that the postmenopausal osteoporosis model had been successfully established [44, 45]. In this study, all OVX-induced rats, including CPB and E_2 treatment, increased body weight from week 4, while all groups, except for the E_2-treatment, had decreased uterus weight. These results confirm previous studies that E_2 treatment reverses the effect of postmenopausal changes to uterus weight, while CPB had no effect in this regard.

Micro-CT is used to analyze the structural properties of bones in three dimensions [46, 47]. Bone density and bone microstructure are indicators used to evaluate bone quality [48]. BV/TV represents the volume of bone within the volume of interest (VOI), whereas SMI refers to the structural morphology index of the cancellous bone [48, 49]. According to a recent study, increased BMD is not sufficient to improve or prevent osteoporosis [50]. Therefore, SMI is a complimentary representative index used for accurate bone quality assessment. In this study, the reduction in BMD, BV/TV, and SMI was improved by the administration of E_2 and CPB-H. These results suggest that CPB can be a treatment for postmenopausal osteoporosis through the prevention of bone loss.

As a result of histological examination, we showed that E_2 and CPB treatment prevented the decrease in the trabecular area, indicating that CPB inhibits bone loss of postmenopausal osteoporosis. IHC staining was used to measure the expression of bone-related factors in tissues. In this study, E_2, CPB-L, and CPB-H groups suppressed the expression of CTK induced by OVX. Furthermore, this also correlates with the findings of the *in vitro* experiments. These results further suggest that CPB inhibits bone resorption. In summary, CPB has antiosteoporotic effects on OVX-induced rats by suppressing BMD and bone resorption markers such as CTK.

The limitations of this study are as follows: (i) MAPK and NF-κB signaling pathways play an important role in NFATc1 and c-Fos activation. In this study, CPB significantly inhibited the expression of NFATc1 and c-Fos, but MAPK and NF-κB pathways were not studied. Therefore, it is still necessary to correlate the inhibitory effect of CPB to the MAPK and NF-κB signaling pathways. (ii) As patients with osteoporosis have already lost a certain amount of bone density, it is important to also promote osteoblast activity to restore the lost bone mass, along with osteoclast activity inhibitors to prevent disease progression. Therefore, future studies should focus on the effects of CPB on promoting osteoblast differentiation. (iii) Treatment of osteoporosis remains focused on postmenopausal osteoporosis in type 1 osteoporosis. However, interest in male osteoporosis and senile osteoporosis is also increasing. Therefore, studies on CPB in other osteoporosis models are also required.

5. Conclusion

In this study, CPB effectively inhibited osteoclast differentiation *in vitro* and prevented bone loss *in vivo*. The mechanisms of inhibition were via suppression of osteoporosis-related protein expression (NFATc1 and c-Fos), gene expression (*Nfatc1*, *Ca2*, *Acp5*, *mmp9*, *CtsK*, *Oscar*, and *Atp6v0d2*), and inhibited bone loss induced in the OVX model. These results indicate that CPB may be useful in the treatment of metabolic bone diseases such as osteoporosis.

Abbreviations

SERM: Selective estrogen receptor modulators
CPB: *Crataegus pinnatifida* Bunge
OVX: Ovariectomized
Micro-CT: Microcomputed tomography
RANKL: Receptor activator of nuclear factor Kappa-B ligand
TRAF6: TNF receptor-associated factor 6
MAPK: Mitogen-activated protein kinase
NFATc1: Nuclear factor-activated T cells c1
DMEM: Dulbecco's modified eagle's medium
α-MEM: Minimum essential medium eagle alpha-modification
DPBS: Dulbecco's phosphate buffered saline
FBS: Fetal bovine serum
BCA: Bicinchoninic acid
E_2: 17β-estradiol
MTS: Cell titer 96® aqueous nonradioactive cell proliferation assay
ABC: Avidin-biotin complex
DAB: 3, 3′-diaminobenzidine
DW: Distilled water
HPLC: High-performance liquid chromatography
SD-rat: Sprague–Dawley rat
BMD: Bone mineral density
BV/TV: Bone volume/total volume
SMI: Structure model index
H&E: Hematoxylin and eosin
EDTA: Ethylenediaminetetraacetic acid
IHC: Immunohistochemistry.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Minsun Kim and MinBeom Kim contributed equally to this work.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (nos. 2020R1A2C1007836 and 2019R1H1A2101224).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6693887, 7 pages
https://doi.org/10.1155/2021/6693887

Research Article

The Efficacy of *Plantago major* Seed on Liver Enzymes in Nonalcoholic Fatty Liver Disease: A Randomized Double-Blind Clinical Trial

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Received 2 January 2021; Revised 21 February 2021; Accepted 15 March 2021; Published 27 March 2021

Academic Editor: Andresa Berretta

Objective. This study aims to evaluate the effects of *Plantago major* (*P. major*) seed on liver enzymes and ultrasound patterns in nonalcoholic fatty liver disease (NAFLD). *Design*. In this randomized double-blind placebo-controlled clinical trial, 74 patients with NAFLD were administered either 2 g *P. major* or placebo twice daily for 12 weeks. All patients were advised to follow the recommendations for daily exercise and diet modification. Levels of liver enzymes as well as other laboratory indexes were measured at the beginning of the study and after 12 weeks. Indeed, the alteration in ultrasound grade was evaluated in NAFLD patients. *Results*. Sixty-three participants completed the study in the intervention and control groups. The mean age of participants was 43.3 (±8.927) and 38.7 (±8.48) years in the intervention and control groups, respectively. *P. major* group showed significant reduction in alanine aminotransferase (ALT) (47.32 ± 21.77 IU/L vs. 50.03 ± 21.19, $P = 0.021$), aspartate aminotransferase (AST) (28.29 ± 10.49 IU/L vs. 32.03 ± 13.30, $P = 0.004$), triglyceride (TG) (200.93 ± 106.741 mg/dl vs. 183.75 ± 73.96, $P = 0.001$), waist circumference (WC) (101.25 ± 9.27 cm vs. 101.18 ± 8.63, $P = 0.027$), and grade of fatty liver in ultrasonography ($P = 0.038$), comparing to the placebo group. There was no significant difference between the two groups regarding serum levels of fasting blood sugar (FBS), high-density lipoprotein (HDL), low-density lipoprotein (LDL), cholesterol, and other outcomes. *Conclusion*. *P. major* supplementation with a daily dose of 2 g for 12 weeks improved serum levels of ALT, AST, and TG in patients with NAFLD. Further studies with a larger sample size are recommended.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a disease with a wide range from a simple accumulation of fat (triglyceride) in liver cells and simple steatosis to the progression to nonalcoholic steatohepatitis (NASH). About 20% of patients with NAFLD develop liver fibrosis, even cirrhosis and liver failure [1]. The high prevalence and chronic nature of NAFLD affect the patients' quality of life and impose a heavy economic burden on society. The prevalence of nonalcoholic fatty liver disease is increasing in the world. In developed countries, about 25% of the total adult population and about 3–10% of children have this disease, and the prevalence of disease among obese children is about 34% [2]. In Iran, at least 30% of the population suffers from this disease [3]. An unhealthy lifestyle is one of the most important risk factors

for NAFLD. Excessive food and calorie intake and lack of enough physical activity often lead to obesity and liver steatosis. Excessive consumption of saturated fats and carbohydrates induces lipogenesis in the liver, inflammation in adipose tissue cells, and insulin resistance in adipose tissue, liver, and skeletal muscle [4, 5]. Besides, an unhealthy lifestyle, which seems to be the most important risk factor for NAFLD, and other factors such as aging, genetics, and intestinal dysbiosis (altered normal intestinal flora) which is thought to be caused by an unhealthy diet may lead to fat accumulation in the liver and the pathogenesis of NASH [6–8]. Besides, the patients with hepatic fibrosis and NASH are also asymptomatic; they may experience fatigue, boredom, right upper quadrant (RUQ) discomfort, or severe symptoms of chronic liver disease [9].

Although liver biopsy is the most specific test to assess the nature and severity of the liver disease, however, liver ultrasound is the easiest diagnostic method for hepatic steatosis [10, 11]. Different clinical trials for testing modern drug candidates of NASH have failed to reach the major findings or have limited therapeutic efficacy. Several agents like nuclear receptor agonists (obeticholic acid, GFT505, and elafibranor), insulin sensitizers (glitazones, pioglitazone, and metformin), and glucagon-like peptide-1 receptor agonists are still in the drug pipeline for NASH [12].

Persian Medicine (PM) has considered a special role for the liver to maintain human health so that the scholars of PM, including Ibn Sina, have considered the liver as one of the chief organs of the body along with the heart and brain [13]. Due to PM, the liver, as the main source of production of humors (phlegm, yellow and black bile, and sanguine) plays an important role in human life and health [14].

Various plants were used to treat liver diseases in PM with different mechanisms. Several herbal remedies show a potential benefit for NAFLD management [15]. Different studies have investigated the efficacy of some of these plants such as turmeric, barberry root, shallot, and rose, on fatty liver [16–19]. *P. major* (Barhang in Persian) is one of the herbs mentioned as a liver tonic in PM literature which can open liver obstructions. *P. major* (plantaginaceae) is a perennial plant that widely grows in the United States, Europe, and Asia and almost throughout Iran [20]. This plant has various pharmacological compounds such as flavonoids, polysaccharides, terpenoids, lipids, iridoid glycosides, and derivatives of caffeic acid which is used to treat various diseases such as constipation, cough, wounds, infection, fever, inflammation, and bleeding [21]. The hepatoprotective effects of *P. major* were confirmed in animal studies [22–24]. Due to our investigations, the efficacy of *P. major* to treat fatty liver has not been studied yet; hence, this clinical trial was designed to evaluate the effects of *P. major* on liver enzymes and ultrasound patterns in NAFLD.

2. Materials and Methods

2.1. Trial Design. This study was a randomized, double-blind placebo-controlled clinical trial with two parallel groups of NAFLD patients. Patients who had the inclusion criteria were included in the study and randomly allocated into two groups through the "block randomization" method. This method was utilized using 4-way blocks and a random number table. Interventions A and B accurately defined six blocks of four. Using random numbers, we carefully selected and typically wrote blocks. Each individual was then assigned to an A or B group. Patients in the intervention group (A) and who were in the placebo group (B) received two capsules, each containing 500 mg of *P. major* seed or placebo, twice a day, 30 minutes before a meal or 2 hours after the meal. Both groups carefully were followed up for 12 weeks.

This study was approved by the Medical Ethics Committee of Iran University of Medical Sciences (Reference number: IR.IUMS.REC.1398.412) which was registered in the Clinical Trials Registry (ClinicalTrials.gov ID: IRCT20191006044993N1).

2.2. Participants. Seventy-four patients with NAFLD referred to Behesht Persian Medicine clinic affiliated to Iran University of Medical Sciences in Tehran, Iran, and other Persian Medicine clinics in Tehran were enrolled. The study was started from December 2019 to August 2020, and the follow-up period was ended in October 2020.

Inclusion criteria were NAFLD patients aged between 12 and 80 years with liver enzymes rising and ultrasound report grades 1 and 2 of fatty liver. Exclusion criteria were patient disliking to enter this study, pregnancy, lactation, anticoagulant drugs consumption, thyroid diseases, spleen diseases, cirrhosis, viral hepatitis and obstructive diseases of liver, uncontrolled diabetes mellitus, being under the treatment of dyslipidemia, lung diseases, malignancy, alcohol consumption, consumption of any drug which affects liver enzymes and liver metabolism like OCP, corticosteroids, salazine, usage of hepatotoxic drugs within the past 6 months, and renal insufficiency (serum creatinine $\geq$1.5 mg/dl).

Patients included in this study should be visited by a gastroenterologist and had a report of ultrasound confirming NAFLD and ruled out other possible causes of liver diseases (alcoholic fatty liver, Wilson, hypothyroidism, infectious, autoimmune, pharmacological and reactive hepatitis, etc.). Patients and investigators were blind to the intervention and control groups.

2.3. Intervention. After signing a written informed consent and taking a complete history of patients at the start of the study, they were randomly divided into two groups of case and control. Patients in both groups were given dietary recommendations due to classical medicine and recommended walking at an average speed for 40 minutes daily. Patients in the treatment group received two capsules (each containing 500 mg *P. major* seed) at 10 a.m. and two capsules at 6 p.m. for a period of 12 weeks. In the control group, patients received placebo capsules (two 500 mg capsules, two times a day) for 12 weeks. *P. major* and placebo capsules were packaged in similar containers and labeled accordingly. Consumption of less than 70% of the drug during the trial was considered as drug intolerance, and the patient was excluded from the trial.

This study's primary outcome was the evaluation of *P. major* on liver enzymes (AST >38, ALT >40) after the 12^{th} week, focusing on ALT changes as the main enzyme showing liver diseases.

Due to similar studies biochemical tests, complete blood count (CBC), FBS, TG, total cholesterol, HDL cholesterol, LDL cholesterol, and specific gravity (SG) of urine were also measured at weeks 0 and 12^{th} [25].

Blood samples and urine analysis were taken from all patients after 12 h of fasting. All participants were evaluated according to blood pressure, height, weight, and waist circumference, at the beginning and the end of the study. The BMI was calculated with the following formula: BMI = kg (weight)/m^2 (height) [26].

A standard flexible tape was used to measure waist circumferences in standing position, and with a standard scale (Omrone with ±0/5 kg); body weight of patients was measured. To avoid measurement error, all measurements were accomplished by the same person.

Photometric assay (Pars Azmoun Company) for the measurement of ALT and AST and enzymatic (glucose oxidase) colorimetric method using the standard kits (Pars Azmoun Company, Iran) to measure FBS were used. Secondary outcome measures were changes in fatty liver's grade (fatty tissue infiltration in the liver by using ultrasound imaging). The severity grade of fatty infiltration is determined by ultrasound due to the standard structure documented in Goldberg textbook:

> Grade 0 is considered to be the normal liver echogenicity.
>
> Grade 1 (mild): echogenicity is slightly increased, with normal visualization of the diaphragm and the intrahepatic vessel borders.
>
> Grade 2 (moderate): echogenicity is moderately increased with slightly impaired visualization of the diaphragm or intrahepatic vessels.
>
> Grade 3 (severe): echogenicity is markedly increased with poor visualization of the diaphragm, the intrahepatic vessels, and the posterior portion of the right lobe [27].

2.4. Drug Preparation. *P. major* seeds were purchased from medicinal plants store in Tehran which were authenticated by a botanist from School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (Voucher no. PMP-1748). Each drug capsule was filled with 500 mg *P. major* seed. In parallel, placebo capsules were filled with 500 mg toasted flour powder and were packaged in similar containers and labeled accordingly under a standard condition.

2.5. Total Phenolic and Flavonoid Content of P. major Seeds. The total phenolic content (TPC) of *P. major* seed was determined using the Folin–Ciocalteu method with some modifications [28].

For the preparation of the calibration curve, 1 ml aliquots of 75, 100, 150, and 200 μg/ml hydroethanolic (50 : 50) gallic acid solutions was mixed with 5 ml Folin–Ciocalteu reagent and 3 ml sodium carbonate (2% w/v). The absorption was read after 2 h at 760 nm and the calibration curve was drawn. One ml of *P. major* seed extract (1 mg/ml) was mixed with the same reagents as described above, and the absorption was measured for the determination of plant phenolic contents. All determinations were performed in triplicate.

The total flavonoid content (TFC) of *P. major* seed was determined using $AlCl_3$ reagent. Briefly, 1 ml of each sample (and/or catechin as the standard), previously dissolved in 90% ethanol, was mixed with 0.2 ml of $NaNO_2$ 5%. After 5 minutes, we added 0.3 ml of $AlCl_3$ solution 3% and 2 ml of NaOH 2M. After 30 min, the absorbance was measured at 510 nm [29].

2.6. Sample Size. The final sample size was calculated using a similar study [30], considering a power of 80% and $\alpha = 0.05$. By the expectation of potential loss to follow-up, 74 patients (37 persons in each group) were enrolled.

2.7. Statistical Analysis. The data were analyzed by SPSS software (version 17). The Kolmogorov–Smirnov test confirmed the normal distribution of variables. The mean ± standard deviation or number and frequency percentage were used to describe the variables. Comparing qualitative variables among groups was made using the Chi-square or Fisher exact test. Quantitative variables were compared among groups using a t-test or Mann–Whitney U test. P values less than 0.05 ($P < 0.05$) were considered statistically significant.

2.8. Safety Assessment. At the beginning of the study, a form was provided to patients in each group for recording possible drug side effects such as gastrointestinal symptoms, constipation, diarrhea, and the effect on libido.

3. Results

3.1. Total Phenolic and Flavonoid Contents. TPC of *P. major* seed was calculated as mean ± standard deviations of μg of gallic acid (GAE) equivalents/mg extract ($y = 0.4393x + 0.0181$, $r^2 = 0.9928$). TPC of *P. major* seed was 4 ± 0.2 mg/gram (mean ± SE $n = 3$).

TFC (as μg catechin equivalents/mg of a sample) for the sample was calculated on the basis of a linear calibration curve obtained using catechin ($Y = 0.0169\ x + 0.3526$, $r^2 = 0.999$). TFC of *P. major* seed was 60 ± 0.22 mg/gram (mean ± SE $n = 3$).

3.2. Clinical Trial Results. 180 patients were enrolled and 106 subjects who had not met the inclusion criteria were excluded (Figure 1).

Seventy-four patients who met the inclusion criteria and agreed to participate in the study were divided into two groups. Thirty-seven patients were assigned to the intervention group and 37 patients to the control group. Six

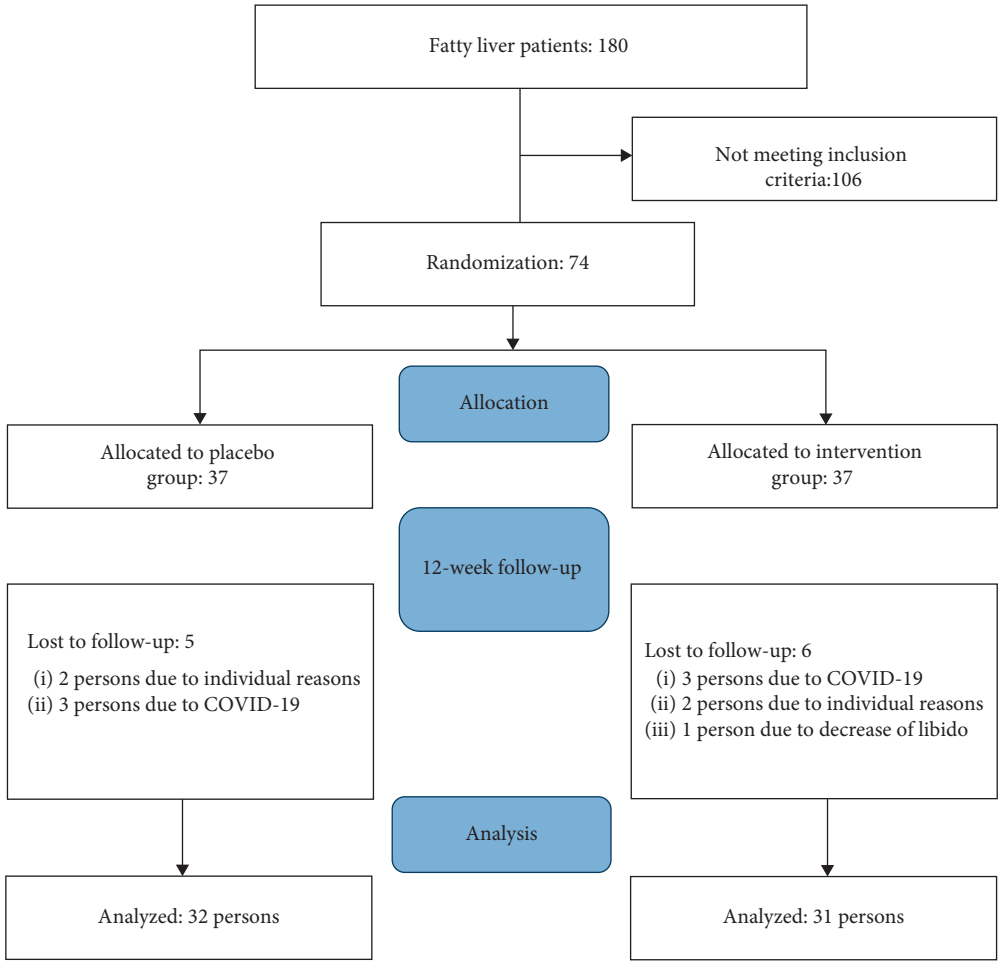

Figure 1: Consort flow diagram of the study.

individuals were excluded from the intervention group for reasons (three patients due to COVID-19, two patients due to personal reasons, and 1 patient due to the decrease of libido) and five individuals left the placebo group (three patients due to COVID-19 and two patients due to personal reasons). Finally, 31 individuals in the intervention group and 32 individuals in the placebo group completed the study which was assessed after 12 weeks.

Baseline characteristics of study groups are summarized in Table 1. Due to demographic characteristics, the mean age of participants was 43.3 (±8.927) and 38.7 (±8.48) years in the intervention and control groups, respectively. There were no significant differences in baseline demographic data and age, gender, and BMI between the two groups (Table 1).

Regarding laboratory indices, there were no significant differences in FBS, grade of NAFLD in ultrasound, ALT and AST, TG, cholesterol, and urine specific gravity levels between the two groups at the beginning of the study (Table 2).

Significant differences were observed due to baseline HDL (P value = 0.029), LDL (P value = 0.036), and platelet (P value = 0.038) (Table 2).

Following the patients in both groups, compliance with lifestyle did not show any significant differences between groups. We followed up the patients in both groups in the terms of compliance with lifestyle modification every week, and reminded them to do the diet and exercise instructions. Weight loss between three to 5% during 12 weeks was considered to assess the compliance with lifestyle and exercise modification between the two groups; at the end of the study, the weight change in the participants of the two groups was about three to 5% (Table 1). The analysis of results after 12 weeks reflected that liver enzymes (ALT and AST) decreased in both groups; however, the percent changes of liver enzymes in the intervention group were significantly higher than the placebo group (ALT, P value = 0.021; AST, P value = 0.004), and TG decreased significantly in the intervention group ($P = 0.001$) (Table 2).

In both groups, anthropometric factors such as weight, WC, and BMI reduced after 12 weeks; especially WC was decreased significantly in the intervention group (P value = 0.027). There was a significant reduction in the grade of NAFLD over the study period in the intervention group compared with the control group (P value = 0.038) (Table 2).

There was no report of severe adverse effects in patients of both groups; only one patient in *P. major* group complained of decreasing libido, which was excluded from the study.

Table 1: Basement characteristics of *P. major* and placebo group.

Variable	Group	Baseline	*P* value	After 12 weeks	*P* value[3]	Percent change	*P* value[3]
Gender (M/F)	*P. major*	M = 28, F = 3	0.113[1]				
	Placebo	M = 32, F = 0					
Age (y)	*P. major*	43.3 ± 8.92	0.054[2]				
	Placebo	38.7 ± 8.48					
WC (cm)	*P. major*	104.58 ± 8.89	0.606[3]	101.25 ± 9.27	0.978	3.2 ± 2.3	0.027
	Placebo	103.65 ± 8.88		101.18 ± 8.63		2.33 ± 2.7	
BMI (kg/m^2)	*P. major*	29.68 ± 3.19	0.83[3]	28.74 ± 3.15	0.842	3.15 ± 1.8	0.262
	Placebo	30.0 ± 3.85		29.17 ± 3.76		2.73 ± 3.3	

[1]Fisher's exact test; [2]*t*-test; [3]Mann–Whitney *U* test. WC = waist circumference; BMI = body mass index.

Table 2: Comparing biochemical parameters and NAFLD grades at the baseline and after 12 weeks in *P. major* and placebo group.

Variables	Group	Baseline	*P* value[1]	After 12 weeks	*P* value	Percent change	*P* value[1]
ALT (IU/L)	*P. major*	79.87 ± 36.19	0.068	47.32 ± 21.77	0.474	35.94 ± 22.82	0.042
	Placebo	66.37 ± 25.54		50.03 ± 21.19		19.53 ± 28.72	
AST (IU/L)	*P. major*	43.77 ± 17.24	0.106	28.29 ± 10.49	0.39	29.59 ± 24.31	0.004
	Placebo	37.93 ± 16.80		32.03 ± 13.30		12.09 ± 19.97	
FBS (mg/dl)	*P. major*	99.0 ± 12.02	0.429	103.93 ± 14.41	0.079	−5.48 ± 12.85	0.67
	Placebo	100.43 ± 25.8		103.62 ± 41.95		−2.59 ± 15.23	
Cholesterol (mg/dl)	*P. major*	190.83 ± 42.72	0.559	183.12 ± 42.86	0.726	3.27 ± 15.05	0.157
	Placebo	189.65 ± 35.49		185.68 ± 33.84		1.60 ± 7.65	
TG (mg/dl)	*P. major*	230.03 ± 108.02	0.063	200.93 ± 106.74	0.929	6.81 ± 36.45	0.001
	Placebo	181.31 ± 77.33		183.75 ± 73.96		−5.07 ± 23.97	
HDL (mg/dl)	*P. major*	40.19 ± 7.11	0.029	42.67 ± 6.71	0.05	−7.73 ± 17.04	0.433
	Placebo	36.12 ± 4.03		39.37 ± 5.03		−9.46 ± 12.74	
LDL (mg/dl)	*P. major*	104.90 ± 32.55	0.036	106.41 ± 33.24	0.466	−4.79 ± 33.39	0.326
	Placebo	114.03 ± 26.68		108.62 ± 25.72		4.27 ± 8.56	
Platelet	*P. major*	218.03 ± 63.50	0.038	213.09 ± 64.81	0.018	1.15 ± 14.23	0.773
	Placebo	247.30 ± 73.20		244.28 ± 76.66		−0.84 ± 17.62	
Urine S. G	*P. major*	1020.60 ± 5.19	0.577	1021.40 ± 5.08	0.827	−0.07 ± 0.51	0.607
	Placebo	1021.21 ± 6.90		1021.87 ± 7.80		−0.06 ± 0.44	
Ultrasound grade *n* (%)	*P. major*/placebo	31/32		31/32			
	Grade 0	0/0		3 (9.6)/0			
	Grade 1	9 (29.1)/7 (21.9)	0.572[2]	17 (54.8)/8 (25)	0.038[2]		
	Grade 2	22 (70.9)/25 (78.1)		11 (35.5)/23 (71.9)			
	Grade 3	0/0		0/1 (3.1)			

[1]Mann–Whitney *U* test; [2]Pearson chi-square. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FBS, fasting blood sugar; CHOL, cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SG, specific gravity of urine; negative sign, increase.

4. Discussion

This is the first clinical trial investigating the effect of *P. major* supplementation on NAFLD. In this randomized double-blind clinical trial, after 12 weeks, compliance of lifestyle modifications was the same in both groups. There was no significant difference in BMI between the two groups. Due to various studies, it showed that 5–10% weight loss is recommended for the effectiveness of the dietary intake and physical activity at improving NAFLD [31]. The percentage of weight loss was less than 5% in both groups (Table 1).

The mean ALT and AST enzymes, WC, and TG were significantly decreased. Also, *P. major* leads to a significant reduction in ultrasound grade of fatty liver in patients with NAFLD, compared with placebo. In a study by Ramezani et al., *P. major* showed a significant reduction in ALT and AST enzymes in mice compared to the control group, which supported the present study's findings [32]. Waist circumference measurement was used to diagnose and define central obesity. This type of obesity is related to visceral fat, insulin resistance (IR), and increased free fatty acid levels [33]. WC is significantly decreased in both groups; it was more significant in *P. major* group ($P = 0.027$). This reduction in WC may be related to the improvement of gastric digestion [34] and the reduction of gastrointestinal bloating [35]. *P. major* showed efficacy in the regulation of carbohydrate and fatty acids metabolism in an animal study [32]. In another study, some other species of Plantaginaceae, such as *P. lanceolata* showed effectiveness to prevent obesity in mice by stimulating metabolism throughout visceral fat tissue [36].

Due to PM, as well as in classical medicine, the first step to treat fatty liver disease is lifestyle modification. Both groups were assigned similar diet and physical activity due to

classical medicine supervised by a gastroenterologist. The placebo group also showed a significant reduction in weight, BMI, and liver enzymes at the end of the study, and this confirms that lifestyle modification is the most important factor in the treatment and improvement of NAFLD indices. Although in the early stages of NAFLD, when the prevalence of NASH and advanced fibrosis is low, 5–8% weight loss and a healthy diet may be sufficient for treatment; in the more advanced stages of liver disease, lifestyle modification with medication may be necessary [2].

Several experimental studies indicated the hepatoprotective effects of *P. major* and confirmed this study's findings. *P. major* showed potent antioxidant activity. Natural products with antioxidant properties showed hepatoprotective activity against some liver toxicity [37]. Turel et al. indicated the protective effects of *P. major* on liver cells in mice poisoned by carbon tetrachloride [22]. Mello et al. showed that *P. major* could prevent oxidative damage to mitochondria, known for its hepatoprotective effect against the toxic effects of oxygen radicals [24]. Scarlat et al. showed the protective effects of *P. major* on liver cells in mice poisoned by diclofenac. *P. major* leads to a decrease in triglycerides values [38]. In a similar study, Nasr et al. indicated that methanolic extract of *P. major* had hepatoprotective effects against hepatocyte damage caused by carbon tetrachloride in mice and reduced the levels of liver enzymes and TG in sick mice. In fact, the antioxidants, as well as anti-inflammatory effects of the flavonoid compounds present in *P. major* reveal its hepatoprotective role. Flavonoids are known as antioxidants, free radical scavengers, and antilipoperoxidants leading to hepatoprotection [21]. The anti-inflammatory activity of *P. major* is through an inhibitory effect on lipoxygenase enzyme. This enzyme catalyzes arachidonic acid to produce leukotrienes. Leukotrienes play a role in inflammatory diseases. The anti-inflammatory activity of *P. major* is exerted by flavonoids such as Baikaline and Hispidoline and iridoid glycosides [39]. These findings are consistent with this study's findings.

There were some limitations like the short duration of the study and the use of *P. major* seed as a capsule for better acceptance in patients (in PM its decoction form is recommended). The effect of *P. major* on liver histology (liver biopsy) was unstudied because it was quite invasive. The prevalence of COVID-19 pandemic prevents patients from participating in the study or causes them to leave the study; also, due to COVID-19 pandemic, the patients (who lived in different parts of the city) preferred to go to the nearest medical center for ultrasound (before and after 12 weeks).

5. Conclusions

This randomized, double-blind controlled clinical trial indicated that treatment with *P. major* seed could reduce liver enzymes and improve the grade of fatty liver in NAFLD. Regarding cost-effectiveness and availability of *P. major* and the lack of any reports for serious complications of this plant, *P. major* can be recommended in patients with NAFLD. Further research with larger sample size, as well as long-term follow-ups of patients, can provide a stronger document about the usage of *P. major* as complementary medicine to treat NAFLD.

Data Availability

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

Disclosure

This paper is a part of Ph.D. student dissertation of Sayedeh Ferdows Jazayeri.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by a grant from Iran University of Medical Sciences, Tehran, Iran. The authors sincerely thank all patients and the team that worked on this study, especially Mrs. Abdollahi, Dr. Behrooz Moghbel Al-Hossein, Dr. Amir Mohammadi Dehdezi, Dr. Masood Tavakol far, and Dr. Kamran Mahmoodi.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6617772, 10 pages
https://doi.org/10.1155/2021/6617772

Research Article

Therapeutic Efficacy and Safety of Safflower Injection in the Treatment of Acute Coronary Syndrome

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Received 10 January 2021; Revised 21 February 2021; Accepted 8 March 2021; Published 18 March 2021

Academic Editor: Mahnaz Khanavi

Background. Safflower injection (SFI), a popular Chinese patent drug, is commonly used to treat acute coronary syndromes (ACSs) in China. The research seeks to scientifically estimate the clinical efficacy of SFI for ACS patients. *Methods*. Eight electronic databases were retrieved for eligible research from the founding date to September 8, 2020. Odds ratio (OR) was adopted to assess the total effective rate, ECG improvement, and adverse reaction, and mean difference (MD) was used for assessing the hemorheology indexes as well as the LVEF. *Results*. Sixteen randomized controlled trials involving 1620 sufferers with ACS were incorporated. The outcomes showed that, in comparison to conventional medication alone, SFI combined with conventional treatment remarkably enhanced the total effective rate (OR = 3.66, 95% CI [2.73, 4.90], $P < 0.00001$), ECG improvement (OR = 2.85, 95% CI [2.04, 3.99], $P < 0.00001$), and LVEF (MD = 5.13, 95% CI [3.73, 6.53], $P < 0.00001$). Moreover, SFI combined with conventional treatment significantly decreased hemorheology indexes including BV (MD = −0.95, 95% CI [−1.76, −0.13], $P = 0.02$), HCT (MD = −2.37, 95% CI [−3.25, −1.50], $P < 0.00001$), FIB (MD = −0.44, 95% CI [−0.60, −0.29], $P < 0.00001$), and PAR (OR = −7.65, 95% CI [−10.16, −5.14], $P < 0.00001$). However, no notable contrast was observed to link the experimental and the control team for PV (MD = −0.42, 95% CI [−0.83, 0.00], $P = 0.05$) and adverse reactions (OR = 0.59, 95% CI [0.13, 2.74], $P = 0.50$). *Conclusion*. Despite the limitations that existed in this meta-analysis, the outcomes demonstrated that SFI and conventional combined medication is an effective and relatively safe therapy for ACS sufferers.

1. Introduction

Acute coronary syndrome (ACS) is a category of clinical syndromes resulting from acute myocardial ischemia. These syndromes include ST-segment elevation myocardial infarction, non-ST-segment elevation myocardial infarction, and unstable angina pectoris [1]. The main pathological basis of ACS is the formation of complete or incomplete occlusive thrombosis induced by the breakage or erosion of unstable atherosclerotic plaques in coronary arteries [2]. ACS is mainly characterized by acute onset, rapid progression, and high mortality [3, 4]. Presently, cardiovascular disease (CVD) is attributed to almost a third of global fatalities, and the most serious of them all is ACS, which results in five million hospitalizations and two hundred and seventy billion dollars cost each year in Europe and America [5, 6].

The treatment of ACS mainly includes medication, surgery, and intervention operation [7]. Among them, medication is the popular procedure [8]. Western medicines for the conventional treatment of ACS include statins,

β-blockers, nitrates, calcium channel restrictors, and angiotensin-converting enzyme inhibitors [9]. However, the clinical efficacy of the conventional western treatments is still limited, and the adverse reactions resulted from these cannot be overlooked. For example, side effects such as gastrointestinal reactions, headaches, and hypotension are prone to occur during the treatment of ACS using nitrates [10]. Therefore, how to improve the efficacy of conventional medication on ACS and relieve the adverse reactions is a direction that clinical staff should strive for.

Safflower injection (SFI) is a popular Chinese patent drug that has been authorized by the China Food and Drug Administration. It is prepared from *Carthami flos* by water extraction and alcohol precipitation [11]. The major components of SFI are carthamin yellow, which belongs to chalcones. According to Traditional Chinese Medicine (TCM) theory, the manifestation of ACS is associated with stagnant blood block [12]. However, SFI is good at promoting blood circulation by removing blood stasis, which is contributed to relieve stagnant blood block. Modern pharmacological studies also have indicated that SFI could expand the coronary artery, protect the myocardium, and eliminate free radicals which reduce the appearance of angina [13]. SFI has been linked to absolute efficacy on sufferers with coronary heart disease, hypertension, cerebral infarction, and other CVDs [14].

Along with the development of integration of traditional Chinese and Western medicine, SFI combined with conventional treatment (Western medicines) was increasingly prescribed for treating ACS over the past decades, and some studies showed that it might bring beneficial effects to the patients [15]. However, a higher percentage of the clinical studies have not given enough proof from the small sample sizes. Systematic evidence which could prove the efficacy and safety is demanded extremely. Thus, the meta-analysis was performed by roundly assessing the efficacy of SFI and conventional combined treatment for ACS compared to single conventional treatment, with the hope of providing a statistical record of this combined medication.

2. Methodology

2.1. Search Strategy. The PRISMA statement was used to form a basis for the meta-analysis [16]. Randomized controlled trials (RCTs) were independently searched and retrieved by two investigators (Qiang Lu and Jiamin Xu). The including databases were used from the formation date to September 8, 2020: PubMed, Embase, the Cochrane Library, Web of Science (WOS), China National Knowledge Infrastructure (CNKI), China Biology Medicine disc (CBMdisc), Wanfang Data, and VIP medicine information system (VMIS). Two different strategies were used in the literature search. For the English databases, the following retrieval terms were used in combination: ("Safflower injection" OR "Honghua injection") AND ("unstable angina" OR "acute myocardial infarction" OR "acute coronary syndrome"). The following keywords were searched in combined ways for Chinese databases: ["hong hua zhu she ye (in Chinese)"] AND ["bu wen ding xin jiao tong (in Chinese)" OR "ji xing xin ji geng si (in Chinese)" OR "ji xing guan mai zong he zheng (in Chinese)"]. Research studies published using either English or Chinese were taken into account.

2.2. Inclusion Criteria. After consulting with several cardiologists, the inclusion criteria were formulated as follows: subjects were confirmed to suffer from ACS according to the cardiovascular disease examination method formulated by the Chinese Medical Society (CMA) as well as the American Heart Association (AHA) with randomized controlled trials (RCTs) [17, 18]; all studies enumerated were detailed as RCTs; SFI administered as the single Chinese patent medicine in RCTs; sufferers in the experimental team had administration of combined therapy of SFI and conventional treatment, while those in the control team got the conventional treatment alone; outcomes of each study had not less than one of these indices: total effective rate, electrocardiogram (ECG) improvement, hemorheology indexes including blood viscosity (BV), hematocrit (HCT), fibrinogen (FIB), plasma viscosity (PV), and platelet aggregation rate (PAR), left ventricular ejection fraction (LVEF), and adverse reactions.

2.3. Exclusion Criteria. This was formulated as follows: reviews, case report, animal experiments, editorials, and unrelated clinical studies; research studies were found not to be RCTs or diagnosis standards were unclear; studies containing patients diagnosed with stable angina; the interference of ACS sufferers was not accordant; and for the studies with information replication, the subsequent publications were considered as data fraud and were then denied once the authors were unreachable.

2.4. Data Extraction and Quality Assessment. Details on relevant studies which include author names, issuance year, sample capacity, intervening measures, and outcomes were generalized. In accordance to the Cochrane Handbook for Systematic Reviews of Interventions, quality evaluation of the incorporated research studies was separately carried out by the researchers (Qiang Lu and Qian Li) using the risk of bias table from Review Manager 5.3 [19]. From this information, seven types of biases were derived. Each of them was evaluated using three levels: low risk of bias, unclear, and high risk of bias. "Low risk of bias" shows the illustration of procedures was sufficient or accurate, whereas "high risk of bias" indicates insufficient or inaccurate. When insufficient detail was presented in the research and we could not decide whether it is "high risk" or "low risk," the object was described as "unclear." Data extraction and study evaluation inconsistencies were judged through requited analysis or discourse with a third party (Xiaobo Yang).

2.5. Statistical Analysis. Review Manager 5.3 (Cochrane Collaboration) was employed to process the extracted data from the relevant studies [19]. Resulting measures which include total effective rate, ECG improvement, and adverse

reactions were referred to as dichotomous variables. These were accorded to be the odds ratio (OR) having a 95% confidence interval (CI). The hemorheology indexes (BV, PV, HCT, FIB, and PAR) and LVEF were continuous variables that were given as the mean difference (MD) with 95% CI. Chi-square analysis was used to examine the heterogeneity among studies, and the I^2 statistic was employed to evaluate the level of heterogeneity. A fixed-effect model was employed to process data with low heterogeneity ($P > 0.1$ and $I^2 < 50\%$), and data having high heterogeneity ($P < 0.1$ or $I^2 > 50\%$) were assessed by a random-effect model [20]. The risk of publication bias was illustrated in the selected studies using a funnel plot.

3. Results

3.1. Study Selection. One hundred and fifty-three possible data from Chinese databases were selected in the initial analysis, and similar data were not recovered from English databases. Eighty-five replicated articles were removed as a result of overlapping of the database scope. A sum of 68 studies was acquired for title or abstract check up, and 27 research studies were eliminated due to irrelevant subjects. Forty-one articles were put aside to examine complete information.

In the inspection of complete information, 25 pieces of research were excluded as a result of the following: 7 studies were single-arm designs, diagnosis in 6 researches was unclear, 9 studies brought up unsuitable interferences, and 3 trials severally presented similar records with another publication. In the end, there were sixteen pieces of study used for this meta-analysis (Figure 1).

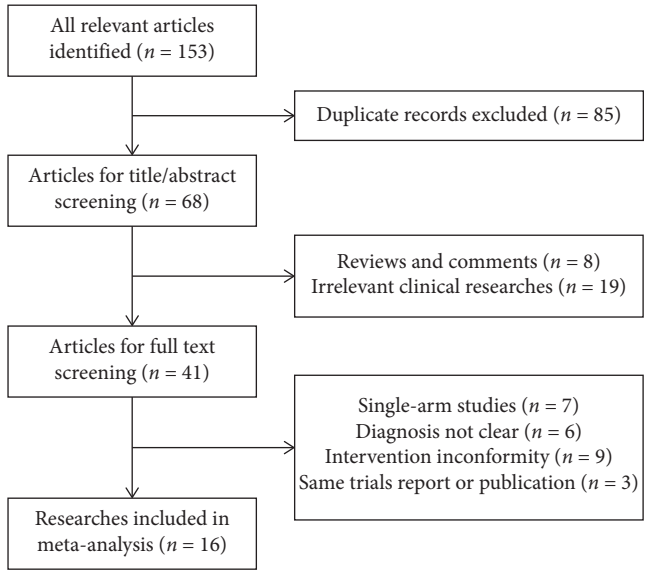


Figure 1: Flow chart of trial searching and screening for this meta-analysis.

3.2. Study Features. Due to SFI-based treatments being mainly used in Chinese Medicine or Integrative Medicine, 16 relevant studies consisting of 1620 sufferers were documented in Chinese databases from 2003 to 2020. The experimental team had 832 patients, while the control team had 788 patients. The total number of males (59.1%) is higher than that of females in the included studies, and the mean age of all the patients was approximately 60.9, ranging from 37 to 83. All the adopted trials were RCTs with a contrast between the SFI and conventional combined treatment and single conventional treatment, and there were some similarities and differences in the conventional treatments. The dosage of SFI was between 15 to 40 mL/day by intravenous drip, and most researchers documented the span of drug administration to be 2 weeks. An absolute dissimilarity was not seen evident between the two groups from fundamental data (Table 1).

3.3. Quality Assessment of the Eligible Studies. The methodological quality of the selected research studies was judged by the Cochrane risk of bias assessment and presented universally low. All the included studies were parallelly designed [21–36]. Eleven of the 16 trials identified the allocation sequence generation in the absence of indicating the concrete procedure [21, 22, 25–30, 33–35]. Only 4 research studies [23, 24, 32,36] showed that they were randomly grouped in accordance to the random number table procedure. All the selected studies did not explain allocation secrecy, blinding of sufferers, and result evaluation. Five trials [23, 25, 27, 32, 36] were at a low risk of attrition bias for giving whole outcome information. Twelve pieces of study [21, 23–26, 28–32, 35, 36] documenting the result of comprehensive indexes indicated a low risk of reporting bias. The risk of bias graph is described in Figure 2.

3.4. Total Effective Rate. Fourteen of 16 research studies [21–23, 25–33, 35, 36] compared the total effective rate between SFI together with conventional medication and conventional medication alone. Meta-analysis of the 14 researches employing a fixed-effect model showed that the combined administration of SFI and conventional medication notably enhanced the total effective rate than single conventional medication in treating ACS (OR = 3.66, 95% CI [2.73, 4.90], $P < 0.00001$). No statistically notable heterogeneity ($P = 1$, $I^2 = 0\%$) was detected among studies individually (Figure 3).

3.5. ECG Improvement. Six of the included research studies [21, 23, 28, 29, 31, 32] reported ECG improvement. Meta-analysis using a fixed-effect model indicated that the number of participants with ECG improvement increased remarkably in the experimental team contrasted to the control team (OR = 2.85, 95% CI [2.04, 3.99], $P < 0.00001$). There was no statistically notable heterogeneity ($P = 0.19$, $I^2 = 33\%$) in the meta-analysis (Figure 4).

3.6. Hemorheology Indexes. BV, PV, HCT, FIB, and PAR were regarded as indexes of blood rheology recorded in the relevant studies. Four of the tests [24, 26, 30, 35] mentioned

Table 1: Characteristics of the included studies.

Study ID	Number (E/C)	Gender (E/C)	Intervention	Control (conventional treatment)	SFI dosage	Duration	Outcome measures
Cao et al. [21]	60/60	67/53	SFI + control	Aspirin and nitroglycerin	20 mL, q.d.	14 days	TER and ECG improvement
Zheng [22]	30/28	38/20	SFI + control	Aspirin, nitroglycerin, metoprolol, ACEI, isosorbide dinitrate, etc.	20 mL, q.d.	10 days	TER
Hang [23]	104/96	123/77	SFI + control	Aspirin, nitrates, ACEI, statins, metoprolol, etc.	20 mL, q.d.	10 days	TER and ECG improvement
Ding and Dong [24]	20/16	22/14	SFI + control	Aspirin, nitrates, ACEI, etc.	20 mL, q.d.	14 days	HR indexes
Du [25]	30/30	34/26	SFI + control	Aspirin, nitrates, β-blockers, calcium antagonists, etc.	20 mL, b.i.d.	10 days	TER and HR indexes
Zhang and Zhao [26]	60/60	80/40	SFI + control	Aspirin, isosorbide dinitrate, metoprolol, etc.	20 mL, q.d.	14 days	TER and HR indexes
Jin et al. [27]	44/40	51/33	SFI + control	Aspirin, isosorbide mononitrate, statins, low molecular heparin, etc.	40 mL, q.d.	14 days	TER
Zhang [28]	41/41	54/28	SFI + control	Aspirin, nitrates, β-blockers, calcium antagonists, etc.	20 mL, q.d.	15 days	TER and ECG improvement
Ma [29]	80/60	73/67	SFI + control	Aspirin and nitrates	20 mL, q.d.	15 days	TER, HR indexes, and ECG improvement
Wang et al. [30]	36/36	43/29	SFI + control	Aspirin, nitrates, calcium antagonists, etc.	20 mL, q.d.	14 days	TER and HR indexes
Yan [31]	70/70	82/58	SFI + control	Aspirin, isosorbide mononitrate, simvastatin, etc.	30 mL, q.d.	14 days	TER and ECG improvement
Zhu et al. [32]	32/28	45/15	SFI + control	Aspirin, nitrates, clopidogrel, statins, low molecular heparin, etc.	40 mL, q.d.	14 days	TER and ECG improvement
Chen [33]	46/46	50/42	SFI + control	Aspirin, atorvastatin, etc.	20 mL, q.d.	14 days	TER and ARs
Cao [34]	39/39	47/31	SFI + control	Aspirin, atorvastatin, β-blockers, etc.	20 mL, q.d.	15 days	LVEF
Zhang [35]	98/98	103/93	SFI + control	Aspirin, isosorbide mononitrate, ACEI, calcium antagonists, etc.	20 mL, q.d.	14 days	TER and HR indexes
Zhao et al. [36]	42/40	45/37	SFI + control	Aspirin, simvastatin, clopidogrel, etc.	15 mL, q.d.	28 days	TER, HR indexes, LVEF, and ARs

E, experimental group; C, control group; SFI, safflower injection; ACEI, angiotensin-converting enzyme inhibitors; q.d., once a day; b.i.d., twice a day; TER, total effective rate; ECG, electrocardiogram; HR, hemorheology; LVEF, left ventricular ejection fraction; AR, adverse reactions.

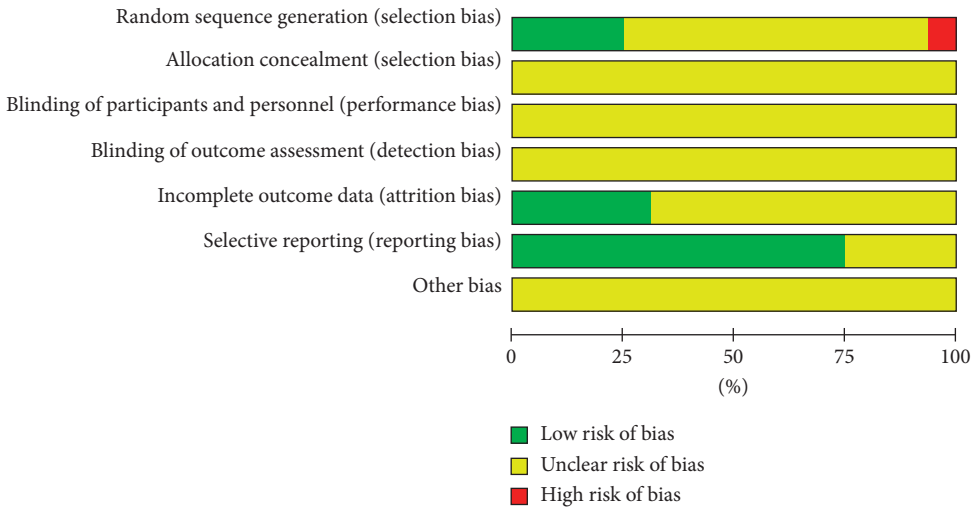


Figure 2: Methodological quality evaluation for the risk of bias in the eligible researches.

the detection of BV. Remarkable heterogeneity was discovered among the studies ($P < 0.00001$, $I^2 = 96\%$); therefore, a random-effect model was employed to perform a meta-analysis which indicated that SFI and conventional combined administration notably lessened BV (MD = −0.95, 95% CI [−1.76, −0.13], $P = 0.02$) (Figure 5(a)).

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, fixed, 95% CI
Cao et al., 2003	58	60	51	60	3.4	5.12 [1.06, 24.79]
Zheng, 2004	22	30	13	28	7.1	3.17 [1.06, 9.52]
Hang, 2007	87	104	53	96	17.8	4.15 [2.15, 8.01]
Du, 2009	29	30	24	30	1.6	7.25 [0.82, 64.46]
Zhang and Zhao, 2009	56	60	44	60	5.8	5.09 [1.59, 16.31]
Jin et al., 2010	40	44	30	40	5.7	3.33 [0.95, 11.66]
Zhang, 2010	37	41	28	41	5.4	4.29 [1.26, 14.60]
Ma, 2011	73	80	42	60	8.3	4.47 [1.73, 11.58]
Wang et al., 2012	33	36	25	36	4.1	4.84 [1.22, 19.21]
Yan, 2012	65	70	60	70	8.5	2.17 [0.70, 6.70]
Zhu et al., 2012	30	32	24	28	3.2	2.50 [0.42, 14.83]
Chen, 2014	40	46	34	46	8.8	2.35 [0.80, 6.94]
Zhang, 2018	88	98	73	98	14.8	3.01 [1.36, 6.68]
Zhao et al., 2019	38	42	29	40	5.6	3.60 [1.04, 12.48]
Total (95% CI)		*773*		*733*	*100.0*	*3.66 [2.73, 4.90]*
Total events	696		530			

Heterogeneity: $chi^2 = 3.35$, $df = 13$ ($P = 1.00$); $I^2 = 0\%$
Test for overall effect: $Z = 8.70$ ($P < 0.00001$)

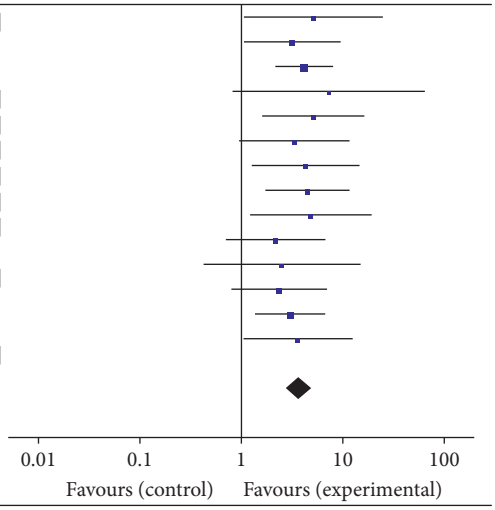


Figure 3: Forest plot of total effective rate of SFI plus conventional treatment compared to single conventional treatment for ACS. *I2* and *P* are the criteria for the heterogeneity test, ◆: pooled odds ratio, –■–: odds ratio, and 95% CI.

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight (%)	Odds ratio M-H, fixed, 95% CI
Cao et al., 2003	47	60	34	60	17.9	2.76 [1.24, 6.15]
Hang, 2007	79	104	44	96	26.7	3.73 [2.04, 6.82]
Zhang, 2010	38	41	23	41	4.1	9.91 [2.63, 37.38]
Ma, 2011	42	80	23	60	30.3	1.78 [0.90, 3.51]
Yan, 2012	62	70	58	70	16.1	1.60 [0.61, 4.20]
Zhu et al., 2012	29	32	21	28	5.1	3.22 [0.74, 13.94]
Total (95% CI)		*387*		*355*	*100*	*2.85 [2.04, 3.99]*
Total events	297		203			

Heterogeneity: $chi^2 = 7.41$, $df = 5$ ($P = 0.19$); $I^2 = 33\%$
Test for overall effect: $Z = 6.12$ ($P < 0.00001$)

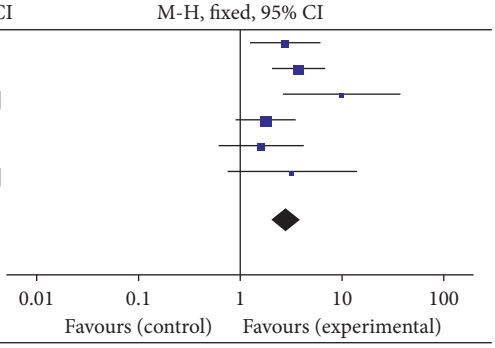
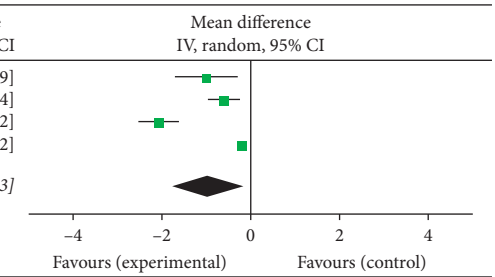


Figure 4: Forest plot of ECG improvement of SFI plus conventional treatment compared to conventional treatment alone for ACS. *I2* and *P* are the criteria for the heterogeneity test, ◆: pooled odds ratio, –■–: odds ratio, and 95% CI.

Study or subgroup	Experimental Mean	Experimental SD	Experimental Total	Control Mean	Control SD	Control Total	Weight (%)	Mean difference IV, random, 95% CI
Ding and Dong, 2008	4.32	1.08	20	5.32	1.08	16	22.4	–1.00 [–1.71, –0.29]
Zhang and Zhao, 2009	4.9	1	60	5.5	1	60	25.7	–0.60 [–0.96, –0.24]
Wang et al., 2012	4.53	1.06	36	6.6	0.9	36	24.9	–2.07 [–2.52, –1.62]
Zhang, 2018	3.9	0.3	98	4.1	0.3	98	27.0	–0.20 [–0.28, –0.12]
Total (95% CI)			*214*			*210*	*100.0*	*–0.95 [–1.76, –0.13]*

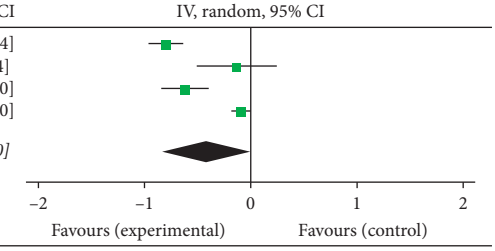
Heterogeneity: $tau^2 = 0.64$; $chi^2 = 70.12$, $df = 3$ ($P < 0.00001$); $I^2 = 96\%$
Test for overall effect: $Z = 2.28$ ($P = 0.02$)



(a)

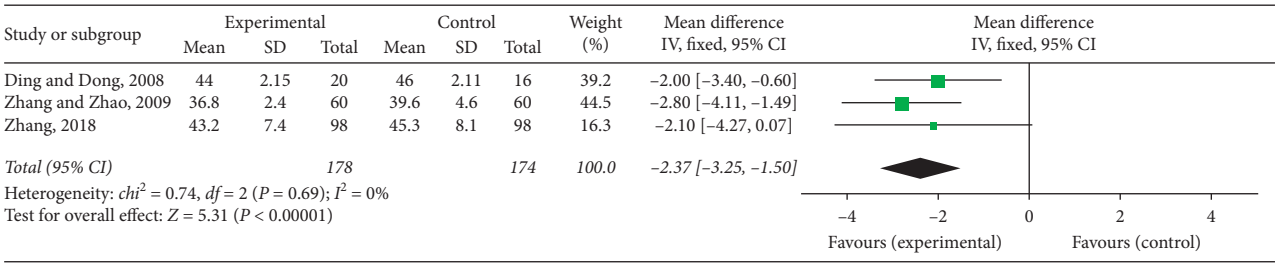
Study or subgroup	Experimental Mean	Experimental SD	Experimental Total	Control Mean	Control SD	Control Total	Weight (%)	Mean difference IV, random, 95% CI
Ding and Dong, 2008	1.3	0.16	20	2.1	0.3	16	26.0	–0.80 [–0.96, –0.64]
Du, 2009	1.68	0.75	30	1.81	0.73	30	22.2	–0.13 [–0.50, 0.24]
Ma, 2011	1.27	0.67	80	1.89	0.66	60	25.1	–0.62 [–0.84, –0.40]
Wang et al., 2012	1.86	0.22	36	1.95	0.16	36	26.7	–0.09 [–0.18, –0.00]
Total (95% CI)			*166*			*142*	*100.0*	*–0.42 [–0.83, 0.00]*

Heterogeneity: $tau^2 = 0.17$; $chi^2 = 66.28$, $df = 3$ ($P < 0.00001$); $I^2 = 95\%$
Test for overall effect: $Z = 1.96$ ($P = 0.05$)

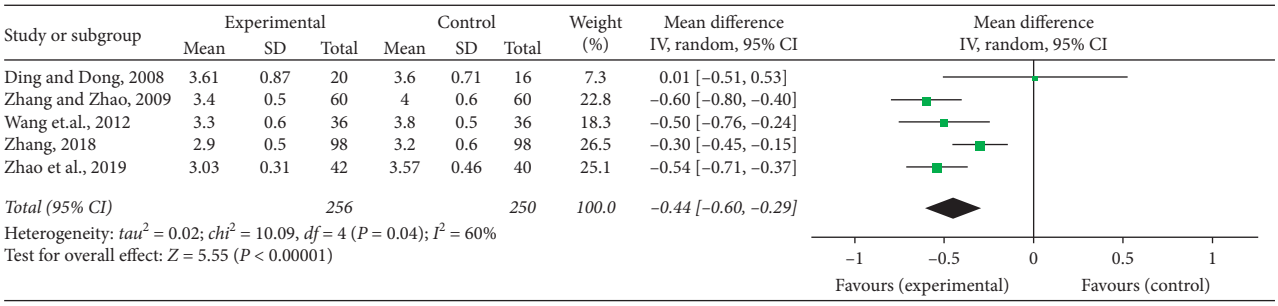


(b)

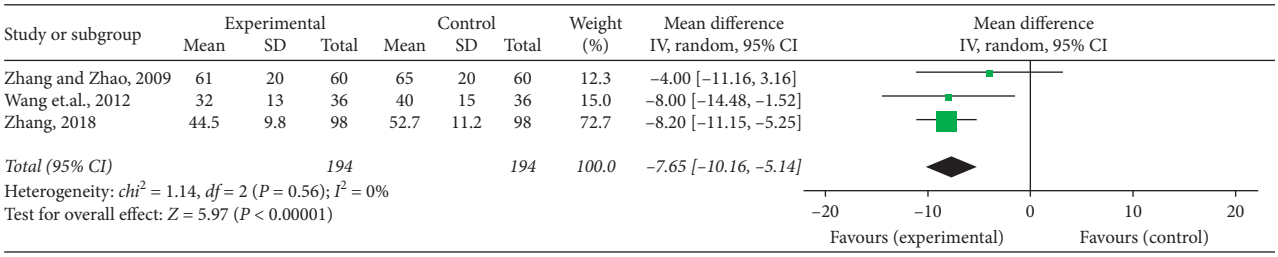
Figure 5: Continued.



(c)



(d)



(e)

Figure 5: Forest plot of comparison in two groups for hemorheology indexes. (a) Blood viscosity; (b) plasma viscosity; (c) hematocrit; (d) fibrinogen level; and (e) platelet aggregation rate. *I*2 and *P* are the criteria for the heterogeneity test, ◆: pooled mean difference, –■–: mean difference, and 95% CI.

Four studies [24, 25, 29, 30] provided the values of PV. There existed dramatically notable heterogeneity ($P < 0.00001$, $I^2 = 95\%$) among individual studies, and a meta-analysis employing a random-effect model manifested no difference linking the PV in the experimental and control teams (MD = −0.42, 95% CI [−0.83, 0.00], $P = 0.05$) (Figure 5(b)).

Three research studies [24, 26, 35] reported the detection of HCT. A fixed-effect meta-analysis indicated SFI together with conventional medication greatly decreased HCT compared to single conventional medication (MD = −2.37, 95% CI [−3.25, −1.50], $P < 0.00001$). There was no notable heterogeneity detected in the research studies ($P = 0.69$, $I^2 = 0\%$) (Figure 5(c)).

Five studies [24, 26, 30, 35, 36] indicated FIB concentration in blood plasma. There was a statistically prominent heterogeneity discovered from the trials ($P = 0.04$, $I^2 = 60\%$). A random-effect meta-analysis was undertaken to indicate that the combination of SFI and conventional medication notably lowered FIB concentration in blood plasma (MD = −0.44, 95% CI [−0.60, −0.29], $P < 0.00001$) (Figure 5(d)).

Three trials [26, 30, 35] recorded the detection of PAR in blood. No heterogeneity was found ($P = 0.56$, $I^2 = 0\%$) among the researches, so a fixed-effect model meta-analysis was conducted. The pooled OR indicated the SFI-conventional combined medication notably decreased PAR in contrast to the conventional medication alone (OR = −7.65, 95% CI [−10.16, −5.14], $P < 0.00001$) (Figure 5(e)).

3.7. LVEF. Two studies [34, 36] mentioned the investigation on LVEF. There was no heterogeneity checked in the meta-analysis, and so, a fixed-effect model was adopted ($P = 0.45$, $I^2 = 0\%$). An OR with 95% CI was used to show the contrast link of LVEF in the experimental and control teams (MD = 5.13, 95% CI [3.73, 6.53], $P < 0.00001$). It indicated that SFI could notably extend LVEF for ACS patients (Figure 6).

3.8. Adverse Reactions. One [29] of the included researches indicated no clear adverse reaction took place after administration of medication, and two [33, 36] indicated occurrences of adverse reactions. These reactions were

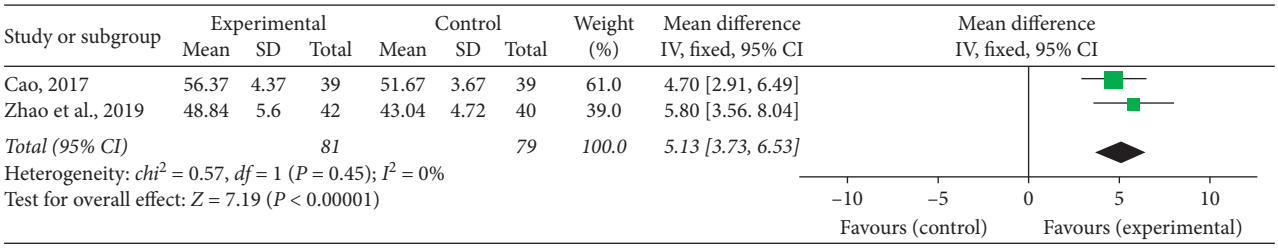


Figure 6: Forest plot of comparison in two groups for LVEF. *I2* and *P* are the criteria for the heterogeneity test, ◆: pooled mean difference, –■–: mean difference, and 95% CI.

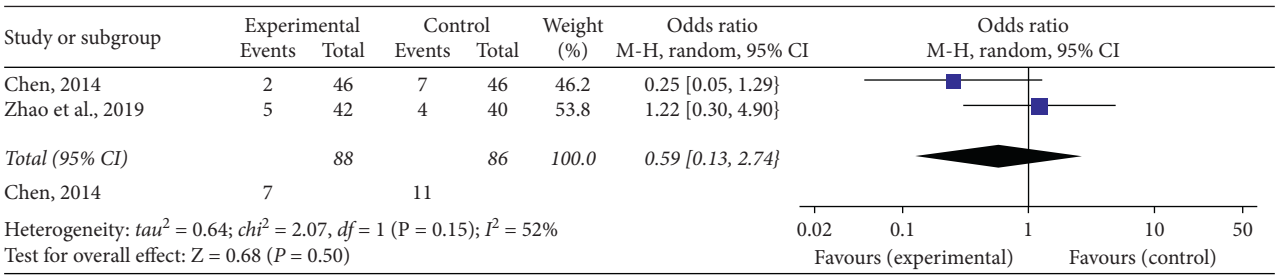


Figure 7: Forest plot of comparison in two groups for the incidence of adverse reactions. *I2* and *P* are the criteria for the heterogeneity test, ◆: pooled odds ratio, –■–: odds ratio, and 95% CI.

characterized by nausea and vomiting, flushing, headache, and diarrhea. A significant heterogeneity ($P = 0.15$, $I^2 = 52\%$) was detected in the researches, so a random-effect model was employed to carry out this meta-analysis. The merged OR with 95% CI indicated no difference in the occurrence of adverse reactions linked the experimental and control teams (OR = 0.59, 95% CI [0.13, 2.74], $P = 0.50$) (Figure 7).

3.9. Publication Bias. A funnel plot was used to estimate the publication bias. There were 14 and 6 pieces of research severally brought into the funnel plots of total effective rate and ECG improvement. As indicated in Figure 8, both of the plots were symmetrical, showing no publication bias in the included trials.

4. Discussion

Cardiovascular disease (CVD) is caused by multiple risk factors such as development standards of life, lifestyle switches, aging of the population, and the gradually changing environment [37]. The morbidity and mortality of such disease has been consistently high, and the burden of prevention and treatment of CVD is increasing. It has become a central public health topic. ACS is the toughest form of CVD with a high handicap rate, mortality rate, and other health conditions which is a serious threat to human health [38, 39]. The main treatment methods of ACS include life intervention, drug treatment, percutaneous coronary intervention, and comprehensive treatment [40, 41]. Percutaneous coronary intervention works well, but it is expensive and exceeds the affordability of many patients. Therefore, relatively inexpensive and effective drug treatment is still a practical solution. However, the therapeutic effects of Western medicines are limited, and some adverse reactions appeared during the period of the treatment. Therefore, more efficacious and safe treatments are quickly needed for these patients in China and throughout the globe.

Over the years, Chinese medics have been seeking for more effective treatments for ACS. TCM has been used to treat coronary heart disease (including ACS) for more than 2000 years. The curative efficacy of Chinese medicines in dealing with ACS is clear and more potent than some Western drugs, and Chinese medicines have less toxicity and fewer side effects. Thus, use of the Chinese medicines on ACS is also as important. Together with the improvement of contemporary pharmacy techniques, all kinds of medicinal preparations for treating patients of ACS that based on classical prescriptions of Chinese medicines have been greatly developed [42]. *Carthami flos* (namely, the dry flowers of *Carthamus tinctorius* L.) is a Chinese traditional medicine, which has been applied in the clinic since the Han Dynasty. Its main function is activating blood to promote menstruation and eliminating stasis to stop the pain [43]. SFI was successfully developed from *Carthami flos* in 1973 and has been widely used for more than 40 years [44]. It has been consistently demonstrated that SFI can effectively treat many CVD [45]. However, there is no extensive and systematic assessment of SFI for the remedy of ACS in accordance with general international standards. Thus, this research intends to give a globally recognized system evaluation of the clinical effect of SFI for the treatment of ACS.

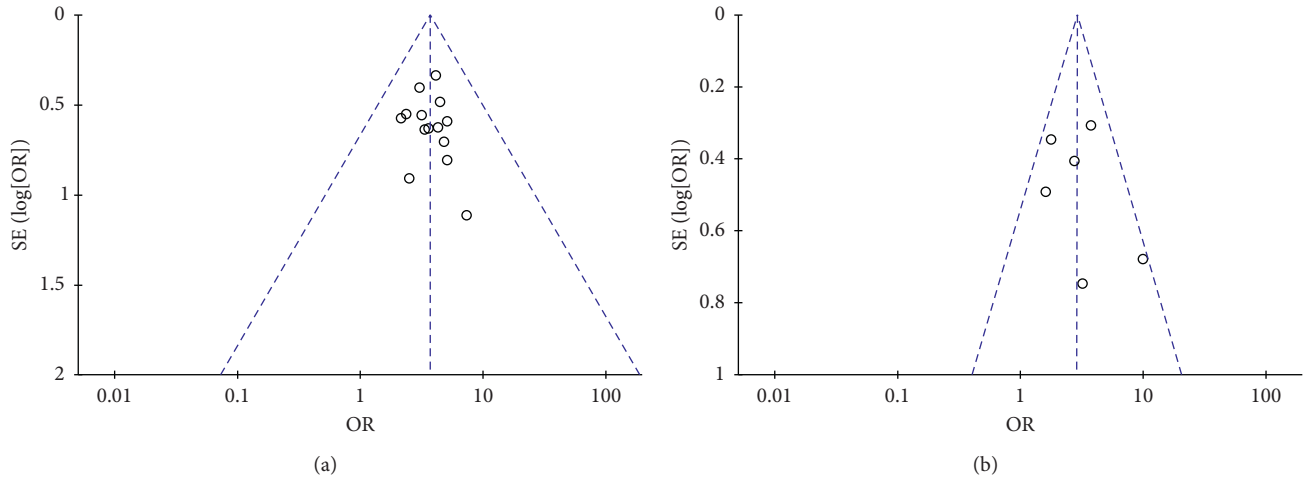


Figure 8: Funnel plot for the publication bias of (a) total effective rate and (b) ECG improvement.

The pathogenesis of ACS mainly involves the atherosclerotic plaque rupture, platelet aggregation, and thrombosis, in which plaque rupture is the dominant initiating event [40]. It was reported that BV could be increased due to elevated hemorheological parameters, such as HCT and FIB. Increased BV may result in high rapture forces at the vascular endothelium and promote the breakage of occlusive plaque [46]. Lee et al. also found that elevated BV in ACS patients was related to coronary plaque rupture; thus, BV may be a therapeutic target for the treatment of ACS [47]. Besides, studies had shown that a pathological platelet aggregation is a critical event promoting intravascular thrombus formation, and the inhibition of platelet aggregation has been a drug development target in ACS [48].

This meta-analysis is the first research conducted to assess the safety and efficacy of SFI for curing ACS systematically. Total effective rate and ECG improvement were used to evaluate the efficacy of SFI for ACS. In comparison to single conventional therapy, SFI-conventional combination therapy was linked with a notably higher total effective rate and ECG improvement ($P < 0.00001$). Hemorheology indexes, such as BV, PV, HCT, FIB, and PAR, were employed to investigate the flow and deformation of blood in ACS patients. In comparison to single conventional therapy, SFI-conventional combination therapy was linked to notably lower BV, HCT, FIB, and PAR ($P < 0.05$). This showed that SFI improved the antithrombotic and anticoagulation actions. LVEF was employed to evaluate the cardiac function of ACS sufferers. Compared to single conventional therapy, SFI-conventional combination therapy was linked with a notably higher LVEF ($P < 0.00001$). There was, however, no contrast in the adverse reactions linking the experimental and control teams ($P = 0.5$). Because SFI did not reduce the incidence of adverse reactions caused by Western medicines, a temporary conclusion could be reached only that SFI is almost safe.

Extensive searching and stern procedures were applied to select trials and look into the medical efficacy and safety linked with SFI administration. However, many possible restrictions were present in the meta-analysis and need to be contemplated. Firstly, despite adoption of a comprehensive searching strategy to lessen the publication bias as far as possible, there was still a certain level of selective bias which this meta-analysis only narrowed down to Chinese and English databases, and no mention of studies written in different languages was made. Secondly, all relevant studies were conducted in China, and the majority of the patients were Chinese. However, population diversity is important when doing such a study for one to get better convincing and well-grounded results. Thirdly, the majority of the relevant studies exhibited relatively low methodological quality. Eleven of the 16 researches employed "randomization," whereas no mention of the particular procedure used was made. Furthermore, all the relevant studies did not indicate allocation concealment and blindness. Fourthly, we did not get more details of the studies from the authors through telephone and electronic mail. Fifthly, there was a statistically notable heterogeneity found in the indexes of hemorheology including BV, PV, and FIB. It is rather hard to investigate the heterogeneity in the results of continuous variables. We cannot conduct a subgroup analyses for the few studies giving hemorheology indexes and also did not find the origins of the heterogeneity after carrying out sensitivity analyses. It can be inferred that the heterogeneity resulted from two or more factors, which include sex, age, and period of therapy. Finally, treatment safety is important to come up with other treatments. However, there were only two of the 16 studies which informed adverse reactions. In light of the restraints that existed in the meta-analysis, high-quality and large-scale RCTs, with a fine design and methodology, are required to study the efficacy and safety of SFI for ACS in time to come.

5. Conclusions

The therapy of ACS has become a global challenge. The combination of Chinese patent drug SFI and conventional medication may bring advantageous effects to improve blood rheology and cardiac function of sufferers with ACS. Thus, it is recommended to consider SFI in the conventional

treatment of ACS. It is worthy of paying attention to the limitations in the meta-analysis. Furthermore, the therapeutic effect and safety of SFI as an adjunctive therapy for ACS still requires methodologically strict studies to prove.

Abbreviations

ACS: Acute coronary syndrome
BV: Blood viscosity
CVD: Cardiovascular disease
ECG: Electrocardiogram
FIB: Fibrinogen
HCT: Hematocrit
LVEF: Left ventricular ejection fraction
MD: Mean difference
OR: Odds ratio
PAR: Platelet aggregation rate
PV: Plasma viscosity
RCTs: Randomized controlled trials
SFI: Safflower injection
TCM: Traditional Chinese Medicine.

Data Availability

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

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (No. 81673845), Guizhou Science and Technology Foundation of China (No. QKHPTRC[2018]5772-020), Science and Technology Planning Project of Guangdong Province (Nos. 2016A020226036 and 2017B030314166), and Science and Technology Research Project of Guangdong Provincial Hospital of Chinese Medicine (YN2018ZD02), Provincial Natural Science Foundation of Guangdong (No. 2019A1515010638), Special Project of State Key Laboratory of Dampness Syndrome of Chinese Medicine (No. SZ2020ZZ03), and Key-Area Research and Development Program of Guangdong Province (No. 2020B1111100010).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6617586, 15 pages
https://doi.org/10.1155/2021/6617586

Research Article

Tian-Huang Formula, a Traditional Chinese Medicinal Prescription, Improves Hepatosteatosis and Glucose Intolerance Targeting AKT-SREBP Nexus in Diet-Induced Obese Rats

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Received 23 October 2020; Revised 21 January 2021; Accepted 5 February 2021; Published 6 March 2021

Academic Editor: Mohammad H.osein Ayati

The progressive increase of metabolic diseases underscores the necessity for developing effective therapies. Although we found Tian-Huang formula (THF) could alleviate metabolic disorders, the underlying mechanism remains to be fully understood. In the present study, firstly, male Sprague-Dawley rats were fed with high-fat diet plus high-fructose drink (HFF, the diet is about 60% of calories from fat and the drink is 12.5% fructose solution) for 14 weeks to induce hepatosteatosis and glucose intolerance and then treated with THF (200 mg/kg) for 4 weeks. Then, metabolomics analysis was performed with rat liver samples and following the clues illustrated by Ingenuity Pathway Analysis (IPA) with the metabolomics discoveries, RT-qPCR and Western blotting were carried out to validate the putative pathways. Our results showed that THF treatment reduced the body weight from 735.1 ± 81.29 to 616.3 ± 52.81 g and plasma triglyceride from 1.5 ± 0.42 to 0.88 ± 0.33 mmol/L; meanwhile, histological examinations of hepatic tissue and epididymis adipose tissue showed obvious alleviation. Compared with the HFF group, the fasting serum insulin and blood glucose level of the THF group were improved from 20.77 ± 6.58 to 9.65 ± 5.48 mIU/L and from 8.96 ± 0.56 to 7.66 ± 1.25 mmol/L, respectively, so did the serum aspartate aminotransferase, insulin resistance index, and oral glucose tolerance ($p = 0.0019$, 0.0053, and 0.0066, respectively). Furthermore, based on a list of 32 key differential endogenous metabolites, the molecular networks generated by IPA suggested that THF alleviated glucose intolerance and hepatosteatosis by activating phosphatidylinositol-3 kinase (PI3K) and low-density lipoprotein receptor (LDL-R) involved pathways. RT-qPCR and Western blotting results confirmed that THF alleviated hepatic steatosis and glucose intolerance partly through protein kinase B- (AKT-) sterol regulatory element-binding protein (SREBP) nexus. Our findings shed light on molecular mechanisms of THF on alleviating metabolic diseases and provided further evidence for developing its therapeutic potential.

1. Background

Obesity and its close consequences, dyslipidemia, impaired glucose tolerance, and insulin resistance, are more common than ever in human history [1]. The progressive increase of the above conditions leads to the rising incidence of type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease (NAFLD), and metabolic syndrome [2]. Overnutrition and lack of physical activities are usually regarded as the key elements of obesity, and a dietary pattern preferring a high-fat high-sugar diet plays a central role among the determinants of obesity in modern society [3, 4]. Epidemiological evidence showed a clear correlation between the increased consumption of a Western high-calorie diet and high-fructose corn syrup (HFCS) and the incidence of obesity and its complications [3–5]. A number of countermeasures have

been applied to fighting against these situations, including active lifestyle modifications, pharmacotherapeutic intervention, and even aggressive bariatric surgery [2, 6]. Noninvasive drugs have been regarded as promising approaches to address the deficit of lifestyle intervention and reduce the severity of bariatric surgery [2, 6, 7]. Although varying in their efficacy and side effect profiles, the existing available approved drugs targeting obesity and its close consequences and medical comorbidities are inadequate. Meanwhile, the complexity of obesity and associated comorbidities underscore the necessity for developing suitable drugs with different benefits in the context of our understanding of the physiopathology of obesity [2, 6]. As is well known, the pharmacological properties of many traditional herbal medicines have been demonstrated according to hundreds of years' experience on humans. In addition, many kinds of natural plant extract derived from them have been developed and used as dietary supplements, though knowledge on their molecular mechanisms is lacking [8, 9]. Therefore, developing novel therapeutics from natural herbal extracts is a promising strategy to address the global health problem of obesity and its consequences; further studies on the precise role and mechanism of potential drugs against the development of diet-induced metabolic diseases will provide better support for their clinical usage [10].

Tian-Huang formula (THF) is a patented and clinically approved Chinese medicinal prescription with hypolipidemic effects. It originated from Traditional Chinese Medicine Fufang Zhenshu Tiaozhi Formula (FTZ) which was derived from Prof. Jiao Guo's 30 years of clinic experience and has been developed into hospital preparations. Due to its excellent cost-effective properties, FTZ capsules have been covered by health insurance in Guangdong Province, China. In the past few years of clinical and experimental study, a more simple but equivalent formula originated from FTZ, namely, THF, was developed [11]. It is composed of *Panax notoginseng* and *Coptis chinensis*, which are both traditional herbal drugs with hundreds of years of usage [12, 13]. Total saponins of *P. notoginseng* (PNS) and total alkaloids of *C. chinensis* (CCA) are believed to be the main active ingredients of *P. notoginseng* and *C. chinensis*, respectively [12, 13]. Gu et al. showed that the active constituents of PNS were ginsenoside Rb_1, ginsenoside Rg_1, ginsenoside Rb, ginsenoside Re, and ginsenoside R_1 [14]. It has been demonstrated that PNS ameliorates hepatic lipid accumulation [15] and hepatic diseases [16]. Nowadays, preparations made from PNS are available and widely used in clinics in China [17]. Meanwhile, berberine, palmatine, and coptisine were reported to be the main active constituents of CCA [18], which has anti-hyperglycemic and anti-hyperlipidemic effects and can be used for anti-diabetic treatment [19, 20]. Our previous rodent model-based experiments showed that THF had therapeutic effects on lipid lowering and anti-atherosclerosis [11]. However, the potential mechanisms for improving glycolipid metabolism of THF have not been fully elucidated.

Metabolomics analysis is emerging as a robust tool for studies on diagnostic biomarkers, fundamental pathogenic mechanisms, and therapeutic targets [21–23] According to biochemical understanding, endogenous metabolites are products of all kinds of life-sustaining biochemical reactions, which can reflect alterations of the bodies' homeostasis. Moreover, use of software such as IPA (Ingenuity Pathways Analysis), MetaCore, and Reactome, algorithmically constructed metabolic networks can be generated to provide more insight than phenotypes and results analyzed individually. They can then provide clues to the pathophysiology of diseases and drug intervention research [24–26].

In the present study, in order to provide mechanistic insight into the therapeutic effects of THF on metabolic disorders, a nontargeted metabolomics analysis and IPA analysis have been performed. Subsequent results validated that THF alleviates glucose intolerance and hepatic steatosis by targeting AKT- (protein kinase B-) SREBP (sterol regulatory element-binding protein) nexus through activating PI3K (phosphatidylinositol-3 kinase) and LDL-R (low-density lipoprotein receptor) involved pathways. Our results shed new light on the underlying mechanisms and present informative evidence that AKT-SREBP nexus is a potential therapeutic target of THF for metabolic disturbances, and furtherly could offer new molecules to therapeutically intervene DIO and its complications.

2. Methods

2.1. Chemicals. High-fructose corn syrup (F55) was supplied by Guangzhou Shuangqiao Co., Ltd. (Guanzhou, China). Nonadecanoic acid was purchased from Sigma-Aldrich (Sigma, San Diego, USA). Methanol, hexane, and chloroform were of HPLC (High Performance Liquid Chromatography) grade and were from Merck (Darmstadt, German). Pyridine, O-methylhydroxylamine hydrochloride, N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), and trimethylchlorosilane (TMCS) were from J&K Scientific Co. Ltd. (Tianjing, China).

2.2. Preparation and Quantitative Profiling of THF. THF was prepared as previously described [11]. In brief, powdered *P. notoginseng* (400 g) and *C. chinensis* (400 g) were separately extracted triply with 70% ethanol at 80°C under reflux, each time for two hours. The extract solution was concentrated in a rotary evaporator to remove ethanol and then dissolved in water and purified using D101 macro-porous resin (Lanxiao, Xi'an). The resulting purified extract was dried in vacuum at 60°C.

The quantitative profiling of THF was performed on an U3000 HPLC with a DAD detector (Dionex, USA). The chromatograph separation was carried out using a Kromasil C18 column (4.5×250 mm, 5 μm in particle size) according to the Pharmacopoeia of People's Republic of China (Ch. P. 2015), and data were recorded and analyzed on Chromeleon Console workstation (for details, see the Supplementary materials). Finally, the contents of eight active components in THF, namely, ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd, ginsenoside Re, notoginsenoside R1, berberine, coptisine, and palmatine, were quantified.

2.3. Animals. Five-week-old male Sprague-Dawley (SD) rats were supplied by Guangdong Medical Laboratory Animal Centre (Guangzhou, China). Rats were housed in a plastic cage (4~5/cage) and a specific pathogen-free facility at controlled temperature of 24 ± 2°C, with relative humidity of 60–70% and a 12-hour light-dark cycle. All animals had free access to water and standard rodent diet during acclimatization. All procedures performed were approved by the Experimental Animals Ethics Committee of Guangdong Pharmaceutical University (No. SPF2017092). All animal studies were conducted in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines for reporting experiments involving animals [27].

2.4. Study Design. After one week of acclimatization, all rats were randomly assigned as control group (Cont, $n = 8$) and test group ($n = 16$). The test group were fed a high-fat diet ad libitum for 14 weeks. The test group had continuous access to a separated bottle with high fructose corn syrup solution (HFCS-55: 55% fructose, and 45% glucose, diluted with distilled water to 12.5% fructose solution). The standard diet provided 20% of calories from protein, 70% of calories from carbohydrate, and 10% of calories from fat, and a digestible energy of 3.85 kcal/g (D12450-B). The high-fat diet provided 20% of calories from protein, 20% of calories from carbohydrate, and 60% of calories from fat, and a digestible energy of 5.24 kcal/g (D12492). The body weight of rats was measured and food intake was recorded every week. During the study period, plasma triglyceride, total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) concentrations were determined at week 8, 12, and 14. At the end of week 14, the test group rats were divided into two groups, the HFF group and vehicle-treated group (HFF, $n = 8$), the HFF-fed and THF-treated group (200 mg/kg; THF, $n = 8$), according to the body weight, plasma TG (triglyceride), and TC levels.

After treating with THF for additional 4 weeks, all the rats were sacrificed after anesthetizing with pentobarbital sodium to collect the blood from the visual abdominal aorta after a 14-hour overnight fast. Blood was conducted with heparin and centrifuged at 4°C, 3000 rpm for 15 min, and then the plasma was aliquoted and stored at −80°C. The rats' livers were rinsed with normal saline, and parts of them were fixed with 4% PFA (paraformaldehyde) for histological examination. All the other liver tissues were snap-frozen in liquid nitrogen and stored at −80°C for qPCR or Western blot analysis. The epididymal white adipose tissues (EpiWAT) were also collected and weighted, and treated like liver tissues.

2.5. Histological Examinations of Liver Tissues and Adipose Tissues. Routine hematoxylin and eosin (H&E) staining of liver tissues and EpiWAT was carried out using 4% PFA fixing, paraffin embedding, and sectioning (4 μm) as described previously [28]. Three or four sections from every tissue sample were stained. The morphology of the liver tissue and EpiWAT was observed under a light microscope of PerkinElmer Vectra 3 (PerkinElmer, USA). The cell number of the same area EpiWAT was counted and compared.

2.6. Biochemical Analyses. Triglyceride, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations were determined using a commercial kit (Shanghai Rongsheng Biotech Co., Ltd., China). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected using a commercial detection kits (Nanjing Jiancheng Bioengineering Institute, China).

2.7. Glucose Homeostasis. At week 19, rats were fasted overnight (12 h) and oral glucose tolerance tests (OGTT, 2 g of 50% glucose/kg body weight) were carried out as described by Wang et al. [29] with minor modification. Blood samples were collected through the tail vein, and levels of blood glucose were determined by ONETOUCH UltraEasy glucometer (Johnson, USA) before (0 min) and after glucose injection at different time points (15, 30, 60, and 120 min). The total area under the curve (AUC) was calculated for OGTT.

Blood samples were collected from the visual abdominal aorta at the end of the study. The fasting glycemia and insulinemia were determined using an ultra-sensitive ELISA kit (Alpco, USA). The homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated based on the following formula: fasting insulinemia (mUI/mL) × fasting glycemia (mM)/22.5.

2.8. Metabolomics Analysis. Experimental nontargeted metabolomics analysis of hepatic tissues was performed as previously described [28]. Briefly, 10 μL of nonadecanoic acid methanol solution (1 mg/mL, w/v), 250 μL of H_2O-MeOH-$CHCl_3$ solution (2 : 5:2, v/v/v), and 50 mg of rat liver were homogenized. Then, the resulting mixture was kept at 4°C for 20 min. Next, the mixture was centrifuged at 14,000 rpm for 15 min at 4°C. Subsequently, 200 μL of supernatant was dried in nitrogen. The residue was derivatized and analyzed as previously reported on 7890B-5977B GC-MS with a HP-5MS column (60 m × 0.25 mm × 0.25 μm, Agilent, MA, USA).

The data analysis was performed as previously described [28]. In brief, all the GC-MS raw data were subjected to batch molecular feature extraction by using MassHunter Profinder_B.08 (Agilent Co., Ltd., CA, USA). Then, the generated data were exported to Excel (Microsoft, Redmond, WA, USA) and used in the subsequent multivariate analysis. All the raw data were stored at Guangdong Metabolic Disease Research Centre of Integrated Medicine, which will be available upon request. Unsupervised PCA (principal component analysis) and supervised OPLS-DA (orthogonal partial least-squares discriminant analysis) analysis were performed on SIMCA-P 13.0 software (Umetrics, Umeå, Sweden) to identify plasma metabolites contributing to the differences between the two groups. All variables were Pareto-scaled prior to analyses. Here, VIP (Variable Importance in Projection) >1.0 and $p < 0.05$ were set as a statistical threshold for discriminating key differential metabolites.

2.9. Molecular Network Construction Using IPA. Molecular network construction using Ingenuity Pathways Analysis (IPA, QIAGEN, Germany) was carried out as previously described [28, 30]. The IPA was applied to construct the metabolic interaction networks by submitting the list of modulated metabolites, the corresponding fold change, and their KEGG identity (http://www.kegg.jp), to online analysis. Based on the Ingenuity Pathway Knowledge Database, the network of interactions among metabolites, protein, and gene was generated.

2.10. Quantitative RT-PCR. RT-qPCR (real-time quantitative reverse transcription-polymerase chain reaction) was performed as previously reported [28, 30]. Briefly, hepatic total RNA was extracted with RNAiso plus reagent (Takara Biotechnology, Dalian, China) and was reversely transcribed to cDNA using PrimeScript™ RT kit with gDNA Eraser (Takara Biotechnology, Dalian, China). mRNA expression levels were determined using SYBR Green method on a PikoReal™ real-time PCR system (Thermo Fisher Scientific Inc., CA). Rat-specific primers for phosphatidylinositol-3 kinase (PI3K), protein kinase B (AKT), insulin receptor substrate 1 (IRS1), sterol regulatory element-binding protein-2 (SREBP-2), low-density lipoprotein receptor (LDLR), proprotein convertase subtilisin/kexin type 9 (PCSK9), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were designed and synthesized by Sangon Biotech (Shanghai, China) (for details, see Supplementary Table S1.) The $2^{(-\Delta\Delta CT)}$method was used to determine the relative expression of mRNA. The amount of each gene was normalized to the amount of rat GAPDH.

2.11. Western Blotting. Western blotting was conducted as previously described [28]. In brief, about 50 mg of rat's liver tissue was homogenized in 500 μL RIPA (radio immunoprecipitation assay) (Solarbio Science & Tech., Beijing, China) supplemented with a PMSF (phenylmethanesulfonyl fluoride) (Solarbio Science & Tech., Beijing, China) and Protease Inhibitor Cocktail (Millipore, Calbiochem, USA). Notably, for phosphorylated protein analysis, phosphatase inhibitors (Beyotime Biotechnology, Shanghai, China) were added while the total protein was extracted. The tissue homogenates were centrifuged at 12000 rpm and 4°C for 10 min and the supernatants were collected. The total protein concentration of the tissue lysates was determined with a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). A total of 30–40 μg protein from each sample was separated on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to poly(vinylidene fluoride) (PVDF) membrane (Millipore, Darmstadt, Germany). The membrane was then blocked with 5% nonfat milk at room temperature for 1–1.5 hours, following incubation with the primary antibodies against PI3K (Rabbit polyclonal 4249S, Cell Signaling Technology, Danvers, MA, USA), AKT (Rabbit polyclonal 4685S, Cell Signaling Technology, Danvers, USA), p-AKT (Rabbit polyclonal 4060S, Cell Signaling Technology, Danvers, USA), p-PI3K (Rabbit polyclonal ab182651, Abcam, Cambridge, UK), SREBP2 (Rabbit polyclonal ab28481, Abcam, Cambridge, UK), PCSK9 (Rabbit polyclonal ab31762, Abcam, Cambridge, UK), and LDLR (Rabbit polyclonal ab30532, Abcam, Cambridge, UK) and β-actin (Cell Signaling Technology, Danvers, USA) at 4°C overnight. Subsequently, the membrane was incubated with appropriate HRP-conjugated secondary antibody for 1 hour and visualized by using an enhanced chemiluminescence kit (Cyanagen, Bologna, Italy). The intensity of the immunoblot signal was detected and quantified using Image Master VDS (SYNERGY Gene Company Limited, Hong Kong, China) with image analysis software (Image Master Total Lab; SYNERGY).

2.12. Statistical Analysis. All data are shown as means ± standard deviation (SD). Data sets that involved more than two groups were assessed by one-way ANOVA followed by Newman–Keuls post hoc tests. $p < 0.05$ was considered statistically significant. For metabolomics analysis, data were normalized to the internal standard and all variables were Pareto-scaled prior to analyses. R (https://www.r-project.org) and GraphPad Prism 6.0 software (GraphPad, CA, USA) were used for statistical analysis and graphics.

3. Results

3.1. Preparation and Quantitative Profiling of THF. Saponins and alkaloids are the main bioactive ingredients of THF, and a traditional ethanol reflux extraction and macroporous resin purification process is effective for THF preparation [20]. As shown in Figure 1, the main active components of *P. notoginseng* root and rhizome (Figure 1(a)) are ginsenoside Rg_1, ginsenoside Rb_1, ginsenoside Rd, ginsenoside Re, and *p. notoginseng* saponin R_1 (Figure 1(d)). The major active components of *C. chinensis* root (Figure 1(b)) are berberine, coptisine, and bamatine (Figure 1(d)). The prepared dry THF is yellow brown powder (Figure 1(c)) and HPLC analysis showed that it was made of ginsenoside Rg1 (23.82%), ginsenoside Rb1 (4.58%), ginsenoside Rd (0.97%), ginsenoside Re (1.03%), *Panax notoginseng* saponin R1 (2.05%), coptisine (4.45%), bamatine (5.11%), berberine (17.01%), and some other unidentified components (41.98%) (Figure 1(e)).

3.2. THF Improved Lipid Accumulation in DIO Rats. HFF diet provides excessive energy and results in obesity. In order to evaluate the protective effects of THF, rats undergoing HFF diet were treated with THF (200 mg/kg) or vehicle. As shown in Figure 2(a), after 7 weeks of HFF-feeding, a significant increase in body weight was observed for the test group compared to control group rats, and the difference became more significant over time ($p = 0.002$, week 14). At the end of week 14, the rats of HFF-fed test group were divided into the HFF-fed group and vehicle-treated group and the HFF-fed and THF-treated group, as described in the methods section. As shown in Figures 2(b) and 2(c), THF treatment lowered the body weight significantly ($p = 0.01$);

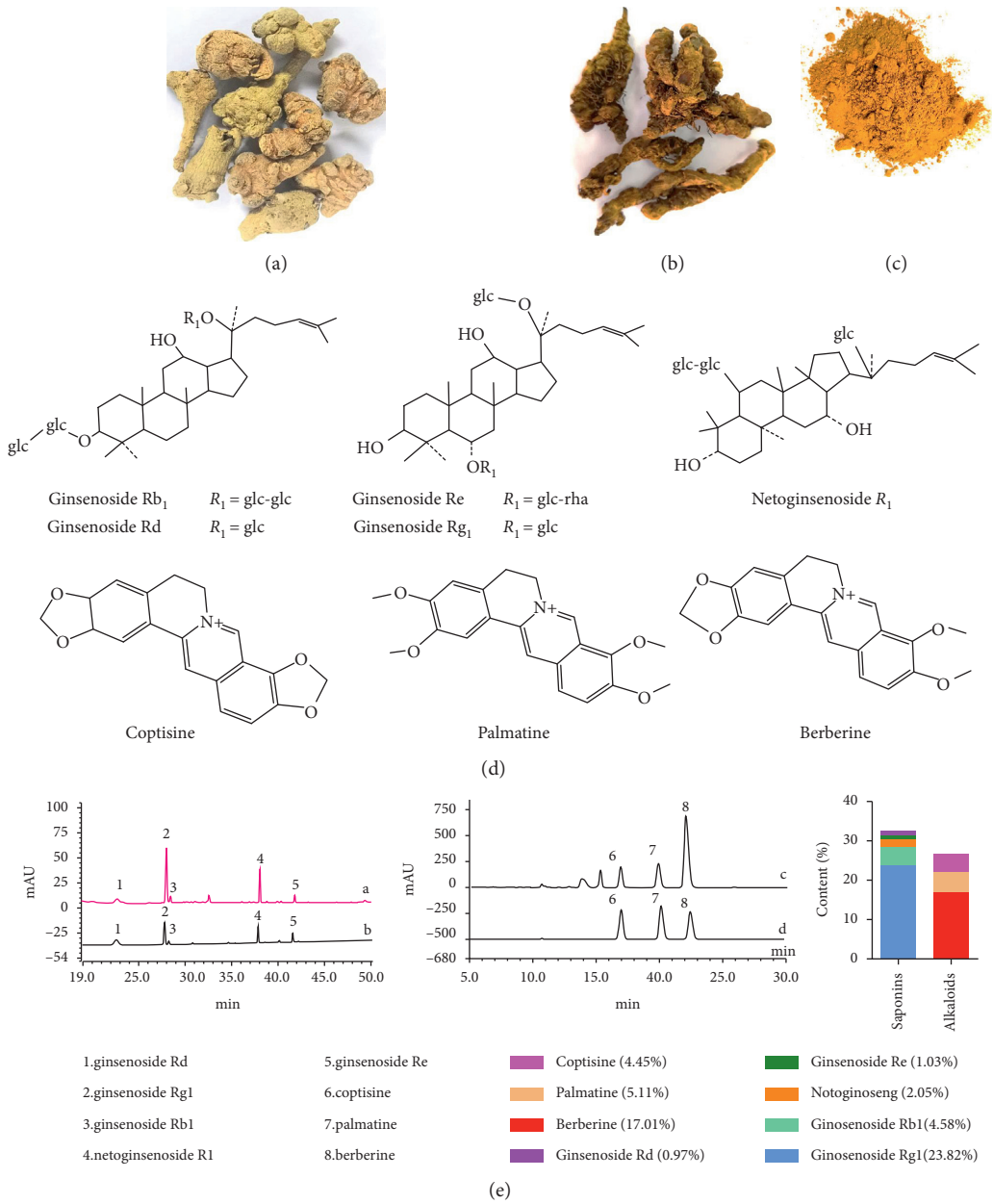

Figure 1: Preparation and quantitative profiling of Tian-Huang formula, a mixed extract of *Panax notoginseng* and *Coptis chinensis*. (a) *P. notoginseng* root and rhizome, (b) *C. chinensis* root, (c) dry powder of Tian-Huang formula (THF), a mixed extract of *P. notoginseng* and *C. chinensis*, (d) chemical structure of eight main components of THF, namely, ginsenoside Rb_1, ginsenoside Rg_1, ginsenoside Rd, ginsenoside R_1, notoginsenoside R_1, coptisine, palmatine, and berberine, and (e) content of abovementioned eight components analyzed by HPLC following the method recorded by Pharmacopoeia of the People's Republic of China (2015).

an obvious tendency towards amelioration of the rats' adiposity was also observed (but was not statistically significant). At the end of the experiment, the rats' plasma levels of TC, TG, LDL-C, and HDL-C showed an obvious rise in the HFF group ($p = 0.0018$, 0.0005, 0.0005, and 0.0011, respectively; Figures 2(d)–2(g)). However, the THF treatment significantly decreased the level of rats' plasma TG ($p = 0.031$) and showed a trend towards decreased levels of TC, LDL-C, and HDL-C although this was not statistically significant (Figures 2(d)–2(g)). Thus, effects of THF were predominantly noted on improving triglyceride metabolism.

Pathological changes of white adipose tissue are part of the typical characteristics of the DIO rats' model [31]. In our results, the EpiWAT weight of the HFF group was elevated significantly compared to that of the control group ($p = 0.0011$) while the THF group showed a significant decrease ($p = 0.0403$; Figure 2(h)). The EpiWAT tissues examined by H&E staining displayed more hypertrophic adipose cells in the HFF group than the control and THF groups; there existed a statistically significant change of the cell number of the same view EpiWAT area among the three groups ($p < 0.01$; Figure 2(h)). Thus, it was also notable that

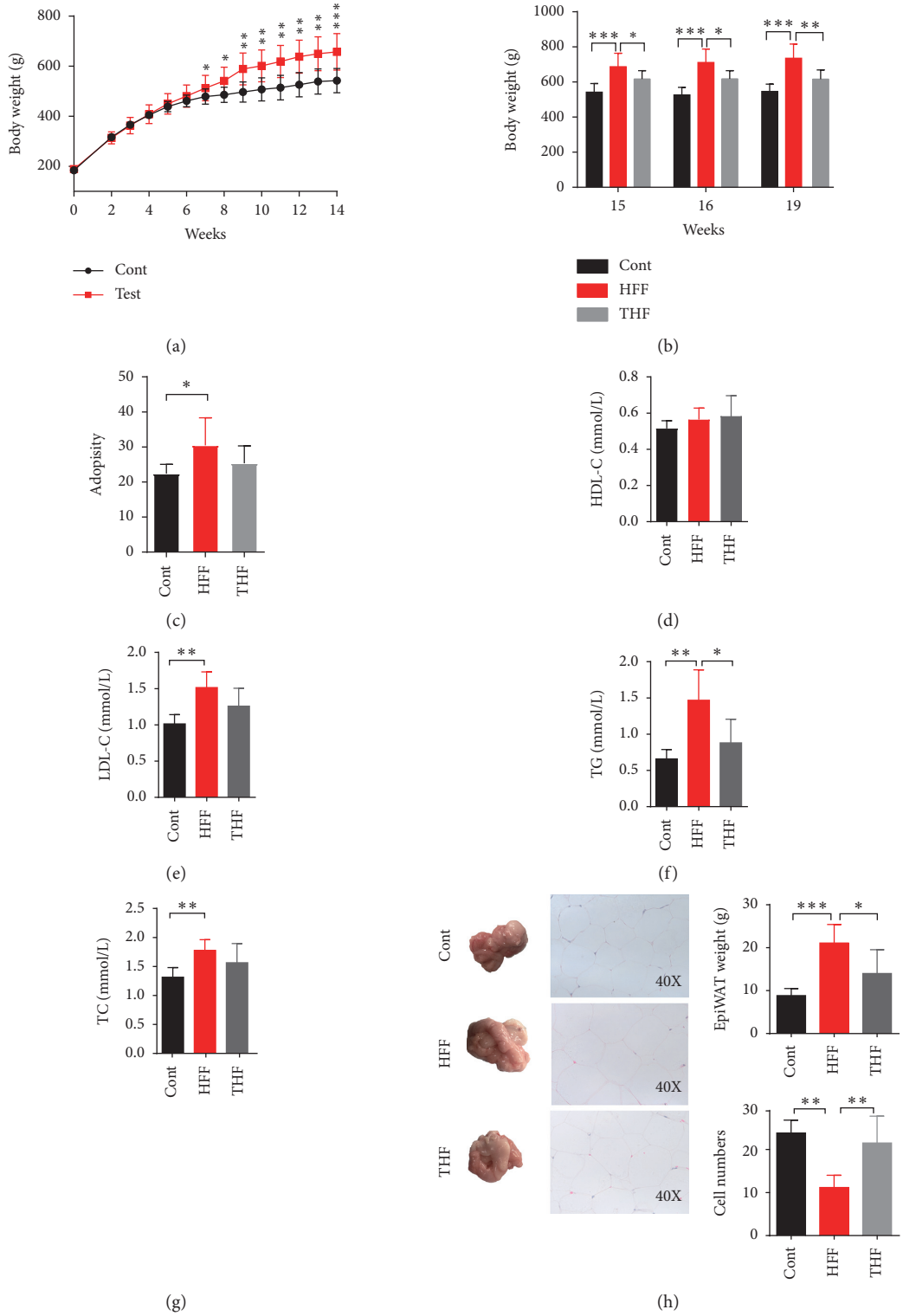

Figure 2: Continued.

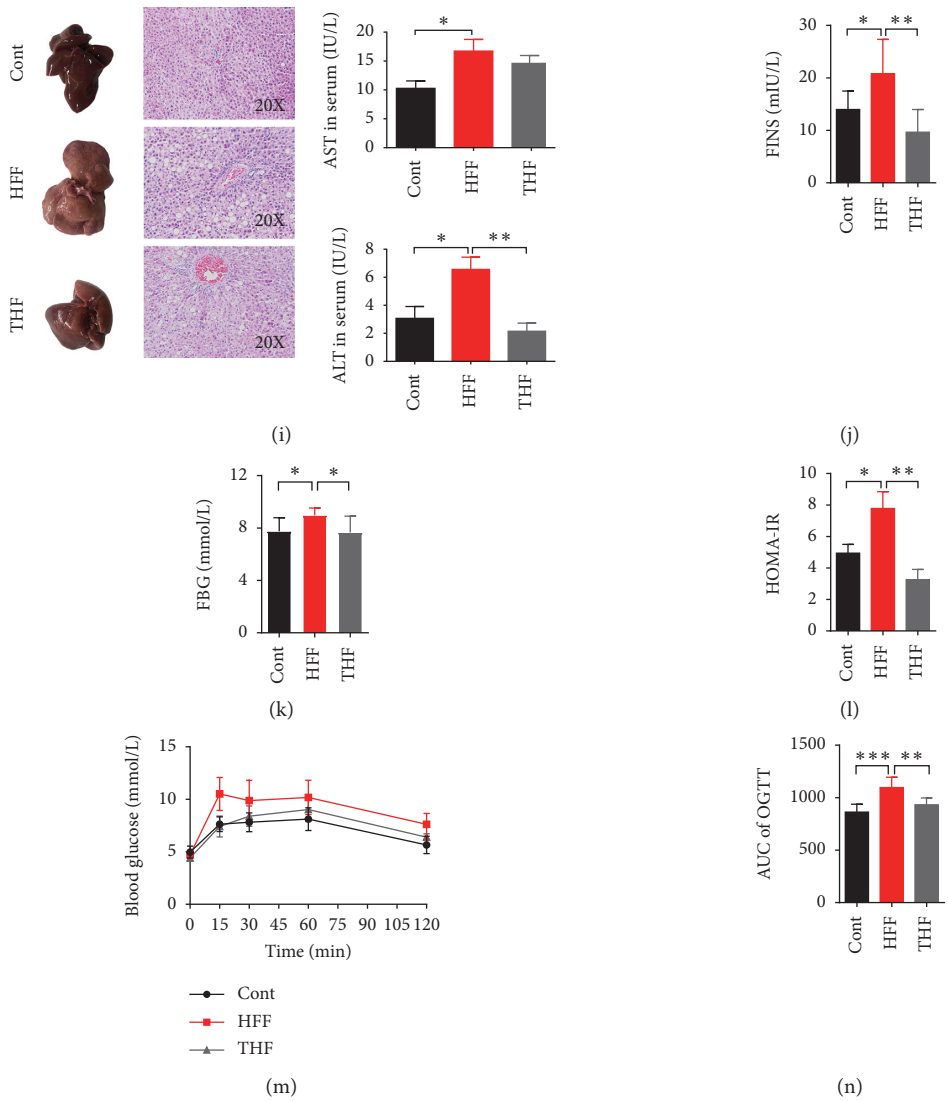

Figure 2: THF improves hepatic steatosis and glucose intolerance of diet induced obese rats. (a) The high-fat high-fructose diet-fed group (test; $n = 16$) and control group (cont; $n = 8$) rats were measured for body weight. (b) At the beginning of week 15, the test group rats were divided into HFF-fed and THF (200 mg/kg) treated group (THF; $n = 8$)) and HFF-fed and vehicle-treated group (HFF; $n = 8$). The control group, the HFF group, and the THF group rats were also measured for body weight from week 15 to week 19 and (c) adiposity at week 19. Plasma (d) HDL-C, (e) LDL-C, (f) TC, and (g) TG levels were also measured. (h) Representative images of EpiWAT and EpiWAT sections with H&E staining; the EpiWAT weight and cells number were measured; (i) representative images of liver and hepatic tissue sections with H&E staining; serum ALT and AST were also measured; (j) FINS and (k) FBG were measured and (l) HOMA-IR index was calculated; (m) oral glucose tolerance tests (OGTT) were carried out, and (n) OGTT-AUC was also calculated. Data are presented as mean ± SD ($n = 6$–8); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. HFF, high-fat high-fructose diet; THF, Tian-Huang formula, a mixed extract of *P. notoginseng* and *C. chinensis*; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; EpiWAT, epididymal white adipose tissue; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FINS, fasting serum insulin; FBG, fasting blood glucose; HOMA-IR, insulin resistance index.

THF could attenuate the hypertrophy of adipose tissue. In addition, the accumulation of fat in the liver is a noted feature in DIO rats [31]. The phenotypes of the whole liver showed that, compared with the normal group, the liver surface color of the HFF group was paler, and the THF group was more normal than the HFF groups (Figure 2(i)). Meanwhile, the results of histological analyses of the liver tissues pathological section stained by H&E showed that there were more lipid droplets and vacuoles in the liver cells of the HFF group, which were larger and accompanied by inflammatory cell infiltration (Figure 2(i)). However, the number of lipid droplets and vacuoles in the THF group was visibly reduced. Furthermore, the serum concentrations of ALT and AST in the HFF group were significantly increased ($p = 0.0156$ and 0.0183, respectively), indicating that the liver cells were damaged. After THF intervention, the ALT level was decreased significantly ($p = 0.0019$), and AST was also reduced, although it was not statistically significant (Figure 2(i)). Collectively, this data demonstrated that a long period of HFF diet resulted in fat accumulation in DIO rats,

and THF (200 mg/kg) treatment could provide an effective reduction.

3.3. THF Improved Glucose Intolerance in DIO Rats. Glucose intolerance always accompanies obesity [1]. In order to investigate whether THF can also improve the glucose metabolism disturbance of DIO rats, homeostasis tests were performed. As expected, the glucose homeostasis tests (Figures 2(j)–2(n)) showed that HFF feeding resulted in glucose intolerance, with altered FINS (fasting serum insulin), FBG (fasting blood glucose), and HOMA-IR. The tests revealed that the HFF group displayed a significant elevation of FINS compared with the control group (Figure 2(j)); similar elevations in FBG (Figure 2(k)) and HOMA-IR (Figure 2(l)) were also noted ($p = 0.0330$, 0.0312, and 0.0355, respectively). Moreover, the results of the OGTTs indicated that there existed an insulin resistance and impaired glucose tolerance in the HFF group rats (Figures 2(m) and 2(n)). However, THF treatment significantly attenuated these conditions; four weeks of THF (200 mg/kg) treatment significantly improved both impaired glucose tolerance and insulin resistance ($p = 0.0066$ and 0.0053, respectively) (Figures 2(j)–2(n)).

3.4. Metabolomics Analysis Revealed Distinct Metabolite Composition in response to THF Treatment. Collectively, the above-mentioned data provide compelling evidence for a protective effect of THF on the glucose intolerance and hepatic steatosis in experimental DIO rats. However, the molecular mechanisms triggered by THF were still unclear. In order to investigate the underlying mechanisms, we conducted nontargeted metabolic profiling of the liver tissues using gas chromatography mass spectrometry (GC-MS, typical total ion chromatograms are shown in Figure 3(a)). Referring to the NIST14.0 database, a total of 215 compounds were detected and quantified by normalizing to the internal standard for all the samples. We used PCA for multivariate analysis of the nontargeted quantitative metabolic profiling data of the control, HFF, and THF (200 mg/kg) groups. The PCA scores plot displayed clear differences between the three groups ($R^2X = 0.867$, $Q^2 = 0.501$) (Figure 3(b)). In addition, OPLS-DA analysis was used to maximize the discrimination between the HFF groups and THF groups. The results revealed a clear separation between the two groups ($R^2X = 0.838$, $R^2Y = 0.956$, $Q^2 = 0.901$); the values of these parameters approached 1.0, indicating a stable model with predictive reliability (Figure 3(b)). Permutation testing demonstrated that the OPLS-DA model was robust ($R^2 = 0.604$, $Q^2 = -0.957$) (Figure 3(b)). Based on a threshold of VIP values >1 and p values <0.05, 32 endogenous molecules, including oxalic acid, lactic acid, 4-hydroxybutanoic acid, palmitic acid, and stearic acid, were found to be key differential metabolites between the HFF groups and the THF groups (for details, see Supplementary Table S2). Using the HMDB (http://www.hmdb.ca) for classification of the 32 metabolites, over 40% were sub-clustered as carbohydrates and carbohydrate conjugates, about 28.13% were amino acids, peptides, and analogs; about 6.25% were alpha-hydroxy acids and derivatives (Figure 3(c)). In terms of cellular locations, these metabolites are primarily located in cytoplasm (21.88%), extracellular (18.75%), mitochondria (15.63%), lysosome (12.50%), peroxisome (12.50), endoplasmic reticulum (6.25%), and Golgi apparatus (6.25%) (Figure 3(d)). Collectively, this series of compounds allowed us to define a metabolomics signature of THF biological actions.

3.5. Pathway Enrichment and Ingenuity Pathway Analysis Hinted AKT and LDLR Involvement in THF Improvement of Glucose and Lipid Metabolism Homeostasis. The algorithmically constructed metabolic networks can generate much more insight than discussing the key metabolites individually. In order to further address the underlying mechanisms of THF-mediated protection on DIO rats, we used specialized software to interpret and visualize the biological changes, altered canonical pathways, and metabolic networks. With the above-mentioned 32 key differential metabolites regulated by THF treatment, pathway enrichment analysis and molecular interaction networks construction were carried out using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) (Figures 4(a) and 4(b)) and Ingenuity Pathway Analysis software (http://www.qiagen.com), respectively [30, 32]. The highest-scoring (IPA score, 42) network targeted by THF involved 12 molecules, in which phosphatidylinositol-3 kinase- (PI3K-) protein kinase B (AKT), mitogen-activated protein kinases- (MAPK-) extracellular regulating kinase (ERK), extracellular regulating kinase 1/2 (ERK 1/2), insulin, proinsulin, pro-inflammatory cytokine, and LDL were algorithmically connected items (Figure 4(c)). Obviously, this network involved regulators that helped THF modulation of the glucose metabolism pathway and lipid metabolism pathway. This hinted that the hypoglycemic and hypolipidemic mechanism of THF might target PI3K and LDL-R dependent pathways, as well as pro-inflammatory related pathways.

3.6. qPCR and Western Blotting Results Supported the Hypothesis That THF Improved Glucose Intolerance and Hepatosteatosis by Potentially Targeting the AKT-SREBP Nexus. Since PI3K-AKT is the canonical pathway of energy metabolism, and SREBP2-PCSK9-LDL-R pathway is reported to play a vital role in hepatic lipids metabolism, and combined with the aforementioned clues from IPA generated molecular networks, we hypothesized that THF improved glucose intolerance and hepatosteatosis in DIO rats by potentially targeting the AKT-SREBP nexus [33]. The mRNA expression and the protein levels of two canonical pathways, namely, PI3K-AKT and SREBP2-PCSK9-LDLR, were thus analyzed using RT-qPCR and Western blotting. As expected, the relative mRNA expressions of PI3K, AKT, and SREBP2 were significantly downregulated in HFF group compared with control group ($p = 0.0066$, 0.0097, and 0.0086, respectively), while they were upregulated after THF treatment compared

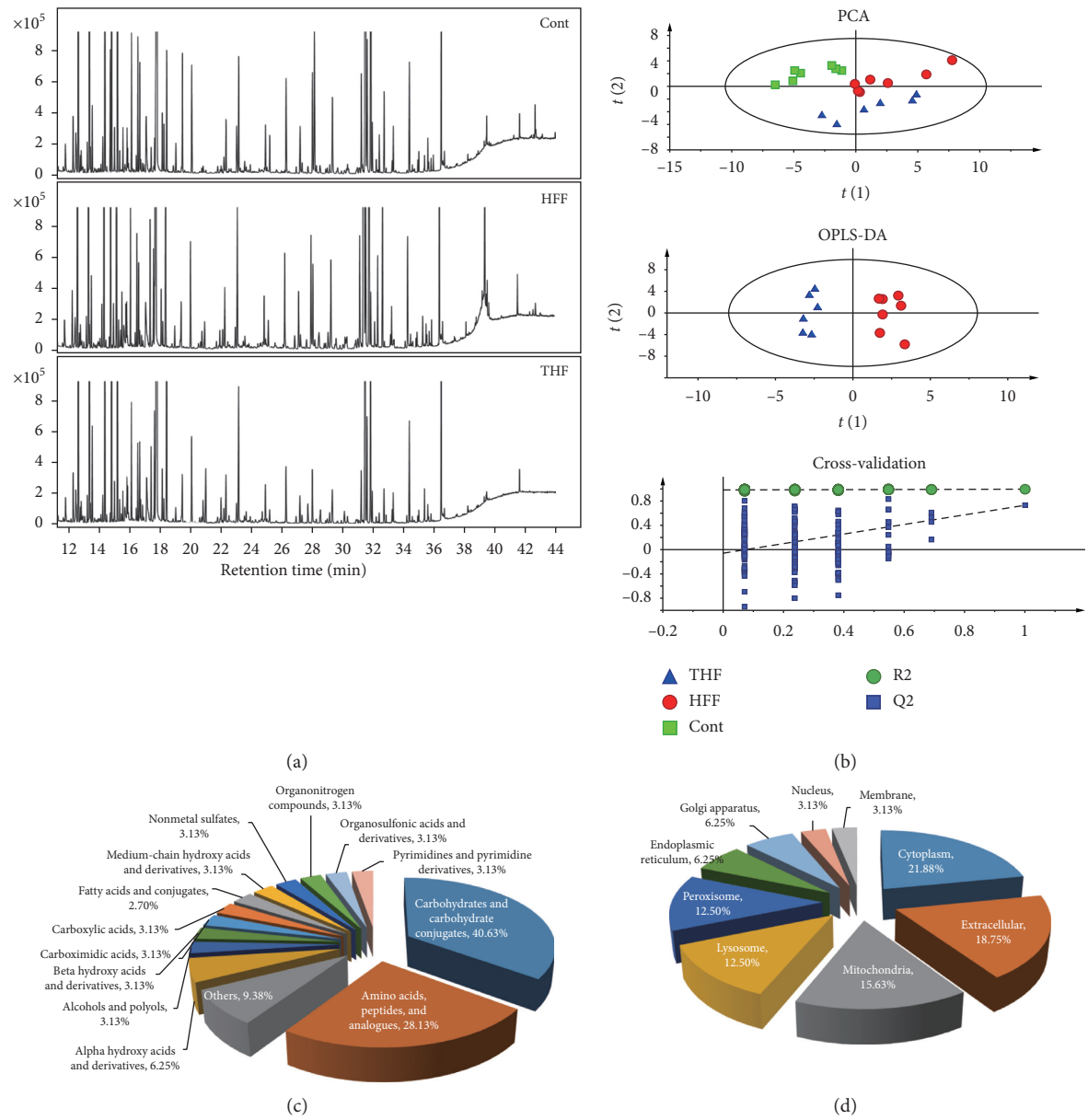

Figure 3: Nontargeted metabolomics analysis presented key differential metabolites between HHF-fed-THF-treated and HFF-fed-vehicle-treated rats. (a) Representative GC-MS total ion chromatograph of plasma from HFF-fed and THF (200 mg/kg) treated group (THF), HFF-fed and vehicle-treated group (HFF), and the control group (Cont) rats. (b) Multivariate statistical analysis of GC-MS metabolic profiling data. PCA scores plot and OPLS-DA scores plot were derived from GC-MS spectra of three groups of rats (THF, blue triangles; HFF, red dot; Cont, green diamond) and statistical validation of the OPLS-DA model by permutation testing. (c) Chemical classification of the key differential metabolites based on the annotations of Human Metabolome Database (http://www.hmdb.ca) and their corresponding percentage. (d) Cellular locations of the key differential metabolites based on the annotations of Human Metabolome Database (http://www.hmdb.ca) and their corresponding percentage. HFF, high-fat and high-fructose diets; THF, a mixed extract of *P. notoginseng* and *C. chinensis*; PCA, principle component analysis; PLS-DA, partial least squares-discriminate analysis; OPLS-DA, pairwise orthogonal projections to latent structures discriminate analysis.

with the HFF group (Figure 5(a)). PCSK9 had the same tendency although it was not statistically significant. In contrast, the relative mRNA expressions of LDLR and insulin receptor substrate 1(IRS1) were upregulated in the HFF group while both of them were downregulated in the THF group (Figure 5(a)). Furthermore, confirming our hypothesis, THF could restore the phosphorylation of PI3K and AKT to homeostatic levels, which were blocked by HFF feeding, as evaluated by Western blotting (Figure 5(b)). Likewise, the protein levels of hepatic SREBP2, PCSK9, and LDLR were also measured. As shown in Figure 5(c), whilst HFF feeding inhibited SREBP2 protein levels, THF treatment resulted in a significant increase ($p = 0.0027$), suggesting that THF supported SREBP2 activity to maintain the homeostatic cascade. Moreover, compared with control group, the

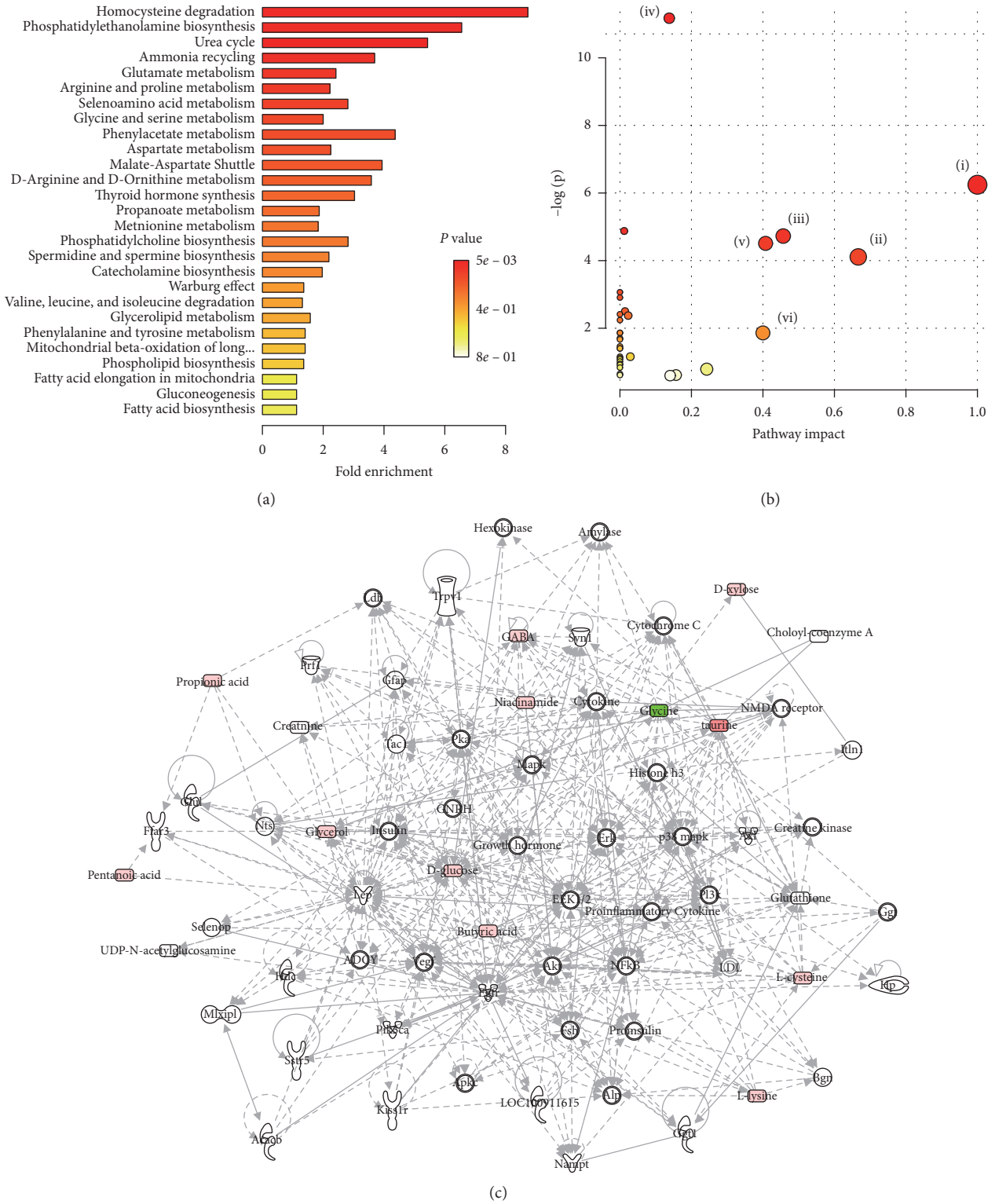

Figure 4: Ingenuity pathways analysis suggested that THF improves the glucose intolerance and hepatosteatosis by regulating PI3K and LDL-R involved pathways. (a) Enrichment analysis performed using the pathway-associated metabolites sets with MetaboAnalyst 4.0. (b). Overview of pathway analysis using Fisher's Exact Test as algorithms with MetaboAnalyst 4.0; (i) phenylalanine, tyrosine, and tryptophan biosynthesis, (ii) valine, leucine, and isoleucine, (iii) alanine, aspartate, and glutamate metabolism, (iv) aminoacyl-tRNA biosynthesis, (v) phenylalanine metabolism, (vi) methane metabolism. (c) Top regulated metabolic network generated by IPA software (Qiagen, Germany) based on the different metabolites discovered through metabolomics analysis hinted that THF improves the glucose intolerance and hepatic steatosis by regulating PI3K and LDL involved pathways. PI3K, Phosphatidylinositol-3 kinase; LDL-R, low-density lipoprotein receptor. HFF, high-fat and high-fructose diets; THF, Tian-Huang formula, a mixed extract of *P. notoginseng* and *C. chinensis*.

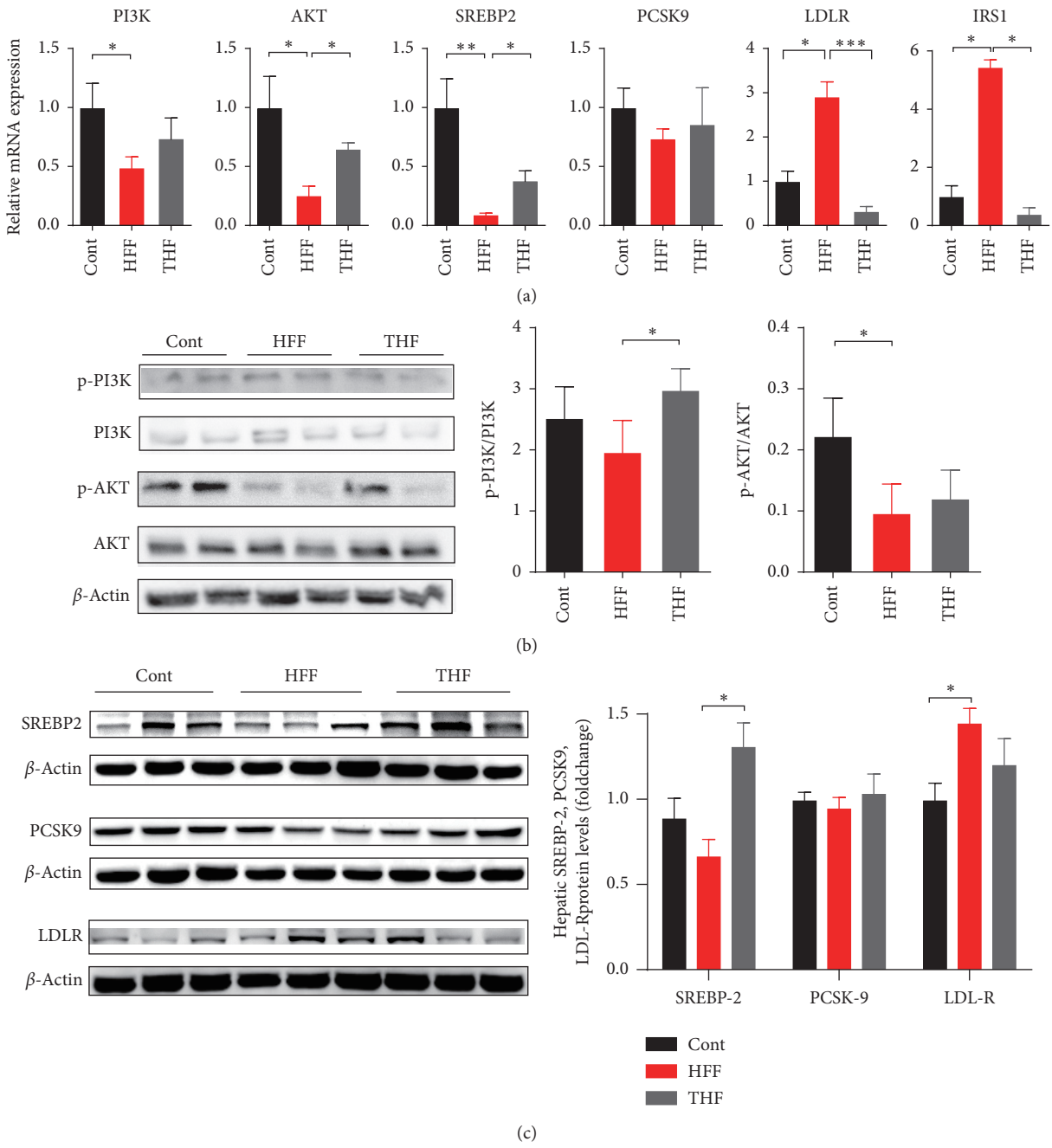

Figure 5: THF improves glucose intolerance and hepatosteatosis in HFF-diet induced obese rats partly through AKT-SREBP nexus. (a) qPCR results showed that the mRNA expressions of PI3K, AKT SREBP2, PCSK9, LDL-R, and IRS1 were improved after treating with THF in DIO rats, indicating that glucose and lipids metabolism pathways were maintained ($n = 6$, $^{*}p < 0.05$ vs. con group; $^{\#}p < 0.05$ vs HFF group). (b) Representative Western blots were shown while β-actin levels were used as a loading control. Western blot results showed the phosphorylation of PI3K and AKT declined in HFF group, indicating that PI3K-AKT pathway was suppressed. THF treatment attenuated the phosphorylation of hepatic PI3K and AKT in DIO rats ($n = 4$, $^{*}p < 0.05$ vs. con group; $^{\#}p < 0.05$ vs HFF group). (c) Representative Western blots were shown while β-actin levels were used as a loading control. Densitometric analysis results showed that HFF decreased the protein levels of SREBP-2 and increased the protein levels of LDL-R, which was reversed by THF treatment ($n = 6$, $^{*}p < 0.05$ vs. con group; $^{\#}p < 0.05$ vs HFF group). PI3K, phosphatidylinositol-3 kinase; AKT, protein kinase B; IRS1, insulin receptor substrate 1; SREBP-2, sterol regulatory element-binding protein-2; PCSK9, proprotein convertase subtilisin/kexin type 9; LDL-R, low-density lipoprotein receptor.

HFF diet induced a marked increase in the protein level of LDLR ($p = 0.0135$), which was attenuated by THF treatment (Figure 5(c)). However, as for the protein level of hepatic PCSK9, there were no fluctuations, and this was in line with data on mRNA expression (Figures 5(a) and 5(c)). Based on these results, we concluded that THF reversed the metabolic disturbance by targeting AKT-SREBP nexus.

4. Discussion

In the present study, we used a simple but robust process to prepare THF following the Ch.P. instructions and animal test demonstrated that THF could improve metabolic disturbance through the AKT-SREBP nexus. Our results proved that THF has valuable therapeutic potential for human DIO and its complications.

We chose the HFF diet induced rat model to evaluate the improvement effect of THF on glycolipid metabolism, because it is a well-accepted model of DIO and its complications, such as dyslipidemia, impaired glucose tolerance, and insulin resistance, and even its more serious consequences, T2DM, NAFLD, and metabolic syndrome [34, 35]. In fact, HFF simulated perfectly the popular Western diet and reflected the dietary preference of a large number of people [34], which can lead to obesity and NAFLD [36].

THF is a traditional Chinese medicinal formula composed of *P. notoginseng* and *C. chinensis*. Given the long traditional medicine history of *P. notoginseng* and *C. chinensis*, quite a few of studies have tried to reveal their pharmacological functions and mechanisms from different standpoints. Guo et al. used PNS to treat diabetes and found its molecular mechanism related to reducing skeletal muscle insulin resistance by regulating the IRS1-PI3K-AKT signaling pathway and GLUT4 expression [37]. Ding et al. investigated the hepatoprotective effects of PNS and found it can improve liver lipid accumulation and oxidative stress and protect acute ethanol-induced liver injury [15]. Zhong et al. also claimed that PNS can promote liver regeneration by activating PI3K/AKT/mTOR cell proliferation pathway and upregulating AKT/Bad cell survival pathway [38]. As for CCA, there are also some studies showing their benefits for therapeutic intervention on obesity and its complications. For example, Choi et al. [39] reported that CCA exerted antiadipogenic activity on 3T3-L1 adipocytes 2 by downregulating C/EBP-α and PPAR-γ; Yang et al. [40] revealed that CCA may help alleviate hyperglycemia in diabetes by promoting glucose uptake by skeletal muscles; and Li et al. [41] concluded that CCA prevent diabetic cognitive deficits most likely by ameliorating disorder of glucose and lipid metabolism, attenuating A-β deposition, and enhancing insulin signaling. Without exception, in this study, we found that THF can significantly improve liver lipid accumulation and impaired glucose tolerance, and reduce fasting blood glucose. It is notable that, during our previous research, the effects of different doses of THF on lipid-lowering were compared and 200 mg/kg dose (THF weight to rat body weight) had the best effect [20–23]. Herein, in the present mechanism investigation, 200 mg/kg of THF treatment was performed.

The endogenous metabolite spectrum reflects phenotypic changes, and accurate regulation of levels of all kinds of endogenous molecules is critical for metabolic homeostasis. Viewed as the list of key metabolites derived from our metabolomics analysis, a HFF diet contributes to marked changes of a series of endogenous molecules, such as 4-hydroxybutanoic acid, oxalic acid, lactic acid and taurine, etc. These changes must be the result of a series of physiological and biochemical reactions and there likely exists some kind of underlying linkage. Using software algorithm-based enrichment and IPA analysis, the full list of modulated key metabolites presented us a molecular network revealing the delicate and intricate influence of THF on rats' metabolism. Along with our knowledge on signaling pathways, we hypothesized that the AKT-SREBP nexus could play a vital role mediated THF's protection, of which the PI3K-AKT pathway and SREBP2-PCSK9-LDLR pathway are familiar pathways related to energy and lipid metabolism [33]. Indeed, some saponin components of the THF described here, such as ginsenoside Rb1 and ginsenoside Rg1, have been reported to target PI3K-AKT pathway to regulate innate immune responses in macrophages [42], to protect IL-1β-induced mitochondria-activated apoptosis [43], to mitigate oxidative stress and apoptosis [44], and to prevent homocysteine-induced endothelial dysfunction [45]. Similarly, as for the other three alkaloid components of THF, namely, berberine, coptisine, and palmatine, literature demonstrates that they can reduce ischemia/reperfusion-induced myocardial apoptosis in diabetic rats [46], can ameliorate insulin resistance in obese rats [47], and can inhibit IL-21/IL-21R mediated inflammatory proliferation [48], through the PI3K-AKT pathway. Moreover, berberine has been claimed to attenuate nonalcoholic hepatic steatosis through the AMPK-SREBP-1c-SCD1 pathway [49], alleviate adipogenesis via the AMPK-SREBP pathway [50], and increase the expression of PCSK9 levels in HFD rats through the SREBP-2 pathway [51]. In line with these, our data show that THF has a strong potential to regulate the AKT-SREBP nexus specifically to improve metabolic disorders upon high-fat high-fructose diet challenge. However, we found that neither the mRNA nor the protein expression levels of PCSK9 were altered, and both the mRNA and the protein expression level of LDLR showed inverse changes. These findings are not completely consistent with the aforementioned literature, which suggests that THF may have different and complicated effects compared to single compounds, possibly affecting some other mediators not focused on in this study but belonging to the AKT-SREBP nexus. Recent studies have shown that berberine significantly upregulated the mRNA level of SREBP-2 in the liver of HFD rats, thereby increasing the levels of PCSK9 and LDLR [51]. In this study, THF increased the expression of SREBP2 and PCSK9 and decreased the expression of LDLR, which was linked with the protein levels assessed by Western blotting analysis. Thus, our study confirmed previous studies and provided novel additional important information regarding the effects of THF on glucose intolerance and lipid accumulation.

Thus, although a full understanding of the mechanisms underpinning THF-mediated protection in DIO associated glucose intolerance and hepatosteatosis requires further work, we propose that THF targets the AKT-SREBP nexus to maintain glycolipid metabolic homeostasis. By targeting the cascades, THF may protect against DIO associated glycolipid metabolic disturbance in a subtle and effective way (Figure 6). Therefore, our study indicates that there is great medicinal potential to be found in THF. Meanwhile, in our future research, the therapeutic potential of the isolated compounds or fractions from THF should be evaluated.

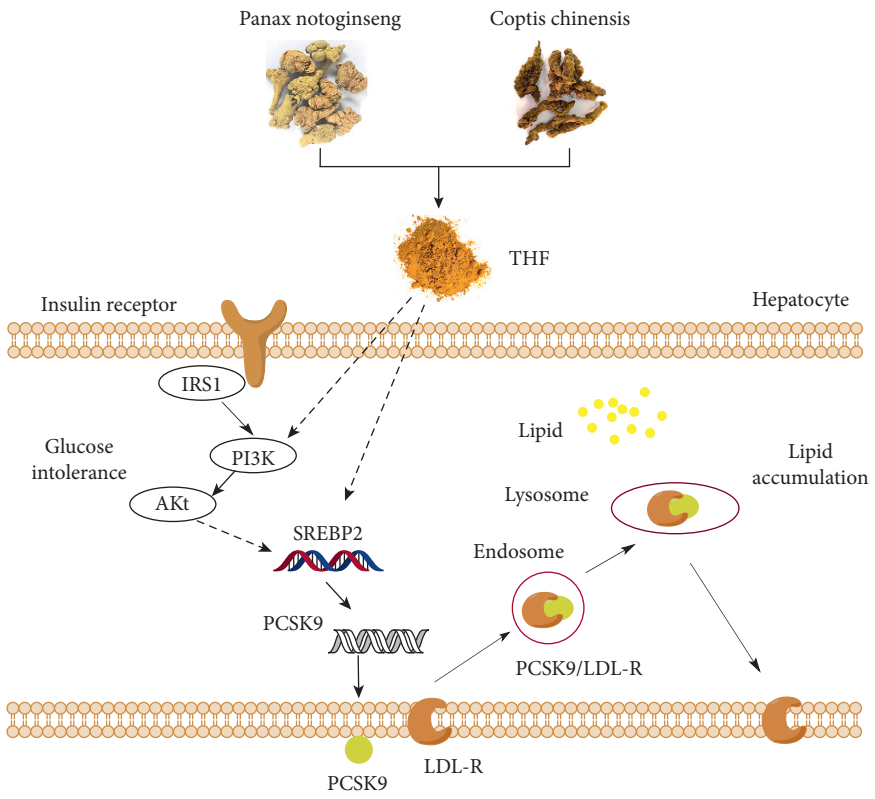

Figure 6: Schematic model summarizing the proposed underlying mechanisms of the THF ameliorates glucose intolerance and hepatosteatosis by maintaining the homeostasis of AKT- SREBP nexus in DIO rats. HFF, high-fat and high-fructose diets; THF, Tian-Huang formula, a mixed extract of *P. notoginseng* and *C. chinensis*; IRS1, insulin receptor substrate 1; PI3K, phosphatidylinositol-3 kinase; AKT, protein kinase B; SREBP-2, sterol regulatory element-binding protein-2; PCSK9, proprotein convertase subtilisin/kexin type 9; LDL-R, low-density lipoprotein receptor.

Furthermore, THF has a long history of usage in Chinese medicine, and it is currently employed in clinic, suggesting that THF might not have significant drawbacks or side effects often associated with other hypoglycemic and hypolipidemic therapies. Thus, the chemical material basis elucidated here might provide more evidence for the rational development of novel drugs to treat human DIO accompanied impaired glucose metabolism and hepatic fat accumulation conditions, even potentially T2DM and NAFLD.

5. Conclusions

In conclusion, this study showed that long-term HFF diet feeding can induce DIO associated glucose intolerance and hepatic steatosis in rats and that THF (200 mg/kg) treatment can ameliorate these conditions. Metabolomics analysis and algorithmically constructed metabolic networks hinted that AKT and LDLR are involved in THF's pharmaceutical effects, and qPCR and Western blotting results supported the hypothesis that THF improves glucose intolerance and hepatosteatosis in DIO rats by potentially targeting AKT-SREBP nexus. Although translation of the finding of rodent experiments to human is not so satisfied, the study sheds light on the therapeutic potential of THF against impaired glucose and lipid accumulation.

Data Availability

All the raw data are stored at Guangdong Metabolic Disease Research Centre of Integrated Chinese and Western Medicine, which will be available from the corresponding author on reasonable request.

Additional Points

High-fat high-fructose diet has deleterious consequences on glucose and lipid metabolism. Tian-Huang formula (THF) is composed of *P. notoginseng* and *C. chinensis*. THF alleviated hepatosteatosis and glucose intolerance in diet induced obese rats. AKT-SREBP nexus are potential targets of THF.

Ethical Approval

All procedures performed were approved by the Experimental Animals Ethics Committee of Guangdong Pharmaceutical University (No. SPF2017092).

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Kun-Ping Li and Yang Yu contributed equally to this work. All authors contributed to the study conception and design. G. J. contributed to conceptualization and funding acquisition. L. K .P., Y. Y., M. Y., and Z. C. M. contributed to material preparation, data collection, and analysis. The first draft of the manuscript was written by L. K. P., and J. E. T. revised and improved the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported in part by the National Natural Science Foundation of China (81530102), Major Basic and Applied Basic Research Projects in Guangdong Province (2019B030302005), and Innovation and Strengthening University Project Subsidized Project of Guangdong Pharmaceutical University (2020KTSCX058).

Supplementary Materials

Supplemental Table S1: the key differential metabolites between HHF-fed-THF-treated and HFF-fed-vehicle-treated rats presented by metabolomics analysis. Supplemental Table S2: primer sequences for RT-qPCR. Supplemental methods: quantitative profiling of THF, a mixed extract of *Panax notoginseng* and *Coptis chinensis*. 1.1: quantitative profiling of *Panax notoginseng* saponins. 1.2: quantitative profiling of *Coptis chinensis* alkaloids. 1.3: linear regression was performed. (*Supplementary Materials*)

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6642600, 13 pages
https://doi.org/10.1155/2021/6642600

Research Article

Network Pharmacology-Based Approach to Comparatively Predict the Active Ingredients and Molecular Targets of Compound Xueshuantong Capsule and Hexuemingmu Tablet in the Treatment of Proliferative Diabetic Retinopathy

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Received 1 January 2021; Revised 16 February 2021; Accepted 18 February 2021; Published 5 March 2021

Academic Editor: Mohammad Abdollahi

Background. Compound Xueshuantong capsule (CXC) and Hexuemingmu tablet (HXMMT) are two important Chinese patent medicines (CPMs) frequently used to treat proliferative diabetic retinopathy (PDR), especially when complicated with vitreous hemorrhage (VH). However, a network pharmacology approach to understand the therapeutic mechanisms of these two CPMs in PDR has not been applied. *Objective*. To identify differences in the active ingredients between CXC and HXMMT and to comparatively predict and further analyze the molecular targets shared by these CPMs and PDR. *Materials and methods*. The differentially expressed messenger RNAs (mRNAs) between normal retinal tissues in healthy individuals and active fibrovascular membranes in PDR patients were retrieved from the Gene Expression Omnibus database. The active ingredients of CXC and HXMMT and the targets of these ingredients were retrieved from the Traditional Chinese Medicine Systems Pharmacology database. The intersections of the CPM (CXC and HXMMT) targets and PDR targets were determined. Then, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed, and the ingredient-target networks, protein-protein interaction networks, and KEGG-target (KEGG-T) networks were constructed. *Results*. CXC contains 4 herbs, and HXMMT contains 19. *Radix salviae* is the only herb common to both. CXC had 34 potential therapeutic targets in PDR, while HXMMT had these 34 and 10 additional targets. Both CPMs shared the following main processes: response to reactive oxygen species and oxidative stress, regulation of blood vessel diameter and size, vasoconstriction, smooth muscle contraction, hemostasis, and blood coagulation. The shared pathways included the AGE-RAGE signaling pathway in diabetic complications, TNF signaling pathway, relaxin signaling pathway, and IL-17 signaling pathway. *Conclusions*. Both CXC and HXMMT include components effective at treating PDR and affect the following main processes: response to reactive oxygen species and oxidative stress, regulation of blood vessels, and blood coagulation. *Radix salviae*, the only herb common to both CPMs, contains many useful active ingredients. The PDR-CXC and PDR-HXMMT networks shared 34 common genes (RELA, HSPA8, HSP90AA, HSP90AB1, BRCA, EWSR1, CUL7, HNRNPU, MYC, CTNNB1, MDM2, YWHAZ, CDK2, AR, FN1, HUWE1, TP53, TUBB, EP300, GRB2, VCP, MCM2, EEF1A1, NTRK1, TRAF6, EGFR, PRKDC, SRC, HDAC5, APP, ESR1, AKT1, UBC, and COPS5), and the PDR-HXMMT network has 10 additional genes (RNF2, VNL, RPS27, COPS5, XPO1, PARP1, RACK1, YWHAB, and ITGA4). The top 5 pathways with the highest gene ratio in both networks were the AGE-RAGE signaling pathway in diabetic complications, TNF signaling pathway, relaxin signaling pathway, IL-17 signaling pathway, and focal adhesion. Additional pathways such as neuroactive ligand-receptor interaction, chemokine signaling pathway, and AMPK signaling pathway were enriched with HXMMT targets. Thus, HXMMT has more therapeutic targets shared by different active ingredients and more abundant gene functions than CXC, which may be two major reasons why HXMMT is more strongly recommended than CXC as an auxiliary treatment for new-onset VH secondary to PDR. However, the underlying mechanisms still need to be further explored.

1. Background

Diabetic retinopathy (DR), a serious complication of diabetes mellitus (DM) caused by microvascular ischemia and hypoxemia, affects approximately 35% of DM patients and an estimated > 90 million people worldwide [1, 2]. The prevalence of proliferative diabetic retinopathy (PDR), a vision-threatening type of DR characterized by retinal neovascular and even vitreous hemorrhage (VH), is nearly 7% [3]. PDR dramatically decreases patients' quality of life and contributes to a massive economic burden. Therefore, it is crucial to develop effective pharmaceutical preparations to treat PDR based on its pathological mechanisms.

For thousands of years, traditional Chinese medicines (TCMs) have been used by Chinese people to treat DM and its complications [4]. Compound Xueshuantong capsule (CXC) and Hexuemingmu tablet (HXMMT) are two important Chinese patent medicines (CPMs) that are frequently used to treat PDR, especially when complicated with VH [5, 6]. According to observations in daily clinical practice and the results of some clinical studies, CXC and HXMMT are crucial auxiliary treatments to eliminate VH secondary to PDR as well as to improve retinal hemodynamics in PDR [6]. Correspondingly, previous experimental studies have shown that CXC and HXMMT may exert protective effects on retinal capillary endothelial cells and nerve cells by regulating multiple pathways [7–9]. However, most CMPs are composed of different types of herbs, and every herb is further composed of multiple active ingredients. Thus, a single CPM may target numerous PDR-related molecules, and the pharmacological mechanisms are complex. Moreover, in clinical practice, HXMMT more effectively eliminates new-onset VH secondary to PDR than HXMMT and is more frequently recommended by TCM doctors for new-onset VH treatment, but its potential mechanisms are poorly understood. Thus, further research is needed to better understand the underlying regulatory and interactive mechanisms of different active ingredients in CXC and HXMMT.

Network pharmacology analysis is a convenient and systematic approach to identify core targets shared by drugs and diseases [10]. It can also be performed to identify potential pathways for disease interventions, providing insight into the complex mechanisms of Chinese herbal formulas used to treat diseases. The aim of our study was to find differences in active ingredients between CXC and HXMMT and to comparatively predict and further analyze the molecular targets shared by these drugs and PDR.

2. Materials and Methods

The general procedure was as follows (described in detail in Section 2.1). First, differentially expressed mRNAs (DEmRNAs) between normal retinal tissues in healthy individuals and abnormal retinal membranes in PDR patients were acquired from the database. The chosen DEmRNAs according to certain criteria were defined as PDR targets (described in detail in Section 2.2). Second, active ingredient screening was, respectively, performed in CXC and HXMMT; thus, the CXC and HXMMT targets were obtained (described in detail in Sections 2.3 and 2.4). Third, the intersections between CXC and PDR targets (CXC-PDR targets) as well as between HXMMT and PDR targets (HXMMT-PDR targets) were determined. Afterward, intersections between CXC-PDR targets and HXMMT-PDR targets were identified. We found that all the CXC-PDR targets were completely included in the HXMMT-PDR targets. Finally, the ingredient-target (I-T) networks, protein-protein interaction (PPI) networks, Kyoto Encyclopedia of Genes and Genomes-target (KEGG-T) networks, and Gene Ontology (GO) analyses were performed for CXC-PDR and HXMMT-PDR targets. A flowchart of the procedure is shown in Figure 1, which provides a detailed description of each step.

2.1. Messenger RNA (mRNA) Data Collection and Differential Expression Analysis. The microarray data used in this study were retrieved from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds/). The mRNA expression data were acquired from dataset GSE60436, which contains 3 samples from normal retinal tissues and 3 from active fibrovascular membranes in PDR patients. The raw expression data were first normalized, and analysis of DEmRNAs was then performed using the limma package based on the R language. The criteria for the selection of DEmRNAs were an adjusted P value of <0.05 and a $|\log2FC|$ value of >1, and the selected DEmRNAs were defined as PDR targets.

2.2. Active Ingredient Screening. A total of 499 Chinese herbs and 12144 chemical ingredients from the Chinese pharmacopoeia (2010) were registered in the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (https://tcmspw.com/index.php), a platform that provides pharmacokinetic characteristics and targets of each ingredient in these herbs. Oral bioavailability (OB) and drug-likeness (DL) are two parameters commonly used to screen active ingredients. The OB is the percentage of the orally administered dose of the unchanged drug that enters the systemic blood circulation, and it is an important pharmacokinetic indicator. DL is used to assess whether the ingredients function as known drugs.

Each herb contained in CXC and HXMMT was searched in the TCMSP database, and all ingredients were obtained. According to most traditional Chinese herbs studies [10–12], an ingredient with an OB of ≥30 and a DL of ≥0.18 was considered an active ingredient, and the targets of these ingredients (CXC and HXMMT targets) were retrieved from the database.

2.3. Network Construction. Cytoscape 3.6.1 (http://cytoscape.org/) was used to generate all visual network diagrams, including the I-T, PPI, and KEGG-T networks.

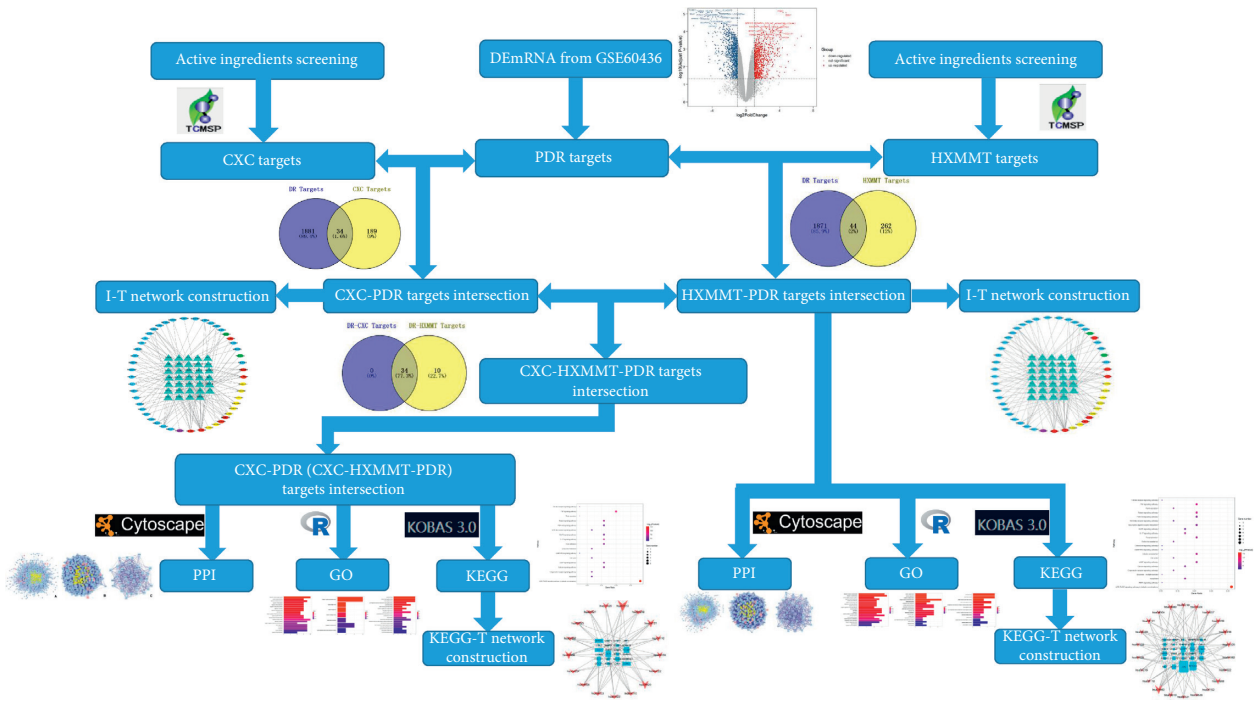


Figure 1: Flowchart of the complete analysis procedure. DEmRNA, differentially expressed messenger RNA; CXC, compound Xueshuantong capsule; PDR, proliferative diabetic retinopathy; HXMMT, Hexuemingmu tablet; I-T, ingredient-target; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; KEGG-T, Kyoto Encyclopedia of Genes and Genomes-target.

The intersections of the formulas' targets (CXC and HXMMT targets) and the PDR targets were determined (CXC-PDR and HXMMT-PDR targets). Accordingly, two I-T networks were constructed. PPI networks were constructed using the Bisogenet 3.0.0 plugin in Cytoscape 3.6.1 based on the following databases: the Database of Interacting Proteins, the Biological General Repository for Interaction Datasets, the Human Protein Reference Database, the IntAct Molecular Interaction Database, the Molecular INTeraction Database and the Binding Database. The CytoNCA plugin was used to perform topological analyses. Degree centrality and betweenness centrality were the measures selected to represent the topological features of each node in the network. Degree centrality represents the number of edges linked by a node, while betweenness centrality represents the proximity of a node to other nodes. KEGG-T networks were constructed after enrichment analyses were performed.

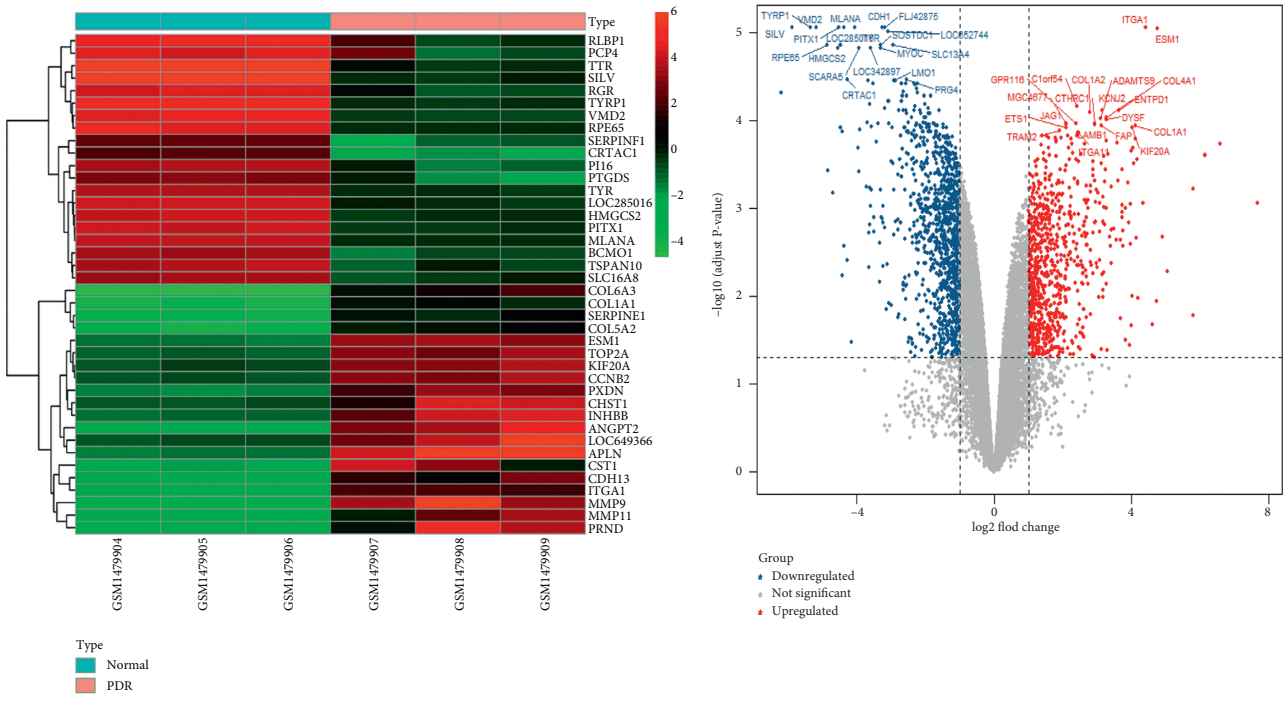
2.4. Functional Enrichment Analysis. GO analyses were performed using the clusterProfiler package based on the R language. The criterion for the selection of GO processes was a P value of <0.05. KEGG pathway analyses were performed using an online biological tool, KEGG Orthology Based Annotation System 3.0 (KOBAS 3.0, http://kobas.cbi.pku.edu.cn). The criteria for the selection of KEGG pathways were a P value of <0.01 and a gene count of ≥3. Visualizations were performed using the ggplot2 R package.

3. Results

3.1. Identification of PDR Targets. Analysis of the microarray dataset GSE60436 showed that 1915 mRNAs (819 upregulated mRNAs and 1096 downregulated mRNAs) were differentially expressed in PDR patients compared with individuals without PDR (Figure 2).

3.2. I-T Network Construction. CXC contains 4 herbs, and HXMMT contains 19 herbs (Table 1). *Radix salviae* is the only herb common to both CPMs. Through screening the active ingredients, we found 34 potential therapeutic targets in PDR for CXC (Figure 3(a)) and the same 34 and 10 additional therapeutic targets in PDR for HXMMT (Figure 3(b)). The ten additional targets of HXMMT were CYCS, APOD, GOT1, PECAM1, ALDH2, COL1A2, CD300A, PTGER2, CHGA, and CD36. Accordingly, the active ingredients and the potential therapeutic targets were used to construct I-T networks for CXC (Figure 4(a)) and HXMMT (Figure 4(b)). Then, the intersections of the PDR-CXC targets and the PDR-HXMMT targets were determined, and the complete set of potential therapeutic targets of CXC was found to be included among the targets of HXMMT (Figure 3(c)).

A PPI network containing 1825 nodes and 36349 edges was constructed for the set of PDR-CXC targets, while another PPI network containing 2004 nodes and 38863 edges was constructed for the set of PDR-HXMMT targets. The screening parameters were degree centrality and betweenness centrality. The thresholds were set at a degree centrality of ≥61 in the first



(a) (b)

Figure 2: Heatmap (a) and volcano plot (b) of DEmRNAs (PDR targets) in microarray dataset GSE60436. The top 20 downregulated and upregulated DEmRNAs are shown in the heatmap and the volcano plot.

Table 1: Components of the compound Xueshuantong capsule and Hexuemingmu tablet [6, 7].

	Herbs
Compound Xueshuantong capsule (CXC)	*Radix salviae (Danshen), Panax notoginseng (Sanqi), Hedysarum multijugum* Maxim. *(Huangqi), Figwort root (Xuanshen)*
Hexuemingmu tablet (HXMMT)	*Radix salviae (Danshen), Chuanxiong rhizoma (Chuanxiong), Radix paeoniae rubra (Chishao), Gentianae radix et rhizoma (Longdan), Scutellariae radix (Huangqin), Cassiae semen (Juemingzi), Chrysanthemi flos (Juhua), Ecliptae herba (Mohanlian), Equiseti hiemalis herba (Muzei), Pollen typhae (Puhuang), Crataegus pinnatifida* Bunge *(Shanzha), Prunellae spica (Xiakucao), Rehmanniae radix praeparata (Dihuang), Plantaginis semen (Cheqianzi), Leonuri fructus (Chongweizi), Fructus ligustri lucidi (Nvzhenzi), Curcumae radix (Yujin), Cortex moutan (Mudanpi), Angelicae sinensis radix (Danggui)*

The names in parentheses are the Chinese names of the components.

screen and a betweenness centrality of ≥600 in the second screen. After the first screen, 350 nodes and 10843 edges were included in the PDR-CXC network, while 386 nodes and 11975 edges were included in the PDR-HXMMT network. After the second screen, 39 nodes and 406 edges were included in the PDR-CXC network, while 51 nodes and 628 edges were included in the PDR-HXMMT network. All the key genes in the PDR-CXC network were also included in the PDR-HXMMT network (RELA, HSPA8, HSP90AA, HSP90AB1, BRCA, EWSR1, CUL7, HNRNPU, MYC, CTNNB1, MDM2, YWHAZ, CDK2, AR, FN1, HUWE1, TP53, TUBB, EP300, GRB2, VCP, MCM2, EEF1A1, NTRK1, TRAF6, EGFR, PRKDC, SRC, HDAC5, APP, ESR1, AKT1, UBC, and COPS5). In addition, RNF2, VNL, RPS27, COPS5, XPO1, PARP1, RACK1, YWHAB, and ITGA4 were included only in the PDR-HXMMT network. The topological screening processes of the PPI networks are shown in Figure 5.

3.3. GO and KEGG Pathway Analyses. The top 20 or all GO processes (when the number of processes was smaller than 20) in the biological process (BP), cellular component (CC), and molecular function (MF) categories were identified (Figure 6). Both CXC and HXMMT may have therapeutic effects on PDR mainly via the following processes in the BP category: response to reactive oxygen species and oxidative stress, regulation of blood vessel diameter and size, vasoconstriction, smooth muscle contraction, hemostasis, and blood coagulation. Additionally, GO terms, such as positive regulation of blood circulation, negative regulation of cell adhesion, extracellular structure organization, and response to lipopolysaccharide, were enriched in HXMMT targets. In the CC category, the therapeutic targets of both CXC and HXMMT were associated mainly with the terms collagen-containing extracellular matrix, fibrillar collagen trimer, and banded collagen fibril. Moreover, HXMMT targets were

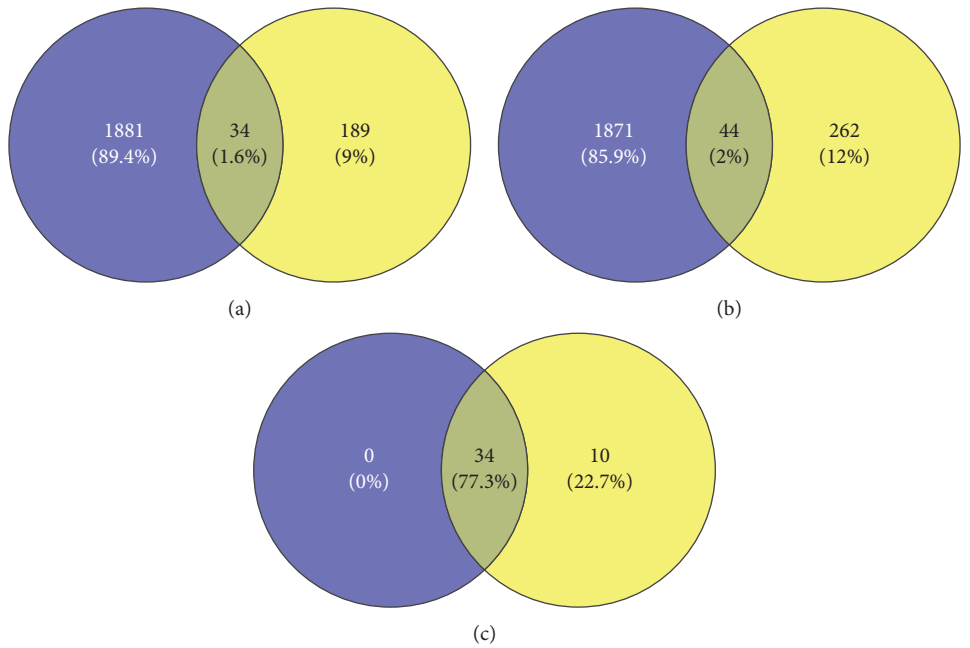


Figure 3: (a) Intersection of compound Xueshuantong capsule (CXC) targets (blue) and proliferative diabetic retinopathy (PDR) targets (yellow). (b) Intersection of Hexuemingmu tablet (HXMMT) targets (blue) and PDR targets (yellow). (c) Intersection of PDR-CXC targets (blue) and PDR-HXMMT targets (yellow).

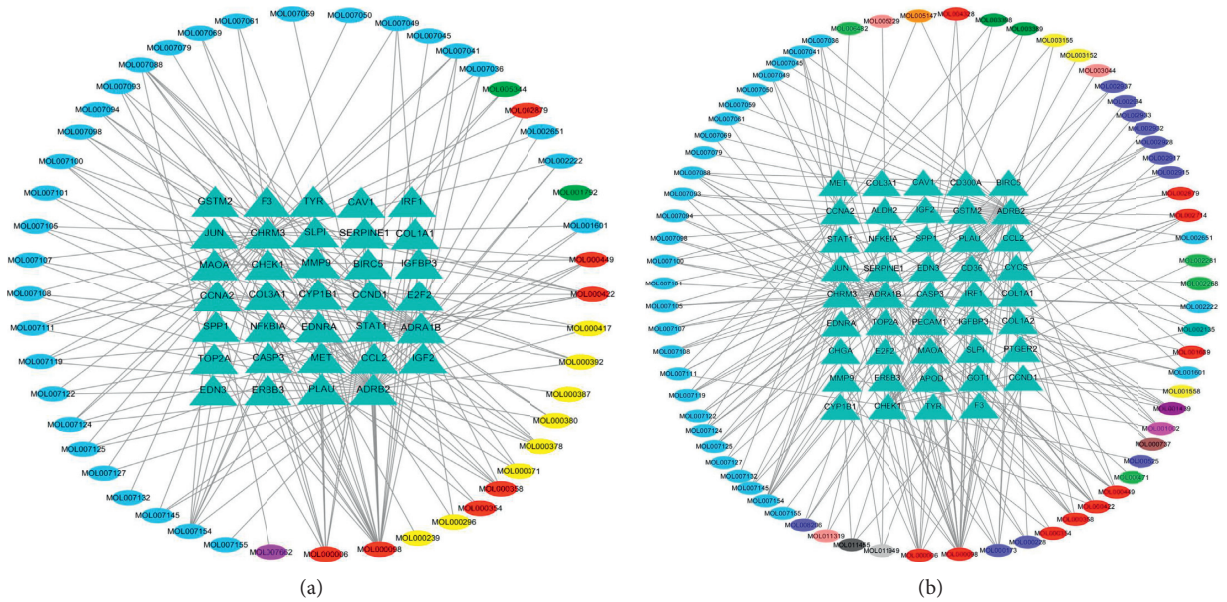
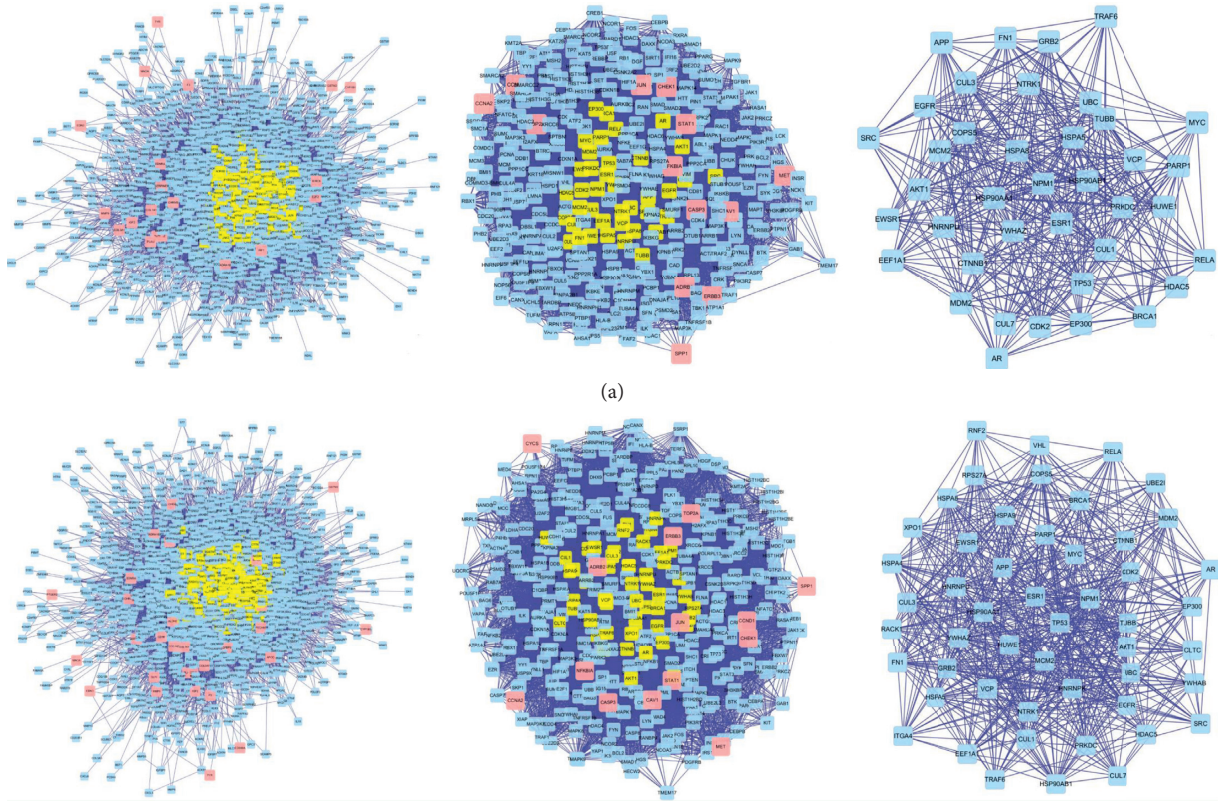


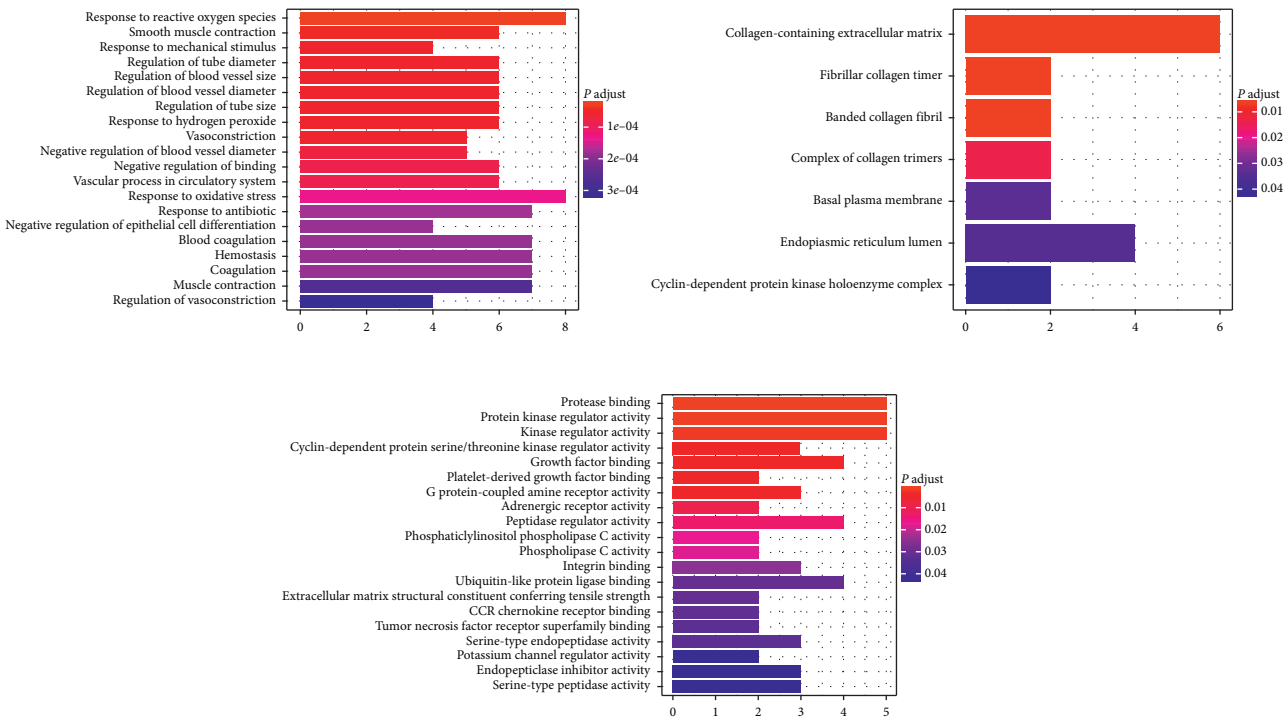
Figure 4: Ingredient-target networks of different Chinese patent medicines. The triangles and circles represent potential therapeutic targets and active ingredients, respectively. (a) Network for the compound Xueshuantong capsule. The blue circles represent active ingredients in *Radix salviae*, the green circles represent active ingredients in *Panax notoginseng*, the yellow circles represent active ingredients in *Hedysarum multijugum* Maxim., the purple circles represent active ingredients in *Figwort root*, and the red circles represent active ingredients in multiple herbs. (b) Network for the Hexuemingmu tablet. The light blue circles represent active ingredients in *Radix salviae*, the bluish green circles represent active ingredients in *Chuanxiong rhizoma*, the light purple circles represent active ingredients in *Radix paeoniae rubra*, the yellow circles represent active ingredients in *Gentianae radix et rhizoma*, the dark blue circles represent active ingredients in *Scutellariae radix*, the light green circles represent active ingredients in *Cassiae semen*, the pink circles represent active ingredients in *Chrysanthemi flos*, the dark green circles represent active ingredients in *Ecliptae herba*, the light gray circles represent active ingredients in *Equiseti hiemalis herba*, the orange circles represent active ingredients in *Fructus ligustri lucidi*, the dark purple circles represent active ingredients in *Pollen typhae*, the dark gray circles represent active ingredients in *Crataegus pinnatifida* Bunge, the brown circles represent active ingredients in *Prunellae spica*, and the red circles represent active ingredients in multiple herbs.



(a)

(b)

Figure 5: Topological screening process of the protein-protein interaction (PPI) networks. Left: original PPI network; middle: PPI network after the first screen; right: PPI network after the second screen. (a) Screening process of the compound Xueshuantong capsule. (b) Screening process of the Hexuemingmu tablet.



(a)

Figure 6: Continued.

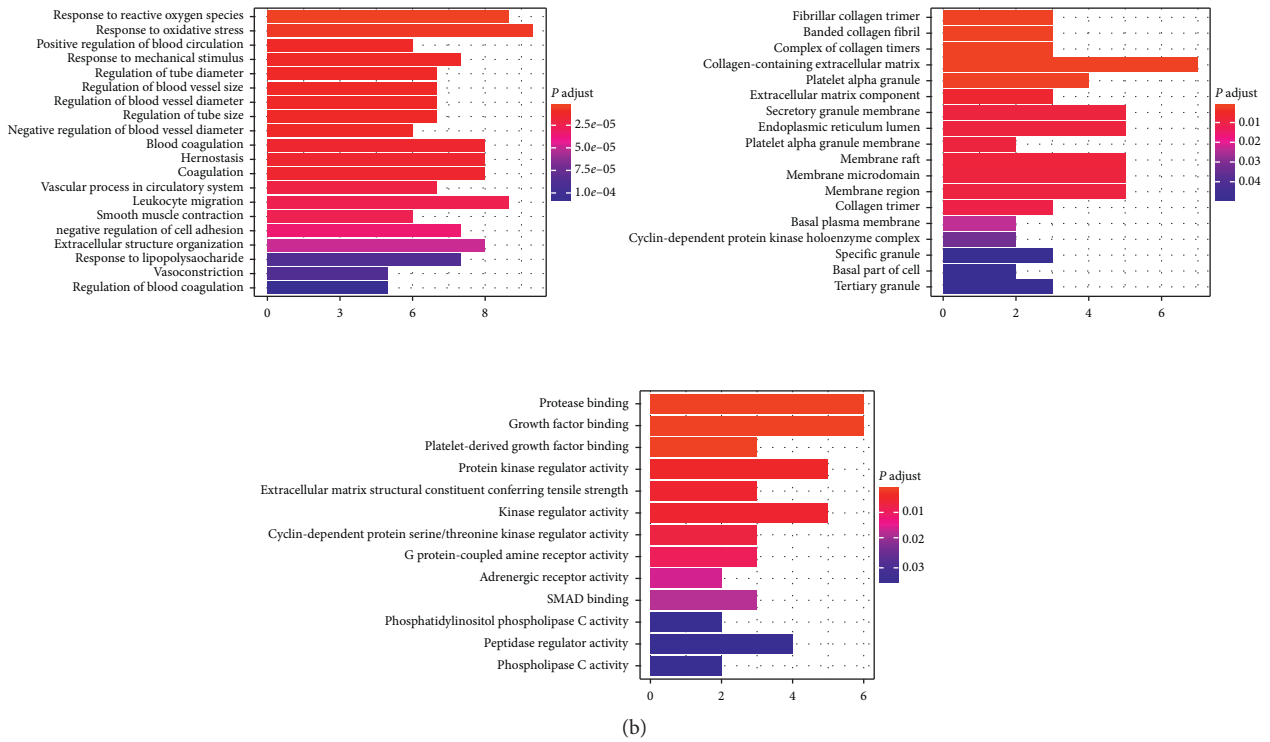


Figure 6: Gene Ontology (GO) analysis results. Left: biological process; middle: cellular component; right: molecular function. (a) GO terms enriched with targets of the compound Xueshuantong capsule. (b) GO terms enriched with targets of the Hexuemingmu tablet.

enriched in the term platelet alpha granule. In addition, CXC and HXMMT targets overlapped in GO MF terms, most of which were related to protein and enzyme binding and regulation.

After KEGG pathway analyses were performed using KOBAS 3.0, possible PDR-related pathways were selected, and visualizations were generated using the ggplot2 R package. All genes and pathways enriched with CXC targets were also enriched with HXMMT targets, and HXMMT had 5 additional target genes: IGFBP3, CYCS, CD36, PTGER2, and COL1A2. The pathway that was the most significantly enriched and had the highest gene ratio in both analyses was the AGE-RAGE signaling pathway in diabetic complications. Other common pathways included the TNF signaling pathway, relaxin signaling pathway, IL-17 signaling pathway, and focal adhesion. Additional pathways, such as neuroactive ligand-receptor interaction, chemokine signaling pathway, and AMPK signaling pathway, were enriched with HXMMT targets. Then, the enriched pathways and their related target genes were used to construct KEGG-T networks for CXC and HXMMT. These results are shown in Figure 7. The most significant pathway and key genes among the PDR treatment targets of CXC and HXMMT are shown in Figure 8.

4. Discussion

The GO terms enriched with CXC and HXMMT targets were similar and focused mainly on the response to reactive oxygen species and oxidative stress, regulation of blood vessels, and blood coagulation.

Radix salviae is an important component that is closely related to the response to reactive oxygen species and oxidative stress. In an in vitro model of hypoxia and reoxygenation, Hu's [13] study showed that *Radix salviae* obviously alleviated cardiomyocyte apoptosis and protected mitochondrial function and cell membrane skeleton integrity in H9c2 cells. In addition, Zhang's experiment in rodents [14] revealed that the Danshen (*Radix salviae*) dripping pill inhibited apoptosis and exerted neuroprotective effects in the retinas of diabetic rats by increasing the expression of Bcl-2, Bcl-2-associated X, and caspase-3 in diabetic rats. Moreover, according to the I-T networks of CXC and HXMMT, *Radix salviae* contained a greater number of active ingredients related to potential therapeutic targets in PDR compared with the other herbal components, suggesting that *Radix salviae* may play a crucial role in PDR treatment. A randomized controlled trial (RCT) performed by Lian and colleagues [5] showed that a *Radix salviae*-containing Chinese herbal product was effective in treating DR and in delaying the progression from non-PDR to PDR by reducing the area of capillary nonperfusion and degree of vascular leakage. Our study identified tanshinone as one of the most important active ingredients of *Radix salviae*. According to previous studies, tanshinone exerts protective effects on retinal pigment epithelium and retinal endothelial cells [15–17].

Regarding circulatory-related effects, CXC is considered to be an effective complementary medicine to treat ischemic vascular diseases, such as cerebral infarction and cardiovascular diseases [18, 19]. Lyu's study [18] showed that CXC

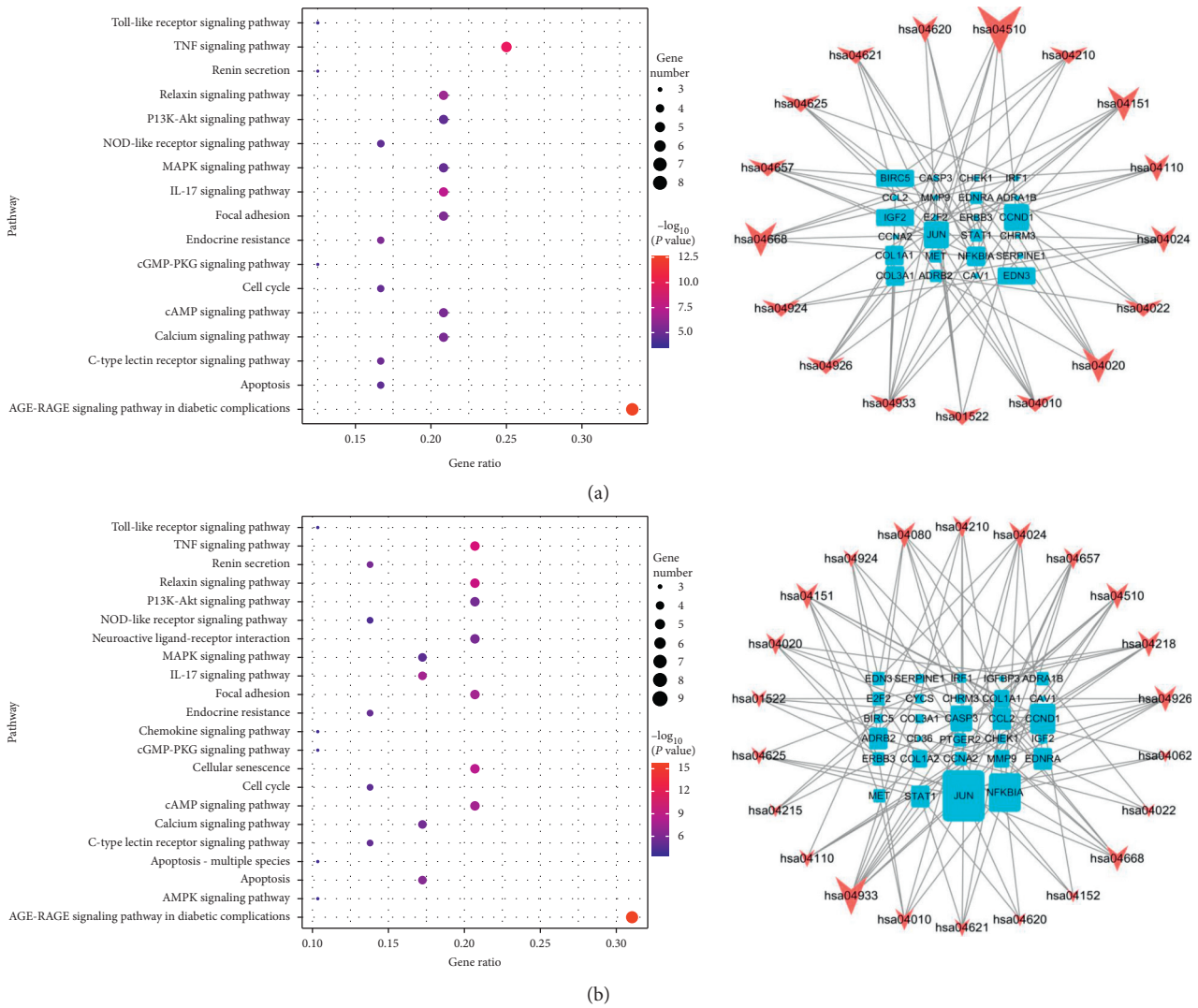


Figure 7: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis results (left) and KEGG-target networks (right). In the right panel, the arrows and squares represent pathways and potential therapeutic targets, respectively. The size of the arrows represents the ratio of the corresponding therapeutic targets, while the size of the therapeutic targets represents the ratio of the corresponding pathways. (a) Pathways enriched with targets of the compound Xueshuantong capsule. (b) Pathways enriched with targets of the Hexuemingmu tablet.

combined with conventional treatments had better clinical effects than conventional treatments alone. Moreover, a significant reduction in the IL-6 and hs-CRP levels was noticed when CXC was combined with conventional treatments. Regarding CXC and DR, some studies [20, 21] have shown that CXC contributes to the attenuation of streptozotocin- (STZ-) induced retinal lesions, including the amelioration of increases in erythrocyte aggregation, plasma viscosity, and acellular vessel and pericyte loss, by reversing the hyperexpression of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1) and endothelin-1 (ET-1) and the hypoexpression of pigment epithelium-derived factor (PEDF) and occludin in the retinas of STZ-induced rats. In addition, Liu's study [22] showed that different core bioactive ingredients in CXC had novel therapeutic uses in managing blood circulation. Panaxytriol and ginsenoside Rb1 were related to red blood cell aggregation, while angoroside C was involved in platelet aggregation. Protocatechualdehyde was related to intrinsic clotting activity, while calycosin-7-O-beta-D-glucoside was related to extrinsic clotting activity. In Sun et al.'s study [23], the systolic and diastolic velocity decreased while the resistance and pulsatility index increased in diabetic rat retinas. Furthermore, they also proved that the protective effects of DR were mediated by coagulation cascades and the peroxisome proliferator-activated receptor (PPAR) signaling pathway. Xing et al.'s study [6] showed that CXC mainly affected blood vessels by protecting high glucose-injured retinal vascular endothelial cells via YAP-mediated effects. However, the effect of HXMMT has seldom been evaluated in DR. Indeed, the only study was conducted by Long et al. [7] in rat models of branch retinal vein occlusion (BRVO), which indicated that HXMMT may alleviate retinal edema by regulating the expression of VEGF-α and improving

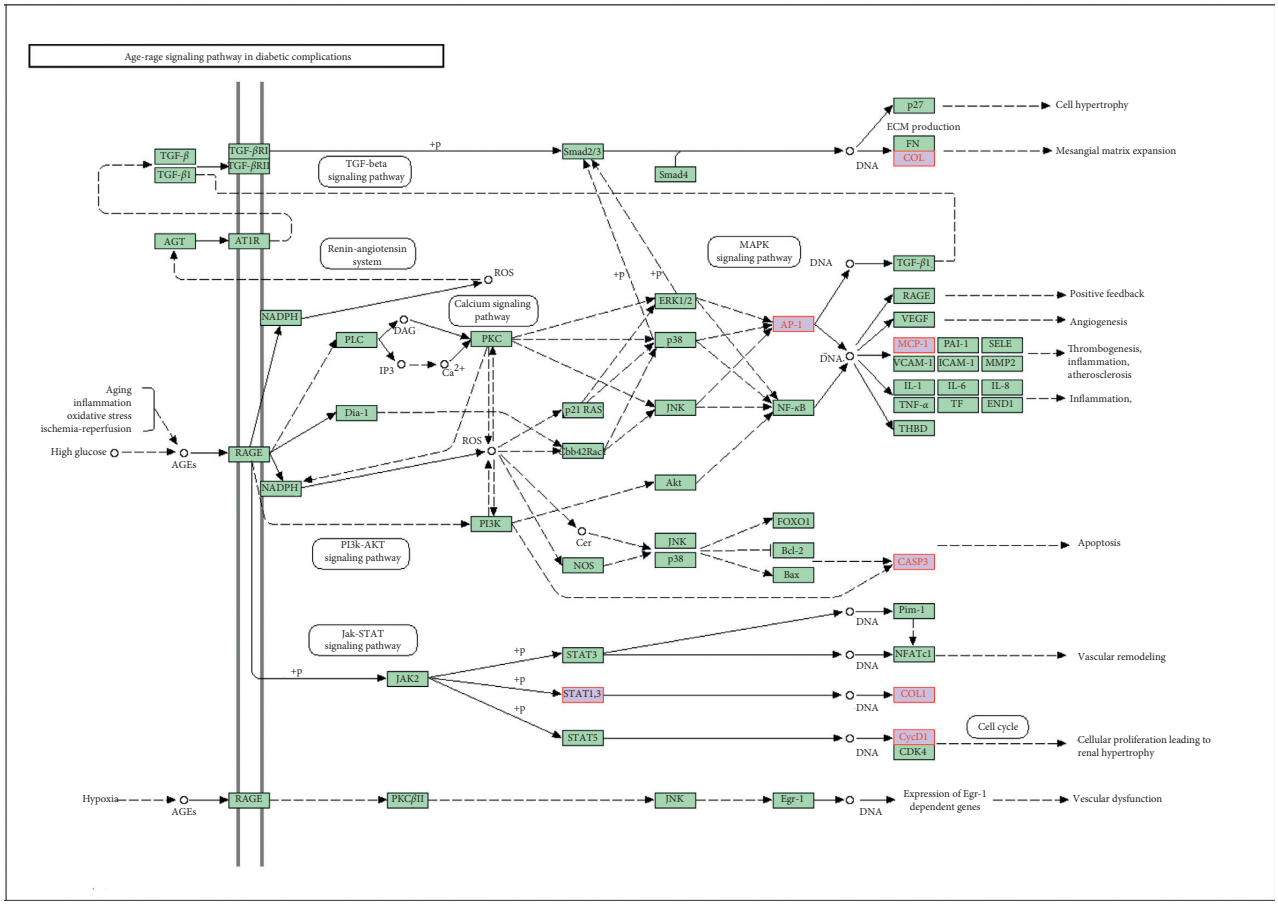


Figure 8: Most significant pathway and key genes (marked in red) among the proliferative diabetic retinopathy treatment targets of the compound Xueshuantong capsule and Hexuemingmu tablet.

microcirculation. Further studies should be performed to clarify the mechanism of HXMMT.

Utilizing a network pharmacology approach, Piao et al. [11] found that MMP9 and IGF-1 (an IGF family member contained in *Radix salviae*) may be key therapeutic targets in DR. Consistent with Piao's result, we found that MMP9 was included in both the CXC and HXMMT I-T networks. Matrix metalloproteinases (MMPs) play an important role in the migration, differentiation, and proliferation of cells [24]. Hyperglycemia may increase the activity of MMP9 and, therefore, provides growth space and nutrients for neovascularization by degrading the basement membrane and relaxing the cell structure [25]. A previous study [26] suggested that MMP9 was upregulated in the DM heart and that knockout of MMP9 in the DM was cardioprotective. Activation of MMPs (MMP-2 and MMP-9) in the retina is an early event in DR. Therefore, activated MMPs increased retinal capillary cell apoptosis and mitochondrial damage [27]. In addition, IGF-2, another IGF family member, was included in our networks but is not targeted by *Radix salviae*. IGF is expressed in many tissues, including the retina, where it is found in cells such as retinal endothelial cells and retinal pigment epithelial cells. IGF is a crucial regulator of cell differentiation and is closely related to blood-retinal barrier breakdown and retinal neovascularization [28, 29]. However, IGF-2 was related to 2 herbal components of CXC (*Huangqi and Sanqi*) and 9 herbal components of HXMMT (*Cheqianzi, Chishao, Huangqin, Mohanlian, Mudanpi, Muzei, Nvzhenzi, Puhuang, and Xiakucao*), implying that different active ingredients may share common therapeutic targets. Combined and stronger therapeutic effects may be exerted on a therapeutic target shared by a greater number of active ingredients. HXMMT contains more components than CXC; therefore, HXMMT may have more therapeutic targets. In addition, many of the genes targeted only by HXMMT but not by CXC were related to circulation and blood coagulation. For instance, CYCS was shown to be involved in blood platelet formation and regulatory processes [30, 31]. APOD, a crucial component of lipoproteins that transports lipids and stabilizes the structure of lipoproteins, was found to also be closely related to angiogenesis, a critical pathophysiological process in PDR [32]. PECAM1 was suggested to play an important role in the maintenance of human vascular endothelial barrier integrity and function [33]. Similarly, our topological analysis showed that some genes targeted only by HXMMT had many other functions. For example, YWHAB may perform specific functions in rod photoreceptors [34]. RACK1 may promote the expression of VEGF in endothelial cells and subsequently facilitate angiogenesis [35]. PARP1, activated by reactive

Table 2: Genes/pathways mentioned in the discussion and whether CXC/HXMMT regulates their functions.

Genes/pathways	Role in PDR	CXC regulates?	HXMMT regulates?
Bcl-2, Bcl-2-associated X, and caspase-3	Apoptosis and neuroprotective effects	Yes [14]	Unknown
VEGF, ICAM-1, ET-1, PEDF, and occludin	Erythrocyte aggregation, plasma viscosity, acellular vessel, and pericyte loss	Yes [20, 21]	Unknown
PPAR signaling pathway	Protective effects	Unknown	Unknown
YAP	Protecting retinal vascular endothelial cells	Yes [6]	Unknown
MMP9 and IGF-1	Regulating retinal capillary cell apoptosis/neovascularization	Unknown	Unknown
APOD	Neovascularization	Unknown	Unknown
PECAM1	Maintenance of human vascular endothelial barrier integrity	Unknown	Unknown
YWHAB	Performing specific functions in rod photoreceptors	Unknown	Unknown
RACK1	Neovascularization	Unknown	Unknown
PARP1	Inflammation, cell death, and retinal disease progression	Unknown	Unknown
AGE-RAGE signaling pathway	Neovascularization/neuroprotection	Unknown	Unknown
Circular RNA COL1A2/miR-29b/VEGF	Neovascularization	Unknown	Unknown
VEGF/PI3K-Akt signaling pathway	Neovascularization	Unknown	Unknown
TNF signaling pathway	Inflammation	Unknown	Unknown

PDR, proliferative diabetic retinopathy; CXC, compound Xueshuantong capsule; HXMMT, Hexuemingmu tablet.

oxygen species, was proven to be involved in inflammation, cell death, and retinal disease progression [36, 37]. In summary, its stronger effects at a given dose and more numerous gene targets may be two major reasons why HXMMT is more strongly recommended than CXC by TCM doctors for treating fresh VH secondary to PDR.

Similar to Li et al.'s research [38], the AGE-RAGE signaling pathway and TNF signal pathway were enriched in CXC in our study. HXMMT and CXC shared many pathways in our study, and the AGE-RAGE signaling pathway in diabetic complications was the most significantly enriched pathway. RAGE is expressed in almost all retinal cells. Retinal Müller cells, the major glial cells in the retina, play a critical role in maintaining the structure and normal functions of the retina, and these cells express high levels of RAGE [39]. In addition, Zong et al.'s study [40] demonstrated that RAGE plays an essential role in retinal neurodegeneration induced by diabetes and that early induction of RAGE expression by hyperglycemia in retinal Müller cells contributes to the increased levels of proinflammatory cytokines, including VEGF (a crucial downstream growth factor in angiogenesis) and monocyte chemoattractant protein-1 (MCP-1), both in vivo and in vitro. Moreover, Hirata et al. [41] found that increased production of VEGF secondary to retinal Müller cell activation may account for neovascularization in PDR. Therefore, the AGE-RAGE signaling pathway may not only provide neuroprotection in DR but also participate in crosstalk between neuroprotection and vascular protection. The difference in the enriched genes between CXC and HXMMT was that one additional gene (COL1A2) was included among the HXMMT targets. COL1A2 has seldom been studied in DR; Zou et al.'s research [42] is the only DR study involving COL1A2 to date. Zou revealed that silencing of circular RNA COL1A2 (circCOL1A2) suppresses angiogenesis during PDR progression by regulating the miR-29b/VEGF axis, suggesting that circCOL1A2 and its related genes may be therapeutic targets in DR.

According to the pathway map, the AGE-RAGE signaling pathway in diabetic complications is also closely associated with the PI3K-Akt signaling pathway and VEGF. The PI3K-Akt signaling pathway is one of the most frequently studied pathways in DR [43–45]. The proliferation, migration, and invasion of retinal vascular endothelial cells, retinal pericytes, retinal pigment epithelial cells, and microglial cells can be regulated through this pathway [46–49]. A series of pathophysiological processes, including oxidative stress regulation, inflammatory response regulation, angiogenesis, and neuroprotective regulation, are also involved. Another common and well-known pathway, the TNF signaling pathway, is closely related to inflammation, which is a crucial process in DR progression [50, 51]. Gao's study [51] revealed that hypoxia inducible factor subtype 1α in diabetic retina is likely to play a role in dysfunction and vulnerability related to DR progression via TNF-α.

The genes and pathways mentioned above and whether CXC/HXMMT regulates their functions are summarized in Table 2.

5. Conclusions

Both CXC and HXMMT include components effective in treating PDR and affect the following main processes: response to reactive oxygen species and oxidative stress, regulation of blood vessels, and blood coagulation. *Radix salviae*, the only herb common to both CPMs, contains many useful active ingredients. The PDR-CXC and PDR-HXMMT networks shared 34 common genes (RELA, HSPA8, HSP90AA, HSP90AB1, BRCA, EWSR1, CUL7, HNRNPU, MYC, CTNNB1, MDM2, YWHAZ, CDK2, AR, FN1, HUWE1, TP53, TUBB, EP300, GRB2, VCP, MCM2, EEF1A1, NTRK1, TRAF6, EGFR, PRKDC, SRC, HDAC5, APP, ESR1, AKT1, UBC, and COPS5), and the PDR-HXMMT network has 10 additional genes (RNF2, VNL, RPS27, COPS5, XPO1, PARP1, RACK1, YWHAB, and

ITGA4). The top 5 pathways with the highest gene ratio in both networks were the AGE-RAGE signaling pathway in diabetic complications, TNF signaling pathway, relaxin signaling pathway, IL-17 signaling pathway, and focal adhesion. Additional pathways, such as neuroactive ligand-receptor interaction, chemokine signaling pathway, and AMPK signaling pathway, were enriched with HXMMT targets. Thus, HXMMT has more therapeutic targets shared by different active ingredients and more abundant gene functions than CXC, which may be two major reasons why HXMMT is more strongly recommended than CXC as an auxiliary treatment for new-onset VH secondary to PDR. However, the underlying mechanisms need to be further elucidated.

Abbreviations

CXC: Compound Xueshuantong capsule
HXMMT: Hexuemingmu tablet
CPM: Chinese patent medicines
PDR: Proliferative diabetic retinopathy
VH: Vitreous hemorrhage
GO: Gene ontology
KEGG: Kyoto encyclopedia of genes and genomes
DR: Diabetic retinopathy
DM: Diabetes mellitus
mRNA: Messenger RNA
DEmRNA: Differentially expressed messenger RNA
I-T: Ingredient-target
PPI: Protein-protein interaction
KEGG-T: Kyoto encyclopedia of genes and genomes-target
GEO: Gene expression omnibus
TCMSP: Traditional Chinese medicine system pharmacology
OB: Oral bioavailability
DL: Drug-likeness
KOBAS: KEGG orthology based annotation system
BP: Biological process
CC: Cellular component
MF: Molecular function
STZ: Streptozotocin
VEGF: Vascular endothelial growth factor
MCP-1: Monocyte chemoattractant protein-1.

Data Availability

No data were used to support this study.

Disclosure

An earlier version of this manuscript has been presented as a preprint following this link: https://www.researchsquare.com/article/rs-55929/v1.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Hongyan Yao and Zijing Li drafted the manuscript and analyzed the data. Danli Xin was responsible for data collection and image editing. Zongyi Zhan collected the data. Zijing Li was responsible for manuscript design.

Acknowledgments

This research was supported by the Natural Science Foundation of Ningbo, China (2015A610311), Ningbo Public Welfare Program (2019C50045), and Clinical Pharmacology Research Foundation of Guangdong Province (2019XQ09).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6688450, 11 pages
https://doi.org/10.1155/2021/6688450

Review Article

Probiotics as a Complementary Therapy for Management of Obesity: A Systematic Review

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Received 21 October 2020; Revised 8 December 2020; Accepted 13 January 2021; Published 23 January 2021

Academic Editor: Vincenzo De Feo

Background. Considering the observed role of probiotics in modulating gut microbiome, probiotics are discussed to be one potential complementary therapy for obesity management in recent years. The aim of the present study was to systematically review the meta-analyses of controlled trials and investigate the effects of probiotics on obesity. *Methods*. A comprehensive search was conducted on PubMed, Web of Science, and Cochrane Library web databases up to May 2020. Inclusion criteria were meta-analyses of controlled trials which evaluated the impact of probiotics on obesity in English language. Meta-analyses done on pregnant women, children, animal studies, or the effect of prebiotics on anthropometric indices were excluded. *Results*. Within 325 recorded studies, 20 studies met the inclusion criteria consisting of 16676 overweight/obese adults with different underlying disorders such as nonalcoholic fatty liver disease (NAFLD), or polycystic ovary syndrome (PCOS). The length of intervention varied from 2 to 26 weeks. Results of meta-analyses have shown a moderate effect of probiotics on body weight in overweight/obese adults: from −0.526 kg/m^2 (95% CI: −0.810, −0.247) to −0.25 kg/m^2 (95% CI: −0.33, −0.17). Body mass index (BMI) was changed from −1.46 kg/m^2 (95% CI: −2.44, −0.48) to −1.08 kg/m^2 (95% CI: −2.05, −0.11) in NAFLD. Probiotics could reduce BMI from −0.36 kg/m^2 (95% CI: −0.74, 0.02) to −0.29 kg/m^2 (95% CI: −0.54, −0.03) in patients with PCOS. *Conclusion*. It seems that the probiotic products could have beneficial effects as an adjunct therapy for care and management of obesity when used in high dose. However, due to heterogeneity of included studies, it is required to confirm our results by more meta-analyses of clinical trials.

1. Introduction

During recent decades, prevalence of overweight and obesity, which are a consequence of more energy receiving and less energy consumption, considerably increased among different age groups [1–3]. Obesity is explained as a body mass index (BMI) of 30 kg/m^2 or more while overweight is defined as a BMI between 25 and 30 kg/m^2 [4]. According to the World Health Organization (WHO), 1.9 billion individuals aged ≥18 years old were overweight (39%) worldwide, 650 million of which (13%) were obese in 2016. Considering that prevalence rate of obesity increased to three times since 1975 [4], obesity is changing to a major global health concern with high burden on healthcare systems [5–7]. Both genetic and environmental factors such as sedentary lifestyle, urbanization, and easy access to high-energy foods are considered as reasons for the rapid rise in the prevalence rate of obesity and overweight [5, 8–10].

Changing lifestyle and personal behaviors are believed as best ways of treating obesity for a long time [3]. New studies which found evidences for concurrent dysbiosis of microbiota and obesity prevalence suggested the possible association between obesity and microbiome [1, 2, 11]. It means the gut microbiome is involved in regulation of energy intake and expenditure.

The underlying mechanisms by which the gut microbiota can contribute to the weight management include many pathways; for instance, saccharolytic gut microbes are able to generate short-chain fatty acids (SCFAs) by fermentation of indigestible polysaccharides. The gut microbiota-derived SCFAs have critical roles in decreasing oxidative stress and inflammation as well as regulating energy consumption. Moreover, SCFAs are responsible for enhancement of satiation through reducing gut motility and stimulating secretion of glucagon-like peptide 1 (GLP1) and peptide YY (PYY) [2, 8, 12].

So, microbial intervention like probiotic foods and supplements could be a novel approach for controlling obesity [11–13]. Probiotics are defined as live microorganisms that are supposed to have positive effects when consumed in acceptable and enough quantities [3, 6]. Using probiotics is suggested as a potential new approach but still doubted for reforming dysbiosis of gut microbiota composition to control obesity through improvement of BMI, body weight (BW), waist circumference (WC), or body fat mass (BFM) [2, 6, 12]. Results of previous studies regarding impact of probiotics on obesity showed contradictions; for instance, some studies did not show any obvious change in anthropometric indices [6, 7, 14, 15]; in contrast, some other studies reported significant changes [1–3, 5, 8, 11–13, 16–22]. These paradoxical and varying results were a motivation for us to look over related studies to answer accurately whether probiotics have beneficial effects on obesity or not. Therefore, the aim of this systematic review study is to gather and review results of meta-analysis studies investigating the effect of probiotics consumption on obesity.

2. Methods

2.1. Search Strategy. PubMed, Web of Science, and Cochrane Library web databases were comprehensively searched for meta-analysis studies evaluating impact of probiotics on obesity recorded up to May 2020. The search terms were "probiotic," "probiotics," "*Lactobacillus*," "*Bifidobacterium*," "obese," "overweight," "obesity," "body weight," "adiposity," "fat mass," "weight," "BMI," "waist circumference," "body mass index," and "meta-analysis". All of the articles were inspected and duplicate ones were removed manually operated by two independent researchers.

2.1.1. Eligibility Criteria and Study Selection. At first, all documents were checked over for titles and abstracts by two researchers independently followed by reviewing the full-text articles based on inclusion and exclusion criteria. The inclusion criterion was meta-analyses performed on controlled trials investigating the impact of various probiotic products on anthropometric indices. The probiotic products consisted of probiotic foods or probiotic supplements. Moreover, probiotic foods are defined as fermented foods that naturally contain probiotics, or have probiotics added to them. The exclusion criteria were (1) meta-analyses that were done on pregnant women, children, or animals and (2) meta-analyses on the effect of prebiotics on anthropometric indices. English language was considered as search limitation.

Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guideline was used for performing this study [23].

2.2. Data Extraction. Data were extracted from the articles including authors and publication year, number and type of included studies in the meta-analysis, participants' characteristics (sample size, age, sex, and underlying disorder), type and dose of intervention, duration of interventions, main outcomes, and reported side effects.

2.3. Quality Assessment. The critical appraisal tool entitled Assessment of Multiple Systematic Reviews (AMSTAR) was used for quality assessment of the included studies [24]. The quality of studies was defined as high quality for scores 8–11, average quality for scores 4–7, and poor quality for scores ≤3. All of the mentioned steps including search, study selection, data extraction, and quality assessment were done by two researchers independently and any disagreement was resolved by discussing until reaching a consensus.

3. Results and Discussion

3.1. Results. After removing duplicate articles (101) from primary recorded studies (325), 224 articles remained to assess their title/abstract and full text. Finally, 20 articles were included in this systematic review (Figure 1). Characteristics of the included meta-analyses are presented in Table 1. All included studies were meta-analysis of randomized controlled trials (RCTs) conducted on both genders of adults for 2–26 weeks [1–3, 5–8, 11–22, 25]. Total sample size was 16676 overweight/obese subjects with different underlying disorders such as nonalcoholic fatty liver disease (NAFLD) or polycystic ovary syndrome (PCOS). Various formats of probiotic products such as fermented foods, fermented dairy products, or supplements including capsules, powder, and sachets which contained single or multiple diverse species were utilized.

Totally, in 12 articles carried on obese or overweight adults from both genders, participants were treated with probiotic capsules, probiotic powder, yogurt, fermented milk, or dough containing various species such as *Lactobacillus, Propionibacterium, Bifidobacterium*, and *Acetobacter* [1–3, 5–8, 11–13, 17, 22]. In most of the studies [1–3, 5, 8, 11–13, 17, 22], significant changes in anthropometric indices were shown. The greatest and the least observed changes in BMI were $-0.526\,kg/m^2$ (95% CI: −0.810, −0.247) and $-0.25\,kg/m^2$ (95% CI: −0.33, −0.17) through

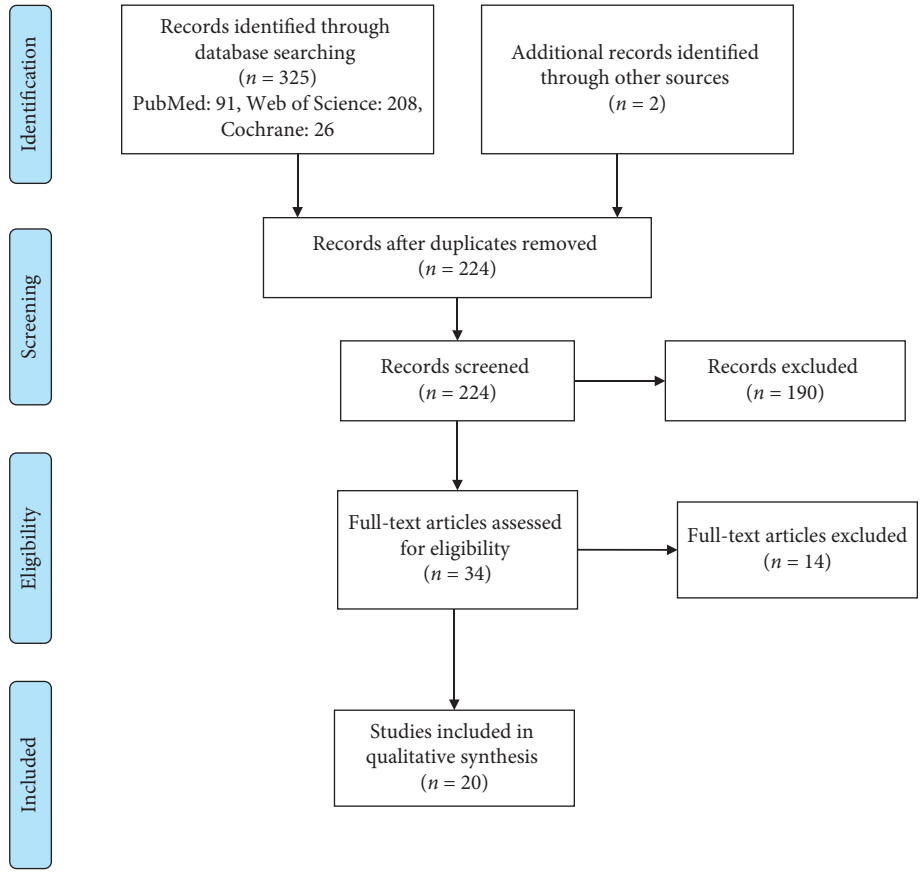

Figure 1: Flow diagram of the study selection process.

3–12 weeks of intervention, respectively [1, 22]. BW was another index which showed significant reduction through probiotic supplementation; regarding that, the greatest reduction was −0.65 kg (95% CI: −1.12, −0.18) and the least reduction was −0.26 kg (95% CI: −0.43, −0.09) during 4–24 weeks [8, 17]. Moreover, other anthropometric indices, including BFM and WC, were reduced in a range from −0.94 kg (95% CI: −1.17, −0.72) to −0.30 kg (95% CI: −0.48, −0.12) [8, 17] and from −2.11 cm (95%CI: −3.543, −0.677) to −0.35 cm (95% CI: −0.81, 0.11), respectively [13, 22].

Six studies out of 20 total included studies were accomplished on both genders of obese adult patients with nonalcoholic fatty liver disease using diverse probiotic products as intervention constituted by various species like *Streptococcus cerevisiae*, *Streptococcus thermophilus*, and *Lactobacillus* spp. [14, 15, 18, 19, 21, 25]. Three of these studies showed no significant change in anthropometric indices [14, 15, 25], contrasting to others [18, 19, 21]. Among the studies displaying beneficial effects on BMI, two of them showed better results when *Bifidobacterium* and *Lactobacillus* species were administered (−1.46 kg/m^2 (95% CI: −2.44, −0.48) and −1.08 kg/m^2 (95% CI: −2.05, −0.11)) [18, 19].

Two meta-analyses among 20 included studies (16, 20) reported effects of 8–12 weeks intervention by probiotic on adult obese women with PCOS. In the study by Hadi et al. [16], probiotic supplementation was observed to be more effective in reducing BW for participants >30 years old but in contrast no special difference was seen in subgroup analyses for age in Tabrizi et al.'s study [20].

Hadi et al. [16] and Tabrizi et al. [20] assessed the effect of probiotics contained in capsules. In both studies, supplements contained different species of *Lactobacillus* and *Bifidobacterium* genera such as *L. acidophilus, L. casei, L. rhamnosus, L. reuteri,* and *B. bifidum*. Both meta-analyses reported change in BMI and BW. BMI was reduced from −0.36 kg/m^2 (95% CI: −0.74, 0.02) to −0.29 kg/m^2 (95% CI: −0.54, −0.03) and also BW showed reduction varying from −1.3 kg (95% CI: −1.93, −0.13) to −0.30 kg (95% CI: −0.53, −0.07) [16, 20].

The effects of probiotics dosage and single/multi-strains were assessed in subgroup analyses of Wang et al.'s [2], John et al.'s [8], and Koutnikova et al.'s [5] studies. Wang et al. found a significant reduction in BW with high dose of probiotics (−0.58 kg, 95% CI: −0.92, −0.23), and a single strain of probiotics (−0.49 kg, 95% CI: −0.92, −0.07). Similar effects were reported for BMI when the high dose was used (−0.29 kg/m^2, 95% CI: −0.46, −0.12) and single strain of probiotics (−0.36 kg/m^2, 95% CI: −0.52, −0.20) [2]. In John et al.'s [8] study, a greater significant reduction in BMI was shown with high dose of probiotics (−0.43 kg/m^2, 95% CI: −0.56, −0.30) compared to low dose of probiotics. When stratified by single vs. multi-strain probiotic supplementation, a significant decrease in BMI (−0.41 kg/m^2, 95% CI:

Table 1: Included meta-analysis studies investigating the effect of probiotics on obesity. Probiotic foods consisted of fermented foods that naturally contain probiotics or foods which have probiotics added to them.

Authors/ published year	Included studies in meta-analysis (*n*)	Type of included studies in meta-analysis	Total sample size (*n*)	Participants' characteristics	Type and dose of intervention	Duration of interventions (weeks)	Main outcomes	Risk of bias assessment	Reported side effects	AMSTAR score
Hadi et al., 2020	3 (all in Iran)	RCTs	180 (intervention: UN, control: UN)	Women with PCOS ≥18 y	Different species of probiotic supplements (10^9 CFU/day)	12	−Sig. ↓weight: −1.3 kg (95% CI: −1.93, −0.13) −Sig. ↓BMI; −0.36 kg/m^2 (95% CI: −0.74, 0.02)	Yes	No side effects	7
Company et al., 2020	17 (11 in Asian countries, 6 in European countries, 1 in Brazil)	DBRCTs	1,106 (intervention: 486, placebo: 62)	Cardio-metabolic disease subjects with obesity, adults ≥18 y, both sexes	Multiple and single species of probiotic supplements within powder and capsule (10^8–10^{11} CFU/day)	4–12	−sig. ↓BW: −0.26 kg (95% CI: −0.43, −0.09) −sig. ↓BMI: −0.35 kg/m^2 (95% CI: −0.48, −0.22) −sig. ↓WC: −0.37 cm (95% CI: −0.52, −0.21) −sig. ↓BFM: −0.30 kg (95% CI: −0.48, −0.12)	Yes	No side effects	11
Cao et al., 2020	31 (22 in Asian countries, 6 in European countries, 3 in Brazil)	RCTs	2051 (intervention: UN, control: UN)	Obese and overweight adults ≥18 y, both sexes	Probiotic foods and supplements and dairy product (10^8–10^{11} CFU/day)	3–12	−sig. ↓BMI: −0.25 kg/m^2 (95% CI: −0.33, −0.17) −sig. ↓WC: −0.99 cm (95% CI: −1.33, −0.66)	Yes	No side effects	9
Xiao M et al., 2019	12 (9 in Asian countries, 3 in European countries)	DBRCTs	693 (intervention: UN, control: UN)	Adults with nonalcoholic fatty liver disease, both sexes	Different species of probiotic supplements (UN amounts)	8–24	−sig. ↓BMI: −1.46 kg/m^2 (95% CI: −2.44, −0.48)	Yes	No serious side effects	10

Table 1: Continued.

Authors/ published year	Included studies in meta-analysis (*n*)	Type of included studies in meta-analysis	Total sample size (*n*)	Participants' characteristics	Type and dose of intervention	Duration of interventions (weeks)	Main outcomes	Risk of bias assessment	Reported side effects	AMSTAR score
Wang et al., 2019	12 (9 in Asian countries, 2 in European countries, 1 in Brazil)	DBRCTs, SBRCTs	821 (intervention: 405, control: 416)	Adults with BMI ≥25 and ≥18 y, both sexes	Multiple and single species of probiotic supplements (10^7–10^{11} CFU/day)	8–26	−sig. ↓BW: −0.55 kg (95% CI: −0.91, −0.19) −sig. ↓BMI: −0.30 kg/m^2 (95% CI: −0.43, −0.18) −sig. ↓WC: −1.20 cm (95% CI: −2.21, −0.19) −sig. ↓BFM: −0.91 kg (95% CI: −1.19, −0.63)	Yes	No side effects	9
Tang et al., 2019	12 (7 in Asian countries, 4 in European countries, 1 in Brazil)	DBRCTs	805 (intervention: 407, control 398)	Adults with nonalcoholic fatty liver disease, both sexes	Multiple and single species of probiotic supplements (varied amounts)	8–24	−sig. ↓BW: −2.31 kg (95% CI: −4.45, −0.16) sig. ↓BMI: −1.08 kg/m^2 (95% CI: −2.05, −0.11)	Yes	No side effects	10
Tabrizi et al., 2019	7 (all in Iran)	RCTs	415 (intervention: 212, control: 213)	Women with PCOS ≥18 y	Probiotic capsule supplements (10^8–10^{10} CFU/day)	8–12	−sig. ↓BW: −0.30 kg (95% CI: −0.53, −0.07) −sig. ↓BMI: −0.29 kg/m^2 (95% CI: −0.54, −0.03)	Yes	No side effects	9
Liu et al., 2019	4 (2 in Iran, 2 in European countries)	RCTs	218 (intervention: 110, control: 108)	Adults with nonalcoholic fatty liver disease ≥18 y, both sexes	Different species of probiotic supplement (varied amounts)	8–12	No significant effect on BMI or WC	Yes	Rare and mild reverse reactions	9

Table 1: Continued.

Authors/ published year	Included studies in meta-analysis (*n*)	Type of included studies in meta-analysis	Total sample size (*n*)	Participants' characteristics	Type and dose of intervention	Duration of interventions (weeks)	Main outcomes	Risk of bias assessment	Reported side effects	AMSTAR score
Koutinkova et al., 2019	68 (26 in Iran)	RCTs, COTs	4015 (intervention: UN, control: UN)	Overweight/ obese/normal weight adults, both sexes	Multiple and single species of probiotic products (varied amounts)	2–28	−sig. ↓BW: −0.39 kg (95% CI: −0.57, −0.21) −sig. BMI: −0.33 kg/m^2 (95% CI: −0.53, −0.12) −sig. WC: −1.01 cm (95% CI: −1.55, −0.48) −sig. BFM: −0.62 kg (95% CI: −0.91, −0.34)	Yes	No side effects	11
Dong et al., 2019	6 (UN countries)	DBRCTs	476 (intervention: UN, control: UN)	Adults with BMI ≥25 and ≥18 y, both sexes	Probiotic capsules and probiotic dairy products (10^6–10^{11} CFU/day)	8–24	No significant effect on BMI or BFM −sig. ↓WC: −0.35 cm (95% CI: −0.81, 0.11)	Yes	No side effects	11
John et al., 2018	18 (15 in Asian countries, 1 in Finland, 1 in Brazil, 1 in Canada)	RCTs	803 (intervention: 443, control: 360	Adults ≥18 y with BMI ≥25, both sexes	Multiple and single species of probiotic food or supplements (10^7–10^{11} CFU/day)	4–24	−sig. ↓BW: −0.65 kg (95% CI: −1.12, −0.18) −sig. ↓BMI: −0.33 kg/m^2 (95% CI: −0.47, −0.18) −sig. ↓BFM: −0.94 kg (95% CI: −1.17, −0.72)	Yes	No side effects	10

Table 1: Continued.

Authors/ published year	Included studies in meta-analysis (*n*)	Type of included studies in meta-analysis	Total sample size (*n*)	Participants' characteristics	Type and dose of intervention	Duration of interventions (weeks)	Main outcomes	Risk of bias assessment	Reported side effects	AMSTAR score
Borgeraas et al., 2018	13 (10 in Asian countries, 1 in European countries, 2 in the US)	DBRCTs, SBRCTs	737 (intervention: 369, control: 368)	Overweight or obese adults ≥18 y	Probiotic dairy products and supplements (10^9–10^{11} CFU/day)	3–12	−sig. ↓BW: −0.6 kg (95% CI: −1.19, −0.01) −sig. ↓BMI: −0.27 kg/m^2 (95% CI: −0.45, −0.08)	Yes	No side effects	11
Dror et al., 2017	15 (7 in Asian countries, 4 in European countries, 2 in Canada, 2 in the US)	RCTs	921 (intervention: 468, control: 453)	Normal or obese or overweight adults ≥18 y	Different species of probiotic dairy and supplements (10^7–10^8 CFU/day)	8–12	−sig. ↓BW: −0.43 kg (95% CI: −0.67, −0.2) −sig. ↓BMI: −0.43 kg/m^2 (95% CI: −0.54, −0.33)	Yes	No side effects	9
Lavekar et al., 2017	3 (2 in European countries, 1 in Asian countries)	DBRCTs	157 (intervention: 79, control: 78)	Adults with nonalcoholic fatty liver disease	Multiple and single probiotic species (varied amounts)	12–24	−sig. ↓BMI: −1.45 kg/m^2 (95% CI: −3.06, 0.16)	Yes	No serious side effects	6
Zhang et al., 2016	25 (UN countries)	DBRCTs, RCTs	1931 (intervention: 977, control: 954)	Adults ≥18 y, both sexes	Multiple and single species of probiotic supplements (varied amounts)	3–24	−sig. ↓BW: −0.59 kg (95% CI: −0.87, −0.30) −sig. ↓BMI: −0.49 kg/m^2 (95% CI: −0.74, −0.24)	Yes	No side effects	10
Gao et al., 2016	6 (4 in Asian countries, 2 in European countries)	RCTs	205 (intervention: 115, control: 90)	Adults with nonalcoholic fatty liver disease, both sexes	Multiple and single species of probiotic sources (NA)	12–24	No significant effect on BMI	Yes	Few side effects	10
Sun et al., 2015	5 (1 in Korea, 1 in Japan, 1 in Iran)	DBRCTs	NA	Obese adults ≥18 y, both sexes	Different species of probiotic capsules and probiotic fermented milk (10^7–10^{10} CFU/day)	8–12	−sig. ↓BMI: −0.526 kg/m^2 (95% CI: −0.810, −0.247) −sig ↓WC: −2.11 cm (95% CI: −3.543, −0.677)	Yes	No side effects	11

Table 1: Continued.

Authors/ published year	Included studies in meta-analysis (*n*)	Type of included studies in meta-analysis	Total sample size (*n*)	Participants' characteristics	Type and dose of intervention	Duration of interventions (weeks)	Main outcomes	Risk of bias assessment	Reported side effects	AMSTAR score
Park et al., 2015	9 (NA)	RCTs	689 (intervention: UN, control: UN)	Obese and overweight adults ≥18 y, both sexes	Probiotic supplements and dairy products (10^6–10^{10} CFU/day)	3–24	No significant effect on BW or BMI	Yes	No serious side effects	9
Ma et al., 2013	4 (1 in Hong Kong, 1 in Spain, 1 in Italy, 1 in the US)	DBRCTs	134 (intervention: 68, control: 66)	Nonalcoholic patients, both sexes	Multiple and single species of probiotic sources (NA amounts)	8–24	No significant effect on BMI	Yes	No side effects	8
Million et al., 2012	5 (UN countries)	DBRCTs	319 (intervention: 257, control: 162)	Lean and obese adults ≥18 y, both sexes	Multiple and single species of probiotic sources (10^7–10^{10} CFU/day)	4–24	No significant effect on BW	Yes	Weight gain in usage of *L. acidophilus*, *L. delbrueckii*, *L. plantarum*	10

BMI: body mass index; WC: waist circumference; BFM: body fat mass; BW: body weight; RCTs: randomized controlled trials; DBRCTs: double blind randomized controlled trials; SBRCTs: single blind randomized controlled trials; COTs: cohort study; PCOS: polycystic ovary syndrome; CFU: colony forming units; UN: unknown; NA: not available; CI: confidence interval.

−0.56, −0.27), BW (−0.77 kg, 95% CI: −1.52, −0.03), and fat mass (−0.95 kg, 95% CI: −1.19, −0.71) was observed by single strain. In subgroup analysis in Koutnikova et al.'s [5] study, a significant effect on BW and BMI when three or more bacterial species were used was observed.

No serious side effect has been reported in the included studies. Just in Million et al.'s study [7], weight gain has been reported after supplementation with some species of *L. acidophilus*, *L. delbrueckii*, and *L. plantarum*. Regarding the quality of included meta-analyses studies, most of the studies had AMSTAR score ≥8 (90%, 18 of 20 articles) which was interpreted as high quality [1–3, 5–8, 11–15, 17–20, 22, 25] and two other studies were classified as medium quality [16, 21].

3.2. Discussion. This systematic review of meta-analyses was aimed at uncovering the effects of probiotics usage on obesity/overweight indices including BW, BMI, BFM, and WC. Results varied from a no significant change to a significant decrease during 2–26 weeks' interventions. Regarding the observed improvement of anthropometric indices, from 20 meta-analyses included in the current study, 10 studies disclosed significant change in BW [2, 3, 5, 8, 11, 12, 16, 17, 19, 20], 14 studies showed significant change in BMI [1–3, 5, 8, 11, 12, 16–22], 6 studies reported change in WC [1, 2, 5, 13, 17, 22], and 4 studies disclosed change in BFM [2, 5, 8, 17]. In the study by Koutinkova et al., an improvement in anthropometric indices was reported when ≥3 species were used [5]. In contrast, John et al. [8] and Wang et al. [2] represented that probiotics containing only single bacterial strain had a considerable decreasing effect on BMI and BW. Moreover, in John et al.'s study [8], it was implicated that even lower dose of interventions can induce a considerable reduction in BMI and BW when interventions continued for more than 12 weeks [8].

Obesity as one of the main health problems predisposes people to cardio-metabolic disorders like type 2 diabetes mellitus and cardiovascular diseases [3]. Probiotic products can affect obesity via some proposed underlying mechanisms [3]. Modulation of gut microbiota composition and function has been suggested as one of these mechanisms. Dysbiosis is common in most obese individuals which can be reformed through probiotic consumption [16]. Moreover, probiotics are able to ameliorate inflammation by inducing secretion of SCFAs and concurrently decreasing number of bacteria producing lipopolysaccharides. SCFAs may lead to regulating energy hemostasis via stimulation of enterocyte receptors and secretion of glucagon-like peptide 1 (GLP1) and peptide YY (PYY) [1, 2, 16]. Furthermore, probiotics stimulate release of glucagon-like peptide 2 (GLP2) which leads to higher expression of tight junction proteins, better gut barrier function, and ultimately more effective inflammation control [26]. Following suppression of inflammation, insulin resistance is also improved in obese individuals [20]. In a meta-analysis study by Companys et al. [17], probiotic supplements together with dairy products generally showed reduction in different anthropometric indices. However, when *L. acidophilus, L. gasseri* SBT2055, and *B. lactis* BB12 have been added to the combination, with intervention for more than 12 weeks at the dosage level of 10^7 to 10^{11} (CFU/day), a bigger reduction in BW, BFM, and BMI was observed. These reductions were even reported in greater amount when participants had low calorie diet synergistically. Zhang et al. reported the greater effects for probiotics supplementation with duration of ≥8 weeks which contained more than 1 species regardless of type of species [3]. In the study of Park S et al., no change in anthropometric parameters was observed via any kind of probiotics therapy but exceptionally when fermented milk including *Lactobacillus* spp. with dosage of 10^8 to 10^9 CFU was utilized, it brought significant changes afterwards [6]. There are controversies in results of meta-analyses investigating probiotics impact on anthropometric indices. Most of meta-analyses showed significant desired changes in BW, BMI, and WC [2, 3, 5, 8, 11–13, 17, 22] and some others did not report any change [6, 7]. These controversies could have originated from differences in duration of interventions, the probiotic dosage, or characteristics of participants. Moreover, the probiotic carriers can play an important role in their efficacy and the effect of probiotics is species- and strain-specific [26]. Since most of the studies used a mixture of different probiotic species, we cannot identify the species with the most considerable effects. The observed effects in studies may be caused by synergistic function of different species used together in probiotics. So, further studies are needed to determine the most effective strains of probiotics with anti-obesity properties.

The safety of probiotic products is a matter of concern especially for infants, people suffering from cancer, critically ill, and immunocompromised patients [27]. The principal reported adverse effects ranged from mild such as gastrointestinal side effects and skin complications to severe including inflammation of endocardium and systemic infections [27]. Therefore, because of mostly unknown probable deleterious effects, it is better to do more research before general recommendation of probiotics [28].

This study has some strengths and limitations. The main strength of this systematic review was comprehensive evaluation of the meta-analyses of high qualified RCTs concerning the anti-obesity effect of probiotic products. Somehow, as a major limitation of the present study, the use of different mixture of probiotic strains made it difficult to draw exact conclusion about each probiotic strain.

4. Conclusion

In conclusion, it seems that using various high-dose probiotic products or supplements could improve overweight/obese indices in participants with different underlying disorders. However, since these products may not be without side effects for all groups, the risk-benefit assessment should be done prior to their prescription.

Data Availability

All data analyzed in this work are supported by the published articles in PubMed, Web of Science, and Cochrane Library web databases, and all data generated are included in this published article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Salman Shirvani-Rad and Ozra Tabatabaei-Malazy equally contributed as first authors. HSE and OTM conceived and coordinated the study. HSE and SSR participated in the design of the study. SSR, OTM, SM, and HSE extracted information from the article and drafted the manuscript. ZHT helped to write and edit the final draft of the manuscript. SHR, ARS, and BL critically reviewed the manuscript and helped in quality assessment. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by Endocrinology and Metabolism Clinical Sciences Research Institute, Tehran University of Medical Sciences, Tehran, Iran.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6666832, 12 pages
https://doi.org/10.1155/2021/6666832

Review Article

The Effects of Oral Consumption of Honey on Key Metabolic Profiles in Adult Patients with Type 2 Diabetes Mellitus and Nondiabetic Individuals: A Systematic Review of Clinical Trials

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Received 6 November 2020; Revised 27 December 2020; Accepted 7 January 2021; Published 23 January 2021

Academic Editor: Mohammad Hashem Hashempur

Objectives. Although several clinical trials have revealed the beneficial effects of honey on metabolic profiles, the results are conflicting. The aim of this study was to systematically summarize the effects of oral consumption of honey on key metabolic profiles in adult patients with type 2 diabetes mellitus (T2DM) and nondiabetic individuals. *Methods*. In total, four electronic databases, including PubMed/Medline, Web of Science, Scopus, and Cochrane library, were searched from 2000 to 31 July 2019 to identify all English language studies that would meet the eligibility criteria. Clinical trials which have examined the effects of oral consumption of any types of honey on anthropometric indices, glycemic status, lipid profiles, and blood pressure in both diabetic and nondiabetic adult subjects were included in the study. *Results*. Of the 7769 possible relevant studies (including 3547 duplicates) identified in the initial search, finally, 13 clinical trials were included in the systematic review. All studies except three had a parallel design. Of 13 studies, 8 trials did not have placebo/control groups. The included studies examined the impact of oral consumption of honey on glycemic status ($n = 12$), anthropometric indices ($n = 6$), lipid profiles ($n = 10$), and blood pressure ($n = 3$). Based on the Jadad scale, 5 studies had acceptable methodological quality, and the remaining ($n = 8$) had low methodological quality. *Conclusion*. The current systematic review showed that oral consumption of honey might have no significant effects on the modulation of metabolic profiles in nondiabetic subjects. In addition, a high intake of honey might increase glucose levels and worsen other metabolic parameters in patients with T2DM. Due to substantial heterogeneity in study design and limited clinical trials, results, however, should be interpreted with great caution.

1. Introduction

According to the report published by the World Health Organization (WHO), cardiovascular diseases (CVDs) are the leading cause of death worldwide, leading to about 17.9 million deaths per year [1]. Obesity, diabetes, and dyslipidemia play the pivotal roles in the incidence of CVDs [2, 3]. For the management of patients at high risk of CVDs, there

are several strategies including taking medicines, lifestyle modifications, adherence to healthy diets [4], and consumption of functional foods [5, 6].

Functional foods can be considered as one of the useful modifiers of CVD risk factors [7]. Such types of food can either improve health status or reduce the risk of various diseases apart from providing nutritional requirements [8]. Leafy greens, berries, soy, fatty fish [9], and honey [10] are examples of functional foods.

Honey, a natural sweetener, is widely available across the world [11]. More than 300 different types of floral honey are available in the world's market places. The appearance, sensory characteristics, and the amount of biochemical components, including glucose to fructose ratio, mineral, and vitamin content of honey, vary based on botanical origin [12]. Honey is a high-carbohydrate food containing monosaccharide (glucose and fructose) and disaccharides [12, 13]. Therefore, its effects on glycemic parameters are exceedingly important, especially for patients with diabetes mellitus (DM) and those suffering from glucose intolerance. Due to differences in physicochemical properties of honey collected from various botanical sources, the glycemic index (GI) of honey varies between 32 and 85 [12].

Honey is beyond a carbohydrate source; it contains numerous components with antioxidant, anti-inflammatory, and antimicrobial characteristics including polyphenols, flavonoids, enzymes, vitamins, and trace elements [12, 14]. In traditional medicine such as traditional Persian medicine (TPM), honey has been used as a complementary therapy for wide ranges of diseases [15] such as liver and vascular diseases. In TPM, honey is called "Angabin" and "Shahd." The type with no wax and transparent red color that is tasty and fragrance has been introduced as the best one [16, 17]. In the conventional medicine, honey has also been used for the treatment of gastrointestinal diseases, healing of ulcers (diabetic ulcers and bedsores), skin diseases, respiratory disorders, and urinary system diseases [15, 18, 19].

Several clinical trials have revealed the beneficial effects of honey on metabolic profiles, including lipid profiles, glycemic status, anthropometric indices, and inflammatory parameters [20–23]. However, the results are conflicting. To the best of our knowledge, no systematic review has been conducted to summarize the effects of oral consumption of honey on metabolic profiles. Accordingly, the primary objective of the present study was to examine the effects of honey on glycemic status, lipid profiles, anthropometric indices, and blood pressure in adult individuals, and the secondary objective was to compare its impacts on patients with type 2 diabetes (T2DM) and nondiabetic individuals and present findings quantitatively, if possible.

2. Methods

The current systematic review was designed based on the Preferred Reporting Items of Systematic Reviews and Meta-Analysis (PRISMA) statement guideline [24].

To identify relevant studies conducted on the effects of honey on metabolic parameters in adult patients with T2DM and nondiabetic individuals, the four electronic databases including PubMed/Medline, Web of Science, Scopus, and Cochrane library were searched from 2000 to July 2019 using both MeSH and non-MeSH terms. Notably, the search was restricted to the English language studies.

As possible relevant studies were exported into Endnote Software (version X8), two independent investigators (M.A and N.N) screened all studies based on their titles and abstracts, and those that were found to be potentially relevant were transferred to the next step in which studies were assessed based on their full-text considering eligibility criteria. Besides, to avoid missing any related studies, the reference lists of all eligible studies were hand-searched.

2.1. Eligibility Criteria. The PICO framework (P, patients/participants; I, intervention; C, comparison; O, outcome) was used to define the inclusion criteria. Accordingly, studies that met the following criteria were included in the review: (i) study population: subjects with T2DM or nondiabetic individuals aged 18 years and over, (ii) intervention: oral consumption of each type of honey, (iii) comparison: the control group receiving a placebo or not receiving it, (iv) outcome: at least one of the following parameters: anthropometric indices (weight, body mass index (BMI), and waist circumference (WC)), glycemic indices (fasting blood glucose (FBG), hemoglobin A1C (HbA1c), insulin, insulin resistance, and insulin sensitivity), and lipid profile (triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)), and (v) duration of intervention equal or longer than 7 days. In addition, only clinical trials with either a parallel or cross-over design were considered eligible in the review.

Human studies with any other design, short-term intervention (less than 7 days), studies on children and adolescents, athletes, other types of diabetes, malignant diseases such as cancer, animal studies, in vitro studies, grey literature (conference papers, theses, and interviews), topical treatments, and studies which examined the effects of honey in combination with other materials were excluded from the study.

2.2. Data Extraction. The characteristics of the included studies were extracted by two independent reviewers (M. A and M. J). The data extraction form included the following information: the first author's last name, year of publication, location, study design, gender, mean age, sample size at baseline and the end of the trail, disease background, dosage of honey, type of honey, duration of intervention, other interventions, adjustments, outcomes, and findings.

When there were insufficient data for the included clinical trials, we contacted the corresponding author via e-mail. Unless an answer was received after three times of contact at the end of each week, it was excluded completely or for a specific parameter. Furthermore, when parameters were reported more than twice, only measurements at baseline and the end of the trial were extracted. Apart from

honey and control groups, data obtained from the other study groups were not extracted.

2.3. Quality Assessment. To assess the quality of the eligible clinical trials, a 5-item Jadad scale was applied [25]. Jadad checklist includes three main items as follows: (i) randomization, (ii) blinding, and (iii) an account of all participants. For the first two items, two scores can be dedicated based on the provided information in the study, and the third one can obtain maximum one score. In general, any clinical trial can obtain a maximum of five scores. In the current systematic review, we considered each study with a minimum of three scores as high quality; otherwise, they were classified into the low-quality group. This section was conducted by two independent reviewers (N. N and M. A).

Any discrepancy in each of procedures was resolved by discussion or consulting with the third reviewer (MH. A) as mentioned earlier.

2.4. Data Synthesis. We found high heterogeneity in the included studies. Studies examined various types of honey and had different control groups, study designs, and study subjects. Given that this heterogeneity could not be solved by subgrouping due to limited studies with similar characteristics, we could not pool the studies to conduct a meta-analysis. Therefore, findings were reported only in a qualitative format.

3. Results

As depicted in Figure 1, a total of 7769 possible relevant studies (including 3547 duplicates) were identified by searching the electronic databases. After screening titles and abstracts, we found 4190 irrelevant studies excluded from the study. In the next step, full-texts of 32 articles were carefully examined. Two studies were also obtained after checking the reference lists. Twenty-one out of 34 full-text articles were excluded due to the following reasons: not clinical trial ($n = 7$), duration of intervention shorter than seven days ($n = 7$), non-English language studies ($n = 3$), and the mixture of honey with other materials ($n = 4$). In total, 13 studies were eligible and included in the qualitative synthesis.

3.1. Study Characteristics. The characteristics of 13 included clinical trials are summarized in Table 1**.** The clinical trials were published between 2008 and 2019 in Asian countries ($n = 10$), European countries ($n = 2$), and the U.S.A ($n = 1$). All studies except three (cross-over) had a parallel design. All clinical trials were randomized, and 7 studies were single or double-blinded. Sample size ranged from −8 to 128 for both genders ($n = 8$), men ($n = 2$) and women ($n = 3$). They were between 18 years old and 62.8 years old. Different types of honey, including Tualang, Acacia, Rapeseed, Kelulut, and Robinia and six Greek varieties, were studied. Types of honey were not reported in some studies ($n = 7$). The effects of honey was examined in healthy ($n = 4$) and unhealthy individuals ($n = 9$), including patients with T2DM ($n = 3$). The remaining were conducted on patients with glucose intolerance ($n = 3$), high TC ($n = 1$), and overweight/obesity ($n = 2$). The duration of intervention in clinical trials with a parallel design ranged from 8 days to 12 months, and the dosage varied between 5 g/day and 80 g/day.

The included studies examined the impact of oral consumption of honey on glycemic status ($n = 12$), anthropometric indices ($n = 6$), lipid profiles ($n = 10$), and blood pressure ($n = 3$). Based on the Jadad scale, 5 studies had high methodological quality (score ≥3), and the remaining ($n = 8$) had low methodological quality (score <3) (Table 2).

3.2. Systematic Review of Studies with Placebo Groups. In 5 clinical trials, the effects of honey on metabolic parameters were compared with placebo or control groups. In a study conducted by Yaghoobi et al., the impacts of natural honey were compared with those of sucrose in both healthy subjects and patients with high risk factors [26]. They found that 70 g/day honey reduced only BMI and FBS with no changes in lipid profile in healthy subjects, while it reduced TG in high risk participants. Changes in other lipid profiles and anthropometric indices were insignificant [26]. Findings of the study by Munstedt et al. revealed that a solution containing 75 g honey only reduced serum levels of LDL-C in women, not men. Comparison of honey with the solution containing 75 g glucose, and fructose showed no changes in TG and TC after 14 days [27]. According to Raatz et al., daily consumption of 50 g/day honey, sucrose, and high-fructose corn syrup (three study groups) increased TG and insulin concentrations in glucose-tolerant and intolerant subjects after 14 days [28]. The mentioned changes were also significant among the study groups. Rasad et al. indicated that compared to sucrose, 80 g/day honey solved in 250 mL water decreased serum levels of FBS with no changes in blood pressure in healthy young subjects after 30 days [29]. Notably, they controlled findings for age, physical activity, and some nutrient intake as confounder factors. Despland et al. compared the effects of consumption of Robinia honey, high free glucose and fructose diet, and low fructose diet (control group) in healthy normal weight men for seven days [30]. All study groups received a weight maintenance diet during the intervention. They found that diet with 25% of total energy from honey or pure fructose-glucose might slightly reduce postprandial blood glucose and insulin, while postprandial TG did not change compared to the control group [30].

Findings were classified into two groups, studies with and without control groups. In the end, the impacts of honey in patients with T2DM were also presented.

3.3. The Systematic Review Section for Studies without a Placebo Group (with a Control Group). A total of seven clinical trials with no placebo groups were included in the study. Majid et al. indicated that natural honey (70 g/days for four weeks) reduced FBS, TG, and LDL-C and increased HDL-C concentrations in healthy young men compared to those continued their usual diet [31]. In their study, Sadeghi

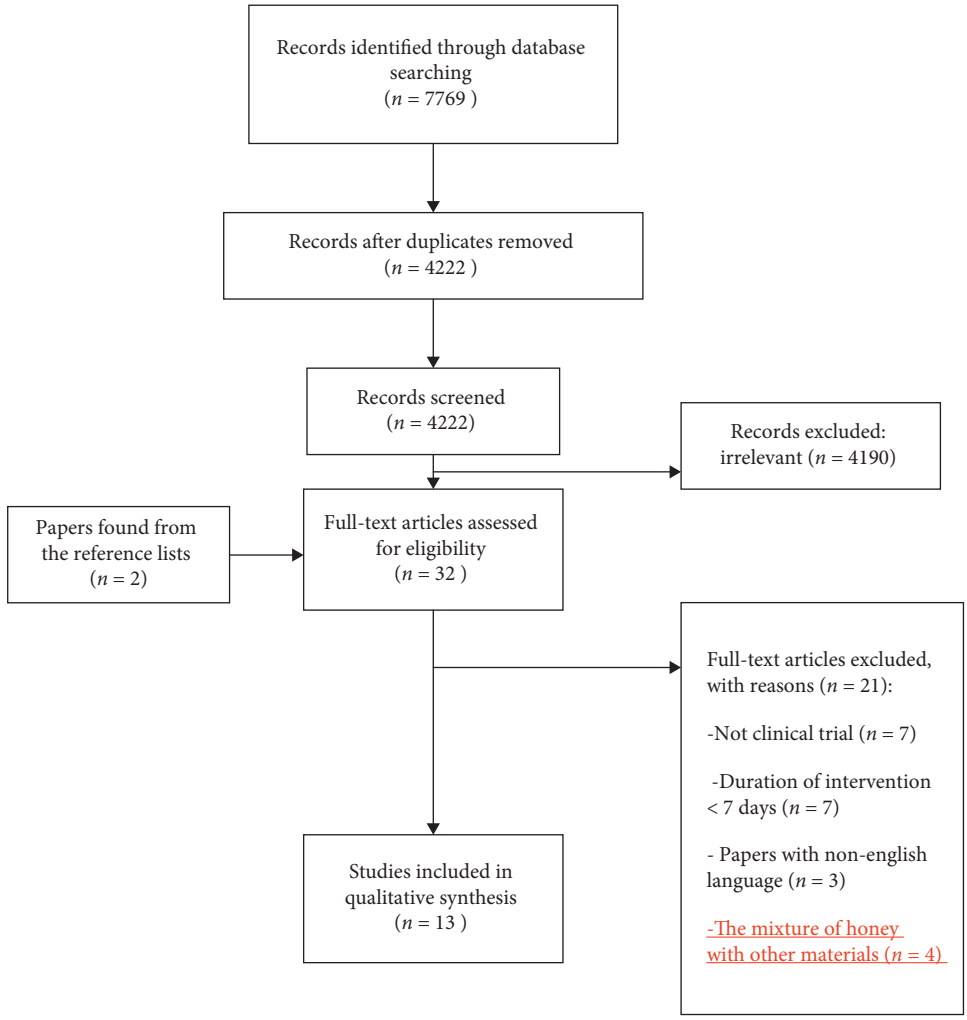

Figure 1: The process to reach the eligible articles.

et al. demonstrated that 50 g/day honey along with a weight maintenance diet increased HbA1c and reduced waist circumference in patients with T2DM compared to those adhered to only a weight maintenance diet after 8 weeks [32]. However, FBS, insulin, homeostatic model assessment for insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI), weight, BMI, hip circumference, and waist-to-hip ratio did not change significantly [32]. Enginyurt et al. compared the effects of three dosages of honey (5, 15, and 25 g/day) with a group receiving no honey. They found that HbA1c was decreased in all intervention groups, but no significant changes were observed in patients with T2DM in terms of lipid profile after 4 months [33]. Mushtaq et al. demonstrated a significant reduction in serum levels of TC, LDL-C, and TG and an increase in HDL-C in groups receiving honey (40 g/day) compared to nonusers of honey in most ethnic groups after 4 weeks. They found that the effects of honey in obese subjects were higher than those in normal weight subjects [23].

Nik Hussein et al. also revealed that 20 g/day Tualang honey along with hormonal replacement therapy (HRT) compared to HRT alone did not have substantial effects on blood pressure, lipid profile, glucose level, BMI, and WC in postmenopausal women after 4 months [34]. Based on the report published by Rashid et al., 30 g/day Malaysian Kelulut honey did not change FBS, lipid profiles, blood pressure, and BMI in patients with IFG after 30 days as compared to control [35]. However, Bahrami et al. demonstrated that natural honey reduced bodyweight, FBS, and LDL-C/HDL-C ratio in patients with T2DM after 8 weeks compared to controls, but no changes were observed in HbA1c and lipid profiles [22]. They examined difference dosages of honey through the intervention, from 1 g/kg/day in the first two weeks to 2.5 g/kg/day in the 7^{th} week and 8^{th} week. Notably, they adjusted findings for baseline values. Rashid et al. showed that consumption of 30 g/day Kelulut honey did not affect the glycemic status, lipid profiles, blood pressure, and BMI compared to those without taking honey after 1 month in patients with impaired fasting glucose [35].

In a clinical trial, the impacts of two kinds of honey (Tualang and honey cocktail) for 12 months and the results showed that honey cocktail increased BMI, while it reduced FBS. In addition, Tualang honey reduced DBP compared to honey cocktail in postmenopausal women [36].

Table 1: The characteristics of the included studies in the current systematic review.

Author/ Year	Location	Study design	Gender (male/ female)	Mean age	Sample size at baseline	Disease background	Sample size at end	Dosage of honey	Duration	Botanical source of honey/type	Other intervention	Adjustment	Findings	
Sadeghi et al./2019	Iran	RCT (cross-over)	Both	57.5	Total (n = 53) Honey (n = 27) Control (n = 26)	T2DM	Total (n = 42) Honey (n = 18) Control (n = 24)	50 g/day	8 weeks (4 weeks wash out)	Milk vetch	Weight maintenance diet for the control and honey group	—	HbA1c WC Waist-to-height ratio Glucose Weight BMI Insulin level HC Waist-to-hip ratio HOMA-IR HOMA-β QUICKI	↔ ↓ ↔
Rashid et al./2019	Malaysia	RCT	Both	51.6	Total ($n = 60$) Kelulut honey ($n = 30$) Control ($n = 30$)	IFG	Total ($n = 64$) Kelulut honey ($n = 27$) Control ($n = 27$)	30 g	30 days	—	—	—	FBS TC HDL-C LDL-C BMI SBP DBP	 ↔
AB Wahab et al./2018	Malaysia	RCT (double-blind, parallel)	F (postmenopause)	58	Total ($n = 100$) Tualang honey ($n = 50$) Honey cocktail ($n = 50$)	T2DM ($n = 12$) HTN ($n = 49$) HLP ($n = 51$)	Total ($n = 98$) Tualang honey ($n = 49$) Honey cocktail ($n = 49$)	20 g/day	12 months	Honey cocktail (contains honey-bee bread-royal jelly)	—	—	FBS BMI DBP	↑ ↓ ↓
Enginyurt O. et al./ 2017	Turkey	RCT	Both	18–80	Total ($n = 64$) Intervention/ patient ($n = 32$) Control/ healthy ($n = 32$)	T2DM (using metformin)	Total ($n = 64$) Intervention/ patient ($n = 32$) Control/ healthy ($n = 32$)	5, 15, and 25 g/ d	4 months	—	—	—	HbA1c (all of dose) TC (doses of 5 and 25 g/d) TG HDL LDL	↓ ↔ ↔ ↓ ↔
Despland et al./2017	Switzerland	RCT (cross-over)	M	—	Total ($n = 8$)	Healthy	Total ($n = 8$)	25% total energy	8 days	—	—	—	Weight Height BMI Fat mass Lean mass SBP DBP Resting heart rate Total TG Chylomicrons-TG LDL Glucose Insulin	 ↔ ↓

Table 1: Continued.

Author/ Year	Location	Study design	Gender (male/ female)	Mean age	Sample size at baseline	Disease background	Sample size at end	Dosage of honey	Duration	Botanical source of honey/type	Other intervention	Adjustment	Findings	
Raatz et al./ 2015	North Dakota	RCT (cross-over)	Both	GT: 38.9 IGT: 52.1	Total ($n = 89$) GT ($n = 52$) IGT ($n = 37$)	GT-IGT	Total ($n = 55$) GT ($n = 28$) IGT ($n = 27$)	50 g carbohydrate from honey, HFCS, and sucrose	2 weeks (2–4 weeks wash out)	—	—	—	Weight BMI Glucose Insulin HOMA_IR-glucose iAUC Insulin iAUC SBP DBP TC HDL-C LDL-C TG	↔
Majid et al./2014	Pakistan	RCT	M	Honey: 20 Control: 20	Total ($n = 70$) Honey ($n = 35$) Control ($n = 35$)	Healthy	Total ($n = 63$) Honey = 32 Control = 31	70 g	4 weeks	—	—	—	FBS TG TC LDL-C HDL-C	↔ ↓ ↓ ↑
Rasad et al./2014	Iran	RCT (double-blind)	M	Honey: 21.53 Sucrose: 24.23	Total ($n = 60$) Honey ($n = 30$) Sucrose ($n = 30$)	Healthy	Total ($n = 60$) Honey ($n = 30$) Sucrose ($n = 30$)	80 g honey in 250 ml water	6 weeks	—	—	Age, physical activity, and some nutrient intake	FBS DBP SBP	↓ ↔ ↔
Nik Hazlina et al./2012	Malaysia	RCT	F	55.4	Total ($n = 82$) Tualang honey ($n = 41$) HRT ($n = 41$)	Healthy postmenopausal	Total ($n = 79$) Tualang honey ($n = 40$) HRT ($n = 39$)	20 g/day	4 months	—	—	Baseline values, age, BMI, WC, and duration of menopause	BMI WC SBP DBP TC TG HDL FBS	↔

Table 1: Continued.

Author/ Year	Location	Study design	Gender (male/ female)	Mean age	Sample size at baseline	Disease background	Sample size at end	Dosage of honey	Duration	Botanical source of honey/type	Other intervention	Adjustment	Findings	
Mushtaq et al./2011	Pakistan	Randomized controlled clinical trial	Both	Normal weight, honey: M, 46.25 and F, 42.8 Normal weight, no honey: M, 42.5 and F, 42.9 Obese, honey: M, 41.27 and F, 40.06 Obese, no honey: M, 39.87 and F, 39.66	Total ($n = 160$) Honey: obese ($n = 40$) and normal weight ($n = 40$) No honey: obese ($n = 40$) and normal weight ($n = 40$)	Obese/normal weight	Total ($n = 128$) Honey: obese ($n = 32$) and normal weight ($n = 32$) No honey: obese ($n = 32$) and normal weight ($n = 32$)	40 g/day	4 weeks	—	—	—	BMI: obese and normal weight, LDL-C and normal weight, HDL Normal weight: female TC, normal weight (male Baloch and Pathan and female Baloch) TC, obese (male Baloch and Punjabi and female Baloch, Punjabi, and Hzara) TG, normal weight (male Baloch and female Punjabi) TG, obese (male all ethnic groups and female Baloch, Pathan, and Punjabi) LDL-C, obese (male all ethnic groups and female Baloch and Pathan) HDL-C, normal weight (male Pathan and Punjabi) HDL-C, obese (male Baloch, Pathan and Hzara, and female Baloch and Pathan)	↔ ↓ ↑
Munstedt et al./2009	Germany	RCT	Both	Honey: 62.6 Honey-comparable sugar solution: 58.8	Total ($n = 60$) Honey group ($n = 30$) Honey-comparable sugar solution group ($n = 30$)	High cholesterol	Total = 60 Honey group: 30 Honey-comparable sugar solution group: 30	75 g	14 days	Mixed blossom honey from Europe, central America, and South America	—	—	Women: LDL General: TG, LDL, HDL, and TG	↔ ↔

Table 1: Continued.

Author/ Year	Location	Study design	Gender (male/ female)	Mean age	Sample size at baseline	Disease background	Sample size at end	Dosage of honey	Duration	Botanical source of honey/type	Other intervention	Adjustment	Findings	
Bahrami et al./2009	Iran	RCT	Both	57.2	Total ($n = 54$) Honey ($n = 28$) Control ($n = 26$)	T2DM	Total ($n = 48$) Honey ($n = 25$) Control ($n = 23$)	First 2 weeks: 1 g/kg/d Second 2 weeks: 1.5 g/kg/d Third 2 weeks: 2 g/kg/d Last 2 weeks: 2.5 g/kg/d	8 weeks	Natural unprocessed honey	—	Baseline values	Bodyweight TG TC LDL-C FBS LDL/HDL HDL-C HbA1c	↓ ↑
Yaghoobi et al./2008	Iran	RCT	Both	41.2	Total ($n = 60$) Honey: 40 Control: 20	Obese or overweight	Total ($n = 55$) Honey: 38 Control: 17	70 g/250 ml tap water	30 days	Natural unprocessed honey	—	—	Normal variable: in subjects with normal values: BMI and FBG Patients with TG >150 mg/dl Bodyweight Body fat TC LDL-C HDL-C	↓ ↔

RCT, randomized controlled clinical trial; T2DM, type 2 diabetes mellitus; WC, waist circumference; BMI, body mass index; HC, hip circumference; HOMA, homeostasis model assessment insulin resistance; QUICKI, quantitative insulin sensitivity check index; FBS, fasting blood sugar; DBP, diastolic blood pressure; HTN, hypertension; HLP, hyperlipidemia; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein; iAUC, incremental area under curve; GI, glycemic index; SBP, systolic blood pressure; FBS, fasting blood sugar; GT, glucose tolerance; ↔, no significant effect; ↓, decrease; ↑, increase.

Table 2: The Jadad score for the included clinical trials ($n = 13$).

Author (year)	Randomization score	Blinding score	Account of patients score	Total score
Sadeghi et al. (2019)	2	1	1	4
Rashid et al. (2019)	0	0	0	0
AbWahab et al. (2018)	2	2	1	5
Enginyurt et al. (2017)	1	0	0	1
Despland et al. (2017)	1	0	1	2
Raatz et al. (2015)	1	0	1	2
Majid et al. (2014)	2	1	1	4
Rasad et al. (2014)	1	1	1	3
Nik Hussein et al. (2012)	1	0	1	2
Mushtaq et al. (2011)	0	0	0	0
Mu¨nstedt et al. (2009)	1	1	1	3
Bahrami et al. (2009)	1	0	1	2
Yaghoobi et al. (2008)	1	0	0	1

3.4. The Effects of Honey in Patients with T2DM. Three clinical trials examined the effects of honey in patients with T2DM [22, 32, 33]. Two clinical trials showed a significant increase in HbA1c following the intake of a minimum 50 g/days honey for 8 weeks [22, 32]. However, Enginyurt et al. showed that following the consumption of 5–25 g/day honey for 4 months, a reduction in HbA1c was observed [33].

4. Discussion

The current systematic review showed that oral consumption of honey might have no positive effects on the modulation of metabolic parameters in nondiabetic subjects. In addition, a high intake of honey might increase glucose levels and worsen other metabolic parameters in patients with T2DM. Due to substantial heterogeneity in study design and limited clinical trials, results, however, should be interpreted with great caution.

To the best of our knowledge, this is the first systematic review on the effects of oral consumption of honey on metabolic parameters in diabetic and nondiabetic individuals. Therefore, we cannot compare our findings with an earlier systematic review. We found a mixture of positive, negative, and null effects on metabolic status following the consumption of honey. This discrepancy might be due to differences in mean age, disease background, gender, BMI at baseline, dosage, duration of intervention, other intervention along with taking honey, and ethnic of participants as well as differences in types of honey obtained from various botanical sources.

In most clinical trials included in the current review, the effects of honey were not compared with those of the placebo. This point can cause considerable bias and affect both the internal and external validities of studies. Although this point is more important for subjective outcomes, it can also lead to overestimating or underestimating the real effects of the intervention for objective outcomes [37]. On the other hand, based on the Jadad scale, the most clinical trials had low methodological quality. Due to this issue, we cannot draw a fix conclusion about the effects of honey on each metabolic parameter.

Another weakness of the most included studies was related to reporting findings without controlling confounders, including baseline characteristics, total energy intake, physical activity, and BMI. The main confounder that can affect findings is total energy intake. Honey is a high-nutrient functional food containing 64 kcal in each tablespoon (about 20 g). Therefore, adding honey to diet, particularly in high dosage with no replacement, no changes in daily calorie intake and physical activity can increase body weight and fat mass due to extra calorie intake. Therefore, metabolic parameters can be influenced by these changes. However, only in the two included clinical trials [30, 32], a weight maintenance diet was recommended along with honey consumption, and in most studies, findings were not reported after adjusting for such confounding factors. The importance of this issue was clarified in the study of Despland et al. They found that a diet containing 25% of total energy intake after designing individualized weight maintenance diets did not show harmful effects on glucose and TG levels, and even, it showed reduction effects [30].

Accordingly, it seems that the negative effects of honey on metabolic parameters reported in some included clinical trials might be related to the added calorie and fructose content of honey. Some studies demonstrated that hypertriglyceridemia was associated with fructose or glucose administered in a hypercaloric diet, while no significant effects were found in a weight maintenance diet [38, 39]. Some previous studies have also showed that fructose can increase TG concentrations and stimulate hepatic de novo lipogenesis [40, 41].

Notably, the amount of fructose and fructose/glucose ratio in honey can contribute to different findings. Based on meta-analyses, postprandial TG can increase following the consumption of fructose greater than 50–60 g/day [42], and an increase in FBS is observed with daily dosage exceeding 100 g [42, 43]. However, the amount of fructose from honey and whole diet was reported in only two studies [28, 30]. The average daily intake of fructose was 95 g in the study of Despland et al. [30]. The fructose content of honey in

another clinical trial was also 40 g/100 g of honey [28]. Fructose to glucose ratio in honey is another possible factor affecting metabolic parameters, and it differs among various types of honey. Among the included clinical trials, only Despland et al. reported the ratio, and it was 1.7 [30]. This ratio may affect both the GI of honey that is important, particularly for patients with DM and glycemic status.

It is possible that some factors, including polyphenols and other antioxidant ingredients of honey, can blunt the harmful effects of fructose on metabolic parameters. Such components are influenced by botanical sources and geographical locations [12]. Therefore, identifying the types of honey with therapeutic effects can be helpful. However, the different types of honey and their nutritional value were provided only in limited studies. Thus, we were not able to report findings based on classifications by the mentioned factors. It seems that producing honey with identified and controlled amounts of components along with adding some nutrients or materials, including probiotics and prebiotics in some cases, can be helpful, particularly for unhealthy subjects.

There were only three studies conducted on patients with T2DM [22, 32, 33]. Although this number is insufficient to conclude how much honey can be allowed for diabetic patients, we can conclude that it is not necessary to delete honey completely from diabetic diets and only restriction and to replace with other carbohydrate sources along with the maintenance of total energy intake is sufficient. One out of three studies conducted on diabetes examined different dosages of honey (5–25 g/days) [33]. The results showed that these dosages not only had no negative effect on metabolic parameters but also reduced HbA1C. However, higher dosage (50 g/day) increased HbA1c in patients with T2DM [32].

It is not fully understood by which mechanisms honey can affect metabolic profiles. However, some potential pathways are suggested. The main possible mechanisms are related to the ingredients of honey, particularly polyphones and flavonoids with anti-inflammatory and antioxidant properties. Through the suppression of inflammatory pathways, reducing free radicals, and helping to rebalance oxidant and antioxidant factors, modulation of glycemic status, lipid profiles, and other metabolic factors can be occur. Other mechanisms are reduction in body weight, increased satiety, delay in gastric emptying, and modulation of appetite hormones including neuropeptide Y and ghrelin due to phenolic components and oligosaccharides content [32], affecting C-peptide, stimulating beta cells of the pancreas due to antioxidant components [35], an increase in uptake of hepatic glucose and glycogen synthesis and storage due to fructose [33].

However, this study has some limitations that should be addressed. They are as follows: (i) due to high heterogeneity in the methodology of the included clinical trials and limited studies with similar characteristics, we were not able to do a meta-analysis, (ii) studies conducted on other metabolic profiles such as inflammatory and antioxidant parameters were not included, (iii) we could not determine cutoff points for dosage of honey with no negative effect on metabolic parameters due to high heterogeneity. However, doing a systematic review on this topic for the first time, examining the quality of studies, providing results separately for both diabetic and nondiabetic subjects were the strengths of this systematic review.

5. Conclusion

The current systematic review revealed that oral consumption of honey might have no beneficial effects on the modulation of metabolic status in nondiabetic subjects. Even high intake of honey might increase glucose levels and worsen other metabolic parameters in patients with T2DM. Due to substantial heterogeneity in study design, low quality in most clinical trials, and limited included studies, results, however, should be interpreted with great caution. More high quality randomized controlled clinical trials on different types of honey (with determined physicochemical properties) with various dosages and longer duration of the intervention are necessary to clarify the effects of honey in diabetic and nondiabetic individuals.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

N. N. and M. H. A. designed and directed the project. N. N. and M. A. provided search strategies and collection of publications in Endnote. M. A. and N. N. screened publications. M. J. and M. A. extracted data. M. A. and N. N. assessed the quality of publications. M. H. A. examined results and interpreted findings. All authors checked and confirmed the draft of the manuscript.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2021, Article ID 6655599, 12 pages
https://doi.org/10.1155/2021/6655599

Research Article

Beneficial Effect of *Taraxacum coreanum* Nakai via the Activation of LKB1-AMPK Signaling Pathway on Obesity

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Received 23 November 2020; Revised 19 December 2020; Accepted 8 January 2021; Published 18 January 2021

Academic Editor: Mahnaz Khanavi

Objective. Liver kinase B (LKB) 1 and AMP-activated protein kinase (AMPK) are master regulators and sensors for energy homeostasis. AMPK is mainly activated via phosphorylation of LKB1 under energy stress. Here, we highlighted the antiobesity effect and underlying mechanism of *Taraxacum coreanum* Nakai (TCN) in connection with LKB1-AMPK signaling pathway. *Methods*. Male C57BL/6 mice were fed on a high-fat diet (60% kcal fat; HFD) to induce obesity. Simultaneously, they received 100 or 200 mg/kg TCN orally for 5 weeks. We measured the body weight gain and liver weight along with liver histology. Moreover, the changes of factors related to lipid metabolism and β-oxidation were analyzed in the liver, together with blood parameters. *Results*. The body weights were decreased in mice of the TCN200 group more than those of the HFD control group. Moreover, TCN supplementation lowered serum triglyceride (TG) and total cholesterol (TC) levels, whereas TCN increased HDL-cholesterol level. Liver pathological damage induced by HFD was alleviated with TCN treatment and accompanied with significant reduction in serum AST and ALT activities. In addition, TCN significantly increased the expression of p-AMPK compared with the HFD control group via the activation of LKB1/AMPK signaling pathway. Lipid synthesis gene like ACC was downregulated and factors related to β-oxidation such as carnitine palmitoyl transferase-1 (CPT-1) and uncoupling protein 2 (UCP-2) were upregulated through peroxisome proliferator-activated receptor (PPAR) α activation. *Conclusion*. Taken together, these data suggest that TCN treatment regulates lipid metabolism via LKB1-AMPK signaling pathway and promotes β-oxidation by PPARα; hence, TCN may have potential remedy in the prevention and treatment of obesity.

1. Introduction

Obesity has continued to be a public health concern across the globe over the past decades. Obesity is primarily caused by the energy imbalance between nutrition and physical activity [1]. It is also at higher risks for developing serious health problems like dyslipidemia, fatty liver disease, hypertension, stroke, diabetes mellitus, osteoarthritis, cancers, respiratory problems, and sleep apnoea [2]. The World Health Organization (WHO) reported that more than 1.9 billion (39% of the world's adult population) in 2016 were overweight and of these over 650 million (about 13% of the world's adult population) were obese. Approximately 2.8 million deaths happened as a result of being overweight or obese [3]. Obesity is related to immune dysregulation and chronic low-grade inflammation [4]. Accordingly, that involves improving the immune response such as regular physical exercise, weight loss though caloric restriction, and use of AMP-activated protein kinase (AMPK) activators.

Reactive oxygen species (ROS) are naturally produced under many metabolic reactions, most of the production of ATP in mitochondria. Above all, ROS regulation is important for the maintenance of cellular homeostasis [5]. AMPK, which is the main sensor of cellular energy status, is activated in response to energy stress such as starvation, hypoxia, exercise, or the stimulation though drug (metformin, thiazolidinediones) and restores energy balance by promoting catabolic process that generates ATP, while inhibiting anabolic process that consumes ATP. Here, AMPK is ultimately activated by upstream kinase (liver

kinase B1; LKB1) in response to stimuli like the increase of AMP/ADP ratio [6]. That is, the phosphorylation at threonine residue (Thr) 172 within the catalytic α subunit by LKB1 requires the process of AMPK activation. The activated AMPK affects energy-consuming pathways such as de novo lipid biosynthesis as well as energy-producing pathways such as lipid oxidation [7]. This series of processes includes the induction of phosphorylation, which means inactivation of acetyl coenzyme A carboxylase (ACC) and the decrease of malonyl-CoA. Thereby, it alleviates the inhibition of carnitine palmitoyl-transferase- (CPT-) 1 and leads to an increase in fatty acid oxidation in the liver. For that reason, AMPK is reported to regulate various metabolic processes dysregulated in classic chronic diseases such as inflammation, obesity, diabetes, and cancer [5]. AMPK is also considered as a promising target for therapeutic and pharmaceutical studies on disorders such as obesity, diabetes, and metabolic syndrome, where the body energy homeostasis is imbalanced [8].

Taraxacum coreanum Nakai (TCN), which is called a white dandelion in Korea and Japan, has been used as a traditional therapeutic agent for various inflammatory diseases such as gastritis, gastric ulcer, and tonsillitis [9, 10]. Its extract has been reported to have protective effects against stomach, liver, and brain damage induced by ethanol, CCl_4, and methionine and choline deficient diets [11–13]. Ultimately, bitter substances are known as stimulating the digestion, while phenolic compounds account for the antioxidative and anti-inflammatory activities [14, 15]. Besides the above-mentioned effects, TCN has been reported to have other biological activities, including antidiabetic, antifungal, and anticancer effects [10]. Compared to roots, the leaf of TCN is characterized by higher polyphenol such as chicoric acid and chlorogenic acid and flavonoids such as luteolin and quercetin [16].

To our knowledge, previous studies have never investigated the antiobesity effect of TCN. Therefore, we selected the best extraction method and evaluated its antiobesity effect and underlying mechanism in connection with LKB1-AMPK signaling pathway with 70% ethanol extract of TCN on an experimental model of obesity.

2. Materials and Methods

2.1. Chemical and Reagents. The protease inhibitor mixture, ethylenediaminetetraacetic acid (EDTA), was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rabbit polyclonal antibody against peroxisome proliferator activated receptor (PPAR) α, mouse monoclonal antibodies against β-actin and histone, and goat polyclonal antibody against mitochondrial uncoupling protein (UCP)-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit monoclonal antibody against LKB1 and phospho-LKB1 and rabbit polyclonal antibodies against acetyl-CoA carboxylase (ACC), phospho-ACC, AMPKα, and phospho-AMPKα were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat polyclonal antibody carnitine palmitoyltransferase- (CPT-) 1 was purchased from Abcam (Cambridge, UK). ECL Western Blotting Detection Reagents and nitrocellulose membranes were supplied by GE Healthcare (Buckinghamshire, UK). Goat anti-rabbit, rabbit anti-goat, and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase- (HRP-) conjugated secondary antibodies were purchased from GeneTex, Inc. (Irvine, LA, USA). All other chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Chicoric acid was acquired from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2. Preparation of Plant Extract. The root and leaf of TCN were collected from Wonilbio (Jecheon, Korea) in 2019. These were identified by Dr Roh SS and were deposited in Daegu Haany University. Ethanolic extracts were made in the following way. Dried leaves and root parts (100 g) were soaked in 70% ethanol (1000 mL) and then left for 1 day at room temperature. Moreover, water extracts were made in the following way. Each dried leaf and root part (100 g) was prepared and extracted with 10 times of distilled water and boiled in 100°C for 2 h. The solvent was dried using a rotary evaporator following filtration with filter paper. We obtained the following yields (Table 1). And, Figure 1 shows the sample extract images obtained by different extraction methods with different parts (leaf and root) of TCN.

2.3. DPPH Radical Scavenging Property. Antioxidant activity determination of AT-mix was performed by the DPPH radical scavenging property according to the method of Hatano et al. [17]. In microwells, 100 μL of an ethanolic solution of the sample (control: 100 μL of ethanol) was added to an ethanolic solution of DPPH (60 μM). After mixing gently and the reaction being carried out for 30 min at room temperature, the optical density was measured absorbance at 540 nm with a Microplate Reader (Tecan M200 PRO, Zürich, Switzerland). The antioxidant activity of each sample was expressed in terms of the IC_{50} (concentration required to inhibit DPPH radical formation by 50%) calculated from the log-dose inhibition curve. The radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging property}\,(\%) = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{blank}}}\right)\right] \times 100. \quad (1)$$

2.4. ABTS Radical Scavenging Property. ABTS radical scavenging activity of the different extracts was measured according to the modified method of Re et al. [18]. ABTS stock solution was dissolved in water to a 7.4 mM concentration. The ABTS radical cation (ABTS) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 16 h at room temperature in the dark. The ABTS solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 415 nm. After adding 95 μL of diluted ABTS solution (A 415 nm = 0.70 ± 0.02) to 5 μL of sample, the mixture was left for 30 min at room temperature in the dark. The optical density was measured absorbance at 415 nm with a Microplate Reader. The radical scavenging property was calculated using the following equation:

Table 1: Extract yields according to different extraction methods of leaf and root of TCN.

Extract methods	Leaf (%)	Root (%)
Water extract	15.5	14.5
30% ethanol extract	19.3	17.0
70% ethanol extract	21.5	11.1

Figure 1: The sample extract images obtained by different extraction methods with leaf and root of TCN. (A) Water extract, (B) 30% ethanol extract, and (C) 70% ethanol extract.

$$\text{ABTS radical scavenging property}\,(\%) = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{blank}}}\right)\right] \times 100. \quad (2)$$

2.5. Total Phenolic and Flavonoid Contents. The total phenolic content of TCN was quantified by mild modification from the method of Folin-Ciocalteu [19]. A 10 μL TCN and distilled water 790 μL were shaken well and then mixed with 50 μL of Folin-Ciocalteu's reagent for 1 min. Then, 150 μL of 20% sodium carbonate solution (Na_2CO_3) was added and the mixture was shaken for 2 h at 20°C. Finally, the absorbance of the resulting color was measured at 765 nm. The total phenolic content was expressed as mg gallic acid equivalents per gram extract. Values presented are the average of three measurements. Flavonoid was extracted and quantified by adaptation of the method of Lister et al. [20]. TCN 100 μL and 1 mL diethylene glycol were mixed well. And then 1 N NaOH 10 μL was added and the mixture was incubated for 1 h at 37°C. Finally, the absorbance of the resulting color was measured at 420 nm. The flavonoid content was expressed as mg naringin equivalents per gram extract. Values presented are the average of three measurements.

2.6. Ex Vivo Lipolysis Assay. The epididymal fat pads were removed from the male mouse (25–30 g) and placed in Hank's balanced salt solution buffer (HBSS, Invitrogen, California, USA). The removed fat pads were shredded (20 mg/piece) and prepared in 1 mL HBSS containing 1% FFA free bovine serum albumin (Sigma-Aldrich, St. Louis, USA) at 150 mg per test tube. Test drug diluted in various concentrations and isoproterenol (7.5 mM, 1.86 mg/mL), which is a positive control drug, were added to the test tube and then incubated at 37°C for 1 h. After 1 h, the supernatant was collected and the amount of glycerol spilled due to decomposition of fat was measured using a glycerol assay kit (Sigma-Aldrich, St. Louis, USA).

2.7. Experimental Animals and Treatment. Male healthy 5-week-old C57BL/6 mice (about 20–25 g) were purchased from DBL Co., Ltd. (Eumseong, Korea). Each mouse was kept at room temperature (23 ± 2°C) and humidity (50 ± 10%) with a 12-hour light/dark cycle. The mice were allowed free access to laboratory pellet chow and water ad libitum. After adaptation (1 week), all experimental mice except normal mice (Normal, $n = 8$) were fed with 60% high-fat diet (HFD; Diet 12492, Research Diets, Inc., New Brunswick, NJ, USA) for 5 days to adapt to a feed. Thereafter, C57BL/6 mice ($n = 32$) fed HFD were randomly divided into four groups ($n = 8$ in each group): HFD control group (Control), *Garcinia cambogia* extract group 200 mg/kg/day (GCE200), and two TCN treatment groups 100 or 200 mg/kg/day (TCN100 or TCN200). The normal and HFD control groups were given water using a stomach tube, while the drug treatment groups were orally administered GCE or TCN daily using a stomach tube for 5 weeks. *Taraxacum* (dandelion) has been used in traditional

Chinese medicine and dietary application; its adverse effects are rare. Oral administration of dandelion did not lead to any toxic sign or death in mice, and LD_{50} was established to be greater than 20 g/kg body weight [21]. For that reason, this experiment was conducted by selecting 200 mg/kg body weight, a concentration frequently used in obesity experiments induced by a high-fat diet. After administration for 5 weeks, each mouse was anesthetized after fasting for 12 h. The serum was immediately separated from the blood by centrifugation and kept at −80°C until analysis.

2.8. Measurement of TG, TC, LDL-Cholesterol, and VLDL-Cholesterol Contents in Serum. The blood was centrifuged at 1,500 × *g* for 10 min at 4°C. Serum triglyceride (TC) and total cholesterol (TG) were conducted spectrophotometrically using commercially available kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). HDL-cholesterol was measured using a commercial kit from Asan Pharm. Co., Ltd., (Hwaseong, Korea, Cat. AM203). LDL-cholesterol was calculated though TG, TC, and HDL-cholesterol levels and VLDL-cholesterol was calculated though TG level.

$$\begin{aligned} \text{LDL} - \text{cholesterol level (mg/dL)} &= \left[\text{TC} - (\text{HDL} - \text{cholesterol}) - \frac{\text{TG}}{5}\right], \\ \text{VLDL} - \text{cholesterol level (mg/dL)} &= \frac{\text{TG}}{5}. \end{aligned} \tag{3}$$

2.9. Preparation of Cytosol and Nuclear Fractions. Protein extraction was performed according to the method of Komatsu with minor modifications [22]. Liver tissues for cytosol fraction were homogenized with ice-cold lysis buffer A (250 mL) containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 1,250 μL protease inhibitor mixture solution. The homogenate was incubated at 4°C for 20 min. And then 10% NP-40 was added and mixed well. After centrifugation (13,400 × *g* for 2 min at 4°C) using Eppendorf 5415R (Hamburg, Germany), the supernatant liquid (cytosol fraction) was separated in a new e-tube. The left pellets were washed twice by buffer A and the supernatant was discarded. Next, the pellets were suspended with lysis buffer C (20 mL) containing 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 1% (v/v) glycerol, and 100 μL protease inhibitor mixture solution suspended and incubated at 4°C for 30 min. After centrifugation (13,400 × *g* for 10 min at 4°C), the nuclear fraction was prepared to collect the supernatant. Both cytosol and nuclear fractions were kept at −80°C before the analysis.

2.10. Immunoblotting Analyses. For the estimation of PPARα and histone, 12 μg of nuclear fraction was electrophoresed using 10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane and then incubated with primary antibodies (PPARα and histone) overnight at 4°C. Then, they were incubated with anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibody for 1 h 30 min at room temperature. In addition, 8 μg proteins of each cytosol fraction of p-LKB1, LKB1, p-AMPKα, AMPKα, p-ACC, ACC, UCP-2, CPT-1, and β-actin were electrophoresed through 8–12% SDS-PAGE. Each antigen-antibody complex was detected by chemiluminescence with Sensi-Q 2000 Chemidoc (Lugen Sci Co., Ltd., Gyeonggi-do, Korea). Band densities were measured using the ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to β-actin or histone. The protein expression levels between the groups are expressed relative to those of the normal group (represented as 1). We followed the methods of Shin et al. [23] and Kim et al. [24] regarding the experimental method.

2.11. Oil Red O Stain in the Liver. The microscopic analysis of the effect of TCN treatment on lipid accumulation of the HFD-fed mouse liver was carried out though Oil Red O stain. The frozen liver tissue was cut into 7 μm with Probe-On-Plus Slides (Thermo Fisher Scientific, Massachusetts, U.S.) and affixed to microscope slides. Sections were reacted with Oil Red O solution buffer for 7 min at 60°C. Then, they were incubated with 85% propylene glycol for 3 min. After rinsing with water, sections were stained with Harris hematoxylin for counterstaining. The stained slices were subsequently observed under an optical microscope and analyzed by the i-Solution (Innerview Co., Seongnam, Korea).

2.12. Statistical Analysis. Data are expressed as mean ± SEM and mean ± SD. Statistical comparisons were performed by one-way ANOVA followed by LSD test (SPSS 25.0 for Windows, SPSS Inc., U.S.A.) and values of $p < 0.05$ were considered significant.

3. Results

3.1. Comparative Evaluation of Antioxidant Efficacy In Vitro for the Selection of TCN Sample Used in This Experiment. DPPH and ABTS radical scavenging activities were performed to compare antioxidant activities according to parts (leaf and root) and extraction methods (water, 30% ethanol, and 70% ethanol) of TCN. Antioxidant activity is expressed

in terms of IC_{50}, and IC_{50} (μg/mL) represents concentration of sample providing 50% of radical scavenging activities like DPPH and ABTS assays. As shown in Figure 2, IC_{50} values of DPPH radical scavenging activity of leaf and root extracted in 70% ethanol extract were 164.06 ± 2.51 and $166.51 \pm 6.3\,\mu$g/mL. Also, IC_{50} value of ABTS radical scavenging activity was 257.12 ± 3.77 and $162.7 \pm 0.6\,\mu$g/mL. The 70% ethanol extract showed better radical scavenging than that of water extract. Subsequently, assays for the content measurement of the phenolic compound or flavonoid were performed. Total phenolic content of 70% ethanol extract (leaf) was the best among the six samples as 25.74 ± 0.17 mg GAE/g of TCN extract. The flavonoid content also had the highest content as 12.53 ± 0.03 mg naringin equivalent (NE)/g of TCN extract. Taken together, 70% ethanol extract (leaf) was most excellent in all antioxidant capabilities.

3.2. Ex Vivo Lipolysis Assay for the Selection of TCN Sample Used in This Experiment. The ex vivo lipolysis assay was performed to compare lipolysis effects according to parts (leaf and root) and extraction methods (water, 30% ethanol, and 70% ethanol) of TCN. As shown in Figure 3, the amount of glycerol spilled of leaf extracted using 70% ethanol extract was $74.63 \pm 0.59\%$ (fold of isoproterenol). Leaf sample extracted using 70% ethanol extract among 6 sample extracts was the most outstanding sample in lipolysis effect.

3.3. Body Weight Gain, Liver Weight, and Food Efficiency Ratio. Table 2 shows body weight gain, liver weight, and food efficiency ratio (FER) during the experimental periods. As shown in Table 2, mean body weights of all groups started without a significant difference between 22.27 g and 22.99 g. The normal group increased slightly in weight gain during the experimental period of 5 weeks and the level showed a significant difference compared with the HFD control group ($p < 0.001$). The GCE200 and TCN200 treated groups were significantly decreased compared with the HFD control group (GCE200; $p < 0.001$, TCN200; $p < 0.01$). The HFD consumption led to the increase of liver weight. Besides, drug treatment significantly decreased (GCE200, TCN200; $p < 0.001$, TCN100; $p < 0.01$). Moreover, the food intake and food efficiency ratio between the HFD supplied groups did not show a significant difference. As a result, TCN treatment decreased body weight gain without a significant change of FER.

3.4. Biochemical Analyses. HFD caused the increase in serum TG, TC, HDL-cholesterol, LDL-cholesterol, and VLDL-cholesterol ($p < 0.001$, respectively). The elevated TG and VLDL-cholesterol levels were significantly reduced by all drugs compared with the HFD control group ($p < 0.001$). Moreover, the augmented TC and LDL-cholesterol levels were significantly lowered by GCE and TCN administration compared with the HFD control group (GCE200; $p < 0.01$, TCN100; $p < 0.05$, TCN200; $p < 0.01$), whereas the administration of GCE and TCN significantly elevated HDL-cholesterol level ($p < 0.001$). Particularly, TG and VLDL-cholesterol in the TCN200 group showed lower values than those of the normal group (Figure 4).

3.5. Histological Changes. As shown in Figure 5, we investigate whether the improved lipid metabolism had changed at the lipid accumulation. We observed Oil red O staining in the liver to evaluate the extent of lipid accumulation. As expected, mice fed only HFD resulted in severe hepatic lipid accumulation, while GCE and TCN administration more effectively improved the pathological condition compared with the HFD control group.

3.6. LKB1/AMPK/ACC Phosphorylation Protein Expressions in the Liver. As shown in Figure 6, p-LKB1/p-AMPK/p-ACC protein levels were significantly decreased in the group fed only HFD compared with the normal group, while the reduced protein levels were significantly increased in both GCE and TCN treatment.

3.7. β-Oxidation-Related Protein Expressions in the Liver. Figure 7 reveals that mice fed HFD remarkably reduced the protein expressions of β-oxidation markers such as PPARα, CPT-1, and UCP-2. However, TCN treatment significantly reversed the decreased PPARα expression. Moreover, its target gene, CPT-1 and UCP-2 by TCN supplementation significantly increased compared with those of the HFD control group.

4. Discussion

Obesity as a growing worldwide concern is determined by abnormal fat deposition, which may have negative effects on general health status. Accordingly, the management of related risk factors like dyslipidemia, hypertension, cardiovascular diseases, type 2 diabetes, and metabolic syndrome through body weight loss is an important concern in obesity control [25]. The various therapeutic approaches such as lifestyle changes, medications, and surgery are introduced for obesity treatment. Despite acquiring partially desirable results, the problem remained unsolved. Therefore, a new approach like the use of safe and effective natural herb seems to be a promising and novel strategy to control obesity and related syndromes because of being able to overcome previous limitations [26].

This work demonstrated for the first attempt the effect and the underlying mechanism of TCN treatment on lipid metabolism and β-oxidation. First of all, DPPH and ABTS radical scavenging activities were performed to compare antioxidant activities according to parts (leaf and root) and extraction methods (water, 30% ethanol, and 70% ethanol) of TCN for the selection of sample to be used in animal testing. Antioxidant activity is expressed in terms of $IC_{50,}$ and IC_{50} (μg/mL) represents half maximal inhibitory concentration of samples to scavenge DPPH and ABTS radical. As shown in Figure 2, IC_{50} values of DPPH radical scavenging activity of leaf and root extracted in 70% ethanol

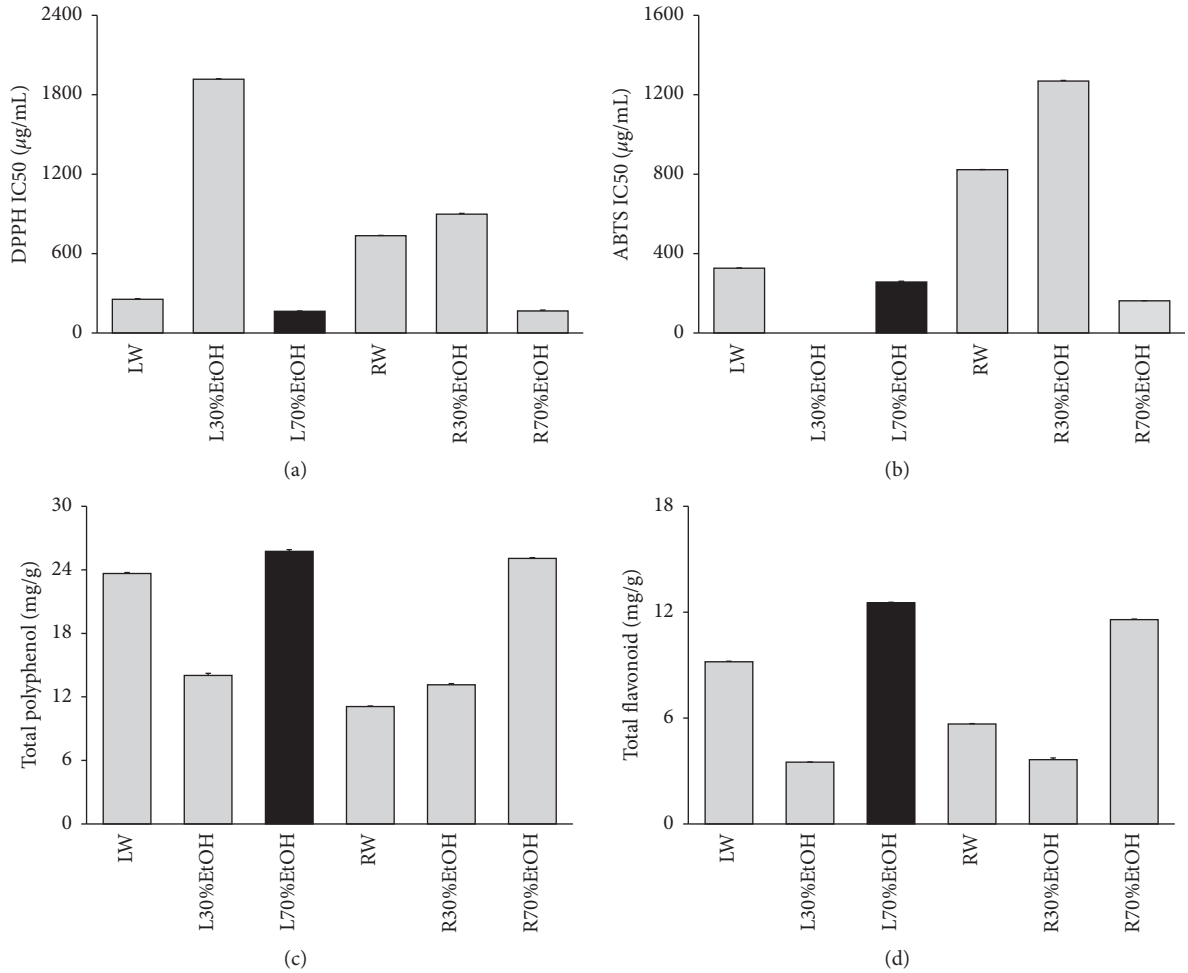


Figure 2: Comparative evaluation of antioxidant efficacy in vitro for the selection of TCN sample used in this experiment. (a) DPPH free radical scavenging activity, (b) ABTS free radical scavenging activity, (c) total polyphenol content, (d) total flavonoid content. LW: water extract of TCN (leaf); L30%EtOH: 30% ethanol extract of TCN (leaf); L70%EtOH: 70% ethanol extract of TCN (leaf); RW: water extract of TCN (root); R30%EtOH: 30% ethanol extract of TCN (root); R70%EtOH: 70% ethanol extract of TCN (root). Data are mean ± SEM. Each experiment was run in triplicate.

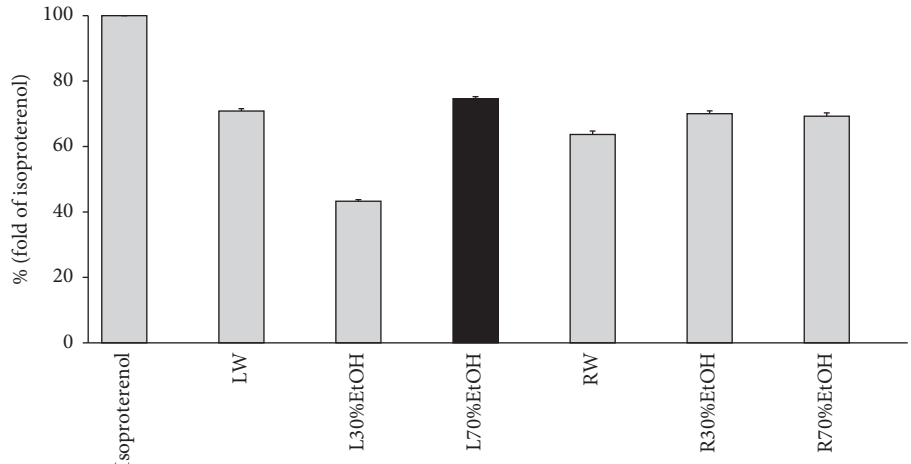


Figure 3: Ex vivo lipolysis assay for the selection of TCN sample used in this experiment. LW: water extract of TCN (leaf); L30%EtOH: 30% ethanol extract of TCN (leaf); L70%EtOH: 70% ethanol extract of TCN (leaf); RW: water extract of TCN (root); R30%EtOH: 30% ethanol extract of TCN (root); R70%EtOH: 70% ethanol extract of TCN (root). Data are mean ± SEM. Each experiment was run in triplicate.

Table 2: Body weight gain, liver weight, and food efficiency ratio.

Group	Body weight			Liver weight	Food intake	Food efficiency ratio
	Initial (g)	Final (g)	Gain (g/day)	(mg/g B.W)	(g/day)	(%)
Normal	22.27 ± 0.26	26.16 ± 0.35	0.11 ± 0.01	30.99 ± 1.71	2.56 ± 0.10	4.47 ± 0.67
HFD-fed mice						
Control	22.92 ± 0.18	32.23 ± 0.75$^{\#\#\#}$	0.27 ± 0.01$^{\#\#\#}$	37.86 ± 3.98$^{\#\#}$	1.91 ± 0.03$^{\#\#\#}$	12.53 ± 0.97$^{\#\#\#}$
GCE200	22.99 ± 0.23	29.09 ± 0.83***	0.19 ± 0.02***	26.12 ± 4.60***	1.94 ± 0.11	10.93 ± 2.99
TCN100	22.94 ± 0.30	30.87 ± 1.08	0.24 ± 1.02	30.83 ± 5.10**	1.99 ± 0.07	12.34 ± 4.39
TCN200	22.91 ± 0.24	29.77 ± 0.62**	0.20 ± 0.01**	27.79 ± 8.87***	1.94 ± 0.06	11.58 ± 3.06

Control: HFD control mice; GCE: GCE 200 mg/kg-treated and HFD-fed mice; TCN100: TCN 100 mg/kg-treated and HFD-fed mice; TCN200: TCN 200 mg/kg-treated and HFD-fed mice. Data are the mean ± SD ($n = 8$). Significance: $^{\#\#\#}p < 0.001$ versus the normal mice and $^{**}p < 0.01$, $^{***}p < 0.001$ versus the HFD control mice.

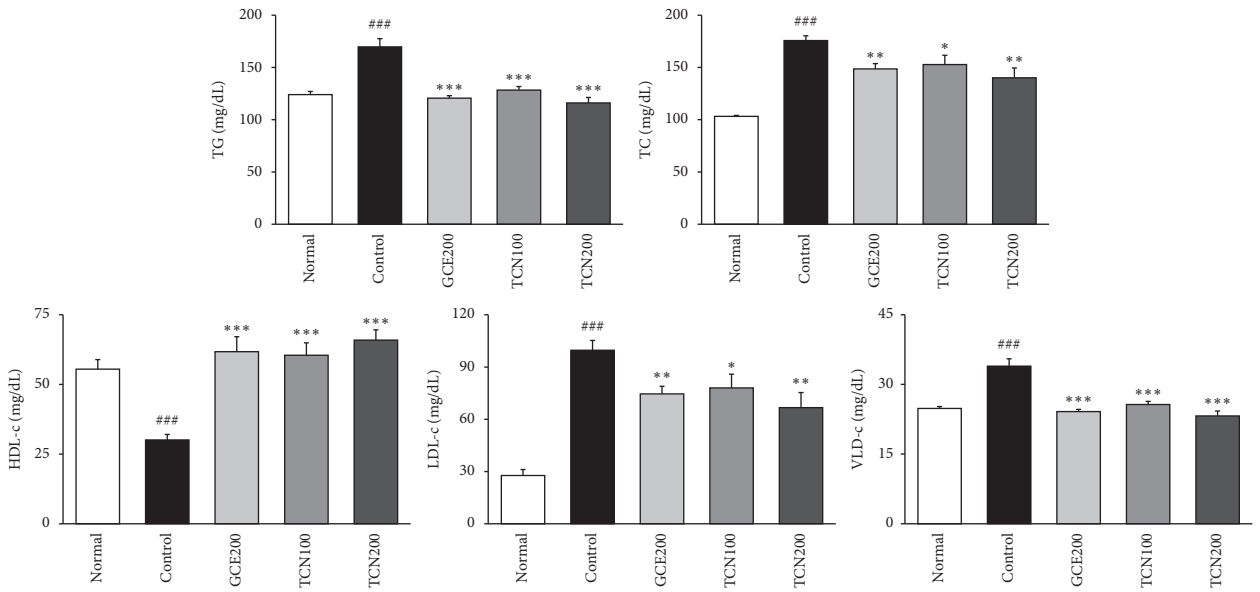
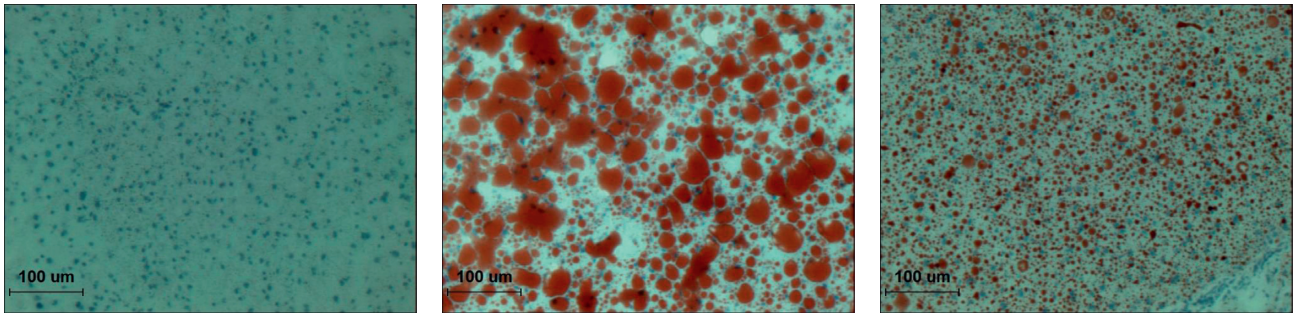


Figure 4: Biochemical analyses. Normal: normal mice; Control: HFD control mice; GCE200: GCE 200 mg/kg-treated and obese mice; TCN100: TCN 100 mg/kg-treated and obese mice; TCN200: TCN 200 mg/kg-treated and obese mice. Data are the mean ± SD ($n = 8$). Significance: $^{\#\#\#}p < 0.001$ versus the normal mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the HFD control mice. TG: triglyceride; TC: total cholesterol; LDL-c: low-density lipoprotein-cholesterol; HDL-c: high-density lipoprotein-cholesterol; VLDL-c: very low-density lipoprotein-cholesterol.



(a) (b) (c)

Figure 5: Continued.

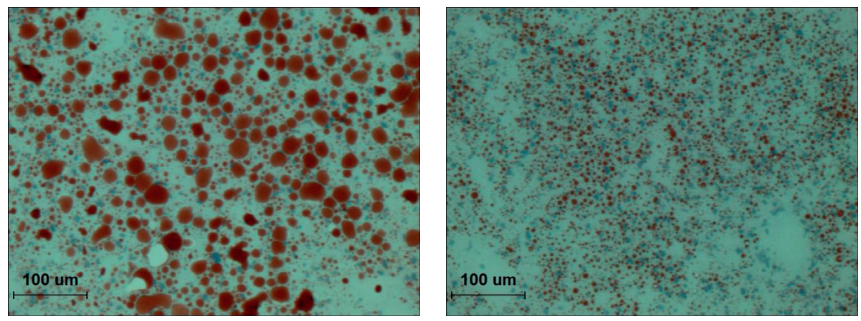


Figure 5: Oil red O staining of hepatic tissue. (a) Normal: normal mice; (b) Control: HFD control mice; (c) GCE200: GCE 200 mg/kg-treated and obese mice; (d) TCN100: TCN 100 mg/kg-treated and obese mice; (e) TCN200: TCN 200 mg/kg-treated and obese mice. ×200.

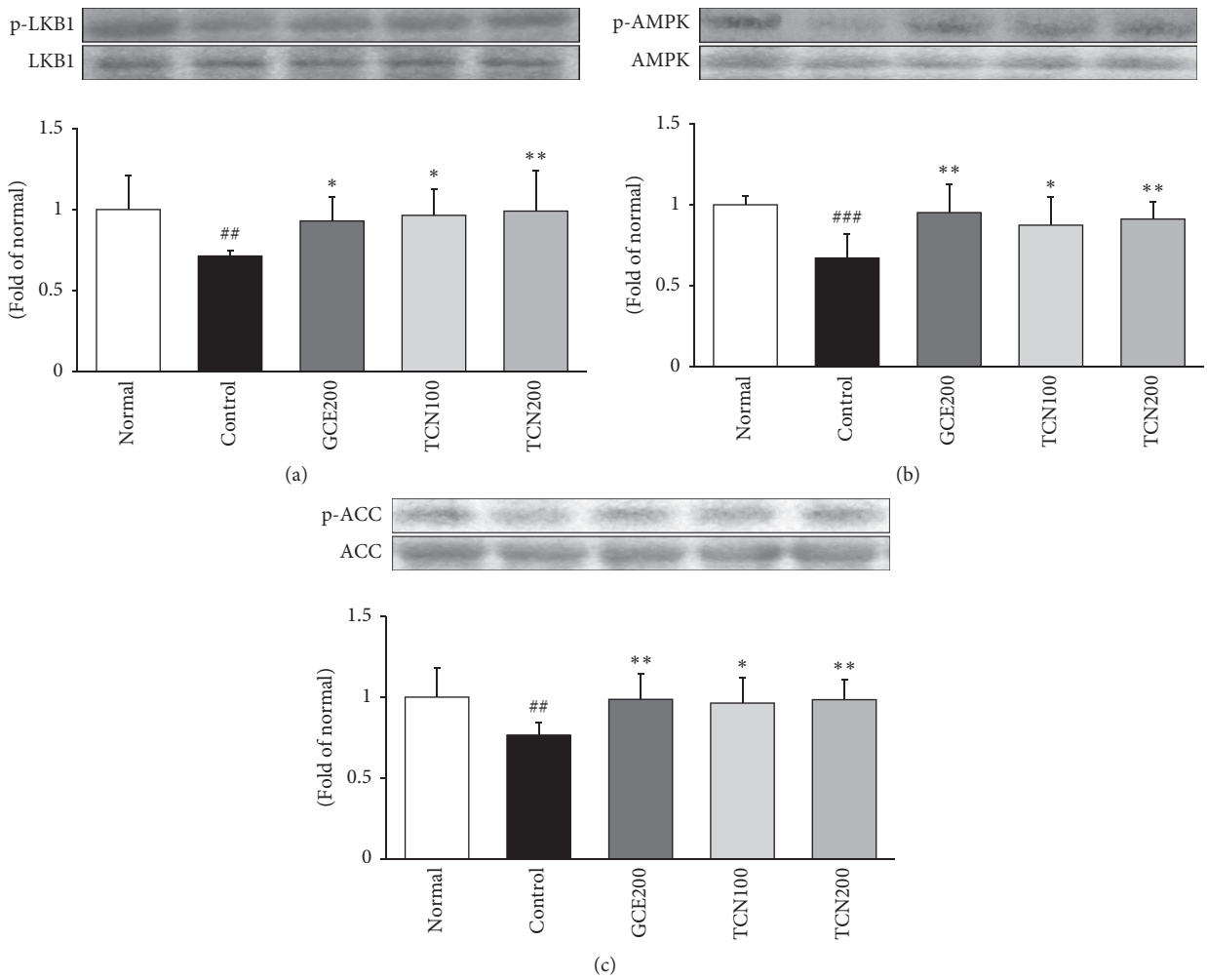


Figure 6: LKB1/AMPK/p-ACC expressions in the liver of mice fed HFD. Western blot analysis of LKB1, phosphorylated LKB1 (p-LKB1), AMPK, phosphorylated AMPK (p-AMPK), phosphorylated ACC (p-ACC), and ACC proteins. Normal: normal mice; Control: HFD control mice; GCE200: GCE 200 mg/kg-treated and obese mice; TCN100: TCN 100 mg/kg-treated and obese mice; TCN200: TCN 200 mg/kg-treated and obese mice. Data are the mean ± SD, ($n = 7$). Significance: $^{*}p < 0.05$, $^{**}p < 0.01$ versus the HFD control mice. The blots shown are representative of three blots from each group of mice.

extract were 164.06 ± 2.51 and 166.51 ± 6.3 μg/mL. Also, IC_{50} value of ABTS radical scavenging activity was 257.12 ± 3.77 and 162.7 ± 0.6 μg/mL. The 70% ethanol extract showed better radical scavenging than that of water extract. Subsequently, assays for the content measurement of the phenolic compound or flavonoid were performed. Total

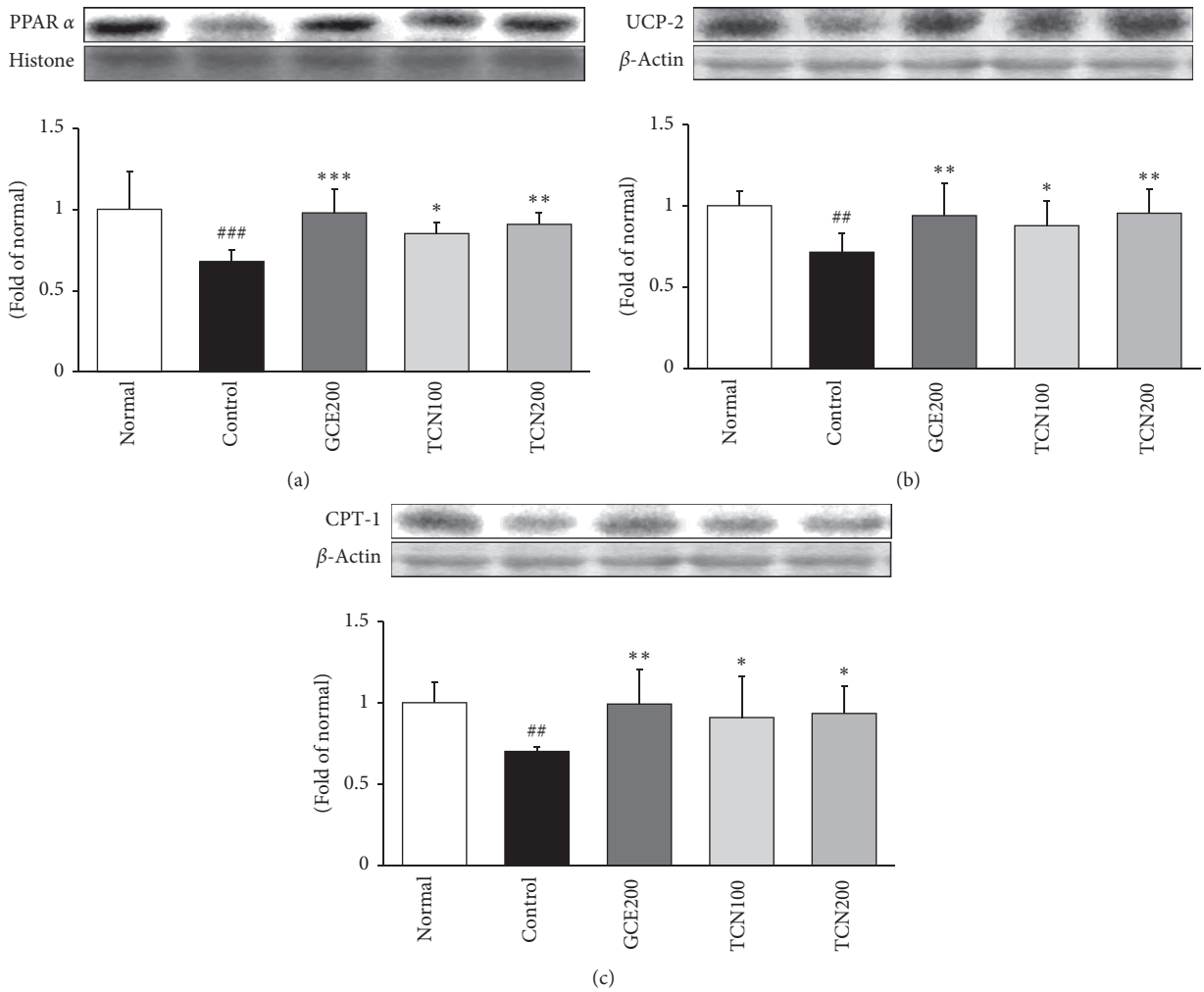


Figure 7: PPARα, UCP-2, and CPT-1 expressions in the liver of mice fed HFD. Western blot analysis of PPARα, UCP-2, and CPT-1 proteins. Normal: normal mice; Control: HFD control mice; GCE200: GCE 200 mg/kg-treated and obese mice; TCN100: TCN 100 mg/kg-treated and obese mice; TCN200: TCN 200 mg/kg-treated and obese mice. Data are the mean ± SD ($n = 7$). Significance: $^{*}p < 0.05$, $^{**}p < 0.01$ versus the HFD control mice. The blots shown are representative of three blots from each group of mice. PPARα: peroxisome proliferator activated receptor α; UCP-2: uncoupling protein 2; CPT-1: carnitine palmitoyltransferase 1A.

phenolic content of 70% ethanol extract (leaf) was the best among the six samples as 25.74 ± 0.17 mg GAE/g of TCN extract. The flavonoid content also had the highest content as 12.53 ± 0.03 mg naringin equivalent (NE)/g of TCN extract. Taken together, 70% ethanol extract (leaf) was most excellent in all antioxidant capabilities. The ex vivo lipolysis assay was performed additionally to compare lipolysis effect according to parts (leaf and root) and extraction methods (water, 30% ethanol, and 70% ethanol) of TCN. As shown in Figure 3, the amount of glycerol spilled of leaf extracted using 70% ethanol extract was 74.63 ± 0.59% (fold of isoproterenol). Leaf sample extracted using 70% ethanol extract among 6 sample extracts was the most outstanding sample in lipolysis effect. As a result, the sample used in this experiment was determined to be 70% ethanol extract (leaf).

Herein, we showed that *Taraxacum coreanum* Nakai (TCN) functioned its antiobesity effect on HFD induced obese mice model and then affected two processes: (1) increased LKB1/AMPK signaling pathway which resulted in the phosphorylation of ACC. P-AMPK activated by LKB1 (AMPK kinases) works to enhance β-oxidation by the phosphorylation of acetyl-CoA carboxylase (ACC), thereby increasing fatty acid degradation. (2) activated expression of PPARα, which is an important regulator of peroxisomal and mitochondrial β-oxidation of fatty acids and lipid metabolism [27].

Table 2 shows body weight gain, food intake, and food efficiency ratio (FER) during the experimental periods. Mean body weights of all groups started without a significant difference between 22.27 g and 22.99 g. Normal mice increased slightly in weight gain during the experimental period of 5 weeks and the level showed a significant difference compared with the HFD control group ($p < 0.001$). GCE200 and TCN200 treated groups were significantly decreased compared with the HFD control group (GCE200; $p < 0.001$, TCN200; $p < 0.01$). Moreover, the food intake and food efficiency ratio among HFD supplied groups did not show a significant difference. As a

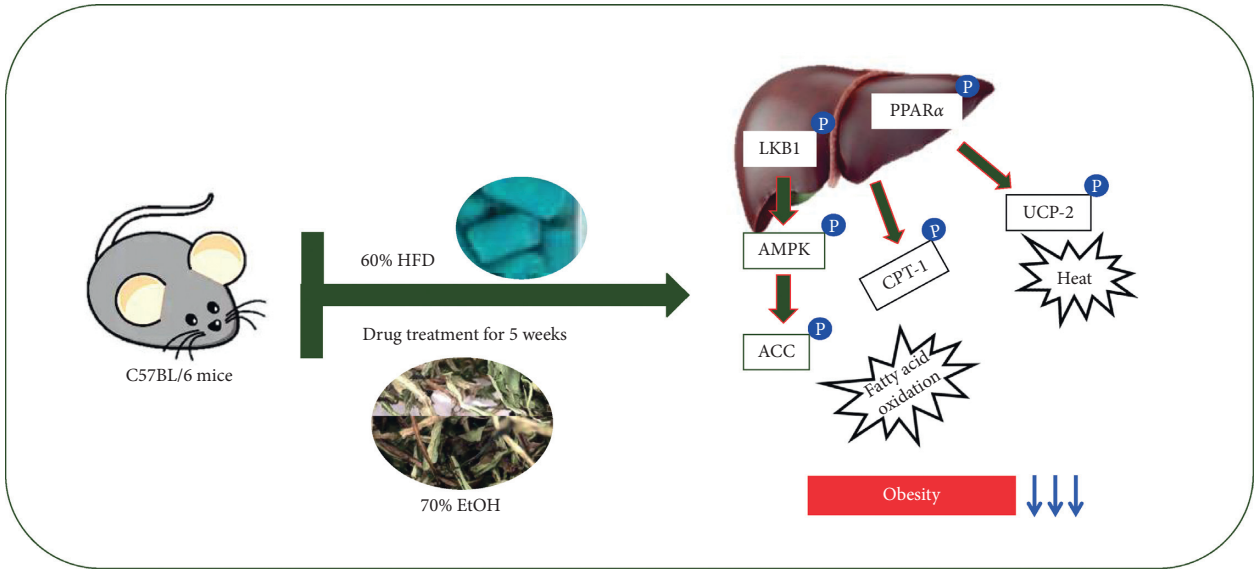


Figure 8: Predicted mechanism after administering TCN in the liver.

result, TCN treatment decreased body weight gain without a significant change of FER. Furthermore, we revealed that TCN-treated HFD mice displayed an outstanding decrease in body weight and improved dyslipidemia; this effect was similar to that of *Garcinia cambogia*. HFD caused the increase in serum TG, TC, HDL-cholesterol, LDL-cholesterol, and VLDL-cholesterol ($p < 0.001$, respectively). The elevated TG and VLDL-cholesterol levels were significantly reduced by all drugs compared with the HFD control mice ($p < 0.001$). Moreover, the augmented TC and LDL-cholesterol levels were significantly lowered by GCE and TCN compared with the HFD control mice (GCE200; $p < 0.01$, TCN100; $p < 0.05$, TCN200; $p < 0.01$), whereas the administration of GCE and TCN significantly elevated HDL-cholesterol level ($p < 0.001$). Particularly, TG and VLDL-cholesterol in TCN200 group showed lower values than those of normal group (Figure 4). TCN treatment significantly reduced serum TG, TC, LDL-cholesterol, and VLDL-cholesterol levels, while it significantly enhanced HDL-cholesterol. In addition, the histological alterations of the liver by Oil Red O staining showed that TCN supplementation obviously reversed the increased lipid accumulation in the liver (Figure 5).

Accumulating evidence of various studies has shown that AMPK plays core roles in regulating lipid metabolism in the liver. Herein, AMPK activation promotes β-oxidation and downregulates fatty acid biosynthesis [28]. Recent studies have reported that AMPK occurs with the phosphorylation of the α-subunit at threonine 172 (Thr172) by upstream kinases (LKB1) because of increase of cellular AMP : ATP ratio. The phosphorlyation of ACC results in decreased malonyl CoA levels. Thereby, a fall in malonyl CoA disinhibits CPT-1, which is a rate-limiting step for the entry into the mitochondria for β-oxidation [29]. This series of pathway is the central effect of AMPK to decrease lipid stores in the liver. The previous reports showed that AMPK phosphorylation levels reduced in the liver after HFD intake [30]. In the current study, we found that p-LKB1/p-AMPK/p-ACC protein levels were significantly decreased in the group fed only HFD compared with the normal group, while the reduced protein levels were significantly increased by TCN treatment (Figure 6). These will increase energy production such as a reduction of energy utilization and an increase of β-oxidation.

Peroxisome proliferator-activated receptors (PPARs) have three different isoforms such as -α, -β, and -γ [31]. The previous studies proposed that PPARα mediated transcription is shown to be co-activated by AMPK [32]. PPARα is involved in fatty acid catabolism including β-oxidation pathway and is most abundant in the liver and adipose tissue [33] activation of the expression of genes associated with the β-oxidation pathway [34]. PPARα expression was positively correlated with the expression of CPT-1. Namely, PPARα regulates the levels of CPT-1, an enzyme essential for β-oxidation of long-chain fatty acids [35, 36]. The present study indicated a similar tendency of PPARα and CPT-1 expressions [37, 38]. Figure 7 reveals that mice fed HFD remarkably reduced the protein expressions of β-oxidation markers such as PPARα and CPT-1. However, TCN treatment significantly reversed the decreased expressions. Furthermore, many studies have reported that PPARα activators upregulate mitochondrial uncoupling protein- (UCP-) 2 expression in the liver. PPARα works to accelerate the thermogenic protein such as UCP-2 as a means for limiting the production of ROS and regulating lipid metabolism [28, 39]. Our result also showed that UCP-2 was significantly upregulated by TCN treatment via PPARα activation.

Besides, several inflammatory markers have been associated with both obesity and risk of adverse outcomes in obesity-associated diseases [40]. Accordingly, the mechanism study underpinning the triggers of such inflammatory responses after TCN treatment could offer another strategy to ameliorate the risk of obesity-associated disease.

5. Conclusions

In this work, we studied the antiobesity effect with the 70% ethanol root extract selected by comparing and analyzing the effects of the extraction method of the leaves and roots of *Taraxacum coreanum* Nakai. Taken together, the current study suggests that TCN supplementation regulates lipid metabolism via LKB1-AMPK signaling pathway and promotes β-oxidation by PPARα, as shown in Figure 8. Hence, TCN may have potential remedy in the prevention and treatment of obesity. However, it is judged that additional studies are needed to determine which effective components of TCN exhibited antiobesity effect.

Data Availability

The datasets used and analyzed in this work are available from the corresponding author upon reasonable request.

Ethical Approval

All animal procedures were approved by the Animal Research Ethics Committee of the Daegu Haany University (Permit number: DHU2020-017).

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Authors' Contributions

M. J. Kim performed the experiments and was responsible for data acquisition. M. R. Shin performed data analysis and drafted the article. J. G. Han provided the test material. H. J. Park and S. S. Roh were responsible for the design of the study. All authors participated actively in carrying out and improving the study, and all authors approved the submission of this article. M. R. Shin and M. J. Kim contributed equally to this work.

Acknowledgments

This research was conducted as a healthcare natural product industry promotion project supported by Chungbuk Technopark and carried out with the support of the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIT) (No. 2018R1A5A2025272).

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