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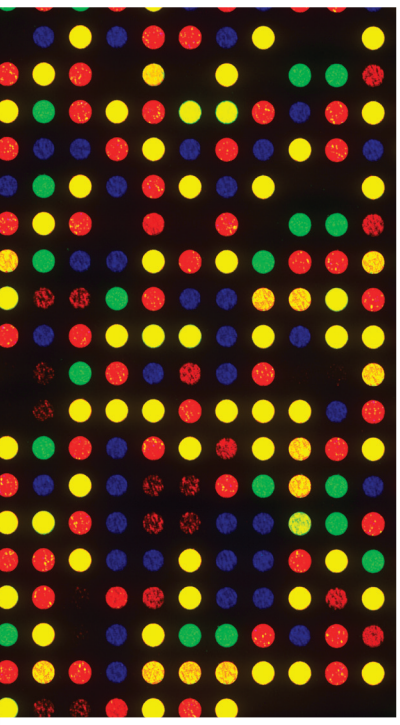
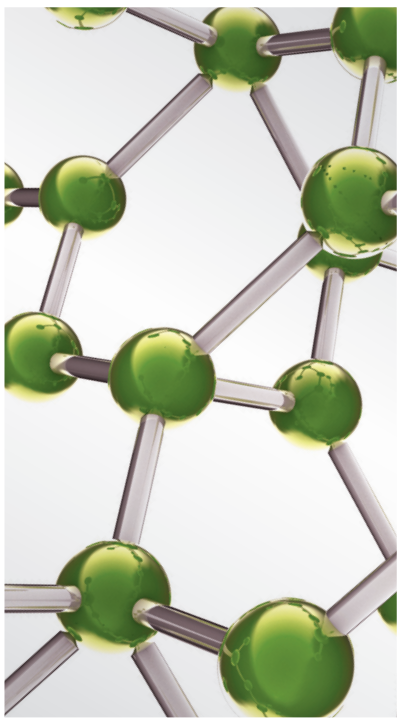
Evidence-Based Medicinal Plants for Modern Chronic Diseases

Guest Editors: Yong-Ouk You, James William Daily III, Tong Ho Kang, Young-Rae Lee, and Serkan Selli



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Editorial Board

Contents

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Editorial

Evidence-Based Medicinal Plants for Modern Chronic Diseases

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Plants have been used as medicines throughout recorded human history by most, if not all, ethnicities and cultures. The knowledge of how to use plants for medicine has been passed down through generations by many means including word of mouth and ancient pharmacopoeias. Modern science has devoted considerable research to characterizing the efficacy and mechanisms of action of many medicinal plants, but this remains an area of vast research potential. This special issue of Evidence-Based Complementary and Alternative Medicine is devoted to publishing the most important recent scientific advances in describing the efficacy, safety, and mechanisms of action of medicinal plants for modern chronic diseases.

Global environmental and human lifestyle changes have dramatically increased the incidence of chronic diseases in the 21th century. Modern chronic diseases cause great human suffering, health care expenses, and lost productivity despite the advances of modern medicine. Evidence-based medicinal plants have great potential as safe and effective alternative medicines for modern chronic diseases. Although medicinal plants are an established part of complementary and alternative medicine with efficacy supported by thousands of years of clinical experiences in treating human diseases, many lack scientific support, since medicinal plants are the products of experience-based medicine. Nevertheless, numerous scientific experiments support the efficacy of medicinal plants.

In this special issue, 3 review articles and 11 research articles were published addressing the safety and efficacy of evidence-based medicinal plants for treating modern chronic diseases, such as diabetes, hypercholesterolemia, obesity, major depressive disorder, chronic infection by antibiotic resistant bacteria, postmenopausal osteoporosis, rheumatoid arthritis, allergy, asthma, chronic inflammation, and myasthenia gravis. These studies include clinical trials with rigorous statistical analyses, phytochemical, pharmacological, and toxicological activity tests in animal models and cell-based studies, analytical characterization of bioactive components in medicinal plants, and investigation of action mechanisms of medicinal plants, all of which are essential for complementary and alternative medicine to have a solid evidence-based foundation.

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Research Article

Crude Ethanol Extract of *Pithecellobium ellipticum* as a Potential Lipid-Lowering Treatment for Hypercholesterolaemia

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If left untreated, hypercholesterolaemia can lead to atherosclerosis, given time. Plants from the Fabaceae family have shown the ability to significantly suppress atherosclerosis progression. We selected four extracts from *Pithecellobium ellipticum*, from the Fabaceae family, to be screened in a 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) assay. The ethanol extract, at a concentration of 500 μg/mL, exhibited superior inhibition properties over the other extracts by demonstrating 80.9% inhibition, while 0.223 μg/mL of pravastatin (control) showed 78.1% inhibition towards enzymatic activity. These findings led to the fractionation of the ethanol extract using ethyl acetate : methanol (95 : 5), gradually increasing polarity and produced seven fractions (1A to 7A). Fraction 7A at 150 μg/mL emerged as being the most promising bioactive fraction with 78.7% inhibition. FRAP, beta carotene, and DPPH assays supported the findings from the ethanol extract as it exhibited good overall antioxidant activity. The antioxidant properties have been said to reduce free radicals that are able to oxidize lipoproteins which are the cause of atherosclerosis. Phytochemical screenings revealed the presence of terpenoid, steroid, flavonoid, and phenolic compounds as the responsible group of compound(s), working individually or synergistically, within the extract to prevent binding of HMG-CoA to HMG-CoA reductase.

1. Introduction

Hypercholesterolaemia is the main contributor to numerous cardiovascular diseases (CVD) with substantial data from studies to support this claim [1, 2]. It is predicted that over a billion deaths will be due to CVD by the earlier half of the 21st century [3]. Mackey and Co. (2007) estimated that approximately 18.1 million deaths were due to cardiovascular disease in 2010 and that we will see an increase of 33.7% by 2030.

Hypercholesterolaemia can lead to atherogenesis. Atherosclerosis is the primary risk factor for coronary heart disease and affects the peripheral arteries and cerebral circulation. Studies relating to cancer and total cholesterol levels have shown that the total cholesterol level is the main risk factor for disease. However, most of the cholesterol is carried in the plasma (60–70%), in the form of low density lipoproteins (LDLs) and it is this which is the main risk factor for disease [4, 5].

Overproduction of reactive oxygen species has been strongly associated to the development of oxidation related conditions like atherosclerosis and cardiovascular diseases [5, 6]. Atherosclerosis begins with the transmigration of oxidized LDLs to the intima (the subendothelial space) which cause injuries to endothelial cells. The injury is described as cellular damage and one result of this type of damage is the loss of

function of the cell [4, 7, 8]. Endothelial cells respond to the injury by becoming sticky which alerts the macrophages to ingest the oxidized LDLs. These macrophages are then overloaded with oxidized LDLs; the appearance of the lipids in the macrophages gives them a foamy appearance; hence they are given the name "foam cells." The accumulation of foam cells leads to the formation of fatty streaks [7]. After some time atheroma takes place, the smooth muscle cells migrate and further restrict the blood flow leading to atherosclerosis. Complex lesions occur with the advancement of inflammatory responses, necrotic core formation, and death of foam cells. Finally, thrombosis occurs when fibrous caps rupture [4, 7–9]. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) is the rate limiting factor for the formation of mevalonate in the cholesterol biosynthesis pathway. This enzyme has been chosen as one of the pharmacological targets to control the production of cholesterol *de novo*. Statins are widely used to treat hypercholesterolaemia because of their ability to exhibit superior suppression of lipid synthesis at a nanomolar scale [10]. The hydrophobic group of a statin is suggested to be the core reason for this nanomolar inhibition constant value, $K_i \sim 1 \times 10^{-9}$ M, while the substrate's K_m value is $\sim 10^{-5}$ M [10, 11]. This gives the enzyme a 10,000-fold higher binding affinity towards statins compared to the substrate [10].

Generally, a statin's heptatonic acid functional group resembles the HMG moiety of the substrate at the molecular level. Istvan and Deisenhofer (2001) proved that the heptatonic acid can be easily competed with and bound at the O5-hydroxyl group of the HMG-like moiety and that it replaced the thioester oxygen atom of the substrate. The conformational flexibility has sterically hindered the substrate from binding with the enzyme. Concurrently, the statin structure extends into the narrow pantothenic acid-binding pocket moiety of coenzyme A which eventually blocks the coenzyme A of the substrate [11, 12]. This is why statins are capable of competing with the substrate.

Besides effectively interfering with cholesterol biosynthesis via inhibiting the reductase enzyme, statins are gaining attention for their antiatherosclerosis effect due to their antioxidant ability. A few of the statins' antioxidant defense mechanisms include reducing lipoprotein cholesterol which reduces substrate oxidation, attenuating development of superoxide anions in endothelial cells to prevent oxidation of LDLs and preventing diffusion of free radicals produced from oxidative stress towards the lipoprotein core [13]. Fluvastatin sodium has shown such ability [14].

Currently available cholesterol lowering drugs such as statins do pose a risk of myopathy by depressing ubiquinone Q_{10} biosynthesis, rhabdomyolysis, and other considerable side effects like comorbidities and several noncardiac effects (gratuitous thoughts, short temper), psychiatric events, and even impairment of the central nervous system [15, 16]. This opens a need for more research into natural product such as plants from the Fabaceae family [2]. *Pithecellobium* sp. shrubs and trees are distributed in the secondary forests and riverbanks of Tropical Asia and Subtropics of America [17–22]. Approximately 200 species have been identified from this genus [23]. This genus exhibited antifungal [24–27], antibacterial [25, 27], anti-inflammatory [28], antiallergic effects [28], insect antifeedant [29], piscicide [30], and antioxidant activity [31–34].

Pithecellobium ellipticum is also known as *Archidendron ellipticum* (Blume) I. C. Nielsen and commonly called kabau (*Jering tupai*) in Malaysia [22]. Wild fruits of *P. ellipticum* are enjoyed by Malaysians in salad and cooking due to their delicious flavor [10]; they are used as an astringent in Indonesia [21], as a remedial shampoo in Java, and as fish poison [35]. This particular plant has not been thoroughly studied for its lipid-lowering properties. A *T. indica* ethanol extract from the same family has been reported to decrease non-HDL cholesterol by 73%, triglycerides by 60%, and even increase HDL cholesterol up to 61% [2, 9]. Besides this, radical scavenging tests disclosed that in some *in vivo* studies the extract improved antioxidant abilities in an animal model [9]. These findings indicate that the properties within the extract are capable of reducing atherosclerosis. The aim of this study is to assess the HMG-CoA reductase inhibition ability, antioxidant properties, and screening for the presence of secondary metabolites from the crude extract of the leaves of *P. ellipticum* (code: UNMC 35L).

2. Results and Discussion

All of the extracts prepared only with the extract and buffer showed absorption at 340 nm in the spectrophotometric analysis (results not shown). This is not a surprise as crude extracts are expected to contain numerous compounds. Therefore, all extracts require colour correction to ensure all possible interfering absorbances contributed by the extracts in the HMG-CoA reductase assay are minimized. This is also important as NADPH molecules absorb light at the same wavelength and their oxidized form $NADP^+$ does not [36]. This is the reason why this enzymatic assay was analyzed at 340 nm and requires minimal interference at 340 nm from the samples analyzed.

Table 1 shows the specific activity and percentage of inhibition of all the crude extracts. Any specific activity obtained lower or near the pravastatin range is considered to have the ability to inhibit HMG-CoA reductase activity. Values near or above the negative control's specific activity implies that the particular sample either is a very weak inhibitor or may elevate the enzymatic activity. The ethanol (E) extract stood out as the extract with the most potential followed by the water (W), ethyl acetate (EA), and finally hexane (H) extract with the least inhibitory capability. The specific activity of the E extract was calculated as being 21.8 times lower than that of the H extract, making it a superior inhibitor to the H extract.

The difference in inhibition competency between E and W extracts was 17.8%. This might be due to the concentration of active compounds present within the E extract being greater or that the competition for the active moiety favors the E extract over the W extract. Another probability would be the binding locations; bonds and presence of inhibitor active moiety involved in the inhibitor-enzyme complex are

Table 1: Percentage of inhibition of UNMC 35L crude extract.

Sample	Specific activity (units/mg protein)	% inhibition
H crude extract[A]	5.668 ± 2.9^{a}	0
EA crude extract[A]	3.638 ± 3.0^{b}	0
E crude extract[A]	0.260 ± 0.6^{ac}	80.9
W crude extract[A]	0.503 ± 0.5^{ad}	63.1
Positive control (pravastatin)[B]	0.298 ± 0.3^{abe}	78.1
Negative control	1.363 ± 0.5^{af}	0

Values with the same letter are significantly different $P < 0.05$ by Tukey's multiple comparison tests.
Results are demonstrated as average ± standard deviation ($n = 2:3$ different experiments).
[A]Crude extracts are prepared at a concentration of 500 μg/mL.
[B]Pravastatin is prepared at a concentration of 0.223 μg/mL.

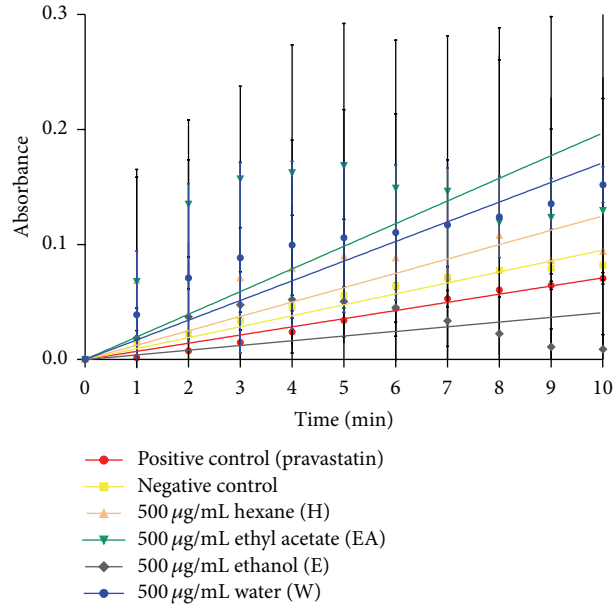

Figure 1: HMG-CoA reductase specific activity of crude extracts.

Table 2: Percentage of inhibition of UNMC 35L ethanol fractions.

Fractions	Specific activity (units/mg protein)	% inhibition
1A[A]	2.744 ± 0.9	0
2A[A]	0.812 ± 0.6	18.0
3A[A]	0.974 ± 0.6	1.6
4A[A]	0.552 ± 1.0	44.2
5A[A]	0.829 ± 0.6	16.3
6A[A]	0.520 ± 0.4	47.5
7A[A]	0.211 ± 0.2	78.7
Positive control (Pravastatin)[B]	0.195 ± 0.3	100.0
Negative control	1.234 ± 0.8	0

All values are not significantly different $P < 0.05$ by Tukey's multiple comparison tests.
Results are demonstrated as average ± standard deviation ($n = 2:3$ different experiments).
[A]1A to 7A fractions are prepared at a concentration of 150 μg/mL.
[B]Pravastatin is prepared at a concentration of 0.223 μg/mL.

sturdier and not easily broken. For example, the constituents in the E extract might have attached firmly at the active pocket, while constituents within the W extract bind weakly instead. Additionally, a different pharmacological activity is contributed by an inhibitor's active moiety, such as that which is present in Pitavastatin [37]. The inhibition value of the E extract at 80.9% was comparable to pravastatin (78.1%) even though the concentration used was distinctively huge. It is essential to realise that even though 500 μg/mL is considered a high concentration, this is the concentration of the crude extract and the active compound within the extract is hypothesized to express this suppression ability at a much lower concentration. Another key finding from Figure 1 is that the W extract which appeared to have some suppressing activity (0.503 units/mg protein) became noticeably weaker over the duration of 10 minutes. The line graph of the W extract is above the negative control line on the graph. This means that the concentration of the active compound within the W extract is exhausted and the activity is reversible, allowing the natural substrate to take over once again and bind with the HMG-CoA reductase.

The finding attained from the extract inhibitory screening has led to the fractionation of the ethanol extract as it had the highest suppressing strength. The resulting fractionation produced 7 fractions labeled 1A–7A. Fractionation was carried out with increasing solvent polarity. Fraction 7A (consisting of the last thirteen vials from fractionation) was found to be the most promising fraction in influencing the HMG-CoA reductase activity (Table 2 and Figure 2).

Fraction 7A had the highest inhibition activity (78.7%) with a specific activity of 0.211 ± 0.2 units/mg protein (Figure 2(a) is expended for viewing purpose). Twofold dilutions of 7A were prepared to reveal the effectiveness of this fraction. It showed an apparent trend with increasing solvent polarity used for fractionation; the fractions achieved higher inhibitory properties as seen from fractions 3A to 7A. In contrast, 5A did not comply with the trend by disclosing only 16.3% inhibition.

Statistical analysis indicated that twofold dilution samples of 7A labeled 7A-1 to 7A-4 actually have no significant difference between them or pravastatin (Figure 2(b)), but pravastatin concentration was kept at 0.223 μg/mL. Despite this, mL7A-2 exhibited higher inhibition activity compared to 7A-1 which was not expected. 7A-3 and 7A-4 fractions did not express any distinct difference to 7A-1. Six different phytochemicals, including saponins, flavanoids, tannins, phenols, terpenoids, and steroids, were screened for, to find out which secondary metabolites were present. Phytochemical screening results for *P. ellipticum* were in agreement with the isolation of saponins from leaves that was reported earlier by Beutler et al. [18]. The hexane extract consisted of flavanoids and terpenoids, while the EA extract had tannins, terpenoids, and steroids. The E extract had positive reactions in all tests except for tannins. Meanwhile, flavanoids

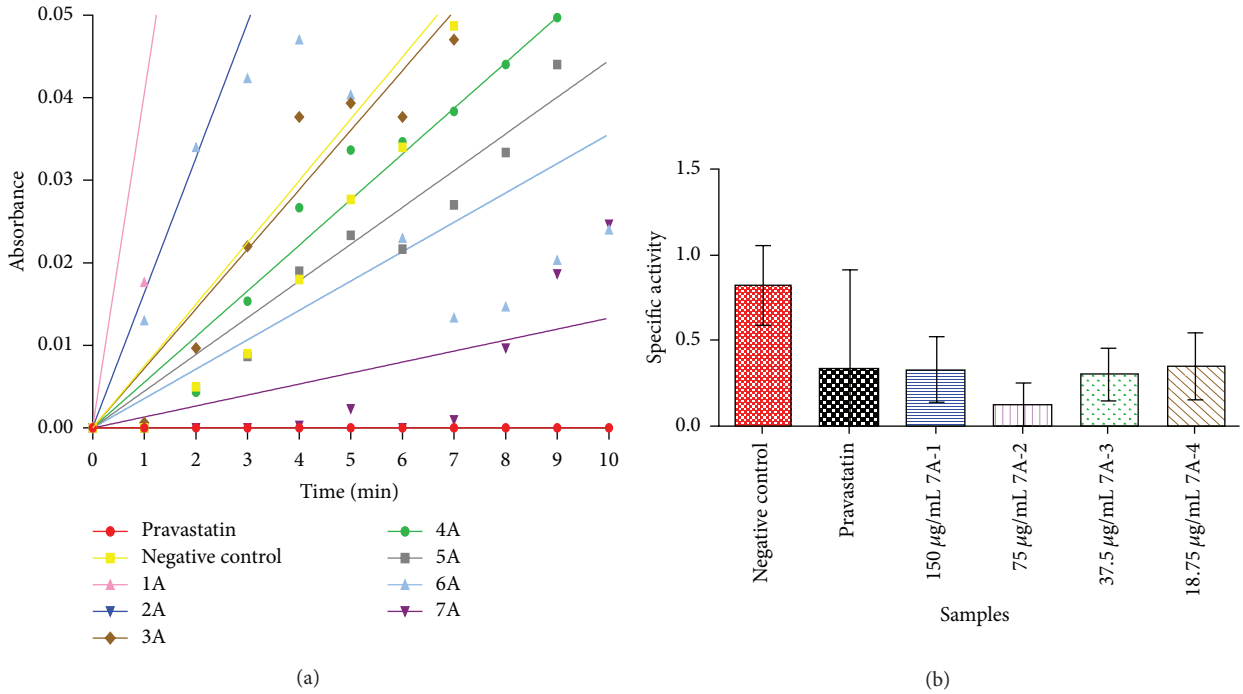

Figure 2: (a) HMG-CoA reductase specific activity on 35L E 1A–7A fractions; (b) HMG-CoA reductase specific activity on UNMC 35L 7A twofold dilution. There is no significant difference with $P < 0.05$ in Tukey's multiple comparison tests. Results are demonstrated as average ± standard deviation ($n = 2 : 3$ different experiments).

Table 3: Phytochemical screenings of UNMC 35L crude extracts.

Types of screenings	Leaves extracts			
	H	EA	E	W
Saponins	−	−	+	+
Flavonoids	+	−	+	−
Tannins	−	+	−	+
Phenolic contents	−	−	+	+
Terpenoids	+	+	+	+
Steroids	−	+	+	−

Results expressed as + and − indicate, respectively, the presence and absence of secondary metabolites within the crude extracts.

and steroids were absented in the W extract. There is a possibility that the flavonoid, phenolic content, terpenoid, and steroid compounds are responsible (Table 3) for effective competition with the substrate, for binding at or blocking the substrate from binding at the enzyme's narrow pocket moiety. Saponins are an exception in this enzymatic discussion because saponins are categorized as a bile acid sequestrant that works within the intestines [38]. Thus, this eliminates the possibility of saponins suppressing the reductase activity in the inhibitory assay that was conducted. Saponins have been reported to elevate the activity and expression of hepatic lipase located at the liver and inhibit the mRNA and expression of fatty acid synthase to reduce lipids in a hyperlipidaemic animal model [38].

Lipid peroxidation involves the forming and spreading of reactive oxygen species (ROS). Lipid hydroperoxides are produced in the presence of oxygen in lipid oxidation. ROS lead to oxidative stress that also induces atherosclerosis and which makes the antioxidant analyses a very important part of our investigation, especially in the case of the E extract in determining whether it truly possesses antioxidant properties. Three antioxidant assays, FRAP, β-carotene bleaching assay, and DPPH, were conducted on the crude extracts and recorded in Table 4. Lipid peroxidation began with Fe^{3+} and tripyridyl triazine (TPTZ). Extracts with antioxidant properties successfully reduce Fe^{3+} to Fe^{2+}. With regards to the FRAP assay values, the E extract had the highest antioxidant activity with 0.843 mmol $FeSO_4$ equivalent/L and followed by the EA, W, and lastly H extract.

Inhibition of oxidation in the β-carotene bleaching assay samples are arranged in decreasing order: W > H > E > EA. EC_{50} of the W extract was 4.631×10^{-5} μg/mL which appeared to be more effective than quercetin and only two times weaker than Trolox. This reveals that the potential antioxidant compound isolated from the W extract might be even more potent than Trolox. Both H and extracts however demonstrated roughly similar abilities in protecting the β-carotene compound; meanwhile the H extract proved to be a poorer antioxidant extract in the FRAP assay with 16.96 times lower antioxidant activity than the E extract.

DPPH results have wider IC_{50} values ranging between 460.930 and 12.738 μg/mL. The EA extract showed the greatest free radical scavenging activity with an IC_{50} of 12.738 μg/mL and this value does not differ much from that of the E extract (approximately 1.09 times more powerful). Trolox (1.09 μg/mL) was 12.7 times stronger than the E

Table 4: Different types of antioxidant analyses.

Crude samples	Type of analysis			
	FRAP		β-Carotene	DPPH
	mmol $FeSO_4$ equivalent/L	R^2	EC_{50} (μg/mL)	IC_{50} (μg/mL)
Hexane	14.301 ± 0.0^{ad}	1.0000	$7.188 \times 10^{-3\,a}$	460.930 ± 0.2^{a}
Ethyl acetate	1.093 ± 0.7^{ab}	0.9812	$1.775 \times 10^{-2\,a}$	12.738 ± 0.4^{ab}
Ethanol	0.843 ± 0.1^{ac}	0.9453	$1.059 \times 10^{-3\,a}$	13.830 ± 0.0^{ac}
Water	$1.240 + 0.3^{ad}$	0.9964	$4.631 \times 10^{-5\,ab}$	145.403 ± 0.1^{ad}
Quercetin	0.003 ± 0.1^{ade}	0.9992	$1.132 \times 10^{-4\,ac}$	0.110 ± 0.0^{ae}
Trolox	0.010 ± 0.0^{adf}	0.9906	$9.730 \times 10^{-5\,ad}$	1.090 ± 0.0^{af}

Values in the same column that are followed by the same letter are significantly different $P < 0.05$ by Tukey's multiple comparison tests.
Results are expressed as means ± S.D ($n = 2:3$ different experiments).

extract. The H extract was the least potent of all (IC_{50} = 460.930 μg/mL). The ethyl acetate and ethanol extracts were ranked as the most and second potent-free radical scavengers, respectively, while the W extract belongs to the intermediate antioxidant group obtaining an IC_{50} of 145.403 μg/mL. The H extract had the lowest free radical quenching capacity. An interesting note is that the E extract was the most potent inhibitor in the HMG-CoA reductase assay and has consistently exhibited strong antioxidant activity in the three assays conducted. However, other extracts demonstrated potent antioxidant activity in certain assays but not in others. No consistently low values were found in the assays for the poorly active extracts except for the H extract which was the weakest in both FRAP and DPPH assays. The ethyl acetate extract had the greatest activity in both FRAP and DPPH assays meanwhile the W extract was, strongest in the β-carotene bleaching assay.

Their antioxidant properties are expected to correlate with the results of the phytochemical screenings. This would be due to phenolic compounds and their derivatives that are capable of preventing auto-oxidation. Phenolic compounds are bioactive substances that are recognized for their antioxidant ability. From the correlation of results, components from 7A fraction might contain antioxidant properties in addition to its enzyme inhibition ability.

This antioxidant compound from the active extracts may potentially be capable of affecting both free radicals from causing oxidative stress and the enzyme activity, reducing atherosclerosis progression in hypercholesterolaemic patients as statins do. Statins have been tested in various antioxidant assays to demonstrate their ability in reducing atherosclerosis in ways other than directly inhibiting HMG-CoA reductase [39, 40]. The antioxidant compound(s) from this plant that is responsible for interrupting HMG-CoA reductase in the assay might behave similar to statins. Reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex could be caused by chain termination of peroxyl and hydroxyl radical formation caused by the antioxidant compounds, especially in the active extract. Lovastatin and simvastatin have been shown to stimulate formation of Fe^{2+} [39].

Statins which are synthesized or naturally isolated have demonstrated different lipid peroxidation and scavenging activity. Another possibility would be that the functional group present within the compound itself from *P. ellipticum* exerts an antioxidant ability like in the case of the enol moiety conjugated to the indole ring of fluvastatin sodium [14]. The hydrophobic side chain of the potential active compound is a contributing factor to the potency of the binding affinity towards HMG-CoA reductase. Furthermore, the same antioxidant compound could also donate an electron to the free radicals through scavenging activity as reported in the cases of simvastatin and rosuvastatin [39].

3. Materials and Methods

3.1. Plant Material and Extraction Procedure. Leaves of *P. ellipticum* were collected from Sungai Congkak Forest, Malaysia. The plant authenticity was verified and herbarium specimen deposited at Forest Research Institute Malaysia (FRIM) with voucher number of PID160610-06. A portion of plant material (232 g) was macerated with hexane overnight in a ratio of 1 : 8 (plant material : solvent) and extracted three times. This extraction process was subsequently repeated using ethyl acetate, ethanol, and finally water. Combined extracts yielded H (0.46%), EA (1.48%), E (1.72%), and W (4.83%).

3.2. Qualitative Analysis by UV-Vis Spectrometer. All the crude extracts were prepared at 500 μg/mL and scanned using PerkinElmer, Lambda 25 model to monitor colour correction at 340 nm. Extracts showing chromophore and auxochrome at 340 nm region were prepared for colour correction.

3.3. HMG-CoA Reductase Assay. HMG-CoA reductase assay kit was purchased from Sigma Malaysia. The kinetic activity measurements were carried out in triplicate and determined by employing the spectrophotometric method from Sigma CS 1090 with minor modifications. 500 μg/mL (crude extracts) and 150 μg/mL and its twofold dilutions (fractions) were dissolved in dimethyl sulphoxide, DMSO (Labscan). Assay kit components were incubated for 15 minutes at 37°C prior to analysis. A different amount of 1 X assay buffer was added (blank: 183 μL, inhibitor: 181 μL, activity: 182 μL, samples: 182 μL, and sample correction: 199 μL), 1 μL DMSO was added to the blank, inhibitor, and activity wells, 1 μL of

pravastatin into inhibitor wells, 4 μL of NADPH, and 12 μL substrate were added into all wells and incubated for another 5 minutes. Finally, 2 μL of reductase enzyme was added. Samples were prepared in duplicate. The kinetic study was analyzed at 340 nm and at 37°C with the shake mode on for 10 minutes using Varioskan Flash (Thermofisher). Specific activity was expressed in unit/mg protein:

$$\frac{\text{unit}}{\text{mg protein}} = \frac{\left(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{control}}\right) \times \text{TV}}{12.44 \times V \times 0.6 \times \text{LP}} \quad (1)$$

12.44 = ε^{nM}—the extinction coefficient for NADPH at 340 nm is 6.22 m/Mcm. 12.44 represents two NADPH consumed in the reaction. TV: total volume of the reaction in mL (0.2 mL for plates), V: volume of enzyme used in the assay (mL), 0.6: Enzyme concentration in mg-protein (mg protein)/mL that is 0.55–0.65 mg protein/mL, and LP: light path in cm (0.55 for plates).

3.4. Fractionation of the Ethanol Extract. The ethanol extract (1.4 g) was chosen for further fraction. Silica 60 (Merck, 0.063–0.200 mm) was used as the packing material with the ratio of 1 g sample : 50 g silica. Starting solvent system was ethyl acetate : methanol (95 : 5) increasing the methanol gradually, followed by ethanol, 1% of acetic acid, and finally 2% of acetic acid in ethanol. A total of 113 fractions were collected and combined into seven major fractions (35L E 1A to 7A) after thin layer chromatography profiles were determined with different ratios of chloroform-methanol, ethyl acetate-methanol, and butanol-acetic acid-water solvent systems using detection under UV light, iodine vapor, vanillin, ferric chloride, and antimony (III) chloride spray reagents. All fractions (150 μg/mL and its two-fold dilutions) were later subjected to the HMG-CoA reductase assay to determine the active fractions.

3.5. Phytochemical Screenings. Several tests were performed to confirm the presence and identity of secondary metabolites within the crude extracts: saponins, flavonoids, tannins, phenolics, steroids, and terpenoids. Approximately 12.5 mg of each extract was prepared.

3.5.1. Test for Saponins (Frothing Method). Each extract was dissolved in 5 mL of purified water in the test tube and covered with cork according to method by H. A. Ibrahim and H. Ibrahim. The samples were sonicated for 15 minutes at 40°C. Samples that had particles were filtered before being shaken vigorously for thirty seconds and then left for another 45 minutes. Persistent froth revealed the presence of saponins [41].

3.5.2. Test for Flavonoids (Modified Shinoda Test). Flavonoid analysis was prepared following the Modified Shinoda Test [42]. All extracts were dissolved in 5 mL of DMSO. 3-4 cm of magnesium turnings and 6 drops of 36% concentrated HCl were added. Various colours such as orange, pink, and red to purple represent flavones, flavonols, 2,3-dihydro derivate, and xanthone, respectively.

3.5.3. Test for Tannins and Phenolic Content (Gelatin Precipitation and Ferric Chloride). This test by Mojab and colleagues used two reagents: gelatin solution for protein precipitation and ferric chloride to confirm the presence of phenolics. Extracts were dissolved in 5 mL purified water and sonicated for 15 minutes at 40°C. The volume was divided into three portions—one for control, gelatin precipitation, and phenolics. White fog or precipitation with 5 mL of 1% gelatin indicates the presence of tannins, while a change from colourless to brownish-green or a blackish-blue colour after 6 drops of 1% ferric chloride indicates the presence of phenolics [43].

3.5.4. Test for Steroids/Terpenoids (Salkowski Test). Both steroid and terpenoid tests were accomplished using the Salkowski test [44, 45]. Every extract was dissolved in 5 mL of DMSO and sonicated for 15 minutes at 40°C. One mL of solution was added to 1 mL chloroform and an equal volume of concentrated sulphuric acid was slowly added down the side of the test tube. The upper layer changed to a red colour and the sulphuric acid layer displayed a yellow colour with green fluorescence. A reddish-brown colour observed at the interface corresponded to the presence of terpenoids, while a blue or green interface indicated the presence of steroid compounds.

3.6. Antioxidant Assays Evaluation. All crude extracts were dissolved in DMSO. Quercetin and trolox were used as standards.

3.6.1. Ferric Reducing Ability Power (FRAP) Assay. The reagents were prepared according to the reports of Benzie and Strain. 180 μg/mL fresh FRAP was pipetted and followed by 20 μL of the sample (in triplicate). The microplate was incubated for 90 minutes prior to being monitored spectrophotometrically at 593 nm through a Dynex Microplate Reader. Concentrations ranging from 1 to 125 mM of ferric sulphate concentrations were prepared for calibration. FRAP values were expressed as the concentration of the extracts that showed an absorbance value equal to 1 mM of $FeSO_4$ [46].

3.6.2. β-Carotene Bleaching Method. This experimental procedure was slightly modified from Miller [47]. Two mg of β-carotene was dissolved in 10 mL $CHCl_3$. Then 2 mL was pipetted into a 100 mL round bottom flask and $CHCl_3$ was later removed. Subsequently, 40 mg purified linoleic acid; 400 mg Tween 80 emulsifier; and 100 mL distilled water were added and shaken. Readings were measured at 490 nm with a Dynex Microplate Reader at 20 minute intervals and incubated at 50°C for four hours. Both the blank and wells without β-carotene solution were used for background subtraction. The following equations were used to calculate

the rate of degradation and antioxidant activity. EC_{50} value calculated represents 50% antioxidant ability:

$$\text{Sample degradation rate} = \ln\left(\frac{\text{Abs}_{t=0}}{\text{Abs}_{t=240}}\right) \times \frac{1}{240},$$

$$\text{Antioxidant activity, \%} = 100\% \times \left(\frac{\text{degradation}_{\text{control}} - \text{degradation}_{\text{sample}}}{\text{degradation}_{\text{control}}}\right). \quad (2)$$

3.6.3. Free Radical Scavenging Activity. Twenty microliters of (0.003 up to 1 mg/mL) samples were added with 180 μL (0.1 mM) DPPH (2,2-diphenyl-1-picrylhydrazyl). Scavenging activity absorbance was performed at 550 nm via Varioskan after incubating for 30 minutes in the dark, at room temperature [48]. The antioxidant activity was presented as the IC_{50} which is 50% of the crude extract concentration required for quenching free radicals. Initially the samples were purple and decoloration to yellow (reduced form of DPPH) disclosed the presence of the scavenging potential of the antioxidant extract [49]:

$$\text{DPPH inhibition, \%} = 100 \times \left(\frac{\text{Abs}_{\text{control}}/\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right). \quad (3)$$

3.7. Statistical Analysis. The statistical analysis was performed using GraphPad Prism 5.02 Software. The data was compared with analysis of variance, ANOVA, and Tukey Multiple Comparison Test as the posttest. Probability value of $P < 0.005$ was selected for significant difference.

4. Conclusions

Through the HMG-CoA reductase assay, the ethanol extract of *Pithecellobium ellipticum* revealed itself to be a promising hypercholesterolaemic lowering agent. The responsible compounds might be flavonoids, phenolics, terpenoids, or even steroids. Negative results in the phytochemical screening might have been due to either the secondary metabolite not being present or being present in a minute amount which could not be detected. Overall, the ethanol extract exhibited good antioxidant ability in the three antioxidant assays. Of course, further tests will be required to confirm whether the active component is also an antioxidant substance. Additional isolations and identifications are being carried out for fraction 7A to find the constituents that are able to influence HMG-CoA reductase activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Clinical Study of Effects of Jian Ji Ning, a Chinese Herbal Medicine Compound Preparation, in Treating Patients with Myasthenia Gravis via the Regulation of Differential MicroRNAs Expression in Serum

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Myasthenia gravis (MG) is an autoimmune disease, of which the pathogenesis has remained unclear. At present, MG does not have any effective treatment with minor side effects. Jian Ji Ning (JJN), a traditional Chinese medicine formula consisting of 11 medicinal plants, has been used in the treatment of MG for many years. The present study aims to determine if the Chinese herbal medicine JJN could lighten the clinical symptoms of patients with MG via the regulation of differential microRNAs (miRNAs) expression in serum. JJN should be orally administered twice a day for 6 months. In the efficacy evaluation adopting the Quantitative Myasthenia Gravis Score (QMG), we found that JJN could improve the clinical symptoms of patients with MG more effectively. Besides, we found that JJN could regulate differential miRNAs expression in serum of patients with MG. Accordingly, we speculate that the effects of JJN on improving clinical symptoms and blood test indicators of patients with MG may be due to its inhibition of apoptotic pathways of some immune cells and its connection with the regulation of serum miRNAs of some patients. In conclusion, we believe that JJN has a reliable curative effect on patients with MG-induced neuropathologic changes.

1. Introduction

Myasthenia gravis (MG) is an antibody-mediated neuromuscular transmission chronic disorder, with an incidence rate of 3–30/1,000,000 people per year. The targets are postsynaptic proteins, mainly involving the skeletal muscle acetylcholine receptor (AchR) and the muscle-specific tyrosine kinase (MuSK) [1, 2]. Some studies have shown that genetic factors played an important role in pathogenesis of MG [3]. In addition, infection with viruses or bacteria, such as poliovirus and *Escherichia coli*, may be involved in the pathogenesis of MG [4, 5]. However, no report has clearly elucidated the fundamental pathogenesis of MG but merely some accepted hypotheses. The main clinical characteristic of this disease is the volatility of skeletal muscle weakness. At present, anticholinesterase drugs, nonspecific immunosuppressants, thymectomy, and plasmapheresis are main treatments for MG [6–8]. Unfortunately, the abovementioned treatments have some serious side effects, such as cardiac arrhythmia, osteoporosis, and hypotension and cannot inhibit the relapse of symptoms of patients with MG and/or achieve complete remission [9]. Therefore, alternative treatments with higher efficacy and fewer side effects are required. Traditional Chinese medicine (TCM) has been practised for many diseases,

including cancer, cardiovascular disease, inflammation, and MG's disease, owing to its long-term efficacy and few side effects [10–14].

Jian Ji Ning (abbreviated as JJN), a traditional Chinese herbal medicine, consists of eleven herbal components, including *Hedysarum multijugum* Maxim. 50 g, *Pseudostellaria heterophylla* (Miq.) Paxex Pax et Hoffm. 25 g, *Atractylodes macrocephala* Koidz. 15 g, *Citrus aurantium* L. 15 g, *Cimicifuga heracleifolia* Kom. 10 g, *Leonurus japonicus* Houtt. 30 g, *Saposhnikovia divaricata* (Turcz.) Schischk. 10g, *Angelica sinensis* (Oliv.) Diels 10 g, *Lycium barbarum* L. 15 g, *Polygonum multiflorum* Thunb 15 g, and *Cornus officinalis* Sieb. 15 g. JJN has been mainly used to treat the autoimmune disease myasthenia gravis for many years. Our previous studies show that JJN is effective in various experiments and clinical treatments related to MG. It can reduce the level of IFN-1, regulate mRNA expression, reduce AChRAb level in blood, improve the severity of myasthenia gravis, promote the growth of new axon in neuromuscular junction (NMJ), increase the number of synaptic vesicles and reduce the synaptic injury in model rat of trial autoimmune MG (EAMG), and effectively regulate the expression levels of some EAMG-related differential proteins [15–22]. However, our understanding of the treatment of MG patients by JJN is still at the molecular level, so that the influence is limited.

MicroRNAs (miRNAs) are small (~22 nucleotides long), noncoding RNAs that mediate posttranscriptional silencing of target genes. In animals, miRNAs usually bind to complementary sites in the $3'$ untranslated region (UTR) of target genes and regulate target gene expression by either translational inhibition or miRNA degradation or both [23]. Ever since the discovery of miRNAs, researchers have been committed to determining biological functions of miRNAs and their related diseases. Dysregulation of miRNAs has been associated with certain human diseases, such as leukemia and heart disease [24, 25]. Moreover, to the best of our knowledge, no report has shown that there are changes in the pattern of differential microRNA expression in serum of MG patients who have received JJN treatment.

According to the above discussion, our study aims at determining whether Chinese herbal medicine JJN could reduce muscle weakness and neuronal apoptosis of autoimmune-induced MG disease via regulation of differential microRNAs expression in serum of patients with MG.

2. Materials and Methods

2.1. Participants. This study was approved by Ethics Committee of the Longhua Hospital Affiliated to Shanghai University of TCM, and all participants were provided with informed consents on this study according to the rules of the Ethics Committee. All of them were Chinese Hans. There were 60 samples in total, including 35 males and 25 females at an average age of 48.70 ± 16.45 years and with a disease duration of 28.03 ± 23.83 month (mean ± SD). All of them were patients who were diagnosed with MG in the Longhua Hospital Affiliated to Shanghai University of TCM and the China Affiliated Hospital of Liaoning University of TCM from November 2010 to April 2012. According to the Osseman classification [26], all MG patients were included in this study because they met the inclusion criteria but not the exclusion criteria. All patients included in this study belonged to class II (33 class IIa; 27 class IIb). Moreover, the study collected peripheral blood samples from 10 healthy individuals as the control group, including 3 males and 7 females at an average age of 45 ± 18 years.

The diagnosis of MG was based on the diagnostic criteria for autoimmune myasthenia gravis in western medicine and the medical diagnosis of flaccidity in TCM [27, 28]. The inclusion criteria are shown as follows: (1) patients with MG belonging to the modified Osseman I, IIA, IIB type; (2) patients diagnosed with spleen-kidney deficiency type flaccidity in TCM, who have one or more cardinal symptoms and secondary symptoms, including pulse and tongue conditions; (3) patients aged from 14 to 75 years; (4) patients informed and willing to participate in this study. The patients with familial MG, congenital myasthenia syndrome (D-penicillamine, interferon induced MG, etc.), allergy (allergic to 2 or more kinds of food or drugs), and serious complications, such as cerebral vascular diseases, renal insufficiency, hematopoietic system diseases, and mental illness, the patients who participated in clinical trials of other drugs in the past month, and the patients who received the treatment with plasma exchange or intravenous gamma globulin in the past three months will be excluded. Ten gender-matched healthy donors with no history of autoimmune disease will be included in this study as the control group. The concomitant use of medicines other than the TCM used in this study is not allowed. After assessment by a neurologist, eligible patients will be assessed by a TCM practitioner to ensure their eligibility.

2.2. Herbal Preparation. In this study, we used Chinese herbal decoctions prepared according to the formula of TCM of Professor Jingsheng Zhang at Affiliated Hospital of Liaoning University of TCM. This formula specific for the TCM syndrome matching certain MG symptoms has been proved to be effective in the early clinical and experimental studies. JJN was supplied by the GMP plant of Benxi Chinese Medicine Factory (Liaoning Province, China). The preparation was a mixture of 11 Chinese herbal medicines as described above. In brief, the drugs were extracted with standard methods according to Chinese Pharmacopoeia (China Pharmacopoeia and Committee, 2000). These crude drugs were soaked in distilled water and boiled for 30 min twice. After that, the drug solution was filtered through a mesh, then concentrated to 4 gmL^{-1} by a vacuum pump at room temperature, and finally stored at −20°C till being used. All herbs were obtained from qualified suppliers in Liaoning Province, China and authenticated at the Chinese Manufacturers Association Testing and Certification Laboratories and the Research and Analysis Laboratory on the basis of standards specified in the Pharmacopoeia of China (2005 edition). These tests included macroscopic and microscopic examination of cross-sections and powders, chemical tests, and/or chromatographic analyses. The screening of heavy metals and pesticides and a microbial limit test were required to ensure the safety of

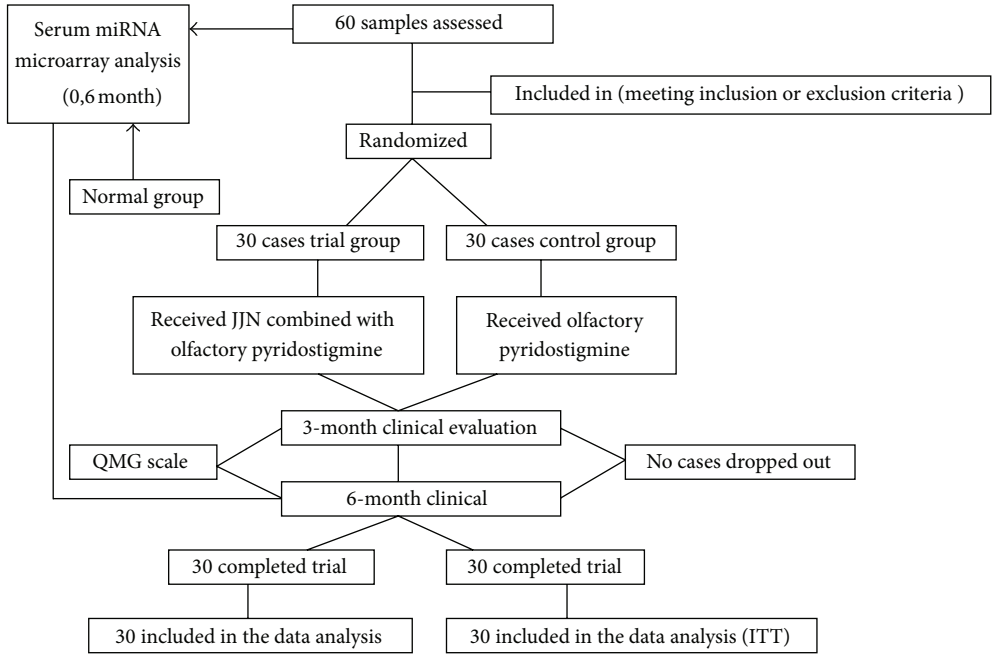


Figure 1: Clinical trial profile.

participants in use. All medicine packages were distributed by an independent researcher in another room after the patients' visits. Then, patients were instructed to dissolve the granules in each package in hot water and drink it twice a day (the daily dose for adults is 250 mL and was administered orally two times a day before each meal). Changes were not allowed in the studied herbal medications during the study.

2.3. Study Design and Therapeutic Method. This is a prospective and randomized controlled study. Random numbers generated by random-list generator software were assigned to the participants. For more confidentiality, we used patients' codes at all follow-up phases. All subjects had been given written information and a verbal explanation concerning the study before they agreed to participate in the study. After one-week baseline assessment, the subjects were randomly assigned into either the treatment combining JJN (usage as above described) with pyridostigmine bromide (60 mg, 4 times a day, manufactured by Sunway Pharmaceutical Co., Ltd., Shanghai, China, approval no. H31020867) or the treatment with pyridostigmine bromide (using treatment allocation codes generated by a statistician and designed to ensure balance of gender, age, and severity of MG between groups (Figure 1)). All the patients were treated with JJN orally twice per day for 6 months, and the dose of JJN remained unchanged. However, the dosage of pyridostigmine bromide can gradually decline to a relatively low maintenance level according to the improvement condition of symptoms of patients. See some literatures for specific methods [29]. The study was started in November, 2010, and finished in April, 2012.

2.4. Preparation of miRNA Microarray. First, put the 5 mL blood sample collected from each subject into an EDTA-supplemented tube and mix it with 3 mL PBS, and then put 6 mL Ficoll-Paque mixture (GE Biosciences, Pittsburgh, PA, USA) into the tube and centrifuge the tube at 2000x rpm for 20 min. The separation method of peripheral blood mononuclear cells is as shown in the literature [30]. The cells are applied to RNA extraction. Total RNA is extracted from lymphocyte cells according to the instructions of the manufacturer of Mirvana miRNA Isolation Kit (Ambion, Carlsbad, USA). Next, we submit the samples collected from 3 MG patients (pretreatment), 2 MG patients (treatment), and 3 controls to Shanghai Biotechnology Corporation for hybridization of Agilent Human miRNA array (v.12.0). Each microarray chip is hybridized with a single sample labeled with either Cy3 or Cy5. The hybridization should follow background subtraction and relevant specifications. The quality control standard of miRNA microarray is 2100 RIN> = 6.0 and 28S/18S> = 0.7. Only when the miRNA microarray is up to the standard, we can move into the next phase.

2.5. miRNA Microarray Data Analysis

2.5.1. MultiClassDif (Multiclassification with Differences) Analysis. We transformed all raw data derived from the miRNA microarray into $\log_2$ with zero mean and unit sample variance and normalized expressions and analyzed them. In order to evaluate the effect of JJN in treating MG patients more accurately, we compared the miRNAs expressions of normal people, MG patients (pretreatment), and MG patients (treatment), respectively. Log ration of 0, a median over all patients that made each patient with a normalized expression

level, was used to find the further normalization of relevant miRNA expression levels. The random variance model (RVM) t-test was used to calculate the weighted differential expressions of miRNAs between the MG (before and after treatment) and the normal. The differential expressions of miRNAs with the fold-change >1.5 and $P < 0.05$ were considered significant. The analysis aimed at finding out the differential expressions of miRNAs for regulation of all target genes through searching the mirdb database. The heat map analysis and hierarchical cluster analysis of expression data adopted Cluster 3.0 and TreeView programs. The analysis method was MultiClassDif [31–33].

2.5.2. Analysis of the Significant Trend of miRNA Expressions. We selected differential expression genes on the basis of the logical sequence that corrected analysis of variance according to RVM. In accordance with the variation trend that a gene had different signal density under different situations, we identified a unique model expression tendency. The value of the original expression was converted into $\log_2$ ratio. By using the strategy of clustering short time-series gene expression data, we defined some unique profiles. The expression model profiles are related to the actual or the expected number of genes assigned to each model profile. Significant profiles have higher probability than that expected by Fisher's exact test and multiple comparison test [34–36]. We carried out significance analysis of gene expression trend by means of Series Test of Cluster (STC) analysis based on the screening as previously described.

2.5.3. Gene Ontology (GO) Analysis. GO analysis was applied in order to organize genes into hierarchical categories and uncover the miR-Gene regulatory network on the basis of biological process and molecular function [37]. In detail, two-side Fisher's exact test and χ^2 test were often used to classify the GO category, and the false discovery rate (FDR) [38] was calculated to correct the P value. We chose only GOs that had a P value of <0.001 and an FDR of <0.05. Within the significant category, the enrichment Re was given by

$$Re = \frac{n_f/n}{N_f/N}, \quad (1)$$

where n_f: indicates the number of differential genes within the particular category, N_f: indicates the number of differential genes in the entire microarray, n: indicates the total number of genes within the same category, and N: indicates the total number of genes in the microarray.

Afterwards, the metastasis-related network of miRNA-mRNA interaction, representing the critical miRNAs and their targets, was established according to the miRNA degree.

2.5.4. Enrichment Analysis of Target Genes. The significance analysis of inferred miRNA target genes cannot be performed until we demonstrate the significant ($P < 0.05$) difference among the normal, MG (pretreatment), and MG (treatment) samples in the expression of miRNA, accomplish the pathways analysis [39, 40] of these genes on the basis of DAVID online analysis system, and correct P value by FDR. The genome containing less than 5 genes overlapping will be removed from the DAVID analysis. In our analysis, GO terms and pathways with an FDR-adjusted P value of <0.05 will be retained.

2.5.5. To Construct MicroRNA-GO-Network. The miRNA-GO-network was built according to the relationship between significant GOs and genes and the relationship between miRNA expressions and gene expression [41]. The adjacency matrix of miRNA and genes $A = [ai, j]$ was determined by the attribute relationship between GOs and miRNA, and ai, j represented the relation weight of GO i and miRNA j. In the miRNA-GO-network, the circle represented gene, the square represented miRNA, and the edge represented the relationship between gene and miRNA. The center of the network was represented by degree. The degree represented the relationship between one miRNA or GO and the GOs or miRNAs around. The key miRNA and GO in the network had the highest degree.

2.6. Assessment of Clinical Effects. Baseline evaluation was performed before randomization. The 13 Quantitative Myasthenia Gravis scores (QMG-13 including all dimensions and the single index) [42] were often used to monitor the quality of life, the extent of MG and muscle weakness of patients according to the assessment of MG related clinical symptoms. The follow-up visits to the clinical assessment were held for all patients within 3 or 6 months' treatment. The blood collection for serological data was conducted only at the first and the last visit (6 months). The serological data included RNA extraction and renal and liver functions tests. The reported adverse events (AEs) were observed by the patient or the investigator and recorded spontaneously. The severity, outcome, and hypothetical cause of each AE report were assessed and recorded.

2.7. Statistical Analysis. Data were expressed as mean ± SE. It was of statistical significance to use Wilcoxon signed rank test to assess the difference before and after JJN treatment. The difference between the two groups was assessed by means of Mann-Whitney U test. The screening for differential miRNA expression adopted RVM t-test while GO-analysis and Pathway analysis adopted Fisher's exact test and χ^2 test. A P-value <0.05 was regarded as significant. The SPSS version 14.0 (SPSS Inc., Chicago, IL) was used for the statistical computation.

3. Results

3.1. Bioinformatics Analysis

3.1.1. Differentially Expressed miRNAs of MG and Normal. The multiple comparison test based on P value <0.01 and FDR <0.05 revealed a total of 87 significant differential gene expressions in three different genetic screening groups consisting of MG (pretreatment), MG (treatment), and normal samples. Hierarchical cluster analysis revealed that

the differential expression profiles of miRNAs extracted from these samples were roughly classified, respectively. The consecutive heat maps were shown in Figure 2(a) and the significant differential expressions of miRNAs were shown in Figure 2(b). On the heat maps, we noted that there were big changes in the miRNAs expression profiles of MG (pretreatment)/normal, MG (treatment)/normal, and MG (before and after treatment), which indicated that effects of TCM compound preparations JJN in treating MG patients may be realized via regulation of miRNA expression profilles.

3.1.2. STC Analysis. Next, we analyzed miRNA expressions in MG (pretreatment), MG (treatment), and normal samples and observed the significant trend of miRNA expression (Figure 3). Every trend stands for a miRNA group with the similar expression trend. The horizontal axis represents duration or different courses and the vertical axis represents the logarithm of ratio of the control signal value of a miRNA expression. We listed three different sample lines representing three different courses (normal, pretreatment, and treatment). P value represents the significance level of the actual number of randomly distributed miRNAs. When P value is smaller, the impact of the analysis of the trend of miRNA expression will be more remarkable.

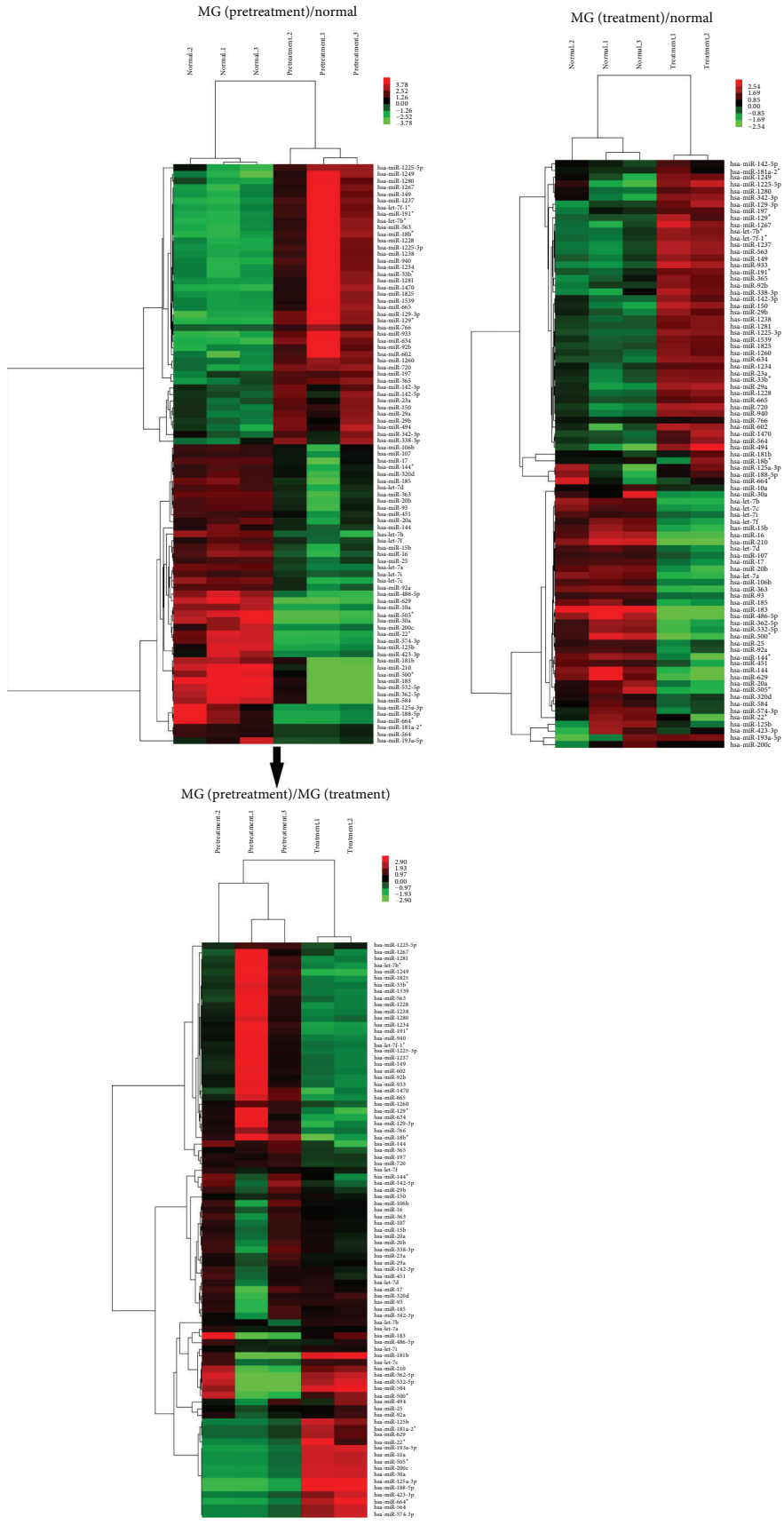
We worked out 16 differential miRNA expression trends by using the significant expressions of gene arrays, including three significant trends ($P \setminus 0.0031$; $0.05/16 = 0.0031$), which were plotted over the color part (up left of Figure 3, zoomed on the low). The significant trend was like Profile no. 5 and the trend change followed (0, 1, 2, 2). "One" or "2" did not represent the actual expression value but symbolized the classification of miRNA expression level. The no. 5 significant trend showed that the level of miRNA expression was drastically upward from normal to pretreatment, but during the course from pretreatment to treatment, the level of miRNA expression appeared downward. From the no. 13 significant trend, we could find that, during the course from normal to pretreatment, miRNA expression was drastically declining, and then the miRNA expression declined to the minimum level and remained unchanged. From the no. 8 significant trend, we could find that, during the course from normal to pretreatment, the miRNA expression was also declining sharply, but after entering the aftertreatment stage, the miRNA expression appeared upward and this trend lasted until the treatment stage. Finally, we selected the most simple no. 5 trend as the best significant trend of this study. After crosstab of target genes of miRNAs and multiClassDif analysis of differential gene expressions of miRNAs, we obtained genes and corresponding miRNAs (data not shown).

3.1.3. GO Analysis and Pathway Analysis. Next, we performed GO analysis to organize the differentially expressed genes and miRNAs on the basis of biological process and function. The results were shown in Figure 4. We found that, compared with the normal group, the differential miRNAs of the MG group (pretreatment) was upward or downward while the MG group (treatment) was upward or downward. The corresponding target genes involved 105 significant differential miRNAs. The points of significance include ($-\mathrm{Lg}\,P \geq 20$) transcription, DNA-dependent, ion transport, multicellular organismal development, chromatin modification, nervous system development, and gene expression. Furthermore, we did pathway analysis to find out significant pathways of relevant differentially expressed genes or miRNAs. As described in Figure 5, we found that target genes involved 47 significant pathways in total, including ($-\mathrm{Lg}P \geq 5$) MAPK signaling pathway, glioma, neurotrophin signaling pathway, axon guidance, ErbB signaling pathway and chronic myeloid leukemia, pathways in cancer, and non-small-cell lung cancer.

Table 1: The key microRNA in network (degree ≥ 50).

MicroRNA	Degree
hsa-let-7b-5p	73
hsa-miR-149-5p	71
hsa-let-7c	70
hsa-miR-93-5p	66
hsa-let-7a-5p	66
hsa-miR-665	65
hsa-miR-16-5p	64
hsa-miR-17-5p	64
hsa-miR-20b-5p	62
hsa-miR-20a-5p	62
hsa-let-7d-5p	62
hsa-miR-940	58
hsa-miR-766-3p	58
hsa-let-7i-5p	58
hsa-miR-106b-5p	56
hsa-miR-15b-5p	54
hsa-miR-107	52

3.1.4. MicroRNA-GO-Network. To further explore the relationship between miRNAs and gene function, we built the miRNA-GO-network among the three groups (Figure 6). As shown in Table 1, the 17 key miRNAs (degree ≥ 50) in the network were hsa-let-7b-5p, hsa-miR-149-5p, hsa-let-7c, hsa-miR-93-5p, hsa-let-7a-5p, and so on. In the miRNA-GO-network, the degree represented the relationship between a miRNA or GOs and the GOs or miRNAs around. The three key miRNAs (degree ≥ 70) in the network, that is, hsa-let-7b-5p, hsa-miR-149-5p, and hsa-let-7c, had a high degree. As we can see from Table 2, the 22 key GOs (degree ≥ 30) in the network were signal transduction, multicellular organismal development, ion transport, and signal transduction in the GO among which multicellular organismal development and ion transport in the GO were more regulated by microRNA. This showed the differences of different samples of MG



(a)

Figure 2: Continued.

	Up	Down	Unchanged
MG (pretreatment)/normal	42	45	
MG (treatment)/(pretreatment)	43/45	35/42	9
Total	87		

Note: down or up denotes significant differential expression miRNAs in MG patients relative to normal

(b)

Figure 2: (a) Heat map of differentially expressed miRNAs between the MG (pretreatment)/normal and MG (treatment)/normal is shown at the horizontal, and the heat map of differentially expressed miRNAs between the MG (pretreatmentand) the MG (treatment) is shown at the vertical. (b) Significant differentially expressed miRNAs of three groups.

Table 2: The key GO in network (degree ≥ 30).

GO_name	Degree
Signal transduction	52
Multicellular organismal development	52
Ion transport	48
Nervous system development	47
Transport	44
Cell adhesion	44
Cell cycle	43
Cell differentiation	41
Positive regulation of transcription, DNA-Dependent	40
Interspecies interaction between organisms	40
Protein transport	39
Chromatin modification	38
Potassium ion transport	37
Regulation of transcription from RNA Polymerase II promoter	36
MRNA processing	35
Proteolysis	34
Regulation of translation	34
Skeletal system development	33
Negative regulation of transcription, DNA-dependent	33
Calcium ion transport	31
Muscle organ development	30
Actin cytoskeleton organization	30

patients before and after treatment using JJN. Among them, hsa-let-7b-5p had the highest degree in the network. Therefore, hsa-let-7b-5p was selected for further study.

3.2. Comparison of the QMG Scoring between the Two Groups before and after Treatment. The result of baseline evaluation was shown in Table 3 and demonstrated the comparison condition between the two groups before randomization. The QMG scores of the trial group and the control group were decreased after treatment. There was a significant difference between the two groups ($P < 0.05$). However, after 3 and 6 months' treatment, the QMG scores of the trial group were apparently lower than those of the control group ($P < 0.05$); moreover, there was also an obvious difference between the two groups compared with the same group before treatment ($P < 0.05$, Table 4).

4. Discussion

As for many autoimmune diseases, it is known that the triggering events involved in MG are not clearly defined. Complex disease pathogenesis of MG has hindered the advancement of our understanding of disease initiation, thus delaying the identification and treatment of susceptible individuals. Obviously, it is necessary to investigate the pathogenesis of MG more deeply and seek more rational and effective treatment for MG, which no doubt becomes a hot spot in the field.

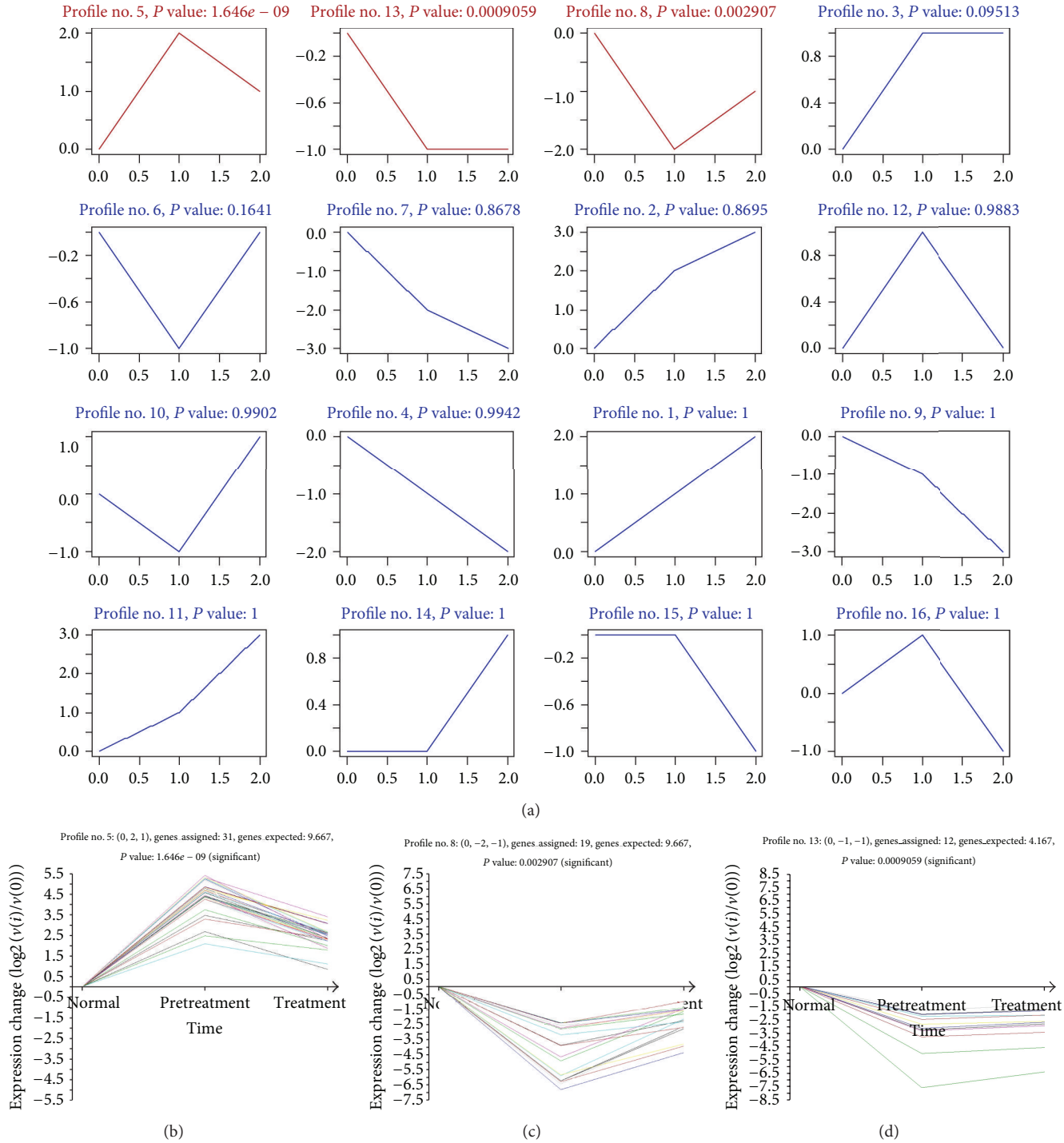


FIGURE 3: Differences in gene expression trends. Expression of each box represents a trend, a trend marked as each box number, such as 5, 8, and 13, lower marked with P value, that is, the trend level of significance, on behalf of the trend test for multiple comparisons after a significant correction level (P value), color-coded for significant trends, such as the trend of 5, 8, and 13. Significant trends of the selection criteria: P value\0.05/16 = 0.0031. As shown above, miRNA expression of differences in a total of 16 species of trends, including the three trends is the expression of significant trends (P \ 0.0031), which are plotted over the color part of the trend. (Color figure online.)

According to the previous studies, miRNAs were small nucleotides of RNA that participate in the regulation of a variety of cellular processes, including cell differentiation, cell cycle progression, and apoptosis. Ever since the discovery of miRNAs, tremendous effort has been devoted to determining the biological functions of miRNAs and their relevance to diseases. Moreover, miRNAs emerge as important factors in tumorigenesis and metastasis and their expression signatures

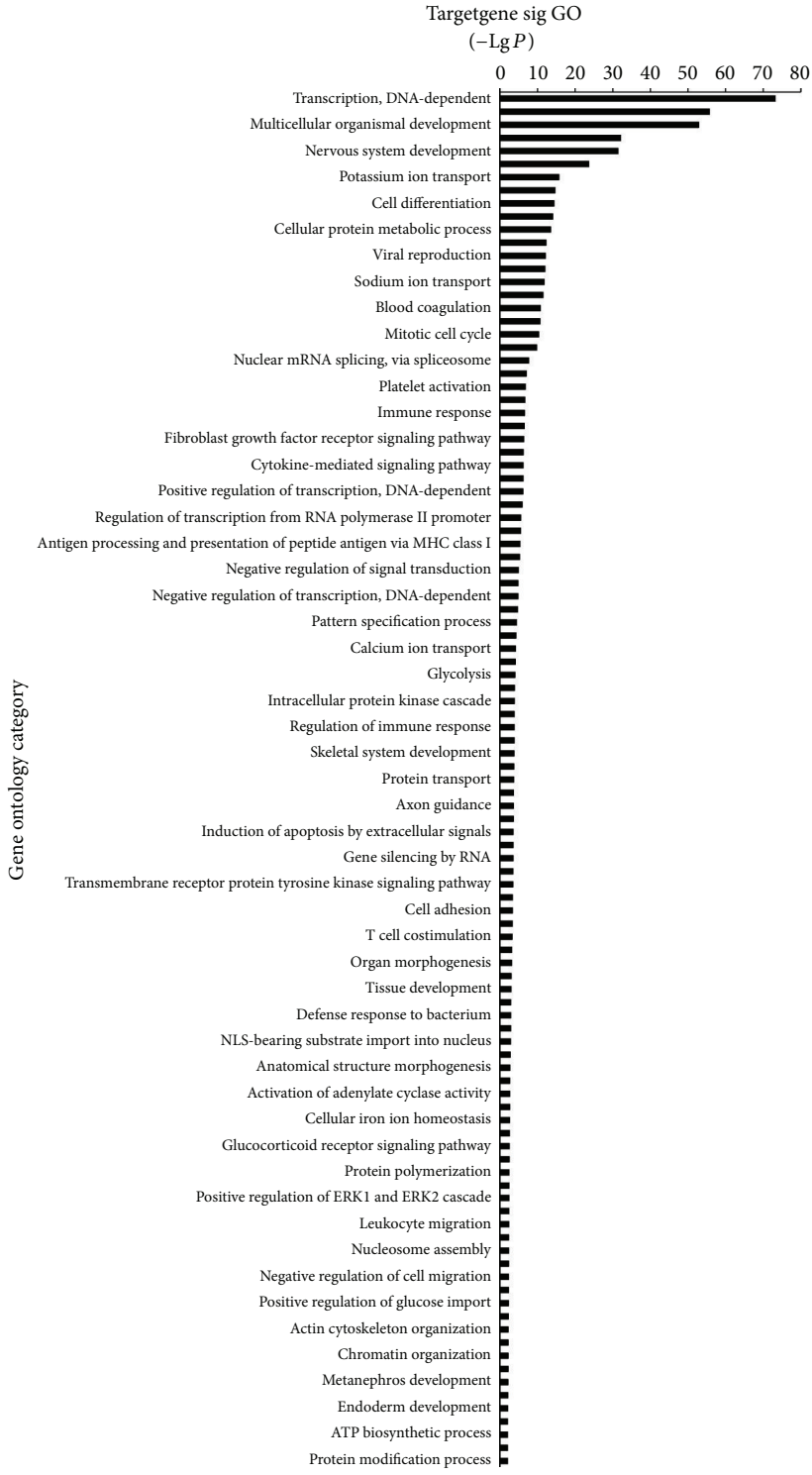


Figure 4: The significant GO functional maps of the target genes. Ordinate is the name of the target gene function and the abscissa is the P value of the negative logarithm. A higher number indicates that the function is more significant.

are associated with the prognosis and progression in chronic lymphocyte leukemia and lung cancer [43, 44]. In addition, bioinformatics analysis reveals that miRNAs can control the expression of one-third of the human proteome [45]. Recent evidence showing miRNA as a micromanager of various stages of immune regulations has generated interest in the involvement of miRNAs in autoimmune disorders. Although still at an early stage in understanding their impact on immunity, miRNAs are changing the way we think about the development of the immune system and

Figure 5: KEGG pathway associations of the target genes of the miRNAs. The enrichment scores of biological processes are shown as –log(P value).

regulation of immune functions [46]. In addition to our preliminary previous study, it seems that no report on the biological consequences of miRNA dysregulation in MG has been characterized, particularly in relation to therapeutic intervention by taking Chinese herbal compound.

TCM is a medical system, and discoveries of ancient Chinese evolved through at least 3000 years of uninterrupted clinical practice. The TCM treatment usually requires a traditional diagnosis method to distinguish the TCM syndrome, which is based on clinical symptoms and signs followed by the use of individualized treatment [47]. What is more, Chinese herbal medicines have been used for thousands of years and are beneficial in prevention and treatment of many diseases, including MG. Greater attention is being given to such medicines due to their varied biological actions and low toxicity. According to our previous studies, JJN, a formula designed according to the TCM theories and clinical experience, has been used to treat MGI (I and II type) patients in China for decades. However, this study is the first report to address the therapeutic effects of JJN on patients induced serum microRNAs expression differences in MG.

In this study, array-based gene and miRNA expression profiling were performed on three sample groups, that is, MG sample group (pretreatment), MG group (treatment) and normal group. First, the findings support the notion that there are 87 significant differentially expressed miRNAs in

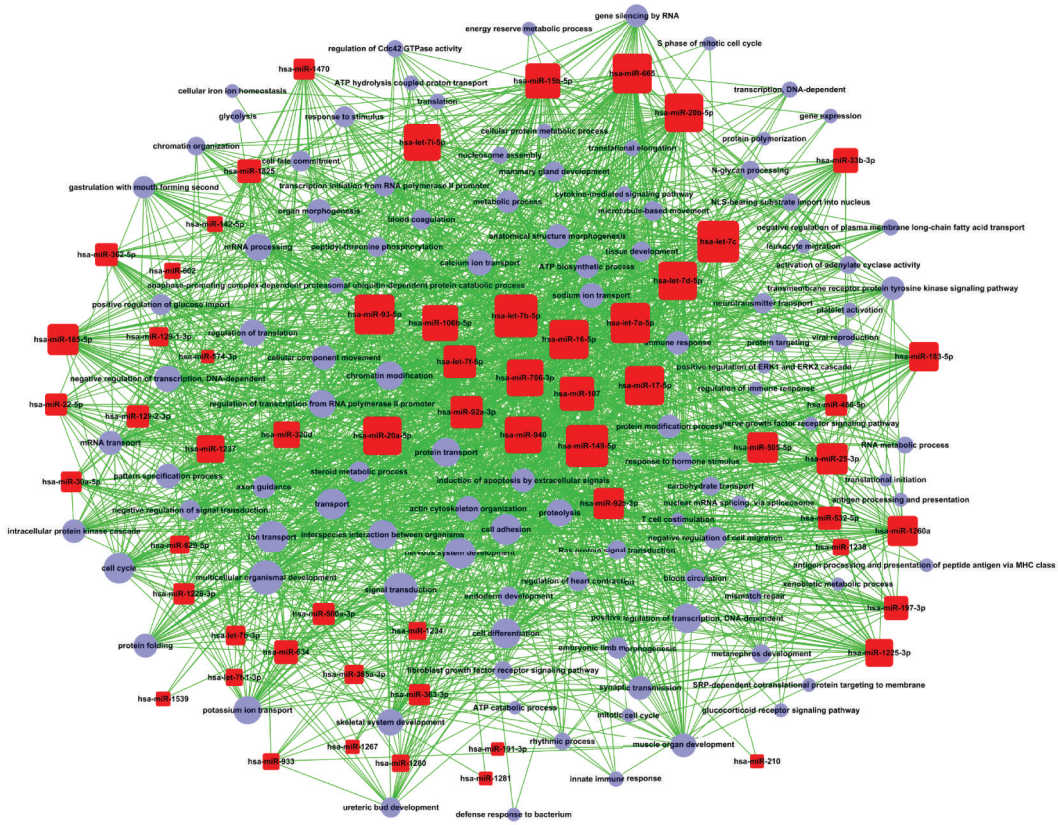


Figure 6: MicroRNA-GO-network. Red rounded rectangle nodes represent microRNA, and light purple cycle nodes represent GO. Lines of edge show the regulatory relations at microRNA and GO. Color and shape indicated no special significance but better distinguish between network MicroRNA and GO. The more MicroRNA regulates GO, the more the area of MicroRNA occupies, similarly, the more GO could be regulated by MicroRNA, the more the area is also occupies. Its quantitative relationship can be provided by the corresponding tables.

Table 3: Comparison of baseline evaluation between the two groups ($\overline{x} \pm s$).

Group	Case	Gender (male/female)	Age (year, range)	Disease course (month)
Normal	10	3/7	45 ± 18	
Trial group	30	12/18*	48.70 ± 16.45 (17–75)**	28.03 ± 23.83$^{\triangle}$
Control group	30	13/17	49.03 ± 15.26 (22–75)	30.43 ± 29.69

Notes: *$P > 0.05$, compared with the control group (gender); **$P > 0.05$ (age), $^{\triangle}P > 0.05$, compared with the control group (disease course).

Table 4: Comparison of evaluation of QMG scoring among the two groups ($\overline{x} \pm s$).

Item	Trial group ($n = 30$)	Control group ($n = 30$)
Pretreatment	10.80 ± 4.92*	12.10 ± 5.70
3 months	7.90 ± 3.82**$^{\blacktriangle}$	10.86 ± 4.98
6 months	6.19 ± 3.57$^{\triangle\blacktriangle}$	9.41 ± 5.18

Notes: *$P > 0.05$, compared with the control group (Pretreatment); **$P < 0.05$ (3 months), $^{\triangle}P < 0.01$ (6 months), compared with the control group; $^{\blacktriangle}P < 0.05$ (3 months and 6 months) compared with the same group before treatment.

the three groups. It means that the MG group (pretreatment), MG group (treatment), and normal group are completely different in the miRNA expression level. Among them, the miRNA expression change in MG group before and after treatment may have occurred due to therapeutic effects of Chinese herbal compound JJN on MG patients. Next, we observed significant trend of miRNA expression in three groups at three different courses (normal, pretreatment, and treatment) and found that the upward or downward trend of MG patients was corrected compared to the normal group, after treatment of Chinese herbal compound JJN. It means that JJN has some therapeutic effects on patients with MG as the prolongation of treatment time. Then, we performed GO analysis and found that there were certain significant differences in miRNAs features between MG group (before and after treatment) and normal group and the corresponding target genes involved those above significant features. It is tempting to speculate that Chinese herbal

compound JJN was applied to the treatment of MG because it had a certain impact on the expression of above significant differential miRNAs features of the human beings and JJN may play a therapeutic role via the regulation of miRNAs expression profile changes in these functions. Furthermore, we applied DAVID analysis to calculate each target gene and conduct pathway analysis, accordingly finding out that target genes involved 47 significant pathways in total. Then, we conjectured that the effect of Chinese herbal compound JJN in treating MG came into play in the abovementioned way. In the biological network, as we all know, one advantage is that the network contains interaction information, which provides an intuitive way to explore gene functions in context using visualization approach. Such holistic approach has advantages for gene expression modules during both disease and its development for their regulation [48], and expression levels are the highest correlation across samples [49]. As a class of gene regulators, combinatorial regulation is an important feature for miRNA. Usually, a given miRNA may have multiple different mRNA targets, whereas a given target gene may also be targeted by multiple miRNAs [50]. In this study, the integrated bioinformatics analysis from gene and miRNA expression profiling suggested that 17 miRNAs, that is, hsa-let-7b-5p, hsa-miR-149-5p, hsa-let-7c, hsa-miR-93-5p, hsa-let-7a-5p, hsa-miR-665, hsa-miR-16-5p, hsa-miR-17-5p, hsa-miR-20b-5p, hsa-miR-20a-5p, hsa-let-7d-5p, hsa-miR-940, hsa-miR-766-3p, hsa-let-7i-5p, hsa-miR-106b-5p, hsa-miR-15b-5p, and hsa-miR-107, were associated with the effect of JJN in treating patients with MG. Among them, hsa-let-7b-5p showed the highest degree in miRNA-GO-network, which was built to explore the association of miRNAs with gene or gene ontology. From our previous studies, we know that key regulatory mechanism by dysregulation of let-7 family was involved in MG, which inhibited IL-10 expression in Jurkat cells. All the results suggest a possible link between the miRNA-mediated mechanisms and the pathogenesis of MG. The reason why the symptoms of MG are partially reversed may be the increase in the level of let-7c in MG patients [25]. Moreover, previous studies have reported that let-7 microRNAs are principal regulators that control major cell functions in various physiological and pathological processes [51]. Collectively, the above-described evidence also suggests that Chinese herbal compound JJN may play a therapeutic role in MG by regulating the let-7 level.

Based on the abovementioned microarray bioinformatics analysis, we obtained that Chinese herbal compound JJN plays its therapeutic effect in treating MG patients by regulating differences in miRNA expression profiles, differences in the certain functions of miRNA expression profiles, changes in miRNA expression pathways, and other ways. Then, from a macroperspective, it is necessary to evaluate the clinical efficacy to further validate therapeutic effect of JJN on MG patients. In this study, on the basis of western conventional treatment, we pulsed Chinese herbal compound JJN to treat MG and found that the QMG score of the trial group was apparently lower than that of the control group before and after treatment; there was a significant difference between the two groups ($P < 0.05$), with statistical significance. MG patients were able to significantly reduce the simple western medicine usage and the clinical symptoms can be effectively certainly alleviated without side effects. In addition, larger samples of previous clinical studies have also proven that the clinical obvious effective rate and effective rate of JJN therapy in MG patients were 71.62% and 87.84%, respectively [19].

In conclusion, through analyzing miRNA microarray data in MG, we provided evidence for the proposition that there were great differences among the MG group (pretreatment), MG group (treatment), and normal group on the basis of MultiClassDif, STC analysis, GO analysis, Pathway analysis, and microRNA-GO-network and indicated that the effects of Chinese herbal compound JJN on treating MG patients might be realized via the regulation of changes in expression profiles of those miRNAs. Meanwhile, Chinese herbal compound JJN provides more clinical advantages than corticosteroids for MG treatments.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bromelain Inhibits Allergic Sensitization and Murine Asthma via Modulation of Dendritic Cells

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The incidence of atopic conditions has increased in industrialized countries. Persisting symptoms and concern for drug side-effects lead patients toward adjunctive treatments such as phytotherapy. Previously, we have shown that Bromelain (sBr), a mixture of cysteine proteases from pineapple, *Ananas comosus*, inhibits ovalbumin (OVA)-induced murine model of allergic airway disease (AAD). However, sBr's effect on development of AAD when treatment is administered throughout OVA-alum sensitization was unknown and is the aim of the present study. C57BL/6J mice were sensitized with OVA/alum and challenged with 7 days OVA aerosol. sBr 6 mg/kg/0.5 ml or PBS vehicle were administered throughout sensitization. Lung, bronchoalveolar lavage (BAL), spleen, and lymph nodes were processed for flow cytometry and OVA-specific IgE was determined via ELISA. sBr treatment throughout OVA-alum sensitization significantly reduced the development of AAD (BAL eosinophils and lymphocytes). OVA-specific IgE and OVA TET$^+$ cells were decreased. sBr reduced CD11c$^+$ dendritic cell subsets, and *in vitro* treatment of DCs significantly reduced CD44, a key receptor in both cell trafficking and activation. sBr was shown to reduce allergic sensitization and the generation of AAD upon antigen challenge. These results provide additional insight into sBr's anti-inflammatory and antiallergic properties and rationale for translation into the clinical arena.

1. Introduction

The incidence of atopic conditions such as asthma, food allergies and atopic dermatitis have increased dramatically in industrialized countries over the last fifty years. Presently, approximately 1 out of 5 Americans suffer from atopic disorders [1], with 1 out of 12 having asthma [2]. Despite major efforts to diagnose and treat these conditions, current conventional medications for allergic disorders are not fully effective. For example, it is estimated that 58% of primary care patients with asthma have poorly controlled asthma [3]. Poor asthma control may result from inadequate assessment or implementation of asthma therapy by healthcare providers or from poor adherence with prescribed therapy by patients [4]. The persistence of symptoms and disease flares, despite medical therapy and the concern for long-term side effects of corticosteroids [5–8] and long-acting beta-2 adrenergic agonists [9], have caused many patients to turn to complementary and alternative medicine (CAM) treatments [10]. A review of 17 articles reported that up to 70–80% of adult asthmatics in the USA use CAM to help control their asthma [11]. Similarly, the reported rates of CAM use in children

with asthma range from 33 to 89% (11). In children, the most commonly used CAM therapies are breathing techniques, vitamins, and herbal products or phytotherapy [12].

One such herbal product with demonstrated anti-inflammatory efficacy is bromelain. Stem bromelain (sBr) is a mixture of cysteine proteases that is derived from the stem of the pineapple plant, *Ananas comosus*. Beneficial effects of sBr have been demonstrated in a variety of inflammatory conditions, including rheumatologic diseases in mice and humans [8–13], experimental allergic encephalomyelitis (a murine model of multiple sclerosis) [14], human allergic rhinitis [15], and murine allergic asthma [16]. In an ovalbumin-(OVA-) induced asthma model [17], we have shown that sBr administered by either intraperitoneal [16] or oral routes [18] inhibits eosinophilic airway inflammation and allergic airway disease, at least in part via proteolytic cleavage of cell-surface CD25 from activated $CD4^+$ T effector cells [19]. In these experiments, sBr was administered prior to and during OVA aerosol challenges (but following OVA antigen sensitization). It is noted that sBr can also affect many other cell surface markers common to T cells and other cell types such as dendritic cells (DC) which could potentially affect their function.

Dendritic cells are professional antigen-presenting cells and are known to take up antigen via specialized endocytic receptors and in response to danger signals and migrate to sites of inflammation [20–22]. Although identifying the specific subsets of DCs which migrate and are responsible for antigen uptake and presentation remains an active area of research, CD44, the receptor for hyaluronic acid, has been shown to be essential for DC migration to regional lymph nodes [23], Th2 skewed T-cell activation [24, 25], and inflammation [26]. Interestingly, sBr has been shown to reduce the expression of CD44 in a variety of models of cell adhesion [27–29] and metastasis [30, 31], thus presenting a plausible mechanism for inhibition of allergic sensitization. Thus, the present study addressed the hypothesis that sBr could inhibit murine sensitization to OVA via modulation of DCs, which play a key role in allergic sensitization.

2. Methods

2.1. Animals. Female C57BL/6J mice, 3–6 months of age, Jackson Laboratory (Bar Harbor, ME), were housed in plastic cages with corncob bedding at 22–24°C with a daily light/dark cycle (light from 06:00 to 18:00 h). Chow and water were supplied *ad libitum*. All protocols were approved by the UConn Animal Care Committee.

2.2. Natural Product Bromelain. For intraperitoneal (i.p.) injections, 60 mg sBr (Vital Nutrients, Middletown, CT) was dissolved in 250 ml PBS. sBr was independently tested for authenticity, potency (2400–2660 GDU g^{-1}), and quality as previously described [16, 18, 19].

2.3. In Vitro Bromelain Studies. sBr was administered in a dose response manner (1–100 μg/mL) to DCs overnight. To obtain DCs, spleens of OVA-alum sensitized mice were digested with Collagenase-D (Roche, Indianapolis, IN) 2 mg/mL for 30 m at 37°C, passed through a 40 μm nylon cell strainer (BD, Bedford, MA) and erythrocytes lysed with Tris-buffered ammonium chloride at room temperature for ~2 min. $CD11c^+$ cells were then isolated with pan-CD11c microbeads (number 130-092-465; Miltenyi Biotech, Auburn, CA). $CD11c^+$ cell isolations yielded $3–5 \times 10^6$ cells/spleen with a purity of >95%. Cells ($0.5–1 \times 10^6$) were cultured in 24 well plates in CO_2 incubator (5%, 37°C). 100 μM E-64 (Sigma, St. Louis, MO) was added to neutralize sBr cysteine protease activity, in selected experiments.

2.4. Bromelain Treatment in OVA-Induced Murine Models of Allergic Airway Disease and OVA/Alum Sensitization. Mice were sensitized with three weekly i.p. or subcutaneous (nape of neck) injections of a suspension containing 25 μg of OVA (grade V, Sigma Chemical, St. Louis, MO) and 2 mg of aluminum hydroxide (alum) in 0.5 mL of saline (Figure 1(a)). OVA-alum was delivered i.p. once per week for 3 consecutive weeks (days −21, −14 and −7) to C57BL/6J mice (Figure 1(b)) PBS or sBr was delivered i.p. (6 mg/kg in 0.5 ml PBS) twice daily, M-F throughout sensitization. In prior studies we determined that sBr (6 mg/kg in PBS) administered i.p. twice daily for 3 consecutive weeks caused no significant elevation in liver enzymes or BAL protein in these animals (see Supplemental Table 1 in Supplementary Materials available at http://dx.doi.org/10.1155/2013/702196).

After sensitization, animals were rested for 1 week and then challenged with 1% aerosolized OVA in 0.9% saline, 1 h per day, for seven days (days 0–7), [16, 19]. Twenty-four hours after the final aerosol exposure, the mice were sacrificed by drug overdose (0.15 mL i.p. injection per 20 g mouse of 13 mg Ketamine HCL, Ketaset-III For Dodge Animal Health, Fort Dodge, IA, USA, and 0.4 mg of xylazine, Tranquived Vedco, St. Joseph, MO, USA) and exsanguination.

Animals were also sacrificed 24 hours after each weekly sensitization (Figure 1(b)); S1 (week 1), S2 (week 2), and S3 (week 3), and tissues (spleen, lymph nodes, and BAL) were processed for assessment of the antigen-specific responses. In selected experiments, a group of mice treated with E64-inhibited-sBr was added as a control for the cysteine protease activity of sBr. The sBr dosages used were based on prior *in vivo* and *in vitro* dose response studies performed in our laboratory [16, 18, 19].

2.5. BAL Cellular Analysis. Lungs were lavaged *in situ* with five 1 mL aliquots of 0.9% saline. BAL fluid was centrifuged (200 g × 10 min), the pellet was resuspended in saline, and total nucleated cells were counted with a hemocytometer using Nigrosin exclusion for viability. Leukocyte differentials were determined using cytocentrifuged (at 900 rpm for 5 min, Thermo Scientific Shandon Cytospin-4, Leicestershire, England, UK) preparations stained with May-Grünwald and Giemsa (Accustain, Sigma, St Louis, MO, USA). The remaining cells were analyzed phenotypically for T-cell subpopulations by flow cytometry.

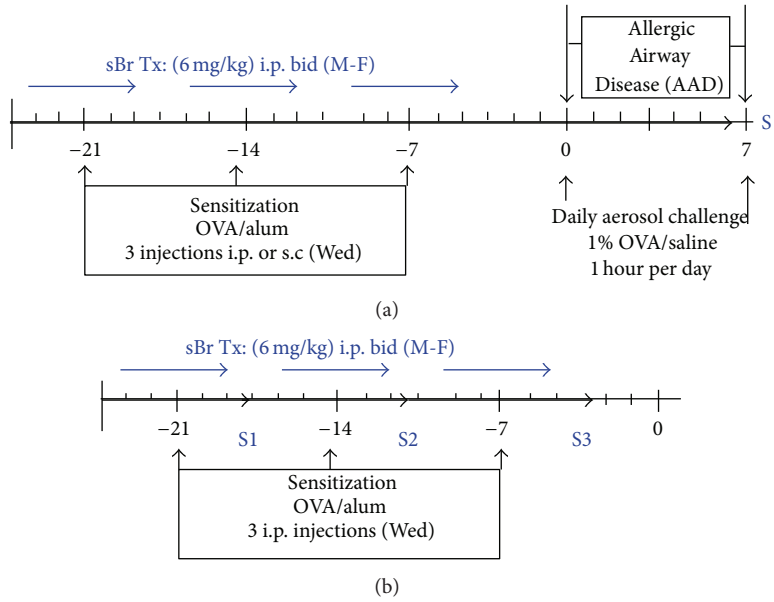

FIGURE 1: Protocols for sBr treatment in murine models of allergic airway disease. (a) Mice (n = 8–10 per group) were sensitized to OVA-alum (i.p.) weekly, for 3 consecutive weeks. sBr (6 mg/kg in 0.5 ml PBS) or PBS was delivered i.p. twice daily. On day 0, mice were challenged with OVA aerosol for seven consecutive days and sacrificed (S) 24 hours later. (b) Sensitization and i.p. sBr treatment were the same as in (a). Animals were sacrificed 24 hrs after each week or treatment; S1 (week 1), S2 (week 2), and S3 (week 3). In selected experiments groups of mice were treated with E64-inhibited-sBr.

2.6. Flow Cytometry. Cells for analysis via fluorescence-activated cell sorting (FACS) were obtained from BAL, homogenized lung tissue, spleen, and lymph nodes. BAL samples were washed in PBS (Dulbecco's Phosphate Buffered Saline, pH 7.4, Sigma, St Louis, MO, USA) and tissues processed and labeled with monoclonal antibodies in standard manner for flow cytometry. The following monoclonal antibodies were used for cellular surface staining: α-CD3 (145-2c11), CD4 (RM4-5), CD8 (53-6.72), α-CD11a (2D7), CD44 (IM7), α-CD62L (MEL-14), CD86 (GL-1), CD11b (M1/70), CD103 (2E7), CD11c (N418), F4/80 (BM8), and MHCII (M5/114.15.2) and were purchased from eBioscience (San Diego, CA) or BioLegend (San Diego, CA). H-2K^b tetramers containing the OVA-derived peptide SIINFEKL were generated in the laboratory as described previously [32]. Enrichment of OVA-TET$^+$ CD8$^+$ T cells from mice was accomplished by processing single cell suspensions from the spleen or pooled lymph nodes (axillary, mandibular, cervical, HLN, ILN, colic, jejunal, and caudal mesenteric). Cells were then stained with both phosphatidylethanolamine- and allophycocyanin (APC)-labeled tetramers and α-CD8 antibody then counter-stained with α-phosphatidylethanolamine microbeads as per the instructions of the manufacturer (Miltenyi Biotec, Auburn, CA). Samples were then run on an AutoMACs (Miltenyi Biotec) magnetic column cell separator. After enrichment, cells were stained with α-CD11a, α-CD62L, α-CD4, α-IA^b, and α-CD11b for 30 minutes at 4°C. Cells were then washed and fixed with 2% paraformaldehyde. Cell samples were acquired with an LSRII cytometer (Becton Dickinson Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). General gating strategies are depicted in Supplementary Figure 3.

2.7. Statistical Analysis. Statistical comparisons between groups were made with analysis of variance and unpaired t-tests using JMP Software (SAS Institute Inc., Cary, NC, USA). All data were expressed as means ± standard error of the mean, and differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Bromelain Administration during Sensitization Prevented Development of Allergic Airway Disease. In the current studies, treatment of mice with bromelain during the sensitization prevented the development of AAD in the animals (Figure 2). Total BAL leukocytes were markedly reduced in sBr-treated AAD mice as compared to control AAD animals (PBS controls 683.8 ± 120 × 10^4; sBr 58.1 ± 16.7 × 10^4 cells; $P < 0.0001$; Figure 2(a)). In regards to the BAL WBC differentials (Figure 2(b)), MACs remained prevalent with sBr treatment (PBS treated 8.0 ± 1%; sBr 92 ± 4%); $P < 0.0001$), lymphs (PBS treated 11.9 ± 1.7%; sBr 3.3 ± 1.8%; $P < 0.0001$), and EOS (PBS treated 78.5 ± 2.8%; sBr 4.3 ± 3%; $P < 0.0001$) were significantly reduced and PMNs (PBS treated 1.6 ± 1%; sBr 0 ± 0%) remained unchanged.

Similar effects were observed in the OVA-alum subcutaneously sensitized mice, with i.p administration of sBr (Supplementary Figure 4). sBr decreased total BAL leukocytes (PSB control 64 ± 8 × 10^5, sBr 18 ± 4 × 10^5; $P < 0.001$) as well as BAL eosinophils (PSB control 56 ± 8 × 10^5, sBr 14 ± 3 × 10^5;

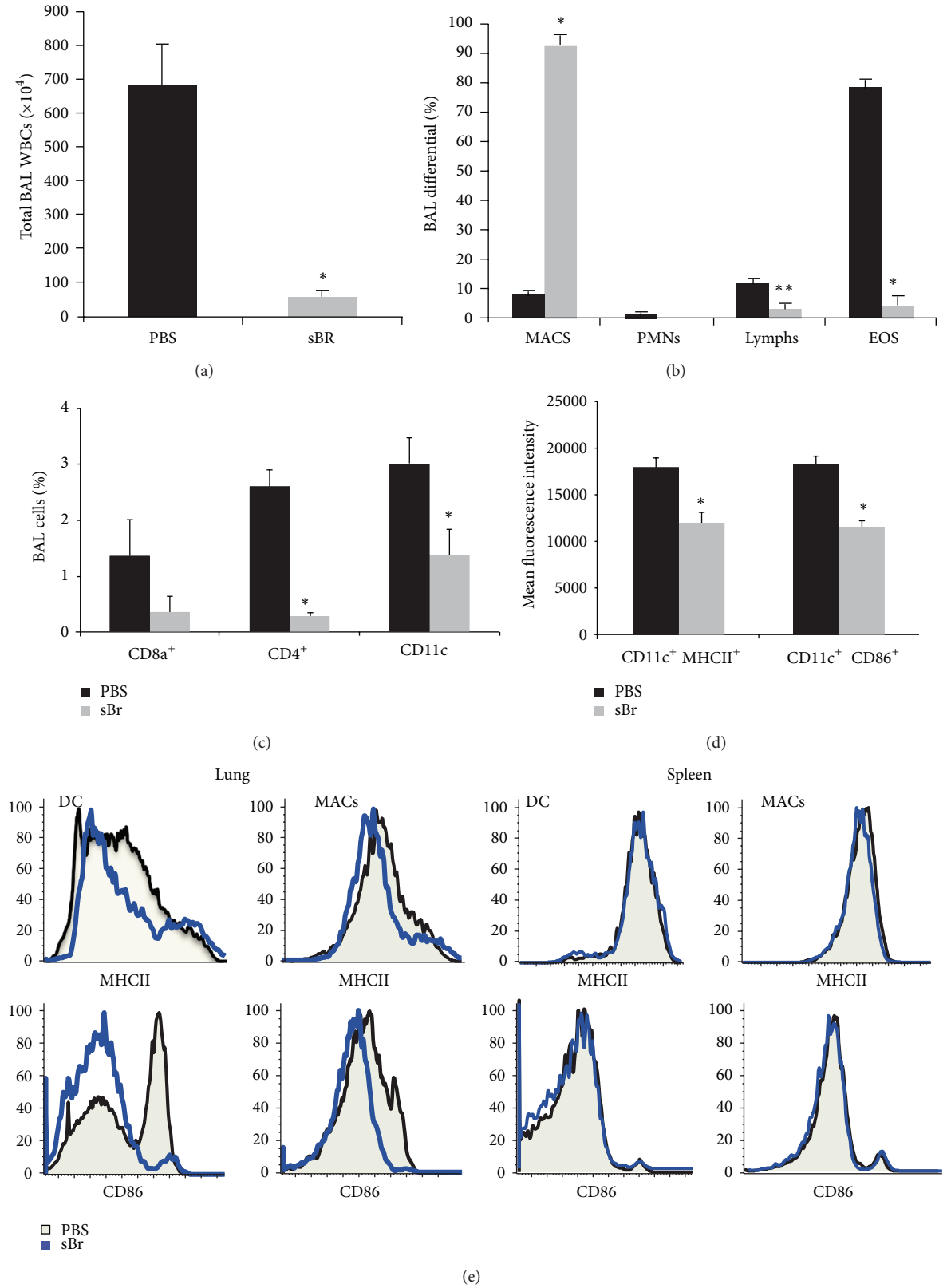

Figure 2: Bromelain treatment through sensitization abrogates the development of AAD upon OVA aerosol challenge. As compared to PBS treated controls, sBr treatment significantly reduced total BAL WBCs (a) as well as lymphocytes and eosinophils (b). sBr also inhibited influx of BAL $CD4^+$ and $CD8^+$ T lymphocytes and % of $CD11c^+$ cells and mean fluorescent intensity of CD86 and MHCII (c). A representative FACS plot (e) compares the MHCII and CD86 expression on lung DCs ($CD11c^+F480^-$) and MACs ($CD11c^+F480^+$) with those in the spleen (n = 8–10 per group).

$P < 0.001$), $n = 8$ animals per group. These observations confirmed that the inhibitory effect of i.p. sBr was not due to allosteric interaction with the i.p. OVA.

3.2. Bromelain Administration during Sensitization Reduced Regional Lymphocytes and Cell Activation after OVA Aerosol Challenge. The above BAL differentials showed that the inhibition of allergic airway disease by sBr treatment was accompanied by an absence of airway lymphocytosis in treated animals. Subsequent FACS analysis demonstrated marked reductions in BAL $CD4^+$ and $CD8^+$ T cells as well as $CD11c^+$ cells in sBr-treated animals (Figure 2(c)). Accompanying the regional reduction in T-cell numbers, the mean fluorescence intensity (MFI) of activation markers MHCII and CD86 were significantly reduced on $CD11c^+$ DCs in lung tissue of sBr-treated mice (Figure 2(d)). A representative histogram (Figure 2(e)) demonstrates that this reduction in receptor expression was predominant in $CD11c^+F4/80^-$ DCs localized to the lung tissue as compared to the spleen.

3.3. Bromelain Administration during Sensitization Inhibited Antigen-Specific Immunoglobulin Production. The prevention of AAD in mice treated with bromelain during OVA sensitization suggested that bromelain interfered with the sensitization process. This consideration was first addressed by measurement of OVA-specific IgE levels after each of the OVA-alum injections. As expected, i.p. sensitization with OVA-alum resulted in the production of OVA-specific IgE. Serum OVA-specific IgE levels increased from non-detectable levels in naïve animals to 869 ± 379 ng/mL following the third i.p. injection (Figure 3). This increase was markedly inhibited in sBr-treated animals, with a final IgE level of only 71 ± 37 ng/mL. The attenuation was due to the proteolytic action of sBr, since treatment of sBr with the antiprotease E64 abolished the effect. Mice treated with E64-treated sBr developed OVA-specific IgE levels of 1165 ± 461 ng/mL after the third i.p sensitization.

3.4. Bromelain Treatment throughout Sensitization Prevented Generation of an OVA Specific $CD8^+$ T-Cell Response. As in Figure 1(b) Protocol-, PBS-, or sBr-treated animals were sacrificed after each weekly OVA-alum i.p. Spleen and nodes (mediastinal, cervical, axillary, brachial, inguinal, and mesenteric) were pooled and enriched for OVA-specific $CD8^+$ T cells via SIINFEKL ($OVA_{257–264}$) loaded tetramer. As compared to PBS-treated controls, sBr treatment significantly limited the expansion of total OVA-TET^+ $CD8^+$ T cells after each weekly OVA-alum i.p. (Figure 4).

3.5. Bromelain Administration during OVA-Alum Sensitization Reduced DCs in the MLNs. The attenuation of both IgE and T-cell responses to OVA demonstrated that sBr modulated pathways involved in allergic sensitization. One key pathway involves antigen presentation by DCs; therefore, we wanted to determine if sBr altered DCs after sensitization. As in Figure 1(b), sBr was administered throughout the 1st OVA-alum i.p., and DCs subtypes were evaluated in the spleen and MLNs. sBr did not affect the number of splenic

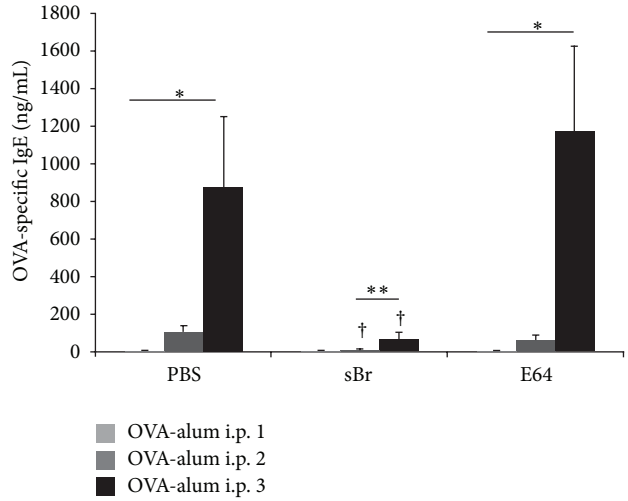

Figure 3: sBr treatment throughout sensitization reduces production of antigen-specific IgE. Peripheral blood of mice (PBS, sBr, or E64-inhibited-sBr) was collected after each weekly sensitization and serum was processed for concentration of OVA-specific IgE. In PBS-treated controls, a significant increase in the concentration of OVA-specific IgE was noted after i.p.'s 2 and 3 when compared to i.p. 1. In sBr-treated groups, the production OVA-specific IgE was delayed and attenuated relative to PBS groups. IgE production was restored in the E64 treatment group. PBS: phosphate buffered saline, E64: E64-inhibited-sBr ($^*P < 0.001$, $^{**}P < 0.05$ between i.p.'s; $^\dagger P < 0.01$ between PBS treated groups; $n = 4$ per group).

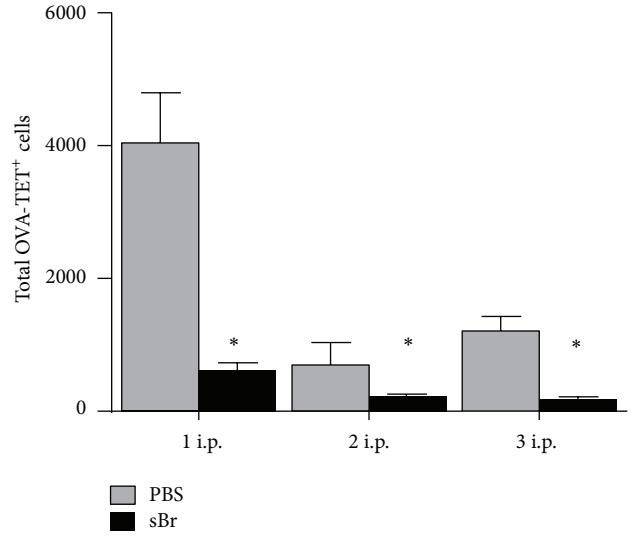

Figure 4: sBr treatment throughout sensitization prevents generation of OVA-specific $CD8^+$ T-cell response. Animals were sacrificed after each weekly OVA-alum i.p. Spleen and nodes were pooled and enriched for OVA-specific $CD8^+$ T cells via SIINFEKL ($OVA_{257–264}$) loaded tetramer. As compared to PBS-treated controls, sBr treatment significantly limited the expansion of total OVA-TET^+ $CD8^+$ T cells after each weekly OVA-alum i.p. ($^*P < 0.001$ compared to PBS; $n = 3–5$ per group).

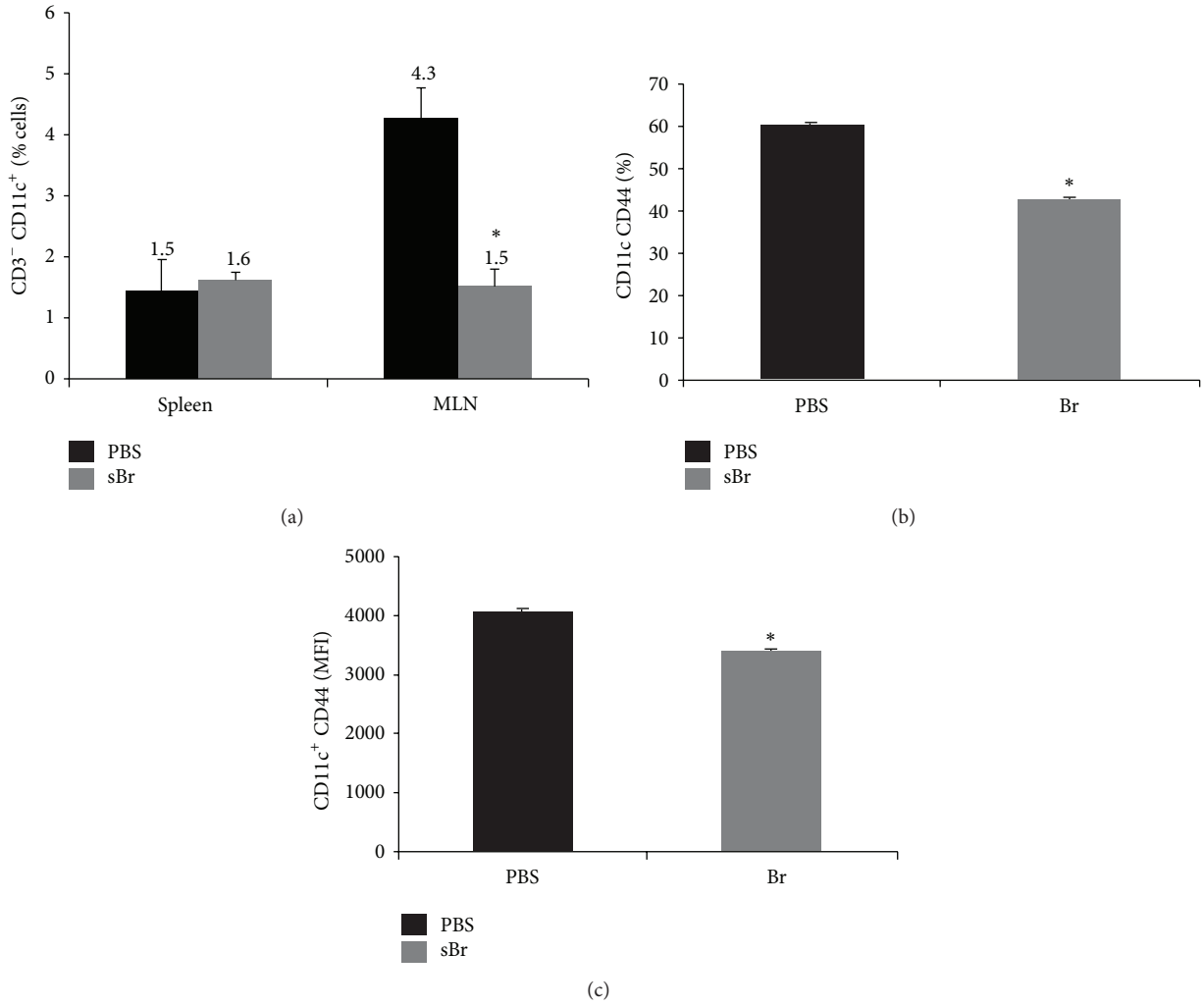

Figure 5: sBr treatment through OVA/alum sensitization reduces DCs in the MLN. Animals were treated with sBr and PBS through the first OVA-alum i.p. Upon sacrifice spleen, and pooled MLN (mesenteric lymph nodes) were processed for analysis via flow cytometry. $CD3^-$ $CD11c^+$ DCs were reduced in MLNs in sBr-treated animals as compared to PBS-treated controls. Both the percentage of $CD44^+$ cells (b) and the mean fluorescent intensity (MFI) of CD44 (c) were significantly reduced in the sBr treatment group as compared to the control PBS group. $^*P < 0.05$; $n = 5$ animals per group.

DCs in OVA-sensitized mice, as assessed by percentage of $CD11c^+$ cells. However, the percentage of DCs in the MLNs was significantly reduced (Figure 5(a)) by sBr administration (PBS 4.3 ± 0.5%, sBr 1.5 ± 0.2%; $P < 0.0001$). Both the percentage (Figure 5(b)) and MFI of CD44 were significantly reduced *in vivo*. In addition, the total $CD11c^+$ cells were reduced in the MLN in CD103 (Supplementary Figure 4(a)), $CDllb^+CD8^+$ (Supplementary Figure 4(b)) and $CDllb^-CD8^+$ (Supplementary Figure 4(c)) subsets.

3.6. In Vitro sBr Treatment of DCs Reduced CD44. Noting that there was a reduction in DCs in the MLNs in addition to CD44 expression with *in vivo* sBr treatment, we wanted to confirm *in vitro* that sBr was having specific effect on DC receptor expression. Therefore, $CD11c^+$ DCs were isolated via positive selection with pan CD11c microbeads and cultured overnight with escalating doses of sBr (1–100 μg/mL) and 100 μM E-64 inhibited sBr 100 μg/mL (Figure 6). As compared to cells in media alone, sBr treatment did not alter the expression of CD11c or MHCII (data not shown). However, CD44 expression was reduced by sBr at doses of 5 μg/mL and greater (Figure 6(b)), with similar reductions in CD44 noted between the 5 and 100 μg/mL doses (Figure 6(c)).

4. Discussion

In previous studies, we demonstrated that i.p. or oral administration of sBr before, during, or after antigen challenge blunts the development of AAD in previously OVA-sensitized mice [16, 18, 19]. The present study extended those findings to demonstrate that sBr given during the sensitization markedly diminished the AAD response to subsequent aerosolized OVA challenge (~90%), despite the absence of sBr treatment during the aerosol challenge period. The BAL of naïve mice

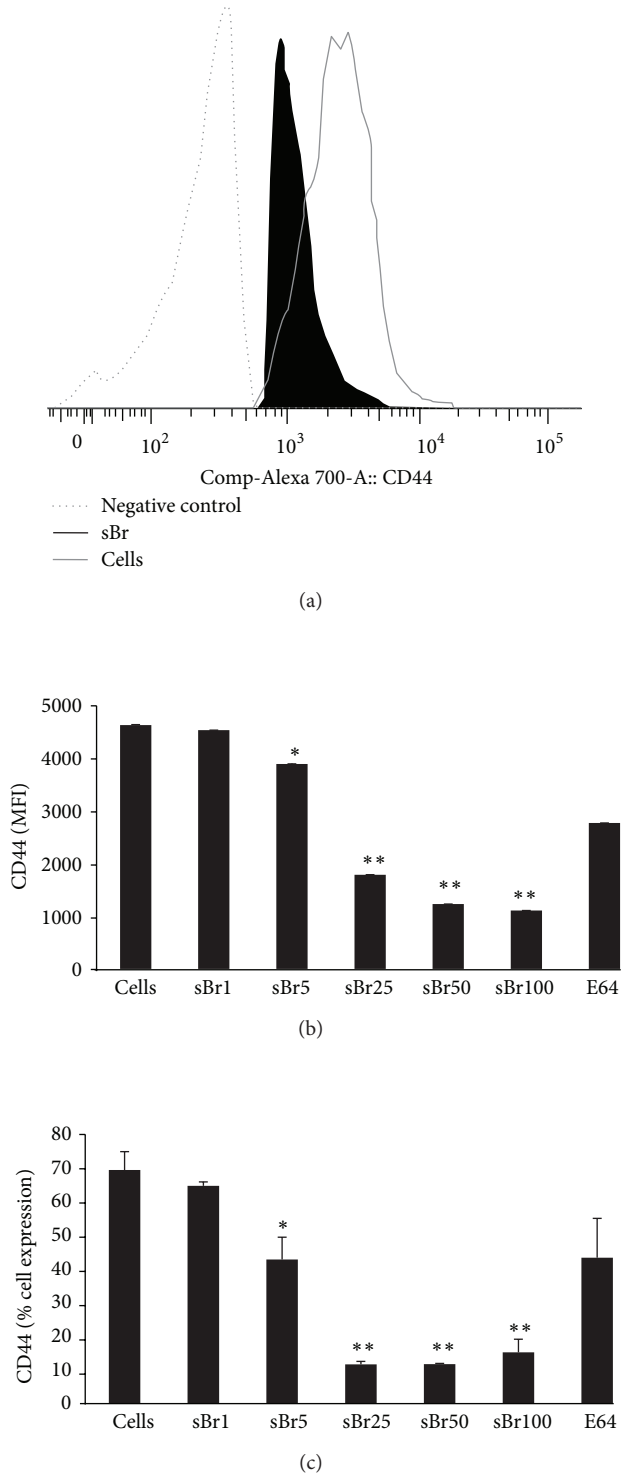

Figure 6: sBr treatment of DCs *in vitro* results in reduced expression of CD44. MLN DCs were selected using Pan DC Beads and cells (5×10^5/well) were cultured overnight. (a) Representative histogram of CD44 expression on cells in media alone (MFI 2668), sBr 100 μg/mL treated cells MFI (1198), and negative control (MFI 244). (b) denotes MFI of CD44 and (c) % cell expression of CD44 on cells alone, sBr (1–100 μg/mL), and (E64 treated sBr 100 μg/mL). Gates were on live, $CD3^-CD11c^+MHCII^+$ cells; MFI: mean fluorescence intensity. $^*P < 0.01$; $^{**}P < 0.0001$ as compared to control cells in media alone. Data represents triplicate wells of duplicate experiments.

and mice undergoing OVA sensitization but no OVA-aerosol challenge consists of >95% macrophages with minimal presence of other cell types [19]. This differential distribution of BAL cell types was not affected during the 3 weeks of sensitization by concomitant administration of sBr (Supplementary Figure 1). In contrast, allergic airway disease (AAD), induced by 3–10 days of aerosolized OVA challenges to sensitized mice, is characterized by marked elevations in eosinophils and lymphocytes [19]. sBr exerted profound inhibitory effects on OVA sensitization itself, resulting in marked reductions in serum OVA-specific IgE and generation of OVA-TET^+ $CD8^+$ T cells. The lack of allergic sensitization was accompanied by a reduction in DCs in the MLN, the percentage of $CD44^+$ cells, and a reduction in expression of CD44, a key modulator of DC activation and migration, *in vitro*.

CD44 is one of the most sensitive surface markers to bromelain degradation. The ~80% reduction in CD44 expression with sBr treatment noted in these *in vitro* studies is similar to the >90% reduction reported by Hale and colleagues in CD44 levels in human peripheral blood lymphocytes incubated for 1 hour in the presence of sBr [27]. CD44 is involved in a number of important biological processes including lymphocyte activation and homing, hematopoiesis, and tumor progression, and metastasis [30]. Of particular relevance to allergies and asthma, it has been shown that CD44 expression on DCs plays a crucial role in DC activation of T cells. The engagement of CD44 molecules expressed on the surface of DCs by specific mAbs or by its extracelluar matrix ligand, hyaluronic acid, induces DC phenotypic and functional maturation [31]. This maturation is associated with increased expression of several surface markers, including HLA class II molecules, and increased allogeneic T cell stimulatory capacity [33–35]. CD44 receptor activity is induced by antigen stimulation in antigen-sensitized spleen $CD4^+$ T cells, and T-cell expression of CD44 is important for the accumulation of antigen-specific Th2 cells in the airway and in the development of AAD induced by antigen challenge [32, 36]. Thus, sBr-induced reduction of CD44 on DCs could have resulted in impaired sensitization to antigen. Future studies will investigate the role of sBr on antigen uptake and presentation in DCs to determine the degree to which DC function is altered.

In summary, the present study demonstrated that sBr attenuated the AAD response when administered throughout sensitization. Additionally, sBr prevented allergic sensitization, which was attributed to reduced accumulation of DCs in the MLNs and decreased CD44 expression of treated DCs *in vitro*. Future research may confirm sBr's role and the specific components within sBr [37] which modulate antigen uptake and presentation in DCs. Both pathways are likely targets in combating allergies. These findings identified an additional inhibitory mechanism of sBr on allergic responses and further support the potential utility of this CAM product in the treatment of allergies and asthma.

Conflict of Interests

The authors have no conflict of interests.

Authors Contributions

Drs. Szczepanek, Linda Guernsey, Christine Castater, and Prabitha Natarajan assisted with animal experiments, acquisition of data, and its analysis and interpretation. Drs. Adam P. Matson and Ektor T. Rafti processed animal samples for acquisition of OVA-specific immunoglobulin data as well as analysis and interpretation. Dr. McNamara assisted with OVA-tetramer studies. Drs. Schramm, Thrall, and Silbart participated in adding substantial intellectual content, study design, and helping in original drafting and revising of the paper. The authors also provided comments and suggestions with the paper content.

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Review Article

Cichorium intybus: Traditional Uses, Phytochemistry, Pharmacology, and Toxicology

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The genus *Cichorium* (Asteraceae) is made up of six species with major geographical presence in Europe and Asia. *Cichorium intybus*, commonly known as chicory, is well known as a coffee substitute but is also widely used medicinally to treat various ailments ranging from wounds to diabetes. Although this plant has a rich history of use in folklore, many of its constituents have not been explored for their pharmacological potential. Toxicological data on *C. intybus* is currently limited. This review focuses on the economic and culturally important medicinal uses of *C. intybus*. Traditional uses, scientific validation, and phytochemical composition are discussed in detail.

1. Introduction

The genus *Cichorium* (Asteraceae) consists of six species with major distribution areas in Europe and Asia [1]. In several Asteraceae, inulin, a β-2,1 linked fructose polymer with a terminal glucose residue, functions as a reserve carbohydrate in stems, tubers, and taproots [2]. *Cichorium intybus* L., commonly known as chicory, is an erect fairly woody perennial herb, around 1 m in height with a fleshy taproot of up to 75 cm in length and large basal leaves [1, 3]. Historically, chicory was grown by the ancient Egyptians as a medicinal plant, coffee substitute, and vegetable crop and was occasionally used for animal forage. In the 1970s, it was discovered that the root of *C. intybus* contained up to 40% inulin, which has a negligible impact on blood sugar and thus is suitable for diabetics [4]. To date, *C. intybus* is grown for the production of inulin on an industrial scale [2]. The name of the plant is derived from Greek and Latin. *Cichorium* means *field* and *intybus* is partly derived from the Greek "to cut", because of the leaves, and partly from the Latin *tubus* to indicate the hollow stem [5].

Chicory is a hardy plant and can endure extreme temperatures during both vegetative and reproductive growth stages [1]. When broken, all plant parts exudate a milky latex [3]. *Cichorium intybus* is cultivated for numerous applications and can be divided into four main varieties or cultigroups according to their use [6]: (1) "industrial" or "root" chicory, predominantly cultivated in northwestern Europe, India, South Africa, and Chile, produces the taproot as a coffee substitute or for inulin extraction; (2) "Brussels" or "witloof" chicory is commonly cultivated around Europe as industrial chicory for etiolated buds (chicons) by forcing; (3) "leaf" chicory is used as fresh or cooked vegetables; and (4) "forage" chicory, initially derived from wild chicory commonly found along roadsides and waste areas, has been used since the mid-1970s to intensify herbage obtainability in perennial pastures for livestock.

Cichorium intybus is a medicinally important plant in Eurasia and in parts of Africa. Despite its long tradition of use, the plant is not described in the European Pharmacopoeia or in any official Pharmacopoeia of a European Union member state [5]. However, due to its prevalent distribution, different parts of the plant have been used in traditional medicines globally [7]. Important phytochemicals are distributed throughout the plant, but the main contents are present in the root [1]. This review focuses on the economic and

culturally important medicinal uses of *C. intybus*. Traditional uses, scientific validation, and phytochemical composition are discussed in detail.

2. Traditional Uses

Medicinal plants have been used for centuries and numerous cultures still rely on indigenous medicinal plants to meet their primary health care needs. It is likely that the insightful knowledge of plant-based remedies in traditional cultures advanced through trial and error and that the most important cures were carefully passed from one generation to another [8]. Historically, chicory was grown by the ancient Egyptians as a medicinal plant [9] and it has had a long history of therapeutic use both in areas where it is indigenous and in areas where it has been introduced. The various common or local names describing this plant may be ascribed to the widespread use by different folkloric groups.

Different preparations of this plant are employed to treat various symptoms and ailments (Table 1). The juice is said to be a folk remedy for cancer of the uterus and for tumors [4]. In South Africa, although it is considered a widespread weed, leaves, stems, and roots are made into a tea for jaundice and chicory syrup is used as a tonic and purifying medicine for infants [3]. In Turkey, an ointment is made from the leaves for wound healing [10]. Decoction refers to a preparation that is made by adding cold water to the plant material which is then boiled and allowed to simmer for 5–10 min after which it is strained [8]. Chicory decoctions are traditionally made from individual plant parts and/or from the plant as a whole (Table 1).

According to the European monograph, traditional use of chicory roots includes the relief of symptoms related to mild digestive disorders (such as feeling of abdominal fullness, flatulence, and slow digestion) and temporary loss of appetite [11]. Prior to the wars in Afghanistan, folkloric reports described the use of aqueous root extracts as a light-sensitive plant remedy for malaria. This indigenous knowledge has since been confirmed and the antimalarial compounds of *C. intybus* roots have been identified as the light-sensitive sesquiterpene lactones lactucin and lactucopicrin [12]. The flowers of the chicory plant (*Cichorii flos*) are used as a herbal treatment of everyday ailments such as a tonic and appetite stimulant and as a treatment of gallstones, gastroenteritis, sinus problems, cuts, and bruises [4]. In Italy, the whorls are made into a decoction and used as a depurative [13]. Chicory seeds are one of the main ingredients of *Jigrine,* a commercial product of India used for the treatment of various diseases of the liver [14]. Other plant parts are also used for liver disorders, namely, aerial parts in Bosnia and Herzegovina [15] and roots in Serbia and India [16, 17].

3. Chemical Constituents

Chicoric acid has been identified as the major compound in methanolic extracts of chicory (Table 2) [18]. Aliphatic compounds and their derivatives comprise the main fraction while terpenoids comprise minor constituents of the plant. The flowers of chicory contain saccharides, methoxycoumarin cichorine, flavonoids, essential oils [4], and anthocyanins contributing to the blue colour of the perianth [19]. Table 2 provides a summary of the compounds isolated and identified from chicory. Octane, *n*-nonadecane, pentadecanone, hexadecane, and a tentatively identified compound have been found as principal volatile components [4]. A list of volatile compounds is given in Table 3.

4. Pharmacological Activities

Cichorium intybus presents a little investigated plant in terms of phytochemistry and pharmacology. Over 100 individual compounds have been isolated and identified from this plant (Table 2), the majority of which are from the roots. Most of the pharmacological studies on this plant document the testing of aqueous and/or alcoholic extracts only. Apart from the pharmacologically important activities, the use of *C. intybus* (hairy root cultures) has also been implicated in the phytoremediation of DDT [20].

4.1. Antimicrobial Activity. The antibacterial activity of the organic acid-rich extract of fresh red chicory (*C. intybus* var. sylvestre) was tested against periodontopathic bacteria including *Streptococcus mutans*, *Actinomyces naeslundii,* and *Prevotella intermedia*. The compounds identified from the active extract include oxalic acid, succinic acid, quinic acid, and shikimic acid. All of the organic acids were found to decrease biofilm formation and adhesion of bacteria to the cells, with different levels of efficacy. These compounds also induced biofilm disruption and detachment of dead cells for the cultured substratum [21]. In other reports on the antimicrobial activity of *C. intybus*, the crude aqueous and organic seed extracts were found to be active against four pathogenic microorganisms, namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli,* and *Candida albicans,* and root extracts had pronounced effects on *Bacillus subtilis*, *S. aureus*, *Salmonella typhi*, *Micrococcus luteus*, and *E. coli* [22, 23]. The leaf extract of *C. intybus* also showed a moderate activity against multidrug resistant *S. typhi* [24]. Guaianolides-rich root extracts of *C. intybus* have shown antifungal properties against anthropophilic fungi *Trichophyton tonsurans*, *T. rubrum,* and *T. violaceum* [25]. A sesquiterpenoid phytoalexin cichoralexin isolated from chicory exhibited potent antifungal activity against *Pseudomonas cichorii* [26].

4.2. Anthelmintic Activity. Several studies have been conducted on grazing animals to determine the anthelmintic potential of secondary metabolites present in *C. intybus*. Grossly, it has been concluded that the animals grazing on chicory have a higher performance index and lower incidence of gastrointestinal nematode infestations. In the majority of the experiments, the condensed tannins and sesquiterpene lactones were responsible for anthelmintic activity [27]. Anthelmintic activity of chicory has also been noticed in the case of lambs wherein the total number of abomasal helminths was found to be lesser in the lambs grazing on

Table 1: Traditional medicinal uses of *Cichorium intybus*.

Country	Traditional use(s)	Plant part(s)	Preparation(s)	Reference
Afghanistan	Malaria	Root	Aqueous extract	[12]
Bosnia and Herzegovina	Diarrhea, strengthening the prostate and other reproductive organs, pulmonary cancer, hangover, and purification of biliary tract	Aerial part, flowers, roots	Not stated	[92]
	Liver disorders, spasmolytic, cholesterol, antiseptic	Aerial	Decoction	[15]
Bulgaria	Cholagogue stimulant for gastric secretion, hypoglycemic	Roots, aerial parts	Decoction	[93]
India	Liver disorders	Seeds		[14]
	Diabetes	Whole plant	Not stated	[17]
	Jaundice, liver enlargement, gout, and rheumatism	Root	Decoction	[17]
	Cough relief	Not stated		
Iran	Eupeptic, stomachic, depurative, choleretic, laxative, hypotension, tonic, and antipyretic	Whole plant	Not stated	[94]
Italy	Blood cleansing	Leaves	Not stated	[13]
	High blood pressure	Leaves	Decoction	[95]
	Blood purification, arteriosclerosis, antiarthritis, antispasmodic, digestive	Leaves/roots	Decoction	[96]
	Depurative	Whorls	Decoction	[97]
	Choleretic, hepatoprotective against jaundice, mild laxative, hypoglycemic	Leaves	Decoction, squashed fresh leaves	[93]
Jordan	Internal hemorrhage, sedative in typhoid	Whole plant	Cooking	[14]
Morocco	Renal disease	Aerial/roots	Not stated	[98]
	Kidney disorders, diabetes	Whole plant	Decoction	[99]
Pakistan	Diabetes	Roots	Decoction	[43]
Poland	Digestive complaints and lack of appetite	Roots	Tea	[5]
Serbia	Diarrhea	Flower	Infusion	[100]
	Diuretic, digestive, laxative, anti-inflammatory, liver complaints, reducing blood sugar	Roots	Decoction/tea	[16]
	Cholagogue, digestive, hypoglycemic	Aerial part/root	Not stated	[101]
South Africa	Jaundice, tonic	Leaves, stems, roots		[3]
Turkey	Cancer, kidney stones	Roots	Decoction	[7]
	Wound healing	Leaf	Ointment	[10]
	Hemorrhoids, urinary disorders	Aerial	Tea	[102]

this plant [28]. The condensed tannin and sesquiterpene-rich extracts of *C. intybus* were evaluated for their efficacy against the larvae of deer lungworm, *Dictyocaulus viviparous* and other gastrointestinal nematode larvae using a larval migration inhibition assay. A dose-dependent decrease in the larval motility was observed in both lungworm and gastrointestinal nematodes [29]. The sesquiterpene lactone-rich extracts of *C. intybus* were also found to inhibit egg hatching of *Haemonchus contortus* [30].

4.3. Antimalarial Activity. The infusion of fresh roots of *C. intybus* has a history of use as a remedy for malarial fevers in some parts of Afghanistan. The bitter compounds in the plant, namely, lactucin, lactucopicrin, and the guaianolide sesquiterpenes, isolated from aqueous root extracts of chicory were concluded to be the antimalarial components of the plant. Lactucin and lactucopicrin completely inhibited the HB3 clone of strain Honduras-1 of *Plasmodium falciparum* at concentrations of 10 and 50 μg/mL, respectively [12, 31].

Table 2: Compounds isolated and identified from *Cichorium intybus* (chicory).

Compound	Reference(s)
Lactucin	[12, 31, 103]
Lactucopicrin	[12, 31]
8-Deoxylactucin	[31, 104]
Jacquilenin	[103]
11β,13-Dihydrolactucin	[103]
11,13-Dihydrolactucopicrin	[103, 104]
Crepidiaside B	[103]
Cyanidin 3-O-p-(6-O-malonyl)-D-glucopyranoside	[105]
3,4β-Dihydro-15-dehydrolactucopicrin	[103]
Magnolialide	[103]
Ixerisoside D	[103]
Loliolide	[103]
Cichorioside B	[103, 104]
Sonchuside A	[103, 104]
Artesin	[103]
Cichoriolide	[103]
Cichorioside	[103]
Sonchuside C	[103]
Cichopumilide	[103]
Putrescine	[83]
Spermidine	[83]
β-Sitosterol	[7, 83]
Campesterol	[83]
Stigmasterol	[83]
(7S, 8R)-3′-Demethyl-dehydrodiconiferyl alcohol-3′-O-β-glucopyranoside	[106]
Chlorogenic acid	[19, 44, 106]
3,5-Dicaffeoylquinic acid	[18, 106]
4,5-Dicaffeoylquinic acid	[106]
Crepidiaside A	[106]
Cichoralexin	[26]
Malic acid	[18]
Caffeic acid	[18, 44]
3-Caffeoylquinic acid	[18]
5-Caffeoylquinic acid	[18]
4-Caffeoylquinic acid	[18]
cis-5-Caffeoylquinic acid	[18]
cis-Caftaric acid	[18]
trans-Caftaric acid	[18]
5-Caffeoylshikimic acid	[18]
5-*p*-Coumaroylquinic acid	[18]
Quercetin-3-O-glucuronide-7-O-(6″-O-malonyl)-glucoside	[18]
Kaempferol-3-O-glucosyl-7-O-(6″-O-malonyl)-glucoside	[18]
Dimethoxycinnamoyl shikimic acid	[18]
Kaempferol-3-O-sophoroside	[18]

Table 2: Continued.

Compound	Reference(s)
Isorhamnetin-7-O-(6″-O-acetyl)-glucoside	[18]
5-O-Feruloylquinic acid	[18]
Dicaffeoyltartaric acid (chicoric acid)	[18]
Kaempferol-7-O-glucosyl-3-O-(6″-malonyl)-glucoside	[18]
Delphinidin-3-O-(6″-O-malonyl)-glucoside-5-O-glucoside	[18]
Cyanidin-3,5-di-O-(6″-O-malonyl)-glucoside	[18]
Cyanidin-3-O-(6″-O-malonyl)-glucoside	[18]
Petunidin-3-O-(6″-O-malonyl)-glucoside	[18]
Cyanidin	[18, 105]
Cyanidin-3-O-galactoside	[18]
Cyanidin-3-O-glucoside	[18, 105]
Cyanidin-3-O-(6″-O-acetyl)-glucoside	[18]
Malvidin-3-O-glucoside	[18]
Pelargonidin-3-O-monoglucuronide	[18]
4-O-Feruloylquinic acid	[18]
Apigenin-7-O-glucoside	[18]
Chrysoeriol-3-O-glucoside	[18]
Tricin-3-O-glucoside	[18]
1,3-Dicaffeoylquinic acid	[18]
1,4-Dicaffeoylquinic acid	[18]
3,4-Dicaffeoylquinic acid	[18]
Quercetin-7-O-galactoside	[18]
Quercetin-3-O-(6″-O-malonyl)-glucoside	[18]
Quercetin-7-O-glucoside	[18]
Quercetin-7-O-glucuronide	[18]
Quercetin-7-O-(6″-O-acetyl)-glucoside	[18]
Kaempferide glucuronide	[18]
Kaempferol-7-O-glucoside	[18]
Kaempferol-7-O-rutinoside	[18]
Quercetin-7-O-p-coumaroylglucoside	[18]
Isorhamnetin-7-O-neohesperidoside	[18]
Kaempferol-7-O-(6″-O-malonyl)-glucoside	[18]
Kaempferol-7-O-glucuronide	[18]
Kaempferide-3-O-(6″-O-malonyl)-glucoside	[18]
Kaempferol-3-O-glucuronide	[18]
Kaempferol-3-O-glucuronide-7-O-glucoside	[18]
Kaempferol-3-O-(6″-O-malonyl)-glucoside	[18]
Kaempferol-3-O-glucoside	[18]

Table 2: Continued.

Compound	Reference(s)
Myricetin-7-O-(6″-O-malonyl)-glucoside	[18]
Kaempferol-7-O-neohesperidoside	[18]
Kaempferol-7-O-(6″-O-acetyl)-glucoside	[18]
Kaempferol-3-O-(6″-O-acetyl)-glucoside	[18]
Isorhamnetin-7-O-glucoside	[18]
Isorhamnetin-7-O-glucuronide	[18]
Delphinidin 3,5-di-O-(6-O-malonyl-β-D-glucoside)	[19]
Delphinidin 3-O-(6-O-malonyl-β-D-glucoside)-5-O-β-D-glucoside	[19]
Delphinidin 3-O-β-D-glucoside-5-O-(6-O-malonyl-β-D-glucoside)	[19]
Delphinidin 3,5-di-O-β-D-glucoside	[19]
3-O-p-Coumaroyl quinic acid	[19]
Cyanidin 3-O-β-(6-O-malonyl)-D-glucopyranoside	[105]
Quercetin 3-O-β-D-glucoside	[19]
Oxalic acid	[67]
Shikimic acid	[67]
Quinic acid	[67]
Succinic acid	[67]

4.4. Hepatoprotective Activity. The folkloric use of *C. intybus* as a hepatoprotectant has been well documented. It is one of the herbal components of Liv-52, a traditional Indian tonic used widely for hepatoprotection. In a randomized, double-blind clinical trial conducted on cirrhotic patients, Liv-52 medication reduced the serum levels of hepatic enzymes, namely, alanine aminotransferase and aspartate aminotransferase. It also reduced the Child-Pugh scores and ascites significantly [32]. Another polyherbal formulation, *Jigrine*, contains the leaves of *C. intybus* as one of its 14 constituents. *Jigrine* was evaluated for its hepatoprotective activity against galactosamine-induced hepatopathy in rats. The pretreatment of male Wistar-albino rats with *jigrine* significantly reduced the levels of aspartate transaminase, alanine transaminase, and urea and increased the levels of blood and tissue glutathione. Histopathological examination of the liver revealed that *jigrine* pretreatment prevented galactosamine toxicity and caused a marked decrease in inflamed cells [33].

The aqueous-methanolic extract of the seeds of *C. intybus* has been investigated for the hepatoprotective activity against acetaminophen and carbon tetrachloride-induced liver damage in mice. It was found to decrease both the death rate and the serum levels of alkaline phosphatase, glutamyl oxaloacetate transaminase, and glutamyl pyruvate transaminase [34]. In analogous studies, the antihepatotoxic activity of the alcoholic extract of the seeds and aqueous extracts of the roots and root callus of *C. intybus* was estimated. The oral administration of these extracts in albino rats led to a marked decrease in the levels of hepatic enzymes. Also, histopathological examination of the liver showed no fat accumulation or necrosis after the treatment [14, 35]. Similar studies have established the hepatoprotective effect of esculetin, a phenolic compound, and cichotyboside, a guaianolide sesquiterpene glycoside reported from *C. intybus* [36, 37].

Table 3: The volatile constituents of *Cichorium intybus* (adapted from Judžentienė and Būdienė [4]).

Compound
Octane
Octen-3-ol
2-Pentyl furan
(2*E*, 4*E*)-Heptadienal
1,8-Cineole
Benzene acetaldehyde
n-Nonanal
Camphor
(2*E*, 6Z)-Nonadienal
(2*E*)-Nonen-1-al
n-Decanal
(2*E*, 4*E*)-Nonadienal
n-Decanol
(2*E*, 4Z)-Decadienal
n-Tridecane
(2*E*, 4*E*)-Decadienal
β-Elemene
(*E*)-Caryophyllene
β-Ylangene
Geranyl acetone
(*E*)-β-Farnesene
allo-Aromadendrene
dehydro-Aromadendrene
β-Ionone
Pentadecane
trans-β-Guaiene
(2*E*)-Undecanol acetate
Sesquicineole
(2*E*)-Tridecanol
n-Hexadecane
Tetradecanal
Tetradecanol
2-Pentadecanone
(*E*)-2-Hexylcinnamaldehyde
Octadecane
n-Nonadecane
(5*E*, 9*E*)-Farnesyl acetone
n-Eicosane
n-Octadecanol
n-Heneicosane

The carbon tetrachloride and paracetamol-induced liver toxicities were also found to be counteracted by intraperitoneal administration of crude extracts and fractions of *C. intybus*. The methanol- and water-soluble fractions exhibited marked reductions in serum glutamyl pyruvate transaminase, serum glutamyl oxaloacetate transaminase, alkaline phosphatase, and total bilirubin levels. In the same study, toxicity was induced in rat hepatocytes by incubation with galactosamine and thioacetamide [38].

The phenolic acid-rich seed extract of *C. intybus* was evaluated for its efficacy against hepatic steatosis *in vitro* and *in vivo*. The *in vitro* model of hepatic steatosis was created by incubation of the HepG2 cells with oleic acid leading to intracellular accumulation of fat. The seed extract was effective in decreasing the deposited fat from the cells in case of administration after the initial fat deposition (i.e., nonsimultaneous administration with oleic acid). However, in case of simultaneous administration of seed extract and oleic acid, the extract could not protect the cells from steatosis except at very high doses. The extract also led to the increased release of glycerol (an indicator of triglyceride degradation) in steatotic cells. In case of nonsimultaneous administration, the extract was found to upregulate the expression of SREBP-1c and PPAR-α genes leading to restoration of normal levels of corresponding proteins. In the *in vivo* model of hepatic steatosis, namely, diabetic rats, treatment with seed extract resulted in significant decrease in fat accumulation and fibrosis [39].The hepatoprotective activity of *C. intybus* has been correlated to its ability to inhibit the free radical mediated damage. A fraction prepared from the ethanolic extract of the leaves was assessed for preventive action on the free radical mediated damage to the deoxyribose sugar of the DNA (obtained from calf thymus). A dose-dependent decrease in the DNA damage was observed in the present assay [40].

4.5. Antidiabetic Activity. Chicory has reported antidiabetic activity [17, 41]. Based on the traditional use of *C. intybus* in diabetes mellitus, the hypoglycemic and hypolipidemic properties of the ethanol extract of the whole plant were investigated. Diabetes was induced by intraperitoneal administration of streptozotocin in male Sprague-Dawley rats. The ethanol extract, at a dose of 125 mg/Kg body weight, significantly attenuated the serum glucose levels in the oral glucose tolerance test. A marked decrease in the serum triglycerides and cholesterol was also observed in the extract-treated rats. Hepatic glucose-6-phosphatase activity was found to be reduced in extract-treated diabetic rats as compared to untreated diabetic rats [17]. The antidiabetic effect of the aqueous seed extract of *C. intybus* has also been investigated. Early-stage and late-stage diabetes were differently induced in male Wistar albino rats by streptozotocin-niacinamide and streptozotocin alone, respectively. The treatment with chicory extract prevented weight loss in both early-stage and late-stage diabetic rats. Chicory-treated diabetic animals resisted excessive increase in fasting blood sugar (assessed by glucose tolerance test). Grossly, normalization of blood parameters, namely, alanine aminotransferase, triacylglycerol, total cholesterol, and glycosylated heamoglobin, was seen in these animals. In early-stage diabetic rats, chicory treatment led to the increase in insulin levels pointing toward the insulin-sensitizing action of chicory [42].

Feeding the diabetic Wistar rats with *C. intybus* leaf powder led to a decrease in blood glucose levels to near normal value. *C. intybus* administration also decreased the malondialdehyde (formed by thiobarbituric acid) levels and increased glutathione content. Anticholinesterase activity was restored to near normal, brain lipopolysaccharide decreased, and catalase activity increased [43]. Caffeic acid and chlorogenic acid have been described as potential antidiabetic agents by increasing glucose uptake in muscle cells. Both compounds were also able to stimulate insulin secretion from an insulin-secreting cell line and islets of Langerhans. Another compound, chicoric acid, is also a new potential antidiabetic agent exhibiting both insulin-sensitizing and insulin-secreting properties [44].

4.6. Gastroprotective Activity. C. intybus has been used in Turkish folklore for its antiulcerogenic potency. The aqueous decoction of *C. intybus* roots was orally administered to Sprague-Dawley rats 15 minutes before the induction of ulcerogenesis by ethanol. More than 95% inhibition of ulcerogenesis was observed in the test group [45].

4.7. Anti-Inflammatory Activity. The inhibition of TNF-α mediated cyclooxygenase (COX) induction by chicory root extracts was investigated in the human colon carcinoma (HT 29) cell line. The ethyl acetate extract inhibited the production of prostaglandin E_2 (PGE_2) in a dose-dependent manner. TNF-α mediated induction of COX-2 expression was also suppressed by the chicory extract [46].

4.8. Analgesic Activity. Lactucin, lactucopicrin, and 11β, 13-dihydrolactucin exhibited analgesic action in mice in hot plate and tail-flick tests. In the hot plate test, all three compounds exerted an analgesic effect, with lactucopicrin being the most potent compound. In the tail-flick test, the antinociceptive effects of all the tested compounds (30 mg/kg dose) were comparable to that of ibuprofen (60 mg/kg dose). Lactucin and lactucopicrin were also established to have some sedative action as evident from the decreased spontaneous locomotor activity in mice [47].

4.9. Antioxidant Activity. The DPPH radical scavenging activity of a polyphenols-rich fraction of *C. intybus* has been investigated [48]. The anti- and prooxidant activities of *Cichorium* species were studied in chemical as well as biological systems. In the case of chemical systems, the antioxidant activity of water-soluble compounds in *C. intybus* var. *silvestre* was established in the coupled model of linoleic acid and β-carotene. A pro-oxidant activity of some of the chemical components was recorded initially which notably diminished with time and/or thermal treatment. Thereafter, the antioxidant activity of the raw juice and its fractions persisted. The molecular weight ranges of the antioxidant fractions of raw juice were also identified based on dialysis [49]. Two varieties of chicory, namely, *C. intybus* var. *silvestre* and *C. intybus* var. *foliosum*, have been investigated for their

antioxidant (antiradical) activities in two distinct biological systems. The lipid peroxidation assay has been carried out on microsome membranes of rat hepatocytes after the induction of oxidative damage by carbon tetrachloride. The antiradical activity was expressed as the protective activity against lipid peroxidation and calculated as the percentage decrease in hydroperoxide degradation products. The second biological system used was the cultures of *S. aureus* after treatment with cumene hydroperoxide. The percentage increase of growth of bacteria was noted after the treatment with juices of chicory varieties. In both systems, the juices of chicory varieties showed strong antiradical activities [21, 49].

Red chicory (*C. intybus* var. *silvestre*) was studied for its polyphenol content and the antioxidant activity was evaluated by using the synthetic 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl radical and three model reactions catalyzed by pertinent enzymatic sources of reactive oxygen species, namely, xanthine oxidase, myeloperoxidase, and diaphorase. Total phenolics were significantly correlated with the antioxidant activity evaluated with both the synthetic radical and the enzyme-catalyzed reactions. On a molar basis, red chicory phenolics were as efficient as Trolox (reference compound) in scavenging the synthetic radical [50]. The aqueous-alcoholic extracts of the aerial parts of *C. intybus* also inhibited xanthine oxidase enzyme dose dependently [51]. In another study, along with DPPH radical scavenging activity, *C. intybus* also exhibited inhibition of hydrogen peroxide and chelation of ferrous ion [52].

4.10. Tumor-Inhibitory Activity. The crude ethanolic extract of *C. intybus* roots caused a significant inhibition of Ehrlich tumor carcinoma in mice. A 70% increase in the life span was observed with a 500 mg/kg/day intraperitoneal dose of the tested extract [53]. The aqueous-alcoholic macerate of the leaves of *C. intybus* also exerted an antiproliferative effect on amelanotic melanoma C32 cell lines [54]. Magnolialide, a 1β-hydroxyeudesmanolide isolated from the roots of *C. intybus*, inhibited several tumor cell lines and induced the differentiation of human leukemia HL-60 and U-937 cells to monocyte or macrophage-like cells [55].

4.11. Antiallergic Activity. The aqueous extract of *C. intybus* inhibited the mast cell-mediated immediate allergic reactions *in vitro* as well as *in vivo*. This extract restrained the systemic anaphylactic reaction in mice in a dose-dependent manner. It also significantly inhibited passive cutaneous anaphylactic reaction caused by anti-dinitrophenyl IgE in rats. Other markers of allergic reaction, namely, plasma histamine levels and histamine release from rat peritoneal mast cells, decreased significantly whereas the levels of cAMP increased after the treatment with *C. intybus* extract [56].

4.12. Other Pharmacologically Important Activities. The ethanol extract of the roots of *C. intybus* is reported to prevent the immunotoxic effects of ethanol in ICR mice. It was noted that body weight gains were markedly decreased in mice administered with ethanol. However, the body weight was not affected when ethanol was coadministered with the ethanol extract of *C. intybus*. Similarly, the weights of liver and spleen were not affected when ethanol extract was given along with ethanol. A considerable restoration in the other markers of immunity, namely, hemagglutination titer, plaque forming cells of spleen, secondary IgG antibody production, delayed-type hypersensitivity reaction (in response to subcutaneous administration of sheep red-blood cells to paw), phagocytic activity, number of circulating leucocytes, NK cell activity, cell proliferation, and production of interferon-γ, was registered [57]. The immunoactive potential of an aqueous-alcoholic extract of the roots was established by a mitogen proliferation assay and mixed lymphocyte reaction (MLR). The extract showed an inhibitory effect on lymphocyte proliferation in the presence of phytohemagglutinin and a stimulatory effect on MLR [58].

Chicoric acid has shown vasorelaxant activity against nor-epinephrine-induced contractions in isolated rat aorta strips [59]. A pronounced anticholinesterase activity of the dichloromethane extract of *C. intybus* roots was seen in the enzyme assay with Ellman's reagent. Two sesquiterpene lactones, namely, 8-deoxylactucin and lactucopicrin, also exhibited a dose-dependent inhibition of anticholinesterase [60]. The methanolic extract displays wound healing effect and β-sitosterol was determined as the active compound responsible for the activity, possibly due to its significant anti-inflammatory and antioxidant effects, as well as hyaluronidase and collagenase inhibition [7].

5. Toxicological Studies

Although *C. intybus* has a long history of human use, the high levels of secondary metabolites have shown potential toxicological effects. To evaluate the safety of the root extract of *C. intybus*, Ames test and subchronic toxicity assessment were conducted. The sesquiterpene-rich extract was evaluated for potential mutagenic properties (Ames test) using *Salmonella typhimurium* strains TA97a, TA98, TA100, and TA1535 and *Escherichia coli* strain WP2 *uvrA*. Though cytotoxicity was observed at high extract doses in some strains, mutagenicity was not noted. A 28-day (subchronic) oral toxicity study, conducted in CRL:CD (SD) IGS BR rats, concluded that there was no extract-related mortality or any other signs of toxicological significance [61]. The toxicity evaluation of *C. intybus* extracts has also been done by *Vibrio fischeri* bioluminescence inhibition test (Microtox acute toxicity test). This bacterial test measures the decrease in light emission from the marine luminescent bacteria *V. fischeri* when exposed to organic extracts. The tested extracts showed less than 20% inhibition of bioluminescence and hence were concluded to be safe for human use [54].

6. Clinical Trials

Two clinical studies on chicory roots are reported in the literature, both of which are pilot studies and are therefore considered to be insufficient to support a well-established use indication for chicory root [5]. The first study, a phase 1, placebo-controlled, double-blind, dose-escalating trial, was conducted to determine the safety and tolerability of a proprietary bioactive extract of chicory root in patients with

osteoarthritis (OA) [62]. In general, the treatment was well tolerated. Only one patient who was treated with the highest dose of chicory had to discontinue treatment due to an adverse event. The results of the pilot study suggested that a proprietary bioactive extract of chicory root has a potential role in the management of OA and merits further investigation. The second pilot study was conducted to assess whether chicory coffee consumption might confer cardiovascular benefits; thus, a clinical intervention was performed with 27 healthy volunteers, who consumed 300 mL chicory coffee daily for one week [63]. Depending on the inducer used for the aggregation test, the dietary intervention showed variable effects on platelet aggregation. Whole blood and plasma viscosity were both significantly reduced, along with serum MIF levels, after a week of chicory coffee intake. It was concluded that the full spectrum of the effects was unlikely to be attributed to a single phytochemical; nevertheless, the phenolics (including caffeic acid) are expected to play a substantial role. The study offered an encouraging starting-point to describe the antithrombotic and anti-inflammatory effects of phenolic compounds found in chicory coffee.

In the European Union, there is currently only one registered/authorized herbal medicinal product containing *C. intybus* as single ingredient whilst there are several combination products on the market [5]. The efficacy of herbal medicine Liv-52 consisting of *Mandur bhasma, Tamarix gallica,* and herbal extracts of *Capparis spinosa, C. intybus, Solanum nigrum, Terminalia arjuna,* and *Achillea millefolium* on liver cirrhosis outcomes was compared with the placebo for 6 months in 36 cirrhotic patients. The study concluded that Liv-52 possessed a hepatoprotective effect in cirrhotic patients. This protective effect of Liv-52 can be attributed to the diuretic, anti-inflammatory, anti-oxidative, and immunomodulating properties of the component herbs [32].

7. Cultivation and Sustainable Use

Greeks and Romans began to grow chicory as a vegetable crop 4000 years ago [9]. Since the discovery in the 1970s that chicory root contains up to 40% inulin (polysaccharide), new strains have been created, with inulin content comparable to that of sugar beet [4]. It is a common vegetable in several Western European countries. It is typically grown in a biennial cycle, with a tuberised root produced during the vegetative growth phase [64]. During the first field year, the vegetative growth phase is characterized by the production of a fleshy taproot. The second field year is the generative phase in which the flowering stem is formed and seeds are produced. To produce the eatable leafy vegetable called a chicon, roots are harvested at the end of the first growing period when an appropriate stage of maturity is reached [65]. The application of inulin in the food industry was restricted to the production of coffee substitutes. It was later discovered that inulin could act as a substitute for sugar or fat due to its low caloric value. The most stable form for the commercialization of inulin is the powdered extract for its greater facility of manipulation, transport, storage, and consumption [66].

Chicory is especially attractive as a cash crop since it can reach more than 62 t ha^{-1} under favourable conditions. Inulin content can reach on average 15% of root fresh weight and a yield of 8 t ha^{-1} of inulin is achievable [67]. The USA imports more than 2.3 million kilograms of chicons and 1.9 million kilograms of roasted chicory roots for coffee according to 2002 US Department of Commerce tariff and trade data [61]. Numerous studies have focused on different cultivation aspects of chicory. Chicory is considered one of the most important sources of inulin since it has a high root yield potential and also a high root sugar content [68]. A high root yield, a high inulin content, and especially long inulin chains are preferred [69]. Short chain inulin is used for the production of fructose syrup used in sweetening of cold drinks whereas long chain inulin is used as fat replacer and foam stabilizer in food products and also in the production of carboxymethyl inulin [2]. The effect of fertilizers on the growth, development, and yield of chicory has been well studied. In general, increase of nitrogen (N) increases the growth and ultimately the yield, although a high application of N has a negative effect on especially some of the amino acids. Increased N application at levels of 200 kg N ha^{-1} leads to a decrease in amino acids such as threonine and valine with a pronounced decreased effect on methionine. A level of 100 kg N ha^{-1} is preferred for enhanced quality [70]. In terms of phosphorous (P), it has been established that chicory has at least two inherent patterns of response to low or zero P conditions. One pattern is the classical increase in the length of the smallest diameter roots in response to P deficient conditions. The second pattern is a significant decrease in root tissue density under low P conditions [71]. It has also been investigated as a suitable catch crop since it has the ability to withdraw N, especially nitrate, from the soil, thereby reducing potential leaching. Additionally, it can withstand competition, has a slow juvenile development and vigorous growth after harvest of the main crop, is frost- and winter-hardy, has a well-developed root system, and does not transmit pathogens or pests to other crops [72]. Canopy closure in chicory is advantageous and critical for yield and can be achieved by a larger supply of assimilates to the shoot [73].

Chicory is greatly influenced by the pH of the soil as this affects the availability of nutrients in the soil to the plant. A study conducted by Anguissola Scotti el al. [74] on two soils of pH, 5.7 and 7.0 indicated that the fly-ash or metal availability to the plant was significantly different at the different pH levels. At a low pH a decrease of Zn, Cu, Cd, and Ni was observed and for neutral soils the added metals are more available to plants than those naturally occurring in soils [75]. Chicory is a cold-requiring long-day plant [72]. In a study by Amaducci and Pritoni [68], it was shown that retarding harvest time significantly affects the content and concentration of inulin. Rainfall has proven to be another important factor since the roots contain a higher water content, thereby affecting the concentration of inulin in the roots. The same results have been obtained in a study by Baert [69] where an early sowing date and harvesting time increased the root yield, total sugar content, and inulin chain length. The increase in yield with an earlier sowing date was up to 30% higher with an increase of 10% with an earlier harvest date. The content of free fructose and sucrose increased and the content of free glucose and inulin decreased with a later harvest time [75].

Even though chicory is a cold-requiring plant, cold storage after harvest can cause a strong decrease in free glucose, an increase in free fructose and sucrose, and hydrolysis of inulin [76]. This has also been observed by Ernst et al. [77] where an onset of cooler temperatures and especially colder temperatures during storage resulted in an increase in sucrose and fructose content. It was found that sucrose increased in the roots of chicory about threefold and fructose increased about tenfold within the first few weeks of cold storage after harvest [77]. The effect of storage at reduced temperature has also been studied on the levels of sesquiterpene lactones. Storage at 2°C and 10°C for up to 13 days had no effect on the level of lactucin-like sesquiterpene lactones in the chicons and after 7 days of storage a slight increase of lactucopicrin content was observed [78]. Several types of discoloration, leaf edge damage, and extensive growth of the internal core can occur in the heads of chicory during postharvest storage, which considerably reduces their market value. An atmospheric composition of 10% O_2 and 10% CO_2 in combination with a storage temperature of 5°C was found optimal [64]. Low temperature in the field also hastens and enhances bolting and flowering [79, 80]. The type and cultivar have been identified in numerous studies to be the determining factor during the stages of growth [81]. Suhonen [82] found the highest numbers of bolters in those being planted last, for which the mean temperatures during early growth were the highest.

During the postharvest period, the major polyamines present are putrescine, especially in the oldest leaves, although spermidine is present in considerable amounts, showing a tendency to decrease with the increasing physiological age of the leaves. Free sterol content increases with postharvest and also with physiological age of the leaves. Sitosterol is the major free sterol present, followed by stigmasterol and campesterol [83].

Emergence has been reported as one negative aspect of the crop, which could be addressed by specific breeding programmes [68]. Chicory can also be propagated and grown by means of micropropagation by regeneration of meristematic nodules. The leaves are cultivated *in vitro* and develop into plantlets when transferred to soil [84]. Although chicory can be regenerated *in vitro* from explants, both through organogenesis and somatic embryogenesis, and from protoplasts, no transgenic plants have yet been reported that have been produced [85].

Promising potential utility technologies of the plant have emerged. Chicory roots pulps are an important by-product of the inulin processing industries and are usually used in animal feed. Other applications can be found for these materials since the extraction of chicory pulp yields high levels of pectin, a polysaccharide extensively used in food as a gelling agent, thickening agent, and stabilizer [86]. The crude protein content in chicory is more valuable than in alfalfa. Furthermore the crude lipid in chicory is generally higher than most varieties of alfalfa [9]. It also has a nutritional quality comparable to lucerne, with a mineral content superior for copper and zinc, with similar proportions of protein, lipid, minerals, and other nutrients, and palatable with good digestion with applicability in the poultry and livestock industry [87, 88].

8. Conclusion

Cichorium intybus has a long tradition of use globally. Historically, chicory was grown by the ancient Egyptians as a medicinal plant, coffee substitute, and vegetable crop and was occasionally used for animal forage. This multipurpose plant contains high amounts of proteins, carbohydrates, and mineral elements [9]. Inulin from chicory roots is considered a functional food ingredient as it affects physiological and biochemical processes resulting in better health and reduction of the risk of many diseases [89].

To date, chicory remains an extremely versatile plant, amenable to genetic manipulation, and there is interest shown in genetically engineered chicory to obtain higher yields and create new potentials [1]. The documented indigenous knowledge relating to the various medicinal uses of chicory has been supported by phytochemical isolation and investigations into biological activity. Nonetheless, many of its constituents have not been explored for their pharmacological potential and further research is necessary to gain better understanding of the phytochemicals against various diseases [9]. Toxicological data on *C. intybus* is currently limited; however, considering that the Asteraceae family is a known source of allergic problems, a contraindication for hypersensitivity should be included in the safety data [5]. Recent studies suggest the use of *C. intybus* as a biomonitor for heavy metals [90, 91]; considering that chicory enters the food chain, this plant should be used with caution. The apparent bioactivity of *C. intybus* shown in preclinical studies (both *in vitro* and *in vivo*) is a testament to its historical use in traditional medicine.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Beneficial Effect of 7-*O*-Galloyl-D-sedoheptulose, a Polyphenol Isolated from Corni Fructus, against Diabetes-Induced Alterations in Kidney and Adipose Tissue of Type 2 Diabetic *db/db* Mice

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Traditional medicines are being focused on as possible treatments for diabetes and its complications because of their negligible toxic and/or side effects. In line with this, our group has reported that Corni Fructus, a traditional medicine considered exhibiting beneficial effects on liver and kidney functions, possessed an antidiabetic effect *via* ameliorating glucose-mediated metabolic disorders. To add to these findings, we screened the iridoid glycoside fraction containing morroniside and loganin, and low molecular weight polyphenol fraction containing 7-*O*-galloyl-D-sedoheptulose (GS) from Corni Fructus. To our knowledge, GS is a compound only detected in Corni Fructus, and its biological activity has been poorly understood until now. For these reasons, we examined whether GS has an ameliorative effect on diabetic changes using type 2 diabetic *db/db* mice. Our findings suggest that GS has a beneficial effect on the pathological state of the serum, kidney, and adipose tissue related to diabetic damage.

1. Background

Diabetes is a metabolic disorder known to cause deleterious changes in various tissues exhibited as diabetic complications triggered by hyperglycemia, dyslipidemia, oxidative stress, inflammation, and advanced glycation [1]. Among these pathogenic factors in diabetes, abnormal lipid metabolism and hyperglycemia-induced oxidative and carbonyl stress (so-called lipotoxicity and glucotoxicity) play a central role in the initiation and progression of diabetes-related disease [2]. Chronic hyperglycemia and dyslipidemia cause oxidative stress and inflammatory responses through the formation of advanced glycation end-products (AGEs) [3, 4], activation of the protein kinase C pathway [5, 6], increased glucose flux through the polyol pathway [7], and the accelerated generation of reactive oxygen species (ROS) [8, 9]. The resulting glycative, glycoxidative, and carbonyl lipotoxicity and oxidative stresses play a key role in the pathogenesis of diabetes [10–13]. Therefore, the attenuation of oxidative stress and regulation of hyperlipidemia have been considered as ways to alleviate diabetes and diabetic complications.

Clinical evidence has suggested that the appropriate use of traditional Chinese medicines with modern Western medicinal, or mainstream antidiabetic drugs, can prevent or ameliorate the development of diabetic complications. Many diabetic patients choose alternative therapeutic approaches such as herbal or traditional Chinese medicine along with mainstream antidiabetic drugs, thus making alternative therapy for diabetes very popular [14]. However, these medicines usually have an insufficient scientific basis, and the exact mechanisms behind their beneficial effects are unknown. Therefore, recently, based on a large number of chemical and

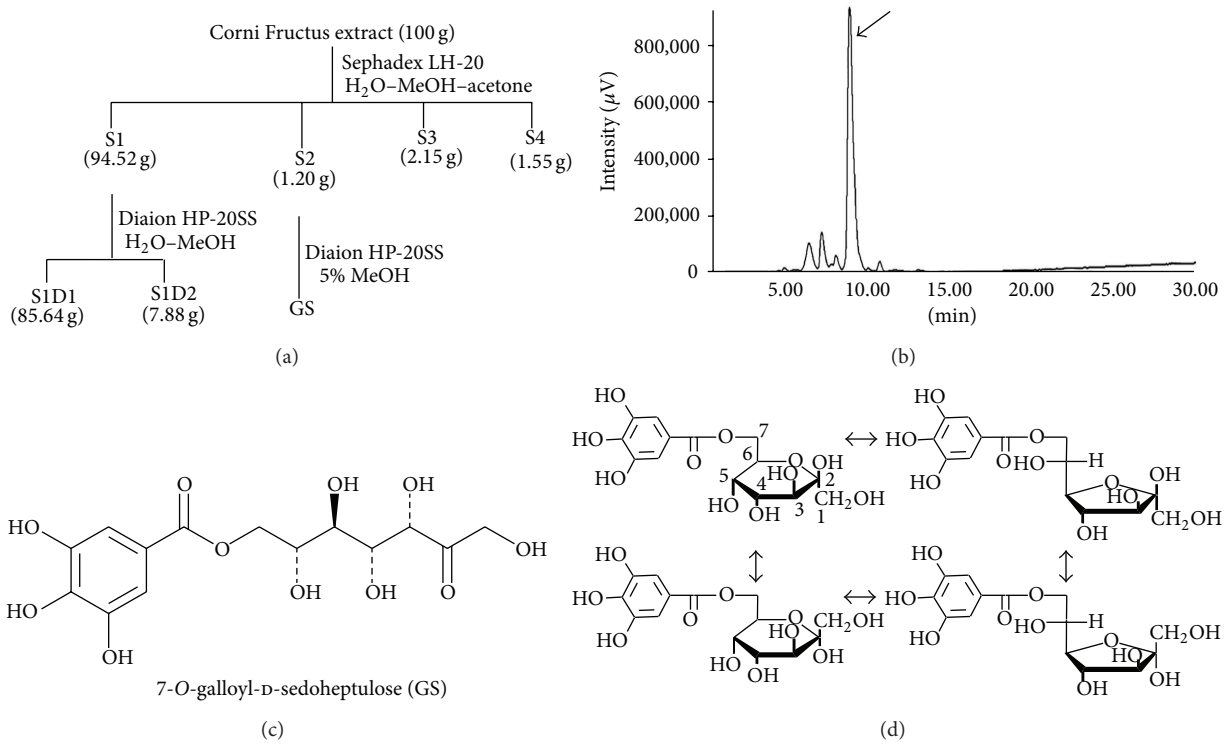

Figure 1: Fractionation of Corni Fructus, HPLC profile of GS, and its structure. (a) Fractionation of Corni Fructus was performed as described in *Biological & Pharmaceutical Bulletin*, vol. 30, no. 7, pp. 1289–1296, 2007. (b) HPLC profile. The large peak shown by the arrow is the structure of GS, as described in (c), and the other peaks represent its four isomers, as described in (d).

pharmacological studies, numerous bioactive compounds have been identified in Chinese medicinal plants for diabetes [15], and we have investigated the mechanism and bioactive constituents of Corni Fructus, the fruit of *Cornus officinalis* Sieb. et Zucc. (Cornaceous), in diabetic animal models.

Corni Fructus is an important crude herb used in Chinese medicine. It is considered to be one of the 25 plant-based drugs most frequently used in China, Japan, and Korea. It is known to exhibit several biological activities, including hypoglycemic, antineoplastic, and antimicrobial effects, and improve liver and kidney functions [16–18]. We previously reported that treatment with Corni Fructus for 10 days suppressed hyperglycemia, proteinuria, renal AGE formation, and related protein expressions, that is, receptor for AGEs (RAGE), nuclear factor-kappa B (NF-κB), transforming growth factor-β_1 (TGF-β_1), and N^{ε}-(carboxymethyl)lysine (CML), in the same way as with aminoguanidine. However, improvement of the renal function, shown *via* serum creatinine and creatinine clearance, was superior to aminoguanidine treatment [19]. In addition, the administration of Corni Fructus inhibited the elevation of both systolic and diastolic blood pressures, and lowered serum total cholesterol levels with a decrease in esterified cholesterol in a diet-induced hypercholesterolemia rat model [20]. Moreover, the atherogenic index was decreased in a dose-dependent manner, suggesting its protective role against cardiovascular disease through regulating cholesterol and lipoprotein levels [20]. Therefore, Corni Fructus was suggested to have beneficial effects on diabetes and diabetic complications.

The discovery of efficacious components is essential for clarification of the precise mechanisms of herbal medicines. However, studies on the biological activities of the active components in Corni Fructus are limited. Therefore, we isolated the major active components of Corni Fructus by employing activity-guided fractionation (Figure 1), and the effects of morroniside, loganin, and 7-*O*-galloyl-D-sedoheptulose (GS) were assessed on glucose metabolism, AGE formation, oxidative stress, and inflammation in type 2 diabetic liver, kidney, pancreas, and adipose tissue to identify their effects and mechanism of action in type 2 diabetes [21–30]. Among the isolated components of Corni Fructus which were suggested to be important contributors to prevent and/or delay the onset of diabetic disease, GS, to our knowledge, is a compound only detected in Corni Fructus [31]. Part of the sugar (sedoheptulose) in GS is ketoheptose, a monosaccharide with seven carbon atoms and a ketone functional group. Sedoheptulose is a seven-carbon ketose sugar originally found in *Sedum spectabile*, a common perennial garden plant. It is often a part of the human diet. This sugar, D-sedoheptulose (I), is a significant intermediary compound in the cyclic regeneration of D-ribulose. It also plays an important role as a transitory compound in the cyclic regeneration of D-ribulose for carbon dioxide fixation in plant photosynthesis.

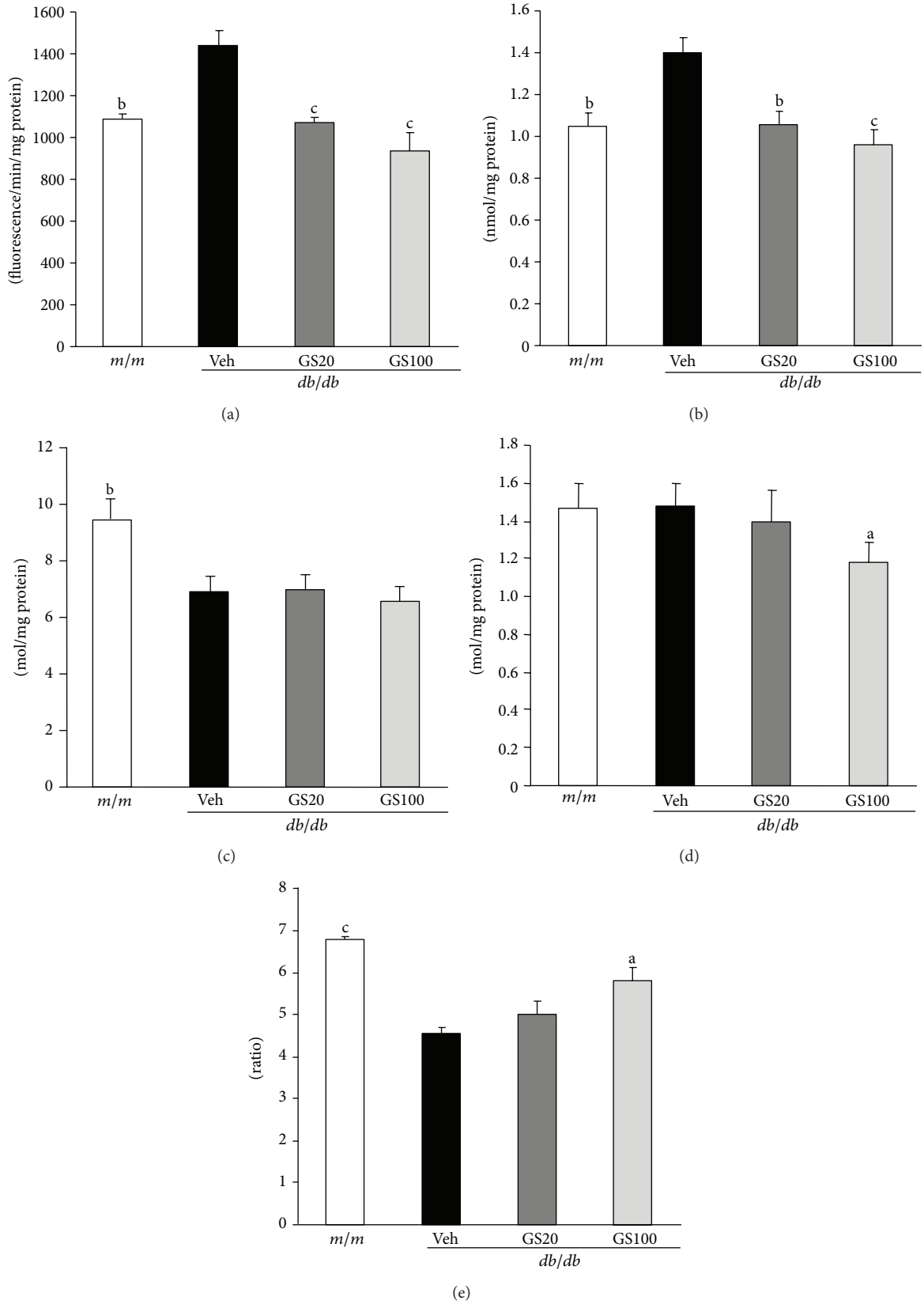

Figure 2: ROS (a), TBARS (b), GSH (c), GSSG (d), and GSH/GSSG (e) levels in the kidney. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated *db/db* mouse values.

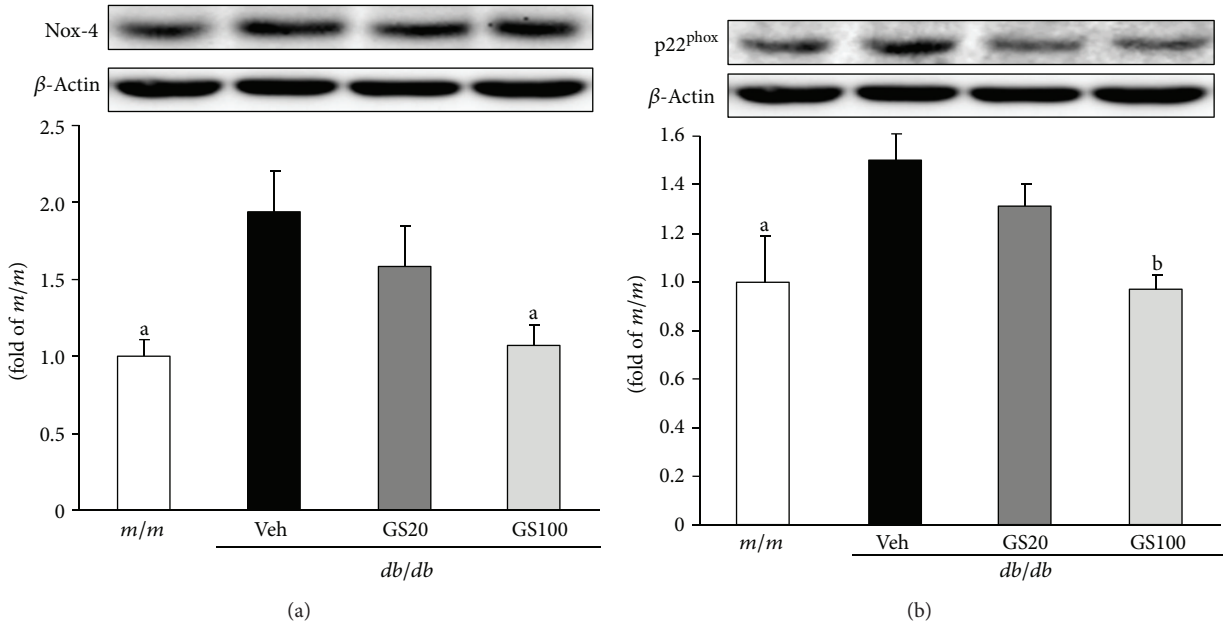

Figure 3: Nox-4 (a) and $p22^{phox}$ (b) protein expressions in the kidney. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated *db/db* mouse values.

Table 1: Glucose, leptin, insulin, and C-peptide in serum.

Group	Dose (mg/kg body weight/day)	Glucose (mg/dL)	Leptin (ng/dL)	Insulin (ng/mL)	C-peptide (pg/mL)
m/m	—	186 ± 25[c]	2.30 ± 0.32[c]	1.82 ± 0.06[b]	177 ± 15[c]
db/db					
Veh	—	791 ± 42	20.24 ± 0.29	3.72 ± 0.45	1,983 ± 277
GS	20	745 ± 31	18.51 ± 0.75	2.68 ± 0.11[a]	1,135 ± 139[a]
GS	100	683 ± 41	17.57 ± 0.87[a]	2.40 ± 0.04[b]	970 ± 142[b]

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS20 mg/kg body weight-treated *db/db* mice; GS100, GS100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

Therefore, this paper presents a review of our recent findings, with emphasis on the therapeutic potential of the polyphenol, GS, of Corni Fructus against diabetic damage in the kidney and adipose tissue.

2. Type 2 Diabetic *db/db* Mice

To investigate the effect of GS, *db/db* mice were used. A spontaneous mutant strain, C57BLKS/J *db/db* mice, has the *db* mutation, a splicing mutation caused by a point mutation in the downstream intron of the leptin receptor gene, and so it is unresponsive to leptin. Leptin is a peptide hormone secreted by adipocytes and is involved in eating behavior and energy homeostasis. For this reason, after birth, the homozygous diabetic (*db/db*) mice show unrepressed eating behavior, become obese, and develop severe insulin resistance associated with hyperinsulinemia and hyperglycemia [32]. In this study, *db/db* mice showed diabetic characteristics, such as hyperglycemia, hyperleptinemia, and hyperinsulinemia, compared with homozygous control (*m/m*) mice, as presented in Table 1. GS administration significantly reduced serum leptin and insulin levels at a dose of 100 mg/kg, while the serum glucose level was slightly decreased without significance. The serum C-peptide level was compared as an indirect biomarker of insulin secretion. As expected, there was a significant increase in the serum C-peptide level in the vehicle-treated *db/db* group, which was closely associated with the increased removal of blood glucose (Table 1). Thus, GS treatment prevents diabetes in *db/db* mice, as evidenced by improved insulin sensitivity through the maintenance of normal insulin and glucose levels and the preservation of insulin and C-peptide levels in the serum, meaning that GS can ameliorate impaired glucose and insulin tolerance in *db/db* mice.

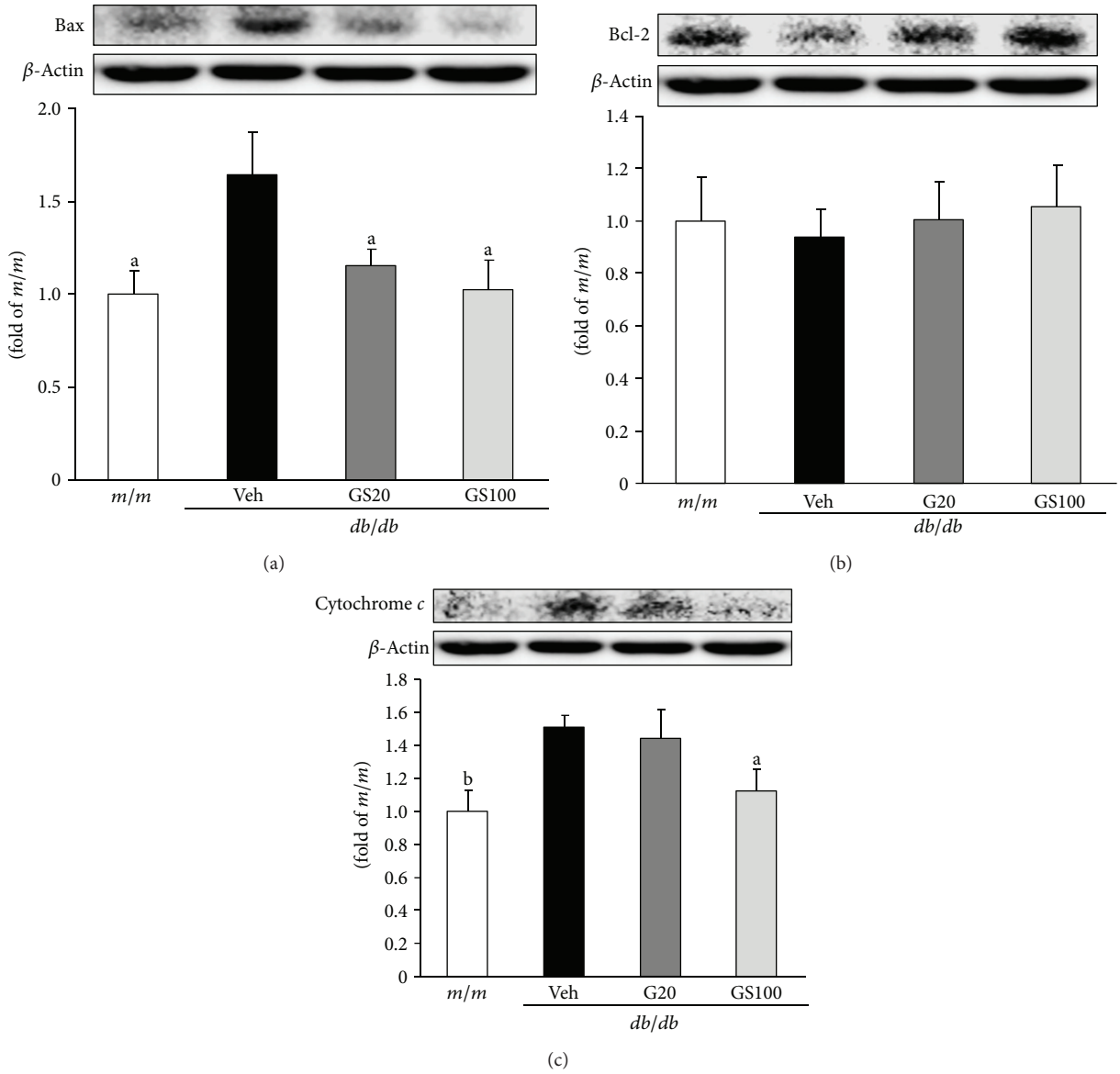

Figure 4: Bax (a), Bcl-2 (b), and cytochrome *c* (c) protein expressions in the kidney. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated *db/db* mouse values.

3. GS Ameliorates Renal Damage Triggered by ROS-Sensitive Pathway of Inflammation and Apoptosis

Initial diabetic renal damage is known to involve hyperglycemia-induced oxidative stress. Increased oxygen and peroxy radicals aggravate tissue oxidative stress, which affects the oxidation of important macromolecules including proteins, lipids, carbohydrates, and DNA chains. Moreover, ROS activates the signal transduction cascade and transcription factors and overexpression of genes and proteins in glomerular mesangial and tubular epithelial cells, leading to pathological changes in the kidney [33]. Therefore, in this study, we investigated the effect of GS on the oxidative stress and ROS-related factors involved in the development of diabetic renal damage using type 2 diabetic C57BLKS/J *db/db* mice.

As shown in Figure 2, GS effectively attenuated oxidative stress *via* a decrease in ROS and thiobarbituric acid-reactive substance (TBARS) levels as well as an enhanced reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio. In addition, increased serum urea nitrogen and creatinine levels associated with an abnormal renal function were significantly lowered by GS treatment.

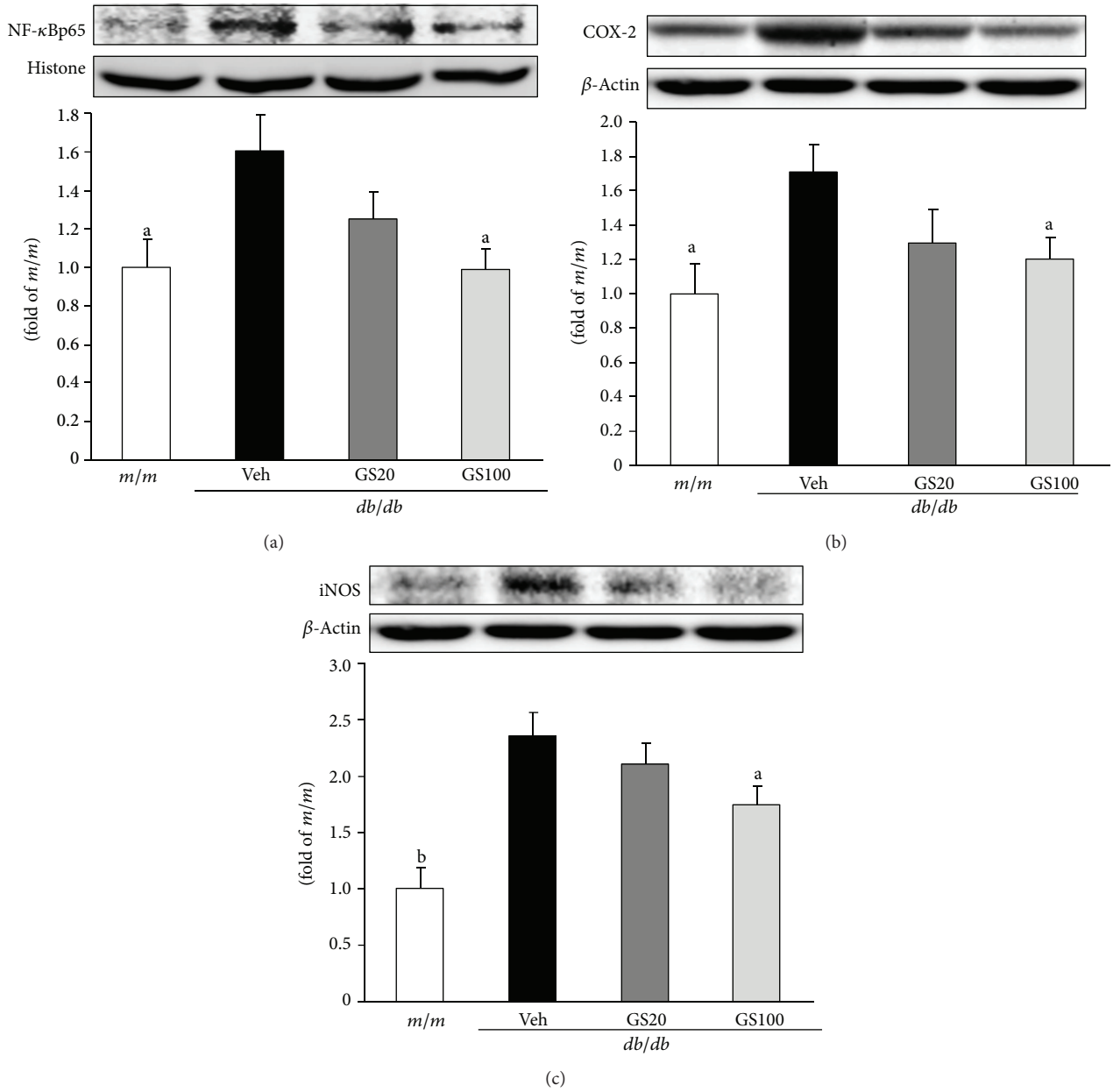

Figure 5: NF-κBp65 (a), COX-2 (b), and iNOS (c) protein expressions in the kidney. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$ versus vehicle-treated *db/db* mouse values.

In the diabetic kidney, enzymatic and nonenzymatic sources of ROS include autoxidation of glucose, transition metal-catalyzed Fenton reactions, advanced glycation, polyol pathway flux, mitochondrial respiratory chain deficiencies, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [34]. Although the origin of increased ROS generation in renal disease is multifactorial, recent studies have focused on the fact that NADPH oxidase mainly participates in the process of ROS generation [35–37]. There is accumulating evidence that nonphagocytic NADPH oxidases are major enzymatic sources of ROS generation in ischemia-reperfusion injury, inflammation, hypertension, and atherosclerosis based on experimental animal and human studies [38, 39]. Also, renal NADPH oxidase expression was reported to be enhanced in glomeruli and distal tubules in the presence of diabetic nephropathy [40]. Structurally, NADPH oxidase comprises the membrane-associated cytochrome b_{558}, composed of one $p22^{phox}$ and one $gp91^{phox}$ subunit and at least four cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTP_{ase} *rac*1 or *rac*2) [41]. In particular, Nox-4 and $p22^{phox}$ were found to be a major source of ROS production in the kidney and could play a role in pathological conditions [35, 42, 43]. Also, in a rodent model of type 2 diabetes (*db/db* mouse), the renal expression of Nox-4 and $p22^{phox}$ was increased, and this was associated with

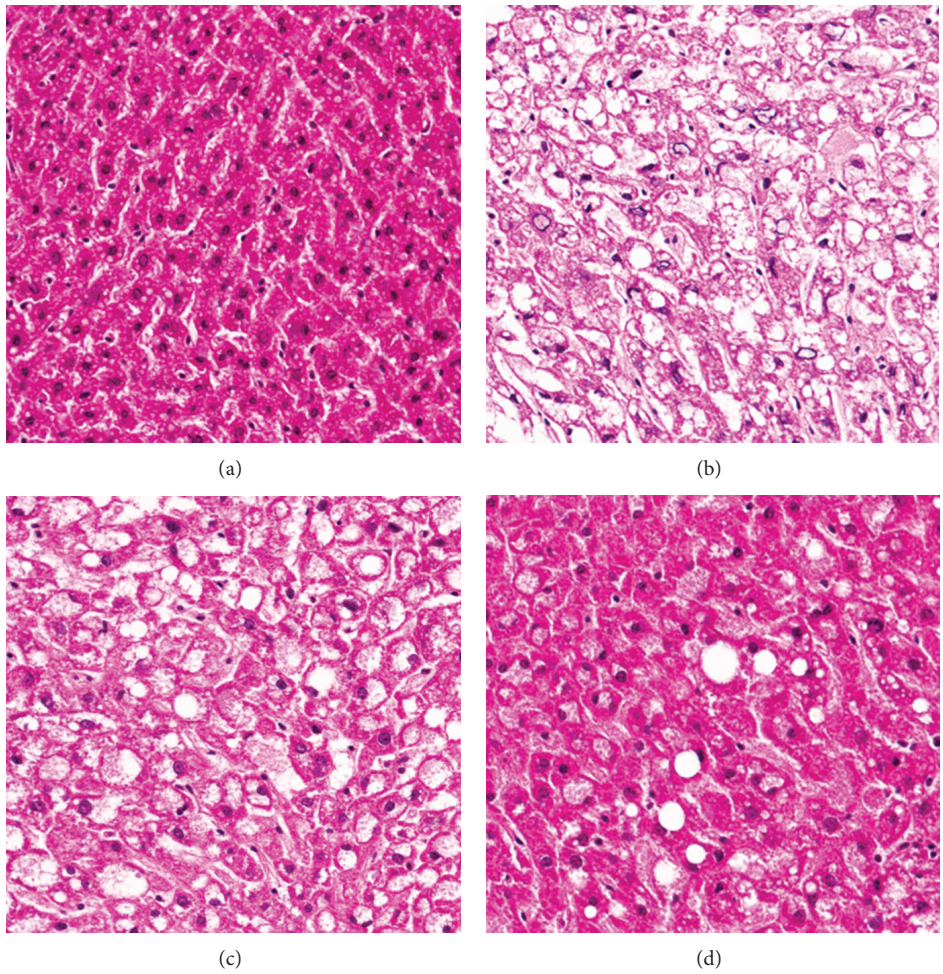

Figure 6: HE staining of the kidney. (a) Misty, (b) vehicle-treated *db/db* mice, (c) GS 20 mg/kg body weight-treated *db/db* mice, and (d) GS 100 mg/kg body weight-treated *db/db* mice. ×200.

ROS-induced renal damage [44]. Therefore, we examined the renal protein expression of Nox-4 and $p22^{phox}$, subunits of NADPH oxidase, to identify the exact mechanism behind the reduction of renal ROS levels in the GS-treated group. In Western blot analysis, Nox-4 and $p22^{phox}$ protein expressions were significantly upregulated in the type 2 diabetic kidney; however, GS 100 mg/kg administration significantly normalized the increased subunits of NADPH oxidase (Figure 3). These results indicate that the inhibitory effect of GS on ROS generation was due to the downregulated expression of NADPH oxidase in *db/db* mice.

Furthermore, ROS has been shown to induce apoptosis in the proximal tubular cells of an animal model of unilateral ureteral obstruction [9]. Apoptotic cells have been detected in both proximal and distal tubular epithelia of human and experimental diabetic kidneys [45], suggesting that apoptosis is also involved in the loss of tubular cells in diabetic nephropathy. Increased mitochondrial superoxide production initiates a range of damaging reactions through the production of H_2O_2, ferrous iron, $^{\bullet}OH$, and $ONOO^{-}$, which can then damage lipids, proteins, and nucleic acids. A number of functional enzymes within the mitochondria are particularly susceptible to ROS-mediated damage, leading to altered ATP synthesis, cellular calcium dysregulation, and the induction of mitochondrial permeability transition, all of which predispose the cell to necrosis or apoptosis. Podocyte apoptosis has been proposed as a new cellular pathomechanism in diabetic nephropathy [46]. Apoptosis is most likely caused by changing the balance in the expression of the anti- and proapoptotic molecules, Bcl-2 and Bax, respectively. While Bcl-2 expression may account for the maintenance of glomerular hypercellularity, Bax expression might be more important in cell loss leading to glomerulosclerosis. Bax forms oligomers, thereby increasing mitochondrial permeability and facilitating the release of cytochrome *c* from the mitochondrial intermembrane space. Once released from the mitochondria, cytochrome *c* further activates apoptosis. In this study, GS administration in *db/db* mice significantly suppressed renal protein expression of Bax and cytochrome *c*, although there was no change in Bcl-2 protein levels among all experimental groups (Figure 4). These results suggest that GS prevents apoptosis-induced renal damage, at least in

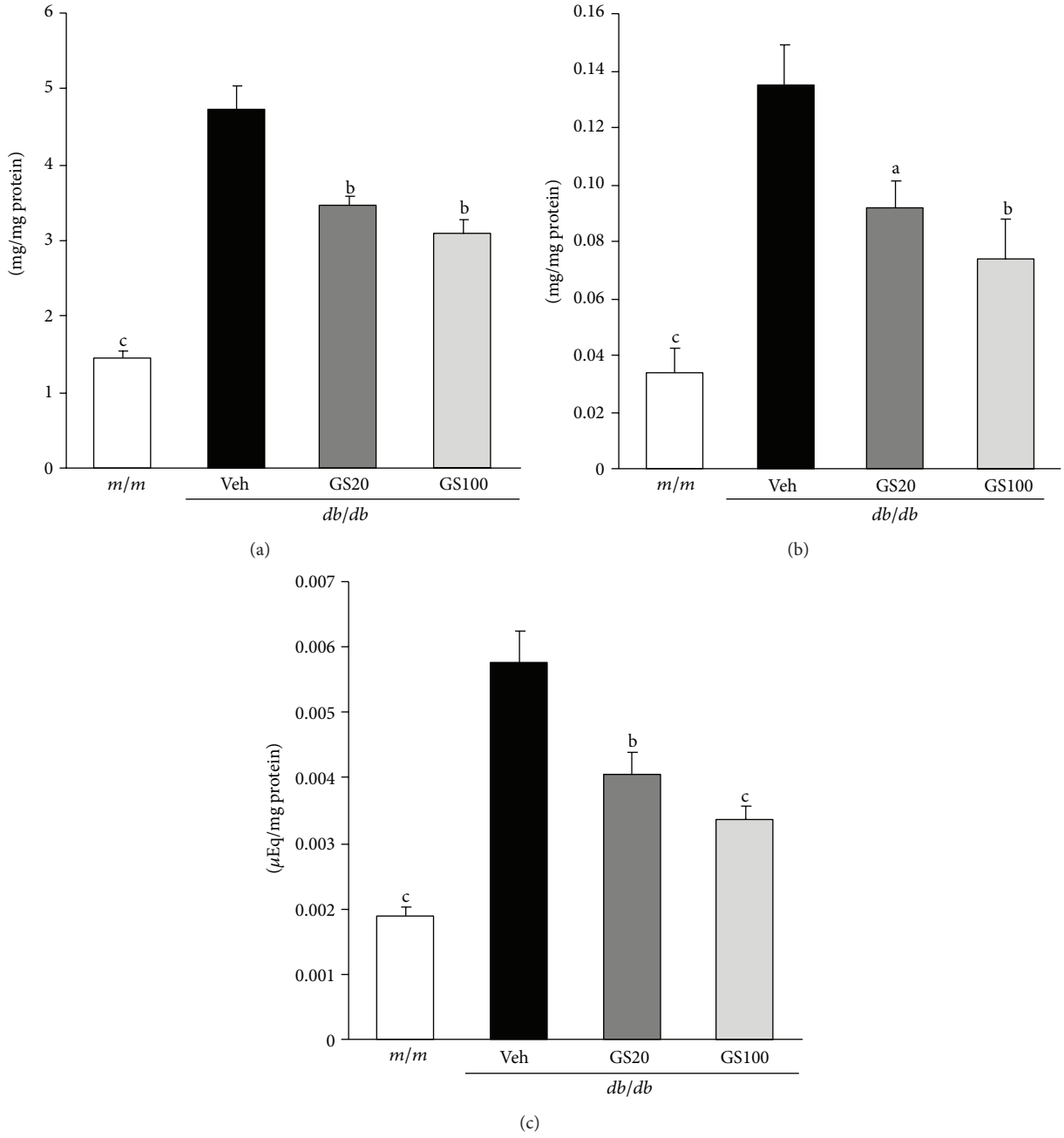

Figure 7: Triglycerides (a), total cholesterol (b), and NEFA (c) contents in the adipose tissue. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

part, through the amelioration of oxidative stress-induced mitochondrial dysfunction.

On the other hand, NF-κB is one of the crosstalk points of multiple signal transduction pathways, and plays a key role in the regulation of transcription and expression of many genes involved in inflammatory responses [47, 48]. For example, enhanced oxidative stress leads to NF-κB transcription and, consequently, induces expressions of its related proinflammatory factors such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [49]. In humans, COX-2 expression is readily detectable in glomerular podocytes of adults [50, 51], and its expression level has been reported to increase during acute renal allograft rejection [52, 53]. In cultured podocytes, COX-2 overexpression led to more marked cytoskeletal disorganization and apoptosis in response to high-glucose stimulation [54]. These changes were ameliorated by treatment with a specific COX-2 inhibitor, indicating that podocyte COX-2 expression increases susceptibility to the development of diabetic nephropathy [54]. Meanwhile, the rapid induction of iNOS

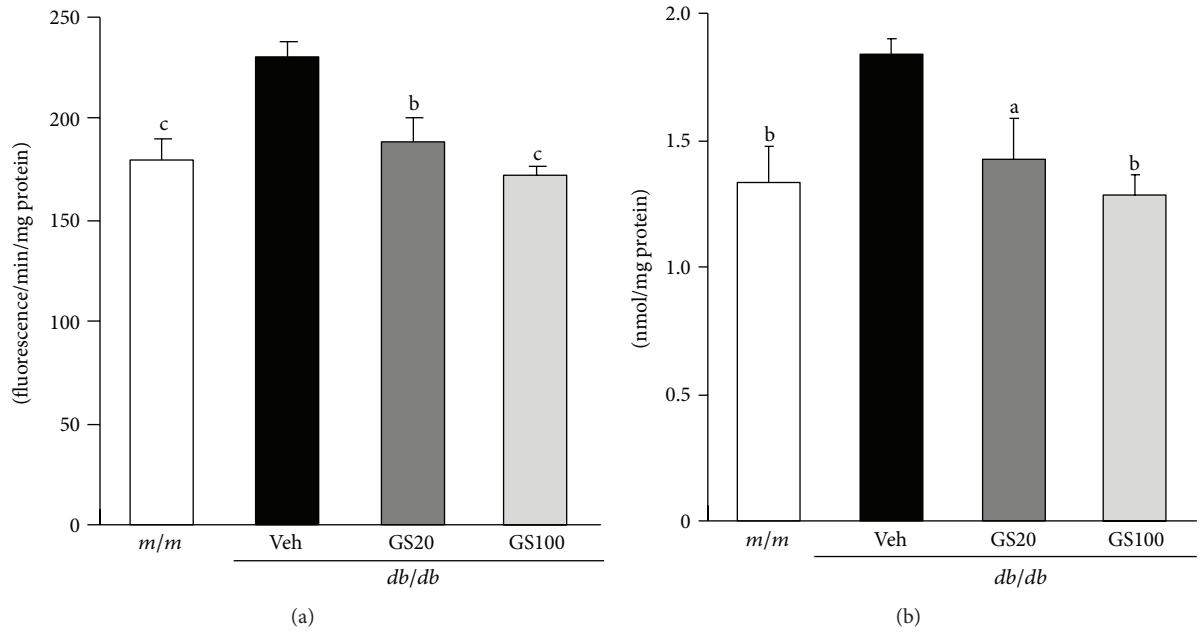

Figure 8: ROS (a) and TBARS (b) levels in the adipose tissue. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

Table 2: Biomarkers associated with lipids, oxidative stress, and inflammation in serum.

Group	Dose (mg/kg body weight/day)	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	NEFA (mEq/L)	HDL-C (mg/dL)	LDL/VLDL-C (mg/dL)	
m/m	—	114 ± 9^{c}	110 ± 8^{c}	0.62 ± 0.02^{c}	51.01 ± 3.55^{c}	522 ± 4^{b}	
db/db							
Veh	—	263 ± 21	186 ± 8	2.55 ± 0.06	80.95 ± 2.49	570 ± 17	
GS	20	198 ± 16^{a}	179 ± 14	1.98 ± 0.04^{c}	90.88 ± 7.68	394 ± 21^{a}	
GS	100	175 ± 8^{b}	163 ± 11	1.56 ± 0.15^{c}	95.88 ± 3.16^{b}	355 ± 13^{c}	
Group	Dose (mg/kg body weight/day)	ROS (fluorescence/min/mL)	TBARS (nmol/mL)	Adiponectin (ng/mL)	Resistin (pg/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)
m/m	—	790 ± 175^{a}	18.33 ± 0.46^{c}	6.32 ± 0.27^{c}	522 ± 4^{b}	117 ± 17^{a}	11.08 ± 0.33^{c}
db/db							
Veh	—	1,563 ± 144	22.48 ± 0.51	3.18 ± 0.09	570 ± 17	261 ± 28	21.27 ± 2.07
GS	20	950 ± 112^{a}	11.88 ± 1.45	3.68 ± 0.12^{b}	394 ± 21^{c}	162 ± 30^{a}	14.90 ± 2.07
GS	100	840 ± 70^{b}	9.06 ± 1.19^{c}	4.63 ± 0.19^{c}	355 ± 13^{c}	133 ± 13^{b}	12.45 ± 1.19^{b}

m/m, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS20 mg/kg body weight-treated *db/db* mice; GS100, GS100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

expression can trigger NO-dependent apoptosis *in vitro*, which appears to result from DNA damage and may be mediated by a p53-dependent apoptotic pathway [55]. iNOS expression is typically absent in unstimulated cells, but is markedly induced by proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 [56, 57]. For that reason, proinflammatory factors such as NF-κB and its transcriptional factors have been important target genes to prevent further renal damage caused by the inflammatory response and apoptosis. In this study, GS administration to type 2 diabetic *db/db* mice caused significant renal protein downregulation of NF-κB, COX-2, and iNOS (Figure 5), suggesting that GS efficiently inhibited renal inflammation-related injury in *db/db* mice.

This study supports the concept that, in hyperglycemia, enhanced oxidative stress, upregulation of NADPH oxidase and apoptosis, and NF-κB-related inflammation are associated with renal damage in type 2 diabetes. GS administration effectively alleviated these unfavorable responses in the presence of diabetic injury of kidney, as shown in

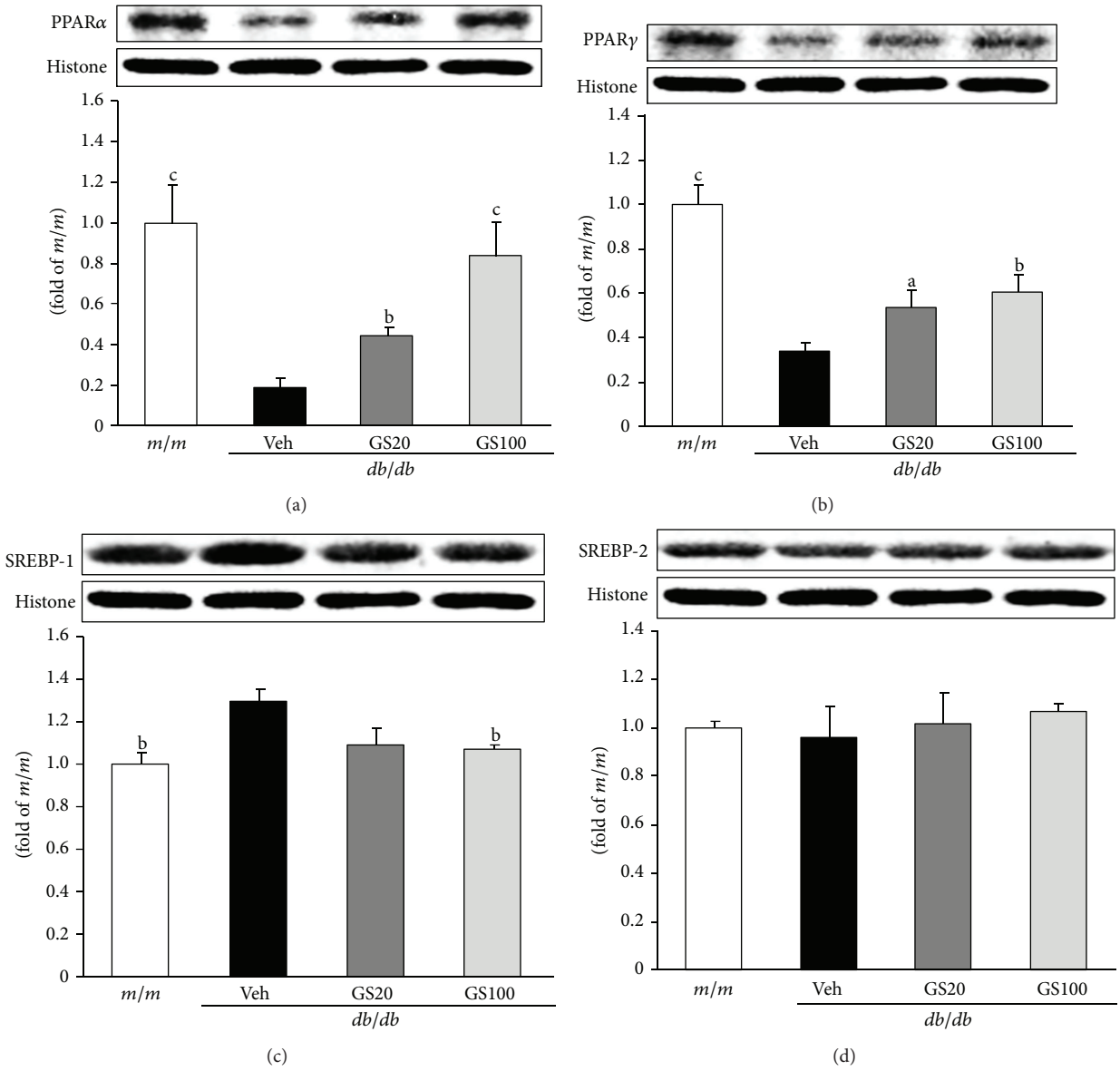

Figure 9: PPARα (a), PPARγ (b), SREBP-1 (c), and SREBP-2 (d) protein expressions in the adipose tissue. *m/m,* Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

Figure 6. Therefore, this study suggests that GS exerts its renal protective potential through the inhibition of oxidative stress-sensitive mechanisms of apoptosis and the proinflammatory response in the kidney of type 2 diabetics.

4. GS Acts as a Regulator of Oxidative Stress, Inflammation, and Fibrosis in Adipose Tissue

Adipose tissue stores energy in the form of lipids and releases fatty acids in response to nutritional signals or energy insufficiency. In addition, adipocytes have endocrine functions, secreting hormones and factors that regulate physiological functions such as the immune response, insulin sensitivity, and food intake [58]. Excessive fat accumulation in the body and white adipose tissue causes obesity and results in an increased risk of many serious diseases, including type 2 diabetes, hypertension, and heart disease. In the present study, we examined whether GS could prevent the gluco- and lipotoxicity of adipose tissue triggered by the ROS-sensitive pathway of inflammation and fibrosis in type 2 diabetic *db/db* mice.

The major biochemical alterations in diabetes are hyperglycemia and dyslipidemia, leading to gluco- and lipotoxicity, which directly or indirectly account for diabetic complications in various organs [59–62]. Longitudinal hyperlipidemia,

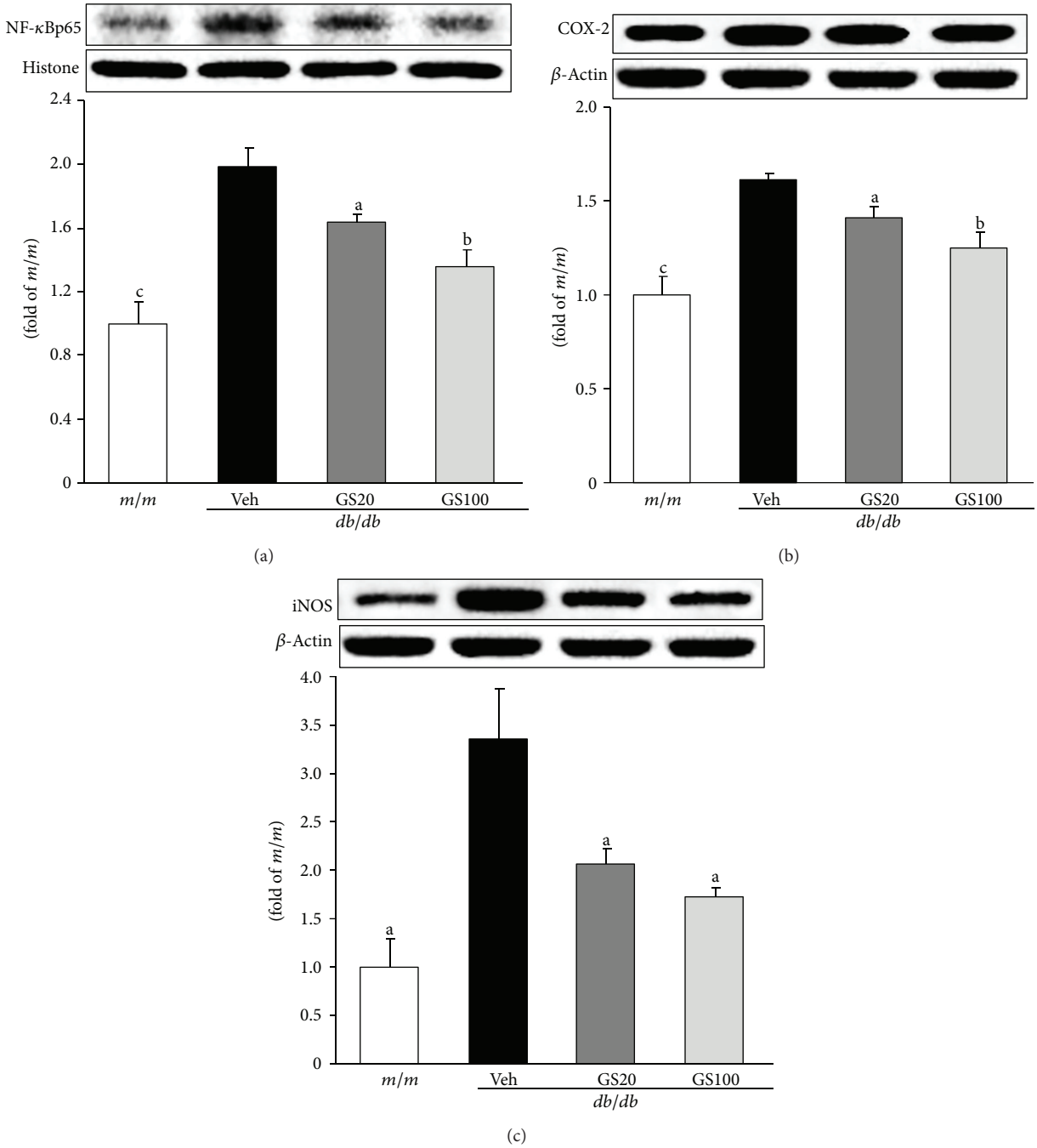

Figure 10: NF-κBp65 (a), COX-2 (b), and iNOS (c) protein expressions in the adipose tissue. *m/m,* Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

which is associated with the abnormal expression of transcriptional factors such as peroxisome proliferator activated receptor (PPAR) α or sterol regulatory element binding proteins (SREBPs) in the nucleus, increases nonesterified fatty acids (NEFA) uptake and accumulations of triglycerides and cholesterol in tissues. Critical toxicity caused by dyslipidemia is also oxidative stress due to impaired antioxidant defense systems and increased ROS generated by the mitochondrial respiratory chain reaction and glucose autoxidation [63–66]. In this study, the concentrations of triglycerides, total cholesterol, NEFA, high-density lipoprotein (HDL) cholesterol, and very low-density lipoprotein (VLDL)/low-density lipoprotein (LDL) cholesterol in the serum, and triglycerides, total cholesterol, and NEFA in the adipose tissue were significantly elevated in *db/db* compared to those in *m/m* mice. The oral administration of GS affected its favorable influences

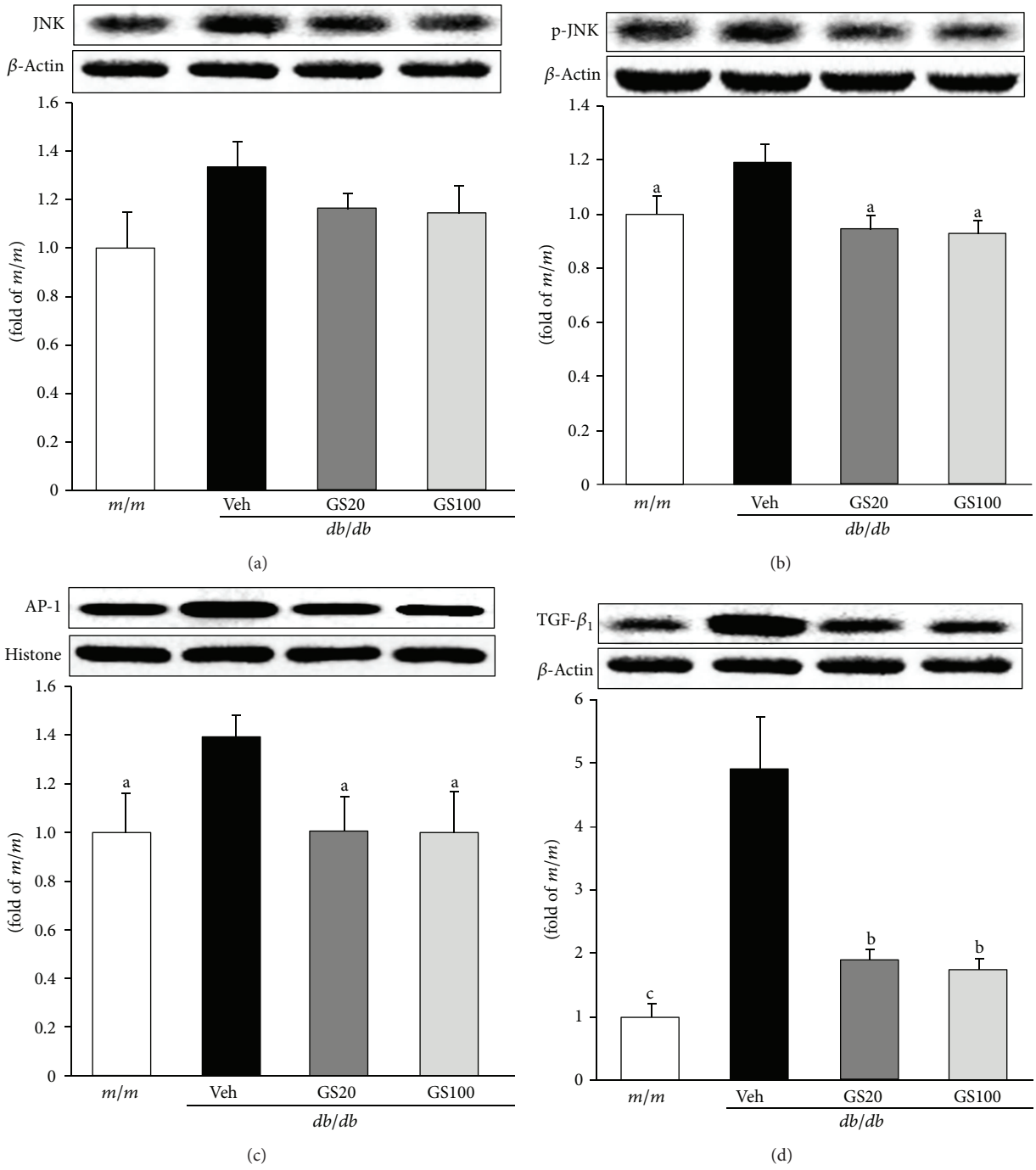

Figure 11: JNK (a), p-JNK (b), AP-1 (c), and TGF-β_1 (d) protein expressions in the adipose tissue. *m/m*, Misty; Veh, vehicle-treated *db/db* mice; GS20, GS 20 mg/kg body weight-treated *db/db* mice; GS100, GS 100 mg/kg body weight-treated *db/db* mice. The results are presented as the means ± S.E.M. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus vehicle-treated *db/db* mouse values.

on the lipid profile of serum and adipose tissue (Table 2, Figure 7). Besides its beneficial effects on lipid metabolism, GS administration promoted antioxidant activity. The elevated ROS and TBARS levels in the serum and adipose tissue were ameliorated nearly to those of *m/m* mice (Table 2, Figure 8). On the other hand, the serum adiponectin level increased on GS treatment, which may be correlated with the decreased serum NEFA level (Table 2). Moreover, lipid metabolism-related protein expressions in the adipose tissue were measured. As shown in Figure 9, protein expressions of transcriptional factors related to lipid regulation, PPARα and PPARγ, were lower in vehicle-treated *db/db* than *m/m* mice, but these decreased expressions were significantly elevated by the 20 or 100 mg GS administration. Also, the elevated SREBP-1 expression in vehicle-treated *db/db* mice was recovered nearly to that of *m/m* mice on 100 mg/kg GS

treatment, suggesting that GS modified lipid metabolism, especially triglyceride synthesis.

Previously, we proposed that the suppression of inflammation is possibly linked to antidiabetic effects [67], and other studies have reported that type 2 diabetes can occur through mechanisms related to the inflammatory state [68]. As inflammation is considered to be a major factor contributing to type 2 diabetes [68], we examined proinflammatory markers including TNF-α and IL-6 in the serum, and found that GS treatment inhibited serum TNF-α and IL-6 (Table 2), indicating that the anti-inflammatory properties of GS result in protection against insulin resistance, consistent with a previous report [69], revealing that the suppression of inflammation *via* the modulation of adiponectin, IL-6, and TNF-α is an important protective factor against insulin resistance. It was reported that NF-κB results in insulin resistance by activating proinflammatory cytokines like TNF-α, IL-6, IL-1β, and resistin, which consequently activates the c-Jun N-terminal kinase (JNK) and NF-κB pathways to create a vicious cycle that exacerbates tissue damage [70].

We further examined proinflammatory NF-κBp65, COX-2, and iNOS protein levels in the adipose tissue of *db*/*db* mice, and found that GS treatment downregulated levels of these proteins (Figure 10), suggesting that GS treatment had antidiabetic effects due to its anti-inflammatory actions. These results showing the amelioration of proinflammatory markers, that is, NF-κBp65 and COX-2 protein expressions, are in parallel with a recent report showing enhanced iNOS protein expression due to NF-κB activation [71]. In addition, it has also been shown that polyphenolic compounds can modulate inflammatory responses *via* the inhibition of COX-2 protein expression through the suppression of JNK activation and inhibition of proinflammatory mediators, like TNF-α, by the attenuation of NF-κB and JNK pathways [72]. GS modulated the activation of JNK pathway (JNK $\rightarrow$ phosphor (p)-JNK $\rightarrow$ activator protein (AP)-1 $\rightarrow$ TGF-β_1) (Figure 11). These data are consistent with a previous report [73] showing that not only the modulation of oxidative stress and consequent activation of the JNK pathway, but also the suppression of inflammation are involved in the development of dysfunction found in adipose tissue in the presence of diabetes, which, therefore, would make these useful therapeutic targets against adipose tissue in diabetes.

One of our significant findings in this study was GS's suppression of diverse proinflammatory cytokines such as TNF-α, IL-6, resistin, and TGF-β_1 that activate the JNK and NF-κB pathways and proinflammatory COX-2 protein expression (Table 2, Figures 10 and 11). In particular, our data showing the suppression of both oxidative stress and inflammation by GS treatment are consistent with our previous report [67], revealing a close relationship between antioxidative and anti-inflammatory actions in diabetes. Thus, based on the results from both our previous and current studies, we suggest a possible mechanism by which the antidiabetic action of GS mediates type 2 diabetes through its dual suppression of oxidative stress and inflammation, as shown in our experiments with *db*/*db* mice. Consecutively, GS could reduce the increased level of TGF-β_1 in the adipose tissue, showing a reduction in fibrosis. These findings suggest that the hyperglycemic control of GS may, at least in part, be derived from the amelioration of disorders such as fibrosis in adipose tissue.

Although the mechanistic details of GS need to be clarified in future studies, our findings support the therapeutic evidence for GS ameliorating the development of diabetic damage in adipose tissue. An important mechanism of GS's antidiabetic effect is its capacity to lower oxidative stress by reducing ROS generation and lipid peroxidation in adipose tissue. Our data further suggest that another critical mechanism of GS's antidiabetic property is its ability to ameliorate inflammation and fibrosis through modulation of the serum TNF-α and IL-6 levels, and oxidative-, inflammation-, and fibrosis-related protein expressions.

5. Conclusion

For patients with type 2 diabetes, hyperlipidemia, and insulin resistance, thiazolidinediones and fibrate drugs, both of which activate PPARs, have been widely used [74], but side effects such as body weight gain with an excess increase of the fat mass have been reported in diabetes patients [75]. Alternatively, traditional Chinese medicines with negligible toxic and/or side effects have been used in East Asia, and, among them, medicines containing Corni Fructus as the main ingredient have been used to treat diabetes. Among the bioactive compounds of Corni Fructus, there is therapeutic evidence for GS ameliorating the development of diabetic damage in the serum, kidney, and adipose tissues. In conclusion, GS, a bioactive compound of Corni Fructus, ameliorates the development of diabetic damage in the serum, kidney, and adipose tissues.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Review Article

Recent Advances in *Astragalus membranaceus* Anti-Diabetic Research: Pharmacological Effects of Its Phytochemical Constituents

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The disease burden of diabetes mellitus is increasing throughout the world. The need for more potent drugs to complement the present anti-diabetic drugs has become an imperative. *Astragalus membranaceus*, a key component of most Chinese herbal anti-diabetic formulas, has been an important prospect for lead anti-diabetic compounds. It has been progressively studied for its anti-diabetic properties. Ethnopharmacological studies have established its potential to alleviate diabetes mellitus. Recent studies have sought to relate its chemical constituents to types 1 and 2 diabetes mellitus. Its total polysaccharides, saponins, and flavonoids fractions and several isolated compounds have been the most studied. The total polysaccharides fraction demonstrated activity to both types 1 and 2 diabetes mellitus. This paper discusses the anti-diabetic effects and pharmacological action of the chemical constituents in relation to types 1 and 2 diabetes mellitus.

1. Introduction

Diabetes mellitus (DM) has been reported as an epidemic and an increasing disease burden throughout the world [1, 2]. It is a chronic disease characterized by high blood glucose levels resulting from defects in insulin production and action. Types 1 and 2 are the most prevalent. Type 1 is characterized by lack of insulin production caused by autoimmune destruction of pancreatic beta cells. Type 2 results from the ineffective use of insulin due to insulin resistance and deficient glucose metabolism [1, 3, 4]. Research for novel anti-diabetic drugs to complement those in present clinical use has intensified over the years.

Plant medicine has been important in present anti-diabetic drug research. The prospects of a number of medicinal plants, herbal formulations, and natural products with anti-diabetic effects have been reported [5–7]. Notable among such is *Astragalus membranaceus* (AM). It is a Fabaceae flowering plant recorded in various pharmacopoeias as a herbal immunomodulator and an anti-diabetic drug. Its roots have been used in many state-approved Chinese herbal formulas for the treatment of diabetes [6, 7]. A recent publication by Wei et al. (2011) [7] identified it as the most frequently prescribed herbal medicine for diabetes treatment in China. Several ethnopharmacological studies have established its pharmacological significance [6, 8]. Recent studies have progressively sought to identify the lead compounds involved in inducing its anti-diabetic effects. Its polysaccharides, saponins, and flavonoids fractions and a number of single isolated compounds have been studied. The pharmacological processes and mechanism of action of these constituents have also been studied [9–11]. This paper considers it as an important anti-diabetic drug prospect. Thus, we review advances in its anti-diabetic research with emphasis on the pharmacological prospects of its chemical constituents in relation to types 1 and 2 DM. The following database systems were considered for data collection: PubMed, SpringerLink, Wiley Online Library, Science Direct, and China National Knowledge Infrastructure (CNKI)-China Academic Journal Network Publishing Database (CAJD).

2. Ethnopharmacology Effects of AM on Diabetes Mellitus

The roots of AM have a long history for the treatment of diabetes-related symptoms in China. In traditional Chinese medicine, it is used to reinforce Qi in order to induce urination, consolidate the exterior, express toxins outward, and make new tissues grow [7, 12]. A number of studies have emphasized its pharmacological relation to diabetes mellitus. Earlier ethnopharmacological studies analyzed various crude extracts for their anti-diabetic activities and their possible pharmacological processes. They were studied as a single extract or as part of a compound formula and were reported to have demonstrated potentials of attenuating DM and their associated complications. They were generally observed to have lowered increasing blood glucose and lipid levels, improved insulin sensitivity, and also corrected several pathological indicators of DM and its complications [6, 13–16]. In a clinical study of the effect of AM on insulin sensitivity, AM decoction reduced fasting blood glucose and homeostatic model assessment (HOMA) levels in type 2 DM patients [14]. Anti-diabetic studies on *Qilan Tangzhining* capsule, a Chinese herbal anti-diabetic formula containing AM, showed its potential to reduce blood glucose levels and improve lipid profiles in streptotozocin-induced diabetic rats [16]. A number of pharmacological processes for inducing these anti-diabetic effects have been suggested. Some of which include the suppression of macrophage- and cytokine-induced inflammatory responses, stimulation of insulin signal transduction, and lowering of the hyperglycemic effects of glucagon in experimental animals. Its mechanism of action has been associated with several enzymes, proteins, and molecular markers such as peroxisome-proliferator-activated receptor gamma (PPARγ), phosphatidylinositide-3-kinase (PI-3-K), and Na^+ K^+-ATPase, among others [10, 14, 16–18]. Further studies have sought to elucidate the phytochemical constituents inducing these anti-diabetic effects.

3. Phytochemical Constituents

Several classes of organic compounds, namely, *Astragalus* polysaccharides, saponins, flavonoids, isoflavonoids, sterols, amino acids, and volatile oils, have been isolated from AM. The polysaccharides, saponins, and flavonoids are the major chemical constituents demonstrating biological activity to DM [19, 20].

3.1. Polysaccharides. The polysaccharides of AM are by extraction methods water-soluble and -insoluble glucans and heteropolysaccharides. Astragalans I, II, and III are polysaccharides extracted by hot water. Astragalan I was elucidated as a neutral heteropolysaccharide containing *D*-glucose, *D*-galactose, and *L*-arabinose in the ratio of 1.75 : 1.63 : 1. It has a molecular weight of 36 kD. Astragalans II and III were α-(1,4)-glucans with molecular weights of 12 kD and 34 kD, respectively [21, 22]. APS I and APS II were isolated by water extraction and alcohol precipitation technique. Structural and content analyses showed that APS I consisted of arabinose and glucose in the ratio of 1 : 3.45. ASP II consisted of rhamnose, arabinose, and glucose in the ratio of 1 : 6.25 : 17.86 [22, 23]. Acidic polysaccharides such as AMem-P, AH-1 and APSID3 have also been isolated [24–27]. AMem-P is a complex acidic polysaccharide with a molecular weight of 60 kD. It consists mainly of hexuronic acid and has terminal and α-1,5-linked-arabinofuranose, terminal and β-1,3-, β-1,4-, β-1,6-linked, 3,6-branched-*D*-galactose, and 2,4-branched-*L*-rhamnose residue groups attached [24]. Other *Astragalus* polysaccharides include AH-2, AE, AEF-1, and AEF-2, and astroglucans A, B, and C [27–29].

3.2. Saponins. The saponin content of AM consists mainly of triterpene saponins. Structurally, they are cycloartane triterpene glycosides with one-to-three sugars attached at the 3-, 6-, and 25-positions. Kitagawa (1983) reported the isolation of several cycloartane triterpenoids such as astragaloside I–VIII [30–32] and isoastragalosides I and II [30]. Astragalosides VII and VIII were elucidated as saponins with oleanane skeleton [32]. Azukisaponin V methyl ester has been isolated and identified as an oleanane-type triterpene saponin [33]. An astragaloside malonate has also been identified as malonylastragaloside [34]. Several other astragalus saponins including isoastragalosides III and IV, astramembrannin II, cyclogaleginoside B, cycloaraloside A, brachyoside B, cyclocanthoside E, cyclounifolioside B [27, 35, 36], and astramembranosides A and B [33] have also been isolated.

3.3. Flavonoids. Flavonoids of varying structures have been isolated from AM. They are mainly in structural groups of flavones, isoflavones, isoflavanones, and pterocarpans. Kaempferol, isorhamnetin, rhamnocitrin, kumatakenin and rhamnocitrin-3-glucoside and quercetin-3-glucoside have been isolated as flavones [27]. Formononetin, ononin, calycosin, calycosin-7-*O*-β-*D*-glucoside-6″-*O*-malonate, 3′-methoxy-5′-hydroxy-isoflavone-7-*O*-β-*D*-glucoside, and (3*R*)-2′,3′-dihydroxy-4′,7-dimethoxyisoflavone have been isolated as isoflavones [27, 37, 38]. The isoflavanones include 2′-hydroxy-3′,4′-dimethoxyisoflavone-7-*O*-β-*D*-glucopyranoside, 2′-hydroxy-3′,4′,7-trimethoxyisoflavone, 2′,7-dihydroxy-3′,4′,7-trimethoxyisoflavone, 3′,4′-dimethoxyisoflavone-7-*O*-β-*D*-glucoside, 8,2′-dihydroxy-4′,7-dimethoxyisoflavone, and 2′,3′,7-trihydroxy-4′-methoxyisoflavone [27]. The reported pterocarpans include 3,9,10-trimethoxypterocarpan, (6a*R*,11a*R*)-10-hydroxy-3,9-dimethoxypterocarpan, and 9,10-dimethoxypterocarpan-7-*O*-β-*D*-glucopyranoside [27, 38, 39].

4. Pharmacological Effects of *Astragalus* Chemical Constituents on Diabetes Mellitus

The polysaccharides (APS), saponins (ASS), and flavonoids (ASF) fractions of AM have been the most studied for their anti-diabetic effects on types 1 and 2 DM. Several single isolated compounds including astragalin, formononetin, astragalosides I, II, and IV, and isoastragaloside I (Figure 1) have also been analyzed. Their pharmacological processes

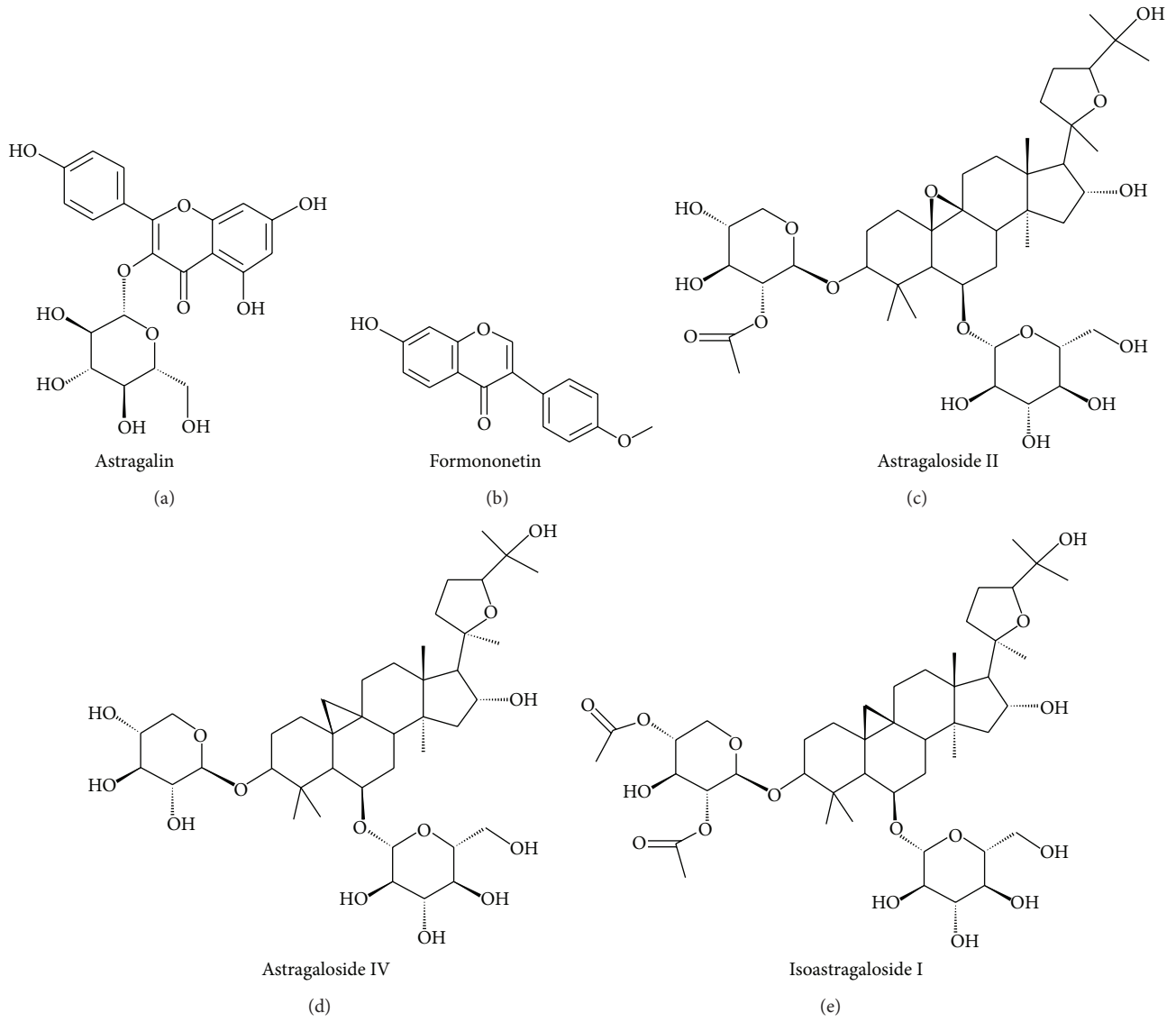

Figure 1: Phytocompounds of *Astragalus membranaceus* demonstrating anti-diabetic effects.

and mechanism of action on types 1 and 2 DM have been reported.

4.1. Type 1 Diabetes Mellitus. Type 1 DM is caused by autoimmune destruction of pancreatic beta cells. The polysaccharides fraction (APS) has been the only constituent demonstrating activity to type 1 DM. It lowered the incidence rate and postponed the onset of type 1 DM in nonobese type 1 diabetes mellitus (NOD) mice [40–42]. It also attenuated autoimmunal insulitis, increased the proliferation of pancreatic beta cells, and decreased apoptotic beta cell mass [43–45]. APS was postulated to have induced immunoprotective effects in type 1 diabetic NOD models. This potential has been widely investigated. Chen et al. (2001) and others evaluated the immunomodulatory effect of APS on $CD4^+$ and $CD8^+$ T cells. APS was observed to have decreased lymphocytic inflammation of pancreatic islets in type 1 noobese diabetic (NOD) mice. It was also reported to have lowered the proliferation of $CD4^+$ and $CD8^+$ T cells [41, 42, 46]. The $CD4^+$ and $CD8^+$ T cells have been implicated in inflammatory response, apoptosis, and autoimmunity leading to type 1 DM [47, 48]. APS may protect pancreatic beta cells from autoimmune destruction through the regulation of inflammatory and apoptotic responses.

4.1.1. Immunomodulation of Inflammatory Response. The anti-inflammatory effect of APS was studied mainly on the secretory cytokines of $CD4^+$ T helper cells. Naive $CD4^+$ T cells differentiate into T helper cells 1 (Th1) and 2 (Th2) for inflammatory response and autoimmunity. The Th1 expresses secretory cytokines such as interferon gamma (IFNγ), tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), and IL-Iβ that induce inflammation and intracellular autoimmune responses. The Th2 is noted for IL-4, IL-5, IL-10,and IL-13

production for extracellular immunity and counteraction of Th1 inflammatory response [49, 50]. APS has demonstrated the potential to lower the expression of Th1 cells and regulate Th1 and Th2 imbalance in *in vivo* diabetic models. Chen and Yu (2004) in molecular immunomodulatory studies reported a possible correction of genetic imbalance of Th1 and Th2 genes and proteins in APS-treated type 1 DM NOD mice. Their studies observed about 5.47% changes in gene expression, of which 17 genes were of functional relation to immunity [51]. Further studies showed that APS demonstrates immunomodulatory effects on Th1 and Th2 cytokines. It was reported to have downregulated the expression levels of Th1 cytokines such as IL-12, TNF-α, and IFNγ and enhanced Th2 cytokines such as IL-4, IL-5, IL-6, and IL-10 [42, 45, 52]. APS also demonstrated a significant lowering effect on Th1/Th2 ratio [44, 53], an important apoptotic index that measures relatively lowered levels of Th1 per Th2 cytokines as an indication for reduced intracellular autoimmunity and inflammatory response [54]. The effect of APS on other inflammatory markers such as peroxisome-proliferator-activated receptor gamma (PPAR-γ), superoxide dismutase (SOD), and nitric oxide (NO) has also been studied. APS significantly enhanced the gene expression of PPAR-γ in a time- and dose-dependent manner [53] and promoted SOD anti-oxidation in type 1 DM models [42, 55]. It also lowered the expression of inducible nitric oxide synthase (iNOS) [42, 55]. PPAR-γ, NO, iNOS, and SOD among a variety of functions also play various roles in the stimulation and regulation of inflammatory response [56].

The effect of astragalin, a flavonoid isolate of AM, on apoptotic cytokines has also been studied. It showed an inhibitory effect on the production levels of TNF-α, IL-1, and IL-6 [57]. It was reported to have repressed the expression of these Th1 cells via NF-κB inhibition. It has also been shown as exhibiting inhibitory effects on proinflammatory mediators similar to quercetin. It was shown to have attenuated the production of nitric oxide (NO) and repressed the expression and production levels of iNOS and cyclooxygenase-2 (COX-2) in J774A.1 mice macrophages [57, 58].

4.1.2. Promotion of Antiapoptotic Response. APS has exhibited the potential to regulate a number of apoptosis-related proteins and enzymes. It demonstrated significant inhibitory effect on caspase-3 enzyme [45, 59] while enhancing the expression of B-cell lymphoma-2 (Bcl-2) [55] in type 1 DM models. Caspase-3 is noted for apoptosis execution, whereas Bcl-2 has apoptosis regulatory effects. APS was also positively correlated to increased galectin-1 levels in the muscles of type 1 DM mice. Its correlation with galectin-1 was further shown to have a negative regulatory effect on $CD8^+$ T cells, an apoptosis-enhancing T cell [60]. APS has also been reported to have lowered the expression of Fas [42, 61, 62]. Fas is a member of the TNF family of receptors that expresses on cells to trigger their apoptosis.

Formononetin, an O-methylated isoflavone, has been reported as inhibiting the activity of caspase-3. It was shown to have reduced caspase-3 levels in INS-1 cells [63]. It also lowered *in vitro* nitric oxide production and apoptotic signaling via a demonstrated inhibition of IL-1β and reduction of Bax/Bcl-2 ratio. It was also shown to have inhibited the activation of nuclear factor-kappaB (NF-κB) [63].

4.2. Type 2 Diabetes Mellitus. Type 2 of DM is caused by insulin resistance and deficient glucose metabolism. All of the major constituents of AM have been shown to differentially lower high blood glucose levels and body weight and improve impaired glucose tolerance in type 2 diabetic models [64–67]. The postulated pharmacological processes include various glucose transportation and insulin signaling pathways that lead to insulin sensitivity and restoration of the proliferative ability of the pancreatic beta cells.

4.2.1. Promotion of Intracellular Glucose Transportation. The polysaccharides fraction has exhibited potentials of reducing hyperglycemia through the induction of glucose translocation enzymes and proteins. It has been studied as a promoter of increased glucose transporter protein-4 (GLUT4) levels. In a molecular expression study of the effect of APS on GLUT4, APS increased the expression and translocation of GLUT4 in skeletal muscle and adipose tissues [64, 68]. The GLUT4 is an insulin-regulated intracellular transporter noted for the mediation of glucose translocation into muscle and fat cells. Liu et al. (2010) analyzed the effect of APS on the GLUT4/protein kinase B (PKB) glucose transportation pathway in the skeletal muscles of insulin-resistant KKAy mice. APS was reported to have partially restored lowered activation levels of PKB and GLUT4 translocation [64].

4.2.2. Regulation of Glucose and Lipid Metabolism. Increased levels of circulating glucose, free fatty acids, and accumulation lipids in nonadipose tissues have been implicated in the development of insulin resistance and type 2 DM [69]. APS, ASS, and ASF have all shown differential regulatory effects on several glucose- and lipid-metabolizing enzymes, proteins, and receptors. The polysaccharides fraction has been the most widely studied. It has been shown to have enhanced the phosphorylation and activation of hepatic glycogen synthase and regulated the expression and activation of adenosine monophosphate-alpha (AMP-α) and acetyl-CoA carboxylase to alleviate glucose accumulation in *in vitro* skeletal muscle cells and KKAy mice models [65]. It also exhibited an upregulatory effect on the levels of adiponectin [70] and its receptor, adipo-R1 [71], in type 2 DM rats. It promoted the expression and activation of adenosine monophosphate protein kinase (AMPK) and its alpha-subunit, AMPK-alpha [65, 71, 72]. Adiponectin and AMPK are important activating factors for glucose and lipid metabolism in the liver, muscles, and adipocytes. Increased levels of their activity have been associated with reduced risk for type 2 DM [73, 74]. Other studies have demonstrated APS as regulating glucose and lipid metabolism through the promotion of peroxisome proliferator-activated receptor-(PPAR-) alpha activity and inhibition of the autonomic neurotransmitter neuropeptide-Y (NPY). The PPARs are

a family of ligand-dependent transcription factors that control energy homeostasis through the regulation of carbohydrate and lipid metabolism. PPAR-alpha potentiates fatty acid catabolism and reduces circulating lipids [75]. APS enhanced the gene and protein expression of PPAR-α and improved the lipoprotein profiles of streptozotocin-induced diabetic hamsters [76]. Neuropeptide-Y is an autonomic neurotransmitter that induces increased food intake leading to obesity and type 2 DM. Chen et al. (2011) reported lowered levels of increased blood glucose and body weight in relation to neuropeptide-Y in streptozotocin-induced diabetic rats. APS was reported to have reduced the mRNA expression levels of neuropeptide-Y and its receptor neuropeptide-Y2 protein [77]. The effect of APS on aldose reductase, a glucose-metabolizing enzyme target implicated in high-glucose-induced diabetes complications [78], has also been studied. APS had no significant inhibitory effect on aldose reductase [79].

The saponins (ASS) and flavonoids (ASF) fractions exhibited their antagonizing effects on ascending blood glucose levels in type 2 DM rats through a common adiponectin and AMPK-metabolizing pathway. They increase the genetic and cellular expression of AMPK, adiponectin, and adipo-R1 levels in the liver and skeletal muscle of diabetic rats [70, 71]. The expression levels of AMPK and adipo-R1 induced by the saponins were reported to be more pronounced in the skeletal muscles than in the liver, whereas the flavonoids showed an increased effect in the liver than in the skeletal muscle [71].

Several *Astragalus* saponins isolates have been studied. Astragaloside II and isoastragaloside I exhibited regulatory effects on adiponectin and AMPK action. They significantly increased adiponectin levels and promoted the activation of AMPK in type 2 DM mice. Their induction of increased adiponectin levels was reported to be independent of PPARγ, an adiponectin agonist [75, 80]. The *Astragalus* saponins astragalosides I and IV have demonstrated inhibitory effect on aldose reductase. They downregulated its activation levels to ameliorate accumulation of advanced glycation endproducts in both erythrocytes and nerve cells of diabetic rats [81].

The comparative effects of formononetin and calycosin isoflavonoids on the peroxisome-proliferator-activated receptors activation system have also been studied. Formononetin was reported to be more potent activator of PPARγ-induced differentiation of 3T3-L1 preadipocyte than calycosin [18]. PPARγ plays crucial role in the differentiation and maturity of fat cells [75].

4.2.3. Alleviation of ER Stress and Induction of Insulin Signal Transduction. Stress responses in the endoplasmic reticulum (ER) have been associated with increased β-cell apoptosis rates, reduced beta cell mass, lowered insulin production, and increased insulin resistance in type 2 DM patients. APS has been reported as a negative regulator of key ER stress indicators such as phosphorylated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF-6), glycogen synthase kinase 3 beta (GSK3β), and XhoI site-binding protein 1 (XBP1) in type 2 diabetes models. It relieved ER stress in type 2 DM SD rats through a significant decrease in the expression of PERK and inhibition of ATF-6 activity [82]. It also reduced the levels of the transcription repressor protein XBP1 and GSK3β in KKAy mice [83]. The inhibitory effect of APS on ATF-6 was further studied in relation to protein tyrosine phosphatase-1-B (PTP1B), a negative regulator of insulin-receptor signal transduction. ATF-6 inhibition was positively correlated with lowered expression and activation levels of PTP1B in experimental animals [67, 84, 85]. APS may have indirectly promoted insulin signaling via ER stress alleviation. Other insulin signaling studies have reported the upregulatory effect of APS on insulin receptors. APS was shown to have increased the levels of insulin receptor substrate-1 (IRS-1) and its beta transmembrane receptor (IR-β) subunit in muscle cells [84]. IRS-1 s key role in insulin signal transduction. Lowered levels of IRS-1 have been associated with increased susceptibility to type 2 DM [86, 87]. APS has also demonstrated regulatory effects on resistin, an insulin-resistance protein [88, 89]. It decreased the mRNA and protein expression levels of resistin in type 2 DM Wistar rats.

5. Pharmacological Prospects and Concluding Remarks

The anti-diabetic potential of *Astragalus membranaceus* has been progressively studied in the recent past. Its crude extracts have been reported in several ethnopharmacological studies as potential prospect for further anti-diabetic studies. Recent studies have analyzed its phytochemical constituents in elucidating its pharmacological significance to types 1 and 2 DM. Its polysaccharides, saponins, and flavonoids fractions and several isolated compounds have been studied. They all exhibited differential potentials of correcting the characteristic defects of inadequate insulin production, secretion, and action on target cells. The total polysaccharides fraction demonstrates significant activity to type 1 DM. It protects pancreatic beta cells from intracellular (autoimmune) cell death via the immunomodulation of several inflammatory and apoptotic cytokines, enzymes, and proteins. It demonstrated the potential to modulate T helper cells 1 and 2, reduce inflammatory response, and promote antioxidant activities towards antiapoptotic protection of pancreatic beta cells. Astragalin and formononetin also demonstrated regulatory effects on various inflammatory and apoptotic indicators.

The polysaccharides, saponins, and flavonoids fractions all exhibited significant activities to type 2 DM. They generally induce their hypoglycemic effects through various insulin sensitizing pathways. They all demonstrated regulatory effects on AMPK and adiponectin and its receptor adipo-R1. Astragaloside II and isoastragaloside I isolates were also associated with this effect. The polysaccharides fraction has been most extensively studied in relation to type 2 DM. It promotes insulin sensitization through various coordinated pathways towards intracellular glucose transportation, insulin signal transduction, and protection of pancreatic beta cells from apoptotic death. It promoted the PKB/Akt and -PPAR-α and -γ systems, activated insulin receptors, and regulated ER stress-related proteins and enzymes.

The PKB/Akt system differentially coordinates PKB to glycogen synthase kinase 3 (GSK3), GLUT4, apoptotic caspases, IR, and IRS-1, among others, to induce glucose transportation and cell proliferation. The phosphorylation and activation of PKB lead to increased IRS-1 and GLUT4 activity for glucose translocation and insulin signaling. Its activation also results in the inactivation of GSK3 and caspase proteases to inhibit apoptosis [90, 91]. Stress-induced apoptosis in the endoplasmic reticulum of pancreatic and liver cells has also been related to reduced insulin production and increased insulin resistance [92, 93]. APS notably exhibited a negatively regulatory effect on PERK, ATF-6, and XBP1 ER stress indicators. The PPAR are a family of ligand-dependent transcription factors that control energy homeostasis through the regulation of carbohydrate and lipid metabolism [94]. They are also involved in the regulation of inflammatory responses [56, 75]. PPAR-alpha [75] and PPAR-gamma [94] have been associated with the regulation of DM. APS demonstrated activity to both of them to alleviate high blood glucose levels. The demonstrated regulatory effects of APS on these systems suggest its importance and prospects for further research and development for diabetes therapy.

Further studies on more single-compound isolates are important to understand the overall mechanisms and processes of anti-diabetic effects as well as their structure-activity relationships.

Conflict of Interests

The authors have no conflict of interests in this paper.

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Research Article

The Efficacy of Red Ginseng in Type 1 and Type 2 Diabetes in Animals

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Diabetes mellitus (DM) is one of the most modern chronic metabolic diseases in the world. Moreover, DM is one of the major causes of modern neurological diseases. In the present study, the therapeutic actions of Korean red ginseng were evaluated in type 1 and type 2 diabetic mouse models using auditory electrophysiological measurement. The comprehensive results from auditory brainstem response (ABR), auditory middle latency response (AMLR), and transient evoked otoacoustic emission (TEOAE) demonstrate auditory functional damage caused by type 1 or 2 DM. Korean red ginseng improved the hearing threshold shift, delayed latencies and signal intensity decrease in type 2 diabetic mice. Type 1 diabetic mice showed a partial improvement in decreasing amplitude and signal intensity, not significantly. We suggest that the Korean red ginseng has a more potent efficacy in hearing loss in insulin resistance type 2 diabetes than in type 1 diabetes.

1. Introduction

Diabetes mellitus (DM) is one of the most common modern chronic metabolic diseases in the world. Moreover, DM is a cause of modern chronic neurological disease. Recently, many studies have reported that hearing impairment could be caused by DM [1]. In our previous study, hearing impairment was found in streptozotocin (STZ-) induced diabetic mouse models as caused by type 1 diabetes. STZ-induced DM may impair the auditory pathway from the peripheral auditory nerve to the midbrain in mouse models [2]. Additionally, chronic hyperglycemia and obesity found in type 2 diabetic mouse models may lead to early sensorineural hearing loss [3].

Recently, investigations have revealed an improvement in hearing after natural product treatment in hearing-impaired subjects with diabetic hearing loss [4, 5]. In our previous study, coffee or trigonelline ameliorated the hearing threshold shift and delayed the latency of the auditory evoked potential in diabetic hearing impairment [4]. Moreover, diosgenin was observed to clearly cause improvements in diabetic auditory impairment in the hearing threshold, latencies, and otoacoustic emission of electrophysiologic evaluation [5].

Korean red ginseng (RG), as an alternative medicine, has been widely used in the Republic of Korea, Japan, and China and is produced by steaming and drying fresh Korean ginseng (*Panax ginseng* C.A. Meyer). This suggests that a chemical transformation of the active physiological properties of ginseng takes place during the production process [6]. RG and its ginsenosides possess multiple pharmacological actions for treating various diseases and conditions including liver regeneration [7], cerebral ischemia [8], and hypertension [9]. Im et al. reported that Korean red ginseng extract could play an antioxidative role in cisplatin-induced ototoxicity through the inhibition of caspase-3 expression in HEI-OC1, an auditory cell line [10]. We reported on a recovery effect of red ginseng on noise-induced hearing loss in mice [11]. Although the antidiabetes effect of red ginseng has been previously reported [12, 13], no study of red ginseng efficacy in diabetic hearing impairment has been reported previously.

In the present study, the therapeutic actions of red ginseng were evaluated in type 1 and type 2 diabetic mouse models

using auditory electrophysiologic measurements. To assess the efficacy of red ginseng in diabetic hearing impairment in type 1 and type 2 diabetes, we performed auditory function evaluations as follows: otoacoustic emission (OAE) for the measurement of cochlear functions, auditory brainstem response (ABR) for the measurement of peripheral auditory functions, and auditory middle latency response (AMLR) for the measurement of central auditory functions.

2. Materials and Methods

2.1. Korean Red Ginseng Extract. RG extract was obtained from the Korea Ginseng Corporation (Taejon, Republic of Korea). Korean red ginseng extract (crude saponin 70 mg/g, solid component 60%, or more) contained Rb1 (0.46%), Rb2 (0.23%), Rc (0.28%), Rd (0.09%), Re (0.12%), Rf (0.10%), Rg1 (0.07%), Rg2 (0.14%), Rg3 (0.12%), Rh1 (0.10%), and other minor ginsenosides.

2.2. Animal. All of the experimental procedures were performed in accordance with the Principles of Laboratory Animal Care (NIH publication, no. 80-23, revised 1996) and the Animal Care and Use Guidelines of Nambu University, Republic of Korea. Eight-week-old adult male ICR mice were used in the type 1 diabetic mice model, nondiabetic ICR mice were used as the control, and eight-week-old adult male *Lepr*$^{(+/+)}$*C57BL/KsJ (dbdb)* mice were used as the type 2 diabetic mice model. Male *Lepr*$^{(+/-)}$*C57BL/KsJ (dbh)* littermates were followed concurrently and served as the appropriate control (Jung-Ang Lab Animal, Seoul, Republic of Korea) in this study. The mice were housed individually with a 12 h/12 h light/dark cycle with food and water *ad libitum*.

2.3. Induction of Diabetes Mellitus. To induce the type 1 diabetic mice, the ICR mice were injected streptozotocin (STZ) dose of 120 mg/kg. Seven days later, mouse blood glucose values were determined to confirm DM induction.

2.4. Treatment of Red Ginseng. The experimental mice were divided into eight groups ($n = 10$/group). The nondiabetic ICR mice (T1-Con) and the STZ-induced mice (T1-DM) were treated orally once daily with 0.5 mL of distilled water. STZ-induced mice were treated orally once daily with red ginseng 100 mg/kg (T1-R100) and red ginseng 200 mg/kg (T1-R200). The *dbh* mice (T2-Con) and the *dbdb* mice (T2-DM) were treated orally once daily with 0.5 mL of distilled water. The *dbdb* mice were treated orally once daily with red ginseng 100 mg/kg (T2-R100) and red ginseng 200 mg/kg (T2-R200). Solutions containing red ginseng in distilled water were prepared daily immediately prior to treatment. Red ginseng treatments were performed once daily for 8 weeks.

2.5. Blood Glucose Level Measurement. Glucose was determined in blood samples obtained by pricking the tail of mice and using a strip-operated blood glucose sensor (ONETOUCH Ultra, Inverness Medical Ltd., UK) under the nonfasting status. Type 1 diabetic mice showing ≥300 mg/dL of blood glucose levels at one week after STZ injection were used.

2.6. Auditory Electrophysiologic Evaluation Preparation. All the groups underwent peripheral and central auditory function tests at 8 weeks after red ginseng treatment. The auditory function tests were performed with the mice under anesthesia after an i.m. administration of xylazine (0.43 mg/kg) and ketamine (4.57 mg/kg). The rectal temperature was maintained at 37°C ± 0.5°C using a heating lamp at the time of testing.

For the auditory electrophysiologic test, two-channel recordings (GSI Audera, Viasys Healthcare Inc., USA) were obtained through needle electrodes inserted s.c. at the vertex. Reference electrodes were placed below the pinnas of the left and the right ears, and a ground electrode was inserted into the shoulder. Electrode impedance was in the range of 2 kΩ to less than 5 kΩ for the electrode pairs.

2.7. Auditory Brainstem Response (ABR) Evaluation. For the ABR recordings, alternating clicks (0.1 ms duration) and 4 and 8 kHz tone bursts (TBs) (rise-plateau-fall; 2-1-2 cycles) were delivered through earphones (Etymotic ER-3A) at a rate of 20.1 stimuli/s. Physiological filters were set to pass electrical activity between 100 and 3000 Hz. Monaural responses were recorded for each mouse and averaged in a 10.24 ms time window. One thousand sweeps were collected. To determine the thresholds in ABR recordings, the clicks were reduced in 10 dB steps. When no response was detected, the level increased in 5 dB steps until a response was determined. ABR parameters were evaluated based on the hearing thresholds and interpeak latencies of waves I–IV at a peak sound pressure level (pSPL) of 90 dB.

2.8. Auditory Middle Latency Response (AMLR) Evaluation. For the AMLR measurements, rarefaction clicks (0.1 ms duration) were delivered through earphones at a rate of 9.1 stimuli/s. Filters were set to pass activity between 10 and 250 Hz. An average of 250 sweeps was determined in a 70 ms time window. The parameters of AMLR were evaluated with absolute latencies of wave Pa at a pSPL of 90 dB.

2.9. Transient Evoked Otoacoustic Emission (TEOAE) Evaluation. Cochlear function was determined based on transient evoked otoacoustic emissions (TEOAEs) using ILO v6 (Otodynamics, Hatfield, Hertfordshire, UK). TEOAEs were evoked by 80-μs clicks of 90-dB SPL intensity, with a masking noise in the opposite ear, according to the standard nonlinear ILO protocol. TEOAE responses were evaluated in the frequency domain (FFT) by estimating the signal-to-noise ratios (SNRs) at 2 and 3 kHz.

2.10. Statistical Analysis. Data were analyzed using the Prism 5 Statistical Software package (GraphPad, San Diego, CA, USA). All data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons between the groups were performed using a paired t-test between 0 weeks and

Table 1: Body weights and glucose levels in diabetic mice.

Groups	Body weight (g)		Glucose levels (mg/dL)	
	0 weeks	8 weeks	0 weeks	8 weeks
T1-Con	38.4 ± 1.5	40.8 ± 1.4	136.9 ± 6.8	133.8 ± 6.2
T1-DM	39.6 ± 2.5	38.8 ± 2.8	≥600.00	≥600.00
T1-R100	37.2 ± 2.5	40.0 ± 1.2	≥600.00	≥600.00
T1-R200	37.8 ± 2.0	40.0 ± 3.0	≥600.00	≥600.00
T2-Con	20.5 ± 0.5	23.3 ± 0.5	168.4 ± 0.8	177.8 ± 8.7
T2-DM	42.4 ± 0.9	65.9 ± 1.9***	267.5 ± 19.4	364.5 ± 30.2*
T2-R100	41.7 ± 0.9	65.0 ± 2.3***	310.8 ± 17.5	392.3 ± 15.8*
T2-R200	42.2 ± 1.3	66.9 ± 1.6***	335.6 ± 32.0	364.7 ± 30.7

Nondiabetic ICR mice (T1-Con), STZ-induced diabetic mice as the type 1 diabetic mice model (T1-DM), STZ-induced diabetic mice treated with RG 100 mg/kg (T1-R100), STZ-induced diabetic mice treated with RG 200 mg/kg (T1-R200), *dbh* mice (T2-Con), *dbdb* mice as the type 2 diabetic mice model (T2-DM), *dbdb* mice treated with RG 100 mg/kg (T2-R100), and *dbdb* mice treated with RG 200 mg/kg (T2-R200) groups were evaluated for their body weights and blood glucose levels. The data shown indicate the means ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences from the values at 0 weeks.

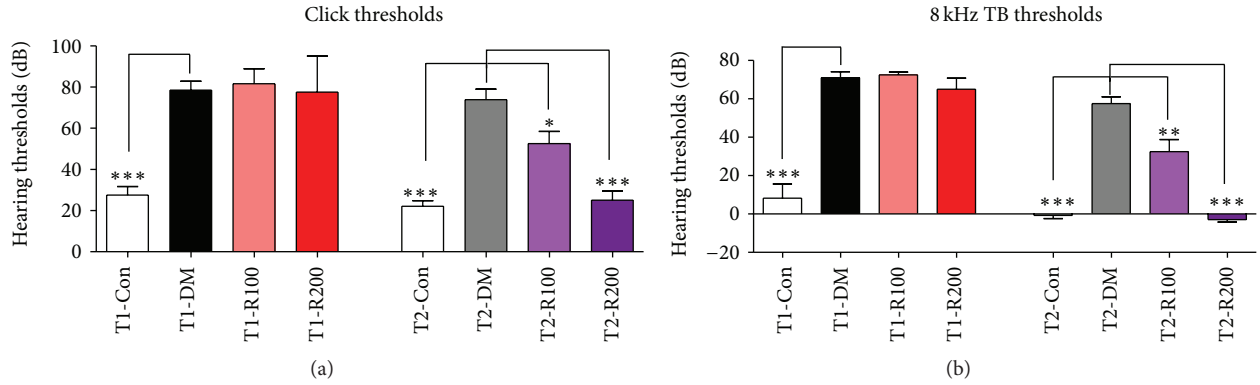

Figure 1: Hearing thresholds of ABR with clicks and 8-kHz TBs in diabetic mice. In this study, the hearing thresholds of ABR after stimulation using clicks (a) and 8-kHz TB stimulations (b) were measured. The data shown indicate the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences between the T1-DM and T2-DM groups (Tukey's multiple comparison *post hoc* test).

8 weeks in body weights and blood glucose levels and one-way repeated measured ANOVA with Tukey's *post hoc* test in hearing thresholds, latencies, and SNRs. Values of $P < 0.05$, 0.01, and 0.001 were considered statistically significant.

3. Results

3.1. Body Weights and Blood Glucose Levels. At 0 weeks and 8 weeks after STZ injection, the body weights and blood glucose levels were evaluated in all the groups (Table 1). The body weights of T1-DM decreased slightly at 8 weeks compared to 0 weeks, whereas the body weights of the T1-Con, T1-R100, and T1-R200 groups increased. The body weights of T2-Con increased slightly at 8 weeks compared to 0 weeks, whereas the T2-DM, T2-R100, and T2-R200 groups significantly increased in body weight from 0 weeks. In the T1-DM, T1-R100, and T1-R200 groups, the mean blood glucose levels 0 weeks and 8 weeks were ≥600 mg/dL, which was the upper measurement limit of the blood glucose analyzer. The blood glucose levels were not decreased in the T1-R100, T1-R200, T2-R100, and T2-R200 groups at 8 weeks. These results show that red ginseng did not improve hyperglycemia in the type 1 and 2 diabetic mouse models used in this study.

3.2. Hearing Thresholds in ABR. To assess the ameliorative effects of the hearing threshold shifts in type 1 and 2 diabetic mice, ABR tests were performed at 8 weeks after red ginseng treatments. In the T1-DM and T2-DM groups, the hearing thresholds significantly increased by 61 dB and 61 dB with clicks and by 63 dB and 58 dB with 8-kHz TBs, respectively, compared to the T1-Con and T2-Con groups at 8 weeks. The hearing threshold shifts in the T1-DM and T2-DM groups may induce damages in peripheral nerve function. In the type 1 diabetic mice, the hearing thresholds of the red ginseng treatment groups (T1-R100 and T1-R200) slightly decreased compared to the T1-DM group. In the type 2 diabetic mice, the hearing thresholds of the red ginseng treatment groups (T2-R100 and T2-R200) significantly decreased by 21 dB and 48 dB with clicks and by 25 dB and 60 dB with 8-kHz TBs, respectively, compared to the T2-DM group (Figures 1(a) and 1(b)). These data indicate that red ginseng suppresses the hearing threshold shifts in the type 2 diabetic mouse model and improves auditory nerve functions, whereas no

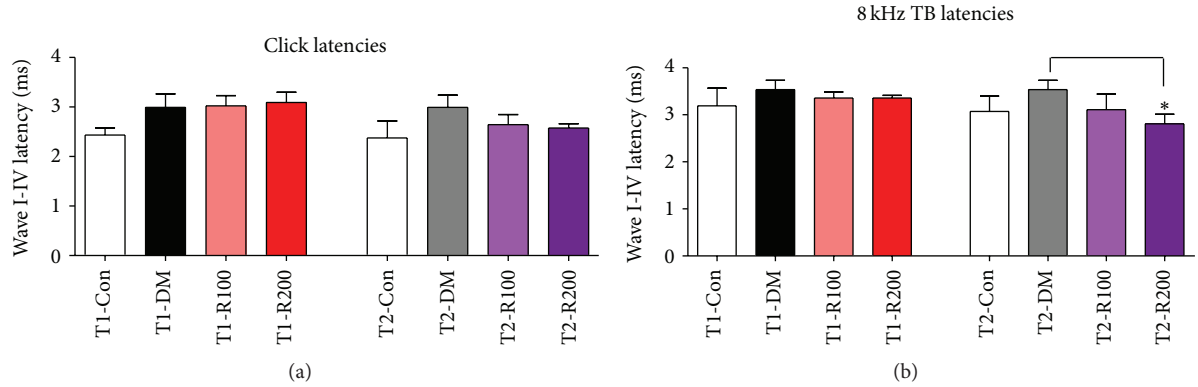

Figure 2: Latencies of ABR with clicks and 8 kHz TBs in diabetic mice. In this study, the wave I–IV latencies of ABR after stimulation using clicks (a) and 8 kHz TB stimulations (b) were measured. The data shown indicate the means ± SEM. $^{*}P < 0.05$ indicates a significant difference from the T2-DM group (Tukey's multiple comparison *post hoc* test).

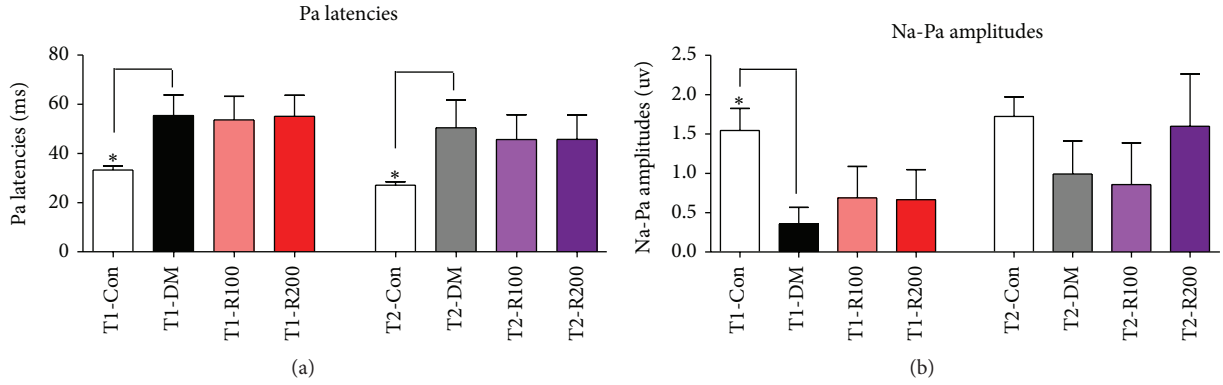

Figure 3: Latencies and amplitudes of AMLR in diabetic mice. In this study, Pa latencies (a) and Na-Pa amplitudes (b) of AMLR after stimulation using clicks was measured. The data shown indicate the means ± SEM. $^{*}P < 0.05$ indicates significant differences between the T1-DM and T2-DM groups (Tukey's multiple comparison *post hoc* test).

improvement was observed in the type 1 diabetic mouse model.

3.3. Peak Wave Latencies in ABR. To assess the ameliorative effects of auditory nerve damage in type 1 and 2 diabetic mice, ABR tests were performed at 8 weeks after red ginseng treatments. In the T1-DM and T2-DM groups, the peak latencies increased compared to the T1-Con and T2-Con groups at 8 weeks. The latency delay in the T1-DM and T2-DM groups may be damaged for peripheral nerve conductivity. In type 1 diabetic mice, the latencies of the red ginseng treatment groups (T1-R100 and T1-R200) were similar to the T1-DM group. In type 2 diabetic mice, the latencies of the red ginseng treatment group (T2-R200) significantly decreased compared to the T2-DM group (Figures 2(a) and 2(b)). These data indicate that red ginseng suppresses latency delay in the type 2 diabetic mouse model and improves auditory nerve functions.

3.4. Pa Latency and Amplitude in AMLR. To assess the ameliorative effects of central auditory function damage in type 1 and 2 diabetic mice, AMLR tests were performed at 8 weeks after red ginseng treatments. In the T1-DM and T2-DM groups, Pa latencies in AMLR significantly increased compared to the T1-Con and T2-Con groups. In type 1 diabetic mice, the Pa latencies of the red ginseng treatment groups (T1-R100 and T1-R200) were similar to the T1-DM group. In type 2 diabetic mice, the Pa latencies of the red ginseng treatment groups (T2-R100 and T2-R200) decreased slightly compared to the T2-DM group (Figure 3(a)). In the T1-DM and T2-DM groups, the Na-Pa amplitudes in AMLR significantly decreased compared to the T1-Con and T2-Con groups. In type 1 diabetic mice, the Na-Pa amplitudes of the red ginseng treatment groups (T1-R100 and T1-R200) slightly increased compared to the T1-DM group, but this increase was not significant. In the type 2 diabetic mice, the Na-Pa amplitudes of the red ginseng treatment groups (T2-R200) increased compared to the T2-DM group, but this increase was not significant (Figure 3(b)). These data indicate that red ginseng suppresses latency delay, decreases amplitude in the type 2 diabetic mouse model, and improves the central auditory functions compared to the type 1 diabetic mouse model.

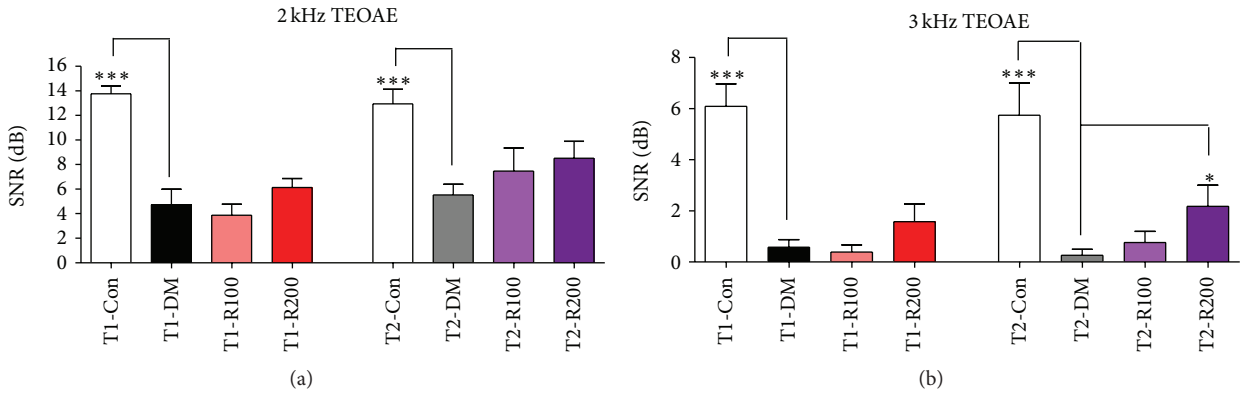

Figure 4: Signal intensities of TEOAEs with 2 and 3 kHz in diabetic mice. In this study, the signal intensity of TEOAEs to noise at 2 kHz (a) and 3 kHz TBs (b) were measured. The data shown indicate the means ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences between the T1-DM and T2-DM groups (Tukey's multiple comparison *post hoc* test).

3.5. Signal-to-Noise Ratios in TEOAE. To assess the ameliorative effects of outer hair cell damages in type 1 and 2 diabetic mice, TEOAEs with 2 and 3 kHz were performed at 8 weeks after red ginseng treatments. The SNRs of 2 and 3 kHz of TEOAEs in the T1-DM and T2-DM groups significantly decreased at 8 weeks compared to the T1-Con and T2-Con groups. In type 1 diabetic mice, the SNRs of the red ginseng treatment groups (T1-R200) slightly increased compared to the T1-DM group. In type 2 diabetic mice, the SNRs of the red ginseng treatment groups (T2-R100 and T2-R200) increased compared to the T2-DM group (Figures 4(a) and 4(b)). These data indicate that red ginseng suppresses the decrease of SNRs in the type 2 diabetic mouse model and improves outer hair cell function in the cochlea compared to the type 1 diabetic mouse model.

4. Discussion

The comprehensive results from ABR, AMLR, and TEOAE demonstrate auditory functional damage caused by type 1 or 2 DM. Red ginseng improved the hearing threshold shift, delayed latencies, and decreased signal intensity in type 2 diabetic mice. Type 1 diabetic mice exhibited a partial improvement in the decrease in amplitude and signal intensity, but these improvements were not significant. The question then remains regarding the reason that there is a significant improvement in auditory function in type 2 diabetes and not type 1 diabetes.

Diabetes mellitus is classified as type 1 or type 2. The metabolic pathologies of type 1 diabetes mellitus (T1-DM) and type 2 diabetes mellitus (T2-DM) are different. T1-DM is caused by the loss of beta cells found in the islets of Langerhans in the pancreas. T2-DM is associated with insulin resistance and relative insulin deficiency [14]. T1-DM has symptoms of hyperglycemia and hypoinsulinemia while T2-DM has symptoms of hyperglycemia and hyperinsulinemia or insulin resistance. Therefore, the neurological diseases associated with T1-DM and T2-DM are different, especially in relation to insulin.

Fukushima et al. [15] have described DM-associated pathology changes within the cochlea that include thickened vessels of the stria vascularis, atrophy of the stria vascularis, and loss of outer hair cells in humans. Altered auditory function in diabetic animals has been examined in both otoacoustic emissions [16] and auditory brainstem responses, as well as in auditory middle latency responses [2]. Current research indicates that DM may cause hearing impairment, but a firm cause-effect correlation according to DM type has not yet been described [17]. A number of studies have attempted to identify the differences in hearing loss in humans with type 1 and type 2 DM [18–20], but, to date, the location of the lesions and the mechanism of deficit have not been established. Recent studies showed distinct differences in the severity of polyneuropathy in patients with type 1 and type 2 diabetes. Progressive axonal atrophy and loss are more serious in patients with type 1 diabetes, while patients with type 2 diabetes exhibit nodal and paranodal degenerative changes as well as more severe downstream effects on neuroskeletal and adhesive proteins [21].

Korean red ginseng (RG) is believed to contain ingredients that possess a variety of health enhancing effects including an antidiabetic effect [22, 23], enhanced erectile function [24], and cognitive enhancement [25]. Recently, RG has been shown to improve cognitive function related to the auditory pathway using electrophysiologic evaluation [26].

Several human studies have reported that the administration of KRG had positive effects on the maintenance of sugar control and insulin resistance in type 2 diabetes mellitus patients [27, 28]. In addition, a previous animal study suggested the potential beneficial effects of KRG on the amelioration of insulin resistance and prevention of T2-DM through the activation of the AMP-activated protein kinase (AMPK) in fat rats [29]. Although the effect of red ginseng on nerve protection has not been reported, the nerve protection efficacy of Rb1, a major ginsenoside of red ginseng, has been reported in many studies [30, 31].

In this study, although red ginseng did not improve hyperglycemia, red ginseng improved diabetic hearing impairments observed in type 2 diabetic mice. We suggest that red ginseng efficacy in the auditory function of type 2 diabetic mice may be related to the improved insulin

sensitivity of red ginseng and protection efficacy of the nerve from the major ginsenoside of red ginseng. Additionally, the mouse strain difference of types 1 and 2 diabetic mice in this study or other factors may have been influenced. More studies will be needed in the future.

5. Conclusions

In the present study, the therapeutic actions of red ginseng were evaluated in type 1 and type 2 diabetic mouse models using auditory electrophysiological measurement. The comprehensive results from ABR, AMLR, and TEOAE demonstrate auditory functional damage caused by type 1 or 2 DM. Korean red ginseng improved the hearing threshold shift, delayed latencies, and signal intensity decrease in type 2 diabetic mice. Type 1 diabetic mice showed a partial improvement in decreasing amplitude and signal intensity, not significant. We suggest that the Korean red ginseng has a more potent efficacy on hearing loss in insulin resistance type 2 diabetes than in type 1 diabetes.

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Research Article

Antiobesity Effect of Caraway Extract on Overweight and Obese Women: A Randomized, Triple-Blind, Placebo-Controlled Clinical Trial

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Caraway (*Carum carvi* L.), a potent medicinal plant, is traditionally used for treating obesity. This study investigates the weight-lowering effects of caraway extract (CE) on physically active, overweight and obese women through a randomized, triple-blind, placebo-controlled clinical trial. Seventy overweight and obese, healthy, aerobic-trained, adult females were randomly assigned to two groups ($n = 35$ per group). Participants received either 30 mL/day of CE or placebo without changing their diet or physical activity. Subjects were examined at baseline and after 90 days for changes in body composition, anthropometric indices, and clinical and paraclinical variables. The treatment group, compared with placebo, showed a significant reduction of weight, body mass index, body fat percentage, and waist-to-hip ratio. No changes were observed in lipid profile, urine-specific gravity, and blood pressure of subjects. The results suggest that a dietary CE with no restriction in food intake, when combined with exercise, is of value in the management of obesity in women wishing to lower their weight, BMI, body fat percentage, and body size, with no clinical side effects. In conclusion, results of this study suggest a possible phytotherapeutic approach for caraway extract in the management of obesity. This trial is registered with NCT01833377.

1. Introduction

Proper nutrition is necessary to keep the body healthy and functioning normally. The addition of extra calories in the diet induces fat accumulation, leading to overweight and obesity. According to the World Health Organization (WHO), excess body weight and obesity are recognized as a body mass index (BMI) greater than 25 kg/m^2. According to this report by WHO, "globesity," as a foodborne illness, is a rapidly growing global problem, which is maximizing the risk of various health problems, such as type 2 diabetes, cardiovascular diseases (CVD), musculoskeletal disorders, and cancer. Overweight and obesity are associated with high morbidity and mortality, resulting in considerable health care costs and other economic and social impacts on the society. Since 1980, obesity has almost doubled worldwide and is recognised as one of the leading causes of death. In 2008, over 1.4 billion adults, predominantly women, were overweight or obese. Finally, more people die because of being overweight and obese than those who are underweight, and this disease state is the fifth main reason for mortality and the sixth for health problems globally. Management of obesity is therefore a public health necessity [1–5].

Obesity is associated with multiple macro- and microenvironmental factors. It is manageable by several different approaches, including pharmaceutical drugs, traditional medications, and surgery. Of these approaches, the use of medicinal plants is increasingly popular and is preferable to conventional chemotherapeutic methods [6, 7]. Early indigenous people faced with various forms of illness and health problems discovered a wealth of valuable healing agents in their local flora. There are a frequently number of potential advantages linked to the use of medicinal plants, including accessibility, safety, effectiveness, affordability, reliability, and acceptability, typically with minor adverse effects and lower costs [8]. Moreover, medicinal plants are naturally available sources with potentially beneficial biological and pharmacological effects and are easy to consume, whereas there is still doubt about the other more invasive therapeutic modalities for obesity, such as surgery [9].

Specific phytochemical constituents present in medicinal plants may assist in regulating weight and body fat through modifying metabolic pathways at the molecular level which are responsible for signalling adipogenesis, lipolysis, and so forth [10]. Hence, they are able to play multiple roles simultaneously in a network pharmacology approach to disease alleviation.

Caraway (*Carum carvi* L.) is a well-known medicinal plant, which was traditionally recommended by the great ancient scientist of the eleventh century Ibn Sina (also known as Avicenna) for weight loss, and is used widely for culinary purposes in Asia and Europe [11]. This plant is from the Apiaceae (formerly Umbelliferae) family and is used in traditional medicine as a remedy for a range of health problems, especially stomach ache, burping and flatulence, and intestinal spasms [12–17]. Caraway seeds contain multiple phytochemical constituents, including fatty acids, essential oils, and volatile phenolic compounds which are used in industry and medicine [18–22]. These bioactive ingredients present in caraway seeds induce a range of different biological benefits, including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities, which offers promising therapeutic potential to alleviate several human diseases [23–29].

Previous studies have established an association between the moderate consumption of caraway-derived metabolites with a lower incidence of diabetes, dyslipidaemia, hypertension, liver dysfunction, reproductive hormone imbalance, osteoporosis, cancer, gastrointestinal, and inflammatory diseases [13, 30]. In addition, *in vitro* and *in vivo* studies have demonstrated the hepatoprotection and safety of caraway ingredients for use in pharmaceuticals and food products [30–32]. A further study showed a plausible, multitargeted, antiobesity effect of caraway on animals through modifying the gene expressions associated with inflammation and adipogenesis [33]. Accordingly, this activity of caraway was examined in a clinical study of overweight and obese subjects as a dietary intervention, in combination with physical activity, in a homogenous population of physically active, adult women selected to evaluate the reliability of the earlier indications in a formal clinical environment.

2. Methods

2.1. Study Design and Study Population. The clinical study reported was a randomized, triple-blind, placebo-controlled, clinical trial, with a duration of three months, and was designed and conducted to evaluate the weight-lowering effect of the caraway seed extract (CE) compared with placebo. Obese and overweight women with a BMI (body mass index) of 25–39.9 kg/m^2 and ages between 20 and 55 years were eligible for this study. Volunteers were recruited at a fitness centre in Yazd, Iran, and were doing moderate aerobics training for 180 minutes/week, with an estimated energy expenditure of 1000–1200 kcal/week.

Subjects with a history of extreme weight loss through surgery or abnormal diet and the presence of diagnosed, severe health problems including hypertension, CVD, dyslipidaemia, clinical depression, diabetes mellitus, and thyroid diseases, using alcohol, cigarette, or any medication or supplements which might have an effect on metabolism or appetite, having a history of allergy to the medicinal plant extract or placebo products, and also pregnant and lactating women were specifically excluded from the study cohort. This interventional study was registered with the clinicaltrial.gov protocol registration system with the Protocol no. NCT01833377 and was approved by the Medical Ethics Committee of the University of Malaya Medical Centre (UMMC) on June 20, 2012 (no. 925/15). All of the entered subjects signed an approved, written consent form at the initiation of the study.

Nutritional consultation was provided at baseline entry into the protocol and during the treatment regimen. Participants were encouraged to follow a healthy lifestyle habit and were advised not to make any significant changes to their diet and routine physical activity during the three-month period of the study protocol.

2.2. Randomization and Blindness. The seventy (70) qualified and allotted subjects were randomized into two equal-sized groups of 35-35 subjects through the online randomization program (http://www.randomization.com/). Investigators, subjects, and the data collectors were masked to the treatment regimens. A statistician, who was not directly involved in the establishment of the groupings and the design of the trial, was provided with the codes and the data for analysis (triple-blind). The bottled CE and placebo samples were coded by the coinvestigator who was not involved in the study, and the sample of CE (500 mL) or placebo was provided for the subjects in sealed PET bottles every two weeks. The eligible study subjects were randomly allocated to consume either a 30 mL sample of the active CE or placebo product, once a day, 20 minutes before lunch for 3 months.

2.3. Preparation of Herbal Extract and Placebo. The CE samples obtained from the Baharan Company, Yazd, Iran (Industrial Ministry License no. 28/1232 and Health Ministry License no. 35/10500) were extracted from the seeds of caraway through steam distillation. From each 1 kg of caraway seeds, 10 litres of caraway water extract was produced. Consequently, the amount of caraway in terms of w/v was

0.1 (10%). The analysis of the CE sample used in the study is described below. The placebo was prepared by dissolving edible caraway essence (Givaudan Flavours Co., Kemptthal, Switzerland) in drinking water (1% g/L) which was identical with CE in appearance and flavour. Subjects were provided with measured bottles and were asked to dissolve 30 mL of the placebo or CE with 30 mL of water. Subjects were provided with brochures with written instructions.

2.4. Assessments and Study Outcomes. The study visits were conducted two weeks before the beginning of the trial, at the beginning of the trial (week 0), and every week up to the end of the three months of treatment. The screening and data collection were performed by the investigators and a medical physician. During the intervention, all participants were examined and checked weekly to ensure that the study instructions were being followed, and that intake of samples was occurring according to the regimen. The occurrence of any probable side effects was recorded by the volunteers.

Body weight loss was the primary study outcome. The secondary outcomes included changes in body composition (body fat, body water, and body muscle percentages), anthropometric indices (BMI, height, waist circumference, hip circumference, mid-upper arm circumference, and thigh circumference), serum lipid profile, urine-specific gravity, systolic and diastolic blood pressure, and pulse rate. Safety outcomes also included laboratory assessments and vital signs. In addition, the occurrence of adverse events which might be related to the treatment was identified by the investigator and the physician through physical examination. All measurements were assessed early in the morning with an empty stomach and were performed at baseline and at week 12.

Body weight was measured within 0.1 kg intervals. Participants were weighed in light clothing and without shoes using a bioelectrical impedance analysis (BIA) machine with remote control (Beurer digital diagnostic scale, Model BG63, Ulm, Germany). Other body composition parameters including percentages of body fat (%BF), body water (%BW), and body muscle (%BM) were displayed with 0.1% graduation. BMI (kg/m^2) was calculated based on the folowing formula BMI = weight/height^2.

Anthropometric indices including height, waist circumference (WC), and hip circumference were measured to the nearest 0.1 cm, using Seca measuring tape. The waist circumference was measured by placing the measuring tape at the umbilicus point (the site between the lowest rib and the iliac crest); hip circumference (HC) was measured at the maximum circumference over the buttocks (WHO, 2008). Waist-to-hip ratio (WHR) was then calculated by dividing the waist and hip circumferences.

2.5. Clinical Assessments. Vital parameters, including blood pressure and heart rate, were measured by a physician using a calibrated mercury sphygmomanometer, stethoscope, and appropriate cuff sizes on the sitting subject's right arm after a 10 min rest. Systolic, as well as diastolic, blood pressure was defined according to phase I and phase V Korotkoff sounds, respectively. Blood and urine tests were conducted at the reference laboratory of Shahid Sadoughi Hospital in Yazd, Iran in the fasting condition. The biochemical parameters were analysed using the ELITech diagnostics kits (ELITech Group, Puteaux, France).

2.6. Sample Size Calculation. The required sample size was calculated using the sample size formula described by Greenberg et al. [34] with 99% level of confidence, 1% precision, and with a power level of 90%. The primary variable was weight, and the sample size was based on a two-tailed t test. The standard deviation of weight in the study population was anticipated to be 14 kg, which is similar to the weight measurements obtained from previous studies. According to this formula, a total sample of 60 subjects (30 subjects in each group) was required. To enhance the power for identifying significant differences in weight loss of participants from baseline compared to the control group and assuming dropouts and loss to followup during the three-month study intervention period, 10 extra patients were randomized and included. Hence, a total of seventy (70) overweight and obese women with BMI > 25 were recruited for this study.

2.7. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis. The phytochemical constituents present in CE were identified using GC-MS analysis with flame ionization detector (FID) and extracted by HS-SPME with subsequent hexane extraction. The capillary gas chromatographic profiles of the CE constituents were reported as their retention time compared with the MS of standard compounds [35].

2.8. Statistical Analysis. Values for each subject were standardized for each dependent variable to remove outliers using Z-scores, and the normal distribution was tested using the Kolmogorov-Smirnov test. Student's t-test, with a 99% confidence interval, was applied to identify the significant differences in values between groups, and the paired t-test was used to examine mean differences within each group during the three-month treatment period [36]. All statistical analyses were performed using SPSS software version 18.0.0 (SPSS Inc., Chicago, IL, USA), and all data are expressed as mean ± standard deviation (SD); P values less than 0.01 were considered to be significant and equal variances were assumed.

3. Results

3.1. Demographic and Baseline Features of the Subjects. Of the 110 overweight women who originally registered for screening, seventy were deemed eligible to have met the study requirements and constraints. The selected subjects were randomised and assigned equally to the CE and placebo groups. Of the selected overweight women, ten of the subjects—six in the placebo group and four in the CE group-failed to complete the study. At the termination of the study, therefore, sixty of the seventy patients completed the full three months of treatment. The demographic characteristics of the subjects in the study are summarized in Table 1. About 54% of the subjects were overweight and 46% were obese. The mean (SD)

Table 1: Demographics of study participants randomized to the placebo or CE groups ($n = 35$).

Variables	Placebo Mean ± SD	CE Mean ± SD	P value
Age (years)	37.00 ± 7.90	37.23 ± 9.34	0.91
Height (cm)	158.20 ± 4.90	159.74 ± 6.22	0.25
Weight (kg)	74.88 ± 11.70	75.99 ± 11.84	0.70
Body mass index (BMI, kg/m^2)	30.39 ± 4.69	29.24 ± 3.36	0.24
Bone mass (kg)	7.79 ± 1.11	8.05 ± 1.07	0.35
Sleep (hours/day)	7.87 ± 1.61	7.86 ± 1.29	0.97
Physical activity level (PAL, kcal/kg/day)	43.68 ± 2.48	44.39 ± 2.79	0.26
Basic metabolic rate (BMR, kcal/m^2/hour)	1474.42 ± 123.46	1488.00 ± 154.43	0.69
Active metabolic rate (AMR, kcal/m^2/hour)	2241.42 ± 216.27	2176.57 ± 260.47	0.30
Resting energy expenditure (REE, kcal)	1453.21 ± 133.26	1503.11 ± 127.37	0.15
Total daily energy expenditure (TDEE, kcal)	2236.06 ± 206.93	2308.33 ± 193.17	0.17

Table 2: Measured variables (mean ± SD) at baseline and after the three-month intervention period.

Variables	Week 0		Week 12	
	Placebo ($n = 29$)	CE group ($n = 31$)	Placebo ($n = 29$)	CE group ($n = 31$)
Body composition				
Weight (kg)	71.96 ± 10.66	76.86 ± 12.24	72.77 ± 10.84	75.0 ± 12.24*
Body mass index (BMI, Kg/m^2)	28.34 ± 2.59	30.69 ± 4.69	28.50 ± 2.80	29.85 ± 4.70*
Body fat (BF, %)	33.82 ± 2.40	35.43 ± 3.60	34.04 ± 2.47	34.74 ± 3.74*
Body muscle (BM, %)	31.81 ± 1.27	31.42 ± 1.60	31.75 ± 1.29	31.61 ± 1.60*
Body water (BW, %)	48.34 ± 1.89	47.16 ± 2.63	48.12 ± 1.78	47.15 ± 2.67
Anthropometric indices				
Waist circumference (WC, cm)	91.34 ± 7.33	96.02 ± 10.21	91.21 ± 7.90	89.78 ± 8.64*
Waist-to-hip ratio (WHR)	0.87 ± 0.04	0.86 ± 0.06	0.87 ± 0.05	0.83 ± 0.05*
Paraclinical assessments				
Diastolic blood pressure (DBP, mmHg)	74.29 ± 6.0	75.48 ± 7.89	70.97 ± 7.60	75.9 ± 6.80
Systolic blood pressure (SBP, mmHg)	111.25 ± 10.33	112.74 ± 10.40	111.25 ± 9.49	113.39 ± 11.21
Heart rate (beats per minute)	75.21 ± 8.70	78.06 ± 9.11	74.46 ± 8.56	77.51 ± 8.11
Lipid profile				
Cholesterol (mg/dL)	183.33 ± 22.56	209.33 ± 29.87	190.38 ± 51.9	199.0 ± 25.1
Triglyceride (TG, mg/dL)	121.86 ± 41.49	112.81 ± 35.14	145 ± 50.4	124.43 ± 42.6
High density lipoprotein (HDL, mg/dL)	52.95 ± 9.87	55.90 ± 9.57	51.71 ± 7.7	56.71 ± 10.1
Low density lipoprotein (LDL, mg/dL)	106.73 ± 17.67	123.94 ± 28.65	110.77 ± 41.9	125.77 ± 25.9
Urine test				
Urine-specific gravity (USG, g/mL)	1.017 ± 0.006	1.021 ± 0.006	1.018 ± 0.005	1.022 ± 0.006

* $p < 0.01$ significantly different from baseline compared to placebo.

age, body weight, and BMI of the participants were 37.11 (8.6) years, 75.43 (11.7) kg, and 29.82 (4.1) kg/m^2, respectively. The average physical activity level of the participants was 44 (2.6) (kcal/kg/day) with 7.86 (1.4) hours of sleep. No significant differences of all of these features were observed between the two groups. At baseline, there were no significant differences in the body composition, anthropometric indices, clinical and para-clinical assessments of both groups, except for the waist circumference which was at the borderline. All of the participants had abdominal obesity (waist circumference > 88 cm) (Table 2).

3.2. Comparison within and between Groups during the Three-Month Trial. The changes in variables over the three-month intervention period for the CE and the placebo groups are also shown in Table 2. Significant mean weight loss was observed within the CE group after three months of treatment, whereas the average weight in the placebo group was increased. The mean weight loss between the CE group and placebo group was significant. The mean weight in the CE group dropped remarkably as compared with the placebo group. Therefore, this traditional medicine extract probably has a positive effect on lowering body weight. Similarly,

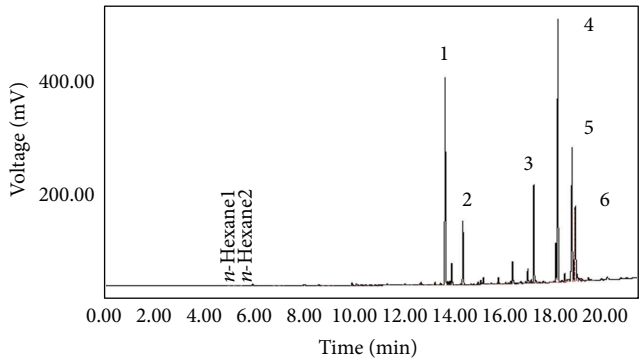

Figure 1: Chromatogram of CE infusion extracted by HS-SPME. Identification of CE volatiles obtained by steam distillation with subsequent hexane extraction, including (1) limonene, (2) γ-terpinene, (3) *trans*-carveol, (4) carvone, (5) thymol, and (6) carvacrol.

the average BMI and %BF in the CE group were significantly decreased. In contrast, in the placebo group, these values increased slightly. Nevertheless, the mean reduction in the BMI and %BF was significant between the CE group and the placebo group.

The percentage of body muscle in the CE group showed a significant increase after intervention. Body water percentage decreased in both groups and was not significant either within or between groups. On the other hand, the WC and WHR were reduced significantly only in the CE group. However, the level of reduction (cm) in all of the anthropometric indices was remarkable between groups. There were no noticeable changes in para-clinical and clinical assessments with either treatment. According to the outcomes of the study, CE showed greater efficacy than did placebo for each primary outcome measure.

3.3. Safety Issues and Adverse Events. No significant changes were observed for heart rate, systolic and diastolic blood pressure, lipid profile, and urine-specific gravity between and within the two groups during the three-month study period. Of the sixty subjects who completed the study, only the placebo participants experienced skin allergy to the placebo product, and no important adverse events were reported during the physical examinations.

3.4. Detection of Phytochemicals Using GC-MS. The principal volatile compounds analyzed by GC-MS following extraction were mostly monoterpenoids, as shown in the chromatogram in Figure 1.

4. Discussion

The weight-lowering property of caraway as a known medicinal plant in Iran was examined in a triple-blind, placebo-controlled, clinical trial in Iranian overweight and obese women. Since diet and physical activity are the two lifestyle principles which induce normal weight, subjects were selected who were regularly performing aerobic exercises during the entire period of study without modifying their diet and lifestyle habits. The results indicated a moderate effect of CE on losing weight, without any severe adverse effects. This finding is consistent with a recent study which reported data of antiobesity effect of this plant in an animal model [33]. Additionally, numerous studies have reported the therapeutic effects of caraway on different diseases such as diabetes mellitus, cardiovascular disease (CVD), and hypertension, which are known as common complications of obesity [37–39]. Altogether, the results of this study suggest a plausible phytotherapeutic approach for the use of caraway seed extract in the management of obesity.

Lowering weight and fat in the subjects in this study may be related to anti-microbial, anti-inflammatory, and antioxidant activities of caraway caused by some of the constituents in caraway, such as carvacrol (polyphenol) and unsaturated fatty acids (UFA) (Figure 2) [19]. These bioactive compounds may balance gut microflora (GM) which help in food digestion and absorption providing intestinal homoeostasis [40]. GM modulates gene expression in the human body involving the host physiology and metabolism, such as obesity mechanisms [41]. Carvacrol, together with UFA, inhibits the growth of pathogenic bacteria, and thus increases the proliferation of GM [42, 43]. In this process, ingredients probably modify GM through activating the expression of some specific genes involved in lipid metabolism inhibiting inflammation and adipogenesis [33, 44]. The balanced GM also inhibits infiltration of macrophages into obese adipose tissues leading to disruption in the conversion of preadipocytes to mature adipocytes, thereby preventing adipocyte differentiation and adipogenesis [45]. UFAs enhance the oxidation of fatty acids leading to lipolysis and fat loss [46, 47]. Caraway constituents also stimulate apoptosis in pre-adipocytes due to their antioxidant activity. They reduce adipose tissue mass through preventing adipogenesis and enhancing lipolysis in adipocytes [10, 48–50]. Further studies are suggested to investigate the effects of these potent components in reversing obesity in overweight and obese women at the molecular level.

In this study, no changes were observed in the subjects' body water during the intervention, whereas body weight and fat mass were decreased, and muscle mass was increased. However, there were also significant changes in body composition in the placebo group, which shows that exercise did not have any interfering and/or synergistic effect on weight and fat loss. This implies that the favourable changes in body composition were probably associated with the bioactive compounds in CE and not necessarily with the physical activity, although it is recognized that there is likely a synergistic effect of exercise on weight and fat loss in the treatment group. It is plausible that the bioproducts formed during lipolysis were converted into muscle induced by physical activity, synergistically, and that the decrease in fat mass and the increase in lean mass are feasibly due to physiological adaptations to exercise [51–53].

To the best of our knowledge, this is the first clinical study to evaluate the effects of CE intake on body composition and anthropometric indices, combined with an exercise program, and examin to antiobesity effects of CE on overweight and obese women during a twelve-week intervention. In addition,

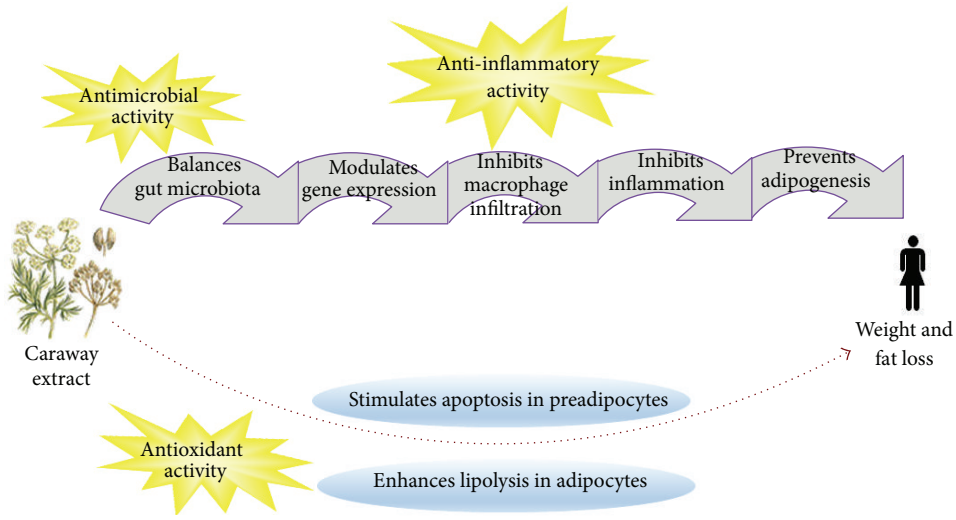

Figure 2: The possible metabolic actions of CE on the human body during weight loss.

this study had three other significant aspects. Firstly, even though the subjects' dietary habits were not modified, a significant weight loss was observed in the CE group, as compared with the placebo group. Secondly, this study was a triple-blind clinical trial which enhances the accuracy of the results and reduces potential bias in the findings. Some limitations of this study should be acknowledged. Firstly, these results are limited to an extract of caraway; therefore, further studies are required to find the anti-obesity effect of other methods of preparation for caraway seed oil extract. Secondly, as physical activity might have a synergistic effect on lowering weight [54], replicating this study on subjects without exercise is recommended. Thirdly, since this study was performed with adult females, in order to have a homogenous population and more reliable data, studies on the anti-obesity effects of CE in males, as well as in obese children, are suggested. Fourthly, in this study, obese or overweight subjects with medical complications, such as metabolic syndrome and cardiovascular disease (CVD), were specifically excluded. Future studies to examine the weight-lowering effects of caraway on overweight and obese patients having obesity complications are proposed. Finally, further studies are suggested to examine these results with different CE doses in order to establish more accurate dosing limitations.

5. Conclusions

From the above results and discussion, it can be concluded that caraway is helpful in the management of obesity because of its bioactive constituents. Although the mechanism of action of the active principle(s) remains to be determined at the molecular level, it is speculated to arise from a prebiotic effect of CE in the gut through balancing its GM growth. Efforts to provide information and understanding about the human use of this medicinal plant and make to the intake of caraway-containing natural and bioactive pharmaceuticals a sustainable dietary practice, along with physical activity, towards a healthy lifestyle, should be continued.

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Research Article

Inhibition of Acute Phase Inflammation by *Laminaria japonica* through Regulation of iNOS-NF-κB Pathway

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Laminaria japonica has been frequently used as food supplements in many of the Asian countries and as a drug in traditional oriental medicine. This research investigated the effects of *Laminaria japonica* extract (LJE) on acute phase inflammation in a carrageenan-induced paw edema model, as assessed by histomorphometric and immunohistochemical analyses. The effect of LJE was also evaluated in Raw264.7 cells stimulated with lipopolysaccharide (LPS) in the aspect of the inhibition of nitric oxide (NO), prostaglandin E_2 (PGE_2), and proinflammatory cytokines production. NO, PGE_2, tumor necrosis factor (TNF)-α, interleukin-1β, and interleukin-6 contents were assayed by ELISA, and inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 expressions were determined by western blot analyses. In rats, LJE treatment inhibited carrageenan-induced paw edema formation and infiltration of inflammatory cells in H&E staining. LJE treatment prevented the ability of LPS to increase the levels of iNOS and COX-2 protein in a concentration-dependent manner. Consistently, LJE suppressed the production of TNF-α, interleukin-1β, and interleukin-6. Treatment of the cells with LJE caused inhibition of inhibitor of κBα phosphorylation induced by LPS, suggesting LJE repression of nuclear factor-κB activity by LPS. In conclusion, this study shown here may be of help to understand the action mechanism of LJE and the anti-inflammatory use of *L. japonica*.

1. Introduction

Over the last few decades, natural products has been known to have beneficial effect to human beings. Some traditional food sources (especially seaweeds) contain high level of essentials for the body, and therefore, are able to maintain good heath by providing various nutrients [1]. *Laminaria japonica* is one of the most famous seaweeds called kombu (Japanese), dashima (Korean), or haidai (Chinese) and commonly consumed in Asian countries such as Republic of Korea, Japan, and China. In these countries, *L. japonica* is widely used as food supplements as well as a drug having effects on treating tumor, relieving phlegm, and regulating urine in traditional oriental medicine. Although it has been used for more than 1000 years as a drug in traditional medicine, the mechanism of beneficial effects was not clearly demonstrated.

Inflammation is a complex biological host response to harmful stimuli and is characterized by the classic signs such as redness, swelling, heat, and pain. A variety of studies has shown that a chronic or acute inflammatory state is closely associated with the pathogenesis of various disorders such as vascular disease, metabolic disease, obstructive pulmonary disease, infectious diseases, and cancer [2–6]. In these inflammatory processes, cytokines have important roles in progression of pathological states such as edema, intra-/intercellular stress, and tissue necrosis [7]. In particular, the proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 and the pro-inflammatory enzymes including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 have been widely accepted with involvement of promoting inflammatory processes [8, 9].

In this study, we investigated the effects of the ethanol extract of *L. japonica* (LJE) as a novel anti-inflammatory candidate to inhibit paw edema formation in a rat model of acute inflammation. Furthermore, this study identifies LJE as a component with the inhibiting effects on the NO, prostaglandin E_2 $(PGE)_2$, and proinflammatory cytokines in

macrophages treated with LPS. In terms of wide applications of *L. japonica* (i.e., food or drugs) and its therapeutic potential, the findings presented here demonstrate the important pharmacology of *L. japonica* and offer the possibility of its therapeutic applications for inflammatory diseases.

2. Materials and Methods

2.1. Preparation of the Ethanol Extract of Laminaria japonica. *L. japonica* was purchased from Daewon pharmacy (Daegu, Republic of Korea). The ethanol extract of *L. japonica* (LJE) was prepared by extracting 200 g of *L. japonica* in 1 L of 100% ethanol for 72 h. The ethanol extracts were filtered through a 0.2 μm filter (Nalgene, New York, NY, USA), lyophilized, and stored at −20°C until use. The amount of LJE was estimated by the dried weight of lyophilized LJE. The yield of lyophilized LJE was 1.19%.

2.2. Analysis of LJE. LJE was analyzed by gas chromatography (GC) and mass spectrometry (MS) (Hewlett-Packard 6890N GC/MS, Palo Alto, CA, USA). The system is equipped with a HP-5MS column (30 m × 0.25 mm). The analyses were performed at 70°C (an initial temperature) and ramped with 2°C/min to 100°C (a final temperature) after equilibration. Injector temperature was 280°C. Carrier gas was helium with a flow of 1 mL/min. From the scanning data of the LJE profiling by GC and MS, we assessed the contents of the three marker components, palmitic acid, myristic acid, and oleic acid. For the analysis of oleic acid, the UPLC (ultra performance liquid chromatography) system (Waters, USA), which was equipped with a pump Waters ACQUITY ultra performance LC system (USA) and a Waters ACQUITY photodiode array detector (PDA), was used. The detection UV wavelength was set at 203 nm. For the palmitic acid and myristic acid, we used GC/MS (i.e., contents of three components in LJE were palmitic acid (89.35 μg/mL); myristic acid (52.05 μg/mL); oleic acid (13.8 μg/mL)).

2.3. Materials. Horseradish peroxidase-conjugated goat anti-rabbit, anti-mouse, and anti-goat IgGs were purchased from KPL (Gaithersburg, MD, USA). Anti-phospho-I-κBα and anti-COX-2 antibodies were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell signaling (Beverly, MA, USA), respectively. Antimurine iNOS antiserum was purchased from Transduction Laboratories (Lexington, KY, USA). Polyethylene glycol #400 (PEG) solution was obtained from yakuri Pure Chemical Co. (Kyoto, Japan). Carrageenan, dexamethasone, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Carrageenan-Induced Paw Edema. Animal studies were conducted in accordance with the institutional guidelines for care and use of laboratory animals [10]. Sprague-Dawley rats at 6 weeks of age (male, 140–160 g) were provided from Samtako Co. (Osan, Republic of Korea), acclimatized for 1 week, and maintained in a clean room at the Animal Center for Pharmaceutical Research, College of Oriental Medicine, Daegu Haany University. Animals were caged under the supply of filtered pathogen-free air, commercial rat chow (Purina, Republic of Korea), and water *ad libitum* at a temperature between 20 and 23°C with 12 h light and dark cycles and relative humidity of 50%. Rats ($N = 24$) were randomly divided into four groups, and thus, each group consisted of six animals. LJE, dissolved in 40% PEG, was orally administered to rats at the dose of 0.1 g or 0.3 g kg^{-1} day^{-1} for 3 consecutive days. Dexamethasone, an anti-inflammatory drug, was used as a positive control [11]. To induce acute phase inflammation in paw, rats were injected subcutaneously into the hind paw with a 1% solution of carrageenan dissolved in saline after vehicle or LJE treatment. The paw volumes were measured up to 4 h after the injection at intervals of 1 h. The hind paw volume was determined volumetrically by measuring with a plethysmometer (Letica, Rochester, MI, USA).

2.5. Histological Process. The hind paw skins—*dorsum* and *ventrum pedis* skins—were separated and fixed in 10% neutral buffered formalin, then, embedded in paraffin, sectioned (3~4 μm), and stained with hematoxylin and eosin (H&E), and after that the histopathological profiles of each sample were observed under light microscope (Nikon, Japan) [12].

2.6. Histomorphometry. The thicknesses of *dorsum pedis* and *ventrum pedis* skins (from epidermis to dermis; keratin layers were excluded) were measured using automated image analyzer (DMI-300 Image Processing; DMI, Korea) under magnification 40 of microscopy (Nikon, Japan) at prepared skin histological samples as mm paw^{-1}, and the infiltrated inflammatory cells were also counted using automated image analyzer as cells mm^{-2} of histological fields under magnification 200 of microscopy according to Kim et al. (2006) and some modifications [12].

2.7. Cell Culture. Raw264.7 cell, a murine macrophage cell line, was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For all experiments, the cells were grown to 80–90% confluency and were subjected to no more than 20 cell passages. Raw264.7 cells were incubated with 1 μg mL^{-1} LPS (*Escherichia coli* 026:B6; Sigma, St. Louis, MO, USA). The cells were incubated in the medium without 10% FBS for 12 h and then exposed to LPS or LPS + LJE for the indicated time periods. LJE being dissolved in dimethylsulfoxide was added to the incubation medium 1 h prior to the addition of LPS.

2.8. MTT Cell Viability Assay. The cells were plated at a density of 5×10^4 cells per well in a 96-well plate to determine any potential cytotoxicity [13]. Cells were serum-starved for 12 h and then were treated with LJE for the next 24 h.

2.9. Assay of Nitrite Production. NO production was monitored by measuring the nitrite content in culture media. This was performed by mixing the samples with Griess

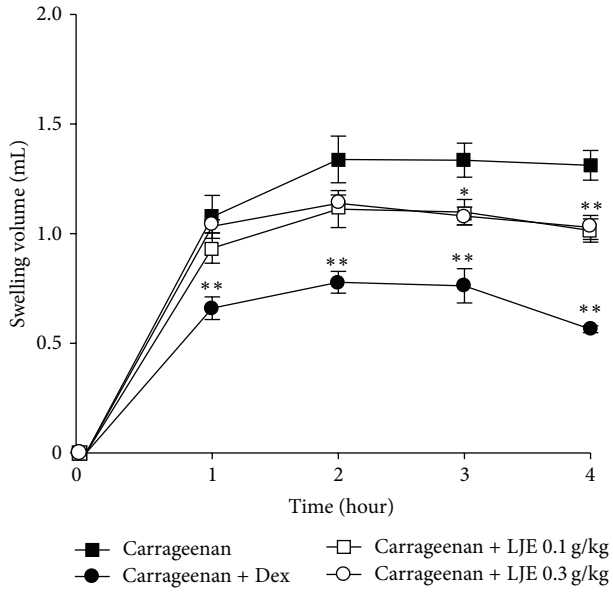

Figure 1: Inhibition of carrageenan-induced paw edema formation by the ethanol extract of *Laminaria japonica* (LJE). LJE was administered for 3 days to rats at the oral dose of 0.1 or 0.3 g kg^{-1} day^{-1} prior to the induction of paw edema. Paw edema was induced by subcutaneously injecting a 1% solution of carrageenan dissolved in saline (0.1 mL per animal) into the hind paw. The thickness of the paw was measured before and 1–4 h after carrageenan injection. Data point represents the swelling volume of the paw, which was standardized with the thickness of the paw volume before carrageenan injection. Dexamethasone (Dex, 1 mg kg^{-1}, p.o.) was used as a positive control. Data represents the mean ± S.E.M. of six animals (significant as compared with carrageenan alone, $^{*}P < 0.05$, $^{**}P < 0.01$).

reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 540 nm after incubation for 10 min.

2.10. Immunoblot Analysis. Cells were lysed in the buffer containing 20 mM Tris·HCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 μg mL^{-1} leupeptin [14]. Proteins of interest were visualized using ECL chemiluminescence detection kit. Equal loading of proteins was verified by actin immunoblottings. At least three separate experiments were performed to confirm changes.

2.11. Enzyme-Linked Immunosorbent Assay (ELISA). Raw264.7 cells were preincubated with LJE for 1 h and continuously incubated with LPS for 24 h. Prostaglandin E_2 (PGE_2), TNF-α, IL-1β, and IL-6 contents in the culture media were measured by ELISA using anti-mouse PGE_2, TNF-α, IL-1β, or IL-6 antibodies and biotinylated secondary antibody according to the manufacturer's instruction (Endogen, Woburn, MA, USA).

2.12. Scanning Densitometry. Scanning densitometry of the immunoblots was performed with an Image Scan & Analysis System (Alpha-Innotech, San Leandro, CA, USA). The area of each lane was integrated using the software AlphaEase version 5.5 (Alpha-Innotech) followed by background subtraction.

2.13. Statistical Analysis. For animal experiments, multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtained data were analyzed by one way ANOVA test followed least significant difference (LSD) test, Kruskal-Wallis *H* test or the Mann-Whitney *U* (MW) test. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, SPSS Inc., USA). Differences were considered significant at $P < 0.05$. In addition, the changes between carageenan control and test material administered groups were also calculated to help the understanding of the efficacy of test material as follows: Percentage Changes Compared with carageenan Control (%) = ((Data of test material treated groups − Data of carageenan control)/(Data of carageenan control) × 100). For cell experiments, one-way analysis of variance (ANOVA) was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data were expressed as means ± 95% confidence intervals (CI). All statistical tests were two-sided.

3. Results

3.1. Inhibitory Effect of LJE on Carrageenan-Induced Paw Edema. To determine inhibitory effects of LJE on acute inflammation, we used the carrageenan-induced paw edema model because this model is widely employed for screening the effects of anti-inflammatory drugs [15].

Because it has been shown that the injection of carrageenan induces acute inflammatory responses including paw swelling and increases in neutrophil infiltration, we focused in this study on the anti-inflammatory effects of LJE in the paw injected with carrageenan. We found that paw edema formation began to be observed as early as 1 h after a carrageenan injection and that the edema induction reached a maximum at 2 h after treatment (Figure 1). Inflammatory paw swellings persisted at least up to 4 h after treatment. Oral administration of LJE at the dose of 0.1 or 0.3 g kg^{-1} day^{-1} to rats significantly blocked the carrageenan-induced paw swellings. We confirmed that dexamethasone treatment (1 mg kg^{-1} day^{-1}, p.o.), a positive control, also effectively decreased edema formation.

3.2. Inhibitory Effect of LJE on Acute Inflammation in Rats. In addition, we determined the effects of LJE on histological profiles of *dorsum pedis* and the *ventrum pedis* skin (Figures 2 and 3). The histomorphometrical measurements of the *dorsum pedis* and *ventrum pedis* skin were listed in Table 1. In the present study, marked increases of infiltrated

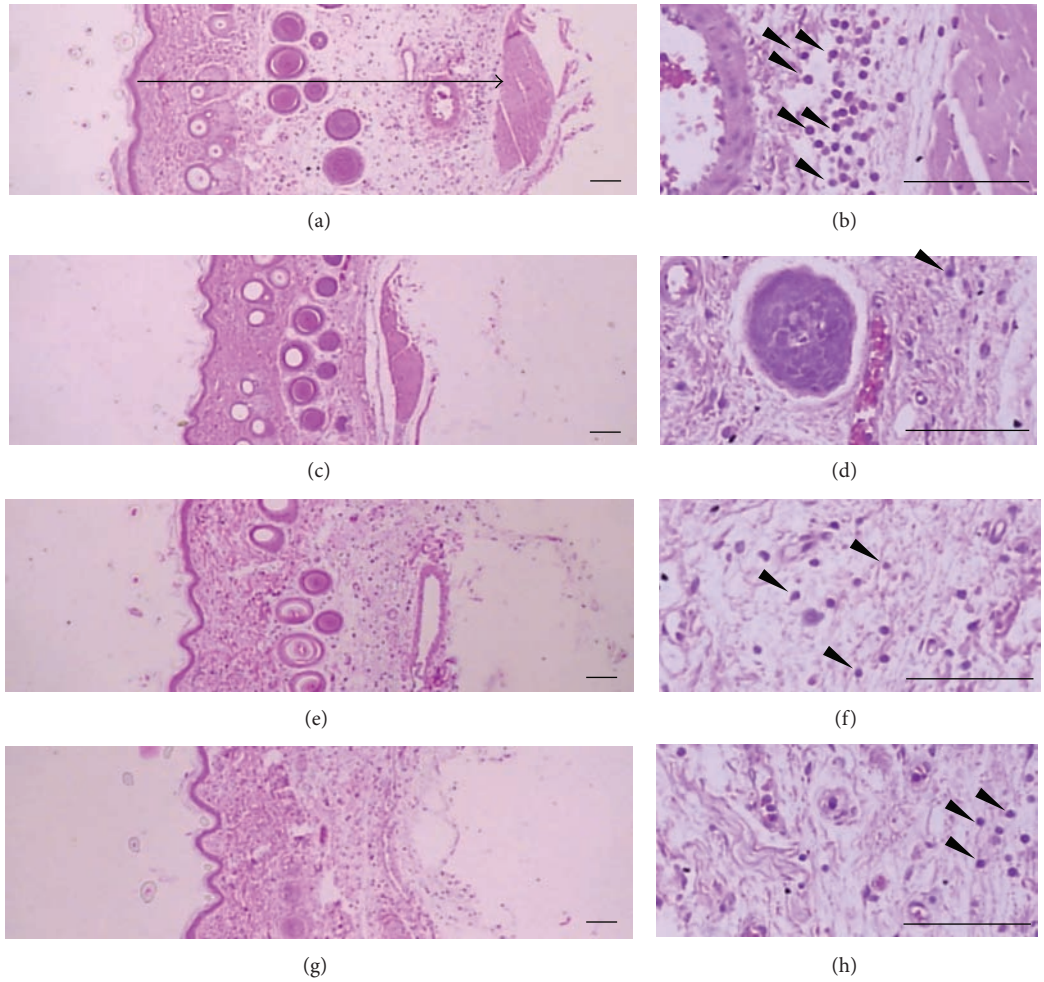

Figure 2: Changes in histological profiles of the *dorsum pedis* skin in carrageenan control (a, b), dexamethasone (c, d), LJE 0.1 g kg^{-1} day^{-1} (e, f), and LJE 0.3 g kg^{-1} day^{-1} (g, h) treated groups. Note that marked increases of skin thicknesses due to edematous changes were detected by carrageenan treatment with increases of inflammatory cell infiltrations. However, these increases of skin thicknesses and inflammatory cell infiltrations were effectively inhibited by treatment with dexamethasone and with two different dosages of LJE 0.1 g kg^{-1} day^{-1} and 0.3 g kg^{-1} day^{-1}, respectively. Arrow indicates total thicknesses measured. Arrow heads showed infiltrated inflammatory cells. All H&E stain; scale bars = 160 μm.

inflammatory cells and of skin thicknesses on both *dorsum* and *ventrum pedis* were detected by treatment of carrageenan (Figures 2 and 3). However, these carrageenan-induced acute edematous inflammatory changes were significantly ($P < 0.01$) inhibited by treatment with both different dosages of LJE and dexamethasone (Table 1). The thickness of *dorsum pedis* skin in dexamethasone and LJE 0.1 and 0.3 g kg^{-1} day^{-1} treated groups was changed as −55.74, −26.07, and −34.24% as compared with carrageenan control, respectively. The thickness of *ventrum pedis* skin in dexamethasone and LJE 0.1 and 0.3 g kg^{-1} day^{-1} treated groups was changed as −30.28, −11.19, and −22.28% as compared with carrageenan control, respectively. The number of infiltrated inflammatory cells on the *dorsum pedis* skin in dexamethasone and LJE 0.1 and 0.3 g kg^{-1} day^{-1} treated groups was changed as −79.73, −47.30, and −54.05% as compared with carrageenan control, respectively. The number of infiltrated inflammatory cells on the *ventrum pedis* skin in dexamethasone and LJE 0.1 and 0.3 g kg^{-1} day^{-1} treated groups was changed as −92.45, −68.09, and −88.81% as compared with carrageenan control, respectively. These results suggest that LJE prevents the acute phase of swelling implicated with inflammation *in vivo*.

3.3. Inhibition Effect of LJE on NO and PGE$_2$ Induced by LPS Treatment. Next, we verified the anti-inflammatory effect of LJE *in vitro*. We determined any possible toxicity of LJE in Raw264.7 cells. MTT assay indicated that cell viability was not affected by LJE treatment up to 100 μg mL^{-1} (Figure 4(a)). Moreover, LJE restored LPS-inducible cell toxicity (Figure 4(b)). 10–100 μg mL^{-1} concentrations of LJE were chosen to determine the effect of LJE on NO and PGE$_2$ production in Raw264.7 cells. Production of the NO and PGE$_2$ was measured in the media of Raw264.7 cells treated with LPS with or without LJE as described in Section 2. LPS treatment (1 μg mL^{-1}, for 24 h) increased NO production by 2.5-fold, which was markedly deceased by concomitant

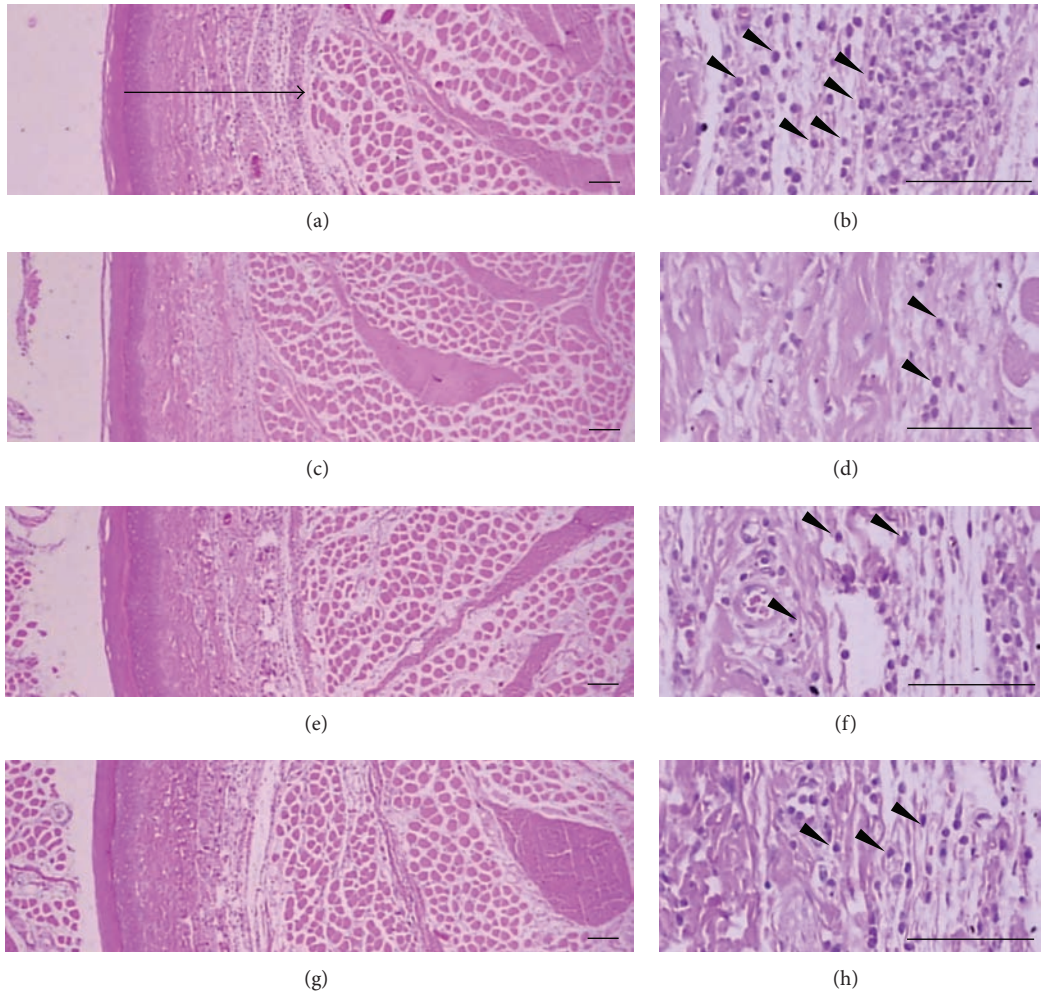

FIGURE 3: Changes in histological profiles of the *ventrum pedis* skin in carrageenan control (a, b), dexamethasone (c, d), LJE 0.1 g kg^{-1} day^{-1} (e, f), and LJE 0.3 g kg^{-1} day^{-1} (g, h) treated groups. Note that marked increases of skin thicknesses due to edematous changes were detected by carrageenan treatment with increases of inflammatory cell infiltrations quite similar to those of *dorsum pedis* skins. However, these increases of skin thicknesses and inflammatory cell infiltrations were effectively inhibited by treatment with dexamethasone and with two different dosages of LJE 0.1 g kg^{-1} day^{-1} and 0.3 g kg^{-1} day^{-1}, respectively. Arrow indicates total thicknesses measured. Arrow heads showed infiltrated inflammatory cells. All H&E stain; scale bars = 160 μm.

treatment with LJE in a concentration-dependent manner (Figure 5(a)). In addition, LJE treatment significantly inhibited PGE_2 production in Raw264.7 cell treated with LPS (Figure 5(b)).

3.4. Inhibition of LPS-Inducible TNF-α, IL-1β, and IL-6 Productions by LJE Treatment. Next, we assessed the effects of LJE on pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Production of the cytokines was measured by using ELISA assays in the media of Raw264.7 cells stimulated by LPS (1 μg mL^{-1}) alone or in combination with LJE. Treatment of the cells with LPS increased the production of the cytokines (Figures 6(a)–6(c)). LJE treatment effectively decreased the production of the cytokines, indicating that the anti-inflammatory effects of LJE might, at least in part, be related to LJE inhibition of LPS-inducible pro-inflammatory cytokines productions.

3.5. Effect of LJE on LPS-Inducible iNOS and COX-2 Expression. iNOS and COX-2 are key enzymes in the aspect of the induction of proinflammatory cytokines. NF-κB is the important transcription factor for the inflammatory genes such as iNOS and COX-2 and is translocated to the nucleus by phosphorylation of I-κBα and subsequent proteolytic degradation of I-κBα subunit [16–19]. We then assessed the protein expression of iNOS and COX-2 by western blotting. Although treatment with LPS significantly induced iNOS and COX-2 expression, LJE treatment (3–30 μg mL^{-1}) prevented the iNOS and COX-2 induction (Figure 7(a)). In particular, LJE treatment at 30 μg mL^{-1} almost completely decreased induction of iNOS expression by LPS, similar to the result using isoliquiritigenin (a known anti-inflammatory flavonoid in licorice) (Figure 7(b)). Moreover, LPS exposure increased phosphorylation of I-κBα, and LJE inhibited LPS-inducible I-κBα phosphorylation (Figure 7(c)). Thus, LJE

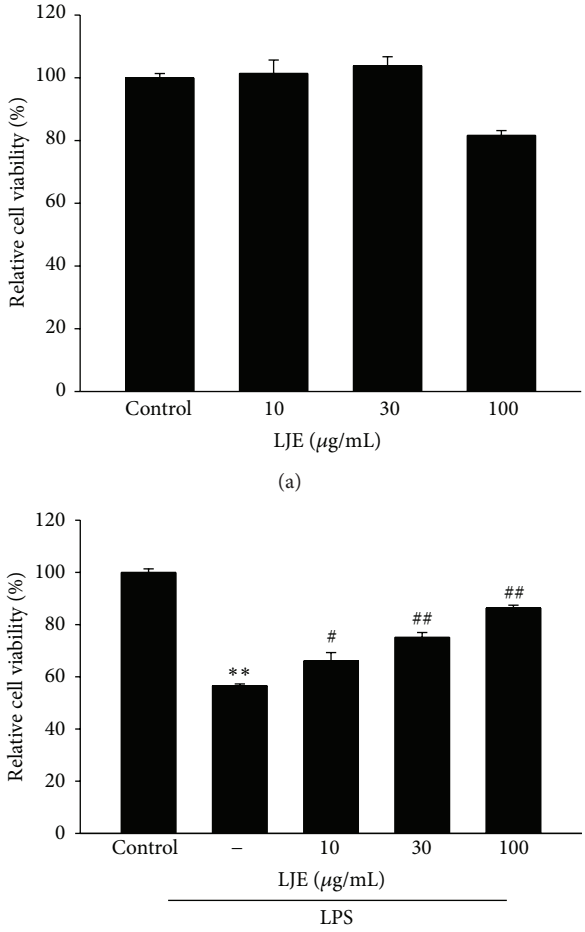

Figure 4: The inhibitory effects of LJE on cell viability. (a) MTT assay. Raw264.7 cells were treated with 10, 30, or 100 μg mL^{-1} LJE. (b) MTT assay. Raw264.7 cells were treated with 10, 30, or 100 μg mL^{-1} LJE for 1 h and continuously incubated with LPS (1 μg mL^{-1}) for 24 h. Data represents the mean ± S.E.M. from three separate experiments (significant as compared with vehicle-treated control, $^{**}P < 0.01$; significant as compared with LPS alone, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

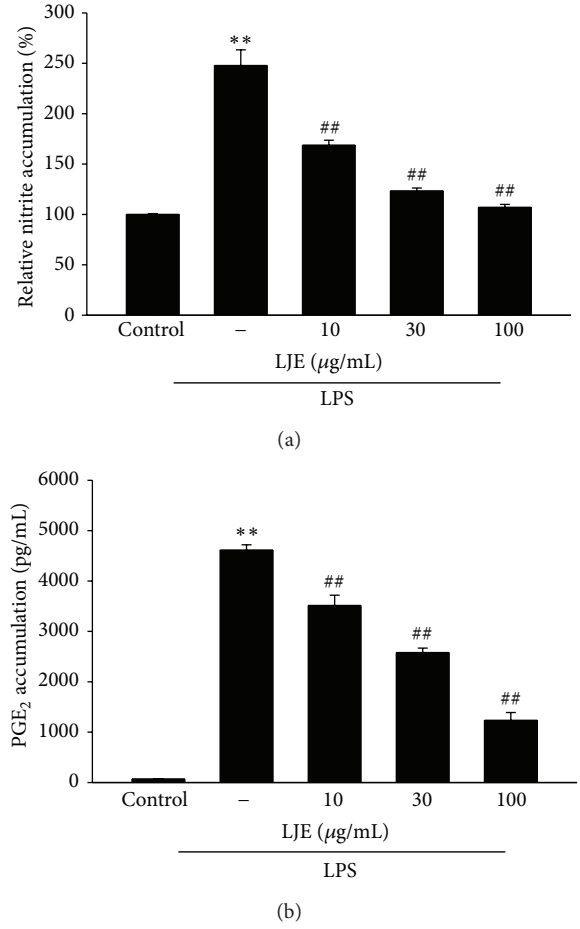

Figure 5: The inhibitory effects of LJE on nitric oxide (NO) and prostaglandin E_2 (PGE_2) induction by LPS. (a) NO and (b) PGE_2 production. Raw264.7 cells were treated with 10, 30, or 100 μg mL^{-1} LJE for 1 h and continuously incubated with LPS (1 μg mL^{-1}) for the next 24 h. NO and PGE_2 concentrations in culture media were monitored, as described in Section 2. Data represents the mean ± S.E.M. from three separate experiments (significant as compared with vehicle-treated control, $^{**}P < 0.01$; significant as compared with LPS alone, $^{\#\#}P < 0.01$).

might prevent iNOS and COX-2 gene induction by inhibiting I-κBα phosphorylation.

4. Discussion

Studies have shown that the extracts of *L. japonica* have multiple bioactivities such as antiallergenic, antioxidant, and anticoagulant effects [20–23]. It has been shown that its ethanol extract has antiallergenic effects and the lipophilic extracts from *L. japonica* have protective effects against oxidant stress [20, 21]. The fucoidan fractions from *L. japonica* have anticoagulant potentials, and the hydrolyzed oligosaccharides from water extract are involved in antiapoptotic activity in mouse thymocytes [22, 23]. Moreover, it is well established that inflammatory and oxidative stress has been implicated in the important factor for the onset of various diseases. Moreover, the pathological process of inflammation may be associated with oxidative stress and the production of free radicals. Therefore, we investigated the effects of LJE on acute inflammation. In this study, we used two different approaches: (1) *in vivo* studies involving paw edema model in rats injected with carrageenan and (2) *in vitro* studies using Raw264.7 murine macrophage treated with LPS.

Swelling is one of the most important symptoms of acute inflammation, which is characterized by an increase in vascular permeability and infiltration of cells. Carrageenan-induced paw edema is commonly used for screening of new anti-inflammatory drug candidates and is a well-established rat model of edema [10]. The intraplantar injection of carrageenan induces inflammatory responses (i.e., increases in paw edema and neutrophil infiltration and development of hyperalgesia) [15]. At the site of inflammation, cytokines have been studied to have an important role in formation of edema by inducing vasodilatation and increasing of vascular

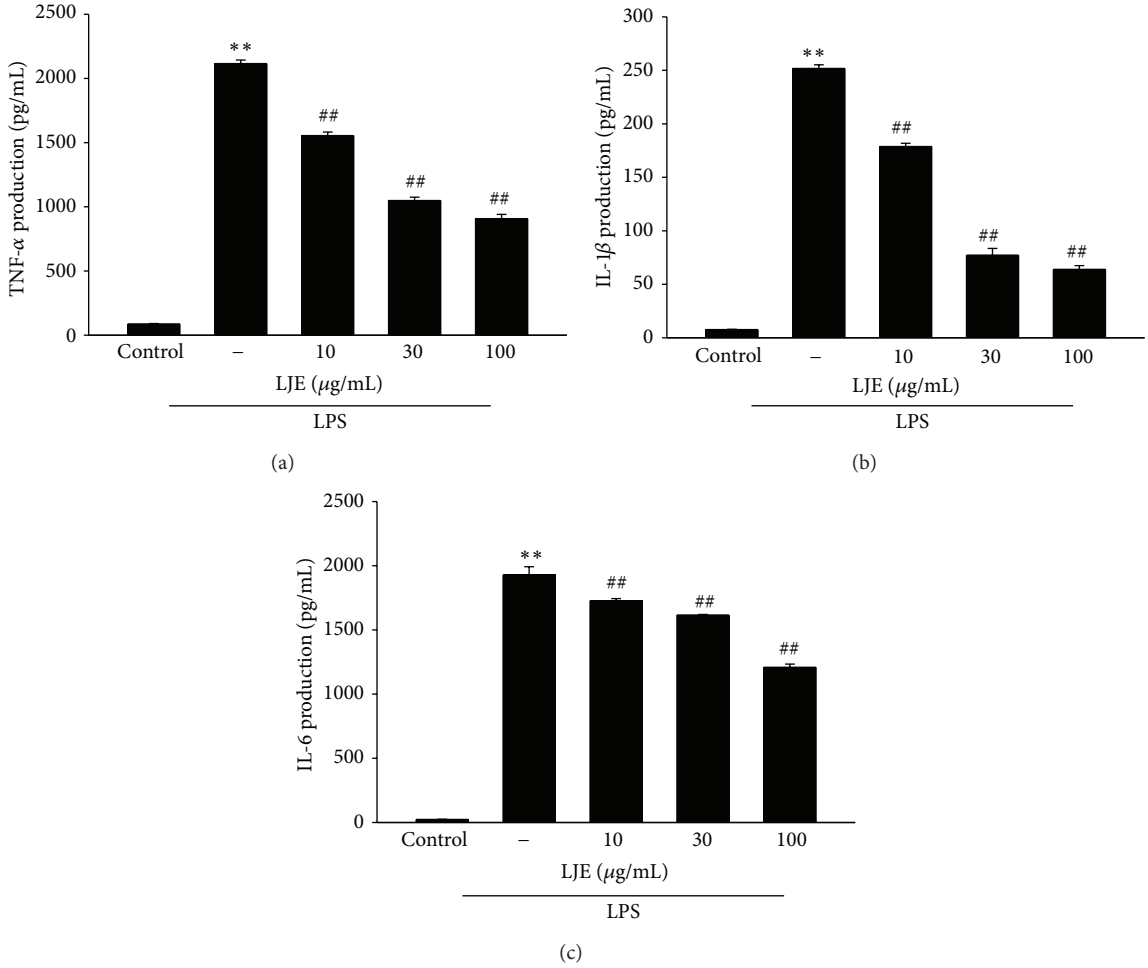

Figure 6: The inhibitory effects of LJE on TNF-α, IL-1β, and IL-6 production by LPS. (a) TNF-α, (b) IL-1β, and (c) IL-6 contents in culture media. Raw264.7 cells were treated with 10, 30, or 100 μg mL^{-1} LJE for 1 h and continuously incubated with LPS (1 μg mL^{-1}) for 24 h. Data represents the mean ± S.E.M. from three separate experiments (significant as compared with vehicle-treated control, $^{**}P < 0.01$; significant as compared with LPS alone, $^{\#\#}P < 0.01$).

Table 1: Changes on the histomorphometrical analysis of hind paw skins in the present study (group summary).

Groups	*Dorsum pedis* skin		*Ventrum pedis* skin	
	Thickness (epidermis to dermis; mm)	Infiltrated inflammatory cells (cells/mm^2 of cutaneous regions)	Thickness (mm)	Infiltrated inflammatory cells (cells/mm^2 of cutaneous regions)
Controls				
Carrageenan	2.424 ± 0.471	118.40 ± 55.25	1.133 ± 0.078	1358.80 ± 332.34
Reference				
Dexamethasone	1.073 ± 0.144[a]	24.00 ± 6.96[c]	0.790 ± 0.136[a]	102.60 ± 38.33[c]
LJE treated as				
0.1	1.792 ± 0.239[a]	62.40 ± 11.15[d]	1.006 ± 0.048[b]	433.60 ± 355.78[d]
0.3	1.594 ± 0.254[a]	54.40 ± 3.98[c]	0.880 ± 0.064[a]	152.00 ± 67.31[c]

Values are expressed as mean ± SD of 5 rat's hind paws.
[a]$P < 0.01$ and [b]$P < 0.05$ compared to carrageenan control by LSD test.
[c]$P < 0.01$ and [d]$P < 0.05$ compared to carrageenan control by MW test.

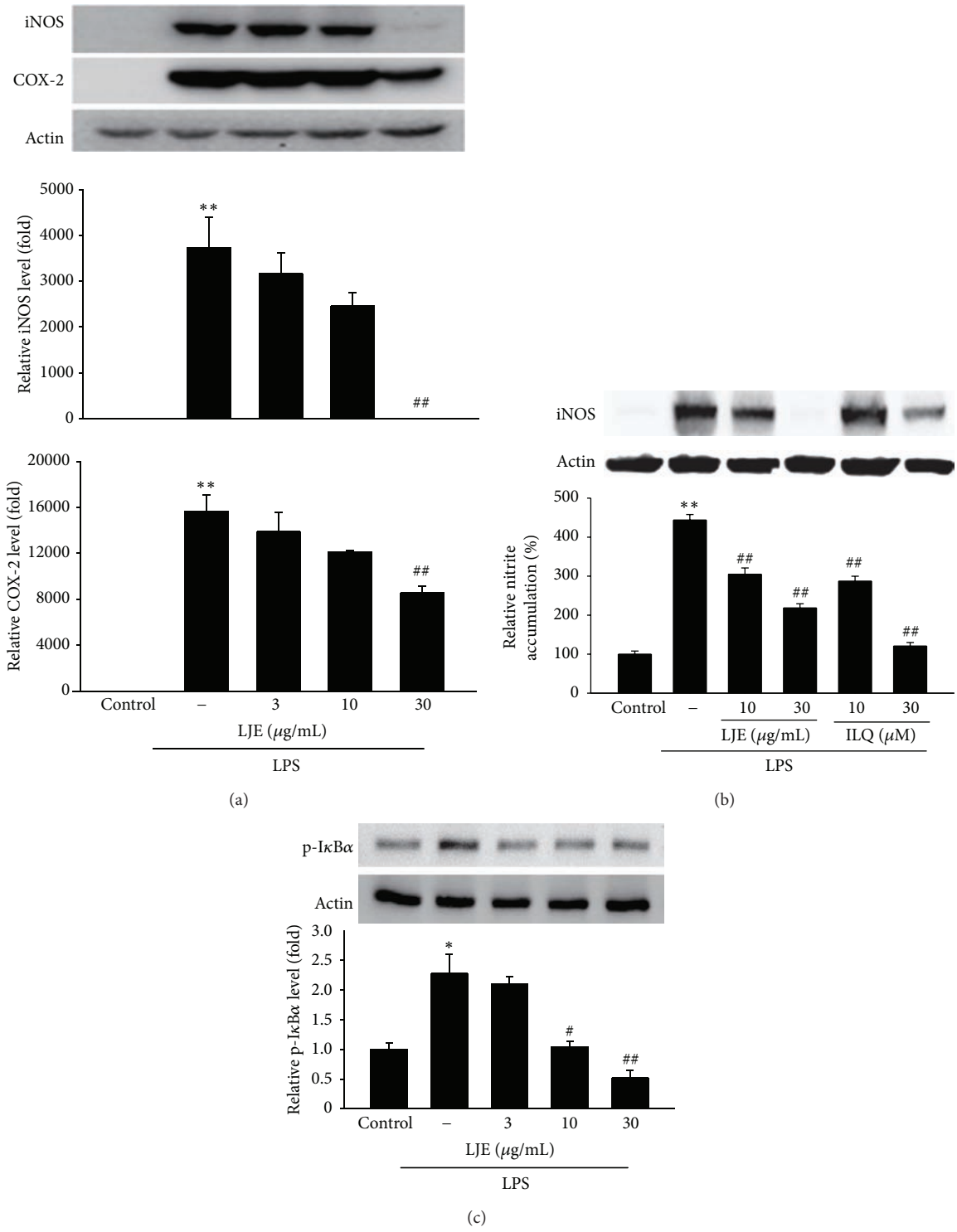

Figure 7: Inhibition of LPS-induced iNOS and COX-2 expression by LJE. (a) Immunoblottings for iNOS and COX-2. iNOS or COX-2 protein levels were monitored 12 h after treatment with LPS (1 μg mL^{-1}) alone or in combination with LJE (3, 10, or 30 μg mL^{-1}). (b) Inhibition of LPS-induced iNOS expression and NO induction by LJE and isoliquiritigenin (ILQ). iNOS protein levels and NO concentration were monitored as described in legends of Figures 7(a) and 5(a), respectively. (c) Immunoblottings for phosphorylated I-κBα (p-I-κBα). The cells were treated with LPS or LPS + LJE for 30 min. The relative protein levels were measured by scanning densitometry. For a, b, and c, data represents the mean ± S.E.M. from three separate experiments (significant as compared with vehicle-treated control, $^{*}P < 0.05$, $^{**}P < 0.01$; significant as compared with LPS alone, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

permeability [24]. In particular, recent studies have shown that carrageenan induces peripheral release of NO as well as PGE_2 [25]. In addition, it has been shown that carrageenan also induces the release of TNF-α, which subsequently promotes IL-1 and IL-6 production in the tissue [26].

Our carrageenan-induced rat paw edema model enabled us to demonstrate the ability of LJE to inhibit paw edema in rats injected with carrageenan. Carrageenan successfully induced paw swelling by subcutaneous injection. As demonstrated in the present study, treatment with LJE significantly decreased the induction of paw edema. In histological analysis, we verified the inhibitory effects of LJE on acute inflammation in rats. In hematoxylin and eosin stating, LJE markedly inhibited skin thicknesses on both *dorsum* and *ventrum pedis* induced by carrageenan. Moreover, administration of LJE also inhibited infiltration of inflammatory cells increased by carrageenan injection. This result demonstrates that LJE could inhibit the acute inflammation in rats.

Macrophages play crucial role in immune defense mechanism during infection and in the process of development of human diseases. LPS, a prototypical endotoxin, can directly activate macrophages, which can produce inflammatory mediators [27–29]. Thus, pharmacological intervention of LPS-inducible inflammatory mediators (e.g., NO, TNF-α, and ILs) is thought as one of the most important strategies to treat various diseases caused by activation of macrophages.

The inflammatory response is mediated by various signaling molecules and enzymatic pathways, among which iNOS and COX-2 are essential enzymes playing an important role in regulating the formation of NO and PGs during inflammation, respectively [6, 7, 24]. Importantly, it has been shown that macrophages from iNOS knock-out mice are protected from acute inflammation induced by LPS/IFNγ [30]. Therefore, we tested in this study whether LJE decreased the level of LPS-induced NO and PG production. Treatment with LJE markedly deceased NO and PGE_2 production in Raw264.7 cell treated with LPS. Moreover, immunoblot analyses revealed that LJE effectively blocked the induction of iNOS and COX-2 proteins. The iNOS and COX-2 promoter comprises the binding sites for transcription factors such as NF-κB and AP-1 [31]. In particular, NF-κB is an essential transcription factor necessary for the iNOS and COX-2 gene transcription.

The pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 are small secreted proteins which mediate and regulate immunity and inflammation. TNF-α is a major mediator in inflammatory responses and can induce innate immune responses by activating macrophages and by stimulating secretion of other inflammatory cytokines [32]. Therefore, TNF-α is thought to be a principal mediator in LPS-inducible tissue injury [33]. IL-1β is another inflammatory cytokine, which is found in the circulation following Gram-negative sepsis, and IL-6 is also an inflammatory cytokine mainly synthesized by macrophages and plays a role in the acute phase response [34, 35]. Here, we evaluated the inhibitory effects of LJE on cytokines in LPS-stimulated macrophages. Our results indicated that LJE significantly inhibited LPS-induced TNF-α, IL-1β, and IL-6 secretions. In addition, it has been reported that carrageenan induces the release of NO, PGE_2, and TNF-α production in the tissue [26]. Our carrageenan-induced rat paw edema model enabled us to demonstrate the ability of LJE to inhibit edema induced by acute inflammation. LJE administration notably decreased the paw edema induction. These results in conjunction with the marked inhibition of LPS-induced NO, PG, and TNF-α productions by LJE in macrophages imply that the antiedematous effects of LJE might result from its inhibition of NO and TNF-α synthesis in the peripheral tissues.

NF-κB, the transcription factor, participates in the regulation of various genes involved in the process of inflammatory and immune responses, cell adhesion, and survival [17–19]. NF-κB activation mediates transactivation of iNOS and COX-2 and many pro-inflammatory genes including TNF-α, IL-1, IL-6, and IL-8 [34–38]. It is well recognized that many anti-inflammatory drugs have inhibitory effects on cytokines by inhibiting NF-κB activation [39]. NF-κB is regulated by its interaction with I-κBα, its inhibitor protein. NF-κB became activated by degradation of I-κBα following I-κBα phosphorylation, and then, the activated NF-κB translocates into the nucleus. Our results showing that LPS caused the phosphorylation of I-κBα, whereas pretreatment with LJE significantly inhibited the process, suggest that LJE might inhibit NF-κB activation due to its inhibition of I-κBα phosphorylation.

Our research results demonstrate that LJE was found to have an anti- inflammatory effect in acute inflammation model using the carrageenan-induced paw edema model *in vivo*. More importantly, LJE exerts anti-inflammatory effects *in vitro*, which results from the inhibition of NF-κB activation in macrophages, thereby, inhibiting the production of iNOS and COX-2 and proinflammatory cytokines. Moreover, we also verified the three main components in LJE including palmitic acid, myristic acid, and oleic acid. Previous studies have shown that various fatty acids have beneficial effects, although each part of LJE has different amount of fatty acid [40, 41]. Therefore, it remains to confirm the effects of three components in LJE on acute phase of inflammation and what is the active compound in LJE. In conclusion, our findings showing the inhibition of paw edema by LJE as well as inflammatory gene induction may be of help to understand the pharmacology and action mechanism of LJE. Although its effects on acute inflammation might be preventive in this study, we showed here the existence of a new candidate for anti-inflammatory herb in terms of therapeutic potential.

Abbreviations

I-κB: Inhibitor of κB
IL: Interleukin
iNOS: Inducible nitric oxide synthase
LJE: *Laminaria japonica* extract
LPS: Lipopolysaccharide
NF-κB: Nuclear factor-κB
NO: Nitric oxide
PGE_2: Prostaglandin E_2
TNF-α: Tumor necrosis factor-α.

Conflict of Interests

No competing financial interests exist.

Authors' Contribution

Seong Kyu Park and Sook Jahr Park equally contributed to this work.

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Research Article

Metabonomic Study on the Antidepressant-Like Effects of Banxia Houpu Decoction and Its Action Mechanism

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The aim of this study was to establish an experimental model for metabonomic profiles of the rat's brain and then to investigate the antidepressant effect of Banxia Houpu decoction (BHD) and its possible mechanisms. Behavioral research and metabonomics method based on UPLC-MS were used to assess the efficacy of different fractions of BHD on chronic unpredictable mild stress (CUMS) model of depression. There was a significant difference between the BHD group and the model group. Eight endogenous metabolites, which are contributing to the separation of the model group and control group, were detected, while BHD group regulated the perturbed metabolites showing that there is a tendency of recovery compared to control group. Therefore, we think that those potential metabolite biomarkers have some relationship with BHD's antidepression effect. This work appraised the antidepressant effect of Banxia Houpu decoction as well as revealing a metabonomics method, a valuable parameter in the TCM research.

1. Introduction

Major depressive disorder (MDD), also called major depression, a highly debilitating and widely distributed illness in the general population and an incapacitating disorder characterized by depressed mood, anhedonia, and altered cognitive function that causes a heavy burden to patients and their families, as well as to society [1]. The lifetime prevalence of MDD is approximately 17% of the population and results in tremendous secondary costs to society [2, 3]. There are typical antidepressant drugs, including selective 5-serotonin reuptake inhibitors (SSRIs), serotonin-noradrenergic reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), and other atypical antidepressants such as monoamine oxidase inhibitors (MAOIs) in clinical treatment [4]. However, the effects of these antidepressants are often inconsistent and many of them often cause side effects, such as apathy, sedation and cognitive disorders, sleep disorders, and sexual dysfunction [5]. Therefore, there is an urgent need for new effective and better tolerable antidepressants.

In the verification of antidepressant drugs, CUMS is considered to be a well-assured animal model [6, 7]. Many behavioral and biochemical changes induced by CUMS are reversible by antidepressant treatments [8]. Therefore, the CUMS model is suitable for investigating the pathophysiology of depression and antidepressant effects of diverse drugs [9]. Metabonomics can be interpreted as a value of alterations in metabolism in biological fluids and tissues of the organism [10, 11], which is a new aspect for elucidation of metabolic reactions of biological systems of any circumstantial impulses [12]. Global metabolites profiling of biofluids has been assigned in the fields of drug discovery, therapeutic scrutinizing, and assessment of drug effectiveness and toxicity [13–16].

Banxia houpu decoction (BHD) is a traditional Chinese herbal prescription, which was first recorded in Jilicn Gui Yao Lue in the Han Dynasty in Chinese history (202BC-220AD). Its recipe is composed of five herbal medicines, *Pinellia ternata* Breit, *Magnolia officinalis* Rehd. et Wils, *Poria cocos* Wolf, *Perilla frutescens* Britt, and *Zingiber officinale*, and is designed for the treatment of liver qi stagnation and phlegm accumulation, in the aspect of traditional Chinese medicine (TCM) [17], and is mainly empirically advocated for the treatment of mental diseases including depression and other disorders [18].

Even though BHD has been applied frequently in Chinese hospitals for many years, the antidepressant researches of BHD were mainly concentrated on behavioral research. Therefore, its definite knowledge is still vague [19]. In addition, little is known about the changes of the whole metabolites in an organism treated with BHD. Therefore, we evaluated the antidepressant action of the decoction in rats to get a better understanding of this empirical formula. In light of this, we performed a UPLC-Q-TOFMS metabonomic approach to characterize the global metabolic profiling of rats brain homogenates in antidepressant studies of BHD to evaluate the pharmacological effect of BHD on CUMS. Meanwhile, some significantly changed metabolites were used to explain the mechanism.

2. Methods and Materials

2.1. Materials and Reagents. Banxia Houpu decoction (*Pinellia ternata* Breit, *Magnolia officinalis* Rehd. et Wils, *Poria cocos* Wolf, *Perilla frutescens* Britt, and *Zingiber officinale*) was purchased from Yifeng drug store (Nanjing, China) and was authenticated according to the standards documented in Chinese Pharmacopoeia by Minjian Qin, a pharmacognosist in our team.

2.2. Preparation of Banxia Houpu Decoction. *Pinellia ternata* Breit (~12 g), *Poria cocos* Wolf (~12 g), *Magnolia officinalis* Rehd. et Wils (~9 g), *Perilla frutescens* Britt (~6 g), and *Zingiber officinale* (~15 g) were accurately weighed and mixed. These herbs were immersed in 8 times volume of water for 1 h. According to the methods of Sun et al. [20], medicinal materials were decocted twice at boiling temperature for half an hour, and then the decocted liquids were centrifuged at 3000 rpm for 5 min. The supernatant was considered as the Banxia Houpu decoction and it was concentrated and then dried in a vacuum oven at 55°C. The yield of dried powder (14.7 g) is equivalent to the 100 g of original dry materials. The final dried powder of BHD was dissolved in distilled water and finally prepared the 1.8 g/mL of BHD. The sample was stored at 4°C. The doses of distilled water extract of BHD were expressed as gram of the original dry materials per kilogram body weight, and the dose (6 g/kg) of distilled water extract of BHD for animals was converted from the human dose.

2.3. Animals and Drug Administration. Male Sprague Dawley rats weighing 180–220 g were purchased from the Experimental Animal Centre of China Pharmaceutical University. The animals were housed in polypropylene cages under standard experimental conditions of room temperature (20 ± 2°C), humidity (50 ± 10%) and light (12-h light/dark cycle, lights on at 7:00 a.m.). Animals were acclimatized for 7 days before any experimentation. All experiments and animal care were performed in compliance with the National Institute of Health Guide (NIH publication no. 80-23, revised 1996) and the PR China legislation for the care and use of laboratory animals. The doses of distilled water extract of BHD were expressed as gram of the original dry materials per kilogram body weight, and the dose (6 g/kg) of distilled water extract of BHD for animals was converted from the human dose.

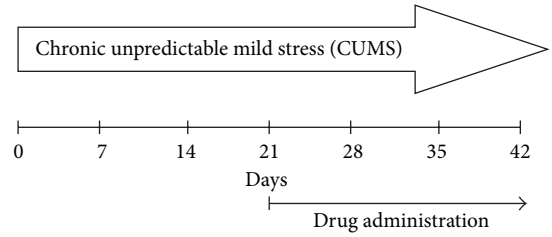

Figure 1: Experimental manipulations rats during the present study.

The animals were divided into three treatment groups as follows: vehicle-control (0.9% physiological saline), vehicle-CUMS (0.9% physiological saline), and CUMS-BHD treatment (6 g/kg). All drugs were intraperitoneally (i.p.) administered once during the last three weeks of the CUMS procedure.

2.4. Chronic Unpredictable Mild Stress (CUMS) Procedure. The CUMS procedure was adopted as described by Willner et al. [21] with slight modifications. The following stressors (10) were used to provoke depressive states: cage tilt (45°, 23 h); soiled cage (100 mL of water spilled onto the bedding (23 h)); withdrawal of food or water (23 h); continuous overnight brilliance; cold water swimming (4°C for 5 min); empty water bottles (23 h); swing on the rocking bed (200 Hz for 5 min; HY-4A, Shunhua Scientific instrument limited Company, China); 2 h behavioral hindrance in a tube (diameter: 8 cm, length: 20 cm); sporadic illumination (light on and off every 2 h). Each animal was exposed to one stress per day individually for 6 weeks. The whole experiment was conducted for 6 weeks, and the procedural succession was as follows: (1) stressors induction: 1–42 days; (2) drug administration: 22–42 days; (3) 1% sucrose utilization test: 40 days; (4) open-field behavior test: 41 days; (5) forced swim test: 42 days (Figure 1).

2.5. Behavior Test

2.5.1. Forced Swim Test. The forced swim test (FST) method was similar to that described by Porsolt and colleagues with minor modifications [22]. Rats were kept in 30 cm of water contained in glass cylinders separately (20 cm in diameter, 50 cm deep), and maintained at 22 ± 1°C. Each animal was forced to swim for 6 min, and the total duration of immobility was measured during the last 4 min. The definition of immobility was the absence of all movements only with motions required to maintain the animal's head above the water. Observers were blind to the group treatment of the rats.

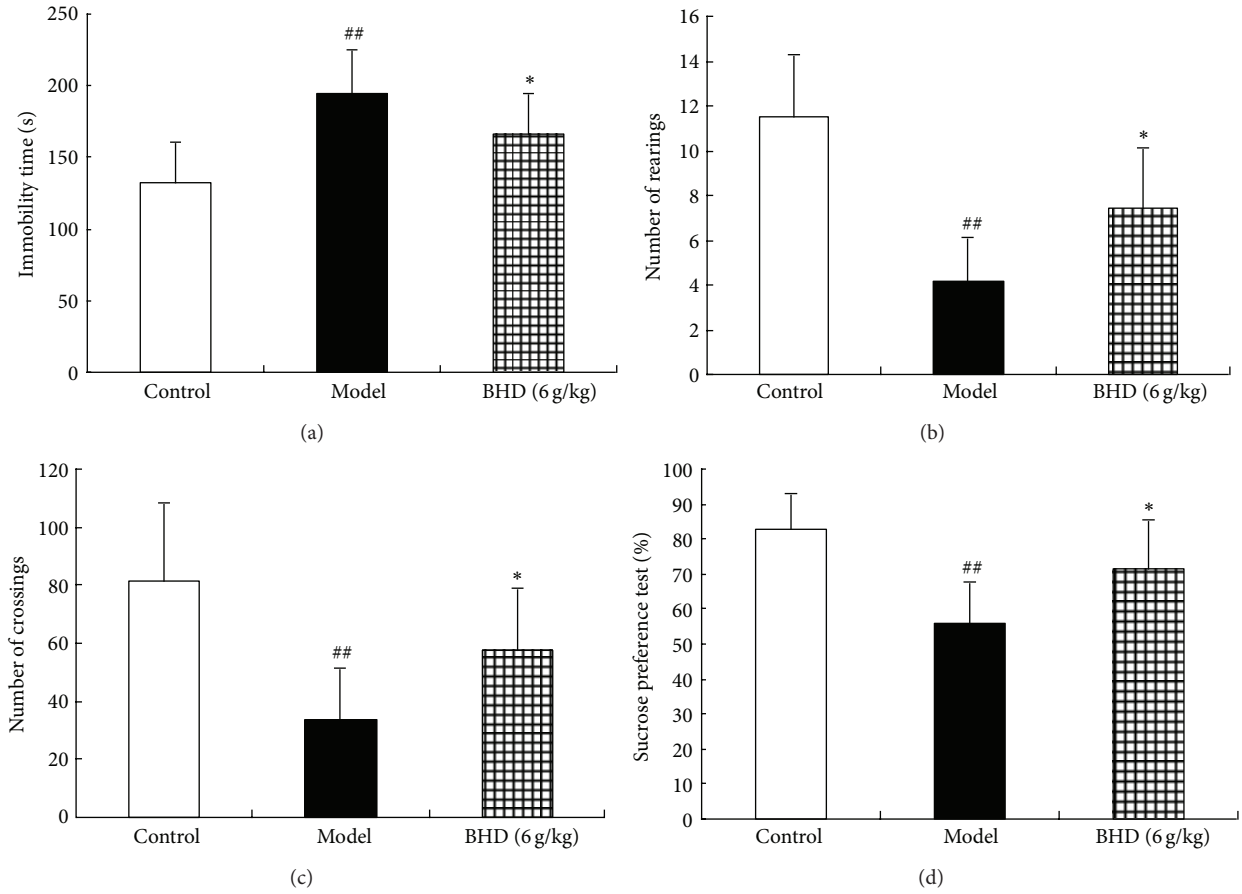

Figure 2: (a) Effect of BHD on forced swimming test after six weeks of CUMS. Each column represented as the mean ± SEM (n = 10–12). $^{\#\#}P < 0.01$ as compared with control group; $^{*}P < 0.05$, compared with model group. (b) and (c) Effects of BHD on locomotor activity of rats after CUMS. (b) Number of rearings during the 5 min session. (c) Number of crossings during the 5 min session. Each column represented as the mean ± SEM (n = 10–12). $^{\#\#}P < 0.01$ as compared with control group; $^{*}P < 0.05$, compared with model group. (d) Sucrose preference of rats after six weeks of CUMS. Percentage of sucrose preference was measured for a period of 1 h, after the 23 h food and water deprivation. Each column represented as the mean ± SEM (n = 10–12). $^{\#\#}P < 0.01$ as compared with control group; $^{*}P < 0.05$, compared with model group.

2.5.2. Open-Field Test. The open-field test was carried out on day 41 between 8:00 am to 12:00 am in a quiet room as previously explained (≤60 dB) [23]. The open-field apparatus was a four-sided 100 cm × 100 cm × 40 cm wooden enclosure, with a white-painted floor, and separated by 25 equal squares with black lines and sidewalls painted black. Tests were performed in a darkened place lit by two 60-W light bulbs which were hanged over the center of the open field. Each rat was placed individually into the center of the arena and permitted free exploration. The numbers of squares crossed by the rats (crossings) and of standing on the hind legs (rearings) were recorded during a test of 4 min. This apparatus was cleansed with a detergent and dried after occupancy by each rat.

2.5.3. Sucrose Preference Test. A sucrose preference (SP) test [21, 24] was applied to operationally define anhedonia [25]. Briefly, 72 h before the test, rats were trained to adapt to 1% sucrose solution (w/v). Two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water for 24 h. At the end of adaptation, rats were deprived of water and food for 24 h, followed by the sucrose preference test, in which rats were housed in individual cages and had free access to two bottles with 200 mL of sucrose solution (1%, w/v) and 200 mL of tap water, respectively. All the procedures were performed in the same cage with minimum disturbances. The SP test was repeated after 40 days of CUMS. The SP was calculated according to the following equation: SP = sucrose intake/(sucrose intake + water intake) × 100.

2.6. Collection and Preparation of Brain Sample. Three rats from each group were decapitated 60 min after the behavioral tests. Brains were removed carefully and quickly stored in liquid nitrogen. The brain tissues were weighed accurately and homogenized in ice-cold methanol (each 1 g brain tissue was mixed with 4 mL methanol). Precisely drawn 1 mL of homogenate was centrifuged at 13,000 rpm for 10 min; the supernatant was decanted and evaporated to give the dry

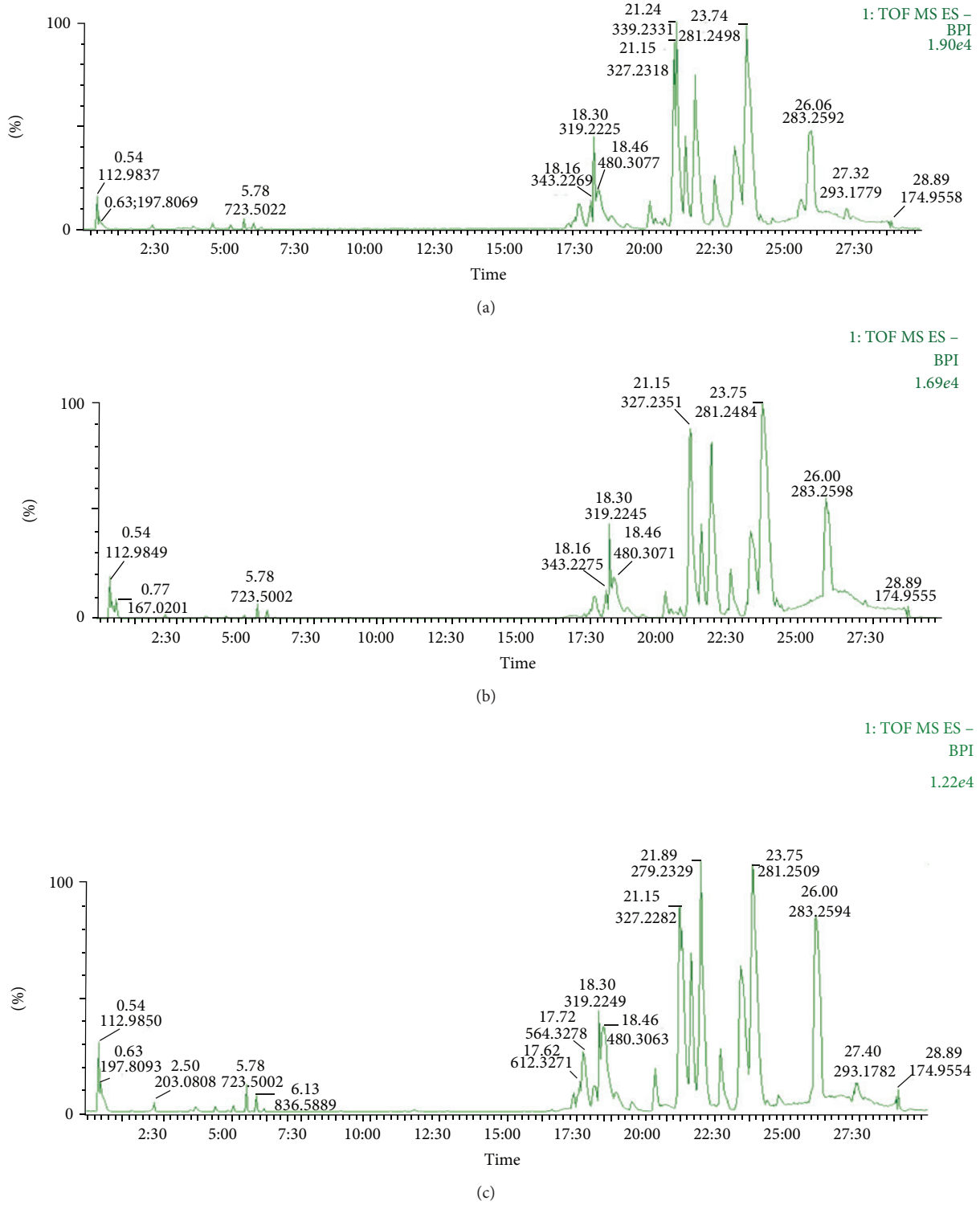

Figure 3: Typical base peak intensity (BPI) chromatograms obtained from rats brain (a) control group, (b) model group, and (c) treatment group in positive mode.

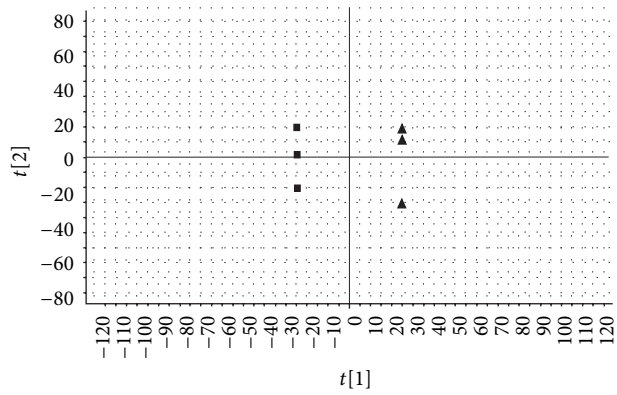

FIGURE 4: Score plot (■) control group rats, (▲) model group rats in positive ion mode from a PCA mode.

powder at 37°C under a gentle stream of nitrogen. The dried residue was then reconstituted in 100 μL of acetonitrile-water (10: 90, v/v). After it was vortexed for 30 s, the content was transferred to 2 mL glass vials and an aliquot of 5 μL was injected for UPLC-MS/MS analysis.

2.7. Liquid Chromatography. UPLC was performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA), consisting of a binary solvent delivery system, an on-line degasser, an autosampler, and a photodiode array detector (PDA) system. An ACQUITY UPLCTM BEH C_{18} (2.1 × 100 mm I.D., 1.7 μm, Waters, Milford, USA) column was used for all the analyses. The mobile phase composed of A (acetonitrile) and B (0.1% formic acid, v/v) with a gradient elution 0–11 min, 5–90% A. The flow rate of the mobile phase was 0.5 μL/min, and the column autosampler temperatures were maintained at 30 and 10°C, respectively.

2.8. Mass Spectrometry. The MS analysis was performed on a Waters ACQUITY Synapt Q-TOF mass spectrometer connected to the Waters Acquity UPLC system via an electrospray ionization interface (ESI). High purity nitrogen was used as the nebulizer and auxiliary gas and argon was used as the collision gas. The Q-TOF mass spectrometer was operated in positive ion mode with a capillary voltage of 3 kV, a sampling cone voltage of 30 V, a cone gas flow of 50 L/h, a desolvation gas flow of 600 L/h, a desolvation temperature of 350°C, a source temperature of 120°C, a collision energy of 6 V, and the full scan spectra from 100 to 1000 Da.

2.9. Data Analysis. Each sample was represented by a total ion current (TIC) chromatogram. The UPLC-MS raw data were processed using the Marker Lynx Applications Manager version 4.1 (Waters Corp. Milford, USA). The raw data were transformed into a single matrix containing aligned peaks with the same m/z retention time pair along with normalized peak intensities and sample name. The three-dimensional data, peak number (RT-m/z pair), sample name, and normalized ion intensity were introduced to SIMCA-P 11.0 software package (Umetrics, Umea, Sweden) for principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Data in the figures or the table are expressed as mean values ± SD. The differences between groups were analyzed by one-way ANOVA or Student's t-tests, with $P < 0.05$ considered as significant.

3. Results

3.1. Analysis of Behavior Test

3.1.1. Forced Swim Test. After 42-day UCMS, the ANOVA test showed a significant effect on groups for forced swim test ($F(2, 27) = 11.6002$, $P < 0.01$). Figure 2(a) shows the effects of oral administration of BHD (6 g/kg) on immobility time in the FST. Chronically stressed rats exhibited a significant increase in immobility time as compared to model animals. The statistical analysis results showed that BHD at a dose of 6 g/kg markedly decreased the immobility time ($P < 0.05$ versus model group).

3.1.2. Open-Field Test. After 41-day UCMS, the ANOVA test showed a significant effect on groups for crossings ($F(2, 28) = 13.5326$, $P < 0.01$) and rearings ($F(2, 28) = 9.8743$, $P < 0.01$). The locomotor activity in the CUMS experiment was observed in the open-field test (Figures 2(b) and 2(c)). The statistical analysis results showed that CUMS led to a pronounced reduction in the number of crossings ($P < 0.01$) and rearings ($P < 0.01$). By the treatment of BHD (6 g/kg), the behavioural changes for the number of rearings and crossings were significantly reversed ($P < 0.05$, $P < 0.05$, resp.).

3.1.3. Sucrose Preference Test. Figure 2(d) shows the effect of BHD treatment on the sucrose preference in the control and the CUMS-treated rats. After 40 days of CUMS, the ANOVA test showed a significant effect on groups for sucrose preference ($F(2, 28) = 12.3124$, $P < 0.01$). The post hoc test revealed that sucrose preference in the stress group was significantly reduced compared with the control group ($P < 0.01$). Simultaneous administration with BHD (6 g/kg) while the rats were exposed to CUMS significantly increased the percentage of sucrose consumption as compared with the model group ($P < 0.05$).

3.2. Pattern Recognition and Identification of Potential Biomarkers. Figures 3 and 4 show the PCA score plot of characteristic UPLC/MS base peak intensity (BPI) chromatograms of rat brain tissue from all the groups demonstrating the distribution among control and model group in two aspects. Obvious separation between the control and model groups suggests that biochemical perturbation was significant in model group. The corresponding loading plot used to identify biomarkers is shown in Figure 5. The ions furthest away from the origin contribute significantly to the clustering of the two groups and may be regarded as the potential biomarkers of chronic unpredictable mild stress-induced depressant. t-test was used to reveal the significant differences of identified metabolites between the model and control group. The significant variables detected in the positive ion mode are summarized in Table 1. Eight endogenous metabolites (lysoPC (18 : 0); lysoPC (16 : 0); PG (16 : 0/18 : 0); behenic acid;

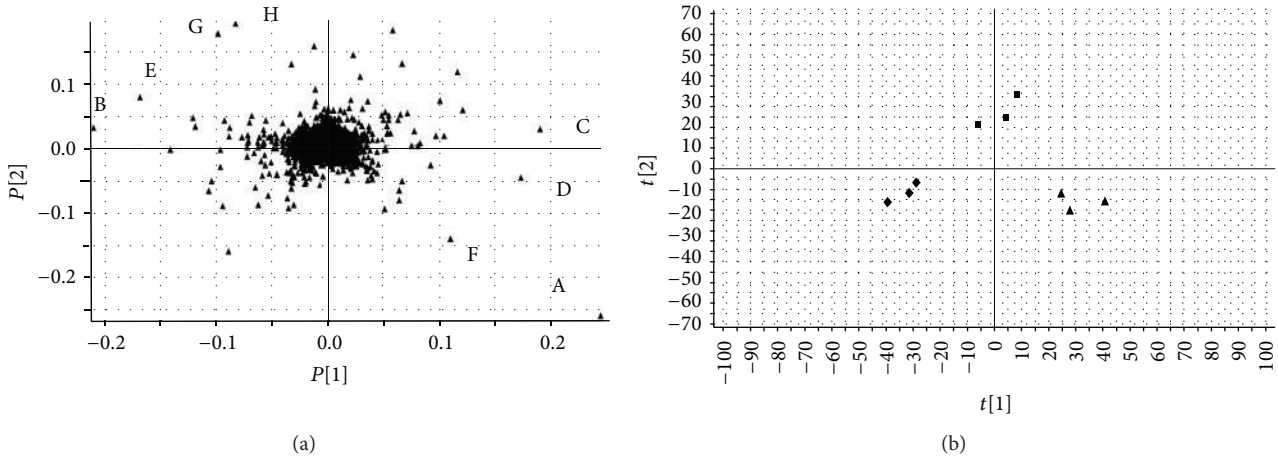

Figure 5: (a) Loading plot in positive ion mode from a PCA model. (A) lysoPC (18 : 0); (B) lysoPC (16 : 0); (C) PG (16 : 0/18 : 0); (D) behenic acid; (E) 8,9-epoxyeicosatrienoic acid; (F) stearic acid; (G) glycine; (H) glutamic acid. (b) Score plot (▲) control group rats, (■) model group rats, and (◆) treatment group rats in positive ion mode from a PLS-DA model.

8,9-epoxyeicosatrienoic acid; stearic acid; glycine; glutamic acid) were tentatively identified by comparing with authentic standards or based on their molecular ion information and corresponding fragments of product ion. The biomarker with retention time and m/z pairs of 18.40–496.3391 was identified as lysoPC (16 : 0). We used it as an illustration to demonstrate the identification process. Under the same UPLC-QTOFMS conditions, we compared standard lysoPC (16 : 0) with sample and we found that peak 18.40–496.3391 and standard have the same RT (Figure 6), so the biomarker at m/z = 496.3391 was identified as lysoPC (16 : 0).

To determine whether the BHD could influence the metabolic pattern of depression in rats, a PLS-DA model was constructed. Figure 5 shows the PLS-DA score plot of three groups in positive ion mode. From the data of Figure 5, after 21 days of BHD treatment, the treatment group showed better improvement which is more similar to the control group than model group in the direction of the first principal component, which implied that BHD has intervened the metabolic process of depressed animals to some extent.

4. Discussion

In the present study, we investigated the metabolic pattern induced by CUMS and the influence of BHD. The CUMS model of rats successfully copied the state of depression by increasing immobility time in the FST and reduction of sucrose intake. The behavior results demonstrated the antidepressant effect of Banxia Houpu decoction.

Subtle changes could be found using a pattern recognition approach, such as PCA and PLS-DA. PCA and PLS-DA are the two most popular pattern recognition methods to gain information for classification and to identify metabolites. PCA, an unsupervised method, is applied as the first step in the separation procedure to filter out the noise, and it reduces the dimension of data to widen the observation. PLS-DA, a supervised method, which has the similar principle with PCA, is used to enhance the classification performance [26].

Eight endogenous metabolites, including amino acids, organic acids, and fatty acids, were detected, and they contributed to the separation of the model group and control group. Lysophosphatidylcholines (lysoPCs, LPC) are a class of compounds that have a constant polar head and fatty acyls of different chain lengths, position, degrees of saturation, and double bond location in plasma. LPC level can be a clinical diagnostic indicator that reveals pathophysiological changes [27]. Lysophosphatidylcholines are products or metabolites of phosphatidylcholines (PCs), which are structural components of animal cell membranes.

Prostaglandin (PG) exists widely in many tissues. In vivo, prostaglandins (PGs) are synthesized by arachidonic acid. Prostaglandin (PG) has complex regulatory effects on the immune system as it can play a positive and positive role in immune regulation. Studies have found that PG plays an important role in neuronal oxidative damage by EP2, which promotes the inflammatory reaction around neurons [28]. More and more evidence indicates that inflammatory processes may play important roles in the pathogenesis of depression [29, 30]. Our study found that CUMS could increase prostaglandin levels in the rats. The level of prostaglandin in model rats can be turnedover by BHD. As a result of that, we inferred that BHD exerts antidepressant-like effect via regulating the PGs level.

Glutamic acid, an excitatory neurotransmitter in the mammalian nervous system [31], was significantly decreased in model group in present study, while glycine, the inhibitory neurotransmitter, was increased in model group. Studies of pathophysiology of depression and neuropharmacology found that patients with affective disorders in glutamate system abnormalities and glutamic acid conversion rate have changed in localized brain regions. The results suggested that the glutamic acid and glycine biosynthesis were affected after CUMS treatment.

Behenic acid, 8,9-epoxyeicosatrienoic acid, and stearic acid are fatty acids, in which 8,9-epoxyeicosatrienoic acid is a metabolite of arachidonic acid. Epoxyeicosatrienoic acid

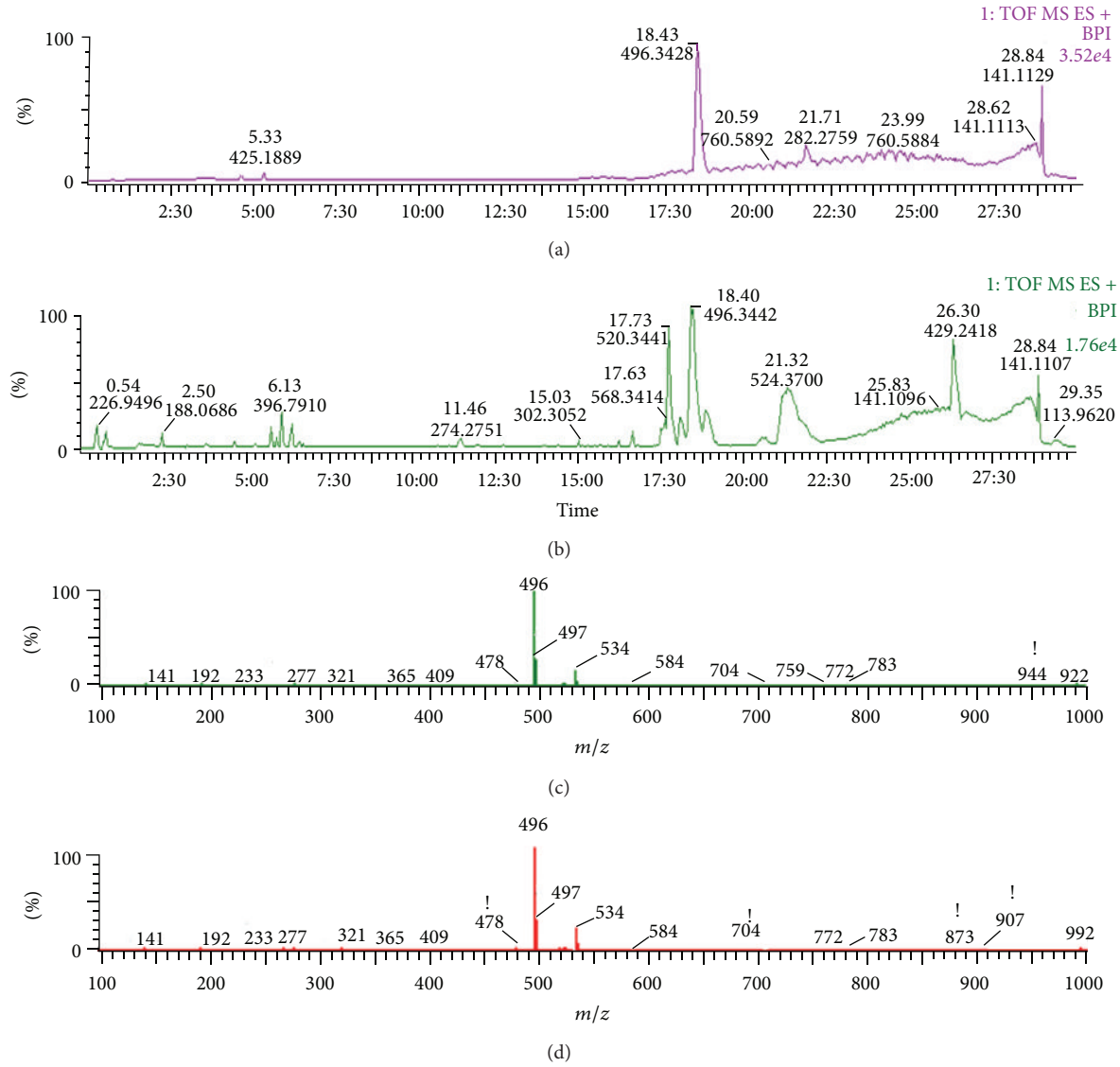

Figure 6: Product ion spectrum of biomarkers at m/z 520.1 in positive ion mode.

Table 1: Identification of biomarkers on ions variations and their trend in positive mode.

RT-m/z	Biomarker identification	Metabolites	Change trend of model group versus control group	Change trend of treatment group versus model group
21.25-524.3714	A: lysoPC (18:0)	$C_{26}H_{54}NO_7P$	↑	↓
18.10-496.3411	B: lysoPC (16:0)	$C_{26}H_{54}NO_7P$	↑	↓
6.11-396.7923	C: PG (16:0/18:0)	$C_{40}H_{79}O_{10}P$	↑	↓
21.22-340.5898	D: behenic acid	$C_{22}H_{44}O_2$	↑	↓
18.28-319.2249	E: 8,9-epoxyeicosatrienoic acid	$C_{20}H_{32}O_3$	↓	↑
26.01-283.2593	F: stearic acid	$C_{18}H_{36}O_2$	↑	↓
25.77-75.0769	G: glycine	$C_2H_5NO_2$	↑	↓
13.26-147.1317	H: glutamic acid	$C_5H_9NO_4$	↓	↑

(EET) is epoxides of arachidonic acid biosynthesized by cytochrome P-450 (CYP450) epoxygenases. EETs function as autocrine and anti-inflammation, ion channel opening, mitogenesis and angiogenesis [32]. The change of fatty acid levels detected in this study may be caused by fatigue induced by physical stressors, which is one of the most frequently represented depressive symptoms in major depressive disorder [33]. In our study, the model group 8,9-epoxyeicosatrienoic acid was reduced, while giving BHD 8,9-epoxyeicosatrienoic acid content levels.

Our study showed that CUMS treated BHD intervention indicated the tendency of turnover in the level of eight endogenous metabolites (lysoPC (18 : 0); lysoPC (16 : 0); PG (16 : 0/18 : 0); behenic acid; 8,9-epoxyeicosatrienoic acid; stearic acid; glycine; glutamic acid). Briefly, BHD displays a remarkable anti-depression effect by adjusting the amino acid metabolism and energy metabolism. However, further investigations are required to identify biochemical and molecular characterization to elucidate the exact antidepression mechanism of BHD.

5. Conclusion

A metabonomics method based on UPLC/MS was developed to establish the metabonomic profiles of rats' brain homogenate to investigate anti-depressive effect of BHD and its mechanism of action. In this work, we found eight potential metabolite biomarkers including LPCs, glycine; glutamic acid, PG; therefore, we assumed that those potential metabolite biomarkers might have some relationship with antidepressant effect of BHD. This work appraised the antidepressant effect of Banxia Houpu decoction as well as revealing a metabonomics method, a valuable parameter in the TCM research.

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Research Article

Methanol Extract of *Artemisia apiacea* Hance Attenuates the Expression of Inflammatory Mediators via NF-κB Inactivation

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Artemisia apiacea Hance is one of the most widely used herbs for the treatment of malaria, jaundice, and dyspeptic complaint in oriental medicine. This study investigated the effects of methanol extracts of *A. apiacea* Hance (MEAH) on the induction of inducible nitric oxide synthase (iNOS) and proinflammatory mediators by lipopolysaccharide (LPS) in Raw264.7 macrophage cells and also evaluated the *in vivo* effect of MEAH on carrageenan-induced paw edema in rats. MEAH treatment in Raw264.7 cells significantly decreased LPS-inducible nitric oxide production and the expression of iNOS in a concentration-dependent manner, while MEAH (up to 100 μg/mL) had no cytotoxic activity. Results from immunoblot analyses and ELISA revealed that MEAH significantly inhibited the expression of cyclooxygenase-2, tumor necrosis factor-α, interleukin-1β, and interleukin-6 in LPS-activated cells. As a plausible molecular mechanism, increased degradation and phosphorylation of inhibitory-κBα and nuclear factor-κB accumulation in the nucleus by LPS were partly blocked by MEAH treatment. Finally, MEAH treatment decreased the carrageenan-induced formation of paw edema and infiltration of inflammatory cells in rats. These results demonstrate that MEAH has an anti-inflammatory therapeutic potential that may result from the inhibition of nuclear factor-κB activation, subsequently decreasing the expression of proinflammatory mediators.

1. Introduction

Inflammation is regarded as a protective response against tissue injury or destruction. During the inflammatory process, a number of inflammatory cells infiltrate into the damaged tissue and produce inflammatory mediators that exaggerate inflammatory responses characterized by redness, swelling, fever, and pain [1]. Nitric oxide (NO), prostanoids, tumor necrosis factor-α (TNF-α), and interleukins (ILs) produced from infiltrated cells or damaged tissue act as pleiotropic effector molecules to amplify acute inflammation and lead to the activation of adaptive immune response. On the other hand, inappropriate inflammation plays a pivotal role in the pathogenesis of a variety of disorders. Thus, it is essential that the inflammatory process must be controlled spaciotemporally to prevent unwanted tissue damage and reduce inflammation-related disorders.

A variety of signaling pathways are involved in transducing the inflammatory response. Among them, nuclear factor-kappa B (NF-κB) is an essential signaling molecule to increase the production of effector molecules related to acceleration of the inflammatory process [2]. The activity of NF-κB is largely dependent on its interaction with inhibitory-κB (I-κB) protein in the cytoplasm. Inflammatory stimuli trigger I-κB phosphorylation through I-κB kinase and degradation via the ubiquitin-proteasome system and the subsequent dissociation of the NF-κB complex from I-κB [2, 3]. Activation of NF-κB in the cytoplasm allows it to translocate into the nucleus, where it binds to the promoter region of target genes that are

associated with immune responses, cell survival, apoptosis, and so on [4].

Because they have relatively fewer side effects, Herbal medications have garnered wide interest as complementary and alternative therapeutics and are becoming more popular. Moreover, novel therapeutic candidates have been screened from plentiful sources of medicinal herbs to manage inappropriate immune responses and developed as anti-inflammatory drugs over the last few decades. To expand the usage of herbal medications, the pharmacological efficacy of medicinal herbs should be evaluated both *in vitro* and *in vivo*, and their molecular mechanisms need to be clarified. Lipopolysaccharide (LPS)-mediated macrophage activation using Raw264.7 cells is a well-established *in vitro* inflammation model [5, 6]. Moreover, carrageenan-induced paw edema formation is regarded as a representative acute inflammation *in vivo* model [7]. Therefore, both combined models have been extensively studied to examine the anti-inflammatory effect of medicinal herbs and to identify the putative molecular mechanism [5, 6, 8].

Artemisia apiacea Hance is one of the most widely used herbs in oriental medicine [9]. Artemisin found in *A. apiacea* Hance is not only a standard treatment worldwide for falciparum malaria but also has a number of pharmacological effects involving anticancer, antiviral, and immunosuppressive activities [10, 11]. Regardless of a number of studies about artemisin and its derivatives, the pharmacological effect of *A. apiacea* Hance itself, the parental herb containing artemisin, has not been fully understood, and little is known about the anti-inflammatory effect of the herb.

This study examined the effect of methanol extracts of *A. apiacea* Hance (MEAH) on the expression of inflammatory mediators in LPS-stimulated Raw264.7 cells and also investigated the *in vivo* effect of MEAH on carrageenan-induced edema formation. With the data, this study was extended to explore the effects of MEAH on the NF-κB signaling pathway as a plausible molecular mechanism.

2. Materials and Methods

2.1. MEAH Preparation and Reagents. A. apiacea Hance was purchased from Daewon Pharmacy (Daegu, Republic of Korea). MEAH was prepared by extracting 400 g of *A. apiacea* Hance in 3 L of 100% methanol for 48 h. The MEAH was filtered through a 0.2 μm filter (Nalgene, NY, USA), lyophilized by a vacuum evaporator, and stored at −20°C until use. The amount of MEAH was estimated from the dry weight of lyophilized MEAH. The yield of lyophilized MEAH was 6.03%. Antibody directed phosphorylated inhibitory-κBα (p-I-κBα), lamin A/C, and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA, USA). Anti-iNOS and anti-COX-2 antibodies were supplied from BD Bioscience (San Jose, CA, USA). Anti-I-κBα, anti-NF-κB (p65 subunit), and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyethylene glycol no. 400 (PEG) was obtained from Yakury Pure Chemical Co. (Kyoto, Japan). Carrageenan, dexamethasone, LPS, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell Culture. Raw264.7 cells, a murine macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. For all experiments, the cells were grown to 80–90% confluency and were subjected to no more than 20 cell passages. The Raw264.7 cells were incubated in medium without fetal bovine serum for 12 h and then subsequently exposed to 1 μg/mL LPS for the indicated time periods (1–24 h). MEAH, dissolved in dimethyl sulfoxide, was added to the incubation medium 1 h prior to the addition of LPS.

2.3. Cell Viability Assay. The cells were plated at a density of 5×10^4 cells per well in 96-well plates to examine the cytotoxicity of MEAH. Cells were serum starved for 12 h and then treated with MEAH in the presence or absence of 1 μg/mL LPS for the next 24 h. After incubation of the cells, viable cells were stained with MTT (0.5 mg/mL, 4 h) according to the previous report [5, 6].

2.4. Assay of Nitrite Production. NO production was monitored by measuring the nitrite content in culture medium [5, 6]. Samples were mixed with Griess reagent (Sigma, St. Louis, MO, USA), and absorbance was measured at 540 nm.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). Raw264.7 cells were preincubated with MEAH for 1 h and continuously exposed to LPS for 18 h. TNF-α, IL-1β, IL-6, and prostaglandin E_2 (PGE_2) production in the medium was measured by ELISA using each antibody and biotinylated secondary antibody according to the manufacturer's instructions (Pierce, Woburn, MA, USA).

2.6. Sample Preparation and Immunoblot Analysis. Whole cell lysates and nuclear extracts were prepared according to previously established methods [5, 6]. Briefly, cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. Cell lysates were collected by centrifugation at 10,000 ×g for 10 min. To prepare nuclear extracts, cells were allowed to swell in 100 μL of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride). After centrifugation of the samples, the pellets containing crude nuclei were resuspended in 50 μL of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and incubated for 30 min on ice. The samples were centrifuged at 15,800 ×g for 10 min to obtain the supernatant containing nuclear extracts. Protein contents of samples were measured using BCA assay

(Pierce, Woburn, MA, USA). Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany). Immunoreactive proteins of interest were visualized by an ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Equal protein loading among the samples in each gel was verified by immunoblotting with an antibody directed against actin or lamin A/C.

2.7. Carrageenan-Induced Paw Edema. Animal studies were conducted in accordance with the institutional guidelines of Daegu Haany University for the care and use of laboratory animals. Sprague-Dawley rats at 6 weeks of age (male, 140–160 g) were provided from Samtako Co. (Osan, Republic of Korea), acclimatized for 1 week, and maintained in a clean room at the Animal Center of the College of Korean Medicine, Daegu Haany University. Animals were caged under the supply of filtered pathogen-free air, commercial rat chow (Purina, Republic of Korea), and water *ad libitum* at a temperature between 20°C and 23°C with 12 h light and dark cycles and relative humidity of 50%. Rats ($N = 20$) were randomly divided into four groups, and each group consisted of five animals. MEAH, dissolved in 40% PEG, was orally administered to rats at a dose of 1.0 or 0.3 g/kg/day for 3 consecutive days. Dexamethasone (1 mg/kg/day, p.o.), a representative anti-inflammatory drug, was used as a positive control [6]. To induce acute phase paw inflammation, rats were injected subcutaneously into the right hind paw with a 1% solution of carrageenan dissolved in saline (0.1 mL per animal) 30 min after vehicle or drug treatment. The paw volume was measured up to 4 h after the injection at intervals of 1 h. The hind paw volume was determined volumetrically by measuring with a plethysmometer (Letica, Rochester, MI, USA). The paw edema volume was defined relative to the paw volume in carrageenan-treated rats at 0 h (i.e., paw edema volume (%) = 100 × (paw volume of treated rat at the indicated time period)/(paw volume of carrageenan-treated rat at 0 h)).

2.8. Histopathology. *Dorsum* and *ventrum pedis* skins were separated and fixed in 10% neutral buffered formalin, then embedded in paraffin, sectioned (3-4 μm), and stained with hematoxylin and eosin. The histopathological profiles of each sample were observed under light microscope (Nikon, Japan) by certified pathologist. The thicknesses of *dorsum pedis* and *ventrum pedis* (from epidermis to dermis, keratin layers were excluded) and the number of infiltrated inflammatory cells were measured using automated image analyzer (DMI-300 Image Processing; DMI, Republic of Korea) according to the previous report with some modifications [12].

2.9. Statistical Analysis. Statistical analyses were conducted using SPSS for Windows (Release 14.0 K, SPSS Inc., USA). Multiple comparison tests among different dose groups were analyzed by one-way ANOVA. The data were expressed as mean ± S.D. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Effect of MEAH on Cell Viability and NO Production in Raw264.7 Cells. Prior to exploring the anti-inflammatory effects of MEAH in cells, any possible toxicity of MEAH was monitored by MTT analyses (Figures 1(a) and 1(b)). MTT assay indicated that treatment with MEAH up to 100 μg/mL for 24 h did not show any toxicity in Raw264.7 cells. However, 300 μg/mL MEAH elicited approximately 40% cell loss. As already reported, 1 μg/mL LPS for 24 h inhibited cell viability slightly [13], and reduction of cell viability by LPS was not changed by pretreatment with 100 μg/mL MEAH. Therefore, 10–100 μg/mL MEAH was chosen for examining the anti-inflammatory effects of MEAH in subsequent experiments. Next, the effect of MEAH on NO production was examined in LPS-stimulated Raw264.7 cells. LPS (1 μg/mL, 24 h) treatment increased the production of NO. However, MEAH pretreatment significantly decreased NO release in a dose-dependent manner compared with LPS-treated cells. Strong inhibition was obtained with 100 μg/mL MEAH-treatment (Figure 1(c)).

3.2. Effect of MEAH on Proinflammatory Mediators Production in Raw264.7 Cells. To investigate the effect of MEAH on the expression of proinflammatory meditators in LPS-stimulated Raw264.7 cells, the secreted levels of TNF-α, IL-1β, IL-6, and PGE_2 were monitored in the medium by ELISA (Figures 2(a)–2(d)). As previously reported [5], LPS-treated cells (1 μg/mL, for 18 h) exhibited significantly increased secretion of proinflammatory mediators (TNF-α, IL-1β, IL-6, and PGE_2) compared with vehicle-treated cells. In contrast, MEAH pretreatment significantly blocked the secretion of TNF-α (Figure 2(a)), IL-1β (Figure 2(b)), and PGE_2 (Figure 2(d)) in a dose-dependent manner compared with LPS-stimulated cells. In the case of IL-6, only 100 μg/mL MEAH pretreatment showed a significant reduction (Figure 2(c)). These results indicate that MEAH inhibited the secretion of proinflammatory mediators in LPS-activated cells, which might result, at least in part, from the perturbation of common signaling pathways involving proinflammatory mediator induction.

3.3. Effect of MEAH on the Expression of Proinflammatory Enzymes in Raw264.7 Cells. To determine whether the reduction of NO is related to the regulation of iNOS expression, immunoblot analyses against iNOS were conducted. No detectable expression of iNOS was observed in vehicle- or MEAH-treated cells, whereas LPS treatment (1 μg/mL) strongly induced the expression of iNOS (Figures 3(a) and 3(b)). MEAH treatment in the presence of LPS decreased the iNOS expression in a dose-dependent manner. In particular, 100 μg/mL of MEAH pretreatment almost completely prevented the iNOS induction by LPS. In our continuing effort to validate the anti-inflammatory effect of MEAH, the expression level of COX-2, the rate limiting enzyme of PG production, was further examined (Figures 3(a) and 3(c)). Like iNOS expression, no basal COX-2 expression was observed in vehicle- or MEAH-treated

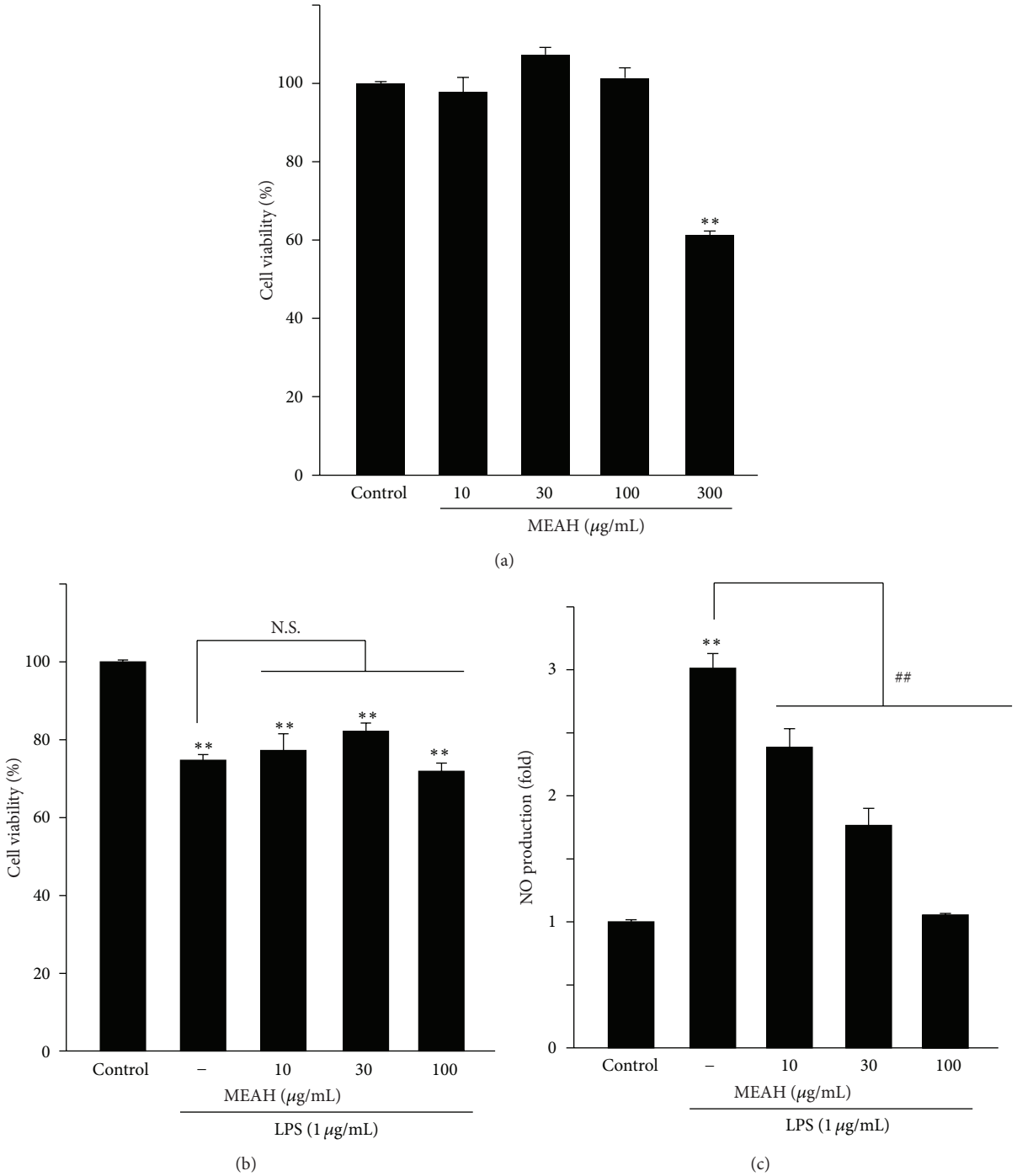

Figure 1: Effect of methanol extracts of *Artemisia apiacea* Hance (MEAH) on cell viability and NO production. Raw264.7 cells were incubated with 10–300 μg/mL MEAH for 24 h (a) or pretreated with 10–100 μg/mL MEAH for 1 h and then continuously exposed to 1 μg/mL LPS for 24 h (b). After incubation, cell viability was assessed by the MTT analysis. Data represent mean ± S.D. of three separated experiments (significant as compared with vehicle-treated control cells, $^{**}P < 0.01$; cell viability of control = 100%). N.S.: not significant. (c) NO production. Raw264.7 cells were pretreated with 10–100 μg/mL MEAH for 1 h and then further incubated with 1 μg/mL LPS for 24 h. NO release in the medium was measured by using Griess reagent. Data represent mean ± S.D. of three separated experiments (significant as compared with control cells, $^{**}P < 0.01$; significant as compared with LPS-treated cells, $^{\#\#}P < 0.01$).

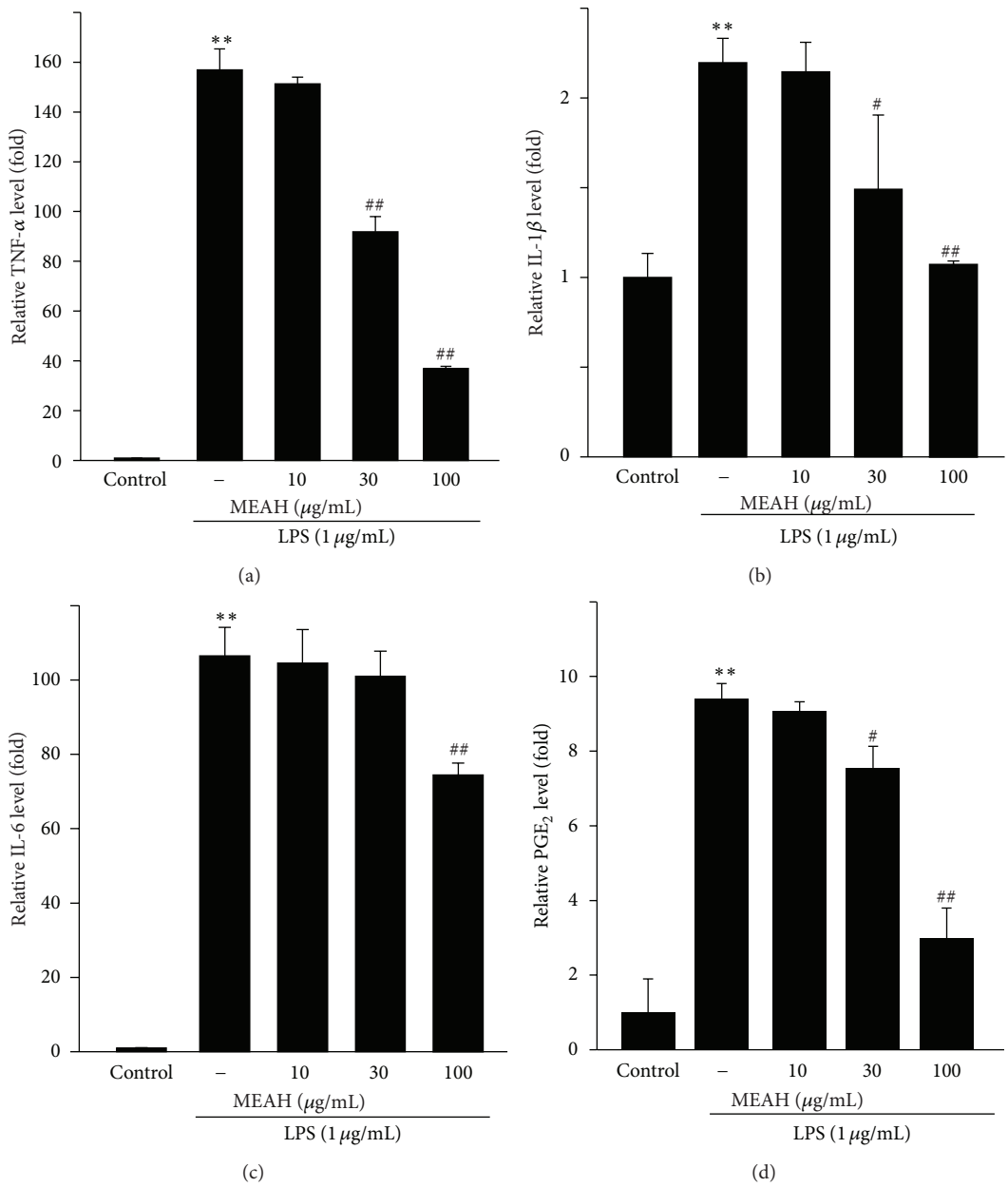

Figure 2: Effect of MEAH on the secretion of proinflammatory mediators in LPS-stimulated Raw264.7 cells. The cells were pretreated with 10–100 μg/mL MEAH for 1 h and then continuously exposed to 1 μg/mL LPS for 18 h. The levels of TNF-α (a), IL-1β (b), IL-6 (c), and PGE_2 (d) were monitored in the medium by using ELISA. Data represent mean ± S.D. of three separated experiments (significant as compared with vehicle-treated control cells, $^{**}P < 0.01$; significant as compared with LPS-treated cells, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$; the level of proinflammatory mediators in control cells = 1).

cells, while LPS-treated cells induced strongly the COX-2 expression. Moreover, pretreatment with MEAH slightly, but significantly, blocked the expression of COX-2. Thus, MEAH inhibited both iNOS and COX-2 expression in LPS-stimulated Raw264.7 cells.

3.4. Effect of MEAH on NF-κB Signaling Pathway in Raw264.7 Cells. As a plausible molecular mechanism for MEAH-mediated inhibition of inflammatory response, the effect of MEAH on the NF-κB signaling pathway was explored (Figure 4). LPS exposure (1 μg/mL) for 1 h facilitated the degradation of I-κBα, phosphorylation of I-κBα, and nuclear accumulation of NF-κB. However, pretreatment with 100 μg/mL MEAH inhibited the degradation of I-κBα. Furthermore, MEAH decreased the LPS-mediated I-κBα phosphorylation and nuclear NF-κB accumulation in a dose-dependent manner. Thus, the anti-inflammatory effect of MEAH might be due to inhibition of the NF-κB signaling pathway.

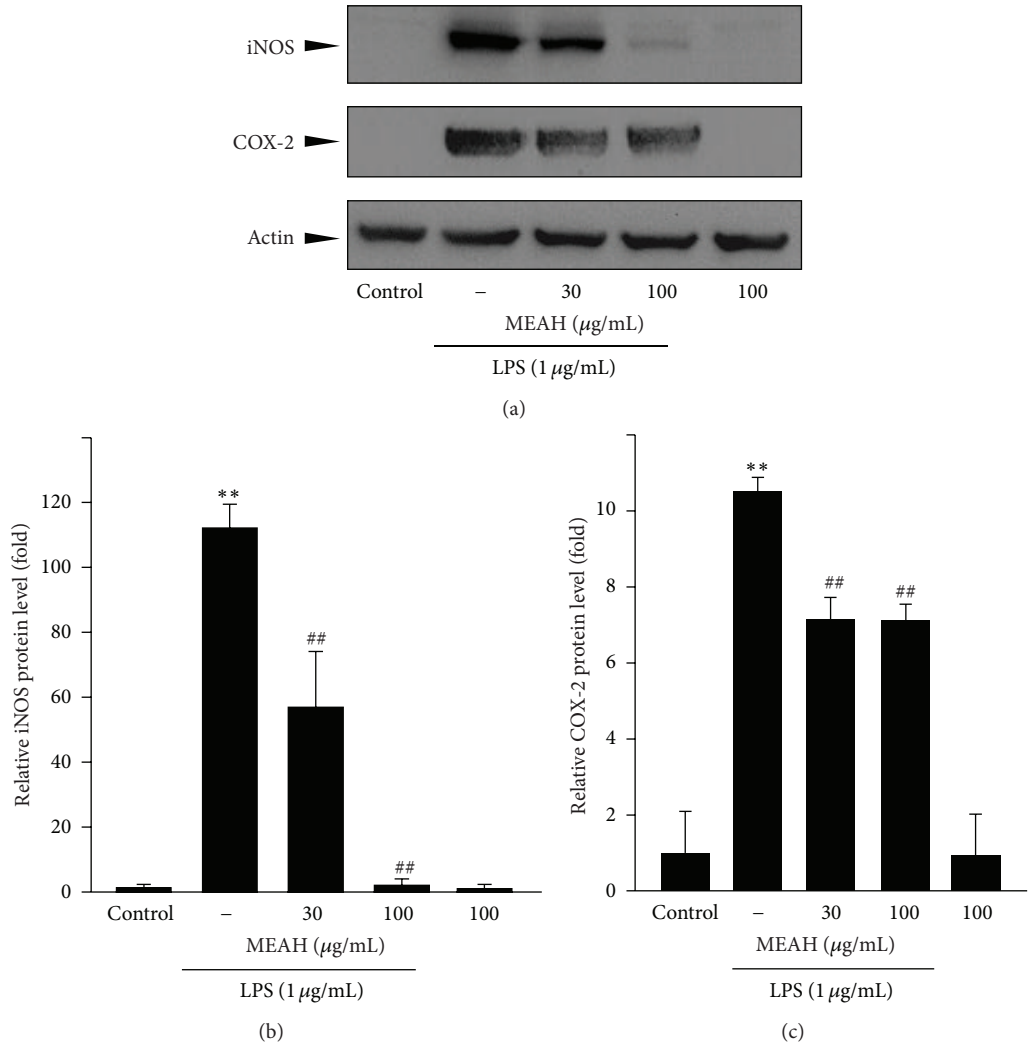

Figure 3: Effect of MEAH on the expression of proinflammatory enzymes in LPS-stimulated Raw264.7 cells. The cells were pretreated with 30 or 100 μg/mL MEAH for 1 h and then continuously exposed to 1 μg/mL LPS for 18 h. iNOS and COX-2 were immunoblotted in the cell lysates. Equal proteins loading among the samples was verified by actin immunoblotting (a). The relative iNOS and COX-2 protein levels were assessed by scanning densitometry (b, c). Data represent mean ± S.D. of three separated experiments (significant as compared with vehicle-treated control cells, $^{**}P < 0.01$; significant as compared with LPS-treated cells, $^{\#\#}P < 0.01$; the expression level of iNOS and COX-2 in control cells = 1).

3.5. Inhibitory Effect of MEAH on Carrageenan-Induced Paw Edema Formation. To explore anti-inflammatory effect of MEAH *in vivo*, the carrageenan-induced paw edema model was used. Results from plethysmometer showed that formation of paw edema began to be observed as early as 1 h after a carrageenan injection. Paw swelling was increased significantly at 2 h and sustained at least up to 4 h after carrageenan injection (Figure 5). Dexamethasone pretreatment (1.0 mg/kg/day, p.o.), a positive control drug, significantly decreased edema formation. Preadministration of MEAH (1.0 or 0.3 g/kg/day) for 3 days decreased carrageenan-induced paw edema, and reduction of swelling volumes persisted for at least 4 h. Reduction of edema volume was not changed significantly among observation times.

3.6. Histomorphometric Effect of MEAH in Carrageenan-Induced Paw Edema. Histomorphometrical measurements of *dorsum* and *ventrum pedis* skin were carried out to further validate the *in vivo* anti-inflammatory effect of MEAH (Table 1). As shown in Figures 6 and 7, marked increases of the *dorsum* and *ventrum pedis* skin thicknesses were detected as results of carrageenan-induced acute edematous inflammations on carrageenan-treated rats, and marked increases of infiltrated inflammatory cells were also detected in both *dorsum* and *ventrum pedis* cutaneous regions, respectively. However, these carrageenan-induced acute edematous changes and inflammatory cell infiltrations were significantly blocked by treatment with two different doses of MEAH (Table 1). The thicknesses of *dorsum pedis* skin in 1.0 and 0.3 g/kg/day MEAH-treated groups were significantly reduced by −23.79%

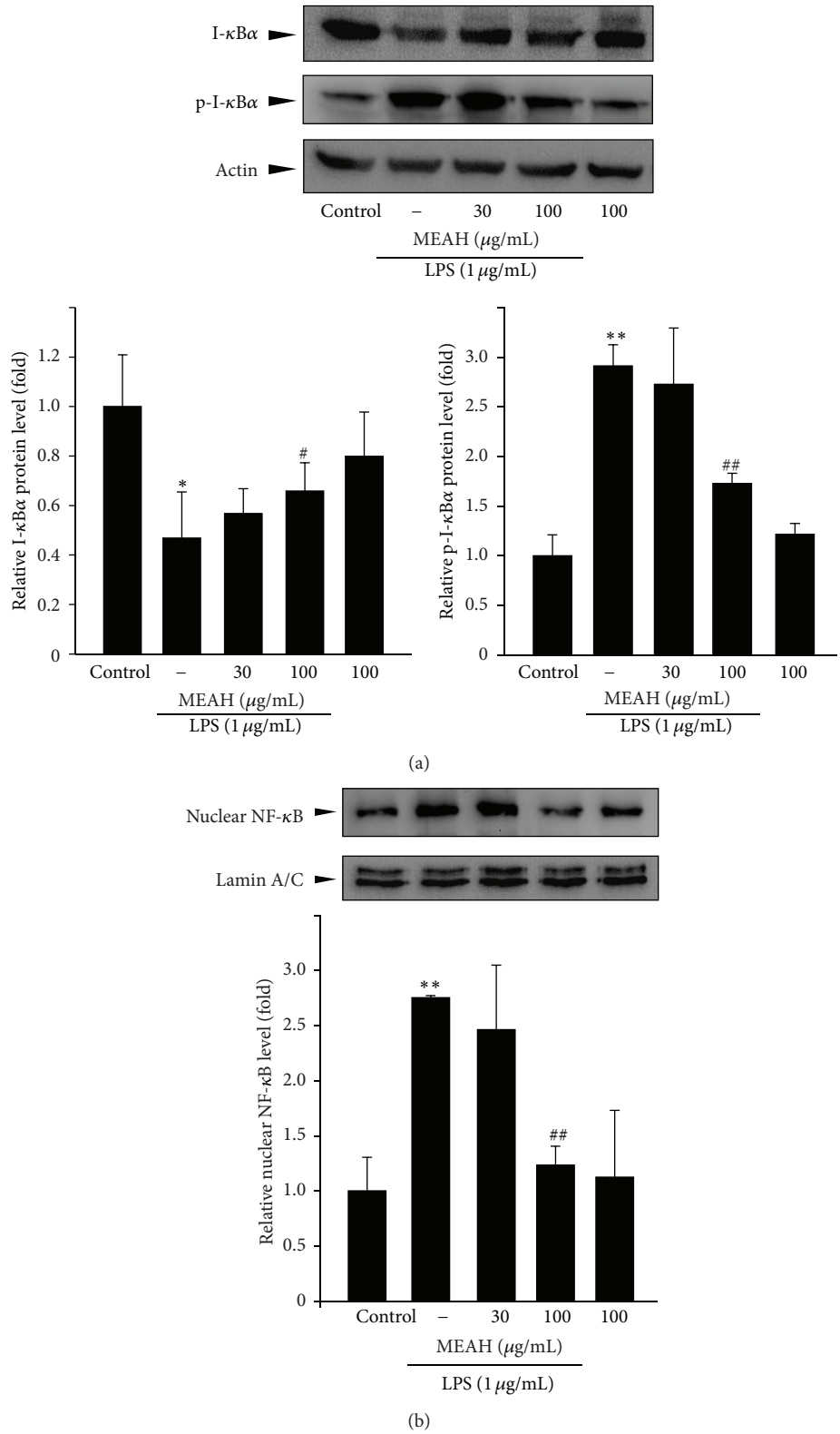

Figure 4: Effect of MEAH on NF-κB signaling pathway in LPS-stimulated Raw264.7 cells. The cells were pretreated with 30 or 100 μg/mL MEAH for 1 h and then continuously exposed to 1 μg/mL LPS for 1 h. Total or phosphorylated I-κBα in whole cell lysates (a) and nuclear NF-κB (b) were assessed by immunoblotting analysis. Antibody against actin or lamin A/C was used for verifying equal protein loading of cell lysates or nuclear fractions, respectively. Data represent mean ± S.D. of three separated experiments (significant as compared with vehicle-treated control cells, $^{**}P < 0.01$, $^{*}P < 0.05$; significant as compared with LPS-treated cells, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$; the expression level of each protein in control cells = 1).

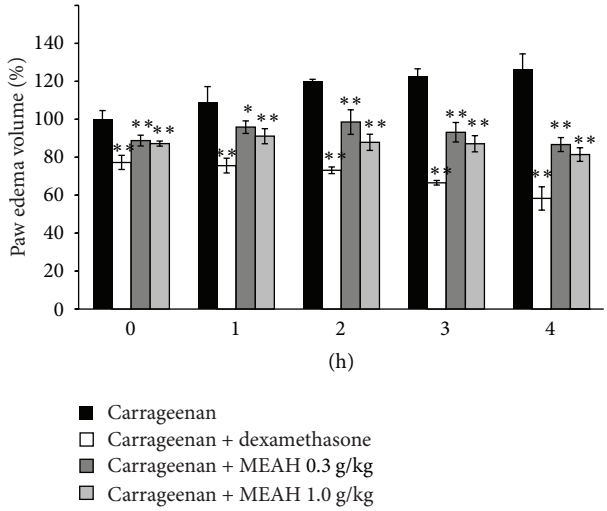

Figure 5: Inhibition of carrageenan-induced paw edema formation by MEAH. MEAH, dissolved in 40% PEG, was orally administered to rats at 0.3 or 1.0 g/kg/day prior to the induction of paw edema for three days. Paw edema was induced by subcutaneous injection of 1% carrageenan solution as described in methods. The swelling volume of paw was measured up to 4 h after 0.5 h carrageenan injection at intervals of 1 h by using plethysmometer. Dexamethasone (1 mg/kg, p.o.) was used as a positive drug. Data represent mean ± S.D. of five animals (significant as compared with carrageenan-treated group, $^{**}P < 0.01$; $^{*}P < 0.05$, paw volume at 0 h in carrageenan-treated rat = 100%).

and −13.12% compared with carrageenan-treated rats, respectively. Moreover, the numbers of infiltrated inflammatory cells in the *dorsum pedis* skin were significantly decreased by −63.41% and −25.97% in 1.0 and 0.3 g/kg/day MEAH-treated groups, respectively (Figure 6 and Table 1). High dose of MEAH treated group (1.0 g/kg/day) further decreased both the thickness of edematous skin and the number of infiltrated inflammatory cells. Similar decreases in skin thickness (−29.02% and −18.93% reduction in 1.0 and 0.3 (g/kg/day) MEAH-treated groups, resp.) and the numbers of infiltrated inflammatory cells (−61.93% and −30.14% reduction in 1.0 and 0.3 (g/kg/day) MEAH-treated groups, resp.) were also observed in *ventrum pedis* skin (Figure 7 and Table 1). Therefore, the results from the carrageenan-induced paw edema model indicate that MEAH effectively attenuated the acute phase of inflammatory swelling and infiltration of inflammatory cells.

4. Discussion

A. apiacea Hance is distributed on river beaches of East Asia. Traditionally, *A. apiacea* Hance has the effects of removing fever from the blood and has been widely prescribed to cure human disorders including malaria, jaundice, skin disorders, and dyspeptic complaints in oriental medicine [9]. The compounds isolated from *Artemisia* species such as terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids, and sterol were shown to have antimalarial, antiviral, antitumor, antipyretic, antihemorrhagic, anticoagulant, antianginal, antioxidant, antihepatitis, antiulcerogenic, antispasmodic, anticomplementary, and interferon-inducing activities [14]. Among them, artemisin and its congeners have been used as standard treatment for malaria. In addition, the therapeutic potential of those compounds as anticancer, antiangiogenesis, antiviral, immunosuppressive, and antifungal agents has been extensively tested [11]. In an effort to identify active constituents of MEAH, MEAH was additionally analyzed by GC-MS. Unfortunately, we could not detect artemisin in MEAH by GC-MS analysis. Our supplementary results indicated that fatty acids and their derivatives are among the major compounds found in MEAH (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/494681). Interestingly, it has been reported that uniphat A60 (methyl palmitate) reduced the inflammatory phenotypes in several experimental animal models [15]. Moreover, uniphat A60 inhibited phagocytic activity and decreased the expression of proinflammatory genes through NF-κB inactivation in macrophage lineage cells [16–19]. Scopoletin found in MEAH also suppressed the production of proinflammatory mediators in LPS-stimulated Raw264.7 cells [20]. Therefore, the compound (or combination of compounds) that accounts for the anti-inflammatory effect of MEAH needs to be clarified in the near future. Regardless of a number of studies about therapeutic potential of the constituents in *A. apiacea* Hance, the pharmacological effect of *A. apiacea* Hance against acute inflammation has not been fully established. As far as new pharmacological aspects of *A. apiacea* Hance, the results from this study clearly showed that MEAH inhibited the expression of proinflammatory enzymes and cytokines in LPS-stimulated Raw264.7 cells and also reduced paw edema in rats injected with carrageenan.

Endotoxins are the prominent initiators of the pathological process of inflammation in the human body. In particular, LPS can directly activate immune cells such as macrophages, which have an important role in immune response [21]. In the progression of inflammation, NO, prostanoids, TNF-α, and ILs are known as the most important mediators produced by macrophages [22, 23]. Among signaling molecules associated

Table 1: Changes in the histomorphometrical analysis of hind paw skins.

Groups	Thickness (epidermis to dermis, mm)	Infiltrated inflammatory cells (cells/mm^2 of dermis)
Dorsum pedis skin		
Carrageenan	2.362 ± 0.168	118.60 ± 10.21
Carrageenan + dexamethasone	0.754 ± 0.082**	31.00 ± 5.96**
Carrageenan + MEAH 1.0 g/kg	1.800 ± 0.128**	43.40 ± 5.23**
Carrageenan + MEAH 0.3 g/kg	2.052 ± 0.090**	87.80 ± 10.71**
Ventrum pedis skin		
Carrageenan	1.268 ± 0.039	1263.60 ± 179.74
Carrageenan + dexamethasone	0.776 ± 0.048**	86.40 ± 12.70**
Carrageenan + MEAH 1.0 g/kg	0.900 ± 0.052**	481.00 ± 55.86**
Carrageenan + MEAH 0.3 g/kg	1.028 ± 0.081**	882.80 ± 109.97**

The thicknesses of *dorsum* and *ventrum pedis* and infiltrated inflammatory cells were measured using automated image analyzer. All values are expressed as mean ± S.D. of five rat hind paws (significant as compared with carrageenan-treated group, $^{**}P < 0.01$).

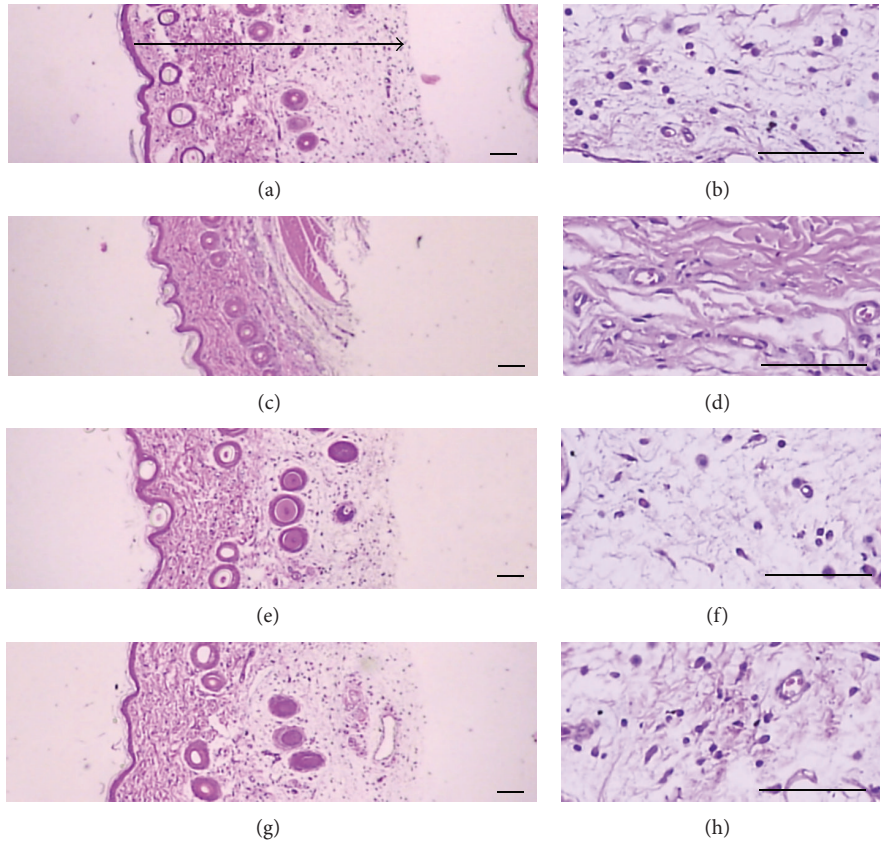

Figure 6: Representative histological images of the *dorsum pedis* skin in carrageenan (a, b), carrageenan + dexamethasone (c, d), carrageenan + 1.0 g/kg MEAH (e, f), and carrageenan + 0.3 g/kg MEAH (g, h) treated groups. After 4 h of carrageenan treatment, *dorsum pedis* skins were separated, and fixed in 10% neutral buffered formalin, then embedded in paraffin, sectioned and stained with hematoxylin and eosin. The arrow indicated total thickness measured (scale bars = 160 μm).

with the production of these inflammatory mediators, iNOS and COX-2 are the most crucial enzymes involved in the production of NO and PGs [24, 25]. In this study, we verified the ability of MEAH to inhibit the production of NO and PGE_2 in LPS-stimulated Raw264.7 macrophage cells. Treatment with MEAH effectively decreased NO and PGE_2 production in the medium. Next, we assessed the level of iNOS and COX-2 proteins by immunoblot analyses. Pretreatment with MEAH significantly inhibited iNOS and COX-2 expression. Although MEAH markedly blocked iNOS induction, its inhibition of COX-2 was slight, but significant, showing that anti-inflammatory activity of MEAH more likely results from its inhibition of iNOS than of COX-2.

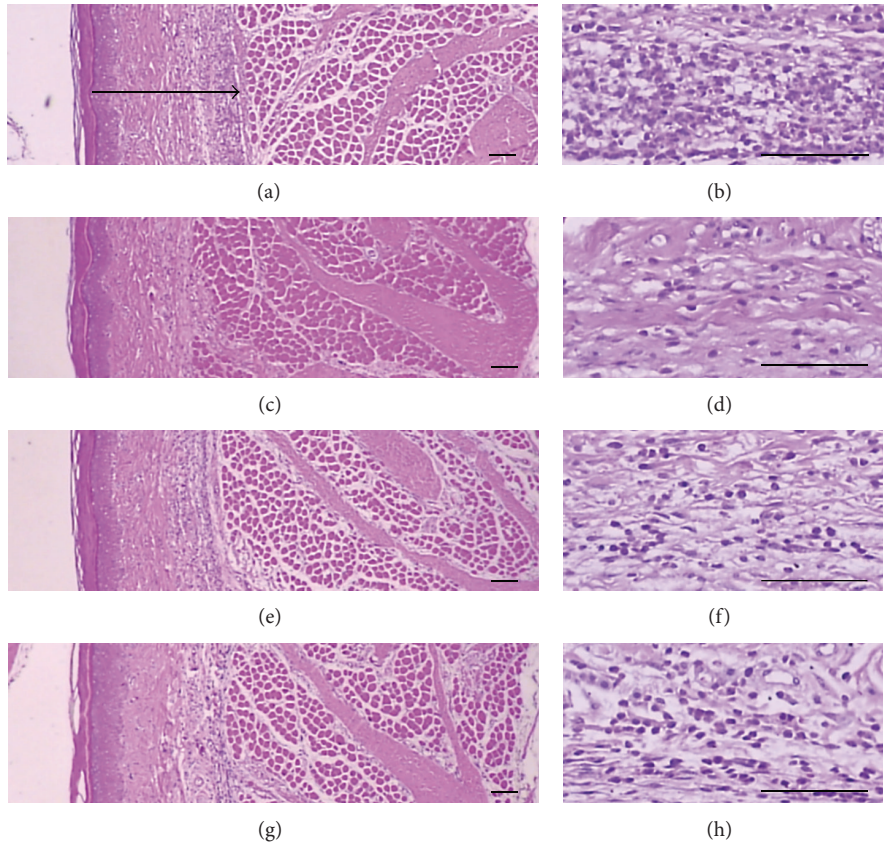

Figure 7: Representative histological images of the *ventrum pedis* skin in carrageenan (a, b), carrageenan + dexamethasone (c, d), carrageenan + 1.0 g/kg MEAH, (e, f), and carrageenan + 0.3 g/kg MEAH (g, h) treated groups. Tissue sections were prepared as described in Figure 6. The arrow indicated total thickness measured (scale bars = 160 μm).

Among the inflammatory cytokines, TNF-α, IL-1β, and IL-6 are the most crucial mediators in the inflammatory process, mediating immunity and activating macrophages [6, 26]. TNF-α, the key mediator in inflammatory responses, can activate immune cells including macrophages [27]. In the process of endotoxin-induced organ injury, TNF-α is regarded as a principal mediator in stimulating secretion of other cytokines. IL-1β and IL-6 are other important inflammatory mediators that are mainly synthesized by immune cells and play a pivotal role in the acute phase of inflammation [28]. They are also mediators of the host inflammatory response in innate immunity in association with gram negative sepsis. We verified the effects of MEAH on secretion of the cytokines in LPS-stimulated Raw264.7 cells. MEAH pretreatment significantly decreased the ability of LPS to induce production of TNF-α, IL-1β, and IL-6 in the macrophages. These results showing that MEAH inhibits inflammatory cytokine production as well as iNOS and COX-2 expression indicate that MEAH has an anti-inflammation role through inactivation of macrophages. Therefore, we next examined the effect of MEAH on the representative transcription factor in association with acute inflammation.

The binding of LPS to the Toll-like receptor-4 recruits a variety of adaptor molecules including MyD88, TIRAP/Mal, TRIF, and TRAM and then activates the NF-κB pathway for the induction of proinflammatory genes [29, 30]. After phosphorylation and subsequent degradation of I-κBα, NF-κB, which is dissociated from the NF-κB/I-κB complex, translocates into the nucleus and then binds to the promoter region for induction of the inflammatory response. Because NF-κB binding to the promoter of iNOS, COX-2, TNF-α, IL-1β and IL-6 genes is essential for the gene induction [28, 31–34], the effect of MEAH on the NF-κB signaling pathway was investigated as a plausible anti-inflammatory mechanism. The results from this study suggest that MEAH pretreatment attenuated the degradation of I-κBα, the phosphorylation of I-κBα, and nuclear accumulation of NF-κB.

In the animal model of acute inflammation, the paw edema model using carrageenan is one of the most well-established models in association with edema formation [7]. This animal model is frequently used for screening of novel anti-inflammatory drugs. In the pathological process of inflammation, swelling is induced by an increase in vascular permeability and results in inflammation by leading to infiltration of inflammatory cells in the sites. Due to the importance of swelling in the inflammation process and the demand for *in vivo* verification of the MEAH effect, we confirmed the ability of MEAH to inhibit swelling of paw injected with carrageenan in rats. Carrageenan treatment significantly induced paw swelling, and administration

of MEAH to rats markedly decreased the induction of paw edema. Carrageenan-induced paw swelling might be related to the release of NO in the peripheral tissue [35]. It has been also reported that carrageenan induces the release of TNF-α and ILs in the tissue [36]. Therefore, the inhibitory effects of MEAH on formation of swelling might, at least in part, be associated with the inhibition of NO and TNF-α productions.

In conclusion, the present results demonstrated that methanol extracts of *A. apiacea* Hance inhibited the production of NO through the inhibition of iNOS expression in LPS-stimulated Raw264.7 cells. In addition, MEAH attenuated the induction of COX-2 and other proinflammatory cytokines (TNF-α, IL-1β, and IL-6) by LPS. Downregulation of the NF-κB signaling pathway was also involved in the decrease in proinflammatory enzymes and cytokines expressions. Furthermore, MEAH treatment decreased carrageenan-induced inflammatory swelling and the number of infiltrated inflammatory cells *in vivo*. The present findings showing the inhibition of inflammatory response both *in vitro* and *in vivo* increase our understanding of the novel pharmacologic aspects of *A. apiacea* Hance and suggest its potential as a novel therapeutic candidate for managing inflammatory disorders.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Ji Choul Ryu and Sang Mi Park contributed equally to this work.

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Research Article

Antibacterial Activity of *Rhus javanica* against Methicillin-Resistant *Staphylococcus aureus*

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In the present study, the leaves of *Rhus javanica* (*R. javanica*) were extracted with ethanol, and we investigated the antimicrobial activity of the ethanol extract of *R. javanica* against methicillin-resistant *Staphylococcus aureus* (MRSA). Control groups were treated with media containing 0.1% DMSO. The ethanol extract of *R. javanica* inhibited the growth of MRSA at concentrations ranging from 0.05 to 0.2 mg/mL and inhibited acid production at concentrations higher than 0.1 mg/mL ($P < 0.05$). MRSA biofilm formation was determined by scanning electron microscopy and safranin staining. The ethanol extract of *R. javanica* inhibited the formation of MRSA biofilms at concentrations higher than 0.05 mg/mL. In confocal laser scanning microscopy, high concentration (0.4–1.6 mg/mL) of *R. javanica* extract showed bactericidal effect in a dose-dependent manner. In real-time PCR analysis, *R. javanica* extract showed the inhibition of the genetic expression of virulence factors such as *mecA*, *sea*, *agrA*, and *sarA* in MRSA. Preliminary phytochemical analysis revealed the strong presence of phenolics. These results suggest that *R. javanica* may be a useful medicinal plant for inhibiting MRSA, which may be related to the presence of phenolics in the *R. javanica* extract.

1. Introduction

Staphylococcus aureus has been reported to cause many diseases, from mild skin infections to more serious invasive infections such as suppuration, abscess formation, pneumonia, and even fatal septicemia in human beings [1]. Prior to the antibiotic era, *S. aureus* was associated with 80% mortality, but the advent of the earliest antimicrobial substances, such as penicillin, contributed to a reduction in mortality [2]. It was reported that over 94% of *S. aureus* strains were susceptible to penicillin at that time. However, antibiotic resistance in *S. aureus* has rapidly developed due to the widespread use of antibiotics [2, 3]. Methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated in the early 1960s [4]. MRSA is resistant to not only methicillin and other β-lactams but also many other antibacterial agents such as macrolides and aminoglycosides [5, 6]. MRSA is one of important causes of modern chronic infectious diseases [3, 5, 6]. One such antibiotic-resistant mechanism involves the production of β-lactamase or penicillin-binding protein 2a (PBP 2a) by MRSA [2]. MRSA forms biofilms on implanted medical devices such as catheters, plates, screws, artificial joints, and cardiac valves in patients [7, 8]. Biofilm formation by MRSA has been reported to increase the antibiotic resistance of MRSA [9]. MRSA metabolizes carbohydrates to produce organic acids, which can stimulate biofilm formation [10, 11]. At present, MRSA is emerging worldwide as one of the most important hospital and community pathogens due to its multidrug resistance. Therefore, new strategies are required to deal with MRSA-associated infections. Recently, scientists have focused on certain natural products with antipathogenic potential as candidates for new antibiotic substances [12, 13].

Rhus javanica (*R. javanica*) is a member of Anacardiaceae and is mainly produced in Korea, China, and Japan [14, 15]. It has been traditionally used to treat dysentery and diarrhea [15]. However, there is little scientific evidence regarding the effect of *R. javanica* on MRSA. In the course of screening for the antibacterial activities of natural products against

MRSA, we recently found that extracts of *R. javanica* exhibit antibacterial activity against this pathogen. In the present study, we show that *R. javanica* has antimicrobial activity against and inhibits biofilm formation by MRSA. We also show the presence of major phytochemicals in *R. javanica*.

2. Materials and Methods

2.1. Materials. Brain heart infusion (BHI) broth was purchased from Difco Laboratories (Detroit, MI, USA). Glucose and dimethyl sulfoxide (DMSO) were obtained from Sigma Co. (St. Louis, MO, USA). MRSA ATCC 33591 was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA).

2.2. Plant Material and Extraction. The leaves of *R. javanica* were obtained from the oriental drug store Dae Hak Yak Kuk (Iksan, South Korea). The identity of the specimen was confirmed by Dr. Bong-Seop Kil at the Department of Natural Science, Wonkwang University (Iksan, South Korea). A voucher specimen (number 05-11) has been deposited at the Herbarium of the Department of Oral Biochemistry in Wonkwang University. Dried leaves (600 g) of *R. javanica* were soaked in 3000 mL of 90% ethanol for 72 h at room temperature. The extracted solution was filtered and evaporated under reduced pressure to yield an ethanol extract of 42.4 g (7.1%). After the extract was thoroughly dried to facilitate complete removal of the solvent, the dry extract was dissolved in DMSO to give the desired stock solution. The final concentration of DMSO applied to culture systems was adjusted to 0.1% (v/v), which did not interfere with the testing system. Control groups were treated with media containing 0.1% DMSO.

2.3. Bacterial Growth and Acid Production. Bacterial growth was determined using a modification of a previously described method [16, 17]. The growth of MRSA was examined at 37°C in 0.95 mL of BHI broth containing various concentrations of the ethanol extract of *R. javanica*. These tubes were inoculated with 0.05 mL of an overnight culture grown in BHI broth (final: 5×10^5 colony-forming units (CFU)/mL), and incubated at 37°C. After 24 h of incubation, the optical density (OD) of cells was measured spectrophotometrically at 550 nm, and the pH of the cultures was determined using a pH meter (Corning Inc., Corning, NY, USA). Three replicates were measured for each concentration of the test extract. NaF (1%) was used as a positive control.

2.4. Biofilm Assay. The biofilm assay was based on a method described previously [18, 19]. *R. javanica* extract was added to BHI broth containing 1% glucose in 35 mm polystyrene dishes (Nunc, Copenhagen, Denmark). The cultures were then inoculated with a seed culture of MRSA (final: 5×10^5 CFU/mL). After cultivating for 48 h at 37°C, the supernatant was removed completely, and the dishes were rinsed with distilled H_2O. The biofilm formed on the surface of the dishes was also stained with 0.1% safranin, and photographed.

2.5. Scanning Electron Microscopy (SEM). The biofilm on 35-mm polystyrene dishes was also determined by SEM using a modification of a previously described method [20]. The biofilm formed on the dishes was rinsed with distilled H_2O and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 24 h. After gradual dehydration with ethyl alcohol (60%, 70%, 80%, 90%, 95%, and 100%), the sample was freeze-dried. The specimens were then sputter-coated with gold (108A sputter coater, Cressington Scientific Instruments Inc., Watford, England, UK). For observation, a JSM-6360 SEM (JEOL, Tokyo, Japan) was used.

2.6. Bactericidal Effect of R. javanica Extract. Bactericidal effect of *R. javanica* extract was determined by confocal laser scanning microscopy. The cultured MRSA in BHI was diluted using BHI media to approximately 1×10^7 CFU/mL. The bacteria (1×10^7 CFU/mL) were treated with high concentrations (0.2–1.6 mg/mL) of *R. javanica* extract at 37°C under aerobic conditions. After 30 min of incubation, the bacteria were washed with PBS and stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA), prepared according to the manufacturer's instructions, for 15 min. Stained bacteria were observed confocal laser scanning microscopy (LSM 510, Zeiss, Germany). This method is based on two nucleic acid stains: green fluorescent SYTO 9 stain and red fluorescent propidium iodide stain which differ in their ability to penetrate healthy bacterial cells. SYTO 9 stain labels live bacteria, in contrast propidium iodide penetrates only bacteria with damaged membranes.

2.7. Real-Time Polymerase Chain Reaction (PCR) Analysis. To determine the effect of *R. javanica* extract on gene expression, a real-time PCR assay was performed. The subminimal inhibitory concentration (0.01–0.1 mg/mL) of *R. javanica* extract was used to treat and culture MRSA for 24 h. Total RNA was isolated from *S. mutans* by using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. Then, cDNA was synthesized using a reverse transcriptase reaction (Superscript; Gibco-BRL). The DNA amplifications were carried out using an ABI-Prism 7,000 Sequence Detection System with Absolute QPCR SYBR Green Mixes (Applied Bio systems Inc., Foster City, CA, USA). The primer pairs that were used in this study were described by previous reports [21–23] and are listed in Table 1. 16S rRNA was used as an internal control.

2.8. Phytochemical Screening. Phytochemical tests of the extract were performed as previously described [24, 25]. Mayer's reagent was used for alkaloids, ferric chloride reagent for phenolics, Molish test for glycosides, Biuret reagent for peptides, Mg-HCl reagent for flavonoids, Libermann-Burchard reagent for steroids, and silver nitrate reagent for organic acids.

2.9. Statistical Analysis. All experiments were performed in triplicate. Data were analyzed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). The data are expressed as the mean ± standard deviation values. The

Table 1: Nucleotide sequences of primer used for real-time PCR in this study.

Gene	Gene description		Primer sequences (5′-3′)
16S rRNA	Normalizing internal standard	Forward	ACTGGGATAACTTCGGGAAA
		Reverse	CGTTGCCTTGGTAAGCC
mecA	Penicillin binding protein 2′	Forward	GTTAGATTGGGATCATAGCGTCATT
		Reverse	TGCCTAATCTCATATGTGTTCCTGTAT
sea	Staphylococcal enterotoxin A	Forward	ATGGTGCTTATTATGGTTATC
		Reverse	CGTTTCCAAAGGTACTGTATT
agrA	Accessory gene regulator A	Forward	TGATAATCCTTATGAGGTGCTT
		Reverse	CACTGTGACTCGTAACGAAAA
sarA	Staphylococcal accessary regulator A	Forward	TGTTATCAATGGTCACTTATGCTG
		Reverse	TCTTTGTTTTCGCTGATGTATGTC

differences between the means of the experimental and control groups were evaluated by Student's t-test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Bacterial Growth Inhibition by R. javanica. In the present study, we investigated the antibacterial activity of the extract of *R. javanica* against MRSA. The bacteria were exposed to 0.01, 0.05, 0.1, and 0.2 mg/mL of the ethanol extract of *R. javanica*. As seen in Figure 1, the extract (0.05–0.2 mg/mL) showed antibacterial activity against MRSA in a dose-dependent manner and resulted in high MRSA growth inhibition at concentration >0.1 mg/mL compared to the control group ($P < 0.05$). The positive control (0.1% NaF) also indicated antibacterial activity. The minimum inhibitory concentration (MIC) for the ethanol extract of *R. javanica* is 0.1 mg/mL. The determination of the MIC revealed the antimicrobial activity of the ethanol extract of *R. javanica* against MRSA.

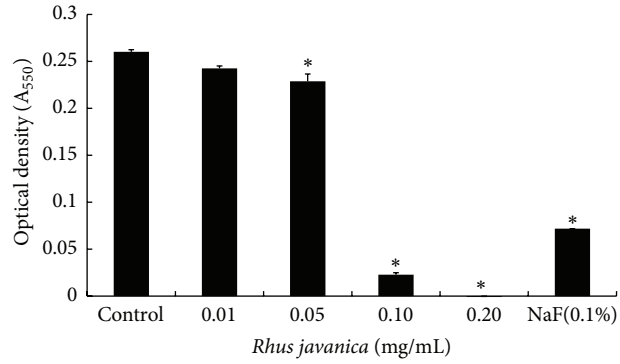

Figure 1: Effect of ethanol extract of *R. javanica* on the growth of MRSA. MRSA was inoculated into BHI broth with various concentrations of *R. javanica* and incubated for 24 h at 37°C. The optical density (A_{550}) was read using a spectrophotometer. Data are mean ± standard deviation. $^*P < 0.05$ compared to the control group.

Table 2: Effect of ethanol extract of *R. javanica* on acid production by MRSA.

Concentration (mg/mL)	pH (before incubation)	pH (after incubation)
Control	7.20 ± 0.05	5.80 ± 0.05[1]
0.01	7.13 ± 0.05	5.13 ± 0.20
0.05	7.13 ± 0.04	5.73 ± 0.36*
0.10	7.13 ± 0.05	6.67 ± 0.23*
0.20	7.13 ± 0.00	7.17 ± 0.40*
NaF (0.1%)	7.20 ± 0.00	7.18 ± 0.20*

[1]Data (pH) are represented as mean ± standard deviation. $^*P < 0.05$ compared to the control group after incubation.

3.2. Inhibitory Effect of R. javanica on Acid Production. Acid productions of the bacteria treated with *R. javanica* extract were also monitored. As summarized in Table 2, there was an obvious decrease in pH in the control group, but the decrease was substantially inhibited in the presence of the extract (0.1–0.2 mg/mL). The decrease in pH was also inhibited in the positive control group (0.1% of NaF). These results demonstrate that *R. javanica* extract may inhibit organic acid production in MRSA.

3.3. Inhibitory Effect of R. javanica on Biofilm Formation. We examined the inhibitory effect of *R. javanica* extract on MRSA biofilm formation by safranin staining. As shown in Figure 2, the extract of *R. javanica* (0.05–0.2 mg/mL) inhibited biofilm formation by MRSA. Biofilm formation was also inhibited in the presence of the positive control (0.1% NaF). SEM photographs underline the results obtained by safranin staining (Figure 3). MRSA attached to and aggregated on the surface of polystyrene 35 mm dishes and visibly formed the biofilm in the control group, but biofilm formation was lower in the presence of *R. javanica* extract at concentrations higher than 0.05 mg/mL. Biofilm formation was also lower in the presence of the positive control.

3.4. Bacteriocidal Effect of R. javanica. Bactericidal effect of *R. javanica* extract was tested by confocal laser scanning microscopy (Figure 4). Bacterial viability was gradually decreased at high concentration (0.4–1.6 mg/mL) of *R. javanica* extract in a dose-dependent manner.

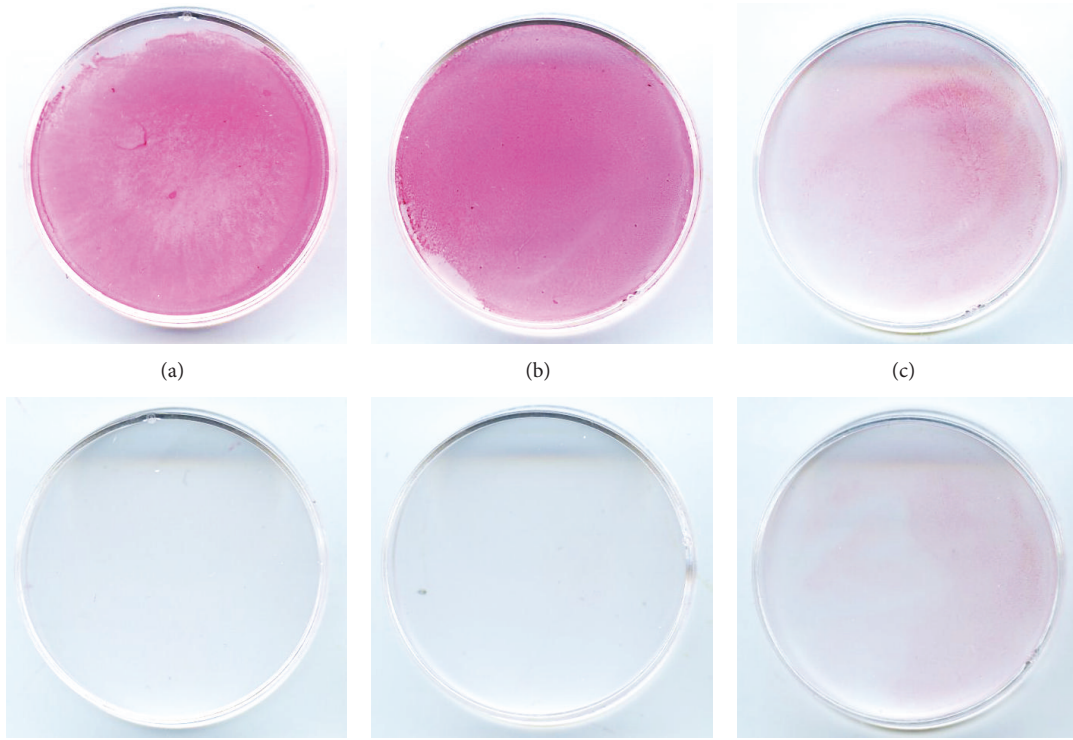

Figure 2: Effect of ethanol extract of *R. javanica* on biofilm formation by MRSA. MRSA was inoculated into BHI broth with various concentrations of *R. javanica* and incubated for 48 h at 37°C. The biofilm formed on the surface of the dishes was also stained with 0.1% safranin and photographed. (a) Control; (b) 0.01 mg/mL; (c) 0.05 mg/mL; (d) 0.1 mg/mL; (e) 0.2 mg/mL; (f) positive control (0.1% NaF).

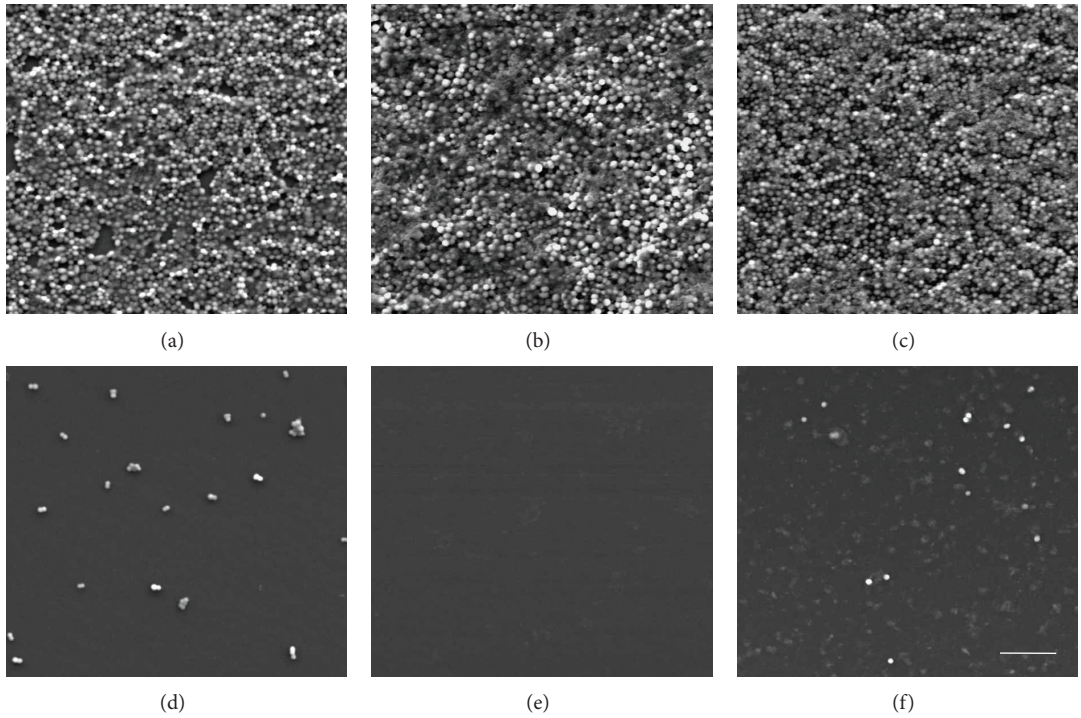

Figure 3: Scanning electron microscopy of MRSA biofilms grown in ethanol extract of *R. javanica*. (a) Control; (b) 0.01 mg/mL; (c) 0.05 mg/mL; (d) 0.1 mg/mL; (e) 0.2 mg/mL; (f) positive control (0.1% NaF); bar = 10 μm.

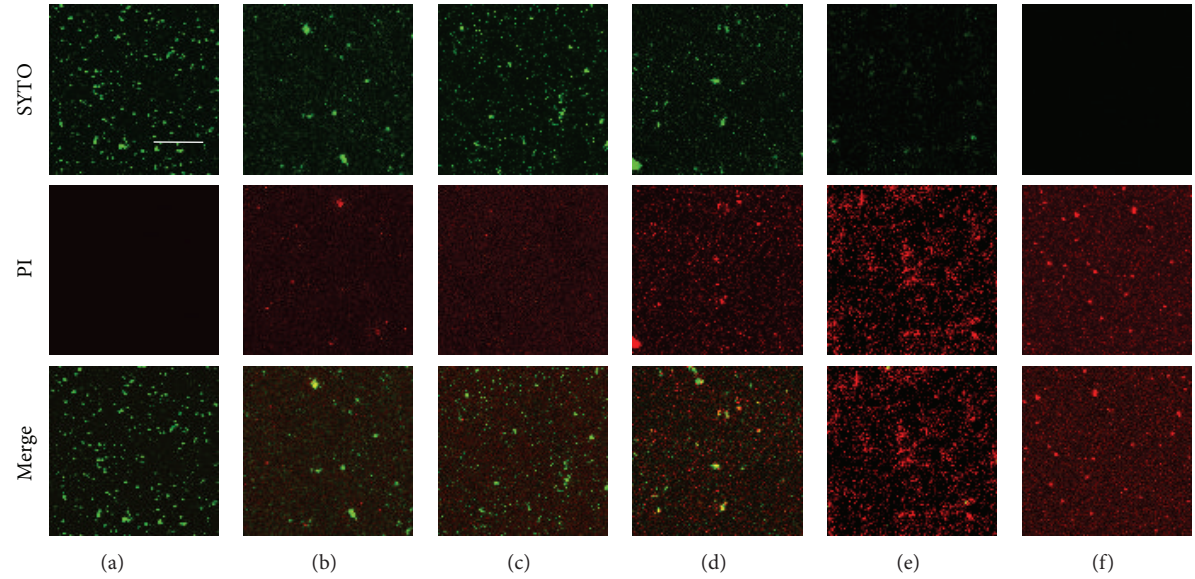

Figure 4: Bactericidal effect of ethanol extract of *R. javanica*. Cultured MRSA was treated with high concentration (0.2–1.6 mg/mL) of *R. javanica* extract and stained with LIVE/DEAD BacLight Bacterial Viability Kit. The stained bacteria were observed by confocal laser scanning microscopy. Treatment with ethanol extract of *R. javanica* decreased green-labeled living bacteria (SYTO 9 stain) and increased red-labeled dead bacteria (PI stain) in a dose-dependent manner. (a) Control; (b) 0.2 mg/mL; (c) 0.4 mg/mL; (d) 0.8 mg/mL; (e) 1.6 mg/mL; (f) positive control (0.1% NaF); bar = 50 μm.

3.5. Inhibitory Effect of R. javanica on Virulence Factor Gene Expression. Real-time PCR analysis was performed to examine the effect of *R. javanica* extract on the genetic expression of virulence factors in MRSA (Figure 5). The expression of *mecA*, *sea*, *agrA*, and *sarA* was significantly decreased in MRSA ($P < 0.05$) when it was treated with *R. javanica* extract.

3.6. Phytochemical Analysis. The results of the phytochemical tests conducted on the ethanol extract of *R. javanica* are shown in Table 3. Preliminary phytochemical analyses revealed the strong presence of phenolics, the moderate presence of glycosides, and the weak presence of flavonoids, steroids (terpenoids), and organic acids. These results suggest that phenolics may have been responsible for the antibacterial activity observed in the present study.

4. Discussion

In the present study, we examined the antimicrobial activity of *R. javanica* extract against MRSA. Our data show that 0.05–0.2 mg/mL of the ethanol extract of *R. javanica* inhibited the growth of MRSA. The fact that the extract of *R. javanica* inhibited MRSA growth provides a scientific rationale for the use of this extract by local inhabitants as an antimicrobial agent. Traditionally, *R. javanica* has been used to treat dysentery and diarrhea in Korea, China, and Japan [15].

Previous studies have shown that MRSA is able to metabolize dietary carbohydrates and thereby produce organic acids [10, 11]. The major organic acid produced by MRSA is acetic acid, which can lower the pH of infected regions and contribute to the formation of firmly adhering biofilm-like microbial communities [10]. In the present study, the extract of *R. javanica* inhibited the decrease of pH induced by MRSA. This result suggests that the extract of *R. javanica* may inhibit carbohydrate metabolism in MRSA.

MRSA has the ability to adhere, colonize, and form biofilms on damaged tissue, implanted medical devices, and prosthetic devices [7, 8]. Biofilms are surface-associated bacterial communities on biological or abiotic substrata. They are enclosed firmly in a self-produced extracellular matrix composed of polysaccharides and proteins. Biofilms are very difficult to remove and are a source of refractory infections. Biofilm formation on the surface of implanted medical devices frequently requires surgical removal of the biofilm, debridement of the surrounding tissue, and long-term antibiotic treatment [7, 8]. The most well-known assay method for the detection of biofilm formation is the tissue culture plate assay method [9]. In our study, the extract of *R. javanica* inhibited biofilm formation by MRSA at concentrations ranging from 0.05 to 0.1 mg/mL, as seen by safranin staining. SEM data of MRSA biofilm formation were consistent with the data from safranin staining. However, the data on biofilm culture were different from the results of planktonic cell growth measurements. The growth of MRSA in planktonic culture was inhibited at concentrations higher than 0.05 mg/mL. A previous study has shown that bacteria in biofilm cultures are physiologically different from planktonic cells of the same organism. Biofilm formation enhances bacterial resistance to both the host defense system and antimicrobials [26]. However, in our study, bacterial resistance against *R. javanica* may be similar within both biofilm cultures and planktonic cultures.

Bactericidal effect of *R. javanica* extract was tested by confocal laser scanning microscopy. Bacterial viability was gradually decreased at high concentration (0.4–1.6 mg/mL) of *R. javanica* extract in a dose-dependent manner. This result

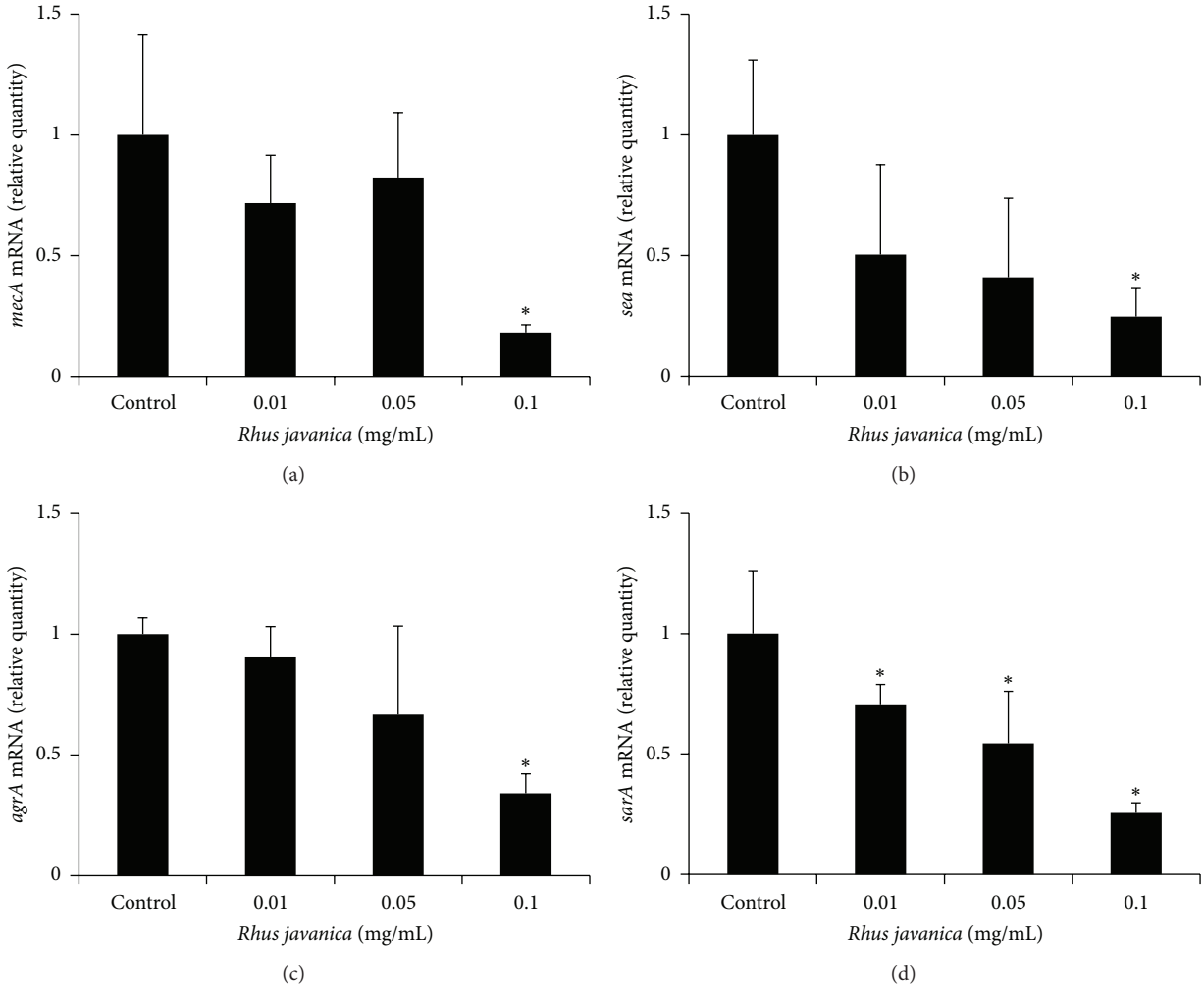

Figure 5: Real-time PCR analysis of expression of several virulence factor genes. MRSA was cultured and treated with sub minimal inhibitory concentration (0.01–0.1 mg/mL) of *R. javanica* extract, and real-time PCR analysis was then performed as described in Section 2. *mecA*, *sea*, and *agrA* expression was significantly inhibited at 0.1 mg/mL of *R. javanica* extract, and *sarA* was significantly inhibited at concentration higher than 0.01 mg/mL. Each value is expressed as a mean ± standard deviation. Significance was determined at $^{*}P < 0.05$ when compared with the control.

Table 3: Phytochemical analysis of the ethanol extract of *R. javanica*.

Plant constituents	Ethanol extract
Alkaloids	−
Phenolics	+++
Flavonoids	+
Glycosides	++
Peptides	−
Steroids, terpenoids	+
Organic acids	+

+++: strong; ++: moderate; +: weak; −: absent.

suggests that high concentration of *R. javanica* extract may be bactericidal on MRSA.

An antibiotic resistance gene, *mecA* encodes PBP2a, which has low affinity to β-lactam antibiotics, so it allow cell-wall biosynthesis despite the presence of β-beta-lactams [2]. In the present study, the effect of *R. javanica* extract on the genetic expression of *mecA* was determined by real-time PCR analysis. The expression of *mecA* was significantly decreased in MRSA when it was treated with *R. javanica* extract.

A virulence factor gene, *sea* encodes Staphylococcal enterotoxin A which is one of major virulence factors in MRSA [27]. Staphylococcal enterotoxin A is one cause of gastroenteritis in human and acts as a superantigen. In this study, the *R. javanica* extract significantly inhibited *sea* expression. *sea* gene expression in MRSA is regulated by global regulators such as *agr* and *sarA* gene [21]. In our study, *R. javanica* extract showed the inhibition of *agrA* and *sarA* expressionin MRSA. *agrA* encodes accessory gene regulator A which positively regulates exotoxin-encoding genes. *sarA* also upregulates expression of virulence factor genes. Previous research has shown that inhibition of *agrA* or *sarA* expression by some chemicals such as thymol or clindamycin reduces transcription of exotoxin-encoding genes [21].

In the present study, suppressive effect of *R. javanica* extract on *sea* gene expression may, in part, be related with inhibitory effect of *R. javanica* extract on *agrA* and *sarA* expression [28].

Previous reports indicate that the leaves of *R. javanica* contain mainly tannins (50–70%) such as tannic acid, pyrogallol, gallic methy lester, syringic acid, protocatechuic acid, and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose [14, 29, 30]. In the present study, we found the strong presence of phenolics, the moderate presence of glycosides, and the weak presence of flavonoids, steroids (terpenoids), and organic acids. These results suggest that phenolics may have been responsible for the antibacterial activity observed in the present study. However, further studies are needed to elucidate the antimicrobial principles underlying the action against MRSA.

In conclusion, our study demonstrated the antimicrobial activity of the ethanol extract of *R. javanica* against MRSA. *R. javanica* inhibited the growth, acid production, and biofilm formation of MRSA. *R. javanica* also showed bactericidal effect and the inhibition of the genetic expression of virulence factors such as *mecA*, *sea*, *agrA*, and *sarA*. Phytochemical analysis revealed the strong presence of phenolics. These results suggest that *R. javanica* may be a useful medicinal plant for inhibiting MRSA, which may be related to the presence of phenolics in the *R. javanica* extract.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Y.-O. You and N.-Y. Choi contributed equally to this work.

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Research Article

Ethanol Extract of *Ulmus pumila* Root Bark Inhibits Clinically Isolated Antibiotic-Resistant Bacteria

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In this study, root bark of *Ulmus pumila* (*U. pumila*) was extracted with ethanol, and then the antimicrobial effects were tested on clinically isolated 12 MRSA strains and 1 standard MRSA strain. *U. pumila* showed antibacterial activities against all MRSA strains. Minimum inhibitory concentration (MIC) of *U. pumila* root bark against all MRSA strains revealed a range from 125 to 250 μg/mL. These results may provide the scientific basis on which *U. pumila* root bark has traditionally been used against infectious diseases in Korea. In real-time PCR analysis, the sub-MIC (64–125 μg/mL) concentrations of *U. pumila* root bark extract showed the inhibition of the genetic expressions of virulence factors such as *mecA, sea, agrA*, and *sarA* in standard MRSA. Phytochemical analyses of *U. pumila* root bark showed relatively strong presence of phenolics, steroids, and terpenoids. These results suggest that the ethanol extract of *U. pumila* root bark may have antibacterial activity against MRSA, which may be related to the phytochemicals such as phenolics, steroids, and terpenoids. Further studies are needed to determine the active constituents of *U. pumila* root bark responsible for such biomolecular activities.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common bacteria in humans. *S. aureus* is normally present in the skin, nasal cavity, or laryngopharynx of healthy men and opportunistically causes a local or systemic infection [1, 2]. However, *S. aureus* is a causative bacteria of nosocomial infection and occupies more than 80% of pyogenic infection such as abscess and septicemia [2–5].

Penicillin was developed in 1941 and has been used as a therapeutic agent indicated for bacterial infections. Since then, numerous antibiotic agents have been developed and are effective against bacterial infections, but the appearance of antibiotic-resistant bacterial strains caused a big problem in the treatment of patients [6, 7]. The appearance of such antibiotic-resistant bacterial strains tends to increase due to the overuse of antibiotics. Antibiotic-resistant strains which became an important issue in the world include methicillin-resistant *Staphylococcus aureus* (MRSA) [8]. These bacteria strains have multidrug resistance showing resistance to various antibiotic agents such as β-lactams, or aminoglycosides. Treatment of patients infected with these bacterial strains is known to be very difficult [9, 10], so MRSA is one of the important causes of modern chronic infectious diseases. It is known that the resistance mechanisms of MRSA to methicillin include (1) production of β-lactamase, which inactivate the β-lactam antibiotics and (2) the possession of *mecA* gene that produces penicillin-binding proteins, such as PBP, PBP2′, or PBP2a, which have low affinity to β-lactam antibiotics [7]. Since MRSA shows resistance to various antibiotics, it is necessary to develop new substances for the treatment of MRSA. Several natural substances may be candidates for new antibiotic substances [11]. We have explored natural substances with antimicrobial effects on MRSA [12–14].

Ulmus pumila (*U. pumila*) is a natural herb that has traditionally been used for the treatment of infections in Korea. *U. pumila,* belonging to the botanical classification

Table 1: Bacterial strains used the test of antibacterial activity.

Strains	Class	*mecA* gene	β-Lactamase activity	Antibiotic resistance pattern
S. aureus ATCC 33591	MRSA	+	+	AM, OX, CF, E
Clinical isolates				
S. aureus (OMS 1)	MRSA	+	+	AM, OX
S. aureus (OMS 2)	MRSA	+	+	AM, OX, E
S. aureus (OMS 3)	MRSA	+	+	AM, OX, CF, E
S. aureus (OMS 4)	MRSA	+	+	AM, OX, E
S. aureus (OMS 5)	MRSA	+	+	AM, OX, CF, E
S. aureus (OMS 6)	MRSA	+	+	AM, OX, E
S. aureus (OMS 7)	MRSA	+	+	AM, OX, CF, E
S. aureus (OMS 8)	MRSA	+	+	AM, OX, CF, E
S. aureus (OMS 9)	MRSA	+	+	AM, OX, CF, E
S. aureus (OMS 10)	MRSA	+	−	AM, OX, E
S. aureus (OMS 11)	MRSA	+	+	AM, OX
S. aureus (OMS 12)	MRSA	+	+	AM, OX

+: positive; −: negative; AM: ampicillin; OX: oxacillin; CF: cephalothin; E: erythromycin.
OMS indicates Staphylococcal strains of Department of Oral and Maxillofacial Surgery, Wonkwang University Hospital.

of Ulmaceae, is distributed in Korea, Japan, northern China, Sakhalin, and East Siberia. This tree grows to 15 meters and its bark is dark brown in color. Owing to its antibacterial and anti-inflammatory reaction, *U. pumila* has been traditionally used for abscess, infection, edema, rhinitis, empyema, and otitis media. It has also been used for gastric and duodenal ulcers as well as gastric cancer [15–17].

In this study, *U. pumila* was extracted with ethanol, and then the antimicrobial effects of *U. pumila* ethanol extract were tested on clinically isolated 12 MRSA strains and 1 standard MRSA strain, and phytochemical analysis was performed.

2. Materials and Methods

2.1. Plant Material and Extraction. The bark of *U. pumila* was obtained from the oriental drug store, Dae Hak Yak Kuk (Iksan, South Korea). The identity was confirmed by Dr. Bong-Seop Kil at the Department of Natural Science, Wonkwang University.

Voucher specimen (number 09-03-26) has been deposited at the Herbarium of Department of Oral Biochemistry in Wonkwang University. Dried bark of *U. pumila* (100 g) was chopped into small pieces and was extracted 2 times with 1000 mL of ethanol for 72 h at room temperature. The filtration of the extracted solution and evaporation under reduced pressure yielded ethanol extracts (9.3 g). After the extract was thoroughly dried for complete removal of solvent, the dry extract was then stored in a deep freezer (−70°C).

2.2. Bacterial Strains. Staphylococcal strains listed in Table 1 were 12 clinical isolates (MRSA) from Wonkwang University Hospital and the standard strain of MRSA ATCC 33591. Antibiotic susceptibility was determined from the size of the inhibition zone, in accordance with guidelines of Clinical & Laboratory Standards Institute (CLSI, 2010), and the used strains were defined as MRSA based on occurrence of the *mecA* gene and their resistance to oxacillin [18]. β-Lactamase activity was also determined using the DrySlide Beta Lactamase test (Difco Laboratories, Detroit, MI, USA) according to manufacturer's specification. After culturing on Mueller-Hinton agar (Difco Laboratories), the bacteria were suspended in Mueller-Hinton broth (Difco Laboratories) and used for inoculation. All MRSA strains used in this study are identified as MRSA [13].

2.3. Disc Diffusion Method. As the first screening, the paper disc diffusion method was used to determine antibacterial activity, which is based on the method described previously [12, 19]. Sterile paper discs (6 mm; Toyo Roshi Kaisha, Japan) were loaded with 50 μL of different amounts (0.25, 0.5, and 1 mg) of the extracts dissolved in dimethyl sulfoxide (DMSO) and were left to dry for 12 h at 37°C in a sterile room. Bacterial suspensions were diluted to match the 0.5 MacFarland standard scale (approximately 1.5×10^8 CFU/mL) and they were further diluted to obtain a final inoculum. After Mueller-Hinton agar was poured into Petri dishes to give a solid plate and inoculated with 100 μL of suspension containing 1×10^8 CFU/mL of bacteria, the discs treated with extracts were applied to Petri dishes. Ampicillin and oxacillin were used as positive controls and paper discs treated with DMSO were used as negative controls. The plates were then incubated at 35°C for 24 h in a incubator (Vision Co., Seoul, Korea). Inhibition zone diameters around each disc were measured and recorded at the end of the incubation time.

2.4. Determination of Minimum Inhibitory Concentrations (MICs). MICs were determined by the agar dilution method, which is based on the method described previously [12, 20]. MICs of ampicillin and oxacillin were also determined. A final inoculum of 1×10^4 CFU/mL was spotted with a multipoint inoculator (Denley Instruments, Sussex, UK) onto agar plates. The plates were then incubated at 35°C for 24 h in the incubator (Vision Co., Seoul, Korea). The MIC was defined as the lowest concentration of extracts at which no visible growth was observed. The minimum concentration of

Table 2: Nucleotide sequences of primer used for real-time PCR in this study.

Gene	Gene description		Primer sequences (5′-3′)
16srRNA	Normalizing internal standard	Forward	ACTGGGATAACTTCGGGAAA
		Reverse	CGTTGCCTTGGTAAGCC
mecA	Penicillin binding protein 2′	Forward	GTTAGATTGGGATCATAGCGTCATT
		Reverse	TGCCTAATCTCATATGTGTTCCTGTAT
sea	Staphylococcal enterotoxin A	Forward	ATGGTGCTTATTATGGTTATC
		Reverse	CGTTTCCAAAGGTACTGTATT
agrA	Accessory gene regulator A	Forward	TGATAATCCTTATGAGGTGCTT
		Reverse	CACTGTGACTCGTAACGAAAA
sarA	Staphylococcal accessory regulator A	Forward	TGTTATCAATGGTCACTTATGCTG
		Reverse	TCTTTGTTTTCGCTGATGTATGTC

Table 3: Antimicrobial activity (mm inhibition zones diameter) of extracts of *Ulmus pumila* against 12 methicillin-resistant *Staphylococcus aureus* (MRSA) and 1 standard MRSA.

Strains	Zone of inhibition (mm)				
	Ulmus pumila (mg)			Ampicillin[a]	Oxacillin[b]
	0.25	0.5	1	10 μg	1 μg
S. aureus (ATCC33591)	10	12	14	10	ND
Clinical isolates					
S. aureus (OMS 1)	11	13	16	12	9
S. aureus (OMS 2)	ND	9	14	11	ND
S. aureus (OMS 3)	9	11	15	11	ND
S. aureus (OMS 4)	10	14	16	11	ND
S. aureus (OMS 5)	10	12	14	11	ND
S. aureus (OMS 6)	11	15	17	10	ND
S. aureus (OMS 7)	ND	10	14	8	ND
S. aureus (OMS 8)	9	13	15	10	ND
S. aureus (OMS 9)	9	12	14	10	ND
S. aureus (OMS 10)	12	15	18	17	ND
S. aureus (OMS 11)	11	14	17	11	ND
S. aureus (OMS 12)	13	16	19	11	ND

ND: no detected activity at this concentration; C: chloroform extract; B: *n*-butanol extract; M: methanol extract; A: aqueous extract.
[a]Ampicillin resistance ≤28 mm.
[b]Oxacillin resistance ≤10 mm.

extracts that inhibited 50% and 90% of the isolates tested was defined as MIC_{50} and MIC_{90}, respectively.

2.5. Real Time Polymerase Chain Reaction (PCR) Analysis. To determine the effect of *U. pumila* extract on gene expression, a real-time PCR assay was performed. The sub-MIC (32–125 μg/mL) of *U. pumila* extract was used to treat and culture MRSA ATCC 33591 for 24 h. Total RNA was isolated from MRSA by using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions and was treated with DNase to digest contaminated DNA. Then, cDNA was synthesized using a reverse transcriptase reaction (Superscript; Gibco-BRL). The DNA amplifications were carried out using an ABI-Prism 7000 Sequence Detection System with Absolute QPCR SYBR Green Mixes (Applied Bio systems Inc., Foster City, CA, USA). The primer pairs that were used in this study were described by previous reports [21–23] and are listed in Table 2. 16S rRNA was used as an internal control.

2.6. Phytochemical Screening. Phytochemical tests of extracts were performed as previously described [24, 25]. Mayer's reagent was used for alkaloids, ferric chloride reagent for phenolics, Molisch test for glycosides, Biuret reagent for proteins, Mg-HCl reagent for flavonoids, Liebermann-Burchard reagent for steroids, and silver nitrate reagent for organic acids.

2.7. Statistical Analysis. All experiments were carried out in triplicate. Data were analyzed using the statistical package for social sciences (SPSS). Differences between means of the experimental and control groups were evaluated by the Student's *t*-test.

3. Results

In this study, the antibiotic effect of ethanol extract of *U. pumila* on clinically isolated MRSA strain 12 and standard MRSA strain 1 (ATCC 33591) was examined. As a result of measuring antibacterial activity of *U. pumila* using the disc diffusion method, *U. pumila* showed antibacterial activities against all strains (Table 3). In all MRSA strains, 1 mg of

Table 4: Minimal inhibitory concentrations (MICs) of the *Ulmus pumila*, ampicillin, and oxacillin against 12 methicillin-resistant *Staphylococcus aureus* (MRSA) and 1 standard MRSA.

Strains	MIC (μg/mL)		
	Ulmus pumila	Ampicillin[1]	Oxacillin[2]
S. aureus ATCC 33591	250	32	256
Clinical isolates			
S. aureus (OMS 1)	250	32	8
S. aureus (OMS 2)	250	32	4
S. aureus (OMS 3)	250	64	4
S. aureus (OMS 4)	250	64	4
S. aureus (OMS 5)	250	32	4
S. aureus (OMS 6)	125	64	16
S. aureus (OMS 7)	250	32	16
S. aureus (OMS 8)	250	32	8
S. aureus (OMS 9)	250	64	8
S. aureus (OMS 10)	125	4	4
S. aureus (OMS 11)	125	64	16
S. aureus (OMS 12)	125	64	4

[1]Ampicillin resistance is an ampicillin MIC of $\geq$0.25 μg/mL.
[2]Oxacillin resistance is an oxacillin MIC of $\geq$4 μg/mL.
OMS indicates Staphylococcal strains of Department of Oral and Maxillofacial Surgery, Wonkwang University Hospital.

U. pumila showed 14–19 mm of inhibition zone and 0.5 mg of *U. pumila* showed 9–16 mm of inhibition zone.

This experimental result was confirmed through MIC measurement (Table 4). Ethanol extract of *U. pumila* showed a range of MICs from 125 μg/mL to 250 μg/mL in all MRSA strains. In most strains, the growth of bacteria was inhibited noticeably from 250 μg/mL of *U. pumila* concentration. The MICs for ampicillin and oxacillin against MRSA strains clinically isolated, which had been used as the positive control, were 4–46 μg/mL and 4–16 μg/mL, respectively. These results showed that the MRSA strains isolated clinically have resistance to ampicillin and oxacillin. From this experimental result, these clinically isolated MRSA strains showed resistance to ampicillin or oxacillin. We performed real-time PCR analysis to examine the effect of sub-MIC (32–125 μg/mL) concentrations of *U. pumila* extract on the genetic expressions of virulence factors in standard MRSA (ATCC 33591). The expressions of *mecA, sea, agrA*, and *sarA* were significantly decreased in MRSA when it was treated with the sub-MIC (63–125 μg/mL) concentrations of *U. pumila* extract (Figure 1).

As a result of phytochemical analysis of *U. pumila,* phenolics, steroids, and terpenoids were detected with a relatively high content; glycosides were detected with a medium level of content; flavonoids, peptides, and organic acids were detected with low content; but alkaloids were nearly never detected (Table 5).

Table 5: Phytochemical analysis of the ethanol extract of *Ulmus pumila*.

Plant constituents	Ethanol extract
Alkaloids	−
Phenolics	+++
Flavonoids	+
Glycosides	++
Peptides	+
Steroids, terpenoids	+++
Organic acids	+

+++: strong; ++: moderate; +: poor; −: absent.

4. Discussion

MRSA, an antibiotic-resistant strain, causes severe complex clinical problems in many parts of the world. Therefore, new agents are needed to treat the MRSA. Some natural products are candidates of new antibiotic substances. Traditionally, *U. pumila* has been used for the treatment of infectious diseases in Korea. In this study, antibacterial activities of ethanol extract of *U. pumila* on clinically isolated 12 MRSA strains and 1 standard MRSA strain were examined.

Antibacterial activities of *U. pumila* were measured by using the disc diffusion method, which were then also confirmed through MIC measurements. *U. pumila* ethanol extract showed antibacterial abilities against all the strains, 12 strains of MRSA isolated clinically and 1 standard strain of MRSA. The fact that *U. pumila* extract suppresses growth of *S. aureus* could provide the scientific basis, that the extract had been used for the treatment of infectious diseases.

According to previous studies, *U. pumila* was known to contain steroidal chemicals such as β-Sitosterol, phytosterol, and stigmasterol; terpenoid chemicals such as friedelin, epifriendelalol, and taraxerol; phenolics such as tannin; and polysaccharides such as starch [16]. In this study, the phytochemical analysis of *U. pumila* showed a result of relatively high content of phenolics, steroids, and terpenoids. This result suggests that the antibacterial activity of *U. pumila* may be related with these chemicals. However, more

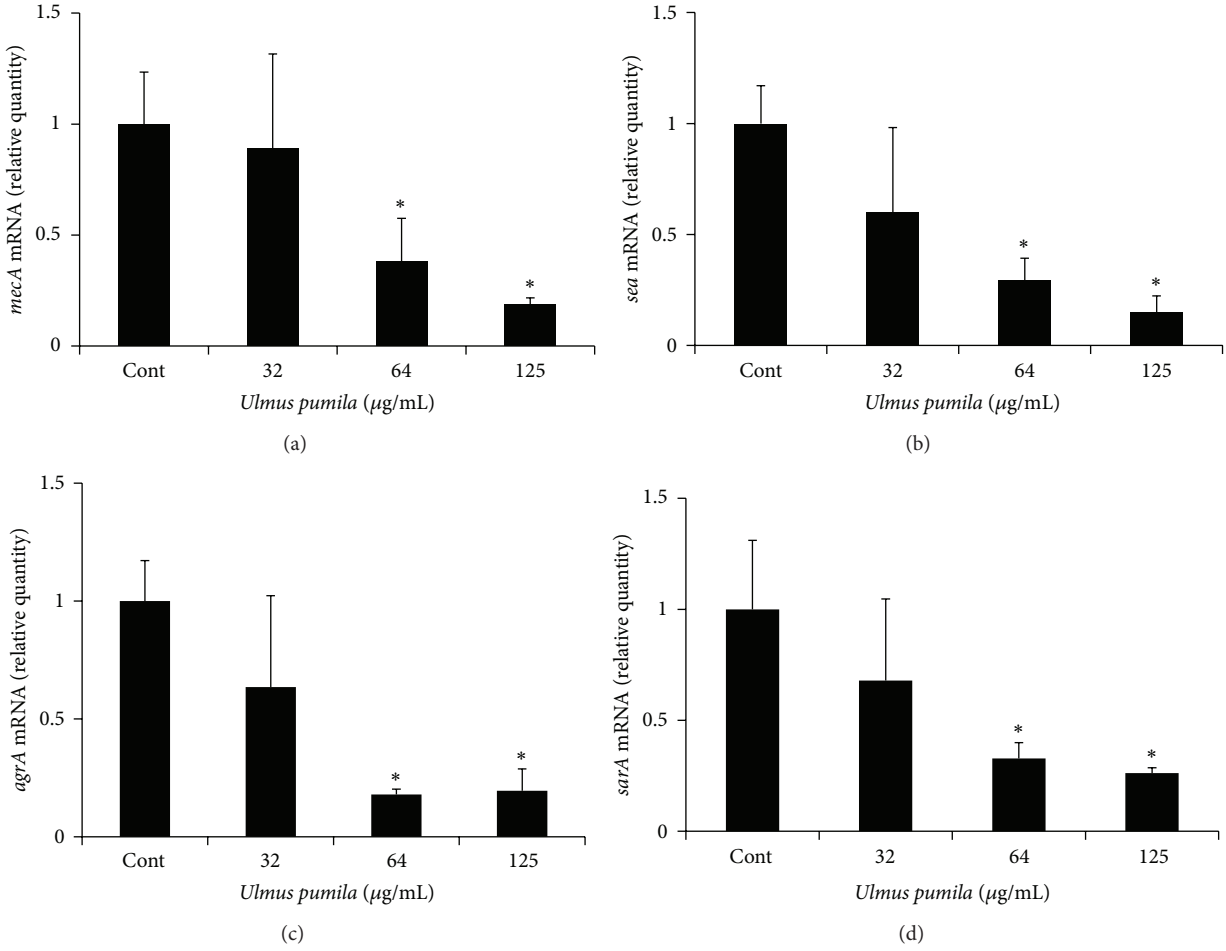

Figure 1: Real-time PCR analysis of expression of several virulence factor genes. MRSA ATCC 33591 was cultured and treated with sub-MIC concentrations (32–125 μg/mL) of *U. pumila* extract, and real-time PCR analysis was then performed as described in the Section 2. *mecA, sea, sarA*, and *agrA* expressions were significantly inhibited at concentration higher than 64 μg/mL. Each value is expressed as a mean ± standard deviation. Significance was determined at $^{*}P < 0.05$ when compared with the control.

additional researches are required to identify the antibacterial components in *U. pumila.*

Several mechanisms by which microorganisms can overcome antimicrobial agents are known. These mechanisms include production of drug insensitive enzymes, modification of targets for drug, and multidrug resistance pump which discharge the antimicrobial agents entered in bacterial cells. *mecA* is the typical multidrug resistance gene, and *fem, llm,* and *sigB* are also the other multidrug resistance genes [26]. Recent studies reported that some medicinal plants contain multidrug resistance inhibitor that is to lower the MIC of antimicrobial agents [27]. In the present study, the effect of sub-MIC (32–125 μg/mL) concentrations of *U. pumila* extract on the genetic expression of *mecA* was determined by real-time PCR analysis. The expression of *mecA* was significantly decreased in standard MRSA (ATCC 33591) when it was treated with concentration higher than 64 μg/mL. Additional investigation is necessary to determine whether *U. pumila* may have multidrug resistance inhibitors.

A virulence factor gene, *sea,* encodes Staphylococcal enterotoxin A which is one of major virulence factors in MRSA [28]. Staphylococcal enterotoxin A is one cause of gastroenteritis in humans and acts as a superantigen. In this study, the sub-MIC concentrations of *U. pumila* extract significantly inhibited *sea* expression. *sea* gene expression in MRSA is regulated by global regulators such as *agr* and *sarA* genes [21]. In our study, sub-MIC (64–125 μg/mL) concentrations of *U. pumila* extract showed the inhibition of *agrA* and *sarA* expressions in MRSA. *agrA* encodes accessory gene regulator A which positively regulates exotoxin-encoding genes. *sarA* also upregulates expression of virulence factor genes. Previous research has shown that inhibition of *agrA* or *sarA* expression by some chemicals such as thymol or clindamycin reduces transcription of exotoxin-encoding genes [21]. In the present study, suppressive effect of *U. pumila* extract on *sea* gene expression may, in part, be related with the inhibitory effect of *U. pumila* extract on *agrA* and *sarA* expressions [29].

In conclusion, *U. pumila* has antibacterial effects against MRSA, which may be related to the phytochemicals such as phenolics, steroids, or terpenoids which are highly present in *U. pumila*.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Yong-Ouk You and Na-Young Choi contributed equally to this work.

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Research Article

Inhibitory Effect of *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura Extract on RANKL-Induced Osteoclast Differentiation

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Chrysanthemum zawadskii Herbich var. *latilobum Kitamura*, known as "Gujulcho" in Korea, has been used in traditional medicine to treat various inflammatory diseases, including rheumatoid arthritis. However, these effects have not been tested on osteoclasts, the bone resorbing cells that regulate bone metabolism. Here, we investigated the effects of *C. zawadskii* Herbich var. *latilobum* Kitamura ethanol extract (CZE) on osteoclast differentiation induced by treatment with the receptor activator of NF-κB ligand (RANKL). CZE inhibited osteoclast differentiation and formation in a dose-dependent manner. The inhibitory effect of CZE on osteoclastogenesis was due to the suppression of ERK activation and the ablation of RANKL-stimulated Ca^{2+}-oscillation via the inactivation of PLCγ2, followed by the inhibition of CREB activation. These inhibitory effects of CZE resulted in a significant repression of c-Fos expression and a subsequent reduction of NFATc1, a key transcription factor for osteoclast differentiation, fusion, and activation *in vitro* and *in vivo*. These results indicate that CZE negatively regulates osteoclast differentiation and may be a therapeutic candidate for the treatment of various bone diseases, such as postmenopausal osteoporosis, rheumatoid arthritis, and periodontitis.

1. Introduction

Bone remodeling and metabolism are maintained by a sophisticated regulation between osteoblasts, bone matrix-forming cells, and osteoclasts, bone-resorbing cells [1, 2]. Imbalance between these cells is implicated in the development of bone diseases accompanied by low bone mineral density and bone destruction, such as postmenopausal osteoporosis, periodontitis, and rheumatoid arthritis (RA), which are caused by excessive differentiation and activation of osteoclasts [3–5].

Osteoclasts are differentiated from hematopoietic macrophage/monocyte lineage precursor cells in several steps, including proliferation, differentiation, fusion, and activation [2]. Together with the macrophage colony-stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL), which is mainly produced by osteoblasts, has been established as a pivotal osteoclast differentiation factor [6, 7]. In RANKL-stimulated osteoclastogenesis, a signal of RANKL binding to its receptor molecules, receptor activator of nuclear factor NF-κB (RANK) expressed on osteoclasts, is transduced into intercellular molecules through TRAF6 adaptor molecule. Thereafter, the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, Akt, and NF-κB are activated via RANKL/RANK interaction [1]. Subsequent upregulation of c-Fos expression, a positive modulator of

osteoclast differentiation, is followed by c-Fos binding to the *NFATc1* promoter region, which induces NFATc1 expression, a master key transcription factor for osteoclastogenesis [8–10]. In addition, RANKL/RANK interaction activates immunoreceptor tyrosine-based activation motif (ITAM) bearing adaptor molecules, such as DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcRγ), followed by activation of phospholipase C-gamma (PLCγ) [11]. Activation of PLCγ leads to the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). Binding of IP_3 to inositol trisphosphate receptors (IP_3Rs) on the endoplasmic reticulum (ER) membrane mobilizes Ca^{2+} from the ER stores to the cytosol, causing $Ca2^{+}$-oscillation, which is important for osteoclast differentiation [12, 13]. Ca^{2+}-oscillation induces the activation of Ca^{2+}/calmodulin-dependent protein kinases (CaMK)IV and cAMP responsive element binding protein (CREB), subsequently leading to induced c-Fos and NFATc1 expression [14]. This CaMKIV/CREB/NFATc1 pathway is also critical to osteoclast differentiation and function [15].

Chrysanthemum zawadskii Herbich var. *latilobum* Kitamura (Compositae), colloquially known as "Gujulcho" in Korea, has been used in traditional medicine for the treatment of various diseases, including cough, common cold, bladder-related disorders, gastroenteric disorders, hypertension, and inflammatory diseases, such as pneumonia, bronchitis, pharyngitis, and rheumatoid arthritis (RA) [16, 17]. *C. zawadskii* Herbich var. *latilobum* Kitamura extract (CZE) has been shown to harbor many pharmacological properties, including anticancer, antiallergic, anti-inflammatory, and antioxidative stress activities, along with protective effects against liver damage [17–22].

Many previously published studies indicate that inflammatory cytokines, including TNF-α, IL-1, IL-17, IFN-γ, and IL-4, which are produced during successful T-cell-based immune responses, directly regulate RANKL expression on osteoblasts as well as osteoclastogenesis and that inflammation affects bone metabolism [1, 3]. Although CZE has an anti-inflammatory activity, the effect of CZE on bone metabolism has rarely been reported, with the exception that linarin, a component of CZE, prevents hydrogen peroxide-induced dysfunction in osteoblastic MC3T3-E1 cells [23]. However, its effect on osteoclasts still remains unclear.

In this study, we investigated the inhibitory effect of CZE on osteoclastogenesis and provided basic mechanisms and possibilities for the use of CZE as a traditional remedy against bone diseases, including osteoporosis, RA, and periodontitis.

2. Materials and Methods

2.1. Experimental Animals. C57BL/6J (Orient Bio Inc., SeungNam, Korea) were used to generate osteoclasts and for all other experiments. All mouse studies were performed using protocols approved by the Animal Care and Use Committee of Wonkwang University.

2.2. Reagents. The 95% ethanol CZE was purchased from Korean Plant Extract Bank (Daejeon, Korea). All cell culture media, fetal bovine serum (FBS), and supplements were purchased from Hyclone (Rockford, IL, USA). Soluble recombinant mouse RANKL was purified from insect cells as described previously [24], and recombinant human M-CSF was supplied by T Kim (KIOM, Daejeon, Korea). Antibodies against p-ERK, p-JNK, p-p38, p-IκBα, p-PLCγ2, p-CREB, ERK, JNK, p38, IκBα, PLCγ2, and CREB were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-NFATc1 and anti-c-Fos antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

2.3. Cell Viability Assay. Cell viability assays were performed using the EZ-Cytox Enhanced Cell Viability Assay Kit, (Itsbio, Korea), following the manufacturer's instructions. Briefly, bone-marrow-derived macrophages (BMMs), which act as osteoclast precursors, were plated in 96-well culture plates at a density of 1×10^4 cells per well with various concentrations of CZE (0, 2, 5, 10, 25, and 50 μg/mL) for 1 day, or they were cultured with 25 μg/mL of CZE under M-CSF treatment for 4 days. Cells were incubated with EZ-Cytox reagent for 4 h at 37°C. After incubation, the optical density was measured using an ELISA reader (Sunrise, Tecan, Switzerland) at 450 nm.

2.4. In Vitro Osteoclast Differentiation. Murine osteoclasts were prepared from bone marrow cells (BM) as previously described [25]. BMs were collected from the tibiae and femora of 6–8-week-old mice by flushing the marrow space with phosphate-buffered saline (PBS). BMs were cultured with M-CSF (30 ng/mL) for 3 days in α-minimal essential medium (α-MEM) containing 10% FBS, and attached cells were harvested and used as osteoclasts precursors (BMMs). To generate osteoclasts, BMMs were cultured with M-CSF (50 ng/mL) and RANKL (100 ng/mL) for 4 days. Fresh α-MEM containing M-CSF and RANKL was replaced on day 3. Cells were fixed with 10% formalin and stained for TRAP. TRAP positive-multinuclear cells ($TRAP^+$ MNCs) containing more than three nuclei were counted as osteoclasts. In some experiments, total TRAP activity using *p*-nitrophenyl phosphate (Sigma, USA) as a substrate was measured at an absorbance of 405 nm as previously described [25].

2.5. Real-Time Quantitative PCR. BMMs treated with or without CZE were cultured with M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days as described above. Total RNA was extracted from cultured cells by using Trizol reagent (Invitrogen, USA) on the indicated days. Then, 1 μg of the total RNA was transcribed to first strand cDNA with random primers using Maxima Reverse Transcriptase (Thermo Scientific, IL, USA) according to the protocol provided by the supplier. Real-time PCR was performed using the VeriQuest SYBR Green qPCR Master Mix (Affymetrix, USA) and StepOnePlus Real-Time PCR Systems (Applied Biosystems, USA). To control for variation in mRNA concentrations, all results were normalized to the GAPDH housekeeping gene. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method, according to the manufacturer's instructions. Primers used in this study are listed in Table 1.

Table 1: Nucleotide sequences of the primers used for real-time PCR in this study.

Gene	Primers
Acp5 (TRAP)	Forward 5′-CTGGAGTGCACGATGCCAGCGACA-3′
	Reverse 5′-TCCGTGCTCGGCGATGGACCAGA-3′
Oscar	Forward 5′-GGGGTAACGGATCAGCTCCCCAGA-3′
	Reverse 5′-CCAAGGAGCCAGAACGTCGAAACT-3′
Cathepsin K (CtsK)	Forward 5′-ACGGAGGCATTGACTCTGAAGATG-3′
	Reverse 5′-GTTGTTCTTATTCCGAGCCAAGAG-3′
Tm7sf4 (DC-STAMP)	Forward 5′-TGGAAGTTCACTTGAAACTACGTG-3′
	Reverse 5′-CTCGGTTTCCCGTCAGCCTCTCTC-3′
ATP6v0d2	Forward 5′-TCAGATCTCTTCAAGGCTGTGCTG-3′
	Reverse 5′-GTGCCAAATGAGTTCAGAGTGATG-3′
Nfatc1	Forward 5′-CTCGAAAGACAGCACTGGAGCAT-3′
	Reverse 5′-CGGCTGCCTTCCGTCTCATAG-3′
Gapdh	Forward 5′-TGCCAGCCTCGTCCCGTAGAC-3′
	Reverse 5′-CCTCACCCCATTTGATGTTAG-3′

2.6. Western Blot Analysis. BMMs were cultured with M-CSF (50 ng/mL) and RANKL (100 ng/mL) in the presence or absence of CZE for the indicated time. The cells were washed with cold PBS and lysed in 100 ul of radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) contained with 1 mM phenylmethylsulfonyl fluoride (PMSF), protease-inhibitor cocktail (Roche, Germany), and phosphatase inhibitor tablets (Thermo Scientific, USA). The cell lysates were cleared by centrifugation at 14,000 ×g for 10 min at 4°C, and the supernatants were collected for immunoblotting. Total lysates (30 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Amersham Hybond-P, GE-Healthcare Life Science, USA). Each membrane was blocked for 2 h with 5% skim milk in TBST (TBS; 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) and then incubated with the 1 : 1000 dilution of the primary antibody. HRP-conjugated IgG (1 : 5000 dilutions) was used as the secondary antibody. The immunoreactive proteins were detected using enhanced chemiluminescence (ECL) detection system (Thermo Scientific, USA), according to the manufacturer's protocols. The bands detected were quantitated with the NIH imaging program (NIH Image 1.62), as previously described [26].

2.7. Measurement of Ca^{2+}-Oscillation. Ca^{2+}-oscillation in osteoclasts by RANKL stimulation was measured as previously described with minor modification [27]. BMMs were cultured on the cover slips with RANKL in the presence or absence of CZE (25 μg/mL). After 24 h of RANKL stimulation, intracellular Ca^{2+} mobilization was measured using the fluorescence Ca^{2+} indicator, Fura-2-acetoxymethyl ester (Fura-2AM, 5 μM; TEFLabs, USA). In some cases, BMMs were cultured with RANKL in the absence of CZE for 1 day, and then treated with CZE to verify the acute effects of CZE at the indicated times. Cells were loaded with Fura-2AM for 50 min at room temperature and placed on a chamber connected with a perfusion system. Unloaded fluorescent dye was washed out with bath solution (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose; 310 milliosmole). With continuous perfusion of bath solution (37°C), the intracellular fluorescence intensity was measured using two excitation wavelengths (340 and 380 nm), and the emitted fluorescence (510 nm) was captured using a CCD camera. Collected images were digitized and analyzed by MetaFluor software (Ratio = F340/F380).

2.8. Statistical Analysis. Data were analyzed using the Student's two-tailed t-test and are presented as mean ± SD values, as indicated. A P value of < 0.05 was considered statistically significant. All experiments were repeated at least twice and representative data are shown.

3. Results

3.1. Effect of CZE on Cell Viability. To assess the cytotoxicity of CZE on osteoclast precursors, BMMs were treated with various concentrations of CZE (0, 2, 5, 10, 25, or 50 μg/mL) for 1 day. Various concentrations of CZE, up to 50 μg/mL, did not affect the viability of BMMs (Figure 1(a)). All tested concentrations of CZE were shown to have viability levels comparable with that of control. In addition, BMMs were treated with 25 μg/mL of CZE for 4 days. Cell viability was measured daily. There was no significant difference of viability between the control and CZE (25 μg/mL)-treated cells during the 4 days of culture (Figure 1(b)).

3.2. Inhibitory Effect of CZE on Osteoclast Differentiation. To investigate the effect of CZE on osteoclast differentiation, BMMs were cultured with various concentrations of CZE under RANKL treatment for 4 days. Osteoclast differentiation was measured by TRAP staining and TRAP solution assay as previously described. $TRAP^+$ MNCs containing more than 3 nuclei and bigger than 100 μm in diameter were counted as mature osteoclasts. CZE treatment dramatically

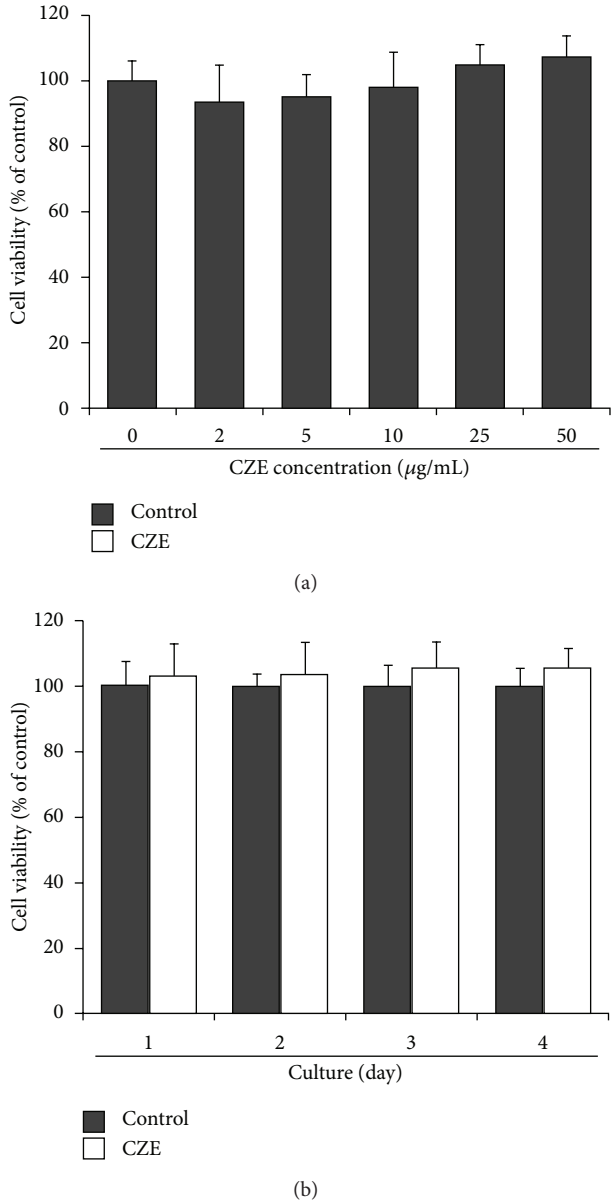

Figure 1: Effects of *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura extract (CZE) on cell viability. (a) BMMs were cultured with indicated concentrations of CZE for 1 day. (b) BMMs were cultured with 25 μg/mL CZE or without (control) for 4 days. Cell viability was measured as described in the materials and methods. Data are the mean of three independent experiments (± SD).

inhibited the formation of mature osteoclasts from BMMs in a dose-dependent manner (Figures 2(a) and 2(b)). When the CZE exceeded 25 μg/mL, mature osteoclasts were rarely formed. In addition, total TRAP activity from mono-, di- and multinuclear osteoclasts was significantly decreased as the CZE concentration increased (Figure 2(c)). These data suggest that the role of CZE is to repress osteoclast differentiation from the precursor cells to mature osteoclasts, and that it is not involved with the osteoclast fusion and activation steps.

To confirm the inhibitory effect of CZE on osteoclast differentiation, the expression of osteoclast differentiation marker genes (*Acp5, Oscar, CtsK, Tm7sf4*, and *Atp6v0d2*) and a master transcription factor for osteoclast differentiation, *Nfatc1*, were measured during RANKL-induced osteoclast differentiation. As shown in Figure 3, CZE significantly inhibited the expression of all tested marker genes and *Nfatc1*. Together with Figure 1, these results indicate that CZE has an inhibitory effect on RANKL-induced osteoclast differentiation and formation without cytotoxicity.

3.3. Suppression of c-Fos and NFATc1 Expression via ERK Inactivation by CZE. In RANKL-induced osteoclast differentiation, RANKL/RANK signaling induces the activation of NF-κB and mitogen-activated protein kinases (MAPKs), followed by c-Fos expression, which results in the induction of NFATc1, a key transcription factor for osteoclastogenesis [1]. Therefore, we investigated the effect of CZE on the regulation of RANKL-induced signaling pathways. First, the BMMs were treated with or without CZE (25 μg/mL) under RANKL and M-CSF treatment, and then, the activation of MAPKs and IκBα was measured by western blot analysis. As shown in Figure 4(a), the phosphorylation of ERK diminished in the CZE-treated cells compared to that of control cells. However, the activation of IκBα and other MAPKs (JNK and p38) was not significantly changed. Next, we measured the expression levels of c-Fos and NFATc1. When CZE was treated, the expression of c-Fos was dramatically repressed 6 h after treatment in osteoclast differentiation (Figure 4(b)). In addition, the induction of NFATc1 was significantly inhibited by CZE treatment (Figure 4(c)), coinciding with mRNA expression patterns (Figure 3). These results indicate that CZE inhibited the expression of c-Fos via the inactivation of ERK in RANKL-induced osteoclast differentiation. This inactivation leads to the repression of the expression of NFATc1, which regulates all the steps involved in osteoclast differentiation, fusion, and activation.

3.4. Breakdown of Intracellular Ca^{2+}-Oscillation and Inhibition of PLCγ2 and CREB Activation by CZE. In addition to MAPK and NF-κB activation, RANKL/RANK signaling also activate phospholipase C gamma 2 (PLCγ2) and induces Ca^{2+}-oscillation, followed by CREB activation [14, 25, 28]. CREB is critical for RANKL-stimulated NFATc1 and c-Fos induction in osteoclast precursors [14]. We first examined whether CZE affects the induction of Ca^{2+}-oscillation by RANKL stimulation. BMMs were cultured with or without CZE under RANKL stimulation for 24 h. Intracellular Ca^{2+} concentration was measured as described previously. Control cells exhibited typical Ca^{2+}-oscillation as shown in Figure 5(a). However, CZE-treated cells showed an irregular Ca^{2+}-oscillation pattern with significantly increased intensity, but without increased frequency (Figure 5(b)). In addition, we acutely added CZE on control cells showing typical Ca^{2+}-oscillation and then measured Ca^{2+} mobilization. As shown in Figure 5(c), Ca^{2+}-oscillation was defective in these cells, with a large Ca^{2+} influx peak after CZE treatment. It seems that CZE may interact with some Ca^{2+} channels, which

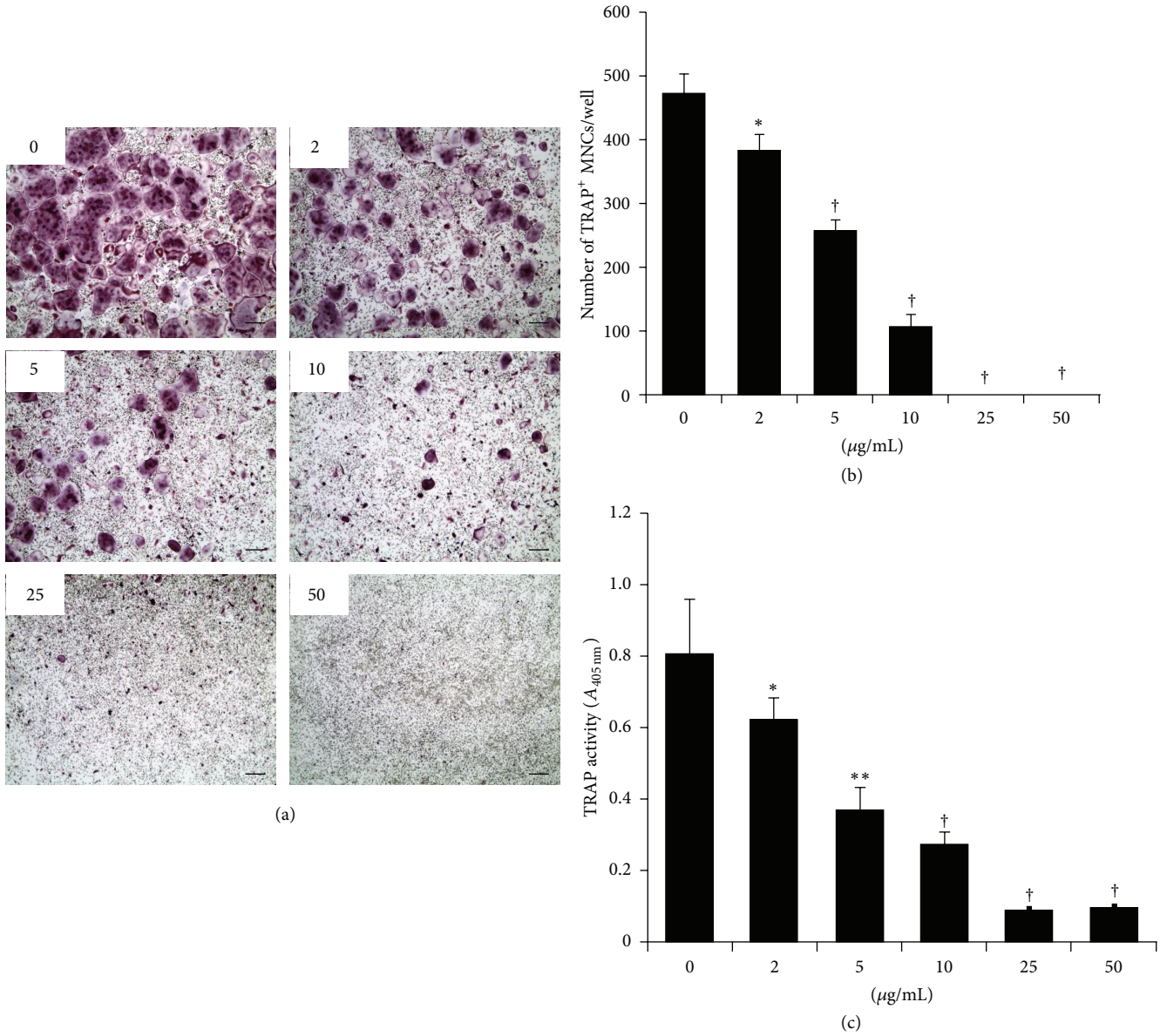

FIGURE 2: Effects of CZE on osteoclast differentiation. BMMs were cultured with various concentrations of CZE under RANKL and M-CSF treatment for 4 days. (a) Osteoclasts were stained for TRAP. (b) $TRAP^+$ multinuclear cells (MNCs) with more than 3 nuclei were counted as mature osteoclasts. (c) Total TRAP activity from $TRAP^+$-mono-, di- and multinuclear cells was measured as described in the materials and methods. Data are expressed as the mean ± SD and are representative of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^\dagger P < 0.001$ versus control (0 μg/mL CZE). Scale bar = 200 μm.

contributes to a substantial Ca^{2+} influx into the cells. We next examined whether RANKL-stimulated PLCγ2 activation is affected by CZE treatment. Phosphorylation of PLCγ2 in CZE-treated cells was significantly inhibited. In addition, the activation of CREB by RANKL was dramatically suppressed during osteoclast differentiation in CZE-treated cells. Collectively, these data demonstrate that CZE regulates not only MAPKs and NF-κB activation, but also PLCγ2 activation and RANKL-induced Ca^{2+}-oscillation, which are important for CREB activation and c-Fos and NFATc1 induction in RANKL-stimulated osteoclast differentiation (Figure 6).

4. Discussion

Although *C. zawadskii* Herbich var. *latilobum* Kitamura has routinely been used as a traditional remedy against several inflammatory diseases and the mechanisms for its anti-inflammatory effects have been studied [16, 17], comparatively little is known about its effect against inflammation-related bone diseases such as RA and periodontitis or on bone cells (osteoclasts and osteoblasts). Only the effect of linarin on osteoblastic MC3T3 cells has been reported that it inhibits cytotoxicity and oxidative damage, and restores the mineralization function of hydrogen peroxide-treated osteoblasts.

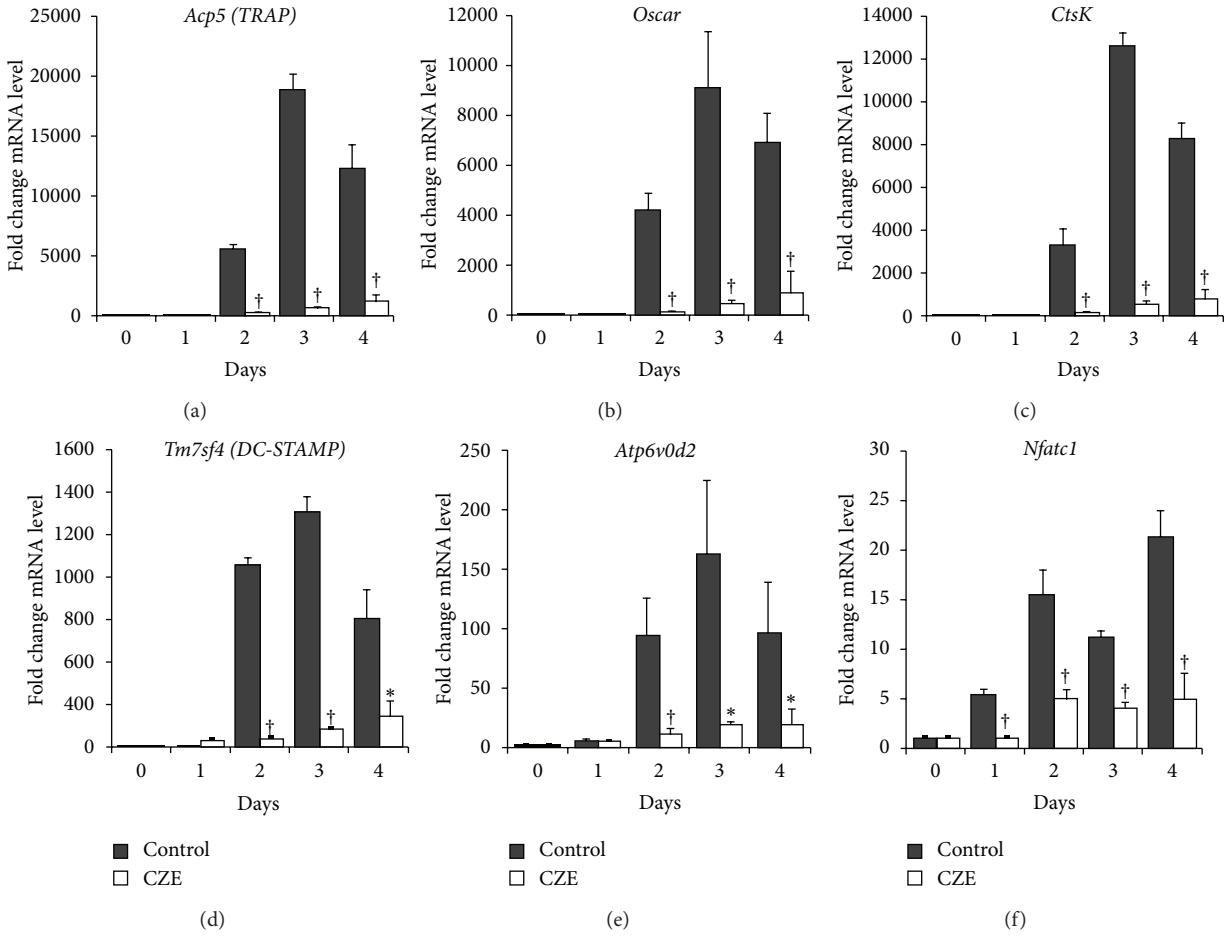

Figure 3: Effects of CZE on the expression of osteoclast differentiation marker genes. BMMs were cultured with RANKL and M-CSF treatment in the presence or absence of CZE (25 μg/mL) for 4 days. The expression of osteoclast differentiation marker genes was measured by real-time PCR. The expression of mRNA levels was normalized with GAPDH and described as fold change of mRNA level. Data are expressed as the mean ± SD and are representative of at least three independent experiments. $^{*}P < 0.05$, and $^{\dagger}P < 0.01$ versus control (0 μg/mL CZE).

Linarin also suppresses RANKL expression induced by hydrogen peroxide and appears to have antiresorptive activity [23]. Here, we have elucidated an inhibitory effect of CZE via the reduction of NFATc1 expression in the differentiation and formation of osteoclasts, which cause bone destruction associated with inflammation-related bone diseases.

Previously, many studies have established that NFATc1 is a critical transcription factor for RANKL-mediated osteoclast differentiation, fusion, and activation. When BMMs are stimulated by RANKL, the expression of NFATc1 is induced through c-Fos and autoamplification by NFATc1 [9, 10]. NFATc1-deficient embryonic stem cells do not form mature osteoclasts by RANKL treatment and overexpression of ectopic ca-NFATc1 in BMMs appropriately induces osteoclast differentiation from BMMs even in the absence of RANKL [9, 25, 29]. Recently, we had reported that NFATc1 is a key regulator of osteoclast fusion, which is an essential step for efficient bone resorption, via upregulation of ATP6v0d2 and dendritic cell-specific transmembrane protein (DC-STAMP), which are known as osteoclast fusion molecules as confirmed by genetic experiments [29]. Moreover, several reports showed that NFATc1 is implicated in the regulation of osteoclast function. The expression of TRAP, Cathepsin K, c-Src, and β3 integrin, which are involved in osteoclast-mediated bone resorption, is regulated by NFATc1 [9, 10, 12]. Furthermore, acidosis and RANKL signals in osteoclasts stimulate bone resorption via activation of Ca^{2+}/calcineurin/NFAT pathway [30]. These results indicate that NFATc1 is a master key regulator of osteoclastogenesis. Therefore, in order to regulate excessive osteoclasts activity, which causes severe bone destruction in bone diseases, it is efficient and essential to control the expression of NFATc1 as a therapeutic target. In this study, our data demonstrate that CZE suppresses the expression of c-Fos and NFATc1 via inactivation of ERK, which contribute to RANKL-induced osteoclast differentiation (Figure 4).

Previous studies elucidated that Ca^{2+}-oscillation for NFATc1 induction is an essential process for osteoclastogenesis, and that ablation of Ca^{2+}-oscillation causes impairment of osteoclastogenesis [12, 31, 32]. RANKL-stimulated Ca^{2+}-oscillation is initiated approximately 24 h after RANKL treatment and is maintained until the formation of mature

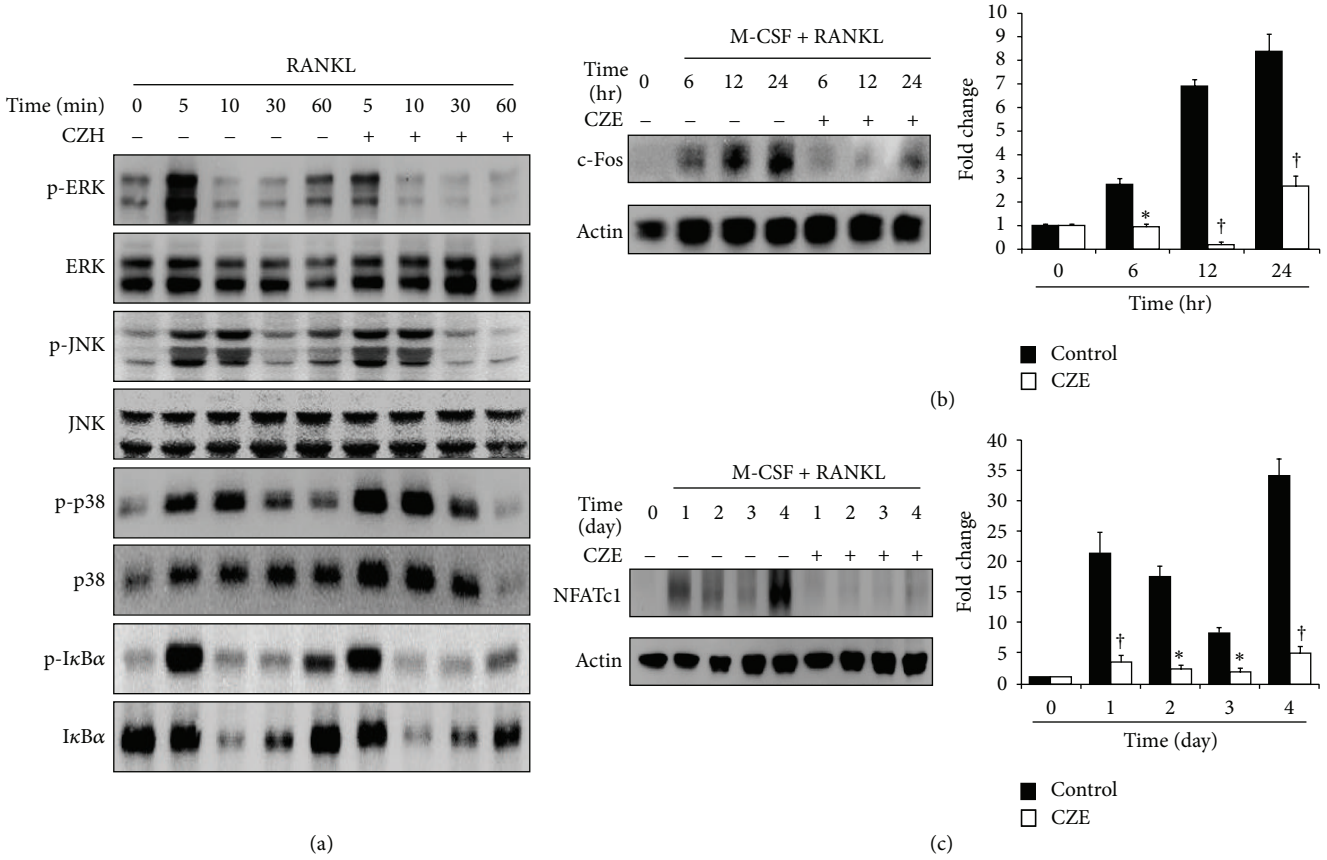

Figure 4: Effects of CZE on RANKL-induced intracellular signaling and the expression of transcription factors in osteoclasts. BMMs were treated with M-CSF and RANKL in the presence or absence of CZE (25 μg/mL) for the indicated time. Lysate (30 μg) was subjected to SDS-PAGE and analyzed by immunoblotting. (a) MAPK (ERK, JNK, and p38) activation was measured by using their respective antibodies. (b)-(c) The expression of c-Fos and NFATc1 was detected by anti-c-Fos and NFATc1 antibody, respectively. Fold change normalized by actin is presented in the right panel. Data are representatively obtained from three independent experiments and are expressed as the mean ± SD. $^{*}P < 0.05$, and $^{\dagger}P < 0.01$ versus control (0 μg/mL CZE).

osteoclasts [15]. It seems that long-term Ca^{2+}-oscillation is helpful for sustaining NFATc1 in the nucleus and for ensuring the transcriptional activation of NFATc1 required for terminal osteoclast differentiation [33]. Intracellular Ca^{2+} originates from the extracellular space through plasma membrane channels or intracellular organelles such as the ER [34]. IP_3 receptors (IP_3Rs), especially IP_3R2 and IP_3R3 in osteoclasts, mediate Ca^{2+} release from the ER to the cytosol in response to IP_3 binding [13]. Recently, it has been reported that the Ca^{2+} influx during osteoclastogenesis is regulated by plasma membrane-localized Ca^{2+} channels, such as Orai1, TRPV4, and TRPV5 [35–37].

In Figure 5, BMMs treated with RANKL for 1 day produced typical Ca^{2+}-oscillation. However, when BMMs were treated with CZE under RANKL stimulation for 1 day, the typical Ca^{2+}-oscillation was interrupted; instead, abnormal biphasic pick, which is thought to be mediated by Ca^{2+} influx, was induced. In addition, when RANKL-stimulated BMMs showing typical Ca^{2+}-oscillation were acutely treated with CZE, an abnormal biphasic pick, implicating dramatically increased intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ was produced, and the typical Ca^{2+}-oscillation by RANKL disappeared. Although it is unknown how CZE induces abnormal Ca^{2+} influx in osteoclasts, CZE definitely ablated the typical Ca^{2+}-oscillation induced by RANKL stimulation. In addition, CZE inhibited PLCγ2 and CREB activation, which are important for osteoclast differentiation, followed by the repression of c-Fos and NFATc1 expression. These results indicate that CZE is an effective regulator of osteoclast differentiation and formation via the control of two main pathways induced by RANKL/RANK binding. In further studies, it will be interesting to explore how CZE regulates Ca^{2+} influx and which Ca^{2+} channel is affected by CZE. Detailed investigations of this mechanism will provide more insights into the effect of CZE on osteoclastogenesis and its relationship with the pathologies of bone diseases, such as osteoporosis, periodontitis, and rheumatoid arthritis.

5. Conclusion

Our results clearly demonstrate that the inhibitory effect of CZE on RANKL-stimulated osteoclastogenesis is mediated

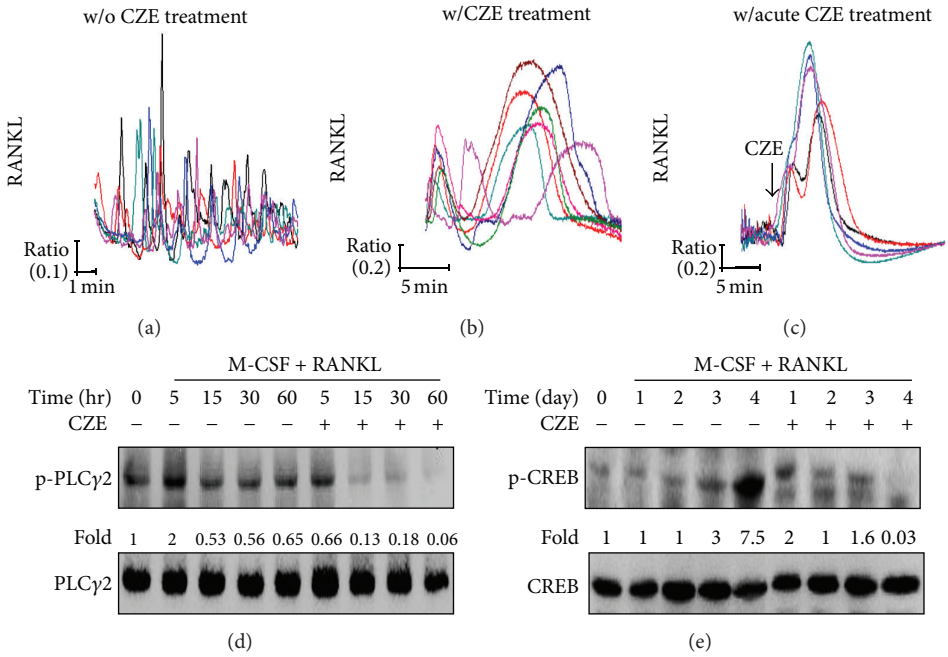

Figure 5: Effect of CZE in RANKL-stimulated costimulatory signals in osteoclasts. BMMs were cultured with M-CSF and RANKL in the presence or absence of CZE (25 μg/mL) for 1 day. (a)-(b) RANKL-induced Ca^{2+}-oscillation was measured with the Ca^{2+} indicator, Fura-2AM. (c) BMMs were cultured with M-CSF and RANKL in the presence of CZE for 1 day. RANKL-induced Ca^{2+}-oscillation by acute treatment of CZE was measured. (d)-(e) BMMs were cultured with M-CSF and RANKL in presence or absence of CZE (25 μg/mL) for the indicated time. Lysate (30 μg) was subjected to SDS-PAGE, and the activation of PLCγ2 (d) and CREB (e) was analyzed by immunoblotting. All data are representative of at least three independent experiments.

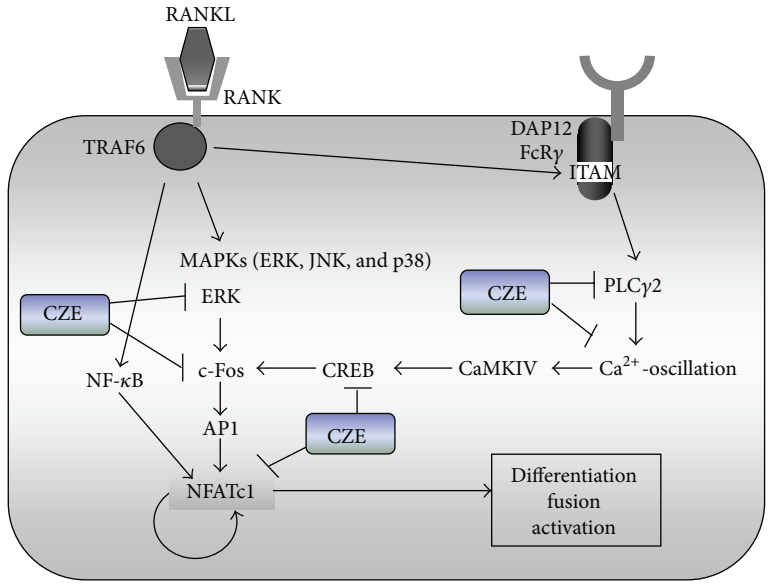

Figure 6: Schematic diagram of the effect of CZE on RANKL-induced osteoclastogenesis. RANKL/RANK interaction may lead to the activation of MAPKs followed by c-Fos expression and alternatively activation of PLCγ2 inducing calcium signaling, which is critical for NFATc1 activation, followed by CREB activation and induction of c-Fos and NFATc1. CZE inhibited both RANKL-induced ERK and PLCγ activation signaling pathways.

by the repression of c-Fos and NFATc1 expression, which are critical for osteoclastogenesis, via ERK and PLCγ/Ca^{2+}-oscillation/CREB signaling in osteoclasts. These findings reveal CZE as a traditional therapeutic agent against inflammatory bone diseases, such as rheumatoid arthritis and periodontitis.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Dong Ryun Gu and Jin-ki Hwang contributed equally to this work.

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