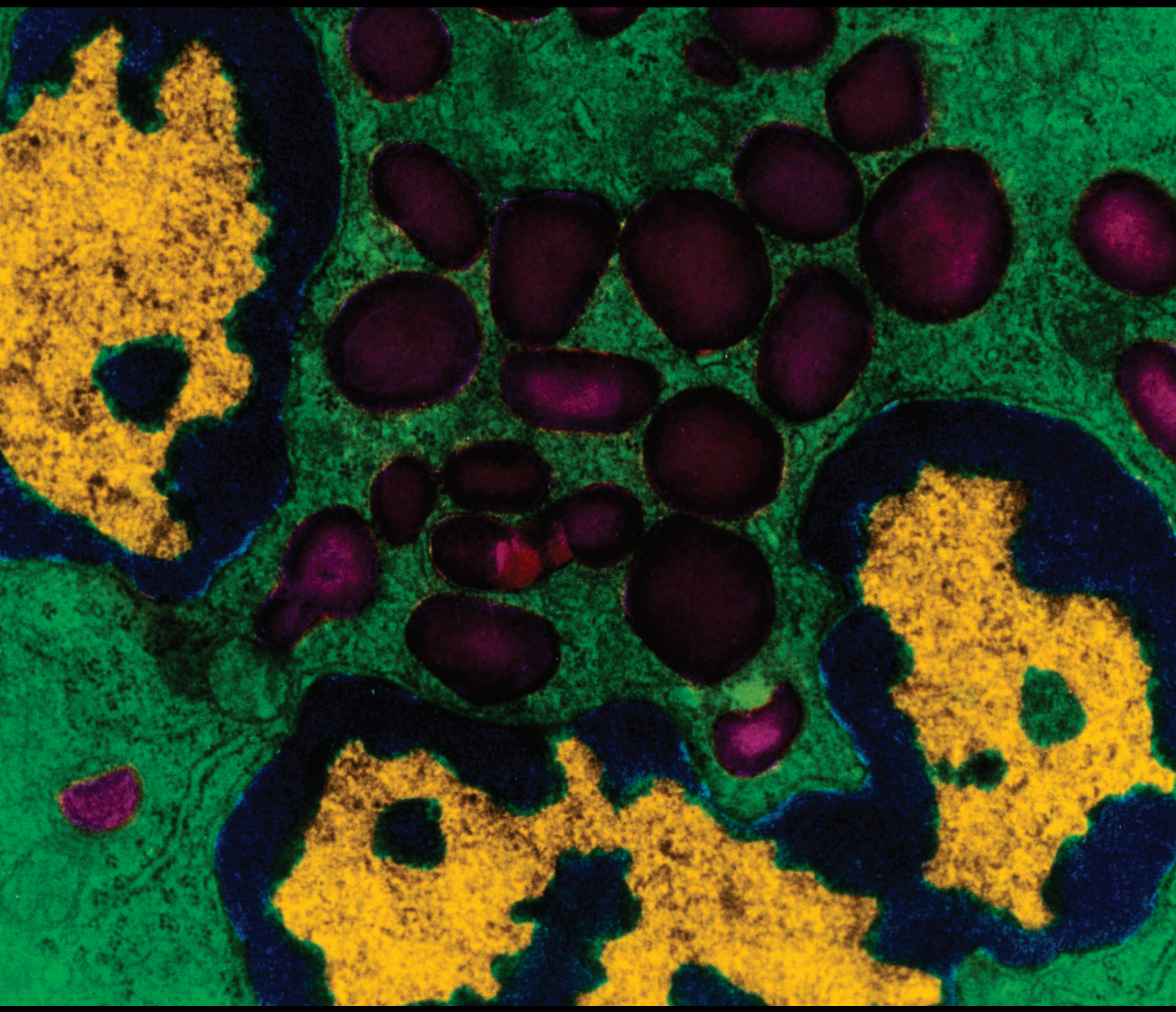
Mediators of Inflammation

Herbal Medicines for Inflammatory Diseases

Guest Editors: Seong-Gyu Ko, Chang Shik Yin, Bing Du, and KyoungHyun Kim



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Editorial

Herbal Medicines for Inflammatory Diseases

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Inflammation plays an essential role in the development of various human diseases including asthma, rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, and tendonitis. Moreover, chronic inflammatory response is a major driving force for the progression of cancer, atherosclerosis, diabetes, obesity, and Alzheimer's disease. When under control, inflammatory process is beneficial in protecting the body from disastrous breakdown whereas inflammatory process is harmful, when unrestrained, resulting in unwanted breakdown of the body. In this sense, inflammation can be a two-edged sword.

Herbal medicines have long been used for preventing or treating diseases including inflammatory diseases and indeed a priceless source of valuable chemical compounds that developed into indispensable drugs in medical practice. Nonetheless, their valuable effects in inflammatory diseases have not been thoroughly investigated. Today, there are plenty of chemical drugs in the shelves of the pharmacy. We may take those modern chemical drugs for granted. However, herbal medicine has been with us from prehistoric days as a rich source of medicinal compounds. Herbal medicine is a valuable constituent of traditional medicine and modern medicine, as will be probably the same in the future.

Considering that inflammation is one of the essential factors in the development of many human diseases, there is an urgent need to expand our scientific understanding for beneficial effects of herbal medicines on inflammatory diseases. Yes, it is true that there is a long way to go to elucidate precise effects of herbal medicines at molecular level. However, it is time to be more aware of the importance and potential of herbal medicine in inflammatory process, considering rapidly expanding its use and application.

This special issue will introduce you to the valuable research reports on herbal medicine in inflammatory diseases, ranging from basic researches to explore roles of herbal medicines against inflammatory diseases, to clinical trials to access roles of herbal medicines for inflammatory diseases, to combinatorial uses of herbal medicines with conventional treatment for inflammatory diseases, to analyses of bioactive compounds in medicinal plant extracts for inflammatory diseases, and to systematic reviews for inflammatory diseases. We hope this timely special issue be a valuable resource to contemporary outstanding researchers.

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Research Article

The Protective Effects of the Supercritical-Carbon Dioxide Fluid Extract of *Chrysanthemum indicum* against Lipopolysaccharide-Induced Acute Lung Injury in Mice via Modulating Toll-Like Receptor 4 Signaling Pathway

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The supercritical-carbon dioxide fluid extract of *Chrysanthemum indicum* Linné. (CFE) has been demonstrated to be effective in suppressing inflammation. The aim of this study is to investigate the preventive action and underlying mechanisms of CFE on acute lung injury (ALI) induced by lipopolysaccharide (LPS) in mice. ALI was induced by intratracheal instillation of LPS into lung, and dexamethasone was used as a positive control. Results revealed that pretreatment with CFE abated LPS-induced lung histopathologic changes, reduced the wet/dry ratio and proinflammatory cytokines productions (TNF-α, IL-1β, and IL-6), inhibited inflammatory cells migrations and protein leakages, suppressed the levels of MPO and MDA, and upregulated the abilities of antioxidative enzymes (SOD, CAT, and GPx). Furthermore, the pretreatment with CFE downregulated the activations of NF-κB and the expressions of TLR4/MyD88. These results suggested that CFE exerted potential protective effects against LPS-induced ALI in mice and was a potential therapeutic drug for ALI. Its mechanisms were at least partially associated with the modulations of TLR4 signaling pathways.

1. Introduction

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are clinical problems induced by acute and excessive inflammatory responses to stimulus in the airspaces and lung parenchyma, involving alveolar-capillary membrane damage, neutrophils recruitment, vascular permeability increase, pulmonary edema, and respiratory failure [1–3]. ALI and ARDS are life-threatening problems, always leading to multiple organ dysfunction syndrome (MODS), with significant incidences and mortalities in critically ill patients [1, 2]. Although the pathologies of ALI have been clearly defined and several candidate therapy strategies have been clinically applied for ALI [3–5], there is still no effective therapy strategy but relatively noteworthy mortality rates [2, 6]. Therefore, to develop novel effective prevention and therapies is urgently needed.

Lipopolysaccharide (LPS), a major constituent of outer membranes of Gram-negative bacteria, has been defined to be a pivotal risky factor and prominent stimulus in the pathogenesis of ALI [3, 7]. It has been defined that LPS challenges induce neutrophil infiltrations, trigger acute inflammatory responses, and generate early lung pathological changes, resulting in the morbidity and development

of ALI [3, 8, 9]. Thus intratracheal instillation of LPS to experimental animals, with clear clinical relations to the process of ALI but no systemic inflammation, is well-suited and reproducible for preliminarily pharmacological researches of novel drugs or other therapeutic agents [8–10]. Once entering into the host, LPS activates toll-like receptor 4 (TLR4), a main sensor of the TLR superfamily acting as transmembrane proteins and signal transduction molecules [5, 7, 11]. TLR4 can activate nuclear factor- (NF-)κB protein through myeloid differentiation factor 88 (MyD88) pathway [5, 11]. Then the activated NF-κB induces the productions of proinflammatory cytokines such as tumor necrosis factor- (TNF-)α, interleukin- (IL-) 1β, and IL-6 [11–13], resulting in recruitment of intravascular neutrophils into the alveolar space and lung parenchyma [14, 15], followed by protease release and reactive oxygen species (ROS) generation which is closely associated with lipid peroxidation aggravation (such as malondialdehyde, MDA) and antioxidant enzyme (such as superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPx) activity decline [8, 14]. Hence, drugs focusing on downregulating the TLR4 signaling pathway and/or inhibiting its related inflammatory responses would provide potential therapeutic effects for ALI [5, 11].

Chrysanthemum indicum Linné (*C. indicum*), a traditional Chinese medicine, has long been used in treatments of several acute respiratory system diseases, including cold, cough, acute bronchitis, acute laryngitis, and acute pharyngitis, with high efficacy and low toxicity [16]. The supercritical-carbon dioxide fluid extract of *C. indicum* (CFE) has been widely applied as a fine material in many TCM preparations, for example, *C. indicum* granules and capsules [17, 18]. Most importantly, CFE was a key ingredient of a TCM recipe named Compound *C. indicum* Soft Capsule (also known as CPZ in previous studies), an anti-influenza drug whose effects were closely associated with anti-inflammatory activity [18]. CFE has also been demonstrated to possess strong anti-inflammatory effects, and the underlying mechanism was related to its upregulations of antioxidant enzymes and downregulations of NF-κB and some proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [17, 18].

Although CFE has shown anti-inflammatory benefits, its potential effects to protect against LPS-induced ALI still remained unclear. Therefore, in this present study, we aimed to evaluate the effect of CFE on LPS-induced ALI in mice. And for potential mechanisms elucidation, the expressions of TLR4/MyD88 and the phosphorylations of NF-κB p65/IκBα were also evaluated.

2. Materials and Methods

2.1. Drugs and Chemicals. Lipopolysaccharide (LPS) and dexamethasone (Dex) were purchased from Sigma Co., Ltd. (St. Louis, USA), and Xianju Pharmaceutical Co., Ltd. (Zhejiang, China), respectively. Phosphate buffered saline (PBS), sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), skimmed milk, Tween-20, and Tween-80 were purchased from Thermo-Fisher Sci. Co., Ltd. (MA, USA). Hexadecyltrimethylammonium bromide (HTAB) and *o*-dianisidine were purchased from TCI Co., Ltd. (Tokyo, Japan). All other chemicals were of the reagent grade.

2.2. Preparation of CFE. The supercritical fluid CO_2 extract of *Chrysanthemum indicum* Linné (CFE) was prepared and provided by the Institute of New Drug Research & Development, Guangzhou University of Chinese Medicine (Lot. 20121104). In our previous chemical analysis of CFE, 35 compounds were identified by GC-MS, and 5 compounds were reconfirmed and quantified by HPLC-PAD (the brief chemical profile of CFE is listed in Table 1) [17]. For experiments in this paper, CFE samples were diluted by 0.5% Tween-80 into the appropriate dose.

2.3. Experimental Animals. Male Kunming (KM) mice (20–25 g) were purchased from Medical Laboratory Animal Center of Guangdong Province (Certificate number SCXK2008-0002, Guangdong Province, China). Animals were kept on 12-hour light/12-hour dark cycles under regular temperature ($22 \pm 2^{\circ}$C) and humidity ($50 \pm 10\%$) with standard diets and clean water *ad libitum*. All animals were sacrificed by lethal sodium pentobarbital injection. All experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine.

2.4. Experimental Designs

2.4.1. Positive Control Setting, Animal Groups, and LPS-Induced ALI. Dex has been frequently used as a positive control agent in various kinds of ALI models in experimental settings. With the definite and proved benefits and effects, Dex is applicable to be the positive control agent for screening and evaluating new therapeutic agents [19–21]. Therefore, Dex was selected as the positive control agent and a common dose of 5 mg/kg (p.o.) used in this study [19–21].

To assess mortality rate, 120 mice were randomly divided into 5 groups ($n = 24$), sham group, LPS group, and CFE (40, 80 and 120 mg/kg) groups. CFE groups were given CFE (40, 80, and 120 mg/kg, p.o.) while sham group and LPS group were given Tween-80, for 7 consecutive days. One hour after the last administration, all animals were anesthetized. And mice from LPS group and CFE groups were given a single intratracheal instillation of 20 mg/kg LPS (10 mg/mL, dilution with PBS; 20 μL/10 g body weight) while mice from the sham group were given an equal volume of PBS. After operation, mice of all groups were monitored and the time when any animal died was recorded every 6 hours up to 120 hours. Then the mortality rate of each group within 120 hours was calculated and compared using the Kaplan Meier methods.

In the other experiments, mice were randomly divided into 6 groups ($n = 30$), sham group, LPS group, CFE (40, 80, and 120 mg/kg) groups, and Dex group (5 mg/kg). CFE groups and Dex group were given CFE (40, 80, and 120 mg/kg, p.o.) and Dex (5 mg/kg, p.o.) once per day for 7 consecutive days, respectively. During this period, sham group and LPS group were given equal volumes of

Table 1: The chemical profile of CFE.

Number	Components	Percentage (%)
1	Camphene	0.475[a]
2	β-Cymene	0.998[a]
3	Eucalyptol	3.091[a]
4	Linalool oxide	0.521[a]
5	α-Thujone	2.186[a]
6	β-Thujone	2.169[a]
7	Isothujol	1.094[a]
8	L-Pinocarveol	0.765[a]
9	d-Camphor	8.582[a]
10	*cis*-Verbenol	4.720[a]
11	*endo*-Borneol	7.845[a]
12	L-4-Terpineol	1.634[a]
13	α-Terpineol	1.022[a]
14	Myrtenol	1.054[a]
15	Cumaldehyde	0.486[a]
16	Bornyl acetate	2.948[a]
17	Thymol	3.071[a]
18	β-Caryophyllene	3.336[a]
19	*cis*-β-Farnesene	2.270[a]
20	α-Curcumene	5.932[a]
21	δ-Cadinene	1.815[a]
22	Spathulenol	1.362[a]
23	Caryophyllene oxide	8.460[a]
24	γ-Eudesmol	1.568[a]
25	T-Muurolol	1.487[a]
26	α-Gurjunene	2.161[a]
27	Aromadendrene	2.280[a]
28	α-Bisabolol	2.289[a]
29	Cubenol	1.742[a]
30	Longifolenaldehyde	2.572[a]
31	α-Bisabolol oxide	2.600[a]
32	Hexahydrofarnesyl acetone	1.212[a]
33	Ethyl hexadecanoate	1.362[a]
34	α-Linolenic acid	2.130[a]
35	Ethyl octadec-9,12-dienoate	2.470[a]
36	Chlorogenic acid	2.110[b]
37	Luteolin-7-glucoside	2.800[b]
38	Linarin	4.830[b]
39	Luteolin	1.140[b]
40	Acacetin	0.880[b]

[a]Identified by GC-MS analysis and the relative percentage calculated by integrated peak area in Agilent MSD Chemstation data analysis program.
[b]Identified and quantified by HPLC-PAD analysis, and the relative percentage was represented by the content quantitatively analyzed with peak areas under the standard curves.

Tween-80. One hour after the last administration, mice were anesthetized via intraperitoneally injecting pentobarbital sodium (30 mg/kg). After that, mice from LPS group, Dex group, and CFE groups were given a single intratracheal instillation of 5 mg/kg LPS (2.5 mg/mL, freshly diluted with PBS; 20 μL/10 g body weight) while mice of sham group were given an equal volume of PBS. In this model, all animals survived for 24 hours after the intratracheal instillation of LPS at the dose of 5 mg/kg, which was optimized and repeatable, based on our preliminary experiments (data were not provided).

2.4.2. Specimen Collections. 24 hours after LPS instillation, 30 mice of each group were randomly divided into 3 parts, 10 mice per part. Part 1 was used for the bronchoalveolar lavage fluid (BALF) preparation and the lung wet/dry weight (W/D) ratio measurement. In brief, after anaesthetization, mouse was surgically exposed the trachea and clamped the right main bronchus. Then the left lung of each animal was lavaged for three times with a total volume of 1.5 mL of Hanks-Balanced-Salt solution using a venous indwelling needle. The BALF recovery rate was more than 90%. Then the mouse was sacrificed, and right lung was harvested for the W/D ratio measurement. Part 2 was used for the lung tissue preparation and the histopathologic evaluation. In brief, after the mouse was sacrificed, the left lung was taken, placed in appropriate amount of precold PBS immediately, and homogenized using a Tissue Lyser II high-throughput tissue homogenization system (Qiagen Co., Ltd., Hilden, Germany). Then the homogenate was centrifuged at 4°C. 100 μL of the supernatant was used for protein measurement and the rest was immediately collected and stored at −80°C for further analysis of MPO, MDA, proinflammatory cytokines (TNF-α, IL-1β, and IL-6), and antioxidant enzymes (SOD, CAT, and GPx). At the same time, the right lung was harvested for the histopathologic examination. Part 3 was left for western-blot assay of TLR4, MyD88, NF-κB p65, and IκBα. In brief, mouse was sacrificed, and the lung tissues were harvested, frozen, and stored in liquid nitrogen immediately.

2.5. Measurement of Lung W/D Ratio. The lung W/D ratio was measured according to the previous study. In brief, the excised right lung was blotted dry and weighed to obtain the "wet" weight, afterwards kept in an oven at 80°C for 48 hours to obtain the "dry" weight. Then the W/D ratio was calculated by the "wet" weight to the "dry" weight.

2.6. Measurement of BALF Protein Contents and BALF Cell Counts. The BALF was centrifuged at 800 ×g for 10 min at 4°C, and the supernatant was collected for measurement of protein content. Measurements of protein contents of BALF were performed using a commercial BCA kit (Beyotime Institute of Biotechnology, Shanghai, China) and expressed as mg/mL BALF. Then sediment cells were resuspended in precold PBS and stained by a Wright-Giemsa kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for cytospin preparations. Counts of the total cells, neutrophils, and macrophages were then double-blindly performed via hemacytometry.

2.7. MPO Assay. The assay of MPO activity was performed via the HTAB method. Briefly, the samples were mixed with KPO_4 buffer (50 mM, pH 6.0) with HTAB (0.5%). After reacting and incubating at 37°C for 15min, the enzyme was assayed by the activity in a H_2O_2/*o*-dianisidine buffer at

460 nm with a Multiskan GO microplate spectrophotometer (Thermo-Fisher Sci., Waltham, USA). Results were expressed as units/mg protein.

2.8. Histopathologic Examination. Biopsies of right lungs were collected, fixed in 4% paraformaldehyde solution, dehydrated, embedded with paraffin, and sectioned into 4 μm. Tissue sections were stained with hematoxylin and eosin kit (H&E, Beyotime Institute of Biotechnology), examined, and photographed using TE2000-S Inverted Microscopes (Nikon Co., Ltd., Tokyo, Japan). According to previous reports, histologic changes including neutrophil infiltration, interstitial edema, congestion (or hemorrhage), and hyaline membrane formation were evaluated and the severity of each change was scored on a scale of 0 (normal) to 4 (severe) by a pathologist blinded to this study. Finally, the overall histologic injury was evaluated according to the sum-scores (0 as normal, 1 to 5 as minimal; 6 to 10 as mild; 11 to 15 as moderate; 16 to 20 as severe). Results were presented as the means of scores of microscopic areas of each group.

2.9. MDA, SOD, CAT, and GPx Assay. The MDA assay was carried out with commercial MDA assay kits (Beyotime Institute of Biotechnology) via the method of thiobarbituric acid reacting substance (TBARS). In Brie, reaction between MDA and TBARS was performed at 100°C, resulting in formation of a red complex TBARS, which could be recorded and measured at 532 nm. SOD, CAT, and GPx were measured using commercially available kits (Beyotime Institute of Biotechnology) according to the manufacturers' instructions, respectively. Briefly, xanthine and xanthine oxidase (XOD) generated superoxide radicals and reacted with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye, which could be measured at 532 nm. CAT was measured by the production of N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinonemonoimine, which would be detected at 520 nm. And GPx was measured by detecting contents of GRd and NADPH, which would be recorded at 340 nm.

2.10. ELISA Assay for TNF-α, IL-1β, and IL-6. TNF-α, IL-1β, and IL-6 were measured using commercially available ELISA kits (*e* Bioscience Co., Ltd., CA, USA). In brief, diluted standards or samples were added to 96-well plates precoated with affinity purified polyclonal antibodies specific for mouse TNF-α, IL-1β, and IL-6, respectively. Then wells were added with enzyme linked polyclonal antibodies and incubated at 37°C for 60 min, followed by final washes for 5 times. The intensities detected at 450 nm were measured after addition of substrate solutions for 15 min. Levels of TNF-α, IL-1β, and IL-6 were calculated according to standard curves.

2.11. Western-Blot Assay for TLR4, MyD88, and NF-κB. Extractions of proteins from the lung tissues were performed with T-PER tissues proteins extractions reagent kits (Beyotime Institute of Biotechnology). Extractions of nuclear and cytoplasmic proteins from the lungs were performed with nuclear and cytoplasmic proteins extractions reagent kits (Beyotime Institute of Biotechnology). Protein contents were measured using BCA protein assay kits and equal amounts of protein were added in per well on 10% SDS PAGE. Then, proteins were separated and transferred into PVDF membranes by an Electrophoresis System (Bio-rad Co., Ltd., Hercules, USA). The resulting membranes were blocked with Tris-buffered-saline containing 0.05% Tween-20 (TBS-T), supplemented with 5% skimmed milk at room temperature for 2 hours, and followed by TBS-T washings. Then membranes were incubated with related specific primary antibodies anti-NF-κB p65 antibody, anti-IκBα antibody (Cell Signaling Technology Co., Ltd., MA, USA), anti-TLR4 antibody, and anti-MyD88 antibody (Santa Cruz Co., Ltd., TX, USA) at 4°C overnight, respectively, followed by washes with TBS-T and incubation with the peroxidase-conjugated secondary antibody at room temperature for 1 hour. The detections of labeling proteins were performed with enhanced-chemiluminescence western-blotting detections kits. And the relative protein levels were normalized to β-actin (Santa Cruz Co., Ltd.) protein as the internal standard.

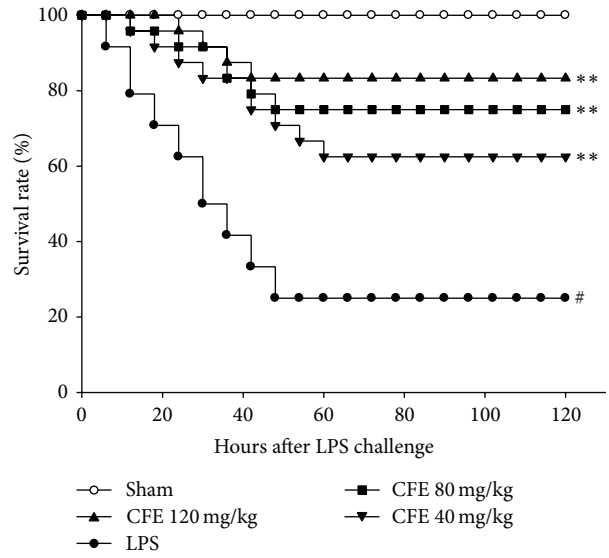

Figure 1: The effect of CFE on survival rates. Data was represented as the mean ± SEM ($n = 24$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the LPS group.

2.12. Statistical Analysis. Data were presented as the mean ± SEM and statistical analyses were performed with Systat Sigma Plot software (version 12.00 for windows). Parametric data were analyzed by one-way ANOVA, followed by Tukey-Kramer test, and nonparametric data were analyzed by Kruskal-Wallis test, followed by Dunn's test. The mortality studies were analyzed by the Kaplan-Meier method. And $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The Effects of CFE on Survival Rates. As shown in Figure 1, compared to the sham group with the survival rate of 100.0%,

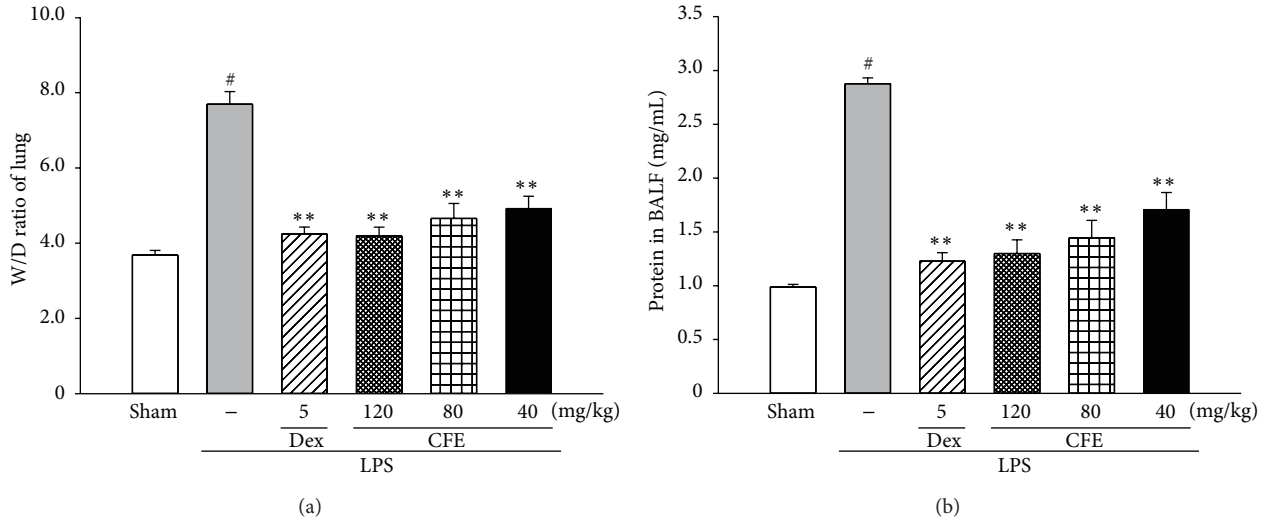

Figure 2: The effects of CFE on lung W/D ratio (a) and BALF protein content (b). Data was represented as the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the LPS group.

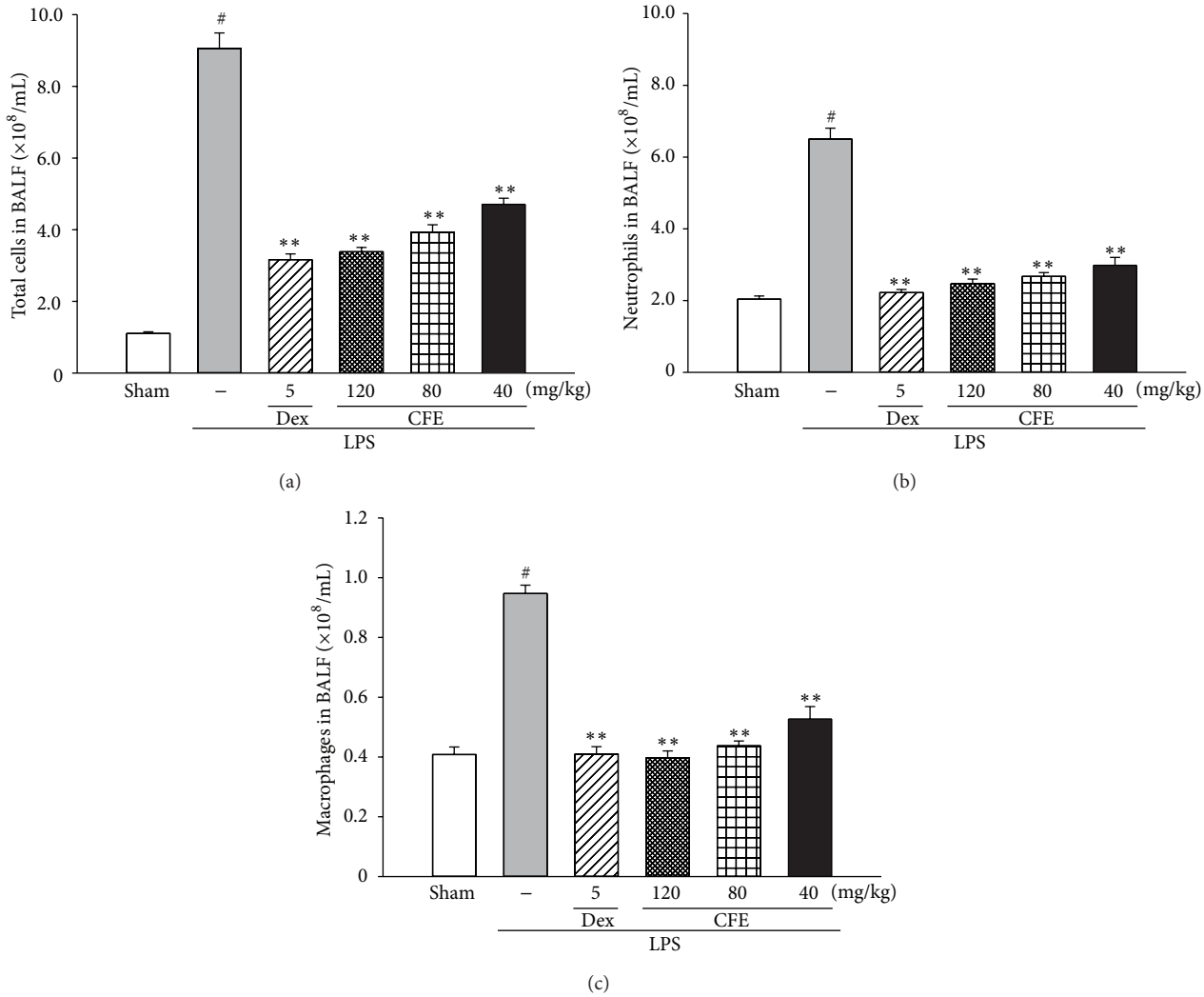

Figure 3: The effects of CFE on total cells (a), neutrophils (b), and macrophages (c) in BALF. Data was represented as the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the LPS group.

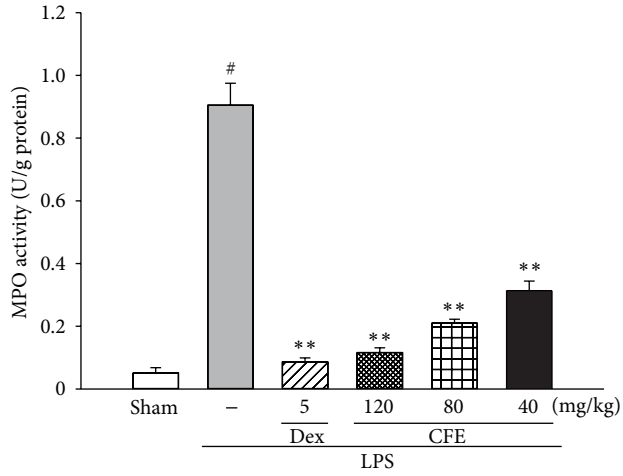

Figure 4: The effects of CFE on MPO activity. Data was represented as the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the LPS group.

LPS challenge markedly declined the survival rate of the LPS group to about 25.0% within 120 hours 11 ($P < 0.01$). Conversely, survival rates of CFE-treated groups (40, 80, and 120 mg/kg) significantly increased to 62.5%, 75.0 %, and 83.3%, respectively, in a dose-dependent manner (for all, $P < 0.01$ versus the LPS group). Data indicated that CFE pretreatment possessed potential prevention against mortality in ALI mice induced by LPS.

3.2. The Effects of CFE on Lung W/D Ratio. The W/D ratio of ALI mice was evaluated to assess the severity of pulmonary edema. And as shown in Figure 2(a), when compared with the sham group, there was a significant increase (approximately 2-fold) in the lung W/D ratio of the LPS group ($P < 0.01$). However, with the pretreatments of CFE (40, 80, and 120 mg/kg), the levels of lung W/D ratio were dose-dependently suppressed, as compared to the LPS group (for all, $P < 0.01$). Data showed that the lung W/D ratio was significantly suppressed by pretreatment with CFE.

3.3. The Effects of CFE on BALF Protein Content. The vascular permeability of the lung in mice was measured by the protein content of BALF. As compared to the sham group, LPS significantly boosted the BALF protein level of the LPS group ($P < 0.01$, Figure 2(b)). On the contrary, the protein contents of CFE groups (40, 80, and 120 mg/kg) were markedly suppressed in a dose-dependent manner, when compared with the LPS group (for all, $P < 0.01$).

3.4. The Effects of CFE on Cell Counts of BALF. As shown in Figure 3, the BALF cell counts of LPS group demonstrated significant increases in the total cells (Figure 3(a)), neutrophils (Figure 3(b)), and macrophages (Figure 3(c)) ($P < 0.01$ versus the sham). However, pretreatments with CFE (40, 80, and 120 mg/kg) and Dex (5 mg/kg) markedly decreased all relevant cell counts in CFE groups and Dex group, respectively, (for all, $P < 0.01$ versus the LPS group).

3.5. The Effects of CFE on MPO Activity. MPO served as a functional index indicating neutrophils infiltration, which represented the levels of MPO-derived oxidants generation and lung tissue damage [5, 8, 14]. As expected outcomes of the LPS group, the MPO activity in lung tissue, was significantly elevated, about 9 folds of the sham group ($P < 0.01$, versus the sham group), which was shown in Figure 4. However, when compared to the LPS group, the MPO activities of the CFE-treated (40, 80, and 120 mg/kg) and Dex-treated (5 mg/kg) groups were significantly inhibited (for all, $P < 0.01$).

3.6. The Effects of CFE on Histopathological Examination. Histopathological analyses were performed to investigate the effects of CFE on physiological parameters. As expected, the sham group displayed normal structures and no histopathological change in lung tissues (Figure 5(a)). On the other hand, with the challenge of LPS, the pulmonary function of LPS group was obviously impaired, with various histopathologic changes including haemorrhage, interstitial edema, thickening of the alveolar wall, and infiltration of inflammatory cells into the lung parenchyma and alveolar spaces (Figure 5(b)). As for experimental groups and positive group, histopathological changes were obviously abated by pretreatments of Dex (Figure 5(c)) and CFE (40 mg/kg, Figure 5(d); 80 mg/kg, Figure 5(e); and 120 mg/kg, Figure 5(f)), respectively, when compared to the LPS group. In addition, similarly inhibitory effects were found in semiquantitative assay by the histologic changes' scorings, which promoted the evaluation of severity of ALI (Figure 5(g)). Results demonstrated that pretreatment with CFE attenuated the severity of lungs injuries of ALI mice induced by LPS and improved the condition of lungs' tissues, in a dose-dependent manner.

3.7. The Effects of CFE on MDA, SOD, CAT, and GPx Levels. Oxidative stress plays vital important roles in the process of ALI induced by LPS, and oxidative damage induces lipid peroxidation of membrane phospholipids and inactivation of antioxidative enzymes (SOD, CAT, and GPx), while culminating in the MDA generation [5, 8, 14]. In the LPS group, MDA level in lung tissue was remarkably increased, compared to the sham group ($P < 0.01$), shown in Table 2. However, groups pretreated with CFE (40, 80, and 120 mg/kg) as well as Dex (5 mg/kg) showed significant declines in the MDA level (for all, $P < 0.01$ versus the LPS group). In addition, the activities of SOD, CAT, and GPx in LPS group were significantly abated, when compared with the sham group (for all, $P < 0.01$), as presented in Table 2. On the contrary, pretreatments with CFE (40, 80, and 120 mg/kg) significantly boosted the SOD, CAT, and GPx activities (for all, $P < 0.01$ versus the LPS group), in a dose-dependent manner.

3.8. The Effects of CFE on TNF-α, IL-1β, and IL-6 Levels. As compared to the sham group, the levels of TNF-α, IL-1β, and IL-6 in LPS group were remarkably raised (for all, $P < 0.01$, Table 3). Administration with CFE (40, 80, and 120 mg/kg) had inhibitory effects on the level of TNF-α ($P < 0.05$, versus

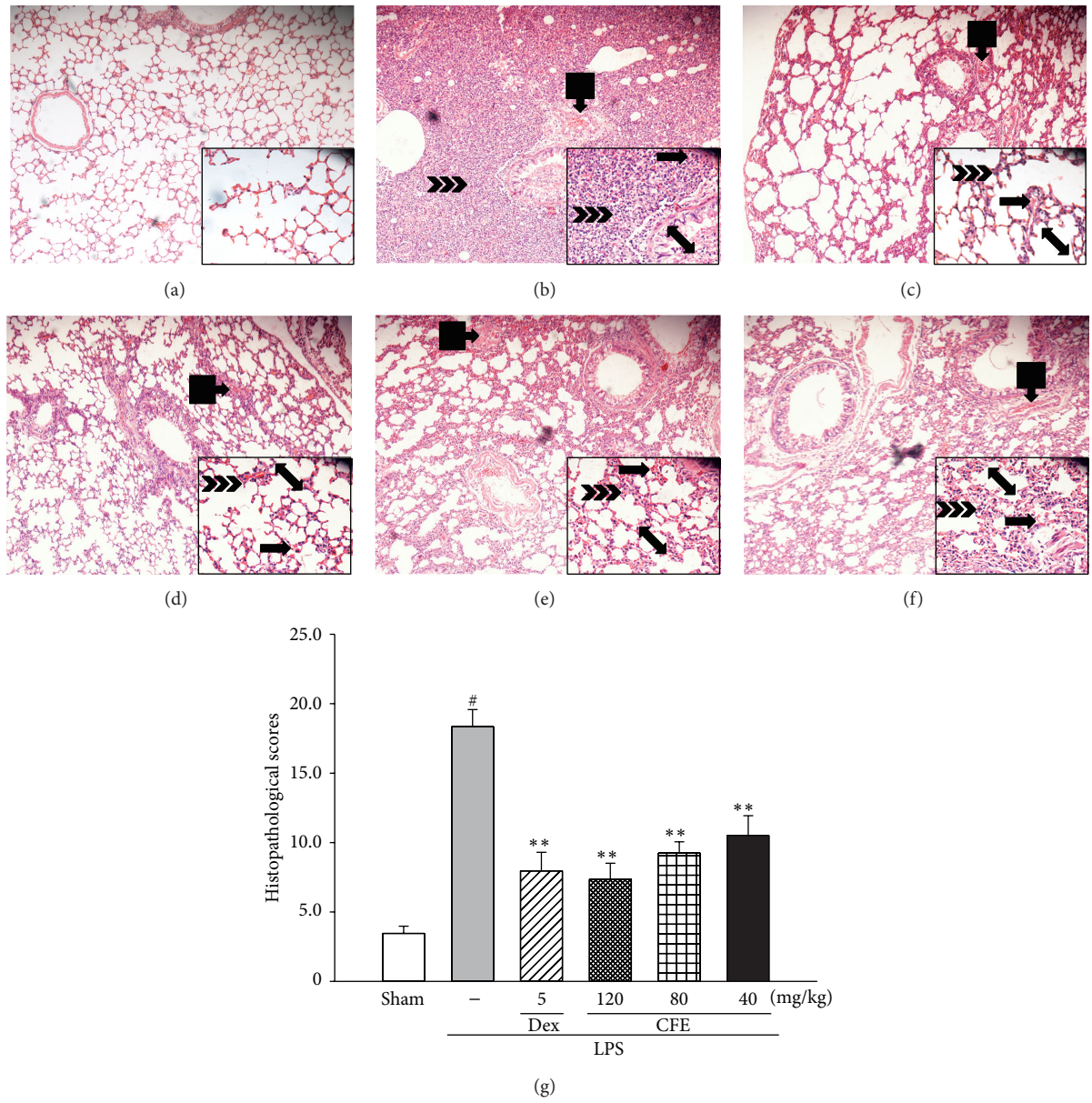

Figure 5: The effects of CFE on histopathological examination. Sham (a), LPS (b), LPS + 5 mg/kg Dex (c), LPS + 120 mg/kg CFE (d), LPS + 800 mg/kg CFE (e), LPS + 40 mg/kg CFE (f), and histologic changes scorings (G) (100x and 400x). Data was represented as the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the LPS group. Haemorrhage: ➡; interstitial edema: ➡; thickening of the alveolar wall: ⟷; and infiltration of inflammatory cells: ⋙.

Table 2: The effects of CFE on LPS-induced the levels of MDA, SOD, CAT, and GPx.

Groups	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Sham	0.29 ± 0.02	1.03 ± 0.08	0.67 ± 0.05	0.32 ± 0.04
LPS	1.18 ± 0.10$^{\#}$	0.31 ± 0.02$^{\#}$	0.11 ± 0.03$^{\#}$	0.10 ± 0.03$^{\#}$
Dex (5 mg/kg)	0.62 ± 0.06**	0.57 ± 0.06**	0.39 ± 0.06**	0.17 ± 0.03**
CFE (120 mg/kg)	0.49 ± 0.06**	0.62 ± 0.05**	0.42 ± 0.03**	0.20 ± 0.02**
CFE (80 mg/kg)	0.53 ± 0.04**	0.53 ± 0.05**	0.38 ± 0.05**	0.17 ± 0.05**
CFE (40 mg/kg)	0.56 ± 0.06**	0.44 ± 0.05**	0.36 ± 0.06**	0.16 ± 0.02**

Data represented the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to LPS group.

Table 3: The effects of CFE on LPS-induced the levels of TNF-α, IL-1β, and IL-6.

Groups	TNF-α (pg/mg protein)	IL-1β (ng/mg protein)	IL-6 (ng/mg protein)
Sham	31.97 ± 2.47	2.36 ± 0.22	5.52 ± 0.74
LPS	123.7 ± 4 9.36$^{\#}$	13. 41 ± 1.13$^{\#}$	20.20 ± 2.47$^{\#}$
Dex (5 mg/kg)	79.81 ± 13.56**	5.28 ± 0.61**	9.66 ± 1.57**
CFE (120 mg/kg)	69.49 ± 9.71**	5. 67 ± 0.83**	10.80 ± 1.69**
CFE (80 mg/kg)	77.43 ± 11.29**	6.08 ± 0.52**	11.96 ± 1.21**
CFE (40 mg/kg)	93.49 ± 6.50*	7. 92 ± 1.24*	12.33 ± 1.09**

Data represented the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to LPS group.

the LPS group). In addition, pretreatment with CFE (40, 80, and 120 mg/kg) downregulated the levels of IL-1β and IL-6, as compared to the LPS group (for all, $P < 0.05$).

3.9. The Effects of CFE on TLR4/MyD88/NF-κB Expressions. In order to probe the potential mechanisms of CFE in protection of LPS-induced ALI in mice, the expressions of TLR4, MyD88, and NF-κB in lungs were further investigated. As shown in Figure 6, data of western-blot displayed that the expressions of TLR4/MyD88/NF-κB signaling pathways were activated by LPS in the LPS group (for all, $P < 0.01$, versus the sham group). However, pretreatments with CFE (80 and 120 mg/kg) inhibited the phosphorylation of IκBα and the expressions of TLR4 and MyD88 (for all, $P < 0.05$, versus the LPS group), despite the fact that the low dose of 40 mg/kg did not reduce the phosphorylation levels of IκBα and MyD88, statistically. On the other hand, in CFE (80 and 120 mg/kg) groups, the expressions of p65 subunit NF-κB in lungs were downregulated in nucleus and upregulated in cytoplasm, and the levels of IκBα increased significantly (for all, $P < 0.01$, versus the LPS group), as shown in Figure 6. In this study, results showed that pretreatment with CFE (80 and 120 mg/kg) possessed simultaneous and efficient downregulations of TLR4 and MyD88-dependent NF-κB signaling pathways, in LPS-induced ALI mice.

4. Discussions

In this present study, we evaluated the effects of CFE on LPS-induced ALI in mice. And data demonstrated that CFE abated LPS-induced lung histopathologic changes, declined the wet/dry ratio and proinflammatory cytokines productions, inhibited inflammatory cells migrations and protein leakages into the lungs, suppressed the levels of MPO and lipid peroxidation, upregulated the abilities of antioxidative enzyme, and downregulated the activations of NF-κB and the expressions of TLR4 and MyD88. These suggested that CFE exerted potential protective effects against LPS-induced ALI.

LPS challenge leads to the leakages of serous fluids into lung tissues, resulting in the pulmonary oedema, a typical symptom of acute inflammatory responses in lung [22]. And pulmonary oedema is always evaluated via measuring a representative index, the lung W/D ratio [22, 23]. Based on the dose-dependent attenuation of the lung W/D ratio in CFE (40, 80, and 120 mg/kg) pretreated groups, pretreatment with CFE showed a significant inhibition of the pulmonary edema in ALI mice. On the other hand, the BALF total protein content, an index of lung permeability, was downregulated by pretreatment of CFE; it also demonstrated that pretreatment with CFE (40, 80, and 120 mg/kg) possessed attenuation action on lung permeability enhanced by LPS. LPS challenge also directly stimulates the infiltrations of inflammatory cells. Particularly, neutrophils migrating into the lung parenchyma and alveolar space have been indicated to have critical roles in the process of ALI [23, 24]. Neutrophils secrete MPO and lead to productions of MPO-derived oxidants and damage of lung tissues [23, 24]. Therefore the infiltration of neutrophils is usually represented by the ability of MPO [23, 24]. As expected, data showed that mice with LPS challenge presented massive infiltrations of inflammatory cells, including neutrophils and macrophages into the lung. However, pretreatment with CFE improved these changes by significantly decreasing the numbers of cells and abating LPS-induced increasing of MPO activity. These results also further proved that neutrophil respiratory burst and lung tissue damage were attenuated by CFE treatment. In addition, results of histopathologic examinations in lung tissues revealed that inflammatory responses and lung injuries in ALI mice were attenuated by CFE treatment in a dose-dependent manner. In summary, results of the alveolar-capillary barrier and inflammatory response as well as histological evidences demonstrated that CFE possessed significantly protective effects on LPS-induced ALI in mice.

Oxidative stress in lung tissue is also an important factor in the pathogenesis of ALI [5, 25, 26]; therefore, oxidative stress in the lung tissue was evaluated. Nowadays, the level of reactive oxygen species (ROS) is known as a classical index of oxidative stress [5, 26, 27]. ROS are chemically reactive molecules containing oxygen and are easy to react with biological macromolecules leading to lipid peroxidation, proteins inactivation, DNAs mutation, and finally tissue damage [5, 26, 27]. In the early stage of ALI induced by LPS, responses of neutrophils, including respiratory burst and degranulation, stimulate cells to rapidly release ROS, such as superoxide radicals ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\bullet^-$) [25, 27, 28]. On the other hand, MPO is catalyzed to produce hypochlorous acid (HOCl) that is categorized as ROS, to respond for LPS challenge and/or kill other pathogens [25, 27, 28]. ROS primarily attack the polyunsaturated-fatty acids of cell and plasma membranes leading to the formations of MDA, a lipid peroxidation product [27, 29]. Thus, the accumulation of MDA is commonly

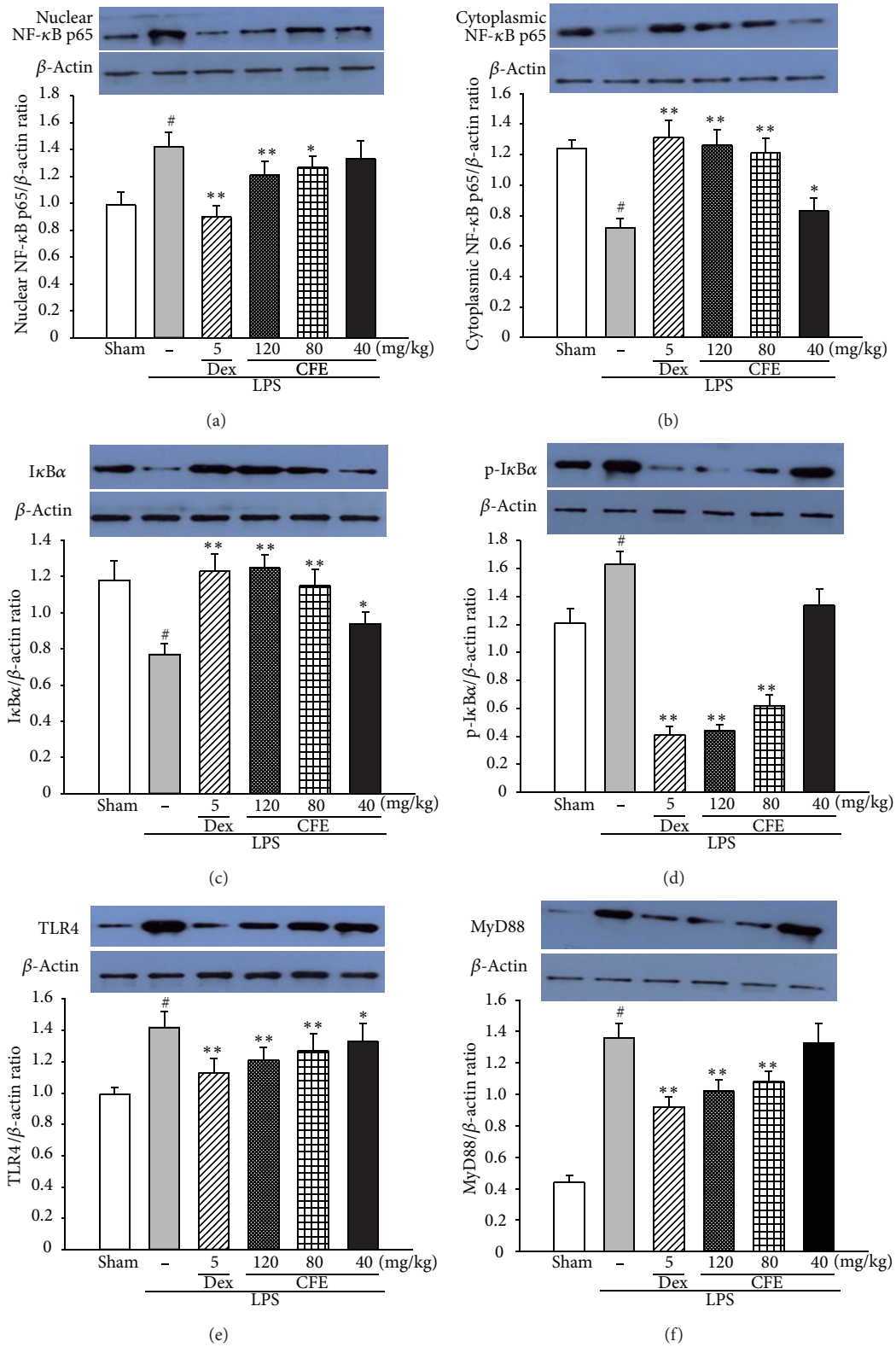

FIGURE 6: The effects of CFE on TLR4/MyD88/NF-κB expressions. The levels of NF-κB p65 in nucleus (a), NF-κB p65 in nucleus cytoplasm (b), IκBα (c), p-IκBα (d), TLR4 (e), and MyD88 (f). Data was represented as the mean ± SEM ($n = 10$). $^{\#}P < 0.01$ compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the LPS group.

used as a marker to manifest the degree of lipid peroxidation, to some extent, the level of oxidative stress and antioxidant status [26, 29]. With the results of MDA assay, pretreatment with CFE was found to have significant inhibitory effects on the formation of MDA, which clearly indicated that oxidative stress in lung tissues of ALI mice was alleviated. In addition, ROS have been proposed to mediate cell damage via a number of independent mechanisms including the inactivation of antioxidant defense systems consisting of a variety of antioxidant enzymes, such as SOD, CAT, and GPx [25, 29, 30]. These enzymes can minimize, scavenge, and eliminate the formation of ROS, protecting the host against oxidative stress-induced damage [30–32]. Hence, effective antioxidants can abate the oxidative stress of ALI by direct elimination of free radicals or by boosting defense systems of antioxidant enzymes [26, 30, 32, 33]. In this study, CFE also markedly upregulated the activities of SOD, CAT, and GPx, which were observed to be markedly decreased in sepsis-induced ALI rodents [34, 35] and in the ALI patients clinically [27, 36]. Therefore, combining with the results of MDA assay, we speculated that CFE could effectively reduce oxidative stress in ALI.

Clinical and experimental studies suggest that LPS induce the activation of alveolar macrophages and endothelial cells, which result in the productions of many proinflammatory and chemotactic cytokines [15, 37]. TNF-α, IL-1β, and IL-6 are well-characterized cytokines involved in the inflammatory responses of ALI [13, 38, 39]. These cytokines, combining with other proinflammatory factors, further stimulate neutrophils' infiltrations to migrate into lung tissues and initiate, amplify, and perpetuate the entire or focal inflammatory responses in ALI [15, 40]. TNF-α, mainly produced by monocytes/macrophages, is the earliest and primary endogenous mediator of the process of an inflammatory reaction and can elicit the inflammatory cascade, cause damage to the vascular endothelial cells, and induce alveolar epithelial cells to produce other cellular factors, such as IL-6 [40, 41]. Elevated TNF-α binds with a TNF-α acceptor in lung tissue, leading to the leakage of enzymes out of the organelle, which causes damage to the lung parenchyma [15]. IL-1β plays a key role in the progression of acute lung injury. It can inhibit fluid transportations across the distal lung epithelium to cause surfactant abnormalities and to increase protein permeability across the alveolar-capillary barrier [13]. IL-6 is one of the most common inflammatory cytokines, and its circulating levels have been shown to be excellent predictors of the severity of acute respiratory distress syndrome of different aetiologies, such as sepsis and acute pancreatitis [15, 40, 42]. In this present study, pretreatment with CFE significantly inhibited the production of TNF-α, IL-1β, and IL-6 in lung. The inhibition of proinflammatory factors productions was in accordance with the protective effects of CFE against histopathologic damage. And the suppression of proinflammatory cytokines by CFE treatment was supposed to contribute to its protective effects against ALI.

NF-κB is an important nuclear transcription factor and plays a pivotal role in immune and inflammatory responses through the regulation of proinflammatory cytokines, chemokines, and adhesion molecules [12, 43, 44]. Uncontrolled activations of the NF-κB pathways were involved in the pathogenesis of many acute and chronic inflammatory diseases, especially ALI [43, 45]. In normal conditions, NF-κB is sequestered in the cytoplasm by IκBs [46, 47]. Once activated, NF-κB p65 dissociates from its inhibitory proteins IκB and translocates from the cytoplasm to the nucleus where it triggers the transcription of specific target genes such as TNF-α, IL-1β, and IL-6 [37, 48]. To detect the inhibitory mechanism of TNF-α, IL-1β, and IL-6 productions, we tested the effects of CFE on NF-κB activation and IκB degradation [37]. With the stimulation of LPS, the levels of phosphorylated IκB protein and nucleus NF-κB p65 protein were remarkably increased. However, this tendency was reversed by CFE pretreatment, as western-blot analysis showed that IκB degradation and NF-κB p65 activation were significantly blocked by pretreatment with CFE at the doses of 80 mg/kg and 120 mg/kg. In addition, we found that 40 mg/kg of CFE did not affect IκB degradations and NF-κB p65 activations statistically, in spite of the fact that it possessed inhibitory tendencies. Therefore, results suggested that the protective effects of CFE against LPS-induced ALI, to some extent, may be attributed to its roles in downregulation of NF-κB pathways.

LPS play key roles in the development and progression of ALI [5, 22, 49, 50]. TLR4, serving as an important pattern recognition receptor of host immune responses and essential upstream sensor for LPS from pathogens and microorganisms, would detect LPS and then trigger the activation of NF-κB and its downstream responses through MyD88 dependent or independent pathways [5, 7, 49, 50]. Thus TLR4 is the fundamental upstream sensor for LPS [7, 11, 49, 51]; it was necessary to probe whether the anti-inflammation action of CFE exerted though TLR4-mediated pathways. Our data showed that the enhanced expressions of TLR4 by LPS challenge were significantly downregulated with pretreatments of CFE at the doses of 80 and 120 mg/kg, which corresponded with the level changes of IκBα, NF-κB p65, and other proinflammatory cytokines, in ALI mice lung tissues. Therefore, in conclusion, as shown in Figure 7, we speculated that CFE could inhibit the binding of LPS to TLR4 in NF-κB signaling pathways, leading to reductions of proinflammatory cytokines productions and attenuations of pulmonary inflammatory responses. However, without any further experiments to eliminate the involvement of MyD88-independent pathway, we could not address and demonstrate the conclusion that TLR4 activates NF-κB via a MyD88-independent pathway. Therefore, explicit regulations of TLR4 signaling pathways by CFE require further studies.

5. Conclusions

The experimental evidence in this study demonstrated that CFE can effectively attenuate the LPS-induced ALI in mice. The protective effects of CFE were associated with the modulations of TLR4 signaling pathways. These experimental results suggested that CFE was a potential therapeutic drug for ALI.

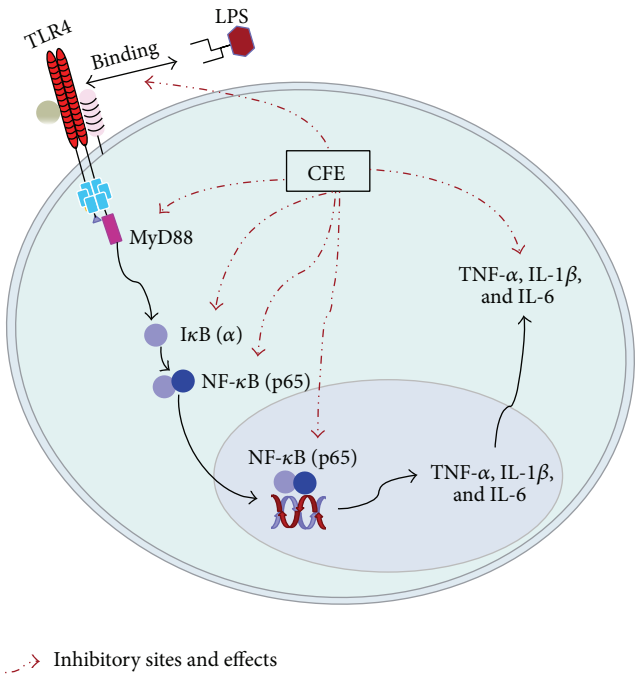

Figure 7: The underlying mechanism of CFE on the TLR4 signaling pathway. The red dotted arrows indicate the inhibitory sites and effects of CEF.

Abbreviations

CFE:	The supercritical-carbon dioxide fluid extract of *Chrysanthemum indicum* Linné
LPS:	Lipopolysaccharide
ALI:	Acute lung injury
ARDS:	Acute respiratory distress syndrome
MODS:	Multiple organ dysfunction syndrome
BALF:	Bronchoalveolar lavage fluid
ELISA:	Enzyme linked immunosorbent assay
Dex:	Dexamethasone
TNF-α:	Tumor necrosis factor-α
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
MPO:	Myeloperoxidase
MDA:	Malondialdehyde
SOD:	Superoxide dismutase
CAT:	Catalase
GPx:	Glutathione peroxidase
TLR4:	Toll-like receptor 4
MyD88:	Myeloid differentiation factor 88
NF-κB p65:	Nuclear factor kappa B p65
IκBα:	Inhibitory kappa B alpha
ROS:	Reactive oxygen species
H&E:	Hematoxylin and eosin.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Xiao-Li Wu and Xue-Xuan Feng contributed equally to this work.

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Research Article

Oral Administration of Herbal Mixture Extract Inhibits 2,4-Dinitrochlorobenzene-Induced Atopic Dermatitis in BALB/c Mice

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CP001 is four traditional herbal medicine mixtures with anti-inflammatory properties. In this study, we investigated the effect of oral administration of CP001 ethanol extract on the 2,4-dinitrochlorobenzene- (DNCB-) induced AD mouse models. For that purpose, we observed the effects of oral administration of CP001 on skin inflammatory cell infiltration, skin mast cells, production of serum IgE, and expression of Th2 cytokine mRNA in the AD skin lesions of DNCB treated BALB/c mice. Histological analyses demonstrated that CP001 decreased dermis and epidermis thickening as well as dermal infiltration induced by inflammatory cells. In addition, CP001 decreased mast cell infiltration in count as well as dermal infiltration induced by inflammatory cells. In the skin lesions, mRNA expression of interleukin- (IL-) 4 and IL-13 was inhibited by CP001. CP001 also reduced the production of IgE level in mouse plasma. In addition, we investigated the effect of CP001 on the inflammatory allergic reaction using human mast cells (HMC-1). In HMC-1, cytokine production and mRNA levels of IL-4, IL-13, IL-6, and IL-8 were suppressed by CP001. Taken together, our results showed that oral administration of CP001 exerts beneficial effects in AD symptoms, suggesting that CP001 might be a useful candidate for the treatment of AD.

1. Introduction

Atopic dermatitis (AD) is a most common chronic inflammatory skin disease, affecting about 10 million people in the world, leading to a significant reduction in quality of life, and its incidence is continuously increasing in westernized countries [1, 2]. The pathogenesis of AD is unknown, but the disease seems to be correlated with specific immune and inflammatory mechanisms. The general characteristics of AD include excessive infiltration of inflammatory cells such as lymphocytes, macrophages, and granulated mast cells into the skin lesions, eosinophilia in peripheral blood, and a high level of serum immunoglobulin E (IgE) [3].

Mast cells are tissue-based inflammatory cells of bone marrow origin, which respond to signals of innate and adaptive immunity. They play a major role in immediate hypersensitivity reaction and are activated through the high-affinity IgE receptor, FcεR [4]. In addition, it has been reported that a large number of mast cells can be found in AD skin lesion. The majority of AD patients have elevated blood IgE level and it is mediated of mast cell activation [5]. Mast cell activation by IgE is release inflammatory mediators, such as histamine, as well as cytokines, including Th2 cytokines, such as IL-4, IL-5, and IL-13. Thus, cell-bound IgE is cross-linked allergens and it is contributed to the development of AD through mediating activation of mast cells localization [6].

Proinflammatory cytokines that are released by activated mast cells, including IL-6 and IL-8, play an important role in allergic inflammation [7]; IL-6 mediates allergic inflammation [8], while IL-8 induces the migration of neutrophils into inflammatory regions as a potent chemotactic cytokine [9, 10].

CP001 is a mixture of four oriental herbal medicines composed of *Houttuynia cordata* Thunb, *Rehmannia glutinosa* Libosch, bark of *Betula platyphylla* var. *japonica,* and *Rubus coreanus* Miq. *Houttuynia cordata* Thunb has long been used in traditional oriental medicine for the treatment of inflammatory diseases. Also, several studies demonstrated that *Houttuynia cordata* Thunb has been associated with a broad range of pharmacological activities, including anti-inflammatory [1], antiviral [11], and anticancer effects [12]. *Rehmannia glutinosa* Libosch has traditionally been used as an ingredient herb in East Asian medicine for the effects of hemostasis, activation of blood circulation, and improvement of kidney function [13]. Several studies indicated that *Rehmannia glutinosa* Libosch has antiallergic effects [14] and anti-inflammatory function [15–17]. *Betula platyphylla* var. *japonica* is known to have antioxidant, anti-inflammatory, and anticancer effects [18] and inhibits the development of AD in NC/Nga mice [19, 20]. *Rubus coreanus* Miq. is a type of red raspberry that grows wild in Korea, Japan, and China. The fruit, known as "*Bokbunja*" in Korean, has been used in traditional oriental medicine for reducing the risk of diseases such as asthma and allergy. It is also known that *Rubus coreanus* Miq. has anti-inflammatory and antioxidative activities [21–23]. These collective observations indicate that CP001 may be good candidate for control of AD and beneficial in the treatment of human allergic disorders. Therefore, in our previous study, we already confirmed that topical application of KM110329 (CP001 modifying herbal mixture) inhibits the atopic dermatitis in ovalbumin- and DNCB-induced mouse model.

Therefore, in this study, we investigated whether 30% ethanol extract of CP001 oral administration has anti-inflammatory activity in 2,4-dinitrochlorobenzene-(DNCB-) induced AD mice model. In addition, we also investigated whether 30% ethanol extract of CP001 has antiallergic effect inhibiting cytokine production in human mast cells, HMC-1.

2. Material and Method

2.1. Preparation of CP001. CP001 was prepared by Hanpoong Pharmaceutical (Jeon-ju, Korea) following good manufacturing practices (GMP) procedure. CP001 is 30% ethanol extracted brown-colored powder, and it is composed of *Houttuynia cordata* Thunb, *Rehmannia glutinosa* Libosch, bark of *Betula platyphylla* var. *japonica*, and *Rubus coreanus* Miq. The powder from the extract was dissolved in distilled water for *in vivo* and *in vitro* experiments.

2.2. Animals. Six-week-old male BALB/c mice were purchased from Orient (Sung-nam, Korea). The mice were randomized into 6 groups (normal, DNCB, and 25, 50, 100, and 200 mg/kg (CP001)), each comprising five mice. All mice were kept under pathogen-free environment and allowed free access to the diet and water. All procedures performed on the mice were approved by the animal care center of Kyung-Hee University (Approval Number KHUASP (SE)-2012-004).

2.3. Induction of AD-Like Skin Lesions and CP001 Treatment. Induction of AD-like skin lesions procedure is described in Figure 1. For that purpose, mice back skin was painted dermally with 200 μL of a 1% DNCB using 1×1 cm patches after shaving. Two weeks after sensitization, the back skin was challenged with 200 μL of a 0.2% DNCB solution twice per week. This procedure was repeated for 2 weeks and CP001 was orally administrated together. At the end of the experiment, mice were sacrificed by CO_2 inhalation, and samples were collected.

2.4. Histological Analysis. A portion of the skin biopsies were fixed in 4% paraformaldehyde (PFA) and embedded in Tissue-Tek optical cutting temperature (O.C.T.) compound (Tissue-Tek, Sakura, AA Zoeterwoude, the Netherlands) on dry ice. Skin sections of 20 μm were cut and stained with hematoxylin and eosin (H & E) for inflammatory cells or with toluidine blue for mast cells counts and examined under light microscopy (Olympus). Mast cells were counted in 10 parts of high-power fields (HPF) at 400x magnification.

2.5. Enzyme-Linked Immune Sorbent Assay. After final CP001 administration, whole blood samples were collected by cardiac puncture for measurement of blood IgE level. The blood was placed in Vacutainer tubes containing EDTA (BD science, NJ, USA) and blood plasma was isolated. Total IgE levels in plasma were determined by sandwich ELISA using the BD PharMingen mouse IgE ELISA set. Briefly, plates were coated with capture antibody in ELISA coating buffer (Sigma-Aldrich) and incubated overnight at 4°C. Plates were washed with PBS-Tween 20 (0.05%) and subsequently blocked (10% FBS in PBS) for 1 h at 20°C. Serial dilutions of standard antigen or sample in dilution buffer (10% FBS in PBS) were added to the plates and plates were incubated for 2 h at 20°C. After washing, biotin-conjugated anti-mouse IgE and SAv-HRP (streptavidin-horseradish peroxidase conjugate) were added to the plates and plates were incubated for 1 h at 20°C. Finally, tetramethylbenzidine (TMB) substrate solution was added to the plates and after 15 min incubation in the dark, a 2 N H_2SO_4 solution was added to stop the reaction. Optical densities were measured at 450 nm on an automated ELISA reader (Versa Max, Molecular Devices, CA, USA). IL-6 and IL-8 levels were measured in HMC-1 supernatant by sandwich ELISA using BD Pharmingen human ELISA set. The sandwich ELISA procedures were performed by following the same protocols described above.

2.6. Cytokine Analysis by Real-Time PCR. Mice skin was immediately frozen in liquid nitrogen and kept at −70°C until use. For real-time PCR assay, mice skin was homogenized with Ultra-Turrax T10 (IKA labortechnik, Seoul, Korea) and RNA extraction was performed using TRIzol (Invitrogen life technologies, NY, USA). RNA content was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). 1 g of total cellular RNA from each sample was reverse transcribed using cDNA synthesis kit (TaKaRa, Japan). Quantitative PCR was performed using SYBR green iMaster and a LightCycler 480 (Roche, Switzerland).

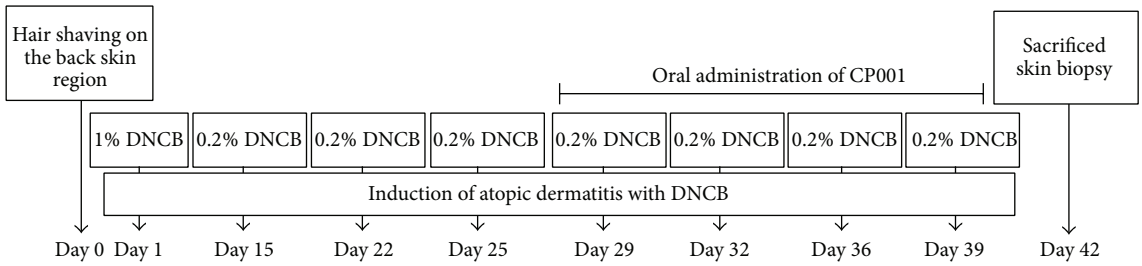

Figure 1: Protocols for induction of atopic dermatitis in mouse model. Shaved dorsal regions of the mice were sensitized with DNCB solution. Male BALB/c mice were epicutaneously sensitized with 200 μl of a 1% DNCB solution on day 1. Two weeks later, dermatitis was induced with 200 μl of 0.2% DNCB solution at the intervals shown in the figure. CP001 was orally administrated from the 3rd week during sensitization together with DNCB.

Table 1

Primer name	Sequence (5′-3′)
IL-4	Forward: AAGAACACCACAGAGAGTGAGCTC
	Reverse: TTTCAGTGTGGACTTCCACTC
IL-13	Forward: AGCATGGTATGGAGTGTGGACCTG
	Reverse: CAGTTGCTTTGTGTAGCTGAGCAG
IL-6	Forward: AACCTTCCAAAGATGGCTGAA
	Reverse: CAGGAACTGGATCAGGACTTT
IL-8	Forward: TCAGTGCATAAAGACATACTCC
	Reverse: TGGCATCTTCACTGATTCTTG
GAPDH	Forward: GAGGGGCCATCCACAGTCTTC
	Reverse: CATCACCATCTTCCAGGAGCG

2.7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Cells were harvested by centrifugation and the pellet was washed with ice-cold PBS. RNA was isolated from the pellet using easy-blue RNA extraction kit (iNtRON Biotech, Republic of Korea) according to the manufacturer's instructions. Isolated RNA content was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). 2 μg of total cellular RNA from each sample was reverse transcribed using cDNA synthesis kit (TaKaRa, Japan). PCR was conducted out in a 20 μL reaction mixture consisting of DNA template, 10 pM of each gene-specific primer, 10x Taq buffer, 2.5 mM dNTP mixture, and 1 unit of Taq DNA polymerase (Takara, Japan). PCR was performed using the specific primer and primer sequences for human IL-6, IL-8, and GAPDH are shown in Table 1.

2.8. HPLC Analysis. Ellagic acid, quercitrin hydrate, and catalpol were purchased from Sigma Chemicals (Saint Louis, MO). Purity of standard compounds was guaranteed to be higher than 95% by HPLC. HPLC grade acetonitrile, methanol, and formic acid were purchased from J. T. Baker (Phillipsburg, NJ). Catalpol, ellagic acid, and quercitrin were chosen as Marker compounds to standardize the extract sample. CP001 was dissolved in distilled water at a concentration of 100 mg/mL and the solution was filtered through a 0.45 μm membrane filter. A 10 μL aliquot of the sample solution was injected into a HPLC system (Agilent Technologies, Palo Alto, CA). The sample was analyzed on a Capcell Pak UG120 C_{18} analytical column (250 × 4.6 mm, 5 μm; Shiseido, Japan).

2.9. Statistical Analysis. Statistical analyses presented as the mean ± standard error of the mean (SEM) and were analyzed for statistical significance using the unpaired Student's t-test. P value < 0.05 was considered statistically significant.

3. Results

3.1. Oral Administration of CP001 Decreases Infiltration of Inflammatory Cells into AD-Like Skin Lesions. To determine whether CP001 decreases infiltration of inflammatory cells into AD-like skin lesions, we performed H & E staining on the skin after oral administration of CP001. We observed infiltration of inflammatory cells into the epidermis and dermis in DNCB group, whereas CP001 decreased such infiltration of inflammatory cells into the skin (Figure 2). Moreover, CP001 (25–200 mg/kg) abrogated skin thickening induced by DNCB (Figure 2). Next, we also performed toluidine blue staining for mast cell observation. Repeated cutaneous application of DNCB increased dermal mast cell number. However, this feature was significantly suppressed by CP001 (Figure 3).

3.2. CP001 Administration Downregulates mRNA Expression of Th2 Cytokines. The Th2 type cytokines are important in an acute phase of AD whereas mixed Th2/Th1 type inflammation is characteristic to a chronic phase of AD. To determine whether CP001 decreases Th2 type cytokines expression, we performed real-time PCR to measure IL-4 and IL-13 levels. We found that oral administration of CP001 decreased IL-4 mRNA expression in AD-like skin lesions (Figure 4(a)). We also found that CP001 administration decreased IL-13 mRNA expression in AD-like skin lesions in a dose-dependent manner (Figure 4(b)). In histology analysis, repeated cutaneous application of DNCB increased dermal mast cell number and this feature was suppressed by CP001 oral administration. Activated mast cells secrete various chemokines and cytokines including IL-6 and IL-8. To determine whether CP001 decreases IL-6 and IL-8 cytokines mRNA levels, we performed RT-PCR analysis in AD-like skin lesions. Oral administration of CP001 did not

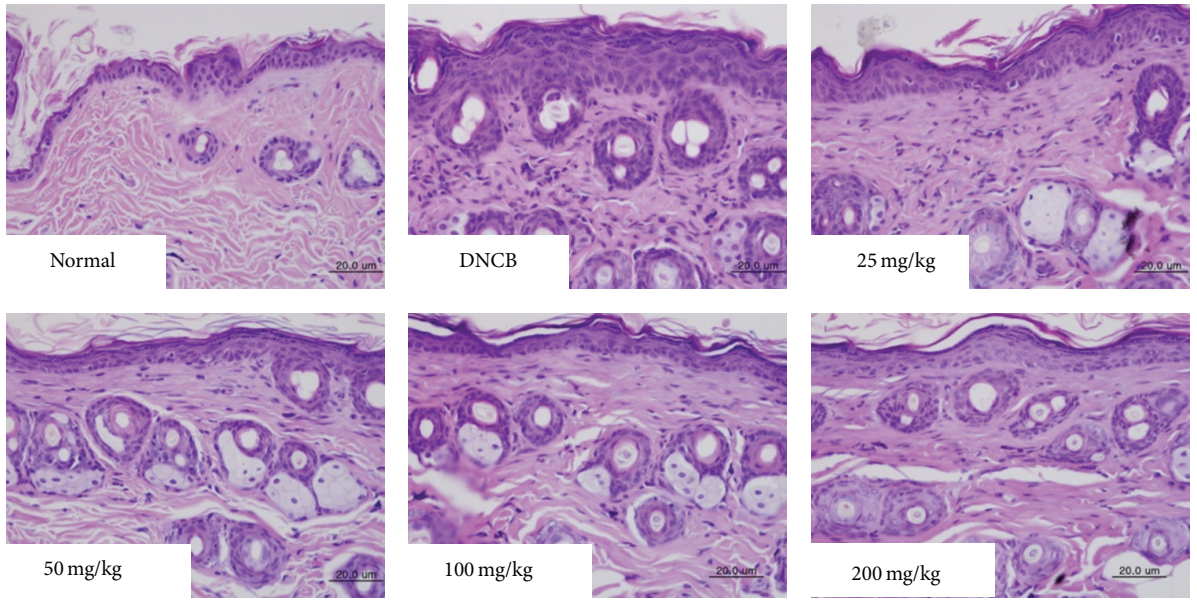

Figure 2: Histological features of AD-like skin lesions treated with CP001. The skin sections were stained with hematoxylin and eosin. Inflammatory cells infiltration into the dermis was measured after treatment with CP001 in the presence of DNCB. Sections were evaluated using microscope at an original magnification of 400x.

affect the suppression of IL-6 and IL-8 mRNA expression in AD-like skin lesions (Figures 4(c) and 4(d)).

3.3. CP001 Administration Downregulates Serum IgE Concentration. Hyperproduction of IgE is a major characteristic of AD and patients with AD often exhibit elevated levels of total and allergen specific IgE antibodies (Abs) in their serum. To further test whether suppression of the progression of AD-like skin lesions by CP001 is associated with serum IgE levels, we performed total IgE ELISA assay. We found that total IgE levels were dramatically elevated in DNCB-treated group compared with normal group. However, increased serum IgE levels induced by DNCB were significantly decreased by CP001 treatment (Figure 5).

3.4. Effect of CP001 on PMA Plus A23187-Induced IL-6 and IL-8 Expression in HMC-1. Next, we investigated whether CP001 affects production of IL-6 and IL-8 in HMC-1. For that purpose, mast cells were pretreated with various concentrations of CP001 for 1 h and then treated with PMA and A23187 for 24 h. The levels of IL-6 and IL-8 in culture supernatants were measured by ELISA assay. We found that IL-6 secretion induced by PMA and A23187 was significantly suppressed by CP001 (Figures 6(a) and 6(c)). We also performed RT-PCR to measure IL-6 and IL-8 mRNA expression in HMC-1. We observed that IL-6 and IL-8 mRNA induced by PMA and A23187 were decreased by CP001 (Figures 6(b) and 6(d)).

3.5. Effect of CP001 on PMA Plus A23187-Induced Th2 Cytokine Expression in HMC-1. CP001 administration decreased IL-4 and IL-13 mRNA expression in AD-like skin lesions (Figure 7(b)). Therefore, we characterized the regulatory effect of CP001 on IL-4 and IL-13 mRNA expression in HMC-1 using RT-PCR. We found that IL-13 expression induced by PMA and A23187 was significantly suppressed by CP001 (Figure 7(a)). IL-4 expression level was not increased by PMA and A23187, but it is suppressed by CP001 (Figure 7(a)).

3.6. HPLC Analysis. To further evaluate the effective compounds of CP001 extract, HPLC analysis was employed. In order to analyze catalpol, the mobile phase consisted of water (W) and methanol (M) and the flow rate was 1 mL/min. The gradient elution program was used as follows. The initial composition of the mobile phase was 97 : 3 (W : M), which was linearly changed to 95 : 5 (W : M) over 1 min and changed to 91 : 9 (W : M) for 9 min. At 11 min, the composition of mobile phase returned to the initial condition, which was maintained for 9 min for column reequilibration. Chromatograms were acquired at 210 nm by UV detection (Figure 8). For ellagic acid and quercitrin, the mobile phase consisted of 0.1% formic acid (F) and acetonitrile (A) and the flow rate was 1 mL/min. The gradient elution program was used as follows. The initial composition of the mobile phase was 90 : 10 (F : A), which was linearly changed to 85 : 15 (F : A) over 5 min and changed to 60 : 40 (F : A) for 35 min. At 41 min, the composition of mobile phase returned to the initial condition, which was maintained for 9 min for column reequilibration. Chromatograms were acquired at 254 nm by UV detection. The retention times of catalpol, ellagic acid, and quercitrin were 6.2, 14.4, and 18.6 min, respectively (Figures 7(a) and 7(b)). The concentrations of catalpol, ellagic acid, and quercitrin in the extract sample were determined using HPLC analysis as described above. The extract was standardized to contain 1.8% catalpol, 0.4% ellagic acid, and 0.3% quercitrin.

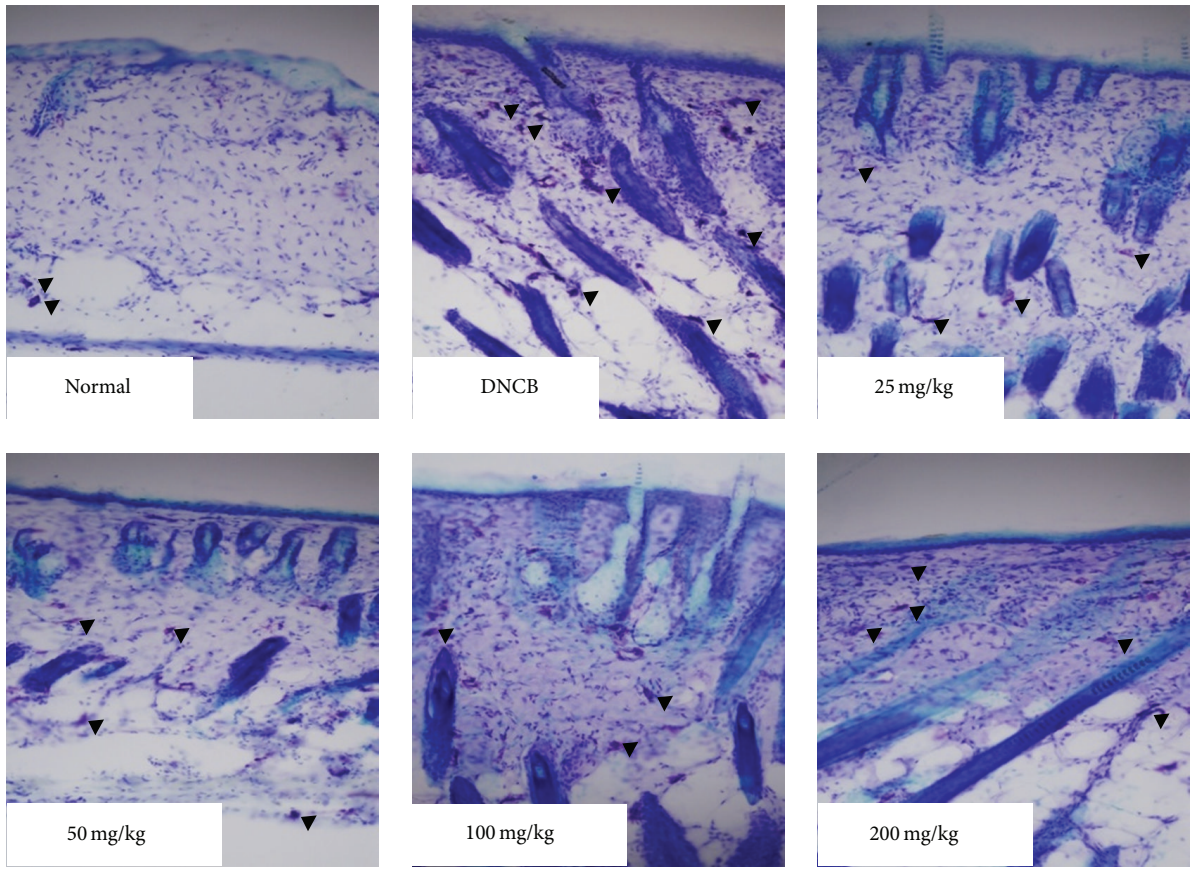

FIGURE 3: The measurement of mast cells number in AD-like skin lesions treated with CP001. The skin sections were stained with toluidine blue for mast cells staining. Sections were evaluated using microscope at an original magnification of 400x. The data are presented as mean ± SD from five animals in each group. $^{*}P < 0.05$.

4. Discussion

AD is a chronic inflammatory disease, which is accompanied by erythema, edema, and scaling in AD skin lesions [24]. Recently, Korean medicine has been the subject of increased interest for its potential in the treatment of inflammatory diseases, including atopic dermatitis and airway inflammation [16, 17, 25, 26]. The present study demonstrates that oral administration of Korean herbal mixture extract, CP001, inhibits DNCB-induced AD. We observed that CP001 decreases infiltration of inflammatory cells into AD-like skin lesions and dermal mast cell number.

Generally, steroid therapy is used for AD treatment, but it cannot be administrated over the long term because of

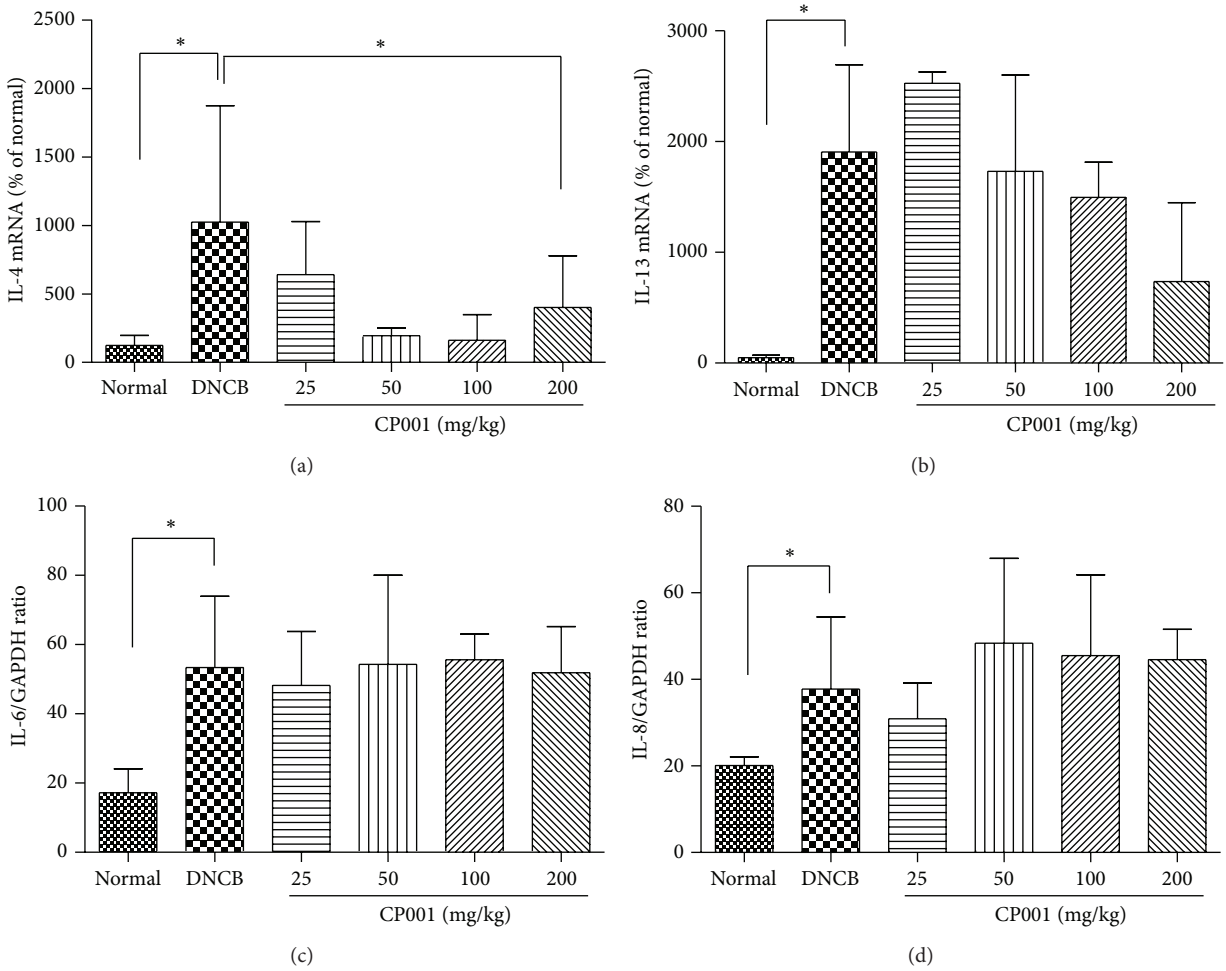

Figure 4: Effect of CP001 on the cytokine mRNA expression in mouse skin tissue. The IL-4, IL-13, IL6, and IL-8 mRNA expression were measured by real-time PCR (a), (b) and RT-PCR (c), (d) in mouse skin tissue. The columns and the error bars represent mean ± SD (n = 5 mice/group). $^{*}P < 0.05$.

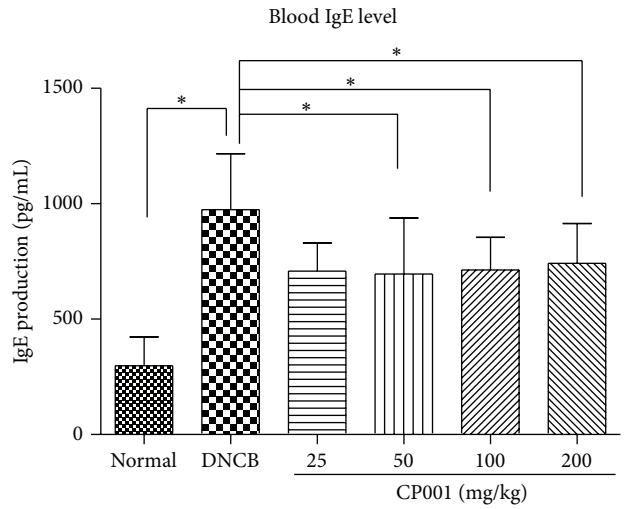

Figure 5: Measurement of plasma IgE level. Total IgE level was determined by ELISA. The columns and the error bars represent mean ± SEMs (n = 4 mice/group). $^{*}P < 0.05$.

the many side effects. Therefore, we find a new drug, which is effective in the treatment of AD without any side effects. Recently, we reported that topical application of KM110329 (CP001 modified drug) reduced ovalbumin- and DNCB-induced atopic dermatitis [23]. Therefore, we were wondering whether CP001 oral administration may inhibit DNCB-induced atopic dermatitis.

Mast cells degranulation can be regulated by the recruitment, trafficking, and function of inflammatory response. For example, IL-4 and IL-13 induce cell adhesion molecules on endothelium which can be recruitment of leukocytes [27–29]. Also, the production of IL-4 cytokine in epidermal cells has been known to be the main factor for initiation of AD [30]. In our data, we show that cytokine production and mRNA levels of IL-4, IL-13, IL-6, and IL-8 were suppressed by CP001 in HMC-1. Also, quantitative real-time PCR of the skin lesions also showed that oral administration of CP001 diminished the mRNA level of IL-4 and IL-13 in the AD-like skin lesions. In addition, we found that CP001 reduces mast cell in

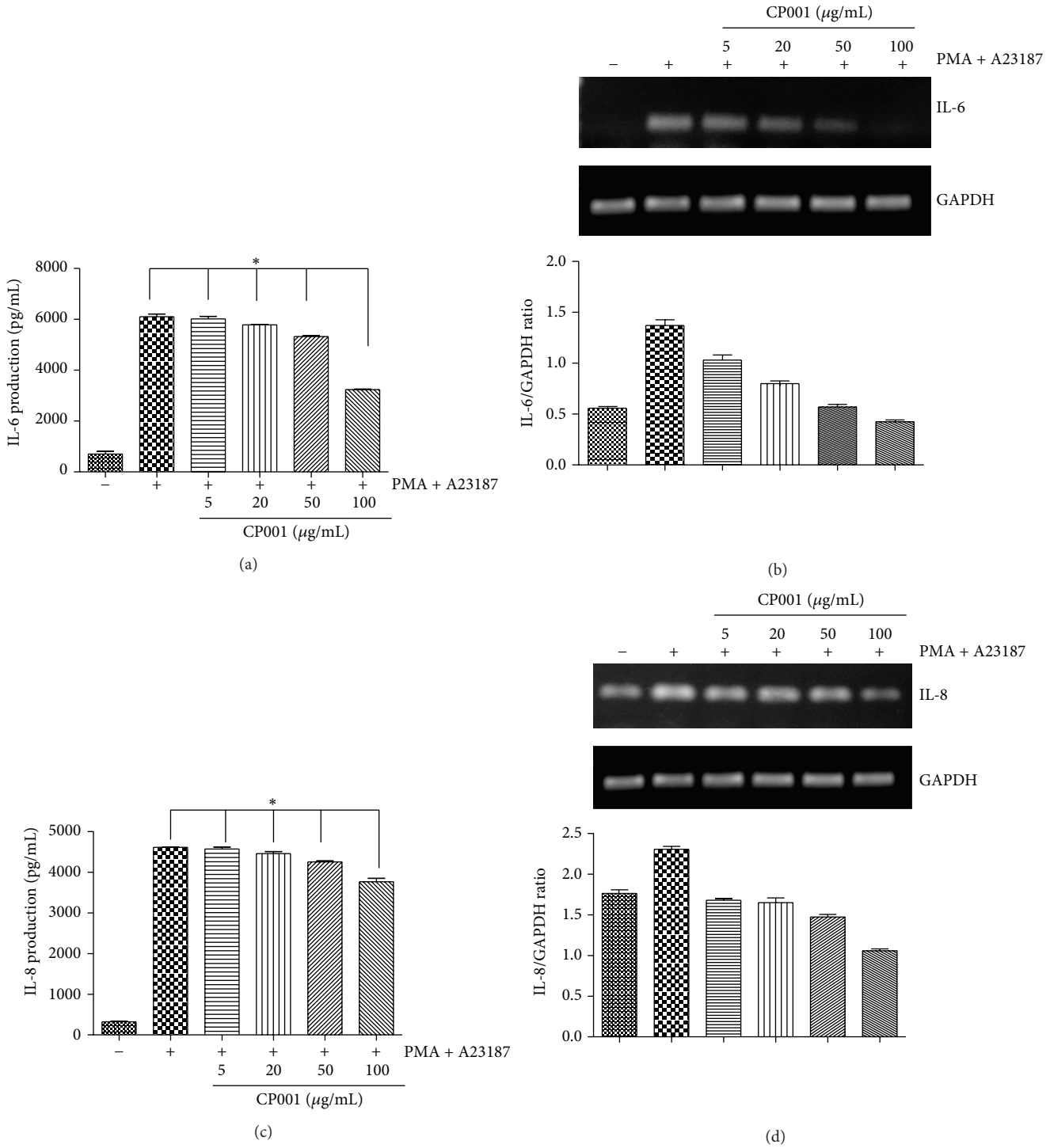

Figure 6: Effect of CP001 on PMA plus A23187-stimulated proinflammatory cytokine expression in HMC-1. HMC-1 were pretreated with various concentrations of CP001 for 1 h and then treated with PMA and A23187 for 24 h. The levels of IL-6 and IL-8 in culture supernatants were measured by ELISA assay (a), (c). The IL-6 and IL-8 mRNA levels were measured by RT-PCR (b), (d). Data represent the mean ± SEMs of three independent experiments.

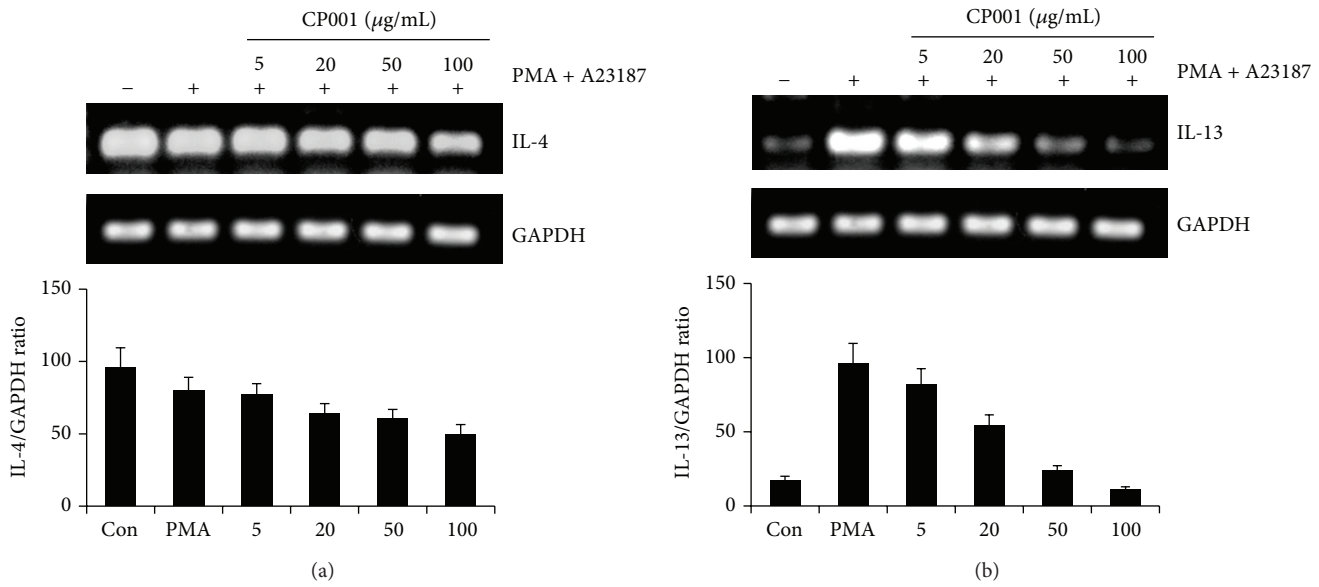

Figure 7: Effect of CP001 on PMA plus A23187-stimulated Th2 cytokine expression in HMC-1. HMC-1 were pretreated with various concentrations of CP001 for 1 h and then treated with PMA and A23187 for 24 h. The IL-4 and IL-13 mRNA levels were measured by RT-PCR (a), (b). Data represent the mean ± SEMs of three independent experiments.

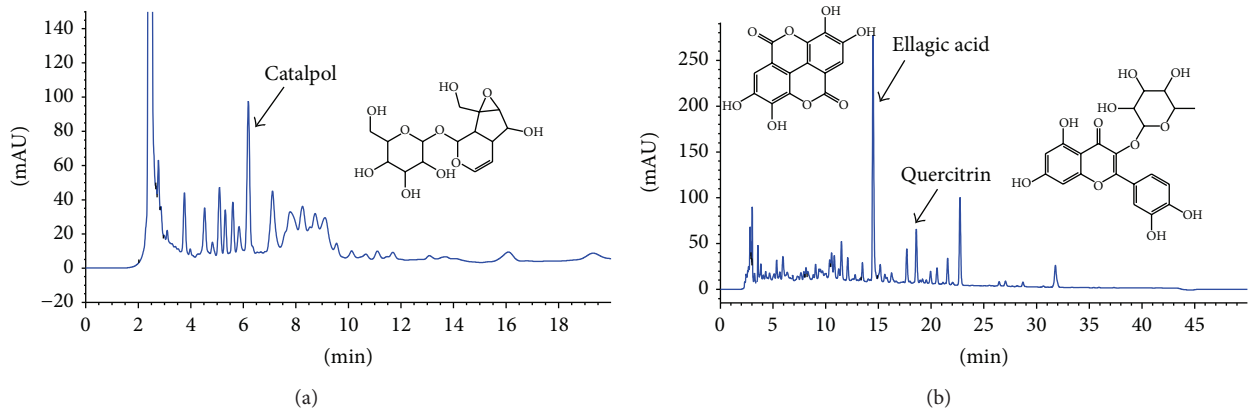

Figure 8: Typical HPLC chromatograms of CP001 for (a) catalpol, (b) ellagic acid, and quercitrin.

DNCB-induced AD-like skin lesion. It seems that inhibition of infiltration of mast cell downregulates secretion of IL-4 and IL-13 cytokines and it may inhibit recruitment of leukocytes. Thus, mast cell may be main factor for suppression of Th2 cytokines in the AD-like skin lesions by oral administration of CP001.

IgE is mediator of mast cell activation and we observed that CP001 oral administration reduced elevated blood IgE levels induced by repeated DNCB sensitization

CP001 also suppressed IL-6 secretion and elevated IL-6 and IL-8 mRNA expression induced by PMA and A23187 in HMC-1. It seems that the reduction of infiltration of mast cells is related to decrease of degranulation of mast cells and maturation of eosinophils suppressing the release of various inflammatory cytokines.

5. Conclusion

Our present study clearly demonstrates that CP001 suppresses the progression of AD induced by DNBC. In addition, inflammatory related cytokine production and mRNA levels of IL-4, IL-13, IL-6, and IL-8 were suppressed by CP001. This suggests that CP001 might be a useful candidate for the treatment of AD.

Conflict of Interests

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

Authors' Contribution

Soon Re Kim and Han-Seok Choi contributed equally to this study.

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Research Article

Effects of Hyeonggaeyeongyo-Tang in Ovalbumin-Induced Allergic Rhinitis Model

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Allergic rhinitis (AR) is an allergic inflammation of the nasal airways. The prevalence of AR is increasing worldwide. We investigated whether Hyeonggaeyeongyo-tang (HYT) is effective to suppress the progression of AR induced by ovalbumin (OVA). Male BALB/c mice were used for this study. Allergic rhinitis was induced by OVA. Treatment with HYT was assessed to study the effect of HYT on allergic rhinitis in mice. Histological analysis, immunohistochemistry, multiplex cytokine assay, blood analysis, and cell viability assay were performed to verify inhibitory effect of HYT on allergic rhinitis. HYT did not show any toxicity maintaining body weight. Food intake was steady without variation in mice. HYT reduced infiltration of inflammatory cells and mast cells into nasal cavity. HYT reduced the levels of cytokines and leukocytes in the blood. HYT decreased the splenocyte cell viability. Antihistamines and steroids are the most common medications used to treat allergic rhinitis. However, long-term use of drug generates resistance or side effects requiring the development of new drug. Our present study clearly demonstrates that HYT suppresses the progression of allergic rhinitis induced by OVA. This suggests that HYT might be a useful drug for the treatment of allergic rhinitis.

1. Introduction

Allergic rhinitis (AR) is an allergic inflammation of the nasal airways and is characterized by sneezing, nasal congestion, nasal itching, and rhinorrhea, in any combination. The prevalence of AR is increasing worldwide. AR affects approximately 60 million people in the United States and the prevalence is about 10–30% in adults and nearly 40% in children [1–3]. A nationwide questionnaire survey conducted with 42,886 Koreans using the International Study of Asthma and Allergies in Childhood (ISSAC) questionnaire demonstrated that 12-month prevalence of 6–12 and 12–15 years of age with AR were 28.8% and 29.1%, respectively [4].

Patients with AR present an inflammatory IgE-mediated response characterized by allergen type 2 helper T cells (Th2) immunological pattern with mast cells and eosinophil activation and release of inflammatory mediators in response to exposure to allergens [5–7]. Allergens are any substance, most often eaten or inhaled, that can be found in a variety of sources, such as dust, pollen, and pet dander. Such allergens (antigens) induce eosinophil recruitment into the tissues and increase serum IgE level [8]. Mast cells contain many granules rich in histamine and heparin. In allergic reactions, mast cells become active when an allergen binds to IgE [9, 10]. In addition, not only mast cells but also B cells play an important role in the pathogenesis of AR [11–16]. Allergen type 2 helper T cells (Th2) play a major role in the regulation of IgE antibody producing B cells, mast cells, and eosinophils. Moreover, Th2 secrete IL-4, IL-5, IL-6, and IL-13 and regulate B cell and eosinophil mediated responses, whereas Th1 produce interferon. Th1/Th2 cells are associated with a series of immune and inflammatory diseases, such as bacterial and viral infectious diseases [17, 18]. T cell-mediated immunity is an important process of elaborating antigen (Ag) specific T lymphocytes to remove viral, bacterial, or parasitic infections or malignant cells. Cytotoxic CD8+ T cells are T lymphocytes that kill infected, damaged, or malignant cells bearing the Ag, while CD4+ T helper cells generate cytokines that can be directly poisonous to the target cells or can stimulate other T cell effector functions and B cell

Table 1: The composition of herbal medicines in HYT.

Composition	Volume
Schizonepeta Spica	0.47 g
Forsythiae Fructus	0.47 g
Saposhnikoviae Radix	0.93 g
Angelicae Gigantis Radix	0.93 g
Cnidii Rhizoma	0.93 g
Paeoniae Radix Alba	0.93 g
Bupleuri Radix	0.47 g
Aurantii Fructus	0.93 g
Scutellariae Radix	0.93 g
Gardeniae Fructus	0.93 g
Angelicae Dahuricae Radix	0.93 g
Platycodi Radix	0.93 g
Glycyrrhizae Radix	0.67 g

antibody production and mobilize powerful inflammatory mechanisms [19].

The traditional herbal medicine Hyeonggaeyeongyo-tang (HYT) is known to treat otitis media, sinusitis, tonsillitis, and a variety of otolaryngology symptoms [20]. In Japan and China, HYT is known as Keigai-rengyo-to or Jing Jie Lian Qiao Tang, respectively. However, its effect on AR has not yet been elucidated. Antihistamines and steroids are the most common medications used to treat AR [21]. However, long-term use of drug generates resistance or side effects requiring the development of new drug [22].

Therefore, in this study, we investigated the effects of HYT on allergic responses in ovalbumin- (OVA-) induced AR mice model. For that purpose, we performed histopathological analysis and measured mast cell numbers. We also measured the levels of cytokines in the serum and the level of leukocytes in the blood. Moreover, we evaluated splenocyte viability.

2. Materials and Methods

2.1. Preparation of Hyeonggaeyeongyo-Tang (HYT). HYT used in this study contains thirteen different herbal medicines: *Schizonepeta Spica* 0.47 g, *Forsythiae Fructus* 0.47 g, *Saposhnikoviae Radix* 0.93 g, *Angelicae Gigantis Radix* 0.93 g, *Cnidii Rhizoma* 0.93 g, *Paeoniae Radix Alba* 0.93 g, *Bupleuri Radix* 0.47 g, *Aurantii Fructus* 0.93 g, *Scutellariae Radix* 0.93 g, *Gardeniae Fructus* 0.93 g, *Angelicae Dahuricae Radix* 0.93 g, *Platycodi Radix* 0.93 g, and *Glycyrrhizae Radix* 0.67 g. They were prepared by Hanpoong Pharm (Jeonju, Republic of Korea) following good manufacturing practice (GMP) procedure. Dried HYT powder was stored in aliquots at −80°C until further analysis (Table 1).

2.2. Animals. Male BALB/c mice (aged 6 weeks) were purchased from Orient (Seoul, Republic of Korea). The average body weight of mice was 26 g. The animals were housed in air-conditioned room with a 12 h light/dark cycle. All mice were allowed free access to food and water. All procedures performed on the mice were approved by the animal care center of Kyung Hee University (Approval number KHUASP (SE)-12-048). The mice were divided into five groups ($n = 8$): group 1, normal saline; group 2, OVA + saline; group 3, OVA + HYT (101 mg/kg); group 4, OVA + HYT (202 mg/kg); group 5, OVA + HYT (404 mg/kg).

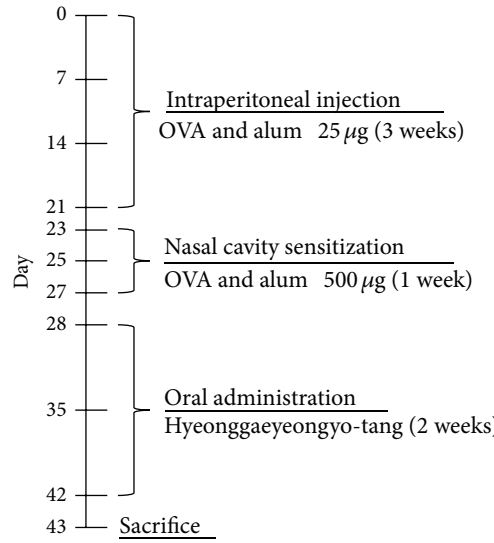

Figure 1: Allergic rhinitis model.

2.3. Sensitization and Treatment. The experimental procedures for allergic sensitization and challenge are summarized in Figure 1. For establishment of OVA-induced AR model, male 6-week-old BALB/c mice were intraperitoneally sensitized with 25 ug of OVA twice a week for three weeks. After three weeks, OVA group of mice were sensitized by intranasal instillation of 500 ug OVA in 30 uL PBS twice a week for one week. Finally, mice were fed with HYT together with OVA sensitization for 2 weeks. At the end of experiment, mice were killed by CO2 inhalation, and samples were collected.

2.4. Histological Analysis. Nasal mucosa samples were immediately fixed with 10% formaldehyde and embedded in paraffin. The sections of the nasal mucosa samples were 4-μm thick. Each section was stained with hematoxylin and eosin (H & E) for inflammatory cells or with toluidine blue (T.B) for mast cells counts and examined under light microscopy (Olympus). Mast cells and inflammatory cells were counted in 10 parts of high power fields (HPF) at 40x, 400x, and 1000x magnification.

2.5. Immunohistochemistry. Expression of CD4+ lymphocytes was detected by immunohistochemical analysis using the anti-CD4+ antibody. The nasal tissues were deparaffinized and rehydrated. After a microwave treatment, the sections were treated with 3% hydrogen peroxide in PBS for 15 min to inhibit endogenous peroxidase activity of blood cells. Following hydrogen peroxide treatment, the nasal sections were incubated with 5% bovine serum albumin (BSA) in PBS, contained a blocking reagent for 1 hour at room temperature. Nasal sections were incubated with mouse monoclonal CD4+ antibody overnight at 4°C and subsequently incubated

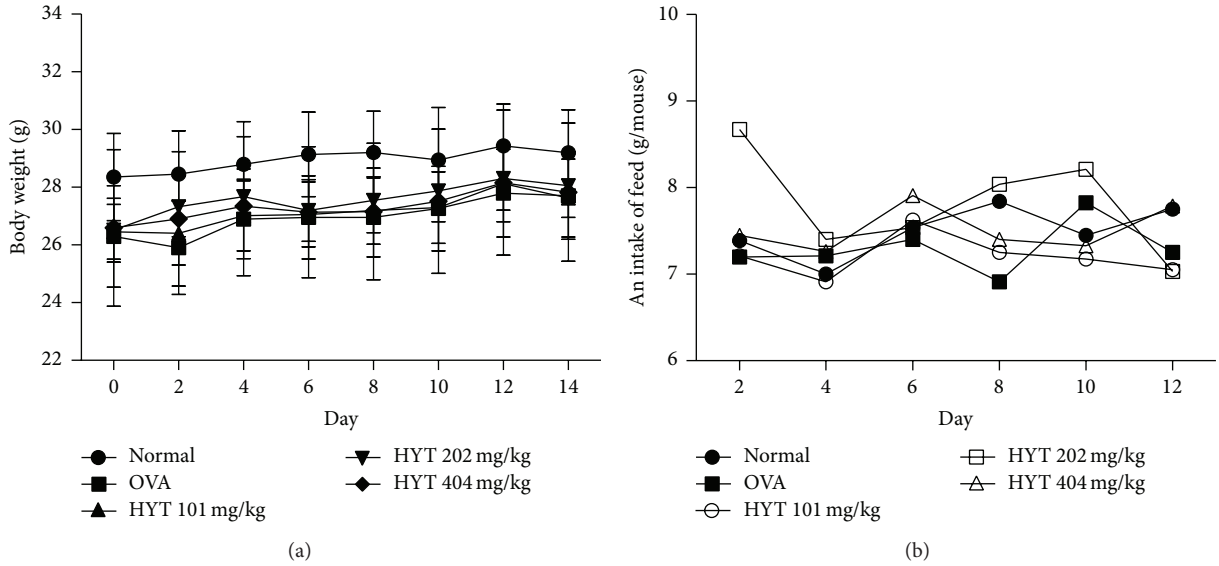

Figure 2: Changes in body weight (a) and food intake (b) during treatments with HYT in OVA-induced AR mice model. Values are expressed as mean ± SEM ($n = 8$).

with secondary biotinylated anti-rabbit IgG for 1 h at room temperature. Sections were treated with avidin-biotin HRP complex (Vectastain ABC kit, Vector Labs, CA, USA) for 30 min at 4°C and stained with diaminobenzidine tetrachloride (DAB) as the substrate. The slides were mounted with an aqueous mounting solution (DAKO, Glostrup, Denmark) and cover-slipped. All the sections were analyzed using an Olympus microscope and images were captured using a digital video camera.

2.6. Multiplex Cytokine Assay. Blood serum was analyzed by the Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The assay was read using a Luminex 100 (Austin, TX) and analyzed using a Bio-Plex Manager software. The mean concentration of cytokines (IL-4, IL-13, and LIF) in supernatants from OVA-stimulated cells over the unstimulated cells (background) was then calculated.

2.7. Blood Analysis. Whole blood samples were collected by cardiac puncture. The blood was placed in Vacutainer TM tubes containing EDTA (BD science, USA). Anticoagulated blood was processed to determine hematological parameters (WBC, lymphocytes, monocytes, eosinophils, basophils, and neutrophils) in a HEMAVET 950 hematology analyzer (Drew Scientific, Inc., Oxford) in accordance with manufacturer' recommendation.

2.8. Splenocytes. Suspension of spleen from normal mice under aseptic condition was prepared by homogenization in RPMI-1640 medium (containing 10% FBS, 1% Ab, and 0.05 mM Mercaptoethanol). The suspension was centrifuged and pelleted. The contaminating red blood cells were removed by using red blood cell lysis buffer (Sigma, USA). Cells were centrifuged and suspended in complete RPMI-1640. Cells were maintained at 37°C in a humidified incubator with 5%.

2.9. Cell Viability Assay. Mice splenocytes (1×10^6 cells/well) were plated in 96-well culture plates and incubated for 24 h. Cells were treated with Con A (2 ug/mL) or HYT (10, 50, 100, 200, 500, 1000, and 2000 ug/mL). After 24 h incubation, 10 uL of WST solution was added to each well of the plate, and the plates were incubated in the dark at 37°C for another 2 h. Optical density was measured at 450 nm using an ELISA plate reader (VersaMax, Molecular Devices, CA, USA).

2.10. Statistical Analysis. All experiment results were expressed as the means ± standard deviations (SD) or means ± SEM ($n = 8$) of at least three separate tests. Student's t-test was used for single variable comparisons, and a P value < 0.05 was considered statistically significant.

3. Results

3.1. Body Weight and Food Intake. Body weight and food intake were monitored throughout the study. We found that normal group shows higher weight as compared to other groups (Figure 2(a)). We also found that HYT did not show any toxicity maintaining body weight (Figure 2(a)). In addition, food intake was steady without variation (Figure 2(b)).

3.2. HYT Reduced Infiltration of Inflammatory Cells into Nasal Cavity. To determine whether HYT reduces infiltration of inflammatory cells into nasal cavity, we performed H & E staining on the nasal mucosa samples. The respective numbers of inflammatory cells in the nasal mucosa in AR mice model were shown to be higher than those in the normal

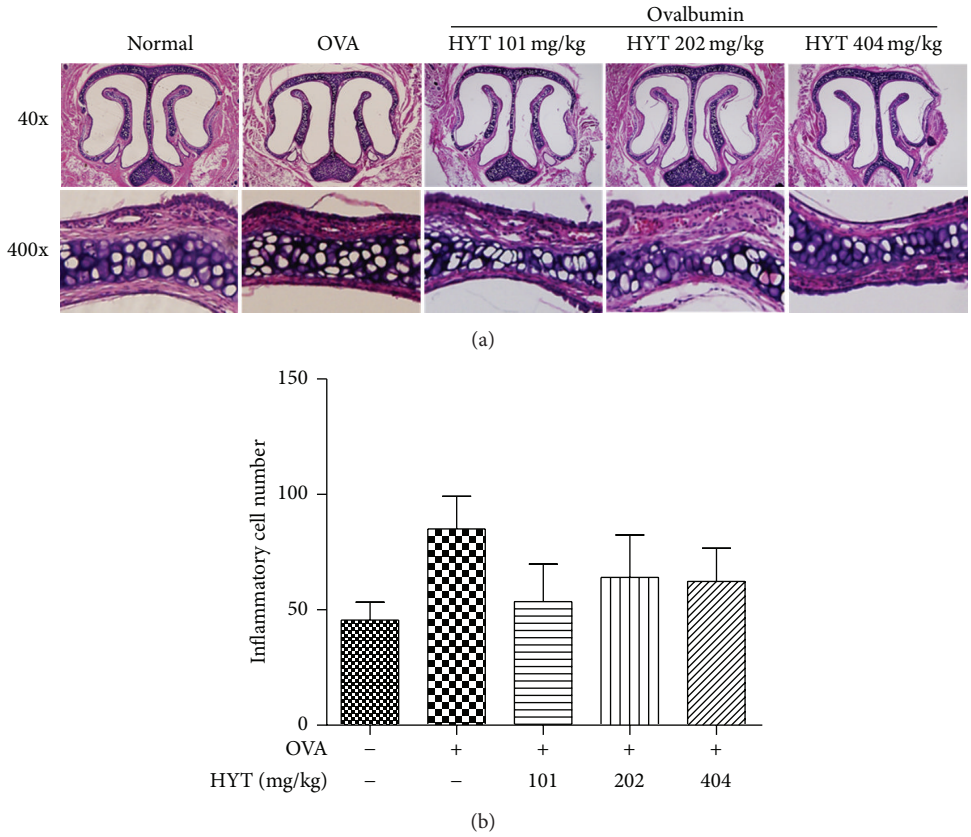

Figure 3: HYT reduced infiltration of inflammatory cells into nasal cavity. The nasal mucosa sections were stained with hematoxylin and eosin. Sections were evaluated using microscope at an original magnification of 40x and 400x. Data are presented as mean ± SEM.

mice. HYT decreased such infiltration of inflammatory cells into nasal cavity (Figure 3(a)). Inflammatory cell numbers under each condition were shown in Figure 3(b).

3.3. HYT Reduced Infiltration of Mast Cells into Nasal Mucosa. Next, we performed toluidine blue staining for mast cell observation. Mast cells play a major role in allergic inflammation [23]. The respective numbers of mast cells in the nasal mucosa in AR mice were shown to be higher than those in the normal mice. HYT decreased such infiltration of mast cells into nasal cavity (Figure 4(a)). Lowest HYT concentration showed the highest effect to reduce infiltration of mast cells into nasal mucosa. Mast cell numbers under each condition were shown in Figure 4(b).

3.4. HYT Reduced Expression of CD4+ Cells into Nasal Cavity. CD4+ T helper cells generate cytokines that can stimulate other T cell effector functions and B cell antibody production [19]. We also performed immunocytochemistry to measure intracellular level of CD4+ (total T cells). We found that OVA increased numbers of CD4+ (total T cells), while HYT decreased them in nasal cavity (Figure 5).

3.5. HYT Reduced the Levels of Cytokines in the Serum. Allergic reactions cause the secretion of various cytokines [23]. Therefore, we measured the levels of cytokines in the blood samples by multiplex cytokine assay. We found that OVA increases the levels of IL-4, IL-13, and LIF, while HYT inhibits such increase (Figures 6(a), 6(b), and 6(c)).

3.6. HYT Reduced the Levels of Leukocytes in the Blood. It is known that allergic diseases activate eosinophils, neutrophils, monocytes, basophils, and lymphocytes [24, 25]. To investigate whether HYT suppresses inflammatory phenomenon, we measured leukocytes levels in cardiovascular blood samples using HEMAVET 950 hematology analyzer. We found that OVA increases the levels of eosinophils, neutrophils, monocytes, basophils, lymphocytes, and WBC, while HYT inhibits such increase (Figures 7(a), 7(b), 7(c), 7(d), 7(e), and 7(f)).

3.7. Effect of HYT on Splenocyte Viability. Finally, we measured the effect of HYT on splenocyte viability. For that purpose, we treated splenocyte with various concentrations of HYT (10, 50, 100, 200, 500, 1000, and 2000 ug/mL) and measured cell viability using WST assay. We found that con A increases cell viability, while HYT decreases it (Figure 8).

4. Discussion

In this study, we investigated the effect of HYT on allergic responses in OVA-induced AR mice. Increase of infiltration

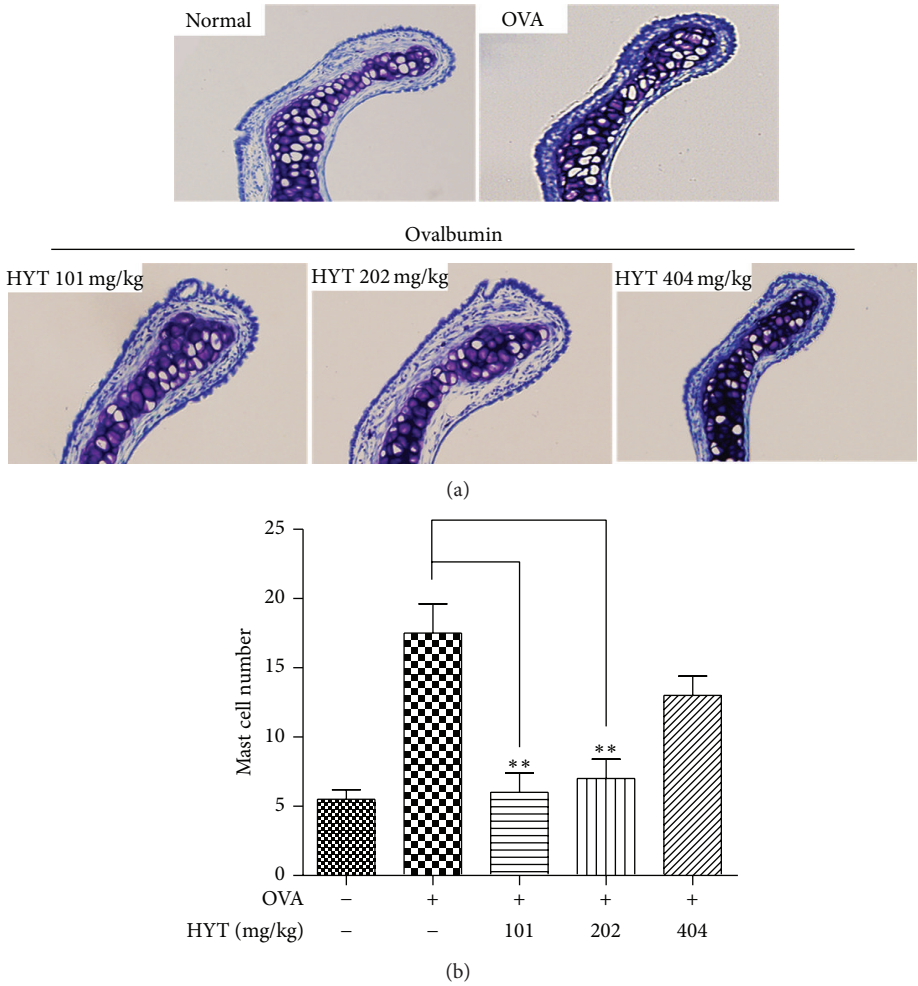

Figure 4: HYT reduced infiltration of mast cells into nasal mucosa. The nasal mucosa sections were stained with toluidine blue. Sections were evaluated using microscope at an original magnification of 200x. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

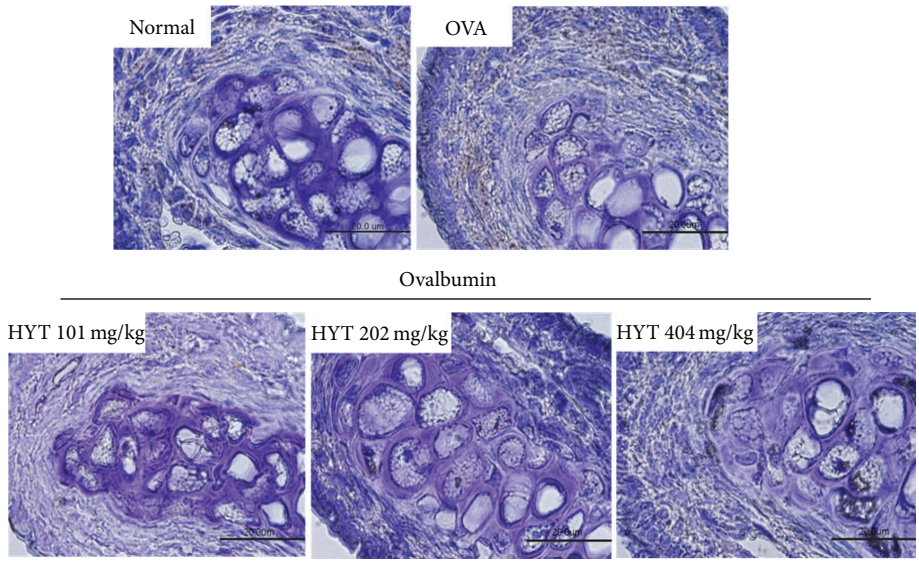

Figure 5: Distribution of CD4+ cells in nasal cavity samples. The nasal cavity sections were immunostained with CD4+ antibody. CD4+ cells show a brown color. Sections were evaluated using microscope at an original magnification of 1000x.

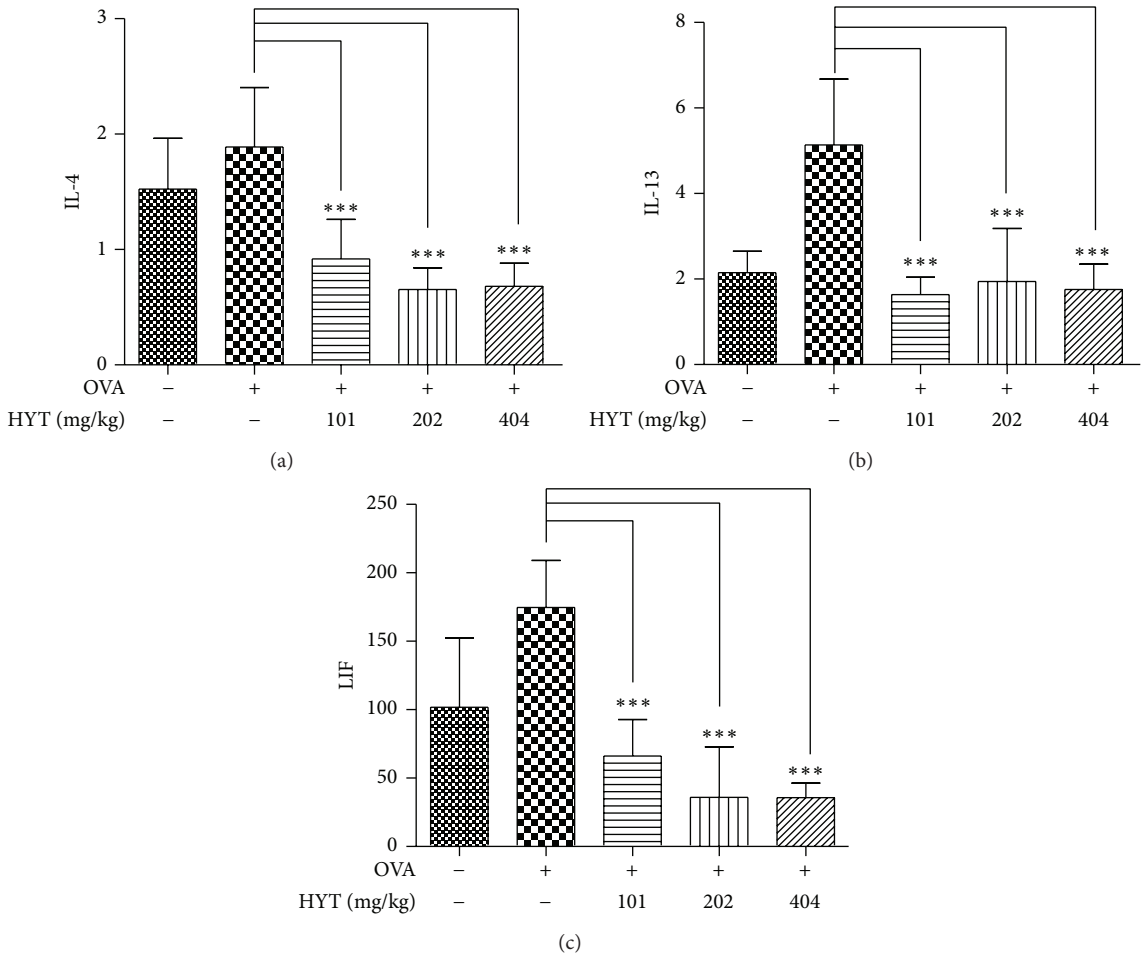

Figure 6: HYT reduced the levels of cytokines in the serum. The cytokines levels were measured by multiplex cytokine assay. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

of inflammatory and mast cells into nasal cavity is known in OVA-induced AR mice. Interestingly, HYT strongly suppressed such increase. Immunohistochemical study showed thatHYT reduced increased number of CD4+ T cells induced by OVA. Moreover, induction of various cytokines due to AR was also suppressed by HYT in mice.

The activation of eosinophils releases a variety of chemicals to cause inflammation and tissue injuries [26, 27]. A variety of cytokines are released by inflammatory stimulation [28–30]. We found that OVA increases the levels of IL-4, IL-13, and LIF, while HYT inhibits such increase. We also found that OVA increases the levels of eosinophils, neutrophils, monocytes, basophils, lymphocytes, and WBC, while HYT inhibits such increase. HYT also decreased cell viability of splenocyte. These data clearly demonstrate that HYT has an efficacy to treat AR.

AR, also known as hay fever, affects approximately 20 percent of people of all ages. The risk of developing AR is much higher in people with asthma or eczema and in people who have a family history of asthma or rhinitis [31]. AR can begin at any age, although most people first develop symptoms in childhood or young adulthood [4]. The symptoms are often at their worst in children and in people in their 30s and 40s. The severity of symptoms tends to vary throughout life; many people experience periods when they have no symptoms at all [32].

The treatment of AR includes reducing exposure to allergens and other triggers, in combination with medication therapy. Nasal glucocorticoids (steroids) delivered by a nasal spray are the first-line treatment for the symptoms of AR. However, we require more effective therapy to treat AR with few side effects. HYT is known to treat otitis media, sinusitis, tonsillitis, and a variety of otolaryngology symptoms [20]. In this study, we verified that HYT could be used to treat AR. Our present study clearly demonstrates that HYT suppresses the progression of AR induced by OVA. This suggests that HYT might be a useful drug for the treatment of AR.

Conflict of Interests

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

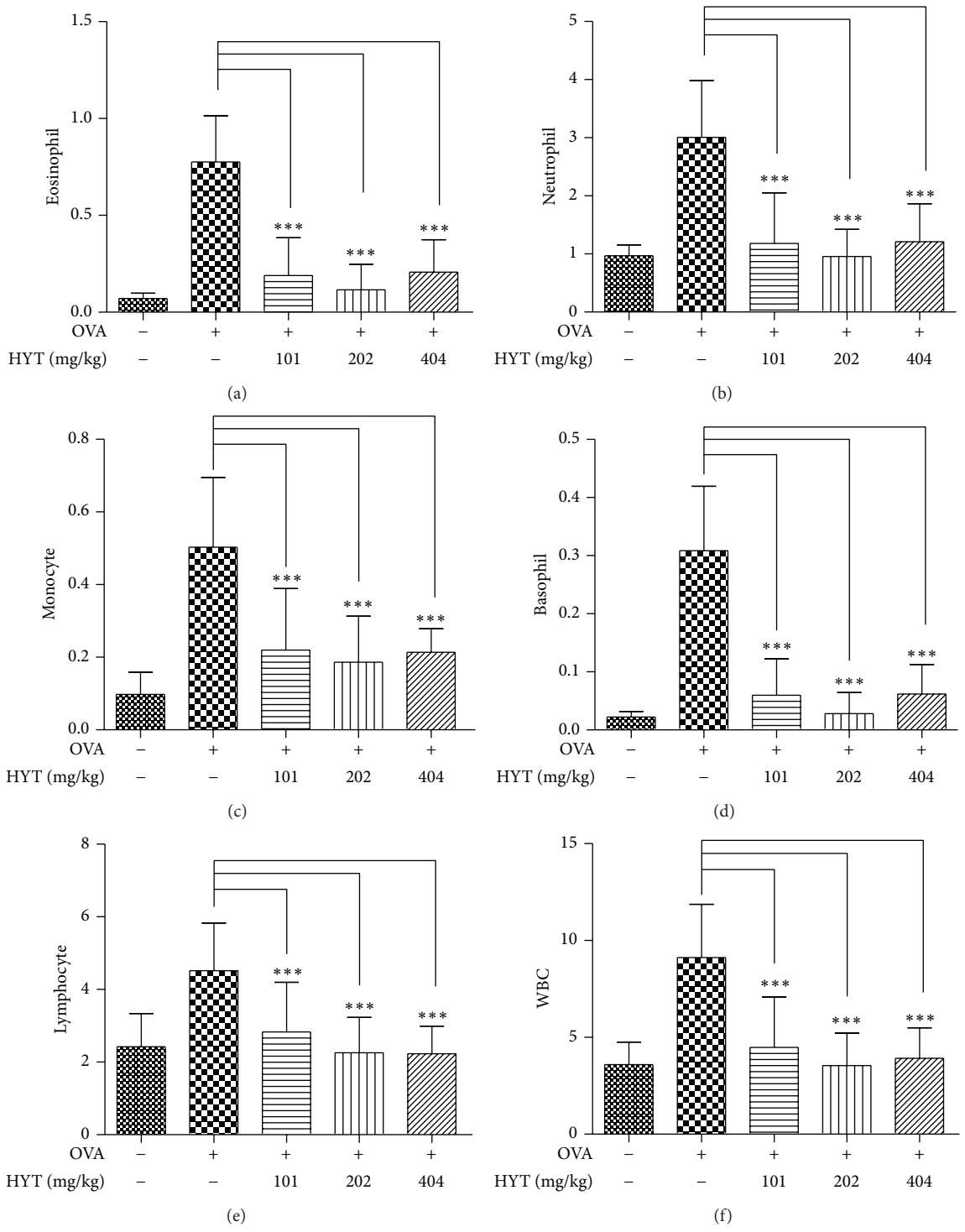

Figure 7: HYT reduced the levels of leukocytes in the blood. Blood samples were analyzed using HEMAVET 950 hematology analyzer. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

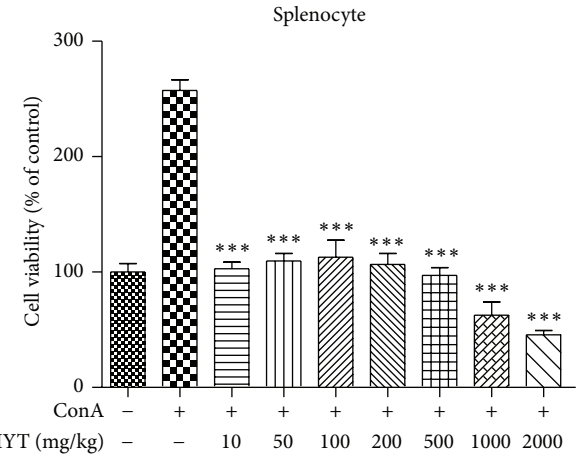

Figure 8: Effect of HYT on splenocyte viability. Splenocytes were treated with various concentrations of HYT (10, 50, 100, 200, 500, 1000, and 2000 ug/mL). Cell viability was measured by WST assay. The columns and the error bars represent mean ± standard deviation (SD). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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Research Article

Puerarin Alleviates Neuropathic Pain by Inhibiting Neuroinflammation in Spinal Cord

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Neuropathic pain responds poorly to drug treatments, and partial relief is achieved in only about half of the patients. Puerarin, the main constituent of *Puerariae Lobatae Radix*, has been used extensively in China to treat hypertension and tumor. The current study examined the effects of puerarin on neuropathic pain using two most commonly used animal models: chronic constriction injury (CCI) and diabetic neuropathy. We found that consecutive intrathecal administration of puerarin (4–100 nM) for 7 days inhibited the mechanical and thermal nociceptive response induced by CCI and diabetes without interfering with the normal pain response. Meanwhile, in both models puerarin inhibited the activation of microglia and astroglia in the spinal dorsal horn. Puerarin also reduced the upregulated levels of nuclear factor-κB (NF-κB) and other proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α, in the spinal cord. In summary, puerarin alleviated CCI- and diabetes-induced neuropathic pain, and its effectiveness might be due to the inhibition of neuroinflammation in the spinal cord. The anti-inflammation effect of puerarin might be related to the suppression of spinal NF-κB activation and/or cytokines upregulation. We conclude that puerarin has a significant effect on alleviating neuropathic pain and thus may serve as a therapeutic approach for neuropathic pain.

1. Introduction

Neuropathic pain is caused by aberrant sensory processing in either the peripheral- and/or the central nervous system (CNS) and can be induced by a variety of factors, including traumatic damage, infection, and diabetes [1]. Abnormal neuronal activity plays a fundamental role in the pathogenesis of neuropathic pain, while current treatments that suppress aberrant neuronal activity generally lack efficacy, in addition to their many undesirable side effects [2]. Recent studies have indicated that immune response of the CNS, in which glial cells are critically involved, plays an important role in the development and persistence of neuropathic pain [3, 4]. Hyperalgesia and allodynia are frequently induced by increased levels of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, in the CNS, and the resulting or accompanying activation of glial cells [5]. Thus cytokines and glial cells in the CNS have been recognized as powerful modulators of nociception and hold potent potential to the control of neuropathic pain [6].

Puerarin, the main constituent of *Radix Puerariae*, has a variety of pharmacology characteristics [7]. Puerarin has been shown to effectively inhibit proinflammatory cytokine production and/or glia cell activation in a variety of diseases [8–10]. We speculated that puerarin could also be effective in the intrathecal treatment of neuropathic pain via inhibition of spinal inflammation; thus the present study was designed to investigate the effects of puerarin on neuropathic pain using two most commonly used rodent pain models (partial sciatic nerve injury and diabetes). In addition to pain, the effects of puerarin on proinflammatory cytokine production and glial activation in the spinal cord were also examined.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats (220–250 g) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The rats were housed in a temperature-controlled room (22–25°C) in plastic cages (5 animals per cage) with a 12-hour light/dark cycle and had free access to food and water. All animal experiments were approved by Xiamen University Animal Care and Use Committee. All efforts were made to minimize the animal suffering and the number of animals used.

2.2. Drugs. For intrathecal injections, Puerarin and fluorocitrate were dissolved in artificial cerebrospinal fluid (ACSF). The solution was completed in distilled water. Puerarin and fluorocitrate and all other reagents were purchased from Sigma-Aldrich.

2.3. Intrathecal Catheterization. For intrathecal drug administration, intrathecal catheterization [11] was performed by advancing the PE-5 catheter 8.5 cm caudally to the lumbar enlargement through an incision in the cisternal membrane of rats that were under isoflurane mask anesthesia. The catheter was externalized and secured to the musculature at the incision site. Rats showing any neurologic dysfunction, such as paralysis or urine incontinence after catheterization, were euthanized. A proper location of the catheter was confirmed by the hind limb paralysis after 10 μL intrathecal injection of 2% lidocaine (Sigma) 1 day after catheterization. Only rats displaying normal grooming, ambulation, and weight gain after catheterization were used in the following study.

2.4. Type 1 Diabetic Neuropathic Pain Model. Diabetes was induced in rats by a single intraperitoneal (i.p.) injection of streptozotocin (STZ, Sigma; 60 mg/kg, i.p.) [12]. Age-matched control rats received an equal volume of vehicle (0.01 M citrate buffer, pH 4.5). Blood was collected via the tail vein at 72 h after STZ injection to measure glucose concentration. Rats with a blood glucose level over 16.67 mmol/L were considered to be diabetic and included in the subsequent experiments. Puerarin (4, 20, and 100 nM), fluorocitrate (1 nM, served as a positive control), or vehicle was administered intrathecally once daily for 7 consecutive days beginning on day 20 after STZ injection. The mechanical withdrawal threshold of the right hind paw was measured before STZ injection (baseline), before drug treatment (predose), and 60 min after each drug administration (postdose) in the morning.

2.5. Chronic Constriction Injury (CCI) Model of Neuropathic Pain. The rat CCI model of neuropathic pain was produced according to the method described before [13]. Adult male Sprague-Dawley rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.). The right common sciatic nerve was isolated at mid-thigh level and loosely ligated using chromic gut suture (5-0) at four sites separated by an interval of 1 mm. For sham surgery, the right sciatic nerve was exposed, but the nerve was not ligated. All animals were allowed 3 days to recover from the surgery. Puerarin (4, 20, and 100 nM), fluorocitrate (1 nM), or vehicle was administered intrathecally once daily for 7 consecutive days beginning on day 4 postoperatively. The mechanical withdrawal threshold was measured before surgery (baseline), before drug treatment (predose), and 60 min after each drug administration (postdose) in the morning.

2.6. Mechanical Allodynia. Mechanical allodynia was measured using a commercially available electronic von Frey apparatus (Model 2390; IITC Life Science Inc., Woodland Hills, CA) as described previously [14] with minor modifications. Rats were placed into a Plexiglas box on a steel mesh floor. Pressure was applied to the center of the hind paw with the von Frey filament in an upward motion until foot withdrawal. The withdrawal threshold was automatically recorded. The maximum strength of the filament was 55 g. The procedure was repeated after approximately 10 min for each daily session, and the average mechanical withdrawal threshold (MWT) was calculated.

2.7. Measurements of NF-κB DNA Binding Activity and Inflammatory Cytokines. NF-κB DNA binding activity measurements were performed using a commercially supplied NF-κB transcription factor binding assay kit (Cayman Chemical, CA, USA) according to the manufacturer's suggested protocol. The assay is based on the principle that only the active form of NF-κB in the sample binds to oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) that is immobilized on the microtiter plate [15]. Briefly, lumbar section of the spinal cord was homogenized in lysis buffer. The nuclear extract was prepared using a nuclear extract kit (Cayman Chemical), and samples of nuclear extract were first incubated overnight at 4°C in wells precoated with a dsDNA sequence corresponding to the NF-κB consensus motif. The NF-κB consensus motif of the assay should bind both human and rat p65. After 5 washes, the samples were incubated overnight at 4°C with primary antibody (rabbit polyclonal, Cayman Chemical) to the p65 subunit of NF-κB. The primary antibody against the p65 subunit of NF-κB used in the assay system is accessible only when NF-κB is activated and bound to its target DNA. Subsequently, samples were incubated for 60 minutes with an HRP-conjugated goat anti-rabbit secondary antibody (Cayman Chemical), followed by colorimetric detection at 450 nm (Multiskan FC, Thermo Scientific). After background subtraction, absorbance measures were referred to a standard curve obtained from a series of duplicate wells containing measured amounts of human recombinant p65 (Cayman Chemical) and then converted to an estimate of the quantity of p65/well, which was normalized by dividing the p65 estimate by the total amount of protein measured in the sample. TNF-α, IL-6, and IL-1β were quantified using ELISA kits (Abcam, USA) according to the manufacturer's instructions.

2.8. Immunohistochemistry. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), perfused intracardially

with 300 mL of 0.9% saline followed by 300 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2–7.4, 4°C). Lumbar spinal segments were removed, postfixed overnight at 4°C, and kept in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) at 4°C. Dissected tissue was mounted in OCT compound and frozen at −20°C. Transverse spinal cord sections (10 μm) were prepared using a cryostat (Microm HM550) and placed in PBS. Sections were washed in 0.01 M PBS twice for 10 min and blocked for 1 h in 5% bovine serum albumin and 0.1% Triton X-100. Free floating tissue sections were incubated overnight at 4°C on a rocker with a rabbit polyclonal antiastroglia marker-GFAP antibody (Abcam; 1 : 1000) or a rabbit polyclonal antimicroglia marker-Iba-1 antibody (Abcam; 1 : 1000). Tissue samples were then washed twice with PBS for 8 min each and incubated with FITC- or Cy3-conjugated anti-rabbit antibody (1 : 300, Jackson Immuno Research Laboratories Inc.) in blocking solution without Triton X-100 for 1 h at room temperature in the dark. Control staining was performed by omitting the primary antibody. Fluorescent images were captured with a digital camera (Olympus). The percentage of positive immunostained area in the dorsal horn was analyzed using Image-Pro software (Plus Version 6).

2.9. Western Blot. Nuclear and cytoplasmic protein was extracted with a nuclear and cytoplasmic extract kit (Cayman Chemical). Protein content of each sample was determined using the BioRad protein assay (BioRad Laboratories) according to manufacturer's protocol. For immunoblot analysis, 25 μg of protein was subjected to SDS-PAGE, transferred to a membrane, and probed with specific antibodies: NF-κB p65 (1 : 5000, Abcam); phospho-NF-κB p65 (Ser 536, 1 : 5000, Abcam); Lamin B (1 : 2000, Abcam); β-actin (1 : 2000, Abcam). Secondary antibody HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, 1 : 5000) were used to detect binding of antibodies. The membrane was incubated with Clarity Western ECL Substrate (Bio-Rad Laboratories, CA, USA), and the target proteins were then visualized and quantitated using a LAS-3000 luminescent image analyzer (Fujifilm). The results were expressed as a relative ratio of the target protein to reference protein.

2.10. Statistical Analysis. The antimechanical allodynia effects were evaluated by the increment of the MWT after drugs treatment and expressed as percentage of maximal possible effect (%MPE): MPE% = [(postdose threshold) − (predose threshold)]/[(baseline threshold) − (predose threshold)] × 100 [16]. It is possible to obtain a negative value of %MPE if the MWT was decreased after treatments, that is, to enhance the allodynic response. Data from the behavior test, ELISA, and immunohistochemistry were analyzed using two-way ANOVA or one-way ANOVA followed by the LSD t-test for post hoc analysis. All of the data are presented as mean ± SEM, and all statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). A P value of <0.05 was considered statistically significant.

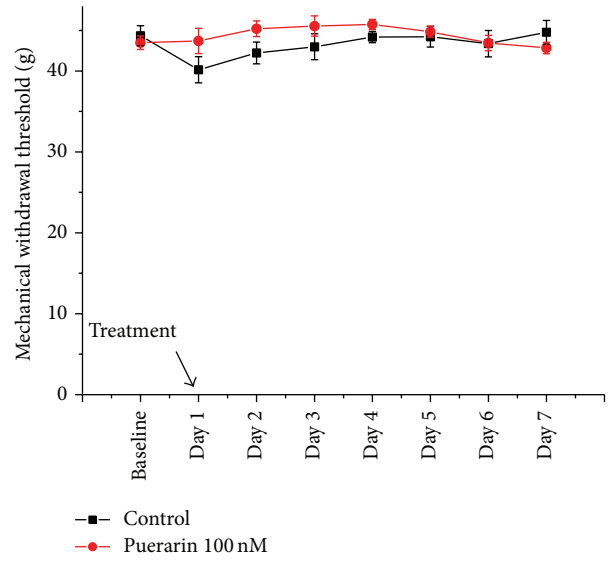

Figure 1: The effects of puerarin on mechanical stimulus test in normal rats. Normal rats received intrathecal 100 nmol puerarin or vehicle. The mechanical withdrawal thresholds (g) of the right hind paws were measured 60 min after drug administration. Data were presented as mean ± SEM. Each group consisted of 5 rats. At 100 nM, puerarin did not affect mechanical withdrawal threshold.

3. Results

3.1. Effects of Puerarin on Pain in Normal Rats. Puerarin (100 nM, i.t., for 7 consecutive days) did not affect MWT (Figure 1).

3.2. Effects of Puerarin on CCI- and Diabetes-Induced Neuropathic Pain. Post hoc tests showed CCI and diabetes significantly decreased ($P < 0.001$, versus sham control) MWT to mechanical stimulation (Figures 2(a) and 2(c), $P < 0.001$, versus sham control), demonstrating the development of mechanical allodynia which persisted for the entire observation period. Comparable to fluorocitrate, puerarin significantly reduced mechanical allodynia in both CCI- and diabetes models compared to the vehicle (Figures 2(a)–2(d)). The MPE of puerarin for CCI- and diabetes-induced neuropathic pain on day 7 was 16.41% ± 5.66% at 4 nM, 43.41% ± 2.75% at 20 nM, and 64.2% ± 3.71% at 100 nM for CCI and 23.93% ± 5.49% at 4 nM, 49.11% ± 4.23% at 20 nM, and 62.07% ± 6.34% at 100 nM for diabetes, respectively (Figures 2(b) and 2(d)). The MPE of fluorocitrate was 69.9% ± 5.48% for CCI and 76.24% ± 5.27% for diabetes, respectively (Figures 2(b) and 2(d)).

3.3. Spinal Microglia and Astroglia Activation in CCI and Diabetic Rats. In CCI groups, staining of the microglia activation marker Iba-1 was barely detectable in the spinal cord in sham control rats (Figure 3(a)). The number of Iba-1 immunoreactive cells significantly increased in the dorsal horn ipsilateral to the CCI injury on day 11 after surgery (Figures 3(a) and 3(c), $P < 0.001$). Puerarin reduced the enhanced Iba-1 immunoreactivity in the spinal cord (Figures

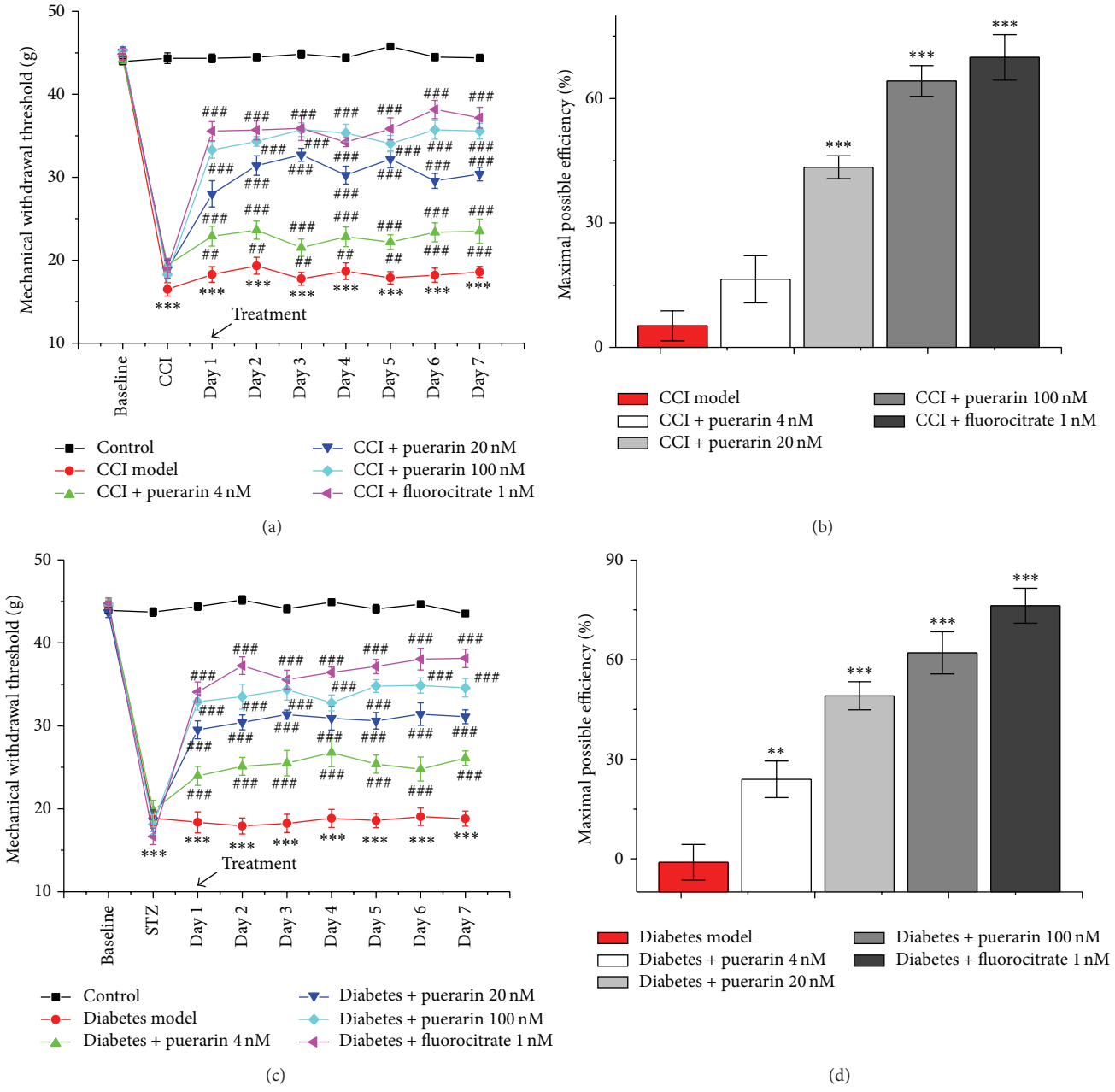

Figure 2: The effects of puerarin on CCI- and diabetes-induced mechanical allodynia. (a), (c) The temporal profile of mechanical withdrawal threshold on CCI- (a) and diabetes- (c) induced mechanical allodynia; (b), (d) the maximal possible efficiency of puerarin on the 11th day after CCI surgery (b) and the 27th day after streptozotocin injection; CCI: chronic constriction injury. Data are presented as mean ± SEM. $^{***}P < 0.001$, model group versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, puerarin treatment group versus model group (one-way ANOVA with *post hoc* LSD *t*-test). Each group consisted of 6–10 rats.

3(a) and 3(c), $P < 0.001$ for puerarin 20, 100 nM). Rats receiving sham CCI surgery showed a low-level staining for GFAP, an astroglia activation marker, in the spinal cord (Figure 3(b)). The number of GFAP immunoreactive cells significantly increased in the dorsal horn ipsilateral to CCI injury on day 11 after surgery (Figures 3(b) and 3(c), $P < 0.001$). Puerarin reduced the increase of GFAP immunoreactivity in the spinal cord caused by CCI (Figures 3(b) and 3(c), $P < 0.001$ for puerarin 20, 100 nM).

As for the diabetic groups, there is a similar trend. A low level and diffuse staining of Iba-1 was noticed in the spinal cord of rats receiving vehicle injection (Figure 4(a)). The number of Iba-1 immunoreactive cells dramatically increased in the dorsal horn on day 27 after diabetes induction (Figures 4(a) and 4(c), $P < 0.001$). Puerarin attenuated the increase of Iba-1 immunoreactivity in the spinal cord in the diabetic rats (Figures 4(a) and 4(c), $P < 0.001$ for puerarin 20, 100 nM). Staining of GFAP in the spinal cord

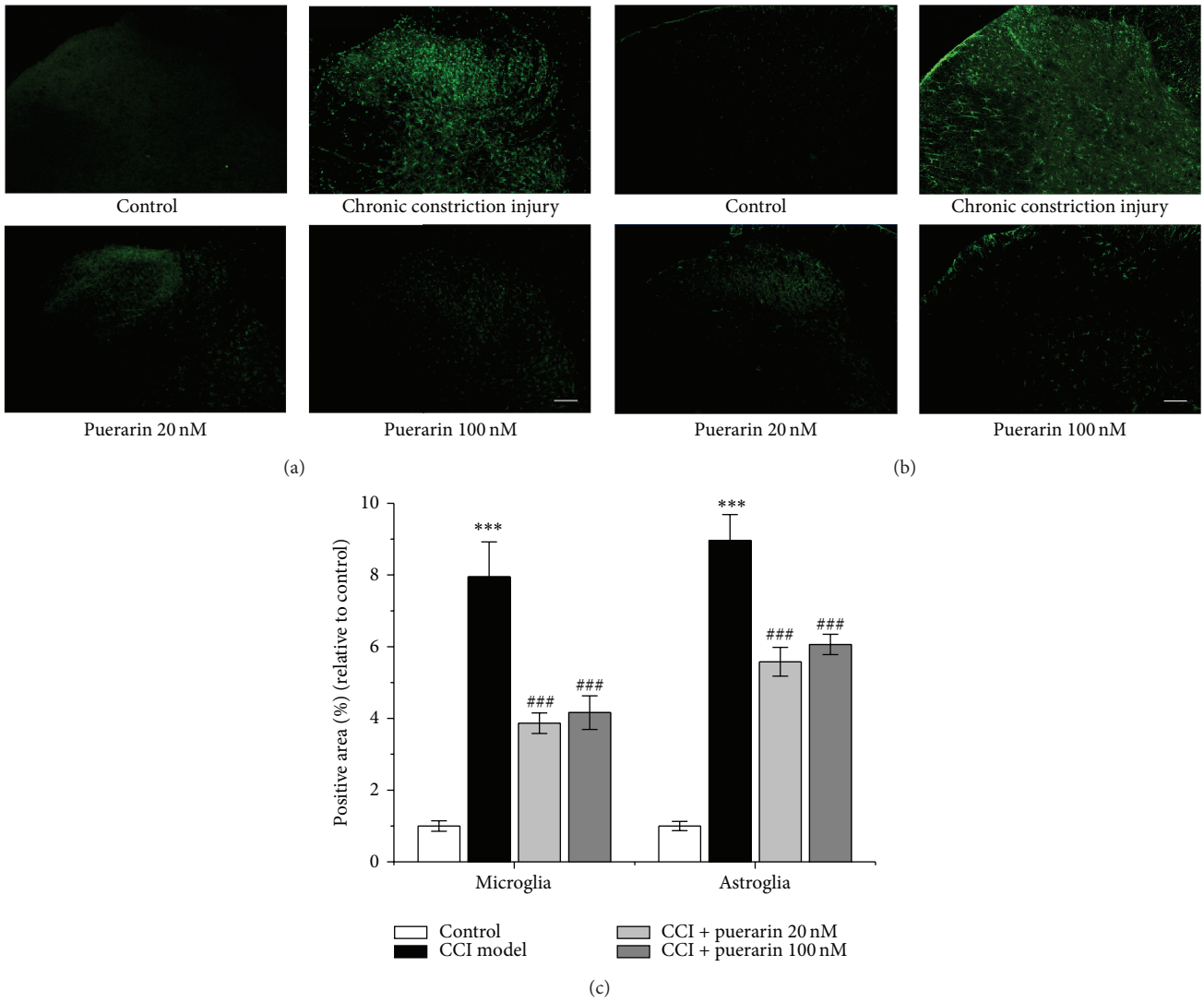

Figure 3: The effects of puerarin on CCI-induced microglia and astroglia activation in ipsilateral spinal dorsal horn of rats. (a) and (b) Representative images of ipsilateral spinal dorsal microglia (a) and astroglia (b) activation in control rats, CCI rats, CCI rats receiving vehicle, 20 and 100 nM puerarin on the 11th day after CCI surgery. Scale bar: 100 μm. (c) Quantification of microglia activation in the spinal cord on the 11th day after CCI surgery. CCI: chronic constriction injury. Data are presented as mean ± SEM. $^{***}P < 0.001$, CCI model versus control; $^{###}P < 0.001$, puerarin versus CCI model (one-way ANOVA with *post hoc* LSD t-test). Each group consisted of 6–10 rats.

was barely noticeable in rats receiving vehicle (Figure 4(b)), while significantly increased in the dorsal horn on day 27 after diabetes induction (Figures 4(b) and 4(c), $P < 0.001$). Puerarin reversed the elevated GFAP immunoreactivity in the spinal cord (Figures 4(b) and 4(c), $P < 0.001$ for puerarin 20, 100 nM).

3.4. Effect of Puerarin on TNF-α, IL-1β, and IL-6 Production and NF-κB Activation in CCI and Diabetic Rats. CCI increased TNF-α, IL-1β, and IL-6 production and NF-κB activation in the spinal cord ($P < 0.001$ versus control, Figure 5). Puerarin attenuated CCI-induced increase of TNF-α, IL-1β, and IL-6 ($P < 0.001$ for IL-1β and IL-6 at 20, 100 nM, $P < 0.01$ and $P < 0.001$ for TNF-α at 20 nM and 100 nM, resp., Figure 5(a)). The elevated NF-κB DNA binding activities were also significantly reduced with puerarin treatment ($P < 0.001$ and $P < 0.01$ at 20 nM and 100 nM, resp., Figure 5(b)). Moreover, the overexpression of NF-κB p65 and p65 nucleus translocation was significantly reduced with puerarin treatment ($P < 0.01$, Figures 5(c) and 5(d)).

With the same pattern, TNF-α, IL-1β, and IL-6 production and NF-κB activation were significantly elevated in the spinal cord of diabetic rats ($P < 0.001$ versus control, Figure 5). Puerarin at 20 and 100 nM decreased the diabetes-induced elevation of TNF-α, IL-1β, and IL-6 ($P < 0.001$, Figure 5(a)) and NF-κB DNA binding activities ($P < 0.01$ and $P < 0.001$ at 20 nM and 100 nM, resp., Figure 5(b)). Besides, the overexpression of NF-κB p65 and p65 nucleus translocation was significantly inhibited with puerarin treatment ($P < 0.01$, Figures 5(c) and 5(d)).

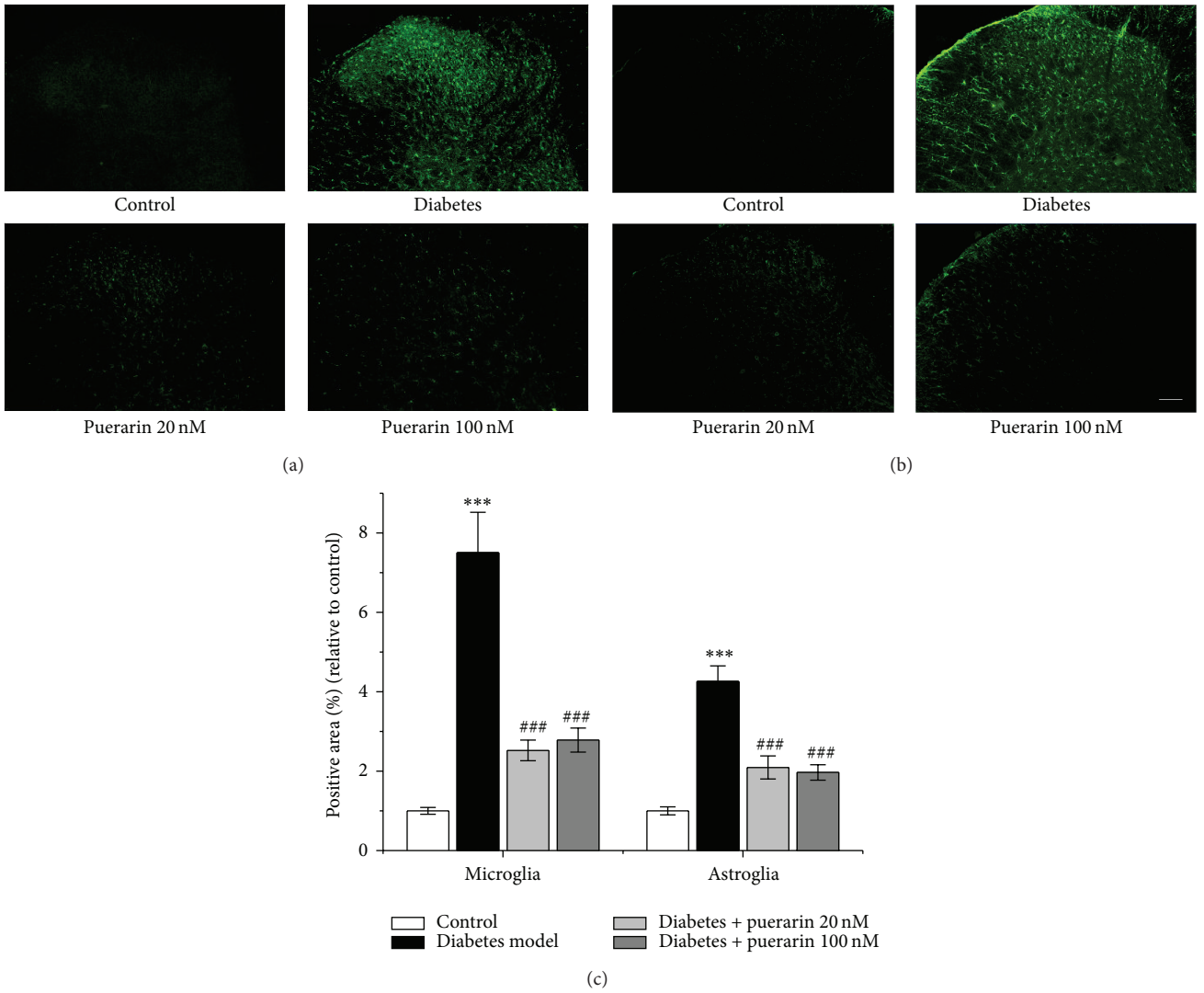

FIGURE 4: The effects of puerarin on diabetes-induced microglia and astroglia activation in ipsilateral spinal dorsal horn of rats. (a) and (b) Representative images of ipsilateral spinal dorsal microglia (a) and astroglia (b) activation in control rats, diabetic rats receiving vehicle, 20 and 100 nM puerarin on the 27th day after streptozotocin injection. Scale bar: 100 μm. (c) Quantification of microglia and astroglia activation in the spinal cord on the 27th day after streptozotocin injection. Data are presented as mean ± SEM. $^{***}P < 0.001$, diabetic rats versus control; $^{###}P < 0.001$, puerarin versus diabetes model (one-way ANOVA with *post hoc* LSD t-test). Each group consisted of 6–10 rats.

4. Discussion

Neuropathic pain is caused by aberrant sensory processing in either the peripheral nervous system and/or the central nervous system and is currently lacking efficacious therapy. We found that consecutive intrathecal administration of puerarin to rats inhibited the mechanical and thermal nociceptive response induced by CCI and diabetes, which might acted through inhibiting the activation of microglia and astroglia in the spinal dorsal horn and reducing the upregulated levels of nuclear factor-κB (NF-κB) and proinflammatory cytokines including IL-6, IL-1β, and TNF-α, in the spinal cord.

Neuropathic pain is characterized by spontaneous pain, increased responsiveness to pain stimuli (hyperalgesia), and pain perceived in response to normally nonnoxious stimuli (allodynia) [1]. A growing body of literature indicates that the enhanced spinal neuroimmune and neuroinflammatory activities initiate and maintain neuropathic pain after the primary nerve injury [6, 17]. Specifically, the proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been strongly implicated in the initiation and development of neuropathic pain after nerve injury [18, 19]. Neuropathic pain is usually accompanied by peripheral and central nervous system damage. Nerve injury leads to a rapid release of pain-related mediators, such as TNF-α, IL-1β, IL-6, and prostaglandins, resulting in inflammatory responses that sensitize the CNS and facilitate pain processing [20]. NF-κB plays a vital role in these processes and is an important mediator in the regulation of proinflammatory cytokines and inflammatory and immune responses [21]. A number of inflammatory mediators (i.e., TNF-α, IL-1β, IL-6, NO, and TGF-1β) that are implicated in the modulation of the neuropathic pain

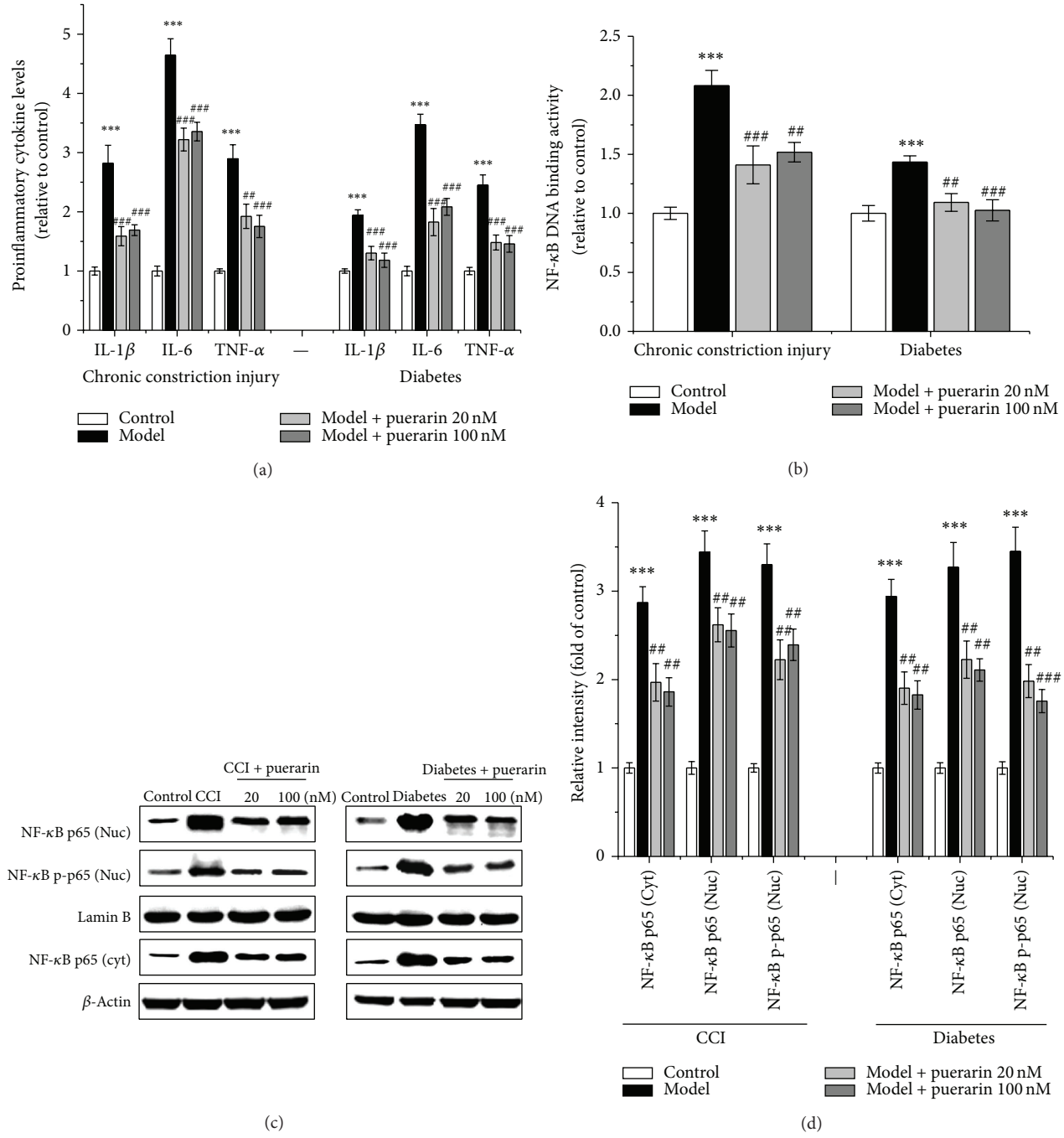

Figure 5: The effects of puerarin on CCI- and diabetes-induced proinflammatory cytokines overexpression (a) and NF-κB overactivation ((b), (c), (d)) on the 11th day after CCI surgery, and on the 27th day after streptozotocin injection in rat spinal cord. Data are presented as mean ± SEM. CCI: chronic constriction injury; Cyt: cytoplasmic; Nuc: nuclear. $^{***}P < 0.001$, CCI and diabetes models versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, puerarin versus CCI and diabetes models (one-way ANOVA with *post hoc* LSD *t*-test). Each group consisted of 6–10 rats.

can activate NF-κB or can be activated by NF-κB [22]. Inhibition of the expression of NF-κB and proinflammatory factors (TNF-α, IL-1β, and IL-6) can alleviate mechanical allodynia and thermal hyperalgesia in chronic constriction injury (CCI) rat model [23]. Taken together, NF-κB is one of the most important transcription factors regulating gene expression of the proinflammatory cytokines [24, 25] and is implicated in the initiation and development of neuropathic pain via a neuron-mediated way of central sensitization and glia cells-mediated expressions of proinflammatory cytokines and pain mediators [6, 23, 26].

Resident astroglia and microglia in the CNS are known to play important roles in neuroinflammation [27]. Spinal glial activation has been demonstrated in a variety of animal pain

models, including models of neuropathic and inflammatory pain [28]. Following stimulation, spinal glia cells proliferate, undergo morphological changes, increase the expression of cell surface receptors, and increase the production and release of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and other cytotoxic products, which in turn enhance pain transmission in the dorsal horn of the spinal cord [28, 29]. Also, the spinal neuroimmune and neuroinflammatory activation is a positive feed-forward loop mediated by proinflammatory cytokines, glia cells, and NF-κB [28].

In the current study, two classic models for neuropathic pain: a sciatic nerve chronic constriction injury model [30] and a diabetic neuropathy model [31, 32], were used. We showed that fluorocitrate, which specifically blocks glial metabolic activity by inhibiting the activity of aconitase, a Krebs cycle enzyme found exclusively in glia [33–36] and served as positive control in this study, reduced the pain response in both models as previous reported [12, 37, 38]. Consistent with the previous studies [5, 23, 27, 39–42], the expression of TNF-α, IL-1β IL-6, and NF-κB p65 (p-p65) was dramatically increased in spinal cord, and the spinal astroglia and microglia were drastically activated in both the CCI injury and diabetic models. The effects of puerarin were dose-dependent and comparable to that of fluorocitrate. Puerarin did not affect the pain threshold in normal rats, suggesting the effects of puerarin on neuropathic pain are specific. The fact that puerarin inhibited the overexpression of spinal TNF-α, IL-1β, IL-6, and NF-κB p65 (p-p65) and spinal glia activation associated with neuropathic pain in our study suggest that the action of puerarin against neuropathic pain involves the regulation of the neuroinflammatory process and the neuroimmune system in general (Figure 6).

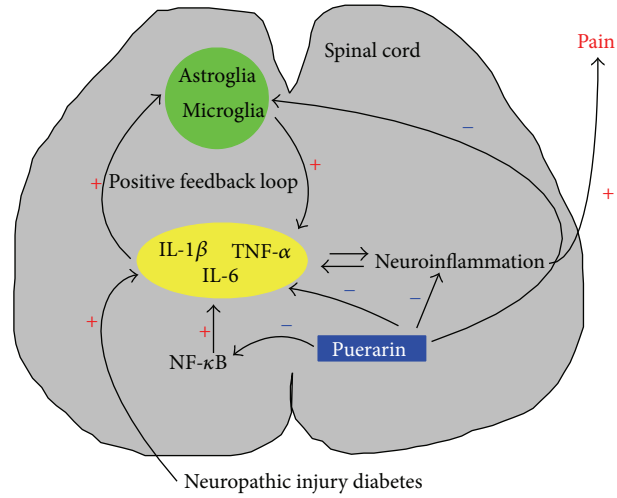

Figure 6: Hypothetical mechanisms of antineuroinflammation activity of puerarin.

5. Conclusion

In summary, intrathecal administration of puerarin produces dose-dependent antinociceptive effects in CCI- and diabetes-induced neuropathic pain. One possible mechanism is the inhibition of neuroinflammatory process and glia activation. The present study also suggests that puerarin is a promising platform for developing novel agents for the treatment of neuropathic pain.

Conflict of Interests

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

Acknowledgments

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Research Article

Boehmeria nivea Attenuates the Development of Dextran Sulfate Sodium-Induced Experimental Colitis

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We examined the therapeutic effect of an ethanol extract derived from *Boehmeria nivea* (Linn.) Gaudich in a mouse model of experimental colitis. Treatment with 70% ethanol extract derived from *B. nivea* (EBN) at a dose of 100, 200, or 500 mg/(kg·d) improved colon shortening, body weight, the disease activity index (DAI), and histopathological score of DSS-induced colitis mice. DSS significantly increased the levels of cyclooxygenase-(COX-) 2 in colon tissue relative to that of the untreated control group. EBN administered at 100, 200, or 500 mg/(kg·d) reduced COX-2 levels in the DSS-treated mice. In addition, EBN decreased the DSS-induced secretion of the inflammatory cytokine interleukin-6 (IL-6) and chemokine monocyte chemotactic protein-1 (MCP-1). Taken together, these data suggest that *B. nivea* extract is effective in preventing colitis.

1. Introduction

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC), are inflammatory diseases. Several anti-inflammatory drugs including sulfasalazine, steroids, and nonsteroidal anti-inflammatory drugs have been used to treat UC [1]; however, these drugs have limited effectiveness, because of their associated side effects and the varying etiology of UC. Experimental colitis models have been used to identify therapeutic agents and elucidate the underlying physiologic mechanisms of UC [2]. The widely employed DSS-induced colitis model recapitulates the histological characteristics of UC [2]. Administration of DSS induces colitis in the rectum that spreads to the anus, resulting in inflammatory changes including crypt distortion, ulceration, and the infiltration of inflammatory cells [2].

Numerous investigators, who have demonstrated that immune dysfunction contributes to the development of UC, have emphasized the importance of COX in UC pathogenesis. COX has three isoforms, COX-1, -2, and -3 [3]. In mammalian cells, COX-1 is constitutively expressed; however, COX-2 expression is induced by the inflammatory response. Recently, COX-3 was shown to be encoded by the same gene as COX-1. The inflammatory response is stimulated by pathogenic infections, resulting in the production of proinflammatory cytokines and chemokines [4]. In IBD patients, proinflammatory cytokines and chemokines such as IL-6 and MCP-1 are upregulated in intestinal mucosa [4]. Thus, inhibiting cytokine production through the use of therapeutic agents is a potential strategy for alleviating the symptoms of IBD.

Natural agents have been proposed by several investigators as a treatment of many diseases including IBD. Mounting evidence shows that the therapeutic efficacy of these natural agents relies on their ability to reduce cytokine production and COX-2 levels in vitro and in vivo [5]. For example, *Serpylli herba* extract reduces both trinitrobenzene sulfonic acid- (TNBS-) or DSS-induced colitis by reducing the levels of tumor necrosis factor- (TNF-) α and IL-6 cytokines and the chemokine MCP-1 [5]. *Solanum tuberosum* L. cv Jayoung epidermis, a color-fleshed potato, reportedly inhibits the

expression of COX-2 and reduces the severity of colitis in DSS-induced mice [6].

Boehmeria nivea (Linn.) Gaudich (Urticaceae) is a flowering plant that is traditionally used to treat several diseases and heal wounds in Asian countries including China, Korea, the Philippines, and Thailand [7, 8]. Previously, we showed that *B. nivea* reduces lipopolysaccharide- (LPS-) induced secretion of proinflammatory cytokines such as TNF-α and IL-6 through the inhibition of p38 and c-Jun N-terminal kinase (JNK) [8]; however, the ability of *B. nivea* to control the effects of DSS-induced colitis has not been investigated. The results of our previous work prompted us to determine whether *B. nivea* exhibits an anti-inflammatory effect in DSS-induced colitis model mice.

2. Materials and Methods

2.1. Reagents. The COX-2 and β-actin antibodies were obtained from Cayman Chemical (Ann Arbor, MI, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. Horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DSS was purchased from MP Biochemicals (Aurora, OH, USA).

2.2. Preparation of B. nivea Extract. B. nivea leaves were provided by the Seocheon County Office (Seocheon, Republic of Korea), where a voucher specimen had been deposited. EBN was prepared from leaves as described previously [8].

2.3. Experimental Colitis. Experimental colitis was induced by 3% DSS as described previously [9]. Briefly, 8-week-old male C57BL/6 mice were obtained from Charles River Korea (Seoul, Republic of Korea) and were housed at a temperature of 22–26°C under a 12 h light/dark cycle with free access to water. After a 1-week period of adaptation, the mice were randomly divided into the following 5 groups (8 mice/group): untreated (no DSS), DSS-treated (3% DSS), DSS + EBN 100 (3% DSS and EBN at 100 mg/kg body weight per day), DSS + EBN 200 (3% DSS and EBN at 200 mg/(kg·d)), and DSS + EBN 500 (3% DSS and EBN at 500 mg/(kg·d)). The untreated group received tap water without DSS for 8 days. DSS alone and DSS + EBN were orally administered for 7 days.

The mice were scored daily with respect to body weight, stool formation, and fecal occult blood. The weight loss, stool formation, and fecal occult blood scores were averaged to determine the DAI. Scores were assigned as follows: weight change (0: <1%, 1: 1–5%, 2: 5–10%, 3: 10–15%, or 4: >15%), fecal occult blood (0: negative, 2: positive, or 4: gross bleeding), and stool formation (0: normal, 2: loose stool, or 4: diarrhea). Scoring was performed as previously described [9]. On day 7, the mice were sacrificed by cardiac puncture under ketamine/xylazine anesthesia and their colon tissues were isolated.

2.4. MCP-1 and IL-6 Quantification. Blood samples were collected and IL-6 and MCP-1 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. Histopathological Analysis. Paraffin-embedded colon sections, fixed in formalin, were stained using hematoxylin and eosin (H&E). The degree of colitis was assessed with respect to the presence of edema, extent of injury, leukocyte infiltration, crypt damage, and loss of goblet cells as described previously [9]. These criteria were scored as follows: inflammation severity (0: none, 1: slight, 2: moderate, or 3: severe), extent of injury (0: none, 1: mucosal, 2: mucosal and submucosal, or 3: transmural), and crypt damage (0: none, 1: damage to the basal third of the crypt, 2: damage to the basal two-thirds of the crypt, 3: only surface epithelium intact, or 4: loss of entire crypt and epithelium).

2.6. Protein Extraction and Western Blot Analysis. Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Total protein was collected and then separated using SDS-polyacrylamide gel electrophoresis. The protein was transferred to the nitrocellulose paper and detected by western blot analysis using specific antibodies.

2.7. Statistical Analysis. Statistical differences of the results were analyzed by Student's t-test between groups. Data show the mean ± standard deviation (SD) of at least 3 independent experiments performed in triplicate. $P < 0.05$ was considered significant.

3. Results

3.1. EBN Improves Colon Shortening, DAI, and Body Weight in DSS-Induced Colitis Mice. Our previous work suggested that *B. nivea* has an anti-inflammatory effect on LPS-induced RAW264.7 macrophages by reducing cytokine levels [8]. Thus, we investigated whether *B. nivea* has an inhibitory effect on an IBD, UC, by using an experimental colitis mouse model. As shown in Figures 1(a) and 1(b), treatment with 3% DSS reduced the colon length, body weight, and DAI relative to that of the untreated group. Administration of EBN mildly reduced colon shortening in the DSS-treated group. The reduction in body weight caused by the 3% DSS treatment was significantly improved by administering EBN at 100, 200, and 500 mg/(kg·d) (Figure 1(c)). The 3% DSS treatment caused diarrhea and rectal bleeding, two criteria that were scored and included in the DAI (Figure 1(d)). The DAI was reduced in mice that had been administered EBN at 100, 200, and 500 mg/(kg·d).

3.2. Histopathological Scores of DSS-Induced Colitis Mice Are Reduced by Administering EBN. We next performed a histological analysis by performing H&E staining. As shown in Figures 2(a) and 2(b), 3% DSS treatment induced an inflammatory response that affected colonic architecture, cell infiltration, crypt shortening or loss, and goblet cell depletion. Administration of EBN significantly reduced the number of

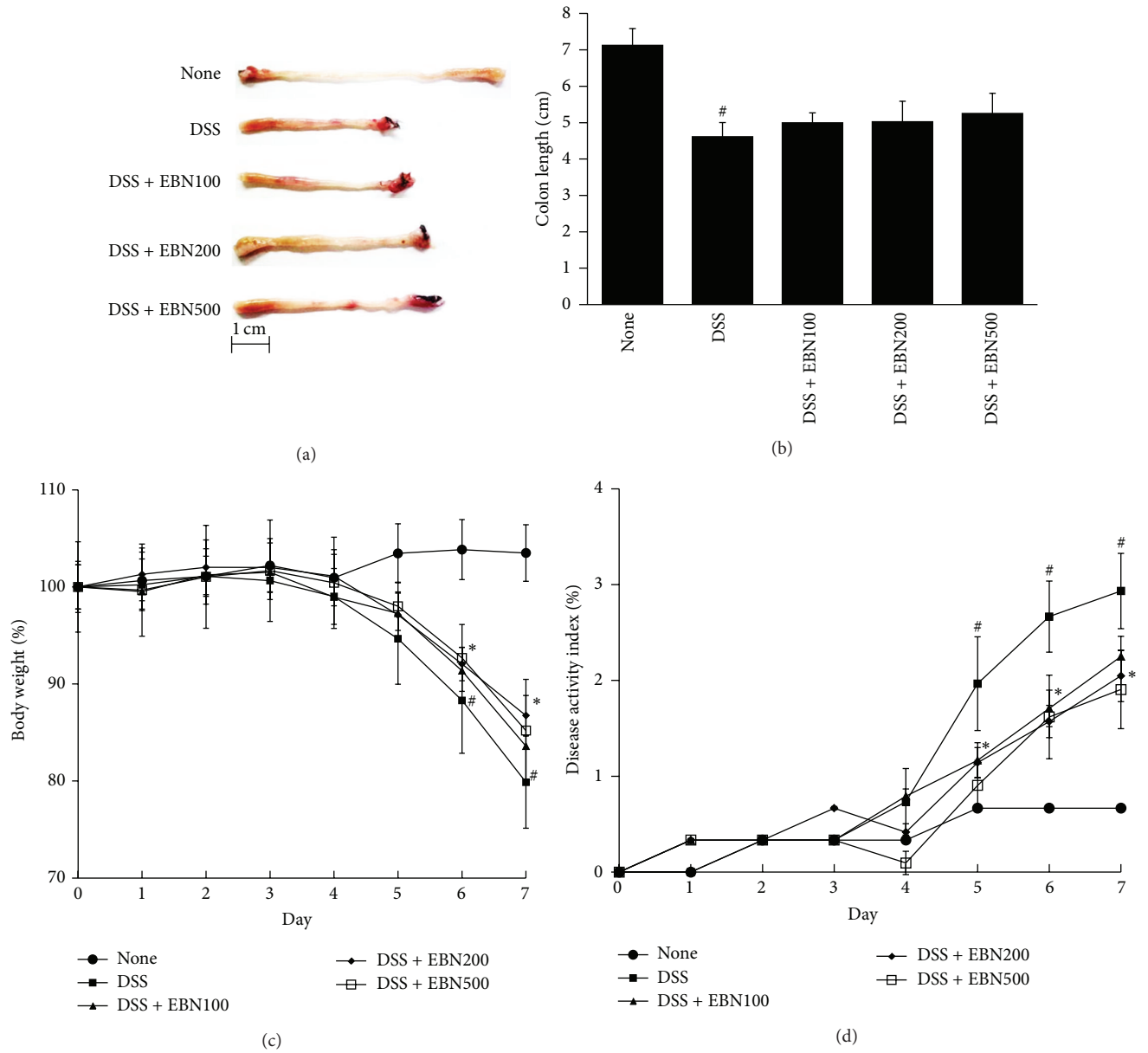

FIGURE 1: The effect of ethanol extract derived from *B. nivea* (EBN) on colon length, body weight, and disease activity index (DAI) in dextran sulfate sodium- (DSS-) induced colitis mice. Mice colons were isolated 7 days after DSS administration and their lengths were measured ((a) and (b)). Body weight was measured daily (c). Clinical scores of each mouse were monitored daily (d). Data from 3 independent experiments is shown and expressed as the mean ± SD ($n = 8$ per group). $^{*}P < 0.05$ versus DSS-treated; $^{\#}P < 0.05$ versus untreated.

infiltrating cells, mucosal injury, and edema. We scored the severity of the inflammation (see Materials and Methods). Mice treated with 3% DSS showed higher histopathological scores than those belonging to the untreated group. The histopathological scores were significantly improved when EBN was administered at 100, 200, and 500 mg/(kg·d) (Figure 2(b)).

3.3. EBN Decreases the Levels of IL-6, MCP-1, and COX-2 in DSS-Induced Colitis Mice. The inflammatory cytokine IL-6 and chemokine MCP-1 play central roles in inflammation [5]. Furthermore, the development of inflammatory diseases such as UC is mediated by COX-2. Thus, we examined whether EBN reduces IL-6, MCP-1, and COX-2 levels in DSS-induced colitis mice. As shown in Figure 3(a), DSS treatment significantly increased the levels of COX-2 in colon tissue relative to that of the untreated control group. Administration of EBN (200 mg/kg and 500 mg/kg) reduced the COX-2 levels that had been elevated in response to DSS treatment. DSS treatment also significantly increased the production of serum IL-6 and MCP-1 relative to controls (Figures 3(b) and 3(c)). Administration of EBN significantly decreased the production of IL-6 and MCP-1 in DSS-treated group (Figures 3(b) and 3(c)). The maximal effect of EBN was

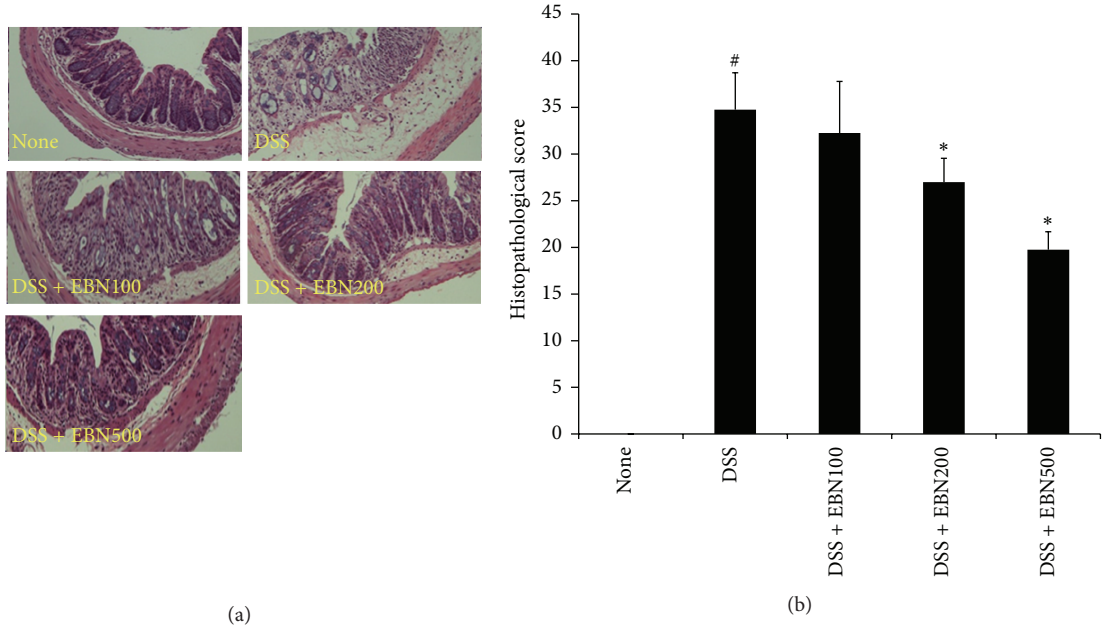

Figure 2: The effect of EBN on the histopathological score in DSS-induced colitis mice. Mice colons were obtained at 7 days post-DSS administration and were stained with H&E. H&E staining was performed on colon sections obtained from mice belonging to the untreated (no DSS), DSS-treated (3% DSS), DSS + EBN 100 (3% DSS and EBN at 100 mg/kg body weight per day), DSS + EBN 200 (3% DSS and EBN at 200 mg/kg·d), and DSS + EBN 500 (3% DSS and EBN at 500 mg/kg·d) groups. Images were obtained at 40x magnification (a). Histopathological scores were analyzed from slides (b). Data from 3 independent experiments is shown and expressed as the mean ± SD ($n = 8$ per group). $^{*}P < 0.05$ versus DSS-treated; $^{\#}P < 0.05$ versus untreated.

obtained with 100 mg/kg. These results suggest that EBN has an anti-inflammatory effect in mice with induced UC, which is mediated, at least in part, by reducing the levels of COX-2, IL-6, and MCP-1.

4. Discussion

Previously, we showed that ethanol extract of *B. nivea* exerts an anti-inflammatory effect through inhibiting p38 and JNK in LPS-induced RAW264.7 macrophage cells [8]. As an extension of that study, we examined the anti-inflammatory efficacy of *B. nivea* in an experimental colitis mouse model. We found that *B. nivea* improves DSS-induced experimental colitis. EBN was able to reduce colon shortening, weight loss, DAI, and histopathological scores associated with DSS treatment (Figures 1 and 2). Furthermore, COX-2 levels were reduced by administering EBN to DSS-treated mice (Figure 3).

B. nivea has traditionally been used in Asian countries to prevent diseases and reduce inflammation [7, 8]. Numerous studies have reported that *B. nivea* is a bioactive agent, exhibiting antifungal and antioxidant properties [7, 8, 10]. The functional properties of natural agents are thought to be associated with their polyphenol content [11]. Our previous work suggests that plant polyphenols possess beneficial effects on health [12]. *B. nivea* contains several polyphenol compounds including chlorogenic acid, rutin, luteolin-7-glucoside, naringin, hesperidin, and tangeretin [8]. Thus, we speculated that *B. nivea* was able to reduce the inflammatory response and that polyphenol compounds are responsible for this effect. Several medications, including sulfasalazine, have been used to treat patients with IBD including UC [1]. Steroids or nonsteroidal anti-inflammatory drugs have also been used to treat IBD [1]. However, these drugs can cause severe side effects in patients. Thus, investigators have proposed that alternative medicines and natural products that exhibit similar activity to sulfasalazine can be used to treat IBD [13, 14]. For example, plant-derived polyphenols have been reported to improve UC. A bioflavonoid derived from the seeds of *Garcinia kola*, kolaviron, exhibits an anticolitis effect by reducing nitric oxide, myeloperoxidase activity, TNF-α, and IL-1β in DSS-induced colitis mice [13]. These effects of kolaviron were comparable to that of sulfasalazine. The flavonoid hesperidin has also been shown to reduce DAI and IL-6 levels in DSS-induced colitis mice [14]. Sulfasalazine reduced DAI and IL-6 level in DSS-induced colitis mice under the same conditions, suggesting that hesperidin can be used to prevent colitis. Furthermore, cytokines including IL-6 were a critical target of both hesperidin and sulfasalazine treatments [14]. Leucocyte infiltration induces mucosal tissue damage in colons, resulting in an inflammatory response involving cytokines and the chemokine MCP-1 [15]. Anticolitis medications such as sulfasalazine significantly decrease colitis and neutrophil/macrophage infiltration by reducing cytokine and chemokine levels [16, 17]. Polyphenol-rich extra-virgin olive oil has been shown to modulate macrophage activation and decrease cytokine production in UC [18]. In this study, DSS increased the serum levels

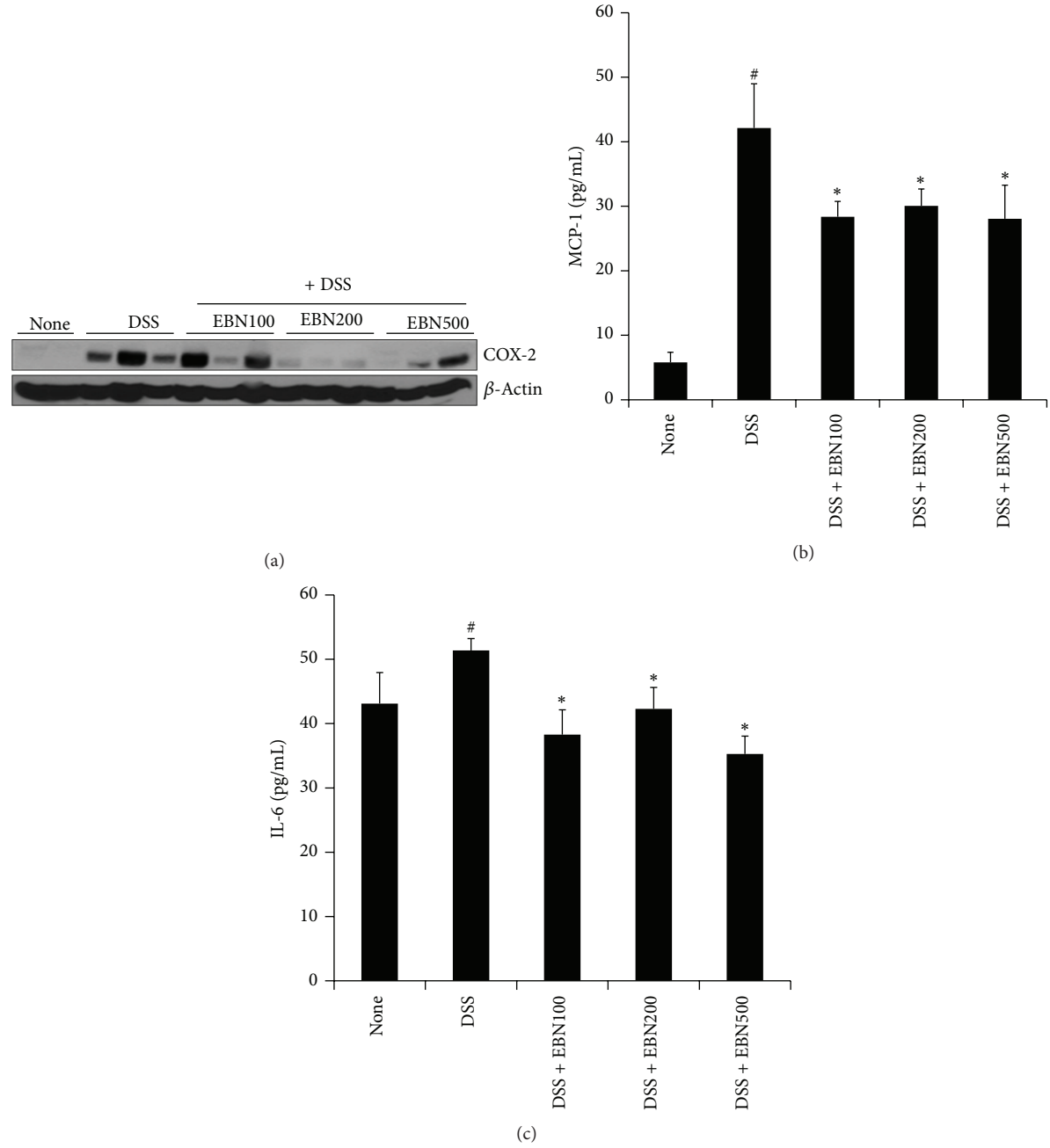

Figure 3: The effect of EBN on IL-6, MCP-1, and COX-2 levels in DSS-induced colitis mice. COX-2 levels were measured in colon tissue lysates by using western blot analysis (a). β-Actin was used as an internal control. Serum levels of IL-6 and MCP-1 were measured using enzyme-linked immunosorbent assays (ELISAs) ((b) and (c)). The levels of IL-6, MCP-1, and COX-2 are shown for the untreated, DSS-treated, DSS + EBN 100, DSS + EBN 200, and DSS + EBN 500 groups. All data are expressed as the mean ± SD. $^{*}P < 0.05$ versus DSS-treated; $^{\#}P < 0.05$ versus untreated.

of IL-6 and MCP-1 relative to control mice (Figures 3(b) and 3(c)). Interestingly, administration of EBN significantly decreased the production of both IL-6 and MCP-1 in the DSS-treated group (Figures 3(b) and 3(c)). These data are consistent with the previous work that suggests sulfasalazine-like natural agents act as inhibitors of cytokine production in UC. Therefore, we hypothesize that EBN is a suppressor of cytokine production and blocks leucocyte infiltration in UC, in a manner similar to sulfasalazine. However, we were unable to elucidate the precise mechanism by which EBN inhibits cytokine production in DSS-induced colitis mice. We also found that administration of EBN reduced the levels of COX-2 that had been increased by DSS treatment (Figure 3(a)). COX-2 is a considerable target for the UC that treatment of DSS induces strong COX-2 expression in colon tissue [19]. Selective COX-2 inhibitors exert a therapeutic effect towards

both TNBS- and DSS-induced colitis by reducing cytokine levels [20]. In this study, we demonstrated that DSS treatment significantly increased the levels of COX-2 in colon tissue (Figure 3(a)), suggesting that COX-2 levels are upregulated in the DSS-induced colitis model. Several studies report that polyphenol-rich extra-virgin olive oil downregulates COX-2 levels [18] and the polyphenol resveratrol decreases COX-2 levels in experimental colitis models [21], suggesting that polyphenols possess anticolitis activity. Here, we demonstrate that EBN, rich in polyphenols such as chlorogenic acid, rutin, luteolin-7-glucoside, naringin, hesperidin, and tangeretin, reduces the levels of COX-2 that were elevated as a result of DSS treatment (Figure 3(a)). Therefore, we hypothesize that polyphenols may be a potential factor for the reduction of colitis by EBN.

5. Conclusion

We demonstrated that an ethanol extract of *B. nivea* exerts an inhibitory effect on colitis pathogenesis, improving colon shortening, body weight, DAI, and histopathological scores in DSS-induced colitis mice. Furthermore, we found that COX-2 levels were reduced by EBN in colon tissues. These findings suggest that *B. nivea* is effective in preventing IBD including UC. Our findings contribute to the understanding of the potential functionality and underlying mechanisms of *B. nivea* bioactivity.

Conflict of Interests

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

Acknowledgments

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Review Article

Plant-Derived Anti-Inflammatory Compounds: Hopes and Disappointments regarding the Translation of Preclinical Knowledge into Clinical Progress

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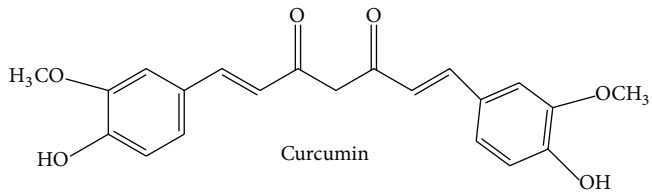
Many diseases have been described to be associated with inflammatory processes. The currently available anti-inflammatory drug therapy is often not successful or causes intolerable side effects. Thus, new anti-inflammatory substances are still urgently needed. Plants were the first source of remedies in the history of mankind. Since their chemical characterization in the 19th century, herbal bioactive compounds have fueled drug development. Also, nowadays, new plant-derived agents continuously enrich our drug arsenal (e.g., vincristine, galantamine, and artemisinin). The number of new, pharmacologically active herbal ingredients, in particular that of anti-inflammatory compounds, rises continuously. The major obstacle in this field is the translation of preclinical knowledge into evidence-based clinical progress. Human trials of good quality are often missing or, when available, are frequently not suitable to really prove a therapeutical value. This minireview will summarize the current situation of 6 very prominent plant-derived anti-inflammatory compounds: curcumin, colchicine, resveratrol, capsaicin, epigallocatechin-3-gallate (EGCG), and quercetin. We will highlight their clinical potential and/or pinpoint an overestimation. Moreover, we will sum up the planned trials in order to provide insights into the inflammatory disorders that are hypothesized to be beneficially influenced by the compound.

1. Introduction

Inflammation is a crucial biological process for maintaining the body's homeostasis. It is indispensible for successfully fighting pathogens and for the repair of damaged tissue. However, inflammatory processes are also involved in the onset and maintenance of many severe disorders, such as rheumatoid arthritis, asthma, chronic inflammatory bowel diseases, type 2 diabetes, neurodegenerative diseases, and cancer [1]. The currently available repertoire of approved anti-inflammatory agents mainly consists of nonsteroidal anti-inflammatory drugs, glucocorticoids, immunosuppressant drugs, and biologicals. Despite this arsenal, therapy is often not effective enough or is hampered by intolerable side effects. Thus, the discovery of new anti-inflammatory compounds is still a great demand on scientists in academia and industry.

Plants were the first source of remedies in human history. In all cultures and through all ages different parts of a huge number of plants were used as drugs against all kinds of ailments. Needless to say, this usage was based on views that are not compatible with nowadays evidence-based medicine. However, different traditional systems, such as European-based plant collections, the Chinese Traditional Medicine, the Kampo system, the Indian Ayurveda, and many more, have evolved by trial and error and have strived toward advancing the appropriate use of plants on the background of their own, specific philosophy of life. One should be aware that in many lesser developed countries traditional medicine is still the only affordable and, thus, accessible way to meet the primary healthcare needs of a large number of patients.

The awareness that one (or more) chemical entity within the plant material is responsible for pharmacological actions and can be isolated for the use as single agent came up in the 19th century in the context of the emerging natural science-based medicine and pharmacy. Prominent plant-derived compounds that were isolated in this period, such as morphine, quinine, colchicine, atropine, pilocarpine, or



Curcumin

Figure 1

theophylline, are still very important in current pharmacotherapy. Due to the developing organic synthesis, plant-derived compounds were among the first lead structures in the history of drug development.

Looking into the list of drugs approved within the last decades demonstrates that plant ingredients are still of importance in drug discovery. Vincristine, vinblastine, and their semisynthetic derivatives, originally isolated from the Madagascar periwinkle (*Catharanthus roseus*), are highly valuable anticancer drugs. Galantamine from the Caucasian snowdrop (*Galanthus caucasicus*), paclitaxel from the Pacific yew (*Taxus brevifolia*), and capsaicin from chili peppers (*Capsicum* species) are further prominent examples of secondary plant metabolites that made it into the clinic. Examples for plant-derived compounds that served as lead structures and/or were chemically modified are salicylic acid (acetylsalicylic acid), morphine (scores of derivatives), camptothecin (topotecan and irinotecan), artemisinin (artemether), and dicoumarol (warfarin).

The number of phytochemical studies describing new substances isolated from plants is huge and rising. Preclinical reports, that is, *in vitro*, cell-based, and animal studies on the action of these substances, are available in an inconceivable quantity. Unfortunately, these studies are often of equivocal quality, especially in the field of anti-inflammatory compounds. Also the review literature is overwhelming and there are many comprehensive publications available that focus on the respective molecular mechanisms [2–7].

Especially for newly isolated compounds, the knowledge is too often based on a very limited number of cell-based assays. From alterations of some prominent mediators of inflammatory processes, most frequently the transcription factor NFκB, the compound is simply judged to be anti-inflammatory without presenting comprehensive *in vivo* data. Animal models are of course indispensible for the analysis of the pharmacological potential of a compound, but these models are used insufficiently and, as commonly known, do not satisfactorily reflect the situation in humans. The major problem is the lack of sound and significant clinical studies. In contrast, prominent and well-known plant-derived compounds, such as curcumin or resveratrol, have extensively been analyzed in clinical trials and have often induced a hype in media (even in scientific ones). However, due to reasons that will be discussed in the respective paragraphs, this knowledge has as yet not led to an approved drug that advances pharmacotherapy.

This minireview will summarize the current situation of 6 selected, prominent, anti-inflammatory compounds that have been tested in humans in recent years: curcumin, colchicine, resveratrol, capsaicin, epigallocatechin-3-gallate (EGCG), and quercetin. We will highlight the potential and/or pinpoint an overestimation of these agents. Moreover, we will sum up the planned registered trials in order to provide insights into the disorders that are hypothesized to be beneficially influenced by the compounds.

2. Plant-Derived Compounds Tested in Clinical Trials

2.1. Curcumin. Curcumin (Figure 1), (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione, also known as diferuloylmethane, is the main ingredient of turmeric (*Curcuma longa*, Zingiberaceae). The Indian spice turmeric has been used for centuries in Ayurvedic medicine against inflammatory disorders. Curcumin was identified in 1910, is yellow in color, and represents a lipophilic polyphenol. It exerts a great variety of actions and is amongst the most frequently investigated natural compounds. Curcumin displays anti-inflammatory, antioxidant, anticancer/proapoptotic, and antibacterial activities due to a plethora of mechanisms. These have recently been reviewed comprehensively [8]. With regard to its anti-inflammatory action, curcumin was reported (i) to inhibit important proinflammatory signaling cascades, such as the NFκB-, MAPK-, COX-, and LOX-pathways [9, 10], (ii) to downregulate the secretion of prominent cytokines, like TNFα, IL-1β, and IL-6 [11], and (iii) to block the expression of cell adhesion molecules (e.g., ICAM-1), which are necessary for the interaction of leukocytes with endothelial cells [12]. Thus, not only in regard to its anti-inflammatory profile, curcumin represents a compound with pleiotropic, multiple modes of action. Fortunately, the compound has been tested in humans. Currently, 77 studies (with known status) dealing with diverse actions of curcumin can be found on http://www.clinicaltrials.gov/. Thereof, 50 studies have been finished and 27 are in their onset (recruiting) phase. In PubMed, almost 100 clinical trials investigating curcumin in an inflammatory context are listed, of which 12 have been published in 2013, 24 in 2012, and 14 in 2011. These facts demonstrate that curcumin is still a hot topic and under intense clinical investigation. The most prominent disorders were rheumatoid arthritis, cancer (e.g., colorectal, pancreatic, breast, prostate, and lung), and inflammatory bowel diseases (ulcerative colitis and Crohn's disease), but there are also studies available, for example, on uveitis, vitiligo, or nephropathies, which reflects the pleiotropic actions of curcumin. A detailed list of these trials can be found in a recent review by Gupta et al. [13].

The recruiting future trials will mainly deal with the action of curcumin on cognitive impairment and—still ongoing—different types of cancer. Also inflammatory conditions will still be investigated. In these trials, curcumin often serves as a dietary supplement or as an adjunct treatment to the standard therapy. The Cochrane Collaboration lists one systematic review (published in 2012) that analyzes the effects of curcumin in ulcerative colitis [12, 14]. The authors conclude that "curcumin may be a safe and effective therapy for maintenance of remission in quiescent ulcerative colitis when given as adjunct therapy. However, further research in form of a large scale methodologically rigorous randomized controlled trial is needed" to really confirm a benefit. This statement perfectly reflects the overall situation. Clinical trials are available; however, they are often too weak and of poor quality to draw a clear conclusion. The major issue is the low number of enrolled patients, which frequently ranges between 10 and 30. It should also be mentioned that curcumin suffers from its very low bioavailability, although considerable progress has been made to overcome this obstacle by technological and chemical approaches [15]. Whether we will see curcumin as an approved (add-on) option for the treatment or prevention of one of the mentioned indications depends on the performance of solid, high-quality, big cohort studies in the future. However, from the wealth of studies, it can at least be concluded that curcumin seems to have a good safety profile; it is well tolerated and nontoxic.

2.2. Colchicine. The tropolone derivative colchicine (Figure 2), N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-6,7-dihydro-5H-benzo[a]heptalen-7-yl]acetamide, is the major alkaloid of the plant *Colchicum autumnale* (Colchicaceae), commonly known as autumn crocus or meadow saffron. Since ancient times, extracts of this plant have been used against gout attacks. Interestingly, the US FDA has only recently (2009) approved colchicine for the treatment of familial Mediterranean fever as well as for the treatment and prevention of acute gout flares. To get this approval, the applying company needed to provide new clinical data and, in return, was given an exclusive marketing agreement, 3 years for the indication gout and 7 years for familial Mediterranean fever (orphan drug status). The mechanisms of action of colchicine are well investigated: the molecular target was identified (tubulin), the binding site was precisely characterized, and the biological consequences of impairing microtubule dynamics were analyzed; comprehensive reviews summarizing these findings are available [16–19]. Noteworthy, colchicine played a crucial role for the initial characterization of microtubules and the tubulin subunits in the 1960s [20, 21]. Despite this huge knowledge and the fact that colchicine is already an approved drug, researchers recently have conducted several clinical studies in order to expand its fields of application. PubMed lists a number of very interesting trials in the field of inflammation-associated pathologies with positive outcome: colchicine was tested as adjunct treatment against acute [22, 23] and recurrent pericarditis [24, 25], for the prevention

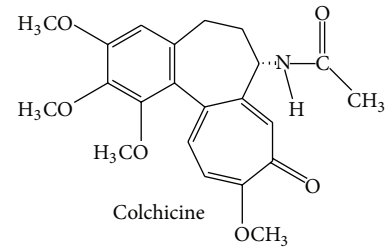


Figure 2

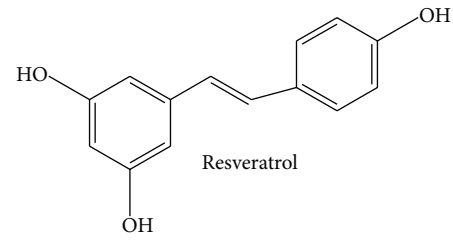


Figure 3

of atrial fibrillation after radiofrequency ablation [26] and for postpericardiotomy syndrome prevention [27]. These large and well-performed studies will surely affect pharmacotherapy guidelines. And the field is still vibrant: 14 clinical trials that are open, that is, in the recruiting phase, are listed on http://www.clinicaltrials.gov/. They intend to investigate the action of colchicine mainly in the areas of cardiology and nephrology, for example, in myocardial infarction, for postpericardiotomy syndrome prevention, or in diabetic nephropathy—all associated with inflammatory processes. Since the number of diseases with an inflammatory component is very large, one might speculate that colchicine will further stay an interesting, not yet fully exploited drug.

2.3. Resveratrol. Resveratrol (Figure 3), 5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol, represents a stilbene derivative and phytoalexin. It can be found in a great number of different plants and dietary products thereof, with peanuts, grapevines, and red wine being the most prominent ones. In parallel to curcumin, resveratrol was found to exert a wealth of pharmacological actions, for example, anti-inflammatory, antioxidant, anticancer/proapoptotic, chemopreventive, and antimicrobial properties. The number of available reviews on these topics is immense [28–37]. Resveratrol is known to have a poor bioavailability, which prompted many groups to work on improvement strategies [38–43]. As a consequence of these interesting *in vitro* and *in vivo* findings, a lot of effort was put into the elucidation of the underlying mechanisms of action. Again, a large number of reviews is available and gives in-depth insights into the mechanisms: resveratrol inhibits the NFκB-, AP-1-, and COX-2-pathway [29, 44, 45] and activates PPAR, eNOS, and SIRT1 [46–49]. However, this gigantic knowledge has as yet not been translated into an approved clinical application. PubMed lists over 40 clinical

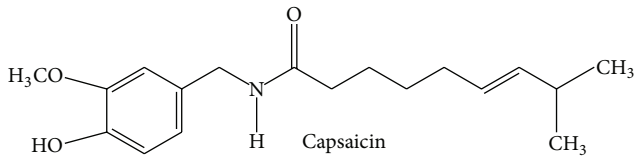


Figure 4

trials on resveratrol in the broad context of inflammation-associated disorders, many of them dealing with diabetes, obesity, and coronary artery disease. These studies often analyze inflammation-related parameters in the plasma (e.g., CRP, TNFα, IL-1β, and IL-6) and in blood cells (e.g., activated kinases or transcription factors) or reported on functional parameters, such as the status of the endothelium [50–55]. Many trials convincingly demonstrate that these parameters are indeed beneficially influenced by resveratrol. However, whether this altered inflammatory status of the patients really results in a clinically relevant improvement of the severity of the diseases or, most importantly, in a reduced occurrence of disease-specific life-threatening events (not to mention mortality) has not been analyzed. According to http://www.clinicaltrials.gov/, 26 clinical trials on resveratrol are planned or are currently recruiting. The main field of interest is type 2 diabetes/metabolic syndrome, followed by polycystic ovary syndrome, nonalcoholic fatty liver disease, and mild cognitive impairment. Research would strongly profit by conducting interventional studies with defined primary outcomes reflecting the stage and/or prevalence of the diseases on a long-term basis.

2.4. Capsaicin. Capsaicin (Figure 4), (E)-N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide, is a very hydrophobic alkaloid produced by chili peppers (*Capsicum* species; Solanaceae) and is responsible for the typical pungency/spiciness of the fruits of the genus *Capsicum*. It has traditionally been used as a topical rubefacient and counterirritant to relieve pain of muscles and joints. A capsaicin 8% cutaneous patch has recently been approved by the authorities in the EU for the use against neuropathic pain in nondiabetic adults and in the US against neuropathic pain associated with postherpetic neuralgia. Interestingly, the research on capsaicin led to the discovery of the transient receptor potential channel vanilloid subfamily member 1 (TRPV1), which is the direct target of capsaicin [56]. TRPV1 is a nonselective cation channel with high preference for Ca^{2+} and is mainly located in nociceptive neurons. It is activated by chemical and physical stimuli, such as heat, low pH, capsaicin, and certain inflammatory mediators [57]. Prolonged activation of TRPV1 by capsaicin is discussed to cause desensitization and, thus, reduced pain sensation [58]. Beyond pain, some few studies also found an anti-inflammatory potential of capsaicin: it can inhibit paw inflammation in arthritic rats [59] and ethanol-induced inflammation of the gastric mucosa in rats [60]. Moreover, capsaicin was reported to inhibit COX-2 activity, iNOS expression, and the NFκB pathway in macrophages in a TRPV1 independent way [61].

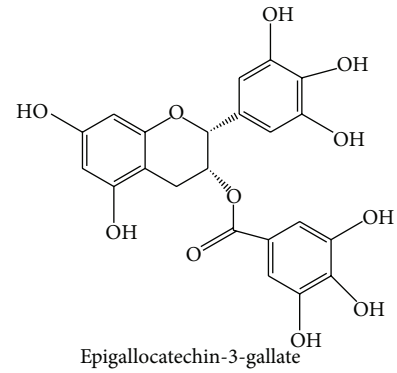


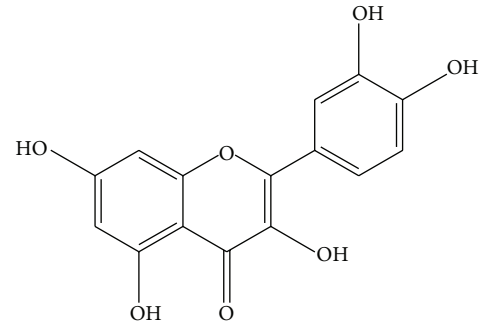
Figure 5

Regarding a clinical influence on inflammatory conditions, capsaicin was evaluated in a recent systematic review by the Cochrane Collaboration: topical capsaicin was reported not to be effective against osteoarthritis [62]. In contrast, one meta-analysis found enough evidence to conclude that capsaicin is effective in the management of osteoarthritis, although the authors pinpointed that there is a paucity of randomized clinical trials [63]. This is in line with a further meta-analysis reporting that capsaicin alleviates osteoarthritic pain [64]. Regarding future therapeutical enhancements, 24 clinical trials are planned or are currently recruiting, as listed on http://www.clinicaltrials.gov/. In most of these studies capsaicin is used as a model substance to induce pain or as a diagnostic tool (provocation test). Trials that investigate the therapeutical potential of capsaicin analyze its potential as preemptive (prior to surgery) analgesic in patients undergoing amputation of a limb and its action on neuropathic pain from critical ischemia (predominantly in hands and feet) and on chronic pain from artificial arteriovenous fistulae (for hemodialysis) in patients with end-stage renal failure. Moreover, capsaicin will be tested against persistent pain after inguinal herniotomy and against the impaired swallow response in stroke patients with oropharyngeal dysphagia. Another study will evaluate the mechanism behind the action of capsaicin against idiopathic rhinitis. No trial deals with anti-inflammatory effects of capsaicin. In summary, in contrast to neuropathic pain, the field of capsaicin and inflammation is not very advanced and will stay on that level in the near future due to a lack of clinical studies.

2.5. Epigallocatechin-3-gallate. Epigallocatechin-3-gallate (Figure 5), [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-3-yl]3,4,5-trihydroxybenzoate, commonly abbreviated as EGCG, is an ingredient of green tea, *Camellia sinensis* (Theaceae). It is the most prominent member of the family of green tea catechins (polyphenols) and accounts for 50–80% of all catechins in a cup of green tea [65]. The number of reports on its biological activity is huge. EGCG was found to exert profound anti-inflammatory, antioxidant, anti-infective, anticancer, antiangiogenetic, and chemopreventive effects [65–69]. Also the knowledge about the cellular and molecular mechanisms

is extremely broad: EGCG promotes cell growth arrest and induces apoptosis by affecting regulatory proteins of the cell cycle and inhibition of NFκB [69–71]. Furthermore, it inhibits growth factor-dependent signaling (e.g., of EGF, VEGF, and IGF-I), the MAPK pathway, proteasome-dependent degradation, and expression of COX-2 [72, 73]. Even molecular targets of EGCG have been identified. It seems to directly interact with and to modulate the character of membrane lipid rafts, which explains the ability to alter signaling processes of growth factor receptors [74–76]. Furthermore, EGCG inhibits telomerase, topoisomerase II, and DNA methyltransferase 1, thereby affecting the functions of chromatin [77–79]. Surprisingly, however, despite the promising preclinical findings and the thorough mechanistic insights, clinical studies in the context of inflammation are largely lacking. One small study analyzed the action of green tea and an extract thereof on biomarkers of inflammation (e.g., adiponectin, CRP, IL-6, IL-1β, sVCAM-1, and sICAM-1) in obese patients with metabolic syndrome. After 8 weeks of treatment, biomarker levels were not changed by green tea [80]. Another trial reported a beneficial impact of topical EGCG treatment on acne vulgaris, which might be at least in part due to anti-inflammatory effects [81]. Interestingly, in 2006, a green tea extract was approved as a prescription drug for the topical treatment of genital and anal warts (condylomata acuminata). This great advancement fueled further research to expand the indications of EGCG. What will be clinically analyzed in the next years? 17 open studies are listed on http://www.clinicaltrials.gov/. EGCG will be tested for its effects on albuminuria in diabetic nephropathy as well as for its action in patients with cardiac amyloid light-chain amyloidosis, with muscular dystrophy of the Duchenne type, with Alzheimer's disease (early stage), with Down syndrome, with fragile X syndrome, with Huntington's disease, and with multiple-system atrophy. Moreover, trials will analyze the potential of EGCG on reactivation of the Epstein-Barr virus in remission patients and on preventing colon polyps in patients at high risk for recurrent colon adenoma. Further studies will examine whether EGCG affects insulin resistance, whether gargling with EGCG prevents influenza infections in teenagers, and whether topical EGCG exerts an anticarcinogenic potential in patients with superficial basal cell carcinoma. Obviously, the trials will not investigate classic inflammatory disorder, although some of the mentioned diseases are associated with inflammatory processes (e.g., Alzheimer's disease or insulin resistance). Nevertheless, it is very likely that EGCG will experience an expansion of its indication in the future.

2.6. Quercetin. Quercetin (Figure 6), 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one, a flavonol, belongs to the class of flavonoids, which is a large family of polyphenols representing very widely spread secondary plant metabolites. Quercetin is found in a great variety of food, such as apples, grapevines, berries, broccoli, red onions, capers, or tea. As with the above discussed compounds, also quercetin exerts a large spectrum of biological effects: anti-inflammatory [82], anti-infectious [83], antioxidant [84],



Quercetin

Figure 6

anticancer/chemopreventive [85, 86], neuroprotective [87], antihypertensive [88, 89], and blood glucose-lowering [90] properties have been reported. Also the mechanisms behind these actions are very broad and have been characterized intensively. Quercetin scavenges reactive oxygen and nitrogen species [84], targets prominent proinflammatory signaling pathways, such as STAT1, NFκB, and MAPK [91, 92], and inhibits infectivity of target cells and replication of many types of viruses [93]. Moreover, phosphodiesterases (PDEs) were suggested to be affected by quercetin [94], as well as topoisomerases I and II [95] and Mcl-1 [86]. Most importantly, quercetin was identified as a broad-spectrum kinase inhibitor [96, 97]. Has all this knowledge been translated into therapy or prevention? Several studies on inflammatory parameters in humans have been performed in the last years: one trial evaluated the effect of quercetin on biomarkers of inflammation depending on the apolipoprotein E genotype of healthy men. Although risk factors of cardiovascular disease were improved, quercetin exerted a slight proinflammatory effect (increased levels of TNFα) [98]. It was reported that quercetin had no action on the levels of the proinflammatory cytokine IL-6 after repeated sprint exercise [99]. In sarcoidosis patients, quercetin reduced markers of inflammation (TNFα and IL-8) [100]. In healthy females, quercetin did not alter blood leukocyte subsets, granulocyte oxidative burst or phagocytosis activity, IL-6, or TNFα plasma levels [101]. Of note, no trial reports on the improvement of clinical parameters of inflammatory diseases (severity and incidence). Almost ten clinical studies are registered on http://www.clinicaltrials.gov/ that are going to use pure quercetin as a pharmacological compound. With respect to inflammatory disorders, quercetin will only be analyzed in two-phase 1-2 trials for its safety and dose-response relationship in chronic obstructive pulmonary disease (COPD). In the field of diabetes, quercetin will be tested in a phase 2 study for an effect on blood glucose and blood vessel function in type 2 diabetes. Quercetin will also be given to obese patients (with or without diabetes type 2) to test its action on glucose absorption (glucose tolerance test). Regarding cancer, it will be tested whether quercetin modulates levels of prostate-specific antigen (PSA) and whether it can prevent prostate cancer. Moreover, in a pilot study, quercetin will be used in children suffering

from Fanconi anemia (safety and pharmacokinetics). Thus, quercetin will undergo very interesting studies that might lead to a profound advancement of knowledge about its clinical efficacy. However, inflammatory diseases are not the main topic of current research.

3. Conclusion

State-of-the-art clinical intervention studies, that is, randomized double-blind placebo-controlled trials, are the gold standard for testing whether a substance has a therapeutical or preventive potential. The field of plant-derived compounds often experiences "hope and hype" phases. Opinions and assumptions about an action in humans are rapidly disseminated once promising *in vitro* and *in vivo* findings have been generated. Unfortunately, the step towards a sound and significant clinical trial is difficult, very laborious, long-ranging, and—most importantly—extremely expensive, which means that it is impossible in many cases. Thus, this research area often suffers from either inadequately performed or a low number of studies. In this minireview, we did try not only to pinpoint deficiencies, but also to highlight positive developments that will hopefully lead to an advancement of prevention or therapy of diseases.

Abbreviations

AP-1: Activator protein-1
COPD: Chronic obstructive pulmonary disease
COX: Cyclooxygenase
CRP: C-reactive protein
EGCG: Epigallocatechin-3-gallate
EGF: Epidermal growth factor
eNOS: Endothelial nitric oxide synthase
IGF: Insulin-like growth factor
iNOS: Inducible nitric oxide synthase
ICAM: Intercellular adhesion molecule
IL: Interleukin
LOX: Lipoxygenase
MAPK: Mitogen-activated protein kinase
NFκB: Nuclear factor κB
PDE: Phosphodiesterase
PPAR: Peroxisome proliferator-activated receptor
PSA: Prostate-specific antigen
SIRT: Sirtuin
sICAM: Soluble intercellular adhesion molecule
STAT: Signal transducer and activator of transcription
sVCAM: Soluble vascular cell adhesion molecule
TNF: Tumor necrosis factor
TRPV1: Transient receptor potential channel vanilloid subfamily member 1
VEGF: Vascular endothelial growth factor.

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

Impact of Wines and Wine Constituents on Cyclooxygenase-1, Cyclooxygenase-2, and 5-Lipoxygenase Catalytic Activity

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Cyclooxygenases and lipoxygenases are proinflammatory enzymes; the former affects platelet aggregation, vasoconstriction, vasodilatation and later the development of atherosclerosis. Red wines from Georgia and central and western Europe inhibited cyclooxygenase-1 (COX-1) activity in the range of 63–94%, cyclooxygenase-2 (COX-2) activity in the range of 20–44% (tested at a concentration of 5 mL/L), and 5-lipoxygenase (5-LOX) activity in the range of 72–84% (at a concentration of 18.87 mL/L). White wines inhibited 5-LOX in the range of 41–68% at a concentration of 18.87 mL/L and did not inhibit COX-1 and COX-2. Piceatannol (IC_{50} = 0.76 μM) was identified as a strong inhibitor of 5-LOX followed by luteolin (IC_{50} = 2.25 μM), quercetin (IC_{50} = 3.29 μM), and myricetin (IC_{50} = 4.02 μM). *trans*-Resveratrol was identified as an inhibitor of COX-1 (IC_{50} = 2.27 μM) and COX-2 (IC_{50} = 3.40 μM). Red wine as a complex mixture is a powerful inhibitor of COX-1, COX-2, and 5-LOX, the enzymes involved in eicosanoid biosynthetic pathway.

1. Introduction

Moderate consumption of wine is associated with reduced incidence of coronary heart diseases [1, 2]. Alcohol present in wine decreases platelet aggregation, resulting in reduced adherence to the endothelial surface of the arteries, blood coagulation, and thrombus formation [3, 4]. Besides alcohol, platelet aggregation could be decreased by the inhibition of cyclooxygenase-1 (COX-1) activity by phenolic compounds present in wine, such as resveratrol [5]. COX-1 catalyzes biosyntheses of thromboxanes, eicosanoids propagating platelet aggregation and vasoconstriction [6, 7]. Therefore, the inhibition of COX-1 (e.g., by aspirin which is a COX-1 selective inhibitor) is proposed for the prevention of cardiovascular diseases [8, 9]. On the other hand, the inhibition of the second cyclooxygenase isoform (COX-2) results in reduced production of prostacyclin which is a vasodilitator and antiaggregatory prostanoid. Therefore, the selective COX-2 inhibitors (coxibs) used as anti-inflammatory drugs increase the risk of heart incidents [10]. The second important biosynthetic pathway leading to the production of eicosanoids is mediated by 5-lipoxygenase (5-LOX). The final product of the 5-LOX pathway, leukotriene B_4 (LTB_4), is a mediator of several inflammatory diseases including atherosclerosis [11]. There are also wine constituents such as quercetin which are able to inhibit 5-LOX activity [12]. In contrast to the reports on positive effects of phenolic compounds, two recent studies describe strong activation of COX-1 and COX-2 catalytic activity by myricetin and quercetin indicating that the wine constituents could also increase production of eicosanoids [13, 14]. In the light of

the mentioned reports, we decided to test wine as a complex mixture of various compounds for its inhibitory potential towards COX-1, COX-2, and 5-LOX. We compared the activity of Georgian red and white wines with wines produced in central and western Europe. In addition, we evaluated the inhibitory activity of 33 phenolic compounds commonly occurring in wine with the aim to determine the contribution of each compound to the overall effect of wine. Finally, *in silico* docking experiments were used to propose a binding mode of the most active compounds.

2. Materials and Methods

2.1. Standards and Chemicals. The tested compounds were purchased from Sigma-Aldrich, Czech Republic (anisic acid, apigenin, caffeic acid, catechin, cinnamic acid, coumaric acid, cyanidin-chlorid, delphinidin-chlorid, 3,4-dihydroxybenzoic acid, epicatechin, ferulic acid, gallic acid, kaempferol, luteolin, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, piceatannol, quercetin-dihydro, resveratrol, salicylic acid, syringic acid, tyrosol, luteolin-7-glucosid, myricetin, naringenin, sinapinic acid, and vanillic acid); HWI Analytik, Germany (chlorogenic acid); Roth, Germany (rutin); and Polyphenols Laboratories AS, Norway (delphinidin 3-O-β-glucopyranoside, malvinidin 3-O-β-glucopyranoside, peonidin 3-O-β-glucopyranoside, and petunidin 3-O-β-glucopyranoside).

Eicosatetraynoic acid (ETYA), ethanol (EtOH), calciumionophor A23187, dimethylsulfoxide (DMSO), arachidonic acid (AA), indomethacin, trypan blue, gentian violet, porcine hematin, L-epinephrine, Na_2EDTA, formic acid, COX-1 from ram seminal vesicles, and human recombinant COX-2 were purchased from Sigma-Aldrich (Czech Republic). Dextran T-500 was purchased from Roth (Germany). Ammonium chloride (NH_4Cl), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl), and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Lachner s.r.o. (Czech Republic) and zileuton was donated by Farmak a.s. (Czech Republic). Potassium chloride (KCl) and sodium hydroxide (NaOH) were purchased from Lachema a.s. (Czech Republic). Tris was purchased from Bio-Rad (Czech Republic). Calcium chloride ($CaCl_2 \cdot 2H_2O$) and acetic acid (CH_3COOH) were obtained from Penta (Czech Republic).

2.2. Wine Samples. Samples of commercial wines from different regions of Georgia, Czech Republic, France, Italy, and Austria were provided by local producers or purchased from supermarkets or wine stores. A total of 26 red wines of the varieties Pinot Noir ($n = 5$), Cabernet Sauvignon ($n = 7$), Cabernet Moravia ($n = 2$), Seperavi ($n = 9$), cuvée of Saperavi and Saperavi Budeshuriseburi ($n = 2$), and Alexandrouli ($n = 1$) and 13 white wine samples of the varieties Chardonnay ($n = 6$), Sauvignon Blanc ($n = 3$), Rkatsiteli ($n = 2$), and cuvée of Rkatsiteli and other local varieties ($n = 2$) were assayed. Detailed information about the tested wines is included in Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/178931). After a preliminary screening, the red wines were diluted in ratio 1 : 9 in water to reach the final concentration of 5 mL/L and white wines were tested undiluted resulting in a concentration of 50 mL/L for the COX-1 and COX-2 assays. Undiluted red and white wine samples were used in the 5-LOX assay resulting in a final concentration of 18.87 mL/L.

2.3. COX-1 and COX-2 Assays. The assay was performed according to the procedure previously described by Reininger and Bauer [15] with COX-1 from ram seminal vesicles and human recombinant COX-2. COX-1 (1 unit/reaction) or COX-2 (0.5 unit/reaction) was added to 180 μL of incubation mixture that consisted of 100 mM tris buffer (pH 8.0), 5 μM porcine hematin, 18 mM L-epinephrine, and 50 μM Na_2EDTA. The wine sample, tested compound diluted in DMSO, 12% ethanol (in case of blanks for the wine samples), or pure DMSO (in case of blanks for purified constituents) was added (10 μL) and the mixture was preincubated for 5 min at room temperature. The addition of 5 μL of 10 μM AA started the reaction. After 20 minutes of incubation at 37°C, the reaction was stopped by 10 μL of 10% formic acid. All samples were diluted 1 : 15 in ELISA buffer and the concentration of (prostaglandin E_2) PGE_2 produced by the reaction was determined by a PGE_2 ELISA kit (Enzo Life Sciences, US) according to the manufacturer's instructions. Absorbance relative to PGE_2 concentration was measured with a microplate reader Tecan Infinite M200 (Tecan Group, Switzerland) at 405 nm. The results were expressed as percentage inhibition of PGE_2 formation against untreated samples (blanks).

2.4. 5-LOX Assay. The assay was perfomed in a slightly modified version of the standard method described previously [16]. Buffy coat (50 mL) obtained from healthy donors was sedimented in 20 mL of dextran solution (6% dextran T-500, 1% NaCl) at 4°C. After one hour, the supernatant was collected and centrifuged at 1600 rpm at 4°C for 10 min and then the supernatant was discarded. The obtained pellet was washed with phosphate buffered saline (PBS, 0.02% KCl, 0.024% KH2PO4, 0.8% NaCl, 0.288% $Na_2HPO_4 \cdot 12H_2O$, pH 7.4) and again centrifuged. The hereby obtained pellet was lysed (0.17% NH_4Cl, 0.2% Tris, pH 7.2) for 5 min at room temperature and then centrifuged at 1400 rpm at 4°C for 5 min. The pellet was washed by PBS again and centrifuged at 1400 rpm at 4°C for 15 min. Finally, the pellet was dissolved in 3 mL of PBS and the cells were tested for the viability. The cells were diluted to the final concentration of 4500 cell/μL.

The incubation mixture consisted of 225 μL of the cell suspension, 10 μL of 2 mM $CaCl_2$, 10 μL of 10 μM ETYA, 5 μL of tested sample (wine, compound dissolved in DMSO, 12% ethanol or pure DMSO in case of blanks), 10 μL of 21 μM calcium ionophor A23187, and 5 μL of 120 μM AA. The reaction was stopped after 10 min incubation at 37°C with 20 μL of 10% formic acid. Samples were diluted 40 times in ELISA buffer and the concentration of LTB_4 was measured using a commercial LTB_4 ELISA kit (Enzo Life Sciences, US) according to the manufacturer's instructions. Absorbance relative to LTB_4 concentration was measured at 405 nm using a Tecan Infinite M200. The results were

expressed as percentage inhibition of LTB_4 formation against untreated samples (blanks).

2.5. Docking. During the docking simulation, 3D conformers of the molecules were placed within the binding pocket of 5-LOX, generating a set of energetically favorable poses. These poses were then ranked according to a score that the docking program assigns to each pose, estimating the binding free energy. The best-ranked pose of each molecule was then further optimized and a 3D representation of its interaction pattern was calculated to analyze the structure-activity relationship.

The possible direct interactions of the active compounds with 5-LOX were simulated, and 3D geometries of the compounds were calculated with Omega 2.2.1. [17]. The docking simulation was performed with the software package GOLD 5.1 (GOLD, UK) using the X-ray crystal structure of 5-LOX from the Protein Data Bank (PDB, code: 3o8y [18]). The A-chain binding site was chosen for the docking. The structure of 5-LOX does not contain a cocrystallized ligand, so the binding site was defined in a 6 Å radius around the catalytic iron (Fe2_1_A). Water molecules inside the binding pocket were set on toggle and spin, which means that the program can either use them as binding partners in the binding site or disregard them if they provoke steric hindrance of ligand binding.

Scoring was performed with the GoldScore scoring function. For structure-activity analysis, the best-ranked resulting docking pose of each molecule was energetically minimized within LigandScout using the Merck Molecular Force Field 94 force field. 3D protein-ligand interaction patterns were generated in LigandScout 3.1. [19] using default settings.

2.6. Statistical Analysis. The COX-1, COX-2, and 5-LOX tests were performed in three independent experiments with two replicates. At least three concentrations were used for the calculation of IC_{50} values of the wine compounds. The inhibition of enzyme activity by wine samples is presented as mean values. IC_{50} values are presented as mean values ± standard error (standard deviation, SD) of the mean.

3. Results and Discussion

3.1. Inhibition of COX-1 and COX-2 by Wine. Red wines tested at the concentration of 5 mL/L showed considerable potential to inhibit COX-1 as well as COX-2 with the efficiencies presented in Table 1. On the other hand, white wines tested at a 10 times higher concentration (50 mL/L) were practically inactive (Table 2). The exceptions were two Georgian samples, Rkatsiteli (sample no. 37), and cuveé of Rkatsiteli + Mtsvane + Kakhuri + Khikhvi + Kisi (sample no. 38), reducing COX-1 and COX-2 activity around 95% and 65%, respectively (Table 2). Results expressed in Tables 1 and 2 also demonstrate that red wines preferentially inhibited COX-1 rather than COX-2 with ratios ranging from 2.1 to 3.7. COX-1 selectivity ratio for aspirin varies between different authors from 1.7 to 42 [20, 21] in cell-free assays. However, the ratios recorded for wine fall within the mentioned range. These results support the hypothesis that wine may act, similar to aspirin, via inhibition of COX-1, in decreased risk of thrombosis. The incidence of cardiovascular diseases (CVD) is also directly influenced by alcohol consumption. While low quantities of ethanol (20–30 g per day) decrease CVD incidence, the overconsumption results in an increased risk [2, 22, 23]. Therefore, the cardioprotective effect of red wine (in moderate doses) could also be explained by the effect of ethanol in combination with preferential inhibition of COX-1 activity.

Comparison of different red wine varieties showed only minor differences. Larger variation in inhibitory activity was observed among the individual samples. For example, semisweet Saperavi (sample no. 19) reduced COX-1 activity only by 38% in comparison to the average 84% of all Saperavi samples. High COX-1 and COX-2 inhibitory activity of two Georgian white wine samples (no. 37 and 38) could be explained by different (Kakhetian) technology used during fermentation (six months fermentation with pomace). It is known that the presence of grape skins, bunch stems, and seeds results in a higher content of phenolic compounds in wine [24].

These results indicate that the processing method influenced inhibitory activity more than the variety of wine or its geographical origin.

3.2. Inhibition of 5-LOX by Wine. Red and white wines were tested for inhibition of 5-LOX at a concentration of 18.87 mL/L (5 μL of undiluted wine in 265 μL of reaction mixture). As with COX inhibition, red wines (Table 1) were stronger inhibitors of 5-LOX than white wines (Table 2), although the difference between red and white was not as pronounced. Red wines which were weak inhibitors of cyclooxygenases (e.g., Cabernet Moravia) were strong 5-LOX inhibitors and vice versa the strong COX inhibitors (cuvée of Saperavi + Saperavi Budeshuriseburi wine) were weak 5-LOX inhibitors. These results indicate that inhibition of COX-1 and COX-2 is influenced by different compounds than the inhibition of 5-LOX or that the same compounds have different effects in on the respective enzymes.

3.3. Inhibition of COX-1 and COX-2 by Wine Constituents. Thirty-three phenolic compounds (phenolic acids, flavonoids, and stilbenes) were investigated for COX-1 and COX-2 inhibitory activity to explain their impact on the overall effect of wines. Only *trans*-resveratrol strongly inhibited COX-1 and COX-2 with respective IC_{50} values 2.27 and 3.40 μM (Table 3). Weak activity was recorded for quercetin (IC_{50} = 43.82 μM) and kaempferol (IC_{50} ~ 60 μM) in case of COX-1.

The final dilution of wine samples (200 times) in the COX assays resulted in estimated concentration of *trans*-resveratrol around 0.06 μM. This concentration is calculated from the value 2.7 mg/L (11.8 μM) which is the mean value of bibliographic data for content of resveratrol in red wine [25]. Evidently, even at a 10 times higher concentration of *trans*-resveratrol in wine sample could not explain the activity considering the IC_{50} values of *trans*-resveratrol. The activity of

Table 1: Inhibition of COX-1, COX-2 (concentration of wine: 5 mL/L), and 5-LOX (concentration of wine: 18.87 mL/L) enzymatic activity by red wines.

Number	Variety and origin	Inhibition against blank (%)		
		COX-1	COX-2	5-LOX
1	Pinot Noir, Czech Rep.	46.13	33.25	79.96
2	Pinot Noir, Czech Rep.	90.25	29.23	77.61
3	Pinot Noir, Austria	70.05	29.20	82.74
4	Pinot noir, France	94.04	52.23	85.01
5	Pinot noir, France	85.57	22.78	84.21
6	Cabernet Sauvignon, Italy	73.52	12.21	81.92
7	Cabernet Sauvignon, Italy	87.20	23.81	83.81
8	Cabernet Sauvignon, France	92.16	37.60	80.96
9	Cabernet Sauvignon, France	92.98	43.14	82.38
10	Cabernet Sauvignon, Czech Rep.	81.42	42.14	77.59
11	Cabernet Sauvignon, Czech Rep.	48.95	34.95	71.36
12	Cabernet Sauvignon, Georgia	77.57	13.68	83.86
13	Cabernet Moravia, Czech Rep.	52.67	13.43	82.56
14	Cabernet Moravia, Czech Rep.	73.68	26.91	85.39
15	Saperavi, Georgia	90.98	25.33	84.86
16	Saperavi, Georgia	90.74	43.73	84.33
17	Saperavi, Georgia	90.31	53.24	80.65
18	Saperavi, Georgia	82.30	35.23	88.29
19	Saperavi, Georgia	38.41	−7.23	62.24
20	Saperavi, Georgia	84.09	25.42	78.68
21	Saperavi, Georgia	95.48	52.95	73.08
22	Saperavi, Georgia	95.13	63.86	80.15
23	Saperavi, Georgia	89.07	28.99	77.79
24	Saperavi + Saperavi Budeshuriseburi, Georgia	92.65	40.27	71.65
25	Saperavi + Saperavi Budeshuriseburi, Georgia	94.35	45.03	72.89
26	Alexandrouli, Georgia	82.27	22.28	79.02

Data is presented as the mean value.

Table 2: Inhibition of COX-1, COX-2 (concentration of wine: 50 mL/L), and 5-LOX (concentration of wine: 18.87 mL/L) enzymatic activity by white wines.

Number	Variety and origin	Inhibition against blank (%)		
		COX-1	COX-2	5-LOX
27	Chardonnay, Czech Rep.	10.91	11.94	41.74
28	Chardonnay, Czech Rep.	9.22	−7.05	47.66
29	Chardonnay, Italy	−5.48	−3.11	47.52
30	Chardonnay, Italy	3.62	4.59	47.44
31	Chardonnay, France	11.26	8.82	51.30
32	Chardonnay, France	17.24	10.95	57.99
33	Sauvignon Blanc, Italy	7.42	−21.85	51.25
34	Sauvignon Blanc, France	19.09	12.43	41.42
35	Sauvignon Blanc, Czech Rep.	15.26	9.82	32.42
36	Rkatsiteli, Georgia	14.37	−10.97	59.24
37	Rkatsiteli, Georgia	94.50	65.61	76.05
38	Rkatsiteli + Mtsvane Kakhuri + Khikhvi + Kisi, Georgia	95.97	63.71	71.93
39	Rkatsiteli + Mtsvane Kakhuri, Georgia	10.32	5.32	60.38

Data is presented as the mean value.

Table 3: IC_{50} values of wine constituents and reference inhibitors for COX-1, COX-2, and 5-LOX.

Compound	IC_{50} ± SD (μM)		Ratio	IC_{50} ± SD (μM)
	COX-1	COX-2	COX-1/COX-2	5-LOX
Resveratrol	2.27 ± 1.17	3.40 ± 0.50	0.67	—
Piceatannol	—*	—		0.76 ± 0.35
Luteolin	—	—		2.25 ± 1.75
Quercetin	—	—		3.29 ± 2.25
Myricetin	—	—		4.02 ± 2.37
Kaempferol	43.82 ± 18.81	—		—
Ibuprofen	13.14 ± 3.84	8.77 ± 2.55	1.49	nt#
Indomethacin	1.61 ± 0.72	10.12 ± 5.66	0.15	nt
Zileuton	nt	nt		4.71 ± 2.83

Data is presented as the mean value ± SD. *IC_{50} > 50 μM concentration; #not tested.

kaempferol and quercetin is negligible and no other inhibitors were identified in our screening. In accordance with the data from the literature, we propose some other compounds which could contribute to the overall effect of wine. We propose that the effect could be caused by (−)-catechin (in our study (+)-catechin was inactive at 50 μM concentration) and (+)-ε-viniferin as Zhang et al. isolated these compounds from grape skins and claimed their COX-1 and COX-2 inhibitory activity [26]. However, they tested these compounds at high concentration 100 μg/mL giving only blurred information about their real potential. Other candidates which could contribute to the activity of wine are proanthocyanidins. Garbacki et al. recorded significant inhibition of both COX forms by a gallocatechin dimer, gallocatechin-epigallocatechin dimer, and gallocatechin trimer at the 10–100 μM concentrations [27]. Since the total proanthocyanidins content in red wine ranges from 250 to 2300 mg/L [28, 29], it seems that these compounds could play a more substantial role in the overall COX.

3.4. Inhibition of 5-LOX by Wine Constituents. Piceatannol, luteolin, quercetin, and myricetin inhibited 5-LOX with better efficiency than the reference inhibitor zileuton (IC_{50} values are stated in Table 3). However, based on the concentrations occurring in the red wines (piceatannol = 5.8 mg/L; myricetin and quercetin = 8.3 mg/L; luteolin = 1.0 mg/L; mean values of bibliographic data adopted from [25, 30]), IC_{50} values and dilution of wine samples in the 5-LOX assay (53 times) only piceatannol (estimated concentration in the assay mixture = 0.44 μM; IC_{50} = 0.76 μM) can contribute to the overall activity of red wines. The role of quercetin (estimated concentration = 0.52 μM; IC_{50} = 3.29 μM), myricetin (estimated concentration 0.34 μM; IC_{50} = 4.02 μM), and luteolin (estimated concentration 0.07 μM; IC_{50} = 2.25 μM) in the overall activity of wines seems negligible due to their low concentration in the assay mixtures. White wines are generally poor in phenolic compounds. Piceatannol, quercetin, myricetin, and luteolin are present in very low concentrations or beneath detection limits in white wines [31, 32]. Although Leifert and Abeywardena recorded the inhibition of 5-LOX (in enzymatic assay) by grape seed extract (IC_{50} = 13 μg/mL) and commercial perpetration "red wine polyphenolic compounds" (IC_{50} = 35 μg/mL), active constituents responsible for its activity were not identified or suggested in their study [33]. However, as in the case of COX enzymes, galloylated proanthocyanidins were able to inhibit 5-LOX activity with IC_{50} ranging from 6.6 to 18.7 μM [34]. This hypothesis works for red wines, but in white wines the proanthocyanidins concentrations are almost 100 times lower [35]. Therefore, the compounds responsible for overall 5-LOX inhibitory activity especially of white wines remain unknown.

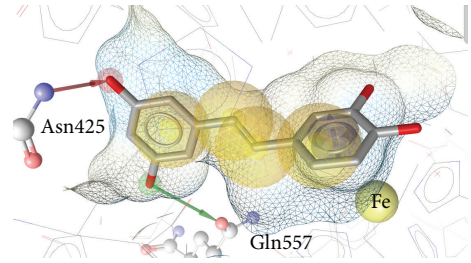

Figure 1: Docking pose of piceatannol in 5-LOX. Yellow spheres signify hydrophobic interactions with the binding pocket. The blue circle marks an aromatic interaction with the binding pocket. The green arrow signifies a hydrogen bond donor interaction with Gln557. The red arrow signifies a hydrogen bond acceptor interaction with Asn425.

3.5. Docking Studies. To further elucidate the mode of inhibition of the most active compounds, they were docked into the crystal structure of 5-LOX. Piceatannol, the most potent 5-LOX inhibitor, showed several interactions with the binding pocket, most notably hydrogen bonds with Asn425 and Gln557 (Figure 1).

Luteolin (Figure 2(a)), quercetin (Figure 2(b)), and myricetin (Figure 2(c)) all displayed a set of very similar interaction patterns. All three compounds coordinated to the catalytic iron and formed stabilizing hydrogen bonds with His367 and Thr364. Quercetin and myricetin also formed hydrogen bonds with Asn407. If a hydroxyl group on the pyrane ring (quercetin and myricetin) is present, a hydrogen bond with Gln363 is formed.

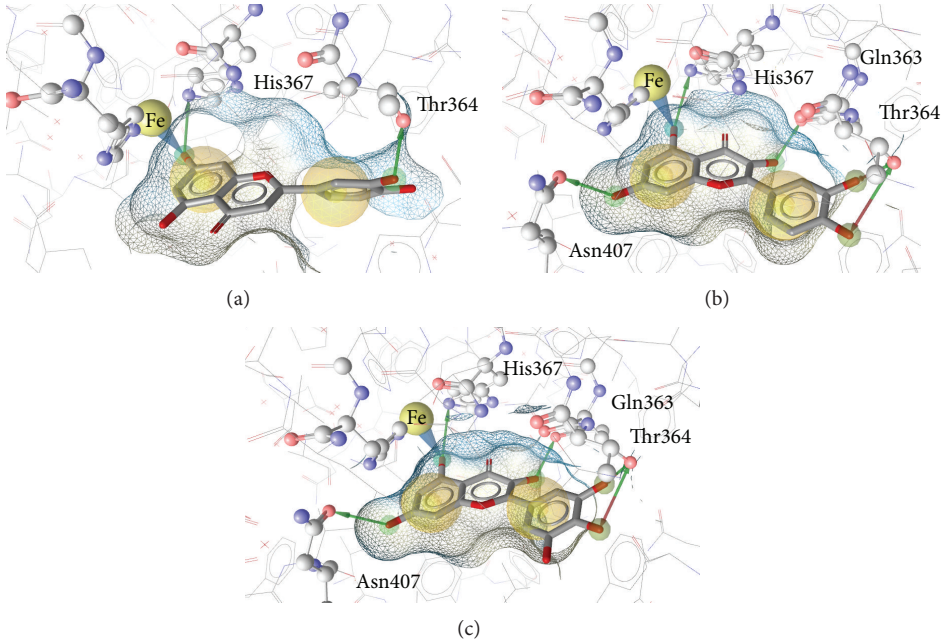

FIGURE 2: Docking poses of luteolin (a), quercetin (b), and myricetin (c). The blue cone marks a metal coordination feature formed with the iron by all three compounds. Additionally, all form hydrogen bonds with His367 and Thr364. Quercetin and myricetin also form H-bonds with Asn407 and Gln363.

In case of COX, we recorded significant activity only for *trans*-resveratrol. Its binding pattern was already described [36, 37]. Here, it should be mentioned that Murias et al. recorded for piceatannol IC_{50} = 4.713 μM and 0.0113 μM for COX-1 and COX-2, respectively, resulting in a COX-2 selectivity index of 417 [5]. In contrast, no inhibition of COX-1 or COX-2 was recorded in our assays. Our results are in concordance with Lee et al. who recorded no inhibition of both COX forms by piceatannol [38] and Gerhäuser et al. who tested COX-1 inhibition with IC_{50} = 81.4 μM [39]. It is difficult to explain why similar studies using enzymatic assays produced so different results. Nevertheless, it should be kept in mind that also piceatannol could be a potential COX inhibitor present in wine. A more detailed study, focusing on piceatannol should reveal the COX inhibitory potency of this interesting compound.

4. Conclusions

Red wines were potent inhibitors of all three tested enzymes with efficacy decreasing from COX-1 through COX-2 to 5-LOX. The evidence that red wine is a better inhibitor of COX-1 than COX-2 could contribute to its cardioprotective effect. White wines were weaker inhibitors of 5-LOX than red wines and did not inhibit COXs. The two exceptions were Georgian samples fermented with pomace (skins, stems, and seeds) by a traditional Kakhetian method. The processing method influenced inhibitory activity more than the variety of wine or its geographical origin. *Trans*-resveratrol proved to be a significant inhibitor of both COX-1 and COX-2, but the activity of this compound alone could not be responsible for overall inhibitory activity of red wines. Similarly, although piceatannol, luteolin, quercetin, and myricetin were potent inhibitors of 5-LOX, considering ratio between their IC_{50} values and their concentration in wine only piceatannol could substantially contribute to the overall activity of red wines. Since the compounds identified in our study could not fully explain the overall activities of wine, we hypothesize, based on the literature data [27, 34], that proanthocyanidins in wine could also contribute to its overall potential. However, further studies are needed for the identification of all COX-1, COX-2, and 5-LOX inhibitors contained in the wine.

Conflict of Interests

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

Acknowledgments

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Research Article

Protective Effect of *Laminaria japonica* with Probiotics on Murine Colitis

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Inflammatory bowel disease (IBD) is a chronically relapsing inflammatory disorder of the gastrointestinal tract. Most IBD treatments are unsatisfactory; therefore, various dietary supplements have emerged as promising interventions. *Laminaria japonica* (LJ) is an edible seaweed used to regulate digestive symptoms. Probiotics have been reported to improve digestive problems and their simultaneous administration with seaweeds has been shown to produce synergistic therapeutic effects. Here, we investigated the effect of LJ combination with probiotics on dextran sodium sulfate-induced colitis model in mice. Aqueous LJ extracts (LJE) at doses from 100 to 300 mg/kg and probiotics at a dose of 300 mg/kg were orally administered for 7 days. Body weight, colon length, histological score, macroscopic damage, and the levels of cytokines IFN-γ, IL-1β, IL-6, IL-10, IL-12 (P40), IL-12 (P70), IL-17, and TNF-α were assessed. LJE alone caused a significant improvement of colitis signs such as colon length, histological score, and IL-1β and IL-6 production. LJE and probiotics demonstrated a synergistic effect by the histological score and levels of IL-1β, IL-6, and IL-12 (P40) but not IFN-γ, IL-10, and IL-12 (P70). In conclusion, LJE was effective in inducing protection against colitis in mice and acted synergistically with probiotics.

1. Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of gastrointestinal tract with two major types including ulcerative colitis (UC) and Crohn's disease (CD). IBD is considered an autoimmune disease and the pathogenesis depends on complex interactions between genetic and environmental factors and innate and adaptive immune mechanisms; the exact cause of IBD is still unknown [1]. Current IBD treatments include aminosalicylates, corticosteroids, inhibitors of tumor necrosis factor alpha (TNF-α), antibiotics, and immunosuppressants [2]. However, these agents have poor tolerability and insufficient therapeutic efficacy; therefore, the need for alternative therapeutic approaches is increasing [3, 4].

Seaweed *Laminaria japonica* (LJ) has been used as a herbal medicine for the treatment of gastrointestinal problems such as vomiting and hemorrhoids in Korea [5]. LJ contains various beneficial ingredients, including alginate and fucoidans known for their dietary benefits and preventive effects on constipation [6] and inflammation [7]. The effects of seaweeds on IBD have been previously shown with *Sargassum pallidum* [8] and *Samiunkyuntang* (a herbal medicine composed of seaweeds *Sargassum pallidum* and *Zostera marina*) [9]. However, the effect of LJ on colitis has not been investigated.

Recent research on new functional food composed of LJ and LAB has shown that the combination exhibits high antioxidant activity and protective effects against liver damage, obesity, hypertension, stress, and insomnia [10, 11].

The lactic acid bacteria (LAB), usually found in decomposing plants and dairy products, comprise a group of Gram-positive, acid-tolerant rods or cocci that exhibit common metabolic and physiological characteristics. Many experimental reports have demonstrated that LAB can improve functions of indigenous microflora and have positive effects on IBD [12–14]. The simultaneous administration of herbal medicines and probiotics containing LAB as a main component has become a popular treatment for many abdominal symptoms in Korea [15]. We have previously shown that the combination of herbal extracts and probiotics may produce a positive synergetic effect in treatment of gastrointestinal diseases [16]. Duolac7S (DUO) is a probiotic mixture containing seven LAB species including *Bifidobacterium*, *Lactobacillus*, and *Streptococcus*. They are easily available, do not require a prescription, and are extensively used to relieve abdominal symptoms [17] by restoring the ecological balance in intestinal microflora [18]. However, in IBD the effect of DUO and its combination with LJ has not been investigated. Here, we applied LJ in a dextran sodium sulfate-(DSS-) induced colitis model in mice and assessed body weight, colon length, and histological damage. Mechanistic studies were conducted by profiling cytokine expression by using the biometric multiplex signaling method. Cytokines including IL-6 and TNF-α, which play a crucial role in acute DSS-induced colitis model [19], were analyzed. We also treated mice with a combination of LJ and DUO in order to investigate a possible synergetic effect on colitis.

2. Materials and Methods

2.1. Animals. Balb/c mice (21-22 g) were obtained from Taconic Korea (Daehan Bio Link, Seoul, Korea). Animals were housed at an ambient temperature of 21°C and 46 ± 10% humidity and allowed food and water *ad libitum*. All mice were acclimated for a week before the experiment. All experimental procedures were conformed to the international guidelines "Principles of Laboratory Animals Care" (NIH publication number 85-23, revised 1985, and Kyung Hee University 2006). The international animal ethical committee of Kyung Hee University approved the experimental protocol (KHUASP (SE)-09-036).

2.2. Preparation of Herbal Water Extract and Probiotics. Dried stem bark of LJ was purchased from Kyung Hee Hanyak Co. (Seoul, Republic of Korea). A sample of 100 g was boiled in water for 2.5 h, filtered, and freeze-dried to a powdered form (code name: PJW-042, yield = 18.01%). The voucher specimen of LJ extract (LJE) was deposited in the herbal laboratory of College of Korean Medicine, Kyung Hee University. LJE was used as a solution in distilled water.

DUO (purchased from Cell Biotech Co., Ltd., Gimpo, Korea) is a probiotic mixture containing multiple viable species of three bacterial genera: three strains of *Bifidobacterium* (*B. brevis*, *B. lactis*, and *B. longum*), three strains of *Lactobacillus* (*L. acidophilus*, *L. plantarum*, and *L. rhamnosus*), and one strain of *Streptococcus* (*S. thermophilus*). Each DUO capsule contains 5×10^9 bacteria (approximately 7×10^8 bacteria for each strain).

To investigate the therapeutic effects of LJE or LJE combination with DUO (LJE + DUO), mice were randomly allocated to seven groups ($n = 7$ per group) and treated with DSS only (control), LJE (100 and 300 mg/kg), DUO (300 mg/kg), and LJE (100 and 300 mg/kg) + DUO (300 mg/kg) or left untreated (normal).

2.3. Induction of Colitis. Male Balb/c mice (7 weeks old) were provided with drinking water containing 5% DSS (USB Corporation, Cleveland, OH, USA) *ad libitum* for 7 days. Treatment groups received LJE, DUO, or LJE + DUO administered twice a day by feeding tube for the duration of the experiment. The animals were sacrificed on day 7 and subjected to pathological analysis.

2.4. Assessment of Mouse Weight and Colon Length. Mouse weight was measured daily from day 0 to day 7 at 9:30 am every day. The colon was isolated immediately after the last weight check. Colon length was measured from the cecum to the anus using a vernier caliper (Mitutoyo, Otopeni, Romania).

2.5. Clinical Parameters. The clinical score was measured by the modified method used in the previous study [20]. Two investigators blinded to the protocol daily assessed the clinical score of DSS treatment. Spontaneous behavior and posture were scored as 4, moving (+++) without hunching; 3, moving (++) without hunching; 2, moving (+) with hunching; 1, moving (±) with hunching; 0, moving (−) with hunching., Coat and piloerection were evaluated as 4, normal state; 3, clean and yellowish (+) without piloerection; 2, yellowish (+) with piloerection (+); 1, dirty and yellowish (+++) with piloerection (++); and 0, yellowish (light brown) with piloerection (+++). Cleaning of perianal region was scored as follows: 4, normal state; 3, with stool (+) trace; 2, with stool (++) and blood (+) trace; 1, with stool (+++) and blood (++) trace; and 0, herniation with blood (+++). The final clinical score was obtained by summarizing all the scores.

2.6. Macroscopic Pathology. The macroscopic score was measured by the modified method of the previous study [21]. Two investigators blinded to the protocol assessed the score at day 7 after DSS treatment. Edema and colon sickness were scored as follows: 4, no edema, colon thickness 0.1-0.2 mm; 3, edema (±), 0.2–0.25 mm; 2, edema (+), 0.25–0.30 mm; 1, edema (++), 0.3–0.35 mm; and 0, edema (+++) > 0.35. Overall health was scored as follows: 4, no bleeding with normal stool; 3, no bleeding with semiformed stool; 2, fecal blood (+) with pasty and semiformed stool; 1, fecal blood (++) with tar stool (+); and 0, bleeding (+++) with tar stool (++). The macroscopic score was obtained by summarizing all scores.

2.7. Histology. Colons were fixed in 10% buffered formalin and embedded in paraffin. Histological sections cut from the paraffin blocks were stained with hematoxylin and eosin.

In a blind fashion, the scoring of histological damage was divided into two categories: inflammatory cell infiltration and ulceration. Inflammatory cell infiltration was assessed in each layer of the colon, including surface epithelium, cryptal glands, stroma, submucosa, and transmural layer and graded on a scale from 0 to 3 (0, none; 1, mild; 2, moderate; and 3, severe). The severity of ulceration was graded histologically on a scale from 0 to 4 (0, none; 1, mild and focal surface; 2, mucosal layer; 3, submucosal layer; and 4, transmural layer) [22, 23]. We modified and established a 0–19 scoring system by summarizing all the scores.

2.8. Collection of Colonic Mucosa and Biometric Multiplex Cytokine Profiling. Isolated colons were snap-frozen and stored at −70°C. The mucosa was scraped from the muscle layer of the colon and weighed using analytical balance (Ohaus Voyager, Parsippany, NJ, USA). A sample of 100 ± 10 mg was dissolved in triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.02% sodium azide, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) and homogenized. The levels of eight cytokines involved in a broad spectrum of immune and inflammatory mechanisms were measured in parallel following the induction of colitis [19].

Interferon (IFN)-γ, IL-1β, IL-6, IL-10, IL-12 (P40), IL-12 (P70), IL-17, and TNF-α in the mucosa samples were analyzed using the biometric multiplex cytokine assay (Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

2.9. Statistical Analysis. All results were expressed as mean values with standard errors. Data were analyzed by one-way ANOVA followed by Dunnett's test to compare treatment groups with the control group. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Body Weight and Colon Length. From day 5, the weight of mice in all groups except normal group has shown a decreasing tendency. Compared with the control group, LJE, DUO, and LJE + DUO groups showed no significant protective effect against weight loss induced by DSS (Figure 1).

However, DSS-dependent colon shortening was significantly inhibited in mice treated with LJE (100 and 300 mg/kg), DUO, DUO + LJE (100 mg/kg), and DUO + LJE (300 mg/kg) ($P < 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.01$, resp.) (Figures 2(a) and 2(b)).

3.2. Clinical Findings. Animals from all groups appeared healthy and active, with relatively clean perianal lesions until day 5, when clinical parameters started to worsen. As shown in Figure 3(a), the treatment with LJE or DUO + LJE caused no significant improvement of the clinical score.

3.3. Macroscopic Findings. Control animals developed clinical signs of colitis such as severe edema of the colon, fecal blood, or tar stool. On the other hand, the cecal diameter in control DSS-treated group was not significantly different to that in healthy animals.

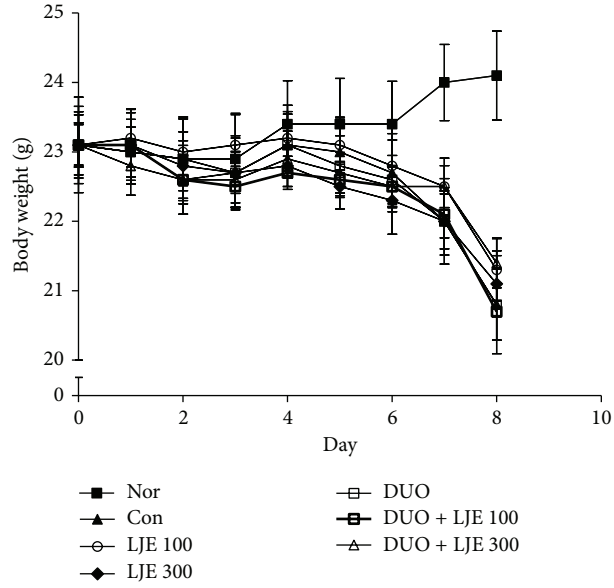

Figure 1: Effects of LJE or DUO on mice weight in DSS-induced colitis. Data are expressed as mean ± S.E.M ($n = 7$). Nor: normal mice without any treatment, Con: mice provided with only 5% DSS treatment, LJE: the water extract of *Laminaria japonica*, DUO: Duolac7S, LJE + DUO: cotreatment of LJE and DUO, 100: the dosage of LJE 100 mg/kg, and 300: the dosage of LJE 300 mg/kg.

The treatment with LJE (300 mg/kg), DUO, and DUO + LJE (300 mg/kg) showed a significant effect compared to other groups ($P < 0.01$, $P < 0.01$, and $P < 0.05$, resp.) as revealed by the macroscopic score (Figure 3(b)). In addition, DUO + LJE-treated mice showed an improvement of the cecal edema compared to any other groups ($P < 0.05$ for both DUO + LJE groups; Figure 3(c)). However, none of the treatments had significant beneficial effects on the cecum diameter (Figure 3(d)).

3.4. Histological Findings. While the surface epithelium, cryptal glands, mucosa, and submucosa in the normal mice were intact (Figure 4(a)-(A)), DSS-treated animals showed severe damage of the surface epithelium, infiltration of the inflammatory cells, and disruption of the cryptal glands (Figure 4(a)-(B)). The pathology of the colonic mucosal layer was improved by the treatment with LJE, DUO, and LJE + DUO, indicating a protective effect against DSS-induced colitis.

The animals administered LJE (100 mg/kg) showed relatively intact surface epithelium, but cryptal glands at the distal colon were disrupted and ulceration similar to the control group was observed (Figure 4(a)-(C)). However, the increase of LJE concentration to 300 mg/kg showed more protective effects and improved DSS-induced pathology. Though infiltrated inflammatory cells and cryptal damage were observed in several mucosal areas, the overall structure of mucosa and submucosa was relatively better preserved (Figure 4(a)-(D)). The DUO-treated group showed more

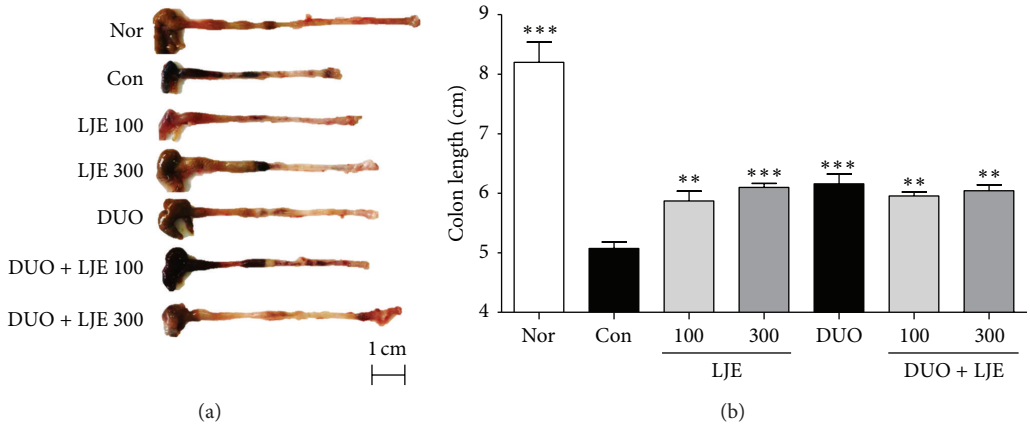

Figure 2: Effects of LJE or DUO on colon length in DSS-induced colitis. (a) The representative colon photos at 7 days on DSS-induced colitis, (b) a graph showing the data of colon length. Data are expressed as mean ± S.E.M (n = 7). Nor: normal mice without any treatment, Con: mice provided with only 5% DSS treatment, LJE: the water extract of *Laminaria japonica*, DUO: Duolac7S, LJE + DUO: cotreatment of LJE and DUO, 100: the dosage of LJE 100 mg/kg, and 300: the dosage of LJE 300 mg/kg; $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group analyzed by one-way ANOVA with Dunnett's post hoc test.

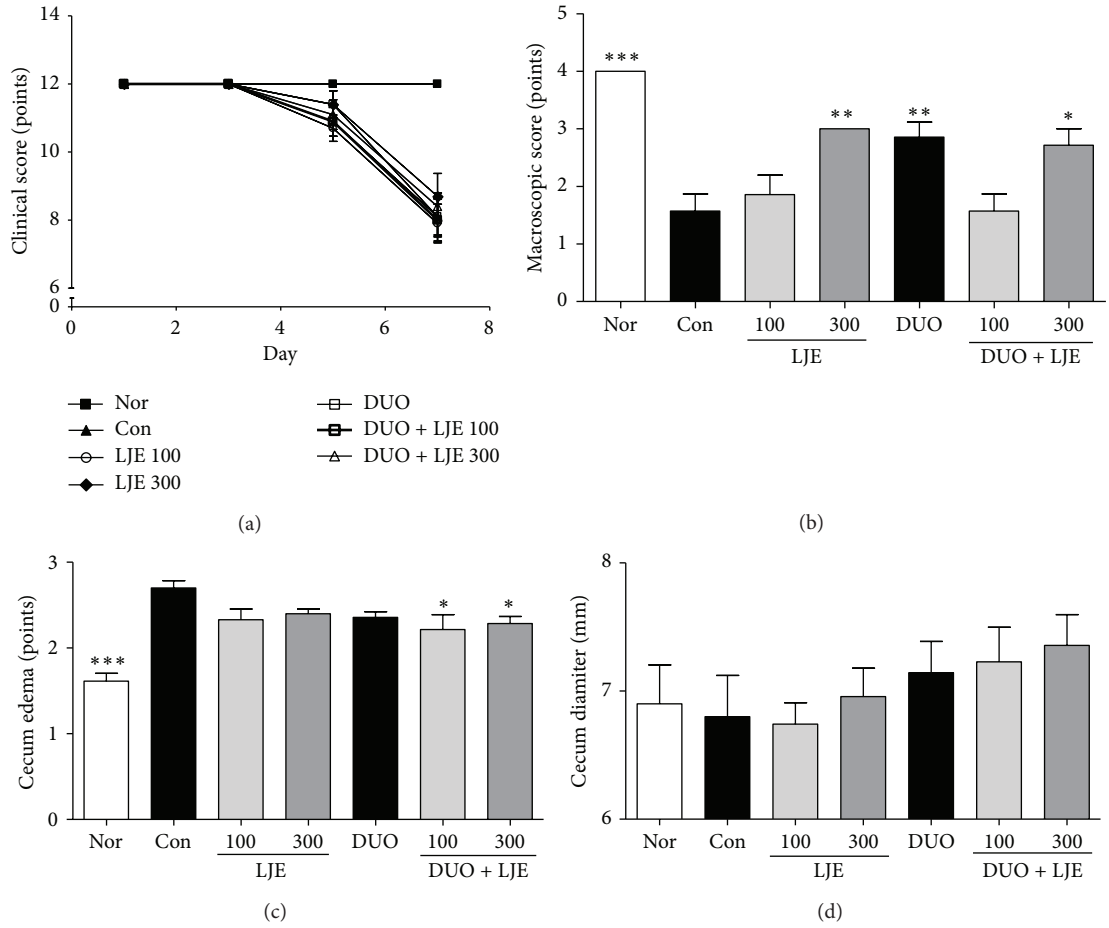

Figure 3: (a) Effects of LJE or DUO on clinical score in DSS-induced colitis. (b) Effects of LJE or DUO on gross macroscopic finding of colon in DSS-induced colitis. (c) Effects of LJE or DUO on cecum edema in DSS-induced colitis. (d) Effects of LJE or DUO on cecum diameter in DSS-induced colitis. Data are expressed as mean ± S.E.M (n = 7). Nor: normal mice without any treatment, Con: mice provided with only 5% DSS treatment, LJE: the water extract of *Laminaria japonica*, DUO: Duolac7S, LJE + DUO: cotreatment of LJE and DUO, 100: the dosage of LJE 100 mg/kg, and 300: the dosage of LJE 300 mg/kg; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group analyzed by one-way ANOVA with Dunnett's post hoc test.

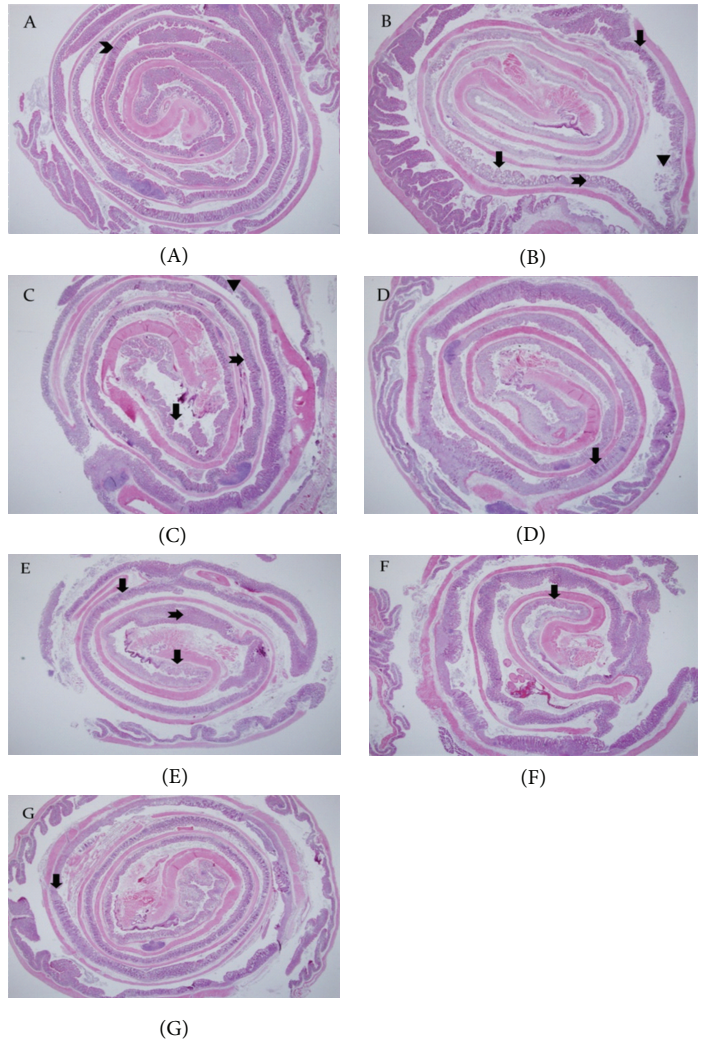

Figure 4: (a) Effects of LJE or DUO on histological findings in DSS-induced colitis. (a)-(A): normal, (a)-(B): control, (a)-(C): LJE 100, (a)-(D): LJE 300, (a)-(E): DUO, (a)-(F): DUO + LJE 100, (a)-(G): DUO + LJE 300, chevron: normal crypt, down arrow: cryptal damage, notches right arrow: infiltration of inflammatory cell, and reversed triangle: ulcer. The whole colon tissues were stained by (H) and (E). The center was the distal part and the boundary was the proximal part of colon (×20). (b) Effects of LJE or DUO on histological scores in DSS-induced colitis. Nor: normal mice without any treatment, Con: mice provided with only 5% DSS treatment, LJE: the water extract of *Laminaria japonica*, DUO: Duolac7S, LJE + DUO: cotreatment of LJE and DUO, 100: the dosage of LJE 100 mg/kg, and 300: the dosage of LJE 300 mg/kg; $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group analyzed by one-way ANOVA with Dunnett's post hoc test.

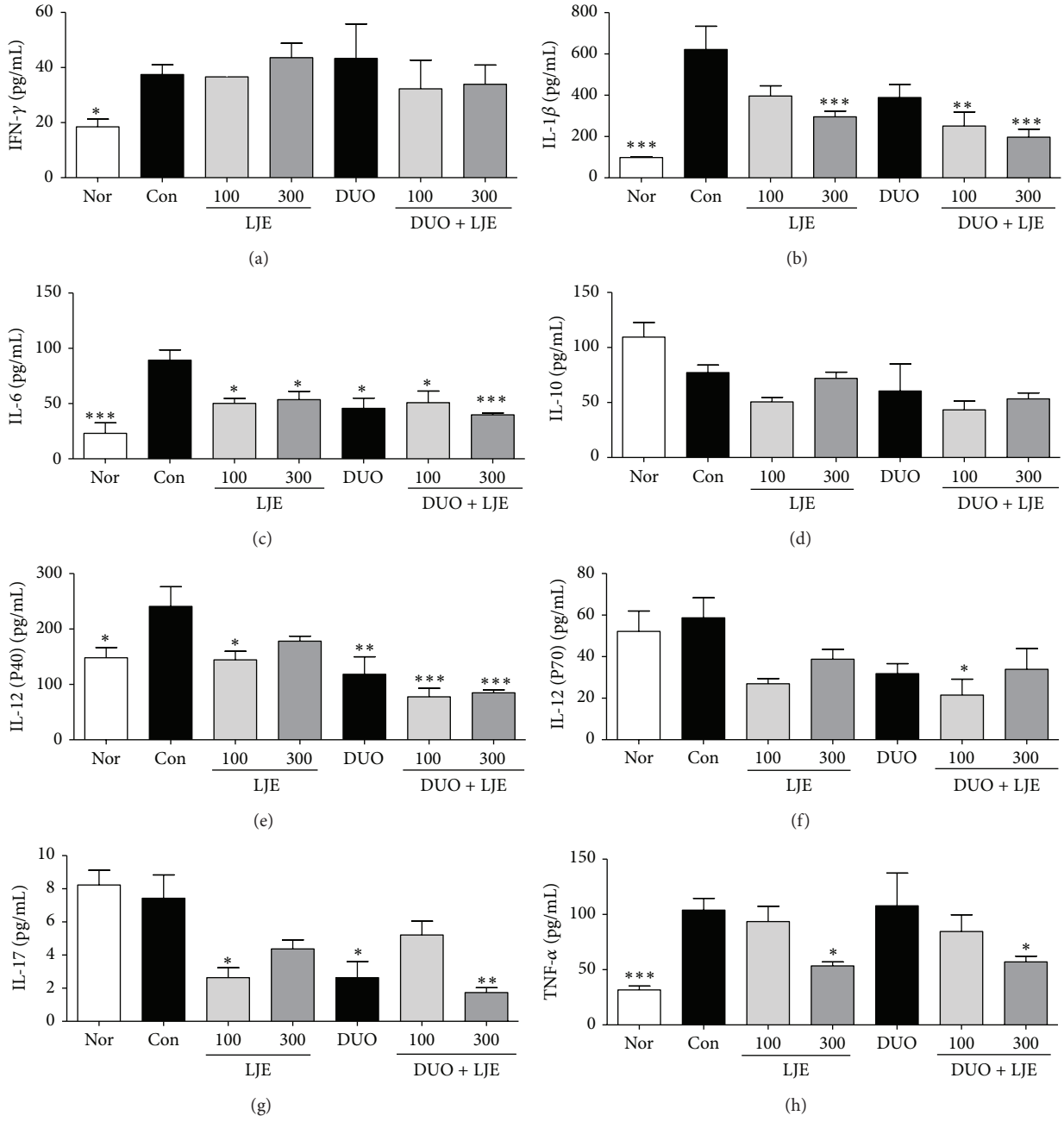

Figure 5: Effects of LJE or DUO on cytokine production in DSS-induced colitis. Data are expressed as mean ± S.E.M ($n = 7$). Nor: normal mice without any treatment, Con: mice provided with only 5% DSS treatment, LJE: the water extract of *Laminaria japonica*, DUO: Duolac7S, LJE + DUO: cotreatment of LJE and DUO, 100: the dosage of LJE 100 mg/kg, and 300: the dosage of LJE 300 mg/kg; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group analyzed by one-way ANOVA with Dunnett's post hoc test. IFN-γ: interferon-γ and TNF-α: tumor necrosis factor-α.

severe damage of cryptal glands and more ulcers compared to the animals treated with LJE (300 mg/kg) (Figure 4(a)-(E)). Both groups treated with the combination of DUO and LJE showed an improvement in the structure of the surface epithelium and cryptal glands compared to the other groups (Figure 4(a)-(F)); it was especially evident for DUO + LJE (300 mg/kg)-treated mice (Figure 4(a)-(G)).

Regarding the histological score, LJE (300 mg/kg) and DUO + LJE (100 and 300 mg/kg) showed significant protection against histological damage caused by DSS administration ($P < 0.01$, $P < 0.001$, and $P < 0.001$, resp.; Figure 4(b)). LJE treatment demonstrated a dose-dependent protection against histological damage and the combined administration of DUO + LJE (300 mg/kg) caused the maximal improvement in histology.

3.5. Changes on Cytokine Levels in Colonic Mucosa. To analyze the influence of LJE and DUO on cytokine

production in colonic mucosa during colitis, we measured the levels of representative cytokines by multiplex profiling after 7 days of DSS administration (Figure 5). IFN-γ level was markedly increased in the DSS-treated group, and the treatments did not cause any significant changes. However, the level of IL-1β in mice administered LJE (300 mg/kg) or combinations of DUO + LJE (100 and 300 mg/kg) significantly decreased compared to that in the control group ($P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.); there was no significant difference with control in the other groups. IL-6 level in the groups treated with LJE (100 and 300 mg/kg), DUO, and DUO + LJE (100 and 300 mg/kg) significantly decreased compared to that of the control group ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.05$, and $P < 0.001$, resp.). All treatment groups showed a statistically significant decrease in IL-6, which was more pronounced in animals treated with DUO + LJE (300 mg/kg). The level of IL-10 was not different among the groups. IL-12 (P40) level in animals treated with LJE (100 mg/kg), DUO, or DUO + LJE (100 and 300 mg/kg) significantly decreased compared to that in the DSS-treated control group ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.001$, resp.); the combination treatment had a stronger effect. However, the treatment with LJE (300 mg/kg) did not change IL-12 levels. The level of IL-12 (P70) in mice treated with DUO + LJE (100 mg/kg) significantly decreased compared to that in the control group ($P < 0.05$); however, no changes were observed in the other treatment groups compared to control. IL-17 levels in the LJE (100 mg/kg), DUO, and DUO + LJE (300 mg/kg) groups significantly decreased compared to that of the control group ($P < 0.05$, $P < 0.05$, and $P < 0.001$, resp.). DUO + LJE (300 mg/kg) showed the strongest effect, while LJE (300 mg/kg) and DUO + LJE (100 mg/kg) did not cause any changes in IL-17 compared to the DSS group. The level of TNF-α in mice treated with LJE (300 mg/kg) and DUO + LJE (300 mg/kg) significantly decreased compared to that of the control group ($P < 0.05$ and $P < 0.05$, resp.), while LJE (100 mg/kg), DUO, and DUO + LJE (100 mg/kg) had no effect.

4. Discussion

In the present study, oral administration of LJE at doses 100 and 300 mg/kg and DUO at 300 mg/kg prevented colon shortening, histological damage and induction of proinflammatory cytokines associated with DSS-induced colitis. The effect of the cotreatment with LJE and DUO showed better protective effect against histological damage and induction of cytokines including IL-1β, IL-6, and IL-12 (P40) compared to the treatment with LJE or DUO alone.

Inflammatory bowel disease (IBD), which comprises Crohn's disease (CD) and ulcerative colitis (UC), is a chronic autoimmune gastrointestinal condition with uncertain etiology. A recent systematic review reported the increase in the worldwide incidence and prevalence of UC and CD, indicating the emergence of IBD as a global disease [24]. The familial aggregation rate is lower in East Asia but higher in West and South Asia; the genetic susceptibility to IBD in Asian population is different from that in Western countries, where it is not associated with NOD2/CARD15 mutations [25]. IBD etiology remains unknown and is thought to be a result of the complex interaction between genetics and environmental factors; some of them are related to intestinal microflora and innate and adaptive immunity [26]. The mucosal immune system exhibits a substantial homeostatic and inflammation-restraining role by secreting and activating various cytokine mediators [27].

In our model of colitis, the treatment with LJE and DUO inhibited the shortening of the colon; it was particularly evident in animals administered LJE (300 mg/kg), DUO, or DUO + LJE (300 mg/kg). Intestinal bacteria produce inflammatory cytokines including IL-6 and TNF-α, which leads to the inflammation of the colonic mucosa, erosion, ulceration, and shortening of the colon [28, 29]. Colon shortening was inhibited by the treatment with LJE or DUO, which also prevented the loss of cryptal glands and epithelial damage. Colon length is considered as a hallmark of experimental colitis, and the histological findings of this study demonstrate that the colon length correlates with the severity of structural damage and the number of infiltrated inflammatory cells. The levels of IL-6 and TNF-α production showed the trend similar to that of the colon length, that is, the highest effect in mice treated with LJE (300 mg/kg) and DUO + LJE (300 mg/kg).

The histological findings demonstrate that the animals receiving LJE, DUO, or LJE + DUO showed a decrease in cryptal damage and inflammation compared to the control group; LJE (300 mg/kg) and DUO + LJE (100 and 300 mg/kg) produced the highest protective effect. In addition, histological scoring revealed the possibility of synergy between LJE and DUO. DSS administration destroys the integrity of the mucosal barrier leading to the disruption of intestinal epithelial layer, mucosal and submucosal ulceration, and infiltration of inflammatory cells [30]. Thus, the protective effect of LJE, DUO, and their combination consists in inhibition of the inflammation-induced damage in intestinal tissues.

The clinical score is considered to reflect the state of gastrointestinal diseases and is used as a reliable assessment tool for IBD patients [18, 31]. DSS-induced colitis in mice presents a variety of clinical symptoms such as diarrhea, bloody stool, or behavioral changes [30]. In this study, LJE or DUO did not improve the clinical score (spontaneous behavior and posture, coat and piloerection, and cleaning of perianal lesion), which might be due to the severity of damage in the intestinal mucosa and/or short period of LJE and DUO administration.

In the current study, LJE dose-dependently improved the macroscopic score, indicating the healing of gross structural damage in mice with colitis. Although the macroscopic score did not entirely corresponded to the results of histological findings, the dose-dependent improvement of macroscopic signs could indicate the possibility of significant effects in the long-term colitis model. Several studies have shown that the macroscopic score could be a useful tool to assess the induction of inflammatory lesions and the severity of

histological damage [21, 32], including intestinal edema and cecal thickness [28].

Weight loss is regarded as one of the major systemic symptoms of the colonic structural damage [30, 33], and previous studies showed that mice treated with DSS for 7 days were rapidly losing weight starting from day 4 [34, 35]. The present results showed a similar pattern of weight loss in the DSS group; however, LJE, DUO, or LJE + DUO did not protect mice against weight loss, possibly because of the severity of DSS-induced intestinal damage.

We have investigated the production of cytokines including IFN-γ, IL-1β, IL-6, IL-10, IL-12 (P40), IL-12 (P70), IL-17, and TNF-α in colonic mucosa of mice with experimental colitis. The levels of IL-1β, IL-6, and TNF-α in mice treated with LJE (300 mg/kg) and DUO + LJE (300 mg/kg) were significantly improved compared to those in other groups. The combined treatment with DUO + LJE inhibited DSS-induced changes in IL-1β, IL-6, and IL-12 (P40) compared to LJE or DUO alone. However, the levels of IFN-γ and IL-10 did not change compared to control. DSS damages the colonic epithelial barrier resulting in subsequent inflammation and induction of cytokine dysregulation, which is thought to be due to the imbalance of T helper (Th) cell subsets, Th1 and Th2 [36]. IBD is characterized by upregulation of Th1 cytokines including IFN-γ, IL-12 (P40), IL-12 (P70), and TNF-α and Th2 cytokines IL-4 and IL-5. Recent studies have also shown that IBD is closely associated with the production of Th17 cytokines such as IL-17 [19, 37]. In an acute model of DSS-induced colitis, mice exhibited an increase in IL-12 and IL-17, suggesting an association with Th1/Th17-dependent mechanisms [19]. Our results show that LJE inhibited IL-17, a representative Th17 cytokine, and Th1/Th17 response-related proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-12 (P40), which is consistent with previous results obtained with *Lonicera japonica* [38]. IL-10 contributes to the differentiation of regulatory T cells (T_{reg}), while suppressing dendritic cell-associated Th1 and Th17 immunity [39]. IL-10 and IFN-γ, which showed no significant change in our experiments, were not elevated in acute and subacute stages of DSS-induced colitis [19].

At the same time, DUO alone also showed an anti-inflammatory effect by regulating IL-6, IL-12 (40), and IL-17. LAB such as *L. rhamnosus* and probiotic mixture composed of *L. acidophilus, B. lactis, L. plantarum,* and *B. breve* have shown protection against DSS-induced colitis [40, 41]. IL-10 was not elevated in our experiments, but several studies have reported that *Lactobacillus*, *Bifidobacterium* and *Streptococcus* species induce the production of anti-inflammatory IL-10, although with different effectiveness [42–44]. Thus, *Bifidobacterium* has been implicated in promoting T_{reg} differentiation and programming T_{reg} cells [45]. In our experiments, the measurements were performed only in intestinal mucosa at day 7 of DSS induction, when the differentiation or movement of T_{reg} cells might not have been completed and T_{reg} cells might still reside in submucosal layers. Plus, the level of IL-10 in mesenteric lymph node or spleen where most T_{reg} cells secreting IL-10 exist was not observed. Therefore, our results do not rule out the possibility that DUO could increase IL-10 levels, which should be evaluated in each step, region, and immunological tissue. DUO also might regulate Th17 and Th1/Th17 response-related cytokines. LJE and DUO showed a synergistic dose-dependent effect on IL-17, IL-1β, IL-6, and IL-12 (P40) production, indicating that LJE + DUO might activate the Th17- and Th1/Th17-dependent pathways similar to LJE and DUO alone. The effect of LJE and DUO on the level of Th17 or Th1/Th17 cytokines correlated with that on histopathology.

Taken together, LJE and DUO inhibited proinflammatory cytokines IL-1β, IFN-γ, TNF-α, IL-6, and IL-12 (P40), which are produced by activated macrophages or dendritic cells in mucosa inducing acute mucosal inflammation. These results correspond to the previous findings, suggesting that in the acute DSS-induced colitis the colonic Th cells exhibit Th17 and Th1/Th17 profiles [36]. The administration of LJE and DUO did not change the levels of IL-10 related to T_{reg} cells. Th1 cytokines have been shown to have a profound influence on the severity of inflammation and infection in mice at the acute phase of DSS-induced colitis, while Th2 cytokines are involved in chronic intestinal inflammation [46].

LJE administration positively affected the colon length, microscopic findings, histological scoring, and proinflammatory cytokine profile, suggesting a mechanism underlying LJE protective effect against cryptal gland loss and epithelial damage. The synergetic effect of LJE and DUO was demonstrated by the histological scoring and the production of cytokines including IL-1β, IL-6, and IL-12 (P40). Therefore, the protective effect of DUO might be associated with Th1 and Th17 differentiation and its neutralization, leading to prevention of cryptal damage, infiltration of inflammatory cells, and ulceration of intestinal tissues.

In the present study, we selected LJE dosage based on standard doses used in humans. In mice, LJE at 300 mg/kg corresponds to 167 mg/kg in humans considering the rate of excretion (10-fold compared to human) and LJE yield (18.01%). As a result, 300 mg/kg of LJE in mice corresponds to approximately 8 g of LJ raw material in a 50 kg human; this concentration is similar to LJ dosage prescribed in clinics [47, 48]. Based on this data, we used LJE in a concentration range from 100 (low) to 300 (high) mg/kg. DUO was approved by the Korean Food and Drug Administration at a dosage of 500 mg twice a day (total 1,000 mg a day), which corresponds to 200 mg/kg of adult body weight (50 kg). Considering this data and mouse excretion rate, in our experiments mice received DUO at a fixed dose of 300 mg/kg, which is similar to a standard human dosage.

It is noteworthy that this study showed a dose-dependent effect of LJE on clinical and biochemical parameters of experimental colitis; in addition, synergetic effects with DUO were observed. Experiments with modified LJE dosage and long-term administration are needed in the future.

5. Conclusions

Our results demonstrated that LJE was effective in protection against colitis in mice and that cotreatment with LJE and DUO showed synergy in DSS-induced IBD model. The possibility of a synergetic effect of LJE and DUO cotreatment was

strongly suggested. LJE at 300 mg/kg was the most effective dose for improvement of IBD symptoms and pathology in mice. LJE and DUO might have a beneficial effect on colitis by regulating Th17- or Th1/Th17-related immune mechanisms. This study presents an evidence of LJE protective effects on IBD, suggesting a possibility for a clinical trial. Further studies on the immune mechanisms induced by LJE should be based on a broader LJE dose range and long-term administration.

Conflict of Interests

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

Authors' Contribution

Seok-Jae Ko and Youngmin Bu contributed equally to the study and are the first coauthors.

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Research Article

PMA Induces Vaccine Adjuvant Activity by the Modulation of TLR Signaling Pathway

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Toll-like receptor (TLR) ligands are being developed for use as vaccine adjuvants and as immunomodulators because of their ability to stimulate innate and adaptive immune responses. Flagellin, a TLR5 ligand, was reported to show potent mucosal vaccine adjuvant activity. To identify ligands that potentiate the adjuvant activity of flagellin, we screened a plant library using HEK293T cells transiently cotransfected with phTLR5 and pNF-κB-SEAP plasmids. The 90% EtOH extract from *Croton tiglium* showed significant NF-κB transactivation in a TLR5-independent manner along with the increase of a flagellin activity. We have studied to characterize an active component from *Croton tiglium* and to elucidate the action mechanisms. Phorbol 12-myristate 13-acetate (PMA) was isolated as an active component of *Croton tiglium* by activity-guided fractionation, column chromatography, HPLC, NMR, and MS. PMA at a range of nM induced PKC-dependent NF-κB activation and IL-8 production in both TLR5− and TLR5+ assay systems. In in vivo mouse vaccination model, PMA induced antigen-specific IgG and IgA antibody responses and increased IL-12 production corresponding to T cell responses in spleen lymphocytes. These results suggest that PMA would serve as an efficacious mucosal vaccine adjuvant.

1. Introduction

Toll-like receptors (TLRs) play crucial roles in innate immunity and can contribute to the development of appropriate adaptive immune responses [1, 2]. To date, 13 members of TLR family have been identified in mammals [3–5], and TLRs received special attention as potent adjuvant receptors [6]. Mammalian TLR5, expressed on epithelial cells and phagocytes such as dendritic cells and macrophages, recognizes flagellins of bacteria and subsequently activates the nuclear factor-kappa B (NF-κB) pathway of host cells [7, 8]. We reported that a *Vibrio vulnificus* flagellin B (FlaB) showed strong mucosal vaccine adjuvant activity [9] and enhanced tumor-specific $CD8^+$ T cell immune responses through TLR5 stimulation in a therapeutic cancer vaccine model [10]. Recently, FlaB combined with TNFα and IFNα was reported to generate potent dendritic cells which produce functionally active cytotoxic T lymphocytes [11].

Flagellin is a highly priced protein adjuvant candidate. To identify ligands that potentiate vaccine adjuvant activity of flagellin, we screened a plant extract library using HEK293T cells transiently cotransfected with phTLR5 and pNF-κB-SEAP plasmids. The 90% EtOH extract from *Croton tiglium* L. (Euphorbiaceae) showed significant NF-κB transactivation. *Croton tiglium* is a plant grown in tropical and subtropical

zones, and the seed of *Croton tiglium* is well known as Ba-Dou (or Badou) in China and Korea. Ba-Dou has been used to treat gastrointestinal disorders, intestinal inflammation, rheumatism, headache, peptic ulcer, and visceral pain [12–14]. The sesquiterpenes and monoterpenes as the main components comprise the great parts of the extracted essential oil from seed. The toxic substances were found mainly in the bark and leaves of *Croton tiglium* and croton oil. In this study, we isolated phorbol 12-myristate 13-acetate (PMA) as an active component from *Croton tiglium* and investigated the action mechanisms in TLR signaling pathways.

2. Materials and Methods

2.1. Cell Culture. HEK293T and Caco-2 cells (ATCC, Manassas, VA) were cultured in Dulbecco modified Eagle medium (DMEM, WELGENE, Korea) supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen, Carlsbad, CA) at 37°C in a 5% CO_2 incubator.

2.2. NF-κB Reporter Assay. HEK293T cell is a human embryonic kidney cell line with SV40 large T-antigen for efficient transfection of plasmids. HEK293T cells seeded at 10^4/well in 96-well plates were transfected with pNF-κB-secreted alkaline phosphatase (pNF-κB-SEAP, InvivoGen, San Diego, California), pIL-8-luciferase [9], or phTLR5 plasmids using Fugene 6 (Roche, Hague Road, Indianapolis). One day after transfection, the cells were replaced with fresh DMEM containing different concentrations of test agents for 1 day. NF-κB-SEAP activities in cell culture supernatants were determined using QUANTI-Blue (InvivoGen) according to the manufacturer's instructions. Recombinant tag-free flagellin, FlaB, was purified as described elsewhere and contaminating lipopolysaccharide (LPS) was removed by using the AffinityPak Detoxi Gel endotoxin removing gel (Pierce Biotechnology, Inc., Rockford, IL) [9].

2.3. Plant Materials. The seeds of *Croton tiglium* were purchased from Chonnam Seangyack Nongob, Hwasun-gun, in April 2011, Republic of Korea. Plant sample was identified botanically by Professor Y. H. Moon. A voucher specimen (SNU2011-04) was deposited at the Herbarium of Seoul National University, Seoul, Republic of Korea.

2.4. Extraction and Isolation from the Seeds of Croton tiglium. The dried seeds of *Croton tiglium* (600 g) were extracted with 90% EtOH (2 L × 3 times) at room temperature. The combined 90% EtOH extract was then evaporated under reduced pressure using a rotary vacuum evaporator (EYELA, Japan). The dried crude extract of *Croton tiglium* (12 g) was suspended in water and divided successively with *n*-hexane (3 × 500 mL), $CHCl_3$ (3 × 500 mL), EtOAc (3 × 500 mL), and *n*-BuOH (3 × 500 mL). The $CHCl_3$ fraction (2.9 g), which showed strong enhanced activity on NF-κB transcription, was chromatographed over a silica gel open column (5 × 40 cm; 63–200 μm particle size, Merck, Darmstadt, Germany) eluting with gradient *n*-hexane/acetone (20 : 1, 10 : 1,. . .,1 : 3, each 200 mL) to yield ten fractions (F1–F10) based on the TLC profile. Fraction F8 (1.05 g) was reapplied to an RP-C_{18} open column (4 × 30 cm; 40–63 μm particle size) with a stepwise gradient of MeOH/H_2O (1 : 2 to 10 : 1) to afford nine subfractions (F81–F89). Finally, subfraction F87 (30.2 mg) was purified by HPLC [OptimaPak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeOH in H_2O containing 0.1% HCO_2H (0–15 min: 85% MeOH, 15–40 min: 95% MeOH, 40–45 min: 100% MeOH)] to yield compound **1** (t_R = 31.3 min, 5.2 mg) (Figure 2).

2.5. IL-8 ELISA in Caco-2 Cells. Caco-2 is a heterogeneous human epithelial colorectal adenocarcinoma cell and constituently expresses TLR5. Caco-2 cells were seeded at 5 × 10^4/well in 48-well plates and were treated with ligands for 8 hours without FBS supplementation. IL-8 in the supernatant was measured by an ELISA kit (BioSource International, Inc., California, USA) according to the manufacturer's instructions.

2.6. Cell Staining and Fluorescence Microscopy. HEK293T cells in 8-well glass chamber plate (Nalge Nunc International, Rochester, NY) were transfected with phTLR5 using Fugene 6 (Roche). The cell culture was replaced with fresh DMEM containing PMA for 6 hours. After fixation for 15 minutes with 3.7% paraformaldehyde, the cells were rendered permeable by incubation in PBS with 0.2% Triton X-100 for 10 minutes. NF-κB p65 protein was detected by immunostaining using a specific antibody (Santa Cruz Biotechnology, Delaware Avenue, Santa Cruz, CA) and Alexa Fluor-488-conjugated anti-rabbit-IgG antibody (Molecular Probes, Invitrogen, Eugene, OR). Fluorescence images were acquired using a fluorescence microscope (DXM1200C, Nikon).

2.7. Inhibition Assay of Pharmacological Antagonists on PMA-Induced NF-κB Activity. To study the action mechanism, various pharmacological inhibitors were tested on PMA-mediated NF-κB activation. Pharmacological inhibitors were used such as Wortmannin, Bay 11-7082, Genistein, GF109203X, PD98059, SB203580, SP600125, and U-73122 (Cell Signaling Technology, Danvers, MA) for the inhibition of phosphoinositide 3-kinase (PI3K), IkB-α phosphorylation, protein tyrosine kinase (PTK), protein kinase C (PKC), MEK1, SAPK2 (p38), jun N-terminal kinase (JNK), and phospholipase C (PLC), respectively.

2.8. Mice Immunization and ELISA. Five-week-old female BALB/c mice were intranasally immunized three times with 10 μL of PBS containing oval albumin (OVA) as an antigen alone or in combination with PMA or FlaB at 7-day intervals. Seven days after the last immunization, feces and serum samples were collected from the immunized mice to assess antigen-specific antibody responses. All animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University. OVA-specific antibodies were determined by ELISA followed by the methods as described elsewhere [9]. Absorbance was read by an ELISA microplate reader (Power Wavex340, NIO-TEK-INS TRUMENTS, INC) at 450 nm.

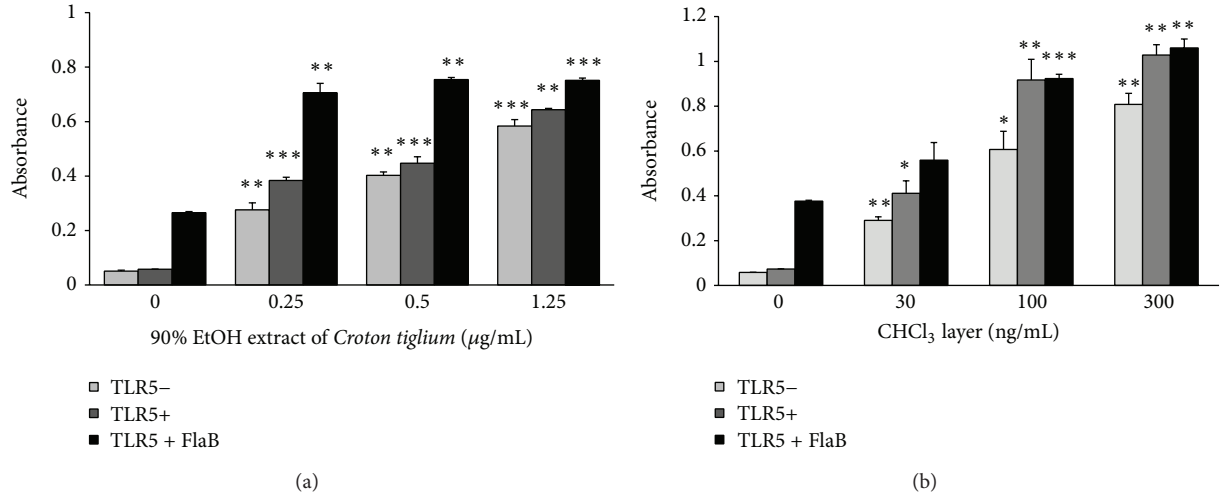

Figure 1: *Croton tiglium* and its chloroform fraction induced NF-κB transcription in TLR5-independent manner and increased the FlaB activity. (a) 90% EtOH extract of *Croton tiglium* increased FlaB-mediated NF-κB transcription. HEK293T cells transiently transfected with pNF-κB-SEAP and phTLR5 were treated with FlaB and 90% EtOH extract of *Croton tiglium* for 1 day. SEAP activities were determined in the cell culture supernatants using QUANTI-Blue. 90% EtOH extract of *Croton tiglium* induced NF-κB transcription in TLR5-independent manner and increased the FlaB activity. (b) The chloroform fraction of *Croton tiglium* increased NF-κB transcription. The chloroform fraction of *Croton tiglium* increased significantly NF-κB transcription in HEK293T cells transiently transfected with pNF-κB-SEAP and phTLR5 regardless of TLR5. The data indicate the mean and SEM from three experiments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

2.9. Cytokine Assay in Splenocytes from Vaccinated Mice. Spleen lymphocytes were prepared from the immunized mice by using lymphoprep according to the manufacturer's instructions (AXIS-SHIELD PoC AS, Norway). The lymphocytes were cultured in RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated with OVA (10 μg/mL) at 37°C for 2 days. The levels of interleukin 12 (IL-12) were measured by using sandwich ELISA kits following the manufacturer's experimental protocols (Biolegend, USA).

2.10. Statistical Analysis. All values are expressed as means ± standard error of the mean (SEM). Statistical comparisons were made using Student's *t*-test. All experiments were repeated three times and the results from a representative experiment were shown.

3. Results and Discussion

3.1. Croton tiglium and Its Chloroform Fraction Induced NF-κB Transcription in TLR5-Independent Pathway and Enhanced FlaB Activity. TLR5 recognizes bacterial flagellin and activates a transcription factor NF-κB hence inducing proinflammatory cytokine production in mammalian cells [7]. We studied to identify herbal medicines that enhanced NF-κB activity of a flagellin, FlaB. TLR5 and NF-κB-SEAP were overexpressed in HEK293T cells by transfection with the plasmids and a plant extract library was screened. The 90% EtOH extract from *Croton tiglium* increased NF-κB transcription in HEK293T cells in TLR5-independent pathway and enhanced the FlaB activity inducing TLR5-dependent NF-κB activation (Figure 1(a)). To identify active components of *Croton tiglium*, the 90% EtOH crude extract was divided into 5 fractions: *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water layers. The chloroform fraction showed a more significant effect than the other fractions on NF-κB activity at concentrations of 30~300 ng/mL (Figure 1(b)).

3.2. Structure Determination and Identification of Active Component Inducing NF-κB Activation from Croton tiglium. In order to isolate an active component, the chloroform fraction from *Croton tiglium* extract was subjected to a succession of chromatographic procedures including silica gel chromatography, RP-C_{18}, and HPLC (Figure 2(a)). Each fraction was tested on NF-κB transcription in HEK293T cells and the activities were shown in Table 1. When phorbol 12-myristate-13-actate [PMA; synonym: 12-*O*-tetradecanoylphorbol-13-actate (TPA)] from Sigma Co. (St. Louis, USA) and an isolated compound (Compound **1**) were coinjected into HPLC, the same retention time (t_R) at 31.3 min suggested that it was an identical compound (Figure 2(b)). For further confirmation of the chemical structure of isolated compound, ^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra of 1 were measured on a Varian Unity Inova 600 MHz spectrometer at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The ^{1}H and ^{13}C NMR spectroscopic data of Compound **1** showed the characteristic signals for an α, β-unsaturated carbonyl group [(δ_H 7.57, 1H, br s, H-1; δ_C 160.7, C-1); δ_C 132.8, C-2; δ_C 208.8, C-3], a trisubstituted double bond [(δ_H 5.66, 1H, d, J = 4.6 Hz, H-7; δ_C 129.2, C-7); δ_C 140.4, C-6], an oxymethine (δ_H 5.39, 1H, d, J = 10.1 Hz, H-12; δ_C 76.5, C-12), and an oxymethylene [δ_H 4.01 and 4.00, AB 2H, J = 12.8 Hz, H_2-20; δ_C 68.0, C-20] of a phorbol ester system [15, 16]. As the NMR and MS data [m/z 616.3980, Micromass

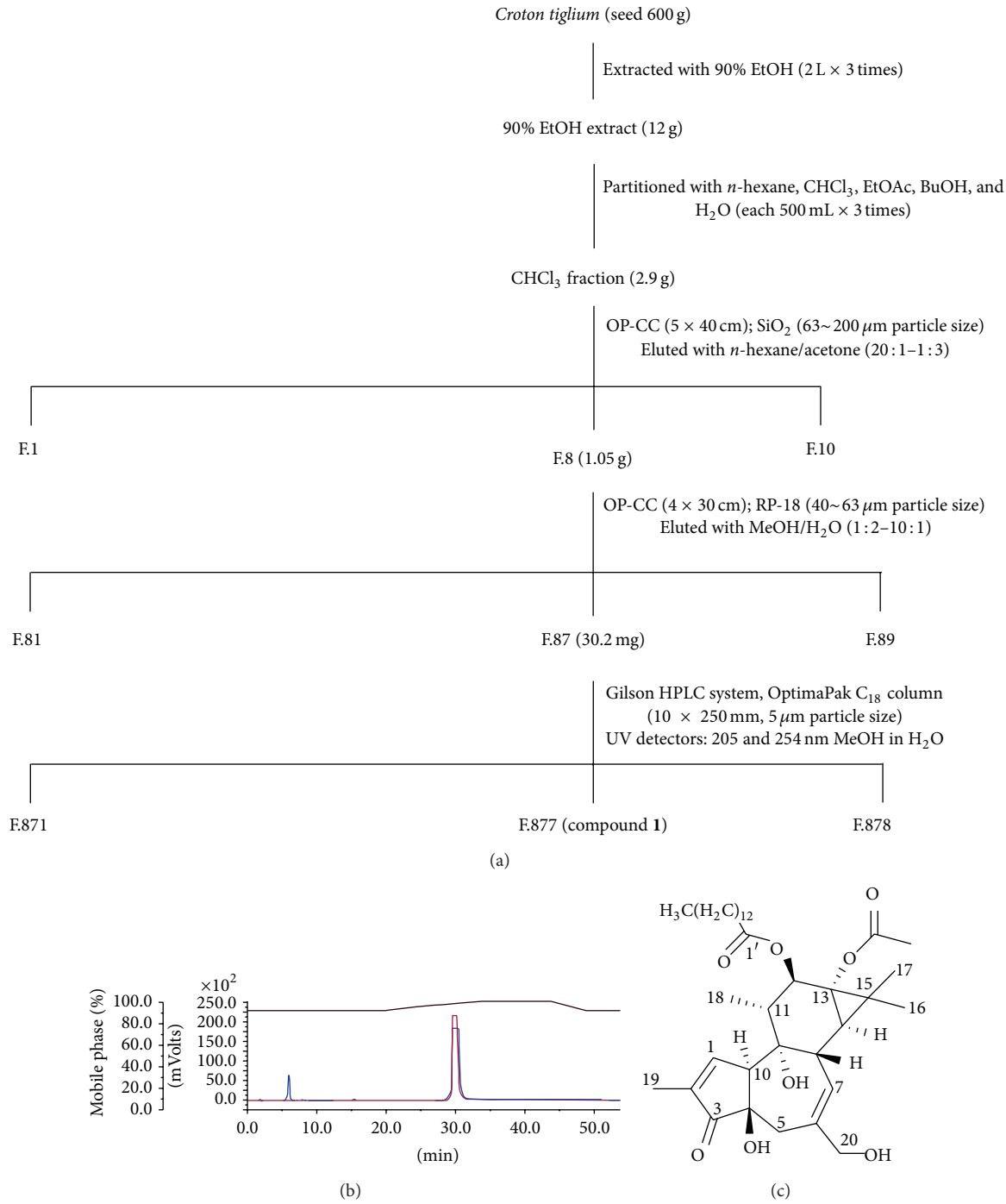

Figure 2: Isolation procedures of an active compound from *Croton tiglium*. (a) Column chromatography and HPLC.Components of the chloroform fraction from *Croton tiglium* were divided using column chromatography. The dried chloroform fraction was eluted on a silica gel column (5 × 40 cm; Merck, 63–200 μm particle size) with a solvent gradient of hexane/acetone (20 : 1 to 1 : 3 ratios) to yield ten fractions (Fr.1–Fr.10). Fr.8 showing the most potent activity was divided into 9 subfractions (Fr.81–Fr.89) using RP-C_{18} column with a solvent gradient of MeOH/H_2O (1 : 2 to 10 : 1). Fr.87 was applied to 8 fractions (Fr.871–Fr.878) using Gilson HPLC system with OptimaPak C_{18} column (10 × 250 mm, 5 μm particle size). For activity-guided fractionation, pNF-κB reporter activities of each fraction were evaluated in HEK293T cells transiently transfected with pNF-κB-SEAP and phTLR5. (b) HPLC comparison of compound **1** and PMA. An isolated compound **1** from *Croton tiglium* was analyzed by coinjection with PMA standard from Sigma Co. (St. Louis, USA) by a Gilson HPLC with the 321-pumps systems; UV/Vis-155; 234-autoinjector; an OptimaPak C_{18} column (10 × 250 mm, particle size 5 μm), using a gradient of methanol and 0.1% formic acid in H_2O as mobile phase. Detection was analyzed with two channels at 205 and 254 nm (blue line; 205 nm, red line; 254 nm). Solvent elution was carried out with a gradient of methanol and 0.1% formic acid in H_2O as mobile phase, at a flow rate of 2 mL/min. PMA and compound **1** had the same retention time at 31.3 minute. (c) The chemical structure. The chemical structure of compound **1** was confirmed by ^1H and ^{13}C NMR spectra (Varian Unity Inova 600 MHz spectrometer) and MS data (Micromass, Wythenshawe, UK).

Table 1: Effects of fractions from *Croton tiglium* on NF-κB transactivation.

Fractions (10 ng/mL)	Absorbance	Fractions (3 ng/mL)	Absorbance	Fractions (1 ng/mL)	Absorbance
Fr.1	0.084 ± 0.004	Fr.81	0.121 ± 0.002	Fr.871	0.080 ± 0.006
Fr.2	0.094 ± 0.008	Fr.82	0.132 ± 0.003	Fr.872	0.082 ± 0.004
Fr.3	0.086 ± 0.004	Fr.83	0.121 ± 0.003	Fr.873	0.120 ± 0.008
Fr.4	0.071 ± 0.004	Fr.84	0.124 ± 0.006	Fr.874	0.092 ± 0.004
Fr.5	0.112 ± 0.001	Fr.85	0.125 ± 0.004	Fr.875	0.077 ± 0.004
Fr.6	0.121 ± 0.002	Fr.86	0.223 ± 0.008	Fr.876	0.103 ± 0.000
Fr.7	0.975 ± 0.086	**Fr.87**	**0.981 ± 0.035**	**Fr.877**	**0.318 ± 0.086**
Fr.8	**0.991 ± 0.026**	Fr.88	0.631 ± 0.016	Fr.878	0.230 ± 0.026
Fr.9	0.110 ± 0.003	Fr.89	0.462 ± 0.036		
Fr.10	0.068 ± 0.003				

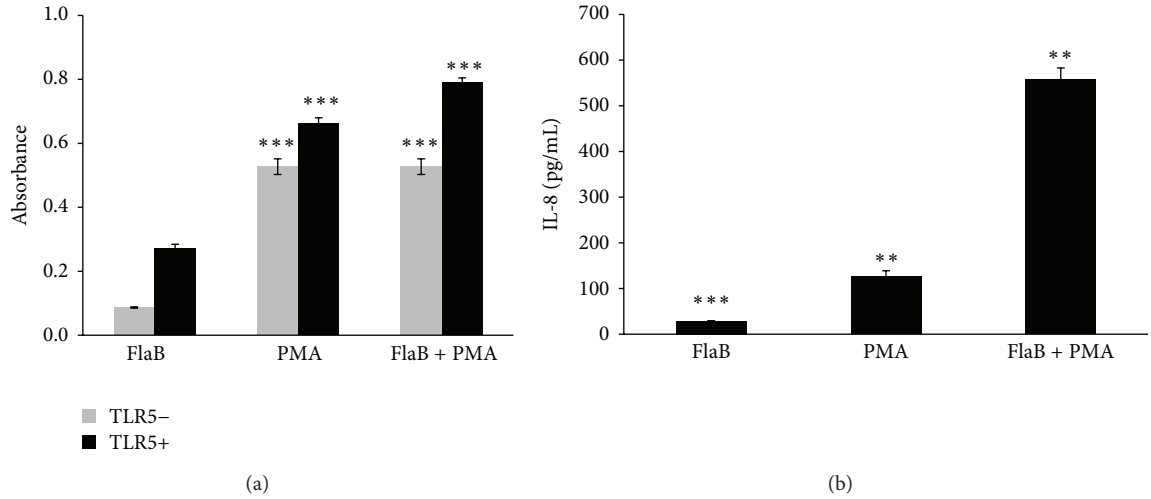

Figure 3: PMA induced NF-κB transactivation and IL-8 production in TLR5-independent pathway. (a) PMA induced NF-κB transactivation. HEK293T cells transfected with pNF-κB-SEAP and phTLR5 plasmids were replaced with the fresh DMEM containing FlaB (20 ng/mL) or PMA (10 ng/mL) and incubated for 1 day. SEAP activity of the cell supernatants was determined in the supernatants using QUANTI-Blue. (b) PMA increased significantly FlaB-mediated IL-8 production in Caco-2 cells. Caco-2 cells were treated with PMA (100 ng/mL) with or without FlaB (1 μg/mL) for 8 hours. IL-8 concentrations in the supernatant were determined by ELISA. PMA increased significantly FlaB-induced IL-8 production. The data indicate the mean and SEM from three experiments ($^{**}P < 0.01$, $^{***}P < 0.001$).

QTOF2 (Micromass, Wythenshawe, UK)] are identical with those reported for PMA [16, 17], Compound **1** was finally determined as PMA (Figure 2(c)).

Compound **1**. Colorless oil; ^{1}H (600 MHz, in $CDCl_3$): δ_H 7.57 (1H, br s, H-1), 5.66 (1H, d, J = 4.6 Hz, H-7), 5.51 (1H, br s, OH-9), 5.39 (1H, d, J = 10.1 Hz, H-12), 4.01 and 4.00 (2H, AB peaks, J = 12.8 Hz, H_2-20), 3.23 (1H, br s, H-10), 3.21 (1H, t, J = 5.5 Hz, H-8), 2.52 and 2.46 (2H, AB peaks, J = 19.3 Hz, H-5), 2.30 (2H, m, H-2′), 2.12 (1H, m, H-11), 2.07 (3H, s, acetyl), 1.76 (3H, dd, J = 2.7, 1.4 Hz, H-19), 1.60 (2H, m, H-3′), 1.18–1.31 [26, (4′–13′ methylene) and 2 × methyl (H-16 and H-17)], 1.06 (1H, d, J = 5.0 Hz, H-14), 0.87 (3H, d, J = 6.4 Hz, H-18), 0.86 (3H, t, J = 6.5 Hz, H-14′); ^{13}C NMR data (150 MHz, in $CDCl_3$): δ_C 160.7 (C-1), 132.8 (C-2), 208.8 (C-3), 73.7 (C-4), 38.6 (C-5), 140.4 (C-6), 129.2 (C-7), 39.1 (C-8), 78.1 (C-9), 56.2 (C-10), 42.9 (C-11), 76.5 (C-12), 65.6 (C-13), 36.2 (C-14), 25.6 (C-15), 23.8 (C-16), 16.8 (C-17), 14.1 (C-18), 10.1 (C-19), 68.0 (C-20), 173.7 (C-1′), 34.6 (C-2′), 25.2 (C-3′), 29.0–29.6 (C-4′ to C-11′), 31.9 (C-12′), 22.7 (C-13′), 14.1 (C-14′), 173.7 and 21.1 (acetyl); EIMS m/z 616.3980 (calcd for $C_{36}H_{56}O_8$, 616.3975).

3.3. PMA Increased Significantly FlaB-Mediated NF-κB Activity and IL-8 Production in TLR5-Independent Pathway. PMA, an active component of *Croton tiglium*, was evaluated whether it could stimulate FlaB-mediated NF-κB activity and IL-8 production. PMA at a low concentration of 10 ng/mL increased NF-κB activity with or without FlaB (20 ng/mL) (Figure 3(a)). PMA also enhanced significantly FlaB-mediated IL-8 production in Caco-2 cells expressing TLR5 constitutively (Figure 3(b)). These results suggest that PMA is an active component of *Croton tiglium* in NF-κB transactivation.

3.4. PMA Induced the Translocation of NF-κB from Cytosol into Nucleus. Like other members of the NF-κB family, p65 resides in the cytoplasm in an inactive form bound

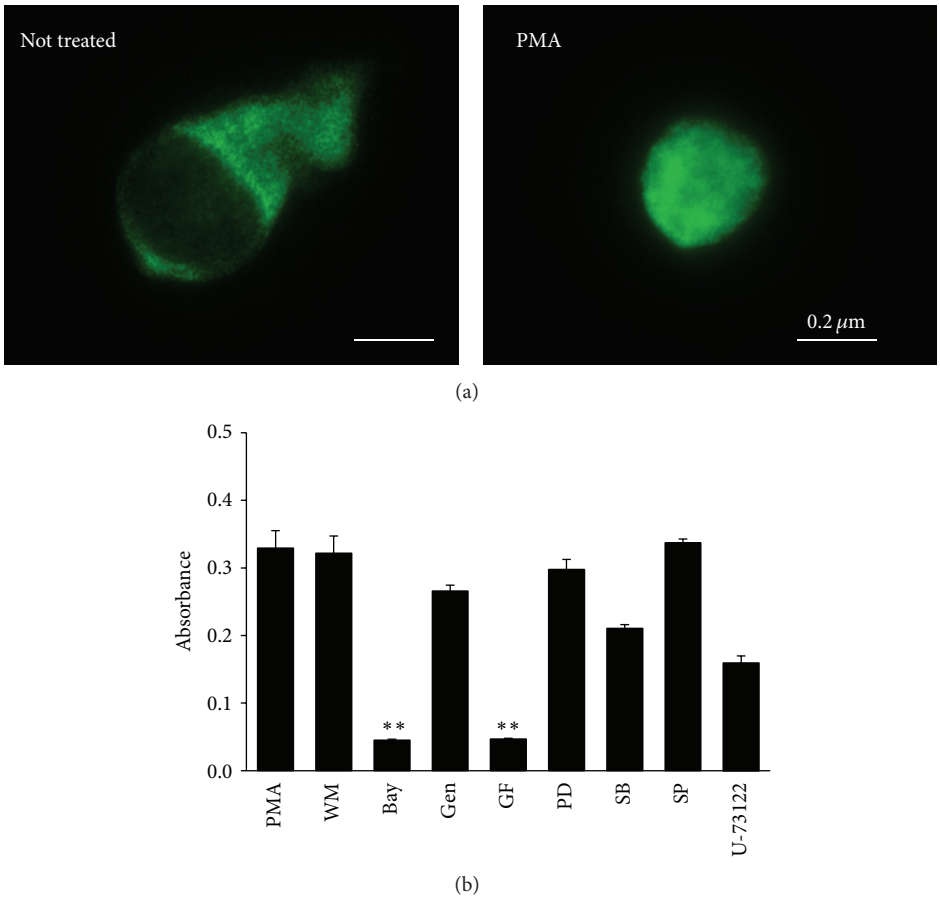

Figure 4: Action mechanism of PMA on TLR signaling pathway. (a) PMA induced nuclear translocation of NF-κB p65 subunit. HEK293T cells were transfected with phTLR5 for 1 day and treated with or without PMA (10 ng/mL) for 6 hours. The cells were fixed, permeabilized, and immunostained with a polyclonal antibody against NF-κB p65, followed by Alexa Fluor-488-conjugated anti-rabbit-IgG antibody. PMA induced nuclear translocation of NF-κB p65 subunit in HEK293T cells. (b) PMA induced NF-κB activation through PKC-dependent pathway. HEK293T cells transfected with pNF-κB-SEAP and phTLR5 plasmids were treated with PMA (10 ng/mL) in the presence of pharmacological inhibitors. PMA-induced NF-κB activity was blocked by the treatment of a PKC inhibitor (GF109203X) and an IκB inhibitor (Bay11-7082). Wortmannin (WM), Bay 11–7082 (Bay), Genistein (Gen), GF109203X (GF), PD98059 (PD), SB203580 (SB), SP600125 (SP), and U-73122. The data indicate the mean and SEM from three experiments ($^{**}P < 0.01$).

to inhibitory IκB protein. Cellular activation results in the nuclear translocation of p50 : p65 for initiating gene transcription. We therefore assessed the nuclear translocation of NF-κB p65 subunit after its activation by PMA treatment (10 ng/mL) to the HEK293T cells by immunostaining. NF-κB p65 was translocated from cytosol to nucleus after PMA treatment (Figure 4(a)).

3.5. A PKC Inhibitor Blocked PMA-Induced NF-κB Activation in HEK293T Cells. To study the action mechanism of PMA, various pharmacological inhibitors were tested on PMA-mediated NF-κB activation. Two hours after inhibitor incubation, the cells were treated with PMA at a concentration of 10 ng/mL. The supernatants were collected after 1 day of PMA treatment and NF-κB-SEAP levels were determined by QUANTI-Blue. PMA-induced NF-κB activation was blocked by the treatment of a PKC inhibitor GF109203X and an IκB-α degradation inhibitor Bay11-7082 (Figure 4(b)). These results indicate that PMA induces NF-κB activation through PKC-dependent IκB phosphorylation.

3.6. PMA Increased OVA-Specific Systemic Immune Response in Mice. BALB/c mice were intranasally immunized three times with antigen OVA alone or in combination with FlaB or PMA under anesthesia. Seven days after immunization, feces and serum samples were collected from the immunized mice to assess antigen-specific antibody responses. OVA-specific IgA and IgG antibodies were significantly increased by the administration of OVA plus PMA or FlaB (Figure 5(a)). These results suggest that PMA has vaccine adjuvant activity.

3.7. PMA Increased IL-12 Production in Spleen Lymphocytes of Vaccinated Mice. Spleen lymphocytes were prepared by using lymphoprep from vaccinated mice and cytokines were analyzed by ELISA. IL-12 production was significantly increased

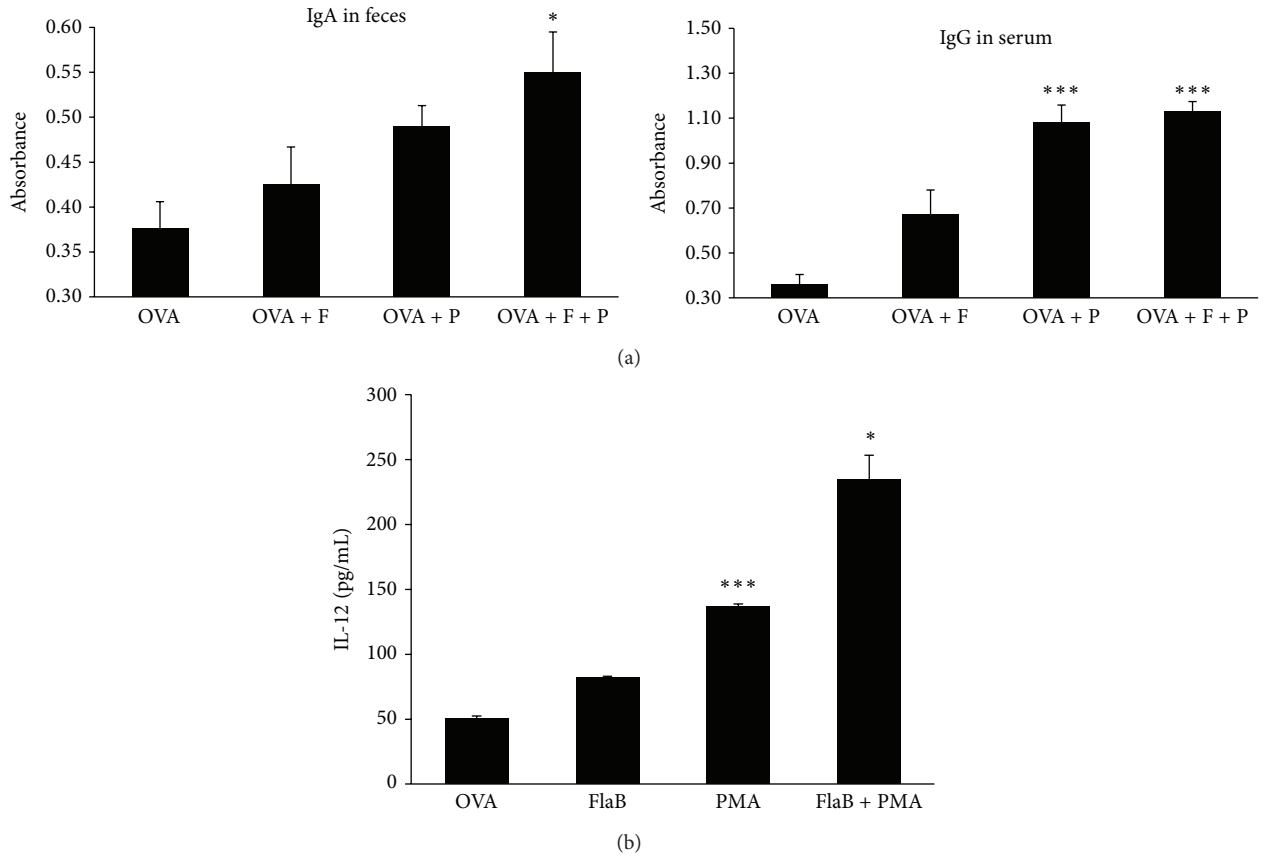

Figure 5: PMA potentiated FlaB vaccine adjuvant activity by the increase of IL-12 production. (a) PMA enhanced significantly OVA-specific IgA and IgG antibody responses. The BALB/c mice were intranasally immunized with OVA (100 μg) alone or OVA in combination with FlaB (F) or PMA (P), three times in 7-day intervals. OVA-specific antibodies were measured by ELISA. Feces IgA and serum IgG levels were increased in OVA plus FlaB and PMA mice group (OVA+F+P). (b) PMA induced IL-12 production in mice spleen lymphocytes. Spleen lymphocytes from vaccinated mice were cultured with OVA (10 μg/mL) at 37°C for 2 days. Cytokine levels were measured by sandwich ELISA kits following the manufacturer's experimental protocols. IL-12 was significantly increased in PMA combined mice splenocytes. The data indicate the mean and SEM from three experiments ($^{*}P < 0.05$, $^{***}P < 0.001$).

in spleen lymphocytes isolated from OVA-vaccinated mice treated with PMA (Figure 5(b)).

4. Conclusion and Discussion

The present study demonstrates the vaccine adjuvant effect of PMA isolated from *Croton tiglium*. To identify herbal medicines potentiating the vaccine adjuvant effect of flagellin, a plant library was screened using HEK293T cells transiently cotransfected with phTLR5 and pNF-κB-SEAP plasmids. Because HEK293T cells do not express any TLRs, the cells transfected with phTLR5 plasmid DNA were used for TLR5-dependent response in the absence of other TLRs. Flagellin, a TLR5 agonist, activated a transcription factor NF-κB only in the presence of TLR5 (Figure 1). In contrast, the 90% EtOH extract of *Croton tiglium* and its chloroform layer significantly stimulated NF-κB transcription both in TLR5− and TLR5+ screening systems (Figure 1). PMA was isolated and characterized as an active component from *Croton tiglium* (Figure 2) by activity-guided fractionation, column chromatography, HPLC, NMR, and MS. PMA induced NF-κB transactivation in a TLR5-independent manner and increased IL-8 production in Caco-2 cells constituently expressing some TLRs (Figure 3). These results indicate that PMA is not a specific ligand for TLR5. The effect of PMA was dependent on NF-κB translocation and PKC activation (Figure 4(b)). Finally, PMA or FlaB enhanced antigen-specific IgG and IgA antibody responses in intranasally OVA-immunized mice (Figure 5(a)). In addition, PMA increased IL-12 production corresponding to T cell responses (Figure 5(b)). The present study suggests that PMA activating PKC of TLR signaling pathway has a possibility for being an efficacious mucosal vaccine adjuvant.

Conflict of Interests

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

Authors' Contribution

Dool-Ri Oh and Hu Won Kang contributed equally to this paper. Young Ran Kim, Joon Haeng Rhee, and Won Keun

Oh participated in research design. Dool-Ri Oh, Hu Won Kang, and Jong-Ro Kim conducted experiments. Young Ran Kim, Sunoh Kim, and In-Kyu Park performed data analysis. Young Ran Kim, Dool-Ri Oh, and Hu Won Kang wrote or contributed to the writing of the paper.

Acknowledgments

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Research Article

Effect of Sipjeondaebo-Tang on Cancer-Induced Anorexia and Cachexia in CT-26 Tumor-Bearing Mice

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Cancer-associated anorexia and cachexia are a multifactorial condition described by a loss of body weight and muscle with anorexia, asthenia, and anemia. Moreover, they correlate with a high mortality rate, poor response to chemotherapy, poor performance status, and poor quality of life. Cancer cachexia is regulated by proinflammatory cytokines such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α). In addition, glucagon like peptide-1 (GIP-1), peptide YY (PYY), ghrelin, and leptin plays a crucial role in food intake. In this study, we investigated the therapeutic effects of one of the traditional herbal medicines, Sipjeondaebo-tang (Juzen-taiho-to in Japanese; SJDBT), on cancer anorexia and cachexia in a fundamental mouse cancer anorexia/cachexia model, CT-26 tumor-bearing mice. SJDBT was more significantly effective in a treatment model where it was treated after anorexia and cachexia than in a prevention model where it was treated before anorexia and cachexia on the basis of parameters such as weights of muscles and whole body and food intakes. Moreover, SJDBT inhibited a production of IL-6, MCP-1, PYY, and GLP-1 and ameliorated cancer-induced anemia. Therefore, our *in vivo* studies provide evidence on the role of SJDBT in cancer-associated anorexia and cachexia, thereby suggesting that SJDBT may be useful for treating cancer-associated anorexia and cachexia.

1. Introduction

Cancer anorexia and cachexia correlate with a high mortality rate, poor response to chemotherapy, poor performance status, and poor quality of life (QOL), particularly in physical, psychological, and social functions in cancer patients [1, 2]. Anorexia, which is characterized by the loss of desire to eat or loss of appetite, causes weight loss and malnutrition in cancer patients [3, 4]. Cachexia is a multifactorial condition described by muscle loss, which is in part associated with loss of body weight, anorexia, asthenia, and anemia [5, 6]. Cachexia occurs in about half of all cancer patients, and lung and upper gastrointestinal tract cancer patients have the highest frequency of cachexia, whereas patients with breast and lower gastrointestinal tract cancer have the lowest frequency of cachexia [7]. In addition, cachexia affects an ability of cancer patients to sustain chemotherapy or radiotherapy [8]. Therefore, it is important to manage anorexia and cachexia in cancer patients. However, current therapies for cancer patients with anorexia and cachexia are limited due to both poor efficacies and side effects of chemotherapeutics [9]. So, different therapeutic options are required to prevent cancer anorexia and cachexia.

Sipjeondaebo-tang (Juzen-taiho-to in Japanese and Shi-Quan-Da-Bu-Tang in Chinese; SJDBT), commonly used traditional herbal medicines in Korea, China, and Japan [10], is composed of 10 species of herbs. SJDBT is prescribed for patients suffering from anemia, fatigue, anorexia, scaly skin, and dryness of the mouth [11–13]. In addition, SJDBT has been known to have anticancer effects [14–19]. Nevertheless, its effect on cancer cachexia is poorly understood. Here, we examined the effect of SJDBT on key parameters of cancer anorexia and cachexia and found that SJDBT ameliorated cancer anorexia and cachexia *in vivo* by altering body and

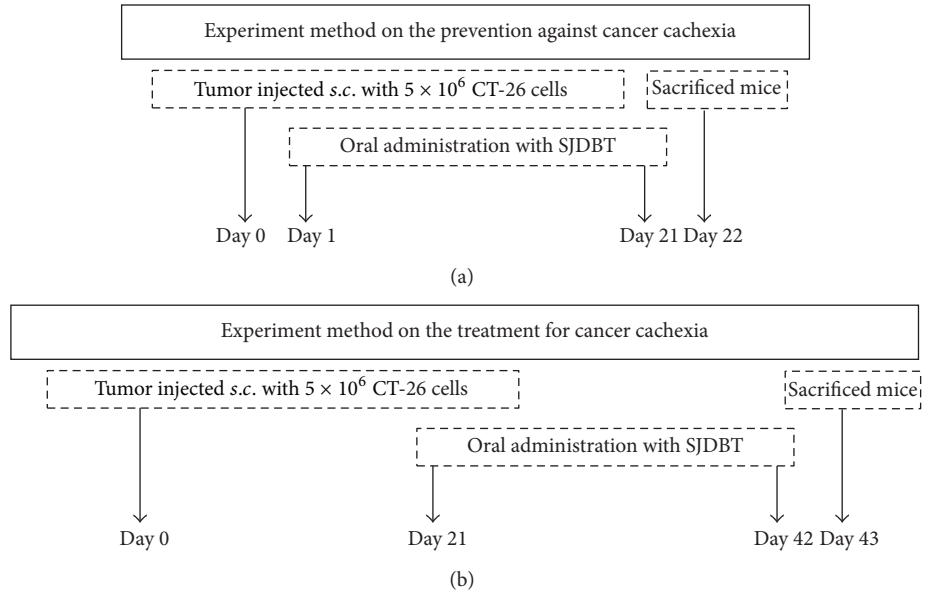

Figure 1: Schedules for prevention and therapy models of mouse cancer-induced cachexia. 5×10^6 CT-26 tumor cells were subcutaneously (*s.c.*) injected into the right flank of BALB/c mice. (a) Experiment method on the prevention: a day after tumor cell inoculation, three doses of SJDBT (L-SJDBT of 6.784 mg/kg, M-SJDBT of 67.84 mg/kg, and H-SJDBT of 678.4 mg/kg) were *p.o.* administrated daily for 21 days. At 22 days after tumor cell injection, mice were sacrificed. (b) Experiment method on the therapy: 21 days after tumor cell inoculation (a day when cachexia was held), three different doses of SJDBT (L-SJDBT of 6.784 mg/kg, M-SJDBT of 67.84 mg/kg, and H-SJDBT of 678.4 mg/kg) were *p.o.* administrated daily for 21 days. At the end of experiments (42 days after tumor cell injection), mice were sacrificed.

muscle weights, food intakes, and levels of cytokines, hormones, RBC, Hb, and HCT in CT-26 tumor-bearing mice suffering from anorexia and cachexia, thereby suggesting that SJDBT may be useful for treating cancer anorexia and cachexia.

2. Materials and Methods

2.1. Preparation of SJDBT. SJDBT were prepared by and obtained from Hanpoong Pharmaceutical Company (Jeonju, Korea) following the good manufacturing practices (GMP) procedures. SJDBT is manufactured as dried powder of hot water extract obtained from 10 herbs (320 g of *Angelica gigas*, 320 g of *Astragalus membranaceus*, 320 g of *Atractylodes japonica*, 320 g of *Cinnamomum cassia*, 320 g of *Cnidium officinale*, 160 g of *Glycyrrhiza uralensis*, 320 g of *Paeonia lactiflora*, 320 g of *Panax ginseng*, 320 g of *Poria cocos,* and 320 g of *Rehmannia glutinosa*; average yield = 28.85%). The dried powders were lyophilized and then dissolved the three different doses (low dose: 6.784 mg/kg, middle dose: 67.84 mg/kg, and high dose: 678.4 mg/kg) in distilled water.

2.2. Animal Study for Cancer Cachexia. Male BALB/c mice were purchased from Central Lab Animal Inc. (Seoul, Korea) at 6 weeks of age, and all mice were kept in pathogen-free environment. Mice were randomized into two groups (prevention or therapy model of cachexia). Each group was divided into subgroups (5 prevention groups: normal, control, low dose SJDBT (L-SJDBT of 6.784 mg/kg), middle dose SJDBT (M-SJDBT of 67.84 mg/kg), and high dose SJDBT (H-SJDBT of 678.4 mg/kg); 6 treatment groups: normal, control, L-SJDBT, M-SJDBT, H-SJDBT, and megestrol acetate- (MA-) treated). Animal studies were approved by the animal care center of Kyung-Hee University (KHUASP (SE)-12-048). The murine CT-26 tumor-bearing mouse was widely used for cancer cachexia model [20]. CT-26 colon carcinoma cell was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and from Dr. Suk-Chan Lee (Department of Genetic Engineering, Sungkyunkwan University, Suwon, Korea). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and 1% antibiotics. The schematic experimental procedure is described in Figure 1. For the prevention model of cancer-induced anorexia and cachexia, mice were injected *s.c.* with CT-26 cells (5×10^6). A day after tumor cell injection, three different doses of SJDBT (L-SJDBT, M-SJDBT, or H-SJDBT) were *p.o.* added daily for 21 days. For the treatment model of cancer-induced anorexia and cachexia, mice were injected *s.c.* with CT-26 cells (5×10^6) and then three different doses of SJDBT (L-SJDBT, M-SJDBT, or H-SJDBT) were *p.o.* added daily for 21 days at 3 weeks after tumor cell injection (Figure 6). MA (Santa Cruz Biotechnology, CA, USA) was used as a positive control for anorexic effect and dissolved in corn oil (100 mg/kg).

2.3. Measurement of Food Intakes and Weights of Whole Body and Muscles. Body weight and food intake were measured every day using an electronic scale. The measured quantity of food intake was divided by the number of mice to determine each intake per animal per day. At the time of sacrifice, the gastrocnemius muscles were dissected and weighted.

2.4. Measurement of Levels of Cytokines and Hormones and Blood Analysis. Whole blood samples were collected by cardiac puncture, and serum was obtained after being

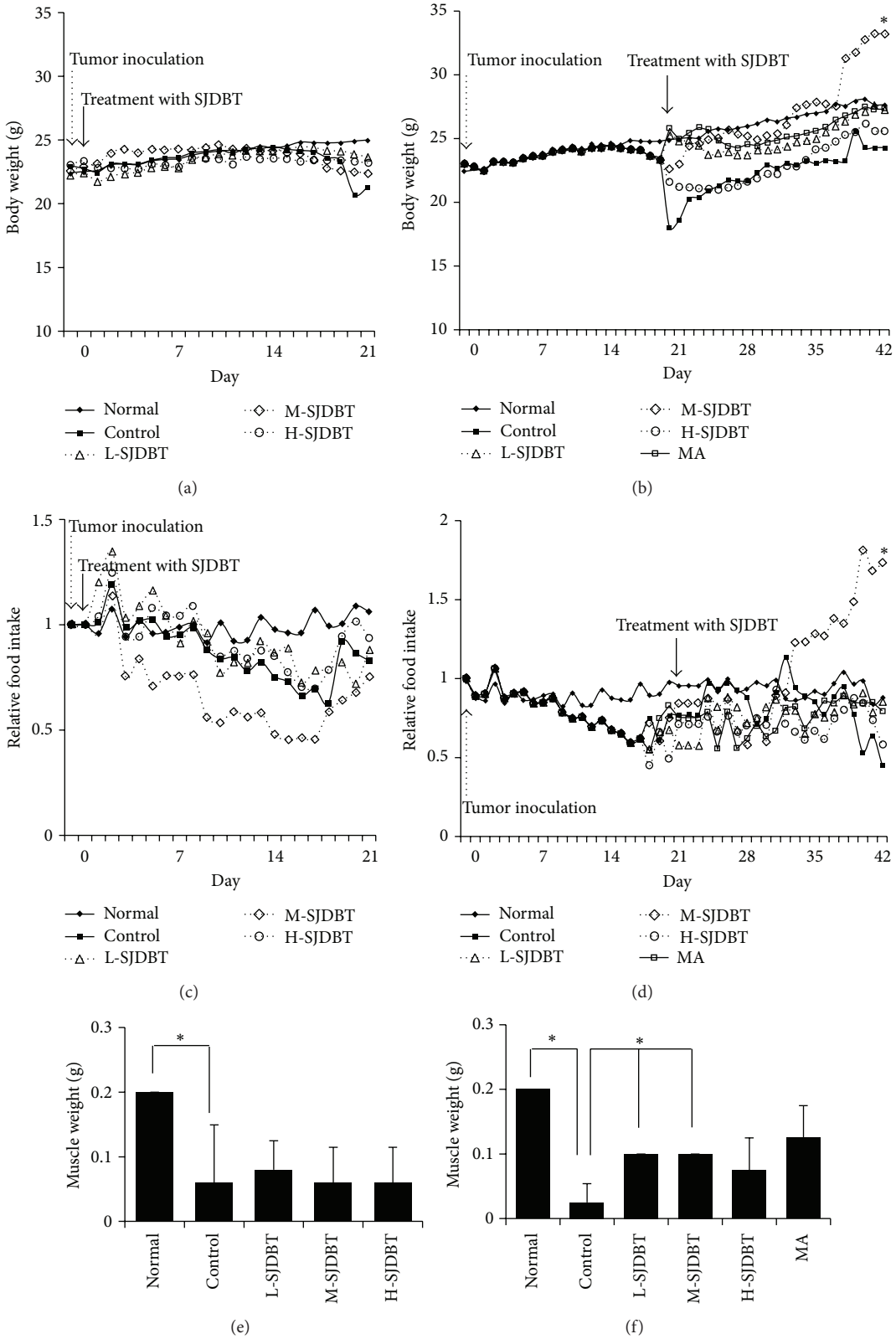

Figure 2: Effect of SJDBT on body weight and food intake in CT-26 tumor-bearing mice. ((a), (b)) Body weight of mice was measured every day at the same time. ((c), (d)) Food intake was measured every day at the same time. The measured quantity of food intake was divided by the number of total mice to determine each intake per animal a day. ◆: healthy normal mice ($n = 5$), ■: untreated control tumor-bearing mice ($n = 5$), △: low concentration (6.784 mg/kg) of SJDBT-treated tumor-bearing mice ($n = 5$), ◇: middle concentration (67.84 mg/kg) of SJDBT-treated tumor-bearing mice ($n = 5$), ○: high concentration (678.4 mg/kg) of SJDBT-treated tumor-bearing mice ($n = 5$), and □: megestrol acetate-treated tumor-bearing mice ($n = 5$). $^*P < 0.05$.

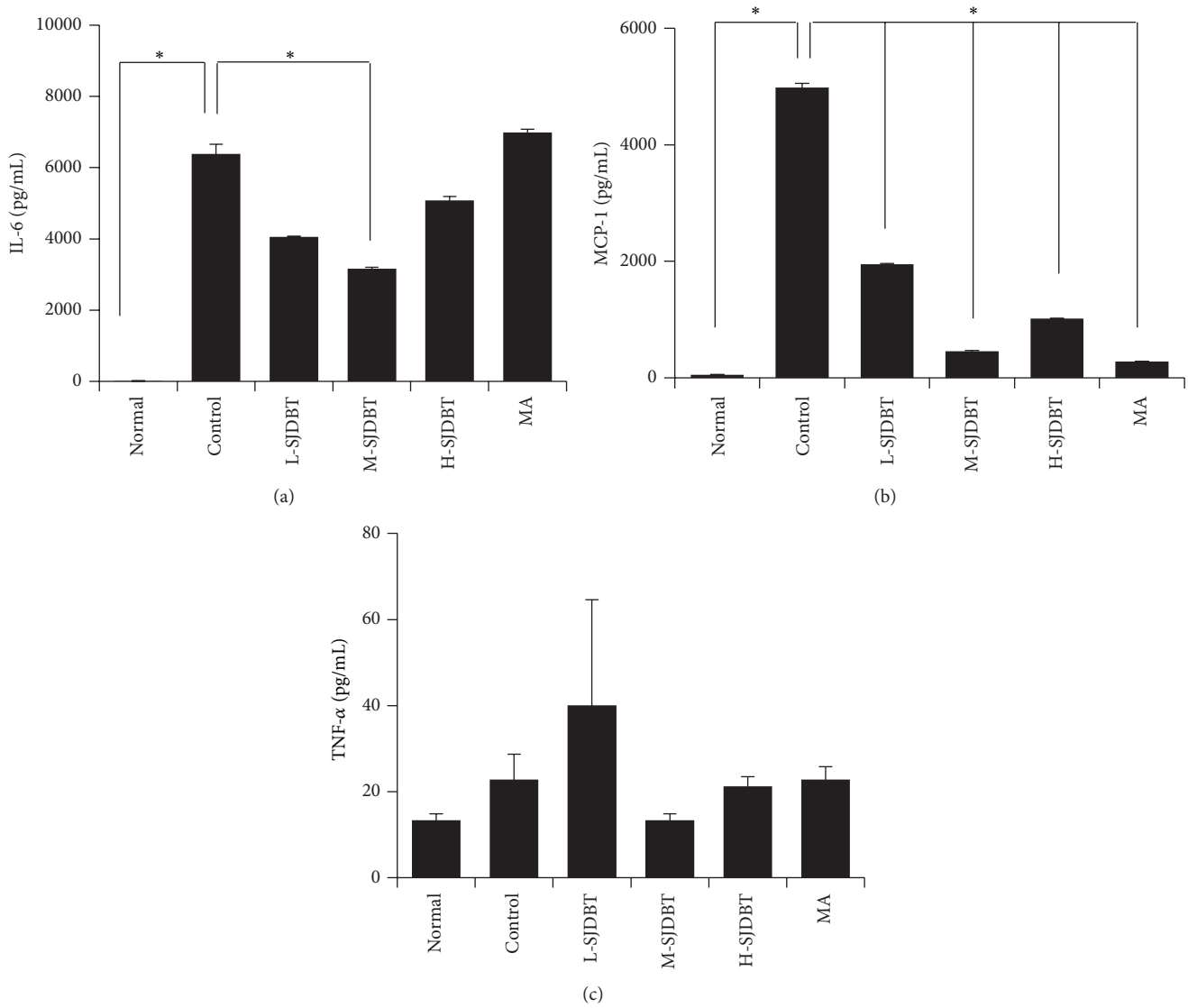

Figure 3: Effect of SJDBT on serums IL-6, MCP-1, and TNF-α levels in CT-26 tumor-bearing mice. ((a), (b)) 21 days after tumor cell injection, SJDBT were *p.o.* administrated every day until 42 days. At the end of experiments, mice were sacrificed. Blood samples were collected by cardiac puncture and serum samples were obtained blood sample after centrifuged. IL-6, MCP-1, and TNF-α level were measured using a Milliplex Mouse Metabolic Magnetic Bead Panel MMHMAG-44K-14. Experiments were performed in duplicate. Bars indicate means and standard deviations. $^{*}P < 0.05$.

centrifuged from the whole blood. Cytokines and hormones level were measured using a Milliplex Mouse Metabolic Magnetic Bead Panel MMHMAG-44K-14 (Millipore, MO, USA) in a Luminex 200. Standards were plotted and concentrations were determined using Milliplex Analyst software version 5.1. The blood samples were placed in Vacutainer TM tubes containing EDTA (BD Science, NJ, USA). Blood analysis was performed using the HEMAVET 950 hematology analyzer (Drew Scientific, Inc., Oxford, CT) in accordance with manufacturer's recommendation.

2.5. Statistics. Data were presented as the mean and standard deviation. *P* values less than 0.05 in two-tailed student's *t*-test or one-way ANOVA were considered statistically significant.

3. Results

3.1. SJDBT Improves Cancer-Induced Weight Loss and Anorexia in CT-26 Tumor-Bearing Mice. To examine whether SJDBT prevents cancer-induced cachexia or treats cancer-induced cachexia, we divided into two groups (Figure 1). A day when CT-26 tumor cells were injected into mice was designated as day 0. We found that control groups in each experimental set compared to the normal group showed reductions of body weights by approximately 14.8% and 36.8%, respectively, at 21 days after tumor cell inoculations (Figures 2(a) and 2(b)), confirming that the injection of CT-26 tumor cells resulted in the loss of body weight. SJDBT was treated for 21 days prior to cancer-induced anorexia and cachexia in the prevention model and treated for 21 days after

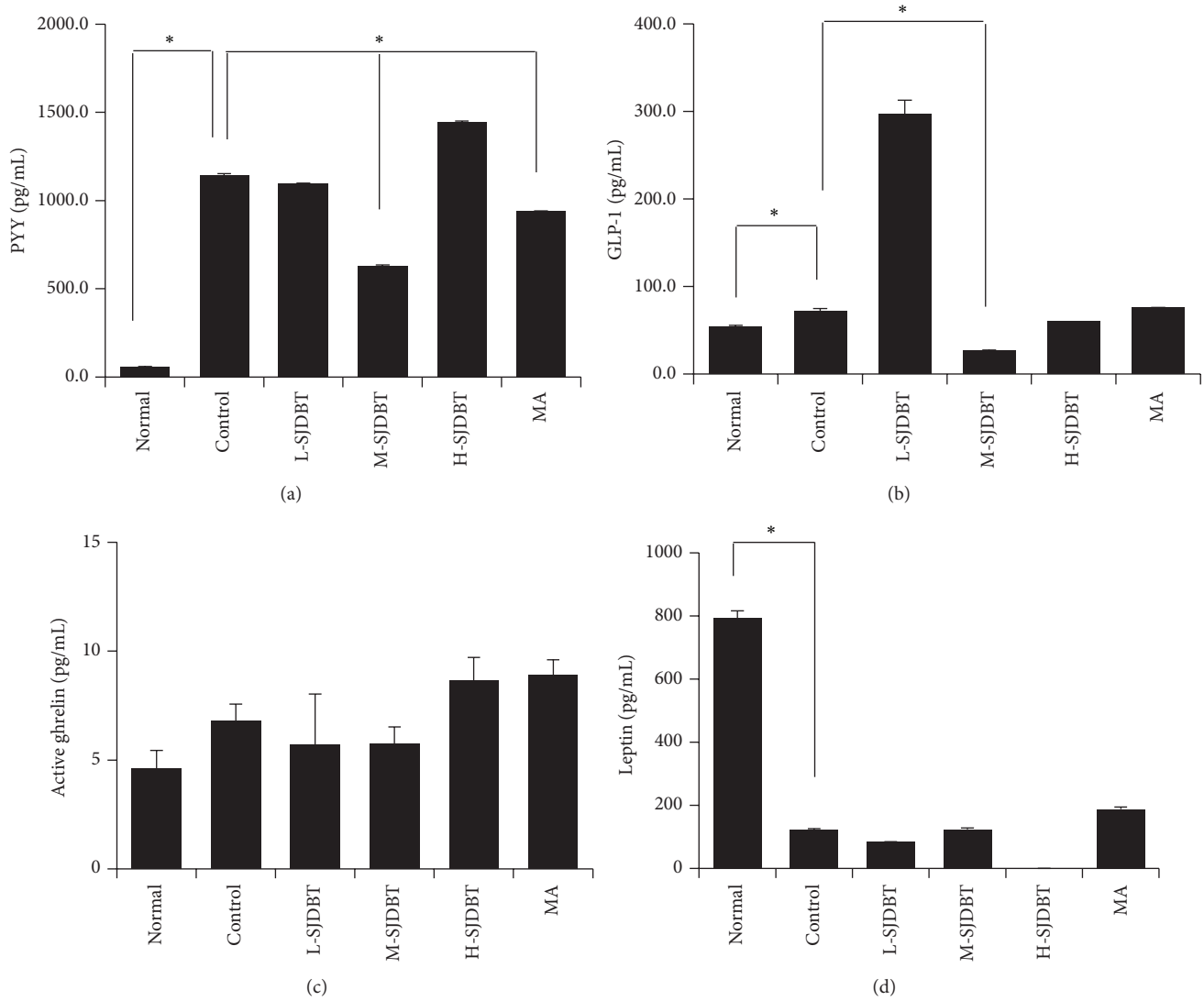

Figure 4: Effect of SJDBT on serums GLP-1, PYY, ghrelin, and leptin level in CT-26 tumor-bearing mice. ((a), (b)) 21 days after tumor cell injection, SJDBT were *p.o.* administrated for 21 days. At the end of experiments, mice were sacrificed. Blood samples were collected by cardiac puncture and serum samples were obtained blood sample after centrifuged. GLP-1, PYY, ghrelin, and leptin level were measured using a Milliplex Mouse Metabolic Magnetic Bead Panel MMHMAG-44K-14. Experiments were performed in duplicate. Bars indicate means and standard deviations. $^{*}P < 0.05$.

cancer-induced anorexia and cachexia in a therapy model. As shown in Figure 2(a), we found that L-SJDBT and H-SJDBT compared to the control increased body weights slightly by approximately 11.1% and 8.9%, respectively, while being not statistically significant. Otherwise, when SJDBT were administered into mice after cancer-induced weight loss, M-SJDBT-treated mice compared to the control significantly increased the weight by approximately 36.6% (Figure 2(b)). Therefore, SJDBT appeared to treat tumor-induced loss of body weight. Next, we performed experiments to determine whether SJDBT improves the appetite in a model of either prevention or treatment. L-SJDBT and H-SJDBT, compared to the control, ameliorated cancer-induced anorexia by approximately 25.3% and 25.1%, respectively, in the prevention model (Figure 2(c)). In addition, M-SJDBT compared to the control significantly increased food intake in the therapy model (Figure 2(d)). The control group compared to the normal decreased muscle weight by approximately 70% at 22 days after tumor cell injection and by approximately 87.5% at 43 days after tumor cell inoculation (Figures 2(e) and 2(f)). Both L-SJDBT and M-SJDBT prevented tumor-induced loss of muscle weight in the therapy model, since muscle weights of tumor-bearing mice treated with each of them were higher than those of tumor-bearing mice untreated by approximately 4-fold (Figure 2(f)). However, those were not effective in the prevention model (Figure 2(e)). Those results indicate that SJDBT may be effective for treating anorexia and for attenuating cachectic phenomenon, loss of muscle weight. Furthermore, our data showed that M-SJDBT was much more effective than MA in the treatment of cancer-induced reduction of food intake, while MA has been used to treat anorexia.

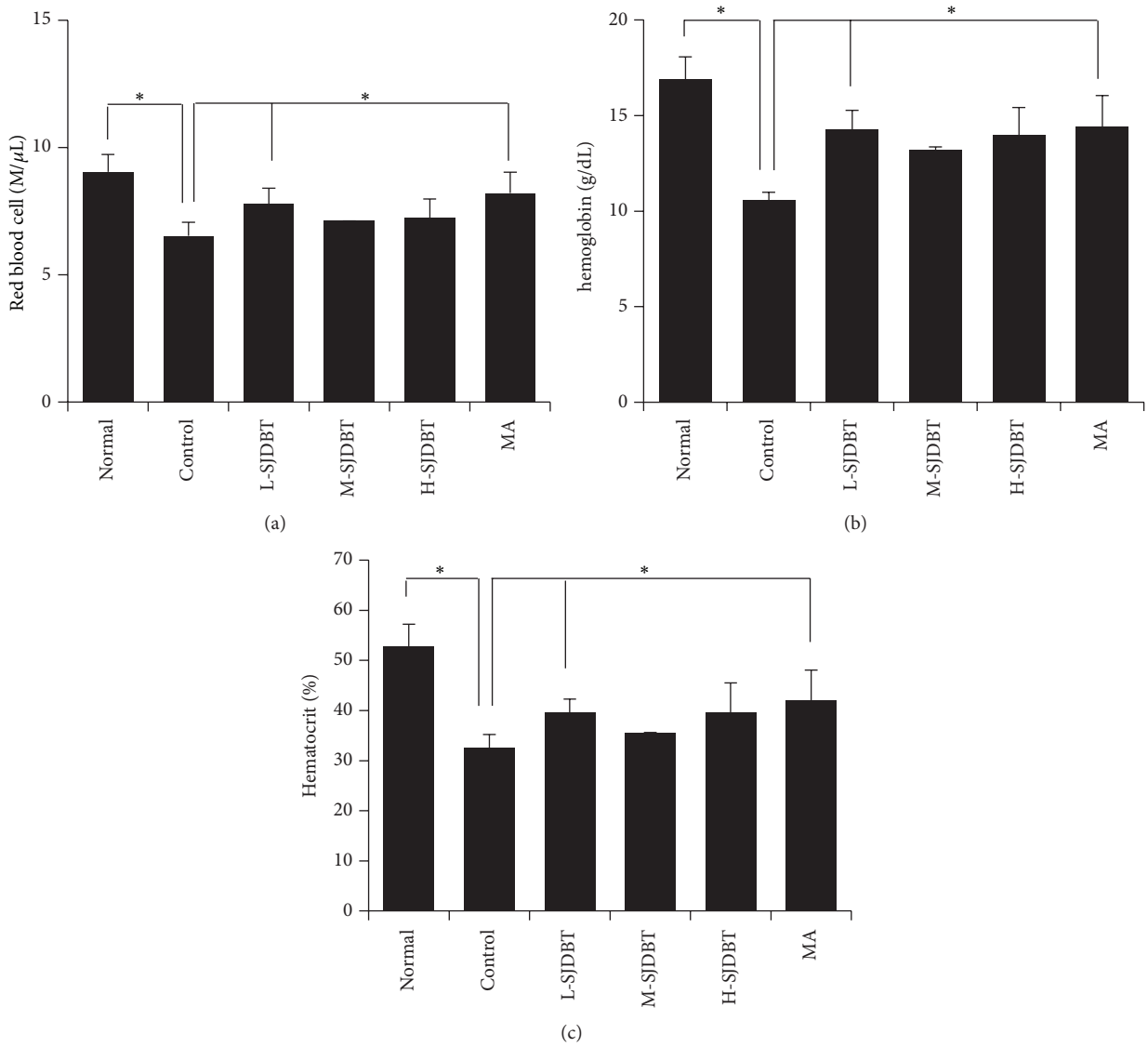

Figure 5: Blood analysis. ((a), (b), and (c)) 21 days after tumor cell injection, SJDBT were *p.o.* administrated for 21 days. At the end of experiments, mice were sacrificed. Whole blood samples were collected by cardiac puncture. The blood was placed in Vacutainer TM tubes containing EDTA (BD science, NJ, USA). Blood analysis was performed using the HEMAVET 950 hematology analyzer (Drew Scientific, Inc., Oxford, CT, USA) in accordance with manufacturer's recommendation.

3.2. SJDBT Suppressed IL-6 and MCP-1 but Not TNF-α in the Therapy Model. As proinflammatory cytokines such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) derived from tumor cells were associated with cancer-related anorexia and cachexia [21, 22], we next examined whether SJDBT affects serum levels of IL-6, MCP-1, and TNF-α. Levels of IL-6 and MCP-1 significantly increased in the control compared to the normal by approximately 480-fold and 91-fold, respectively (Figures 3(a) and 3(b)). In addition, M-SJDBT significantly inhibited cancer-induced induction of IL-6 and MCP-1 by approximately 50.5% and 90.8%, respectively (Figures 3(a) and 3(b)). Furthermore, MCP-1 level was uniquely altered in all tested doses of SJDBT. Whereas the serum level of TNF-α increased slightly in the control compared to the normal by approximately 1.7-fold, M-SJDBT compared to the control reduced TNF-α level by approximately 41.4% while it was not statistically significant (Figure 3(c)). These results suggest that M-SJDBT may ameliorate cancer-induced anorexia and cachexia by altering levels of cytokines such as IL-6 and MCP-1.

3.3. Effects of SJDBT on Levels of Gut Hormones and Leptin in the Therapy Model. As anorexia involves appetite-regulating hormones such as gut hormones (GLP-1, PYY, and ghrelin) and leptin [23, 24], we further examined whether SJDBT affects their levels in serum. Levels of GLP-1 and PYY significantly increased in the control compared to normal by approximately 1.3-fold and 20-fold, respectively (Figures 4(a) and 4(b)). M-SJDBT significantly inhibited cancer-induced induction of both GLP-1 and PYY by approximately 63.2% and 44.9%, respectively (Figures 4(a) and 4(b)). However,

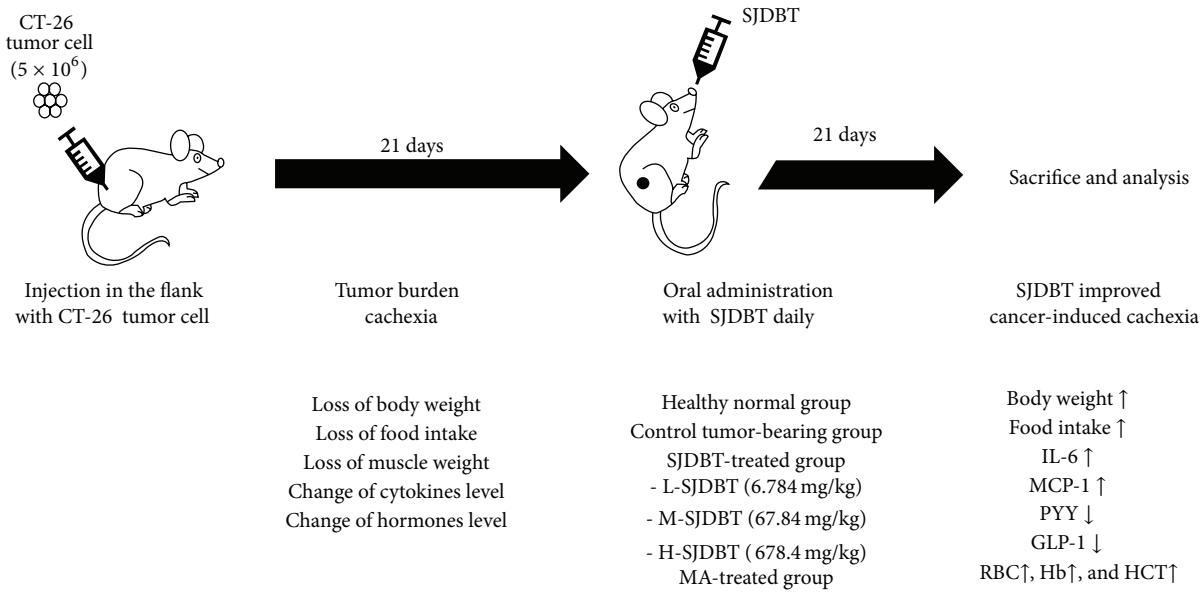

Figure 6: Schematic illustration. To set up the tumor burden cachexia mice model, mice were injected *s.c.* with CT-26 tumor cells. 21 days after tumor cell injection, three doses of SJDBT (L-SJDBT, M-SJDBT, and H-SJDBT) and MA were *p.o.* added daily. After treatment with indications for 21 days, mice were sacrificed and blood samples were analyzed for blood analysis and cytokines and hormones level in serum. SJDBT improved cancer cachexia in mice and inhibited the production of IL-6, MCP-1, PYY, and GLP-1. Therefore, we conclude that SJDBT improves cancer-induced cachexia including weight loss, anorexia, muscle wasting, anemia, and dysregulation of cytokines and hormones.

levels of ghrelin and leptin were not altered by M-SJDBT. Those results suggest that M-SJDBT regulation of GLP-1 and PYY may affect cancer-associated anorexia and cachexia.

3.4. SJDBT Improves Cancer-Induced Anemia in CT-26 Tumor-Bearing Mice. Because cancer-induced anorexia and cachexia are a multifactorial syndrome including anemia [25], we measured levels of red blood cells in blood samples using HEMAVET 950 hematology analyzer. The control compared to the normal accrued anemia, which was measured by numbers of red blood cells (RBC; normal mice versus control mice; 9.04 ± 0.68 M/μL versus 6.53 ± 0.44 M/μL; $P = 0.02$), hemoglobins (Hb; normal mice versus control mice; 16.91 ± 1.15 g/dL versus 10.6 ± 0.38 M/μL; $P = 0.0001$), and hematocrits (HCT; percentage of red blood cells in whole blood; normal mice versus control mice; $52.9 \pm 4.32\%$ versus $32.53 \pm 2.68\%$; $P = 0.03$). L-SJDBT compared to the control significantly increased values of RBC, Hb, and HCT by approximately 18%, 34.7%, and 22%, respectively (Figures 5(a), 5(b), and 5(c)). In addition, MA similarly affected those values, indicating that the treatment of anorexia might be linked to alterations of those values. While M-SJDBT compared to the control increased values of RBC, Hb, and HCT by approximately 7.6%, 24.5%, and 9.4%, respectively, those were not statistically significant. Therefore, those results indicate that L-SJDBT may be a good remedy for cancer-mediated anemia.

4. Discussion

As herbal medicine therapy has been known to be beneficial with high efficacy and safety, market of herbal medicine therapy is growing in Korea, China, and Japan as well as in the United States [26]. Especially, herbal medicines are used for clinical trial and therapy in cancer patients [27, 28]. However, biomedical scientists have limitations to use the herbal medicines due to the lack of evidence even in lab-based experimental studies [28]. For example, SJDBT is widely used for cancer patients suffering from symptoms such as loss of appetite, fatigue, and anemia in Korea, China, and Japan [10, 12, 13, 29]. However, its effect on the loss of either weight or food intake is still unknown. In this present study, we showed SJDBT effect on cancer anorexia and cachexia.

Cancer-induced anorexia and cachexia involve proinflammatory cytokines such as IL-6, MCP-1, and TNF-α [21, 22]. Furthermore, it has been suggested that MCP-1 rather than TNF-α is more important for cancer cachexia [22, 30, 31]. Circulating level of IL-6 is known to be important for keeping body weight and for survival in cancer patients [32–34]. In our study, SJDBT reduced IL-6 but increased MCP-1, while it did not affect TNF-α. Thus, it is plausible that SJDBT may modulate effects of cytokines on cancer-induced anorexia and cachexia. Meanwhile, GLP-1, PYY, ghrelin, and leptin are known as readouts for anorexia/cachexia [35]. SJDBT significantly suppressed GLP-1 and PYY levels, whereas it did not affect ghrelin and leptin. In sum, our data suggest that SJDBT ameliorates cancer-associated anorexia/cachexia by regulating cytokines (IL-6 and MCP-1) and hormones (GLP-1 and PYY). Anemia is tone of common features of cancer cachexia [6, 8, 36]. Especially, anemia is linked to the fatigue and QOL in cancer patients [36]. In addition, Hb level is supposed to be related to QOL [37]. SJDBT increased values of RBC, Hb, and HCT, which further indicates that SJDBT is beneficial for treating cancer anorexia/cachexia.

Cancer-associated anorexia is related to central mechanisms governing food intake [38]. Food intake is controlled by neuroendocrine paths through central nervous system (CNS) [39]. Short-term reaction for food intake is mediated by peptides produced by enteroendocrine cells in the gastrointestinal tract [39]. Incretin hormones such as GLP-1 mediate a response of the pancreas to nutrients, resulting in the reduction of food intake. Likewise, ghrelin is secreted from the stomach and promotes appetite. Leptin mediates long-term reaction for food intake. It is produced mainly from the adipose and stimulates food intake through CNS [40]. Furthermore, cancer-associated anorexia is caused by chronic stimulations via cytokines released from cancer cells or from host cells responding to cancer cells [41]. Cytokines have been known to interrupt with appetite-stimulating signaling [42]. Furthermore, cytokines impair muscle and fat metabolisms, which is tightly linked to cancer-associated anorexia [42, 43]. While cytokines such as TNF-α and IL-6 have been known to mediate cancer cachexia [42], SJDBT reduced IL-6 level without altering TNF-α. IL-6 is known to cause the loss of lean body weight in CT-26 tumor-bearing cachectic mice by inducing proteolytic pathways [44]. In addition, MCP-1, the circulating level of which was altered by SJDBT, has been known to regulate leptin level in the adipose and to mediate cachexia [22, 45]. Therefore, it is plausible that SJDBT may target cytokine production in cancer cells, which may result in modulations of hormone levels. However, we could not exclude possibilities that SJDBT may target multiple sources including cancer cells, cancer-associated immune cells, adipocytes, and hypothalamic neurons. Recent studies have revealed functional relationships between cytokines (IL-6 and MCP-1) and hormones (GLP-1 and PYY) [46–51]. Thus, it remains to be deciphered molecular and cellular mechanisms of SJDBT to ameliorate cancer-associated anorexia and cachexia.

5. Conclusion

Our study demonstrates that the SJDBT ameliorates cancer-induced anorexia and cachexia in CT-26 tumor-bearing mouse model by altering the production of IL-6, MCP-1, PYY, and GLP-1. Our *in vivo* studies first provide evidence on the role of SJDBT in cancer-associated anorexia/cachexia, which suggests that SJDBT may be useful for patients with cancer-associated anorexia/cachexia.

Conflict of Interests

The authors declare that they have no conflict of interests.

Author's Contribution

Youn Kyung Choi, Sang-Mi Woo, and Yee Jin Yun carried out the experiment. Youn Kyung Choi, Ki Yong Jung, and Sang-Mi Woo analyzed data and wrote the paper. Chan-Yong Jun, Jong Hyeong Park, and Yong Cheol Shin assisted in data analyses. Sung-Gook Cho designed experimental concept and revised the paper. Seong-Gyu Ko designed concepts and supervised the study. Youn Kyung Choi, Ki Yong Jung, and Sang-Mi Woo equally contributed to this work.

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Research Article

Protective Effects of Pretreatment with Oleanolic Acid in Rats in the Acute Phase of Hepatic Ischemia-Reperfusion Injury: Role of the PI3K/Akt Pathway

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Oleanolic acid (OA) has been used to treat liver disorders, but whether it can attenuate hepatic ischemia-reperfusion- (IR-) associated liver dysfunction remains unexplored. In the present study, 160 male Sprague-Dawley rats were equally divided into five groups: group SH received neither hepatic IR nor drugs; group IR received hepatic IR without drugs; group CM and group OA received 0.5% sodium carboxymethylcellulose and 100 mg/kg OA, intragastrically, once a day for seven days before the hepatic IR, respectively; on the basis of treatment in group OA, group OA+wortmannin further received 15 μg/kg of PI3K inhibitor wortmannin, intraperitoneally, 30 min before the hepatic IR. Then each group was equally divided into four subgroups according to four time points (preoperation, 0 h, 3 h, and 6 h after reperfusion). Serum ALT activity, IL-1β concentration, and hepatic phosphorylation of PI3K, Akt, and GSK-3β protein expression were serially studied. We found that OA pretreatment improved histological status and decreased serum ALT and IL-1β levels. It also increased p-PI3K, p-Akt, and p-GSK-3β protein expression at all the four time points. Prophylactic wortmannin partially reversed OA's protective effects. The data indicate that OA pretreatment protects liver from IR injury during the acute phase partially through PI3K/Akt-mediated inactivation of GSK-3β.

1. Introduction

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid, OA) is a pentacyclic triterpene acid which exists naturally in vegetable oil, food, and some medicinal herbs [1]. OA provides protection against experimental hepatic injury in rats and has been used as an oral remedy for human liver dysfunction [2, 3]. Pretreatment with OA also considerably decreases augmentation of serum alanine transaminase (ALT) activity and hepatic centrilobular necrosis induced by chemical hepatotoxicants in mice [4, 5]. However, the molecular mechanisms of OA-mediated hepatic protection remain unclear.

Surgeries such as liver transplantation, partial hepatic resection, and hepatic tumor resection may result in hepatic ischemia-reperfusion (IR) injury, which correlates with clinical reduction of liver function [6, 7]. A pathological consequence of the cessation of hepatic blood supply, followed by reperfusion, is cellular damage within the ischemic areas, which induce formation of reactive oxygen species, release of proinflammatory cytokines, and hepatocyte necrosis, ending in liver dysfunction [8]. Despite the evidence of OA protection against myocardial or cerebral IR injury [9, 10], the potential for this compound to attenuate hepatic IR-induced injury remains unexplored.

Glycogen synthase kinase (GSK-3β) activity has recently been identified in a number of studies as crucial in the regulation of the inflammatory response [11, 12]. Its inactivation is also involved in indirubin-3′-oxime-induced protection

against hepatic IR injury in rats [13]. Phosphatidylinositol-3-kinase (PI3K)/protein kinase B(Akt) dependent inhibition of GSK-3β activity plays an important role in the protective effects of carbon monoxide against hepatic IR injury [14]. These promising results prompted us to investigate the possible beneficial effects of OA pretreatment in rats submitted to partial hepatic IR. In a mechanistic approach, activation of the PI3K/Akt pathway in hepatic tissue was also evaluated.

2. Materials and Methods

2.1. Chemicals. OA (purity > 98%) was purchased from Guiyang Pharmaceutical Company. Sodium carboxymethylcellulose (CMC-Na) was purchased from SINOPHARM (Shanghai, China). Wortmannin was purchased from Sigma (St. Louis, USA) and dissolved in dimethyl sulfoxide. Antibodies were purchased from Cell Signaling (Boston, USA). All other chemicals were of highest purity commercially available.

2.2. Animal Care and Hepatic IR Procedure. After approval by the Institutional Animal Care and Use Committee at Nanjing Medical University (Nanjing, China), 160 male SPF Sprague-Dawley rats (230–250 g) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). They were maintained at a 12:12 h light: dark cycle and given water ad libitum.

Each rat was anesthetized with 2.5% isoflurane with a small rodent face mask. Partial hepatic ischemia was conducted by performing a midline laparotomy exposing the liver hilum, and subsequent clamping of portal structures to the left and median lobes with a microvascular clip, yielding 70% hepatic ischemia [15]. The abdomen was covered during the ischemic period. After 60 min of 70% hepatic ischemia, the clip was removed to initiate hepatic reperfusion. The abdominal cavity was stitched with 4-0 silk sutures. The rectal temperature was maintained at 37 ± 0.5°C with a warming pad throughout the surgical process. The rats were injected with buprenorphine (0.1 mg/kg s.c) for postoperative analgesia (five rats, three of them were from anesthetic accident and the remaining two rats from postoperative infection). Therefore, the survival rate in this study was nearly 97%.

The 160 rats were randomly but evenly assigned to one of five groups: rats that received a sham operation (SH group) were submitted neither to the clamping procedure nor to drugs; rats in the IR group underwent IR treatment without drug delivery; rats in the CM group received 0.5% CMC-Na once a day for seven days, followed by IR; and rats in the OA group received 100 mg/kg OA once a day for seven days [16], followed by IR; on the basis of treatment in group OA, group OA+wortmannin further received 15 μg/kg of PI3K inhibitor wortmannin, intraperitoneally, 30 min prior to hepatic IR [17]. OA suspension (20 mg/mL) was made with 0.5% CMC-Na aqueous solution. With regard to daily substance delivery, SH and IR rats received 2 mL physiological saline, the CM group received 0.5% CMC-Na aqueous solution, and the OA group received OA suspension. Moreover, according to different time points for collecting serum and hepatic specimens, each group was further equally divided into the following four subgroups: preoperation (Prep), 0 h, 3 h, and 6 h after reperfusion.

2.3. Hepatic Histological Examination. Rat livers were collected 6 h after IR and were cut into small tissue blocks (about 1 cm in length, width, and height). After the blocks had been fixed with 10% buffered formalin solution, they were embedded in paraffin. Paraffin-embedded sections (10 μm thick) were prepared as usual for histological examination after hematoxylin and eosin staining.

2.4. Measurements of Serum ALT Activity and IL-1β Concentration. Serum ALT activity and the concentrations of IL-1β were measured by using ALT assay kit (Jiancheng Bioengineering, Nanjing, China) and IL-1β ELISA kit (USCN, Wuhan, China), respectively. Blood was taken from the postcaval vein at the four aforementioned time points and then centrifuged for 10 min. ALT activity in the serum was determined as described previously and expressed as international units per liter (U/L) [18]. Production of IL-1β in the serum was measured by ELISA kit according to the manufacturer's instructions.

2.5. Western Blot Analysis. The rats were all sacrificed by decapitation at the four time points. The livers were quickly excised free from any adhering tissues. Total tissue extracts were obtained by lysing the liver in ice-cold RIPA buffer in the presence of a cocktail of protease inhibitors (Roche, Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium orthovanadate). After centrifugation, protein concentrations were quantified using the Bradford method, and 70 μg of protein for each sample was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were subsequently electrotransferred onto polyvinylidene difluoride membranes. The blots were blocked with 5% milk powder and 0.1% Tween 20 in 10 mM Tris-HCl (pH 7.5) at room temperature for 2 h and then incubated with rabbit anti-p-PI3K, p-Akt, Akt, p-GSK3β, and GSK3β (1 : 1000, 1 : 1000, 1 : 500, 1 : 1000, and 1 : 500, resp.) at 4°C overnight. After three washes with TBS-T, the blots were then incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody at a dilution of 1 : 10,000 at room temperature for 1 h. The bound antibodies were visualized using an ECL system and exposed to X-ray films (Kodak, Rochester, NY, USA). The immunoblots were washed briefly and then incubated with a rabbit anti-GAPDH (1 : 2000) for 40 min at room temperature, followed by a horseradish peroxidase conjugated anti-rabbit antibody. GAPDH protein was then visualized and detected as the loading biomarker of proteins mentioned above.

2.6. Data Analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, USA; version 5.00). Data were expressed as means ± SD. For the analysis of normally distributed data, one-way analysis of

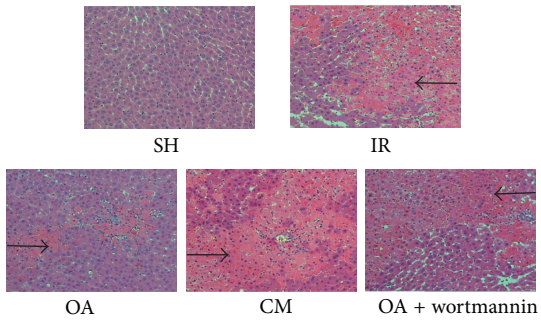

Figure 1: Effects of pretreatment with OA on partial hepatic IR injury evaluated by histological examination of rat liver tissue. The sections of the five groups were prepared at 6 h after reperfusion (HE, ×400). Arrows indicate necrotic areas of liver tissue.

variance was used followed by Dunnett's t multiple comparison tests. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of PI3K with Wortmannin Partially Reversed Protective Effects of OA on IR-Induced Hepatic Injury. Histologically, whereas necrotic areas were significantly decreased in the OA pretreated group at 6 h after reperfusion, the PI3K inhibitor wortmannin worsened the hepatic histological status (Figure 1). Biochemically, the IR procedure strikingly increased serum ALT activity at 3 h and 6 h after reperfusion, indicating that the partial hepatic IR model was established successfully (Figure 2; $P < 0.05$). At the same time points, pretreatment with 0.5% CMC-Na alone (group CM) did not affect serum ALT activity, indicating that CMC-Na, which was used as a suspending agent, had no effects on liver function. In contrast, rats pretreated with 100 mg/kg OA had drastically inhibited serum ALT activity at 3 h and 6 h after reperfusion ($P < 0.05$), which reflected protective effects of OA against IR-induced hepatic injury. When compared with group OA, inhibition of PI3K with 15 μg/kg wortmannin increased serum ALT activity at 3 h and 6 h after reperfusion ($P < 0.05$). However, serum ALT activity at 3 h and 6 h after reperfusion in group OA+wortmannin was still lower than those in group IR and group CM ($P < 0.05$), suggesting that protective effects of OA involved only partially the PI3K pathway.

3.2. PI3K Inhibitor Wortmannin Partially Abrogated Protective Effects of OA on Serum IL-1β Concentrations. Serum IL-1β concentrations were increased following the IR procedure, not only at 0 h after reperfusion, but also at 3 h and 6 h after reperfusion in both IR and CM groups (Figure 3). However, these changes were markedly decreased by OA pretreatment ($P < 0.05$), indicating that OA exerted anti-inflammatory effects against systemic injury induced by partial hepatic IR. Furthermore, suppression of IL-1β concentrations by OA pretreatment actually emerged at 0 h after reperfusion, prior to the decrease in ALT activity. When compared with group

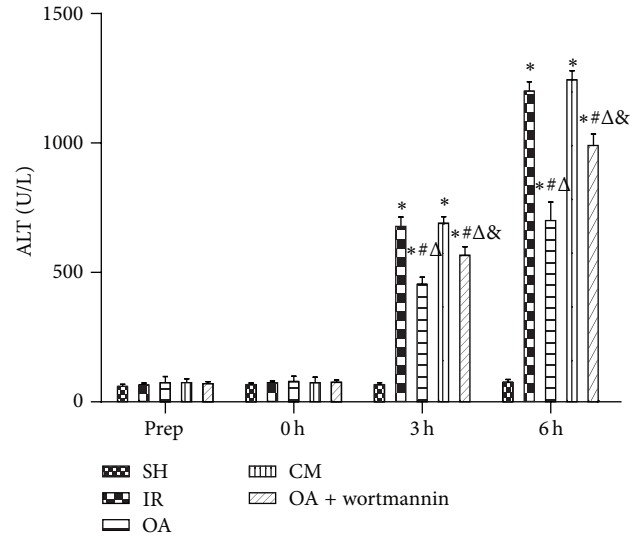

Figure 2: Effects of pretreatment with OA on the activity of ALT in rats induced by partial hepatic IR. Data are represented as means ± SD ($n = 8$). $^{*}P < 0.05$, compared with group SH. $^{\#}P < 0.05$, compared with group IR. $^{\Delta}P < 0.05$, compared with group CM. $^{\&}P < 0.05$, compared with group OA.

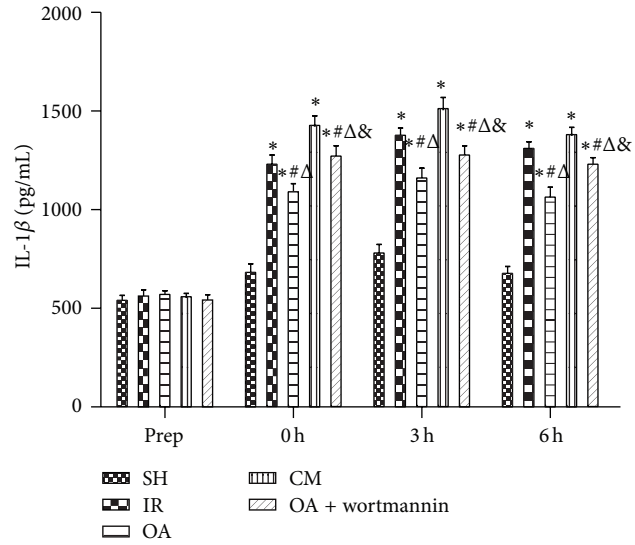

Figure 3: Effects of pretreatment with OA on the release of IL-1β in rats induced by partial hepatic IR. Data are represented as means ± SD ($n = 8$). $^{*}P < 0.05$, compared with group SH. $^{\#}P < 0.05$, compared with group IR. $^{\Delta}P < 0.05$, compared with group CM. $^{\&}P < 0.05$, compared with group OA.

OA, wortmannin increased serum IL-1β concentration at 0 h, 3 h, and 6 h after reperfusion ($P < 0.05$). However, the serum IL-1β concentration at 3 h and 6 h after reperfusion in group OA+wortmannin remained lower than those in group IR and group CM ($P < 0.05$).

3.3. OA Pretreatment Enhances PI3K, Akt, and GSK-3β Phosphorylation. To further evaluate the mechanism of OA-induced protective effects against IR-stimulated hepatic

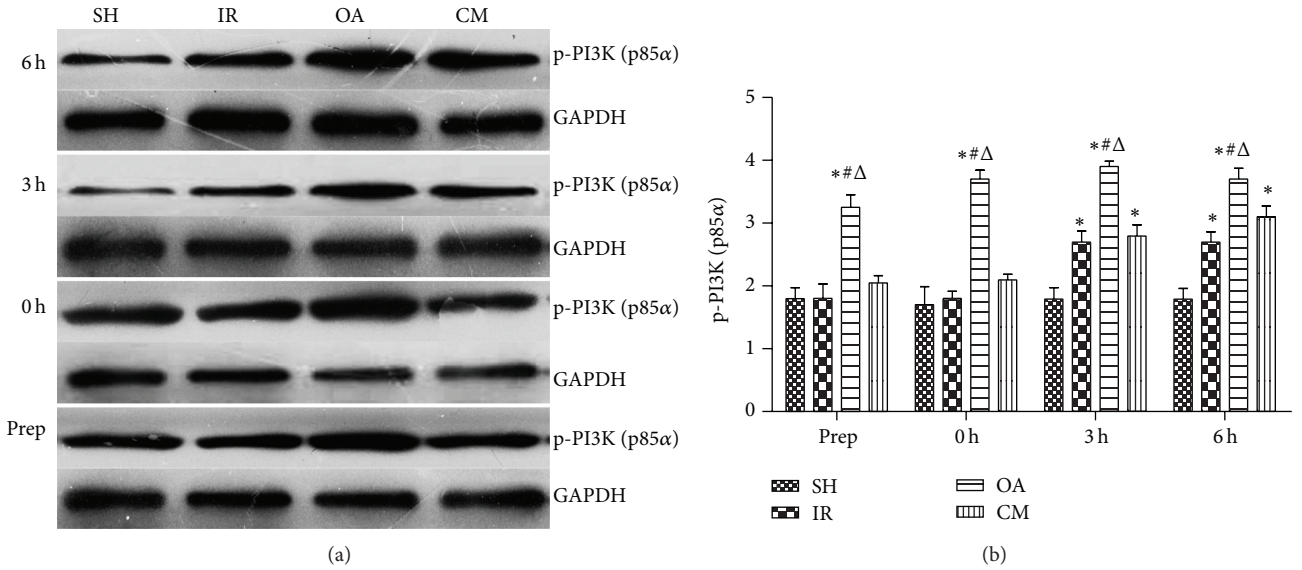

Figure 4: Effects of pretreatment with OA on p-PI3K (p85α) protein expression in rats induced by partial hepatic IR. Expression of p-PI3K (p85α) protein was detected by western blot analysis (a). The GAPDH protein expression was determined as the internal standard. These bands were quantified and analyzed (b). Data are represented as means ± SD ($n = 8$). $^{*}P < 0.05$, compared with group SH. $^{\#}P < 0.05$, compared with group IR. $^{\Delta}P < 0.05$, compared with group CM.

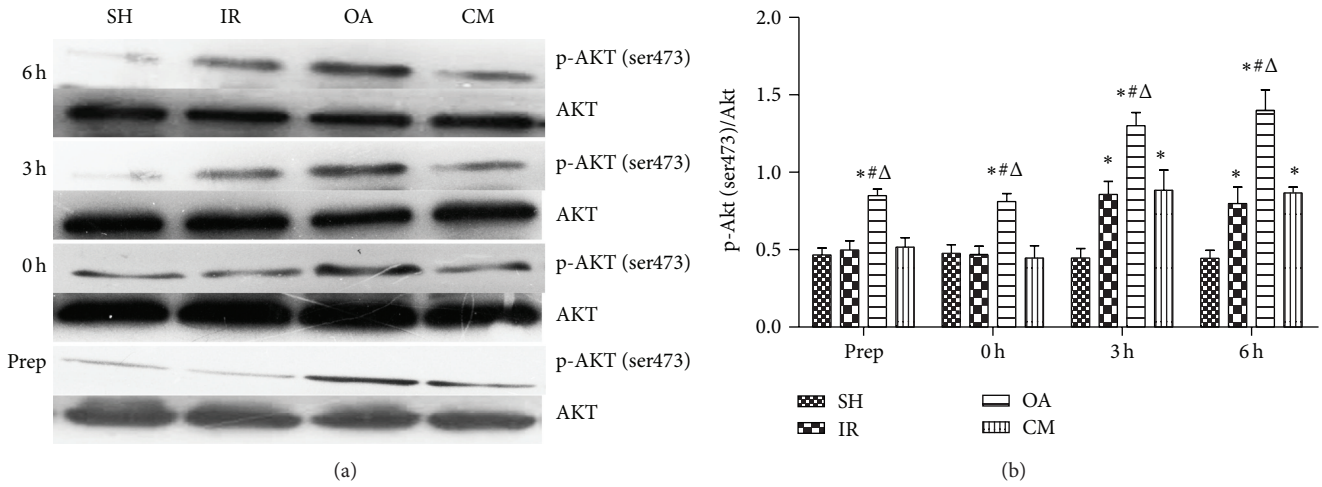

Figure 5: Effects of pretreatment with OA on p-Akt (ser473) and Akt protein expression in rat induced by partial hepatic IR. Expression of p-Akt (ser473) and Akt protein was detected by western blot analysis (a). These bands were quantified and analyzed (b). Data are represented as means ± SD ($n = 8$). $^{*}P < 0.05$, compared with group SH. $^{\#}P < 0.05$, compared with group IR. $^{\Delta}P < 0.05$, compared with group CM.

injury, we explored the levels of p-PI3K, p-Akt, total Akt, p-GSK-3β, and total GSK-3β protein expression (Figures 4, 5, and 6). There were no significant differences in the basal levels of total Akt and total GSK-3β protein expression among the four groups. The IR procedure was followed by increased p-PI3K, p-Akt, and p-GSK-3β protein synthesis at 3 h and 6 h after reperfusion ($P < 0.05$) whether rats were pretreated with CMC-Na or not. Furthermore, OA pretreatment significantly increased p-PI3K, p-Akt, and p-GSK-3β protein synthesis in IR-stimulated rat liver not only at preoperation, but also at 0 h, 3 h, and 6 h after reperfusion ($P < 0.05$). These results indicated that preoperative OA treatment was an important activator of the PI3K/Akt pathway.

4. Discussion

The present work demonstrated that pretreatment with 100 mg/kg OA could reduce the liver injury caused by partial hepatic IR, as indicated by histological improvement and significant decreases in both serum ALT activity and IL-1β level. These results were associated with a marked increase in p-PI3K, p-Akt, and p-GSK-3β protein expression, strongly

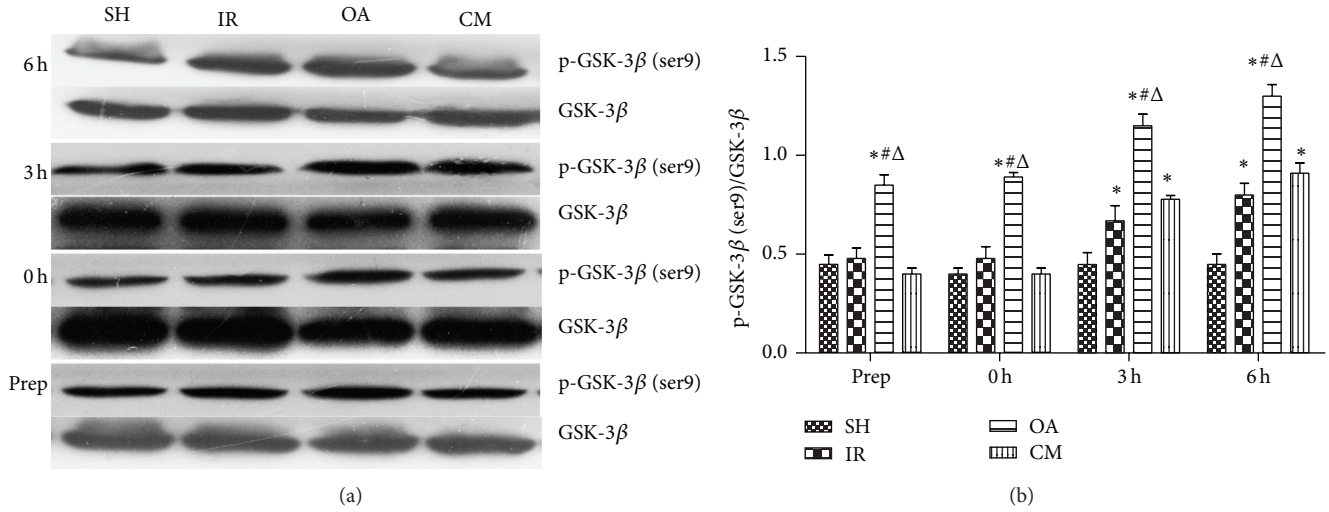

Figure 6: Effects of pretreatment with OA on p-GSK-3β (ser9) and GSK-3β protein expression in rats induced by partial hepatic ischemia-reperfusion (IR). Expression of p-GSK-3β (ser9) and GSK-3β protein was detected by western blot analysis (a). These bands were quantified and analyzed (b). Data are represented as mean ± SD ($n = 8$). $^{*}P < 0.05$, compared with group SH. $^{\#}P < 0.05$, compared with group IR. $^{\Delta}P < 0.05$, compared with group CM.

suggesting that PI3K/Akt-mediated inactivation of GSK-3β may be involved in the protective effect of OA pretreatment towards IR-induced rat liver dysfunction. Inhibition of PI3K with 15 μg/kg of wortmannin partially reversed the OA protective effects on IR-induced hepatic injury, which further supported the involvement of the PI3K/Akt pathway.

The rat model with 70% hepatic IR experienced severe hepatic injury without obvious mesenteric venous hypertension. Mesenteric congestion was avoided by allowing intestinal blood flow through right and caudate lobes, which led to a satisfactory survival rate despite substantial hepatic IR injury.

One major hallmark of hepatic IR is inflammation-induced hepatopathology [19, 20]. At ischemia stage, proinflammatory cytokines, such as IL-1β, were instantly released [21]. After reperfusion, neutrophils, triggered by proinflammatory cytokines, are further activated to cross the endothelial barrier and translocate into hepatic tissue [22]. Hepatic enzymes and reactive oxygen species are then released to aggravate the associated hepatic tissue injury [23–25]. OA exerts anti-inflammatory effects on lipopolysaccharide-induced inflammation by inhibiting hyperpermeability and leukocyte adhesion and migration [26]. It also exerts protective effects against hepatic injury by inducing antioxidant enzymes such as HO-1 [27]. Our results confirmed the development of liver injury by markedly elevated ALT activity and, indirectly, by IL-1β concentrations, whereas OA pretreatment considerably reduced the enhancement of ALT activity and IL-1β concentrations induced by IR. In addition, IL-1β concentrations were suppressed prior to the reduction of ALT activity, suggesting that systemic inflammatory responses induced by partial hepatic IR might be an important promoter for hepatic damage during the acute phase.

The PI3K/Akt pathway has an endogenous negative regulatory function, which limits proinflammatory mediators and chemotactic events by reducing neutrophil infiltration and production of cytokines [28–31]. However, the role of the PI3K/Akt pathway in the modulation of partial hepatic IR-induced injury was poorly understood. In our study, increased expression of the P13K/Akt pathway in the absence of OA may be a compensatory mechanism to counteract cytokine release following IR. The role of PI3K in a given signaling pathway is often examined by the use of pharmacological tools. The PI3K inhibitor wortmannin has been successfully used to clarify the physiological roles of PI3K pathway. In our study, wortmannin partially reversed the protective effects of OA as shown by worsening histological status and increasing serum ALT and IL-1β levels. Our results also showed that OA pretreatment was able to further markedly enhance phosphorylation of PI3K and Akt protein expression not only at the preoperative period, but also at 0 h, 3 h, and 6 h after reperfusion. This finding was consistent with previous studies which have reported that activation of the PI3K pathway protected organs against IR injury [32, 33].

When N-terminal Ser9 residue of GSK-3β is phosphorylated, the phosphate-binding pocket is occupied by an intramolecular interaction with the phosphorylated Ser9. This interaction considerably inhibits GSK-3β activity by changing the conformation of the catalytic site and also by preventing the binding of the primed substrate. The N-terminal Ser9 residue of GSK-3β is within the consensus sequence of the substrates for Akt. Thus, GSK-3β is one of the downstream targets of PI3K/Akt pathway [34]. Inactivation of GSK-3β may result in a decrease of proinflammatory cytokines [35]. A recent study has also shown that GSK-3β inactivation was involved in a propofol-induced protective effect against IR injury in an isolated guinea pig heart [36].

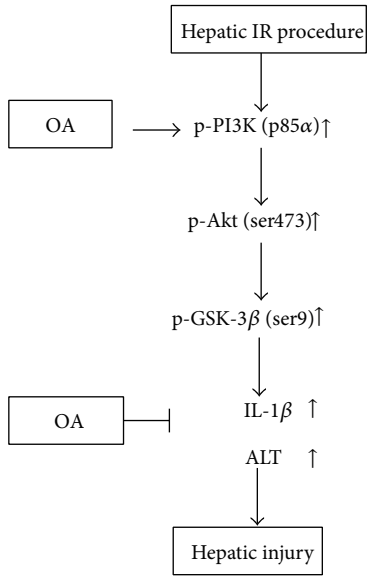

Figure 7: Proposed schematic flow of OA's role in attenuating hepatic IR injury. IR procedure induces activation of PI3K cascades and then results in increases in IL-1β and ALT in the serum. OA can further activate these molecular changes at the PI3K/Akt signaling pathway and results in the decreased serum ALT activity and IL-1β concentration.

Our data showed that the changes in p-GSK-3β paralleled the alterations of serum ALT activity and IL-1β expression. These results also suggested that the PI3K/Akt-mediated inactivation of GSK-3β might play an important role in hepatoprotective action owing to OA pretreatment.

There are some limitations in our study. Firstly, we did not compare the OA effects on hepatic IR injury depending on whether the administration was done before or after IR. Indeed, although pretreatment with OA might exert protective effects in some selective hepatic surgeries, posttreatment may be more practical than pretreatment in clinical use since injury is unexpected. Secondly, PI3K inhibitor wortmannin only partially reversed the protective effects of OA on hepatic IR injury, so that further studies designed to investigate the overall mechanism of the OA protective effects are warranted.

5. Conclusion

Our data suggest that OA pretreatment exerts hepatoprotective effects in a rat model of hepatic IR, likely involving the PI3K, p-Akt, and p-GSK-3β pathways (see schematic flow of OA's role in decreasing hepatic IR injury in Figure 7). We think that these findings may pave the road for future innovative therapeutic strategies in the field of ischemia-reperfusion related liver injury.

Conflict of Interests

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

Authors' Contribution

Bo Gui, Fuzhou Hua, and Jie Chen contributed equally to this study.

Acknowledgments

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