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Pain and Inflammation: Update on Emerging Phytotherapy, Zootherapy, and Nutritional Therapies

Guest Editors: Bamidele V. Owoyele, Musa T. Yakubu, and Roi Treister

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Editorial Board

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 2520371, 1 page
http://dx.doi.org/10.1155/2016/2520371

Editorial

Pain and Inflammation: Update on Emerging Phytotherapy, Zootherapy, and Nutritional Therapies

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Received 15 May 2016; Accepted 15 May 2016

Pain is the most common health challenge that drives patients to consult physicians. Pain and inflammation are usually symptoms of many diseases. Inability to perceive sensory pain may lead to shortened life expectancy and yet excessive pain may result in low quality of life. Despite the arrays of therapies available for the treatment of pain, it is still not tamed and the currently available drugs have their drawbacks. The challenges to amelioration of pain and inflammation have become an impetus for research into alternative and complementary medicines for the treatment of these ailments. The roles of phytotherapy and nutritional therapies in the treatment of varied types of ailments cannot be overemphasized. Actually, most of the commonly available therapeutic drugs for pain, such as opioids and nonsteroidal anti-inflammatory drugs (NSAIDs), were derived from phytotherapy. Hence, giving attention to this issue is highly recommended as it provides a medium for knowledge acquisition for improvement of the treatment of the twin ailment-pain and inflammation.

The researches published in this issue are broad and they accomplished the aim for which the issue was set out. Many manuscripts were received but only those contributing significantly to the subject matter were accepted after thorough peer review. The new insights include topics on systemic models of inflammation contributed by Y. Liu et al., "Effects of Wutou Decoction on DNA Methylation and Histone Modifications in Rats with Collagen-Induced Arthritis," and X. Wu et al., "Protective Effect of Tetrandrine on Sodium Taurocholate-Induced Severe Acute Pancreatitis." Z. Shu et al. reported the anti-inflammatory effects of plant extract "Antibacterial and Anti-Inflammatory Activities of *Physalis alkekengi* var. *franchetii* and Its Main Constituents" as well as R. Boukhary et al., "Anti-Inflammatory and Antioxidant Activities of *Salvia fruticosa*: An HPLC Determination of Phenolic Contents." In another study, I. J. M. Santos et al. reported "Topical Anti-Inflammatory Activity of Oil from *Tropidurus hispidus* (Spix, 1825)." A review was accepted for inclusion in the issue on effectiveness of acupuncture for treating sciatica by Z. Qin et al. A. Rauf et al. reported on a potential cyclooxygenase inhibitor named daturaolone while W. Kim et al. evaluated the anti-inflammatory potential of a new Ganghwaljetongyeum (N-GHJTY) on adjuvant-induced inflammatory arthritis in rats. This issue therefore is a rich resource for all interested in pain and inflammation research and treatment.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 1230294, 10 pages
http://dx.doi.org/10.1155/2016/1230294

Research Article

Evaluation of Anti-Inflammatory Potential of the New Ganghwaljetongyeum on Adjuvant-Induced Inflammatory Arthritis in Rats

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Received 22 October 2015; Revised 22 April 2016; Accepted 24 April 2016

Academic Editor: Ke Ren

Ganghwaljetongyeum (GHJTY) has been used as a standard treatment for arthritis for approximately 15 years at the Korean Medicine Hospital of Dongshin University. GHJTY is composed of 18 medicinal herbs, of which five primary herbs were selected and named new Ganghwaljetongyeum (N-GHJTY). The purpose of the present study was to observe the effect of N-GHJTY on arthritis and to determine its mechanism of action. After confirming arthritis induction using complete Freund's adjuvant (CFA) in rats, N-GHJTY (62.5, 125, and 250 mg/kg/day) was administered once a day for 10 days. In order to determine pathological changes, edema of the paws and weight were measured before and for 10 days after N-GHJTY administration. Cytokine (TNF-α, IL-1β, and IL-6) levels and histopathological lesions in the knee joint were also examined. Edema in the paw and knee joint of N-GHJTY-treated rats was significantly decreased at 6, 8, and 10 days after administration, compared to that in the CFA-control group, while weight consistently increased. Rats in N-GHJTY-treated groups also recovered from the CFA-induced pathological changes and showed a significant decline in cytokine levels. Taken together, our results showed that N-GHJTY administration was effective in inhibiting CFA-induced arthritis via anti-inflammatory effects while promoting cartilage recovery by controlling cytokine levels.

1. Introduction

Arthritis collectively refers to more than 100 rheumatic diseases that are characterized by inflammation, pain, and stiffness in the musculoskeletal system and that range from localized, self-limiting conditions to systemic, autoimmune processes [1]. Arthritis occurs in all age groups and peaks between the ages of 35 and 50, affecting ~1% of the world's population [1, 2]. Rheumatoid arthritis (RA) is a systemic inflammatory disease that attacks the joints by producing proliferative synovitis, leading to destruction of articular cartilage and underlying bone. RA affects approximately 0.5% to 1% adults worldwide, with women being affected two to three times more frequently than men; RA is also associated with a high mortality rate [1, 3].

Autoimmunity and chronic inflammation are activated by an imbalance between pro- and anti-inflammatory cytokines, thereby causing joint damage in RA [4]. RA is characterized by angiogenesis in the synovial membrane, which contributes to the advancement of the disease, as well as the production of inflammatory cells that infiltrate and destroy the synovial tissue [1, 5, 6]. In RA, production of both cytokines and chemokines is induced by macrophage- and fibroblast-derived cytokines [7]. Of these, proinflammatory

interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) are often targeted in RA treatment strategies [8, 9].

Our previous study showed that Ganghwaljetongyeum (GHJTY) can effectively attenuate RA by inhibiting synovial cell proliferation as well as the production of proinflammatory mediators from macrophage-like cells [10]. However, 18 medicinal herbs are included in GHJTY, making it impractical for clinical application. In order to improve the efficacy and convenience of pharmaceutically prescribing GHJTY, we used bioinformatics (http://combio.gist.ac.kr/herding) [11] to select the five medicinal herbs with the greatest potential to treat arthritis. The selected medicinal herbs were *Ostericum koreanum* Maximowicz (Osterici Radix, OK), *Lonicera japonica* Thunberg (Lonicerae Folium, LJ), *Clematis mandshurica* Ruprecht (Clematis Radix, CM), *Angelica gigas* Nakai (Angelicae Gigantis Radix, AG), and *Phellodendron amurense* Ruprecht (Phellodendri Cortex, PA).

A review of the literature revealed that each of the selected herbs is effective in targeting certain aspects of RA pathophysiology. For example, OK inhibits the production of inflammatory mediators by downregulating nuclear factor kappa beta (NF-κB) and mitogen-activated protein kinase (MAPK) activity in lipopolysaccharide-stimulated RAW264.7 cells [12]. Additionally, anti-inflammatory activity of the major constituents of LJ has been shown [13], along with the ability of CM extract to interact with NF-κB, TNF-α, and cyclooxygenase 2 (COX-2) in rats with collagen-induced arthritis [14]. Studies have also shown that AG inhibits focal and systemic inflammation in dinitrofluorobenzene-induced inflammation models and that PA protects against human osteoarthritis by regulating aggrecanases, matrix metalloproteinases (MMPs)/tissue inhibitors of metalloproteinases (TIMP), proinflammatory cytokines, and MAPK pathway signaling [15, 16]. However, the effects of combining these herbal medicines remain unclear.

The purpose of the present study was to evaluate the efficacy and mechanism of action of this new GHJTY (N-GHJTY) using an animal model of RA. Specifically, we used rats with complete Freund's adjuvant- (CFA-) induced arthritis as an experimental animal model used to mimic human RA, as it produces profound systemic inflammation that results in severe joint swelling and remodeling [17]. Moreover, CFA is typically used to investigate therapeutic agents with antiarthritic potential [18]. Our specific aims were to (1) confirm the quality assessment of marker components in N-GHJTY, (2) determine the antiarthritic effects of N-GHJTY by measuring paw edema and observing gross lesions of the paw and knee joint, (3) evaluate potential adverse effects of N-GHJTY by histopathological investigation, and (4) assess the effect of N-GHJTY on proinflammatory cytokines by examining levels of TNF-α, IL-1β, and IL-6.

2. Materials and Methods

2.1. Animals. Adult male Sprague-Dawley rats, weighing 200–210 g, were housed in a room with constant temperature (24–26°C) and humidity (40–60%). Food (Pellet, GMO, Korea) and water were available ad libitum. Animals were acclimated to the laboratory environment for 1 week before the experiment, and all procedures were approved by the Institutional Animal Care and Use Committee of the Dongshin University (2014-03-02).

2.2. CFA-Induced Arthritis and Drug Administration. In the primary adjuvant-induced arthritis model, 0.25 mL of CFA (Sigma, St. Louis, MD, USA) was injected into the left hind knee joint. After 7 days, secondary arthritis was induced by injecting 0.05 mL of CFA under the left hind knee joint and left hind sole. Animals were then divided into the following five groups (n = 6/group): normal, CFA arthritis (CFA-control) and CFA arthritis treated with 62.5, 125, and 250 mg/kg of N-GHJTY extract per day (N-GHJTY-62.5, N-GHJTY-125, and N-GHJTY-250, resp.). Oral administration of N-GHJTY was initiated on the 10th day after arthritis induction and continued for 10 days thereafter. Animals were anesthetized using 2.5% isoflurane and volumes of the hind paw and knee joint were measured using a Digital Plethysmometer (LE7500, Panlab, Spain) 0, 2, 4, 6, 8, and 10 days after oral N-GHJTY administration.

2.3. Preparation of Herbal Materials. The five herbal medicines forming N-GHJTY were purchased from Omniherb Co. (Yeongcheon, Korea), and their origin was taxonomically confirmed by Professor Jong-Kil Jeong in the Department of Herbology at the College of Oriental Medicine, Dongshin University.

The five herbs (OK, LJ, AG, CM, and PA) were combined in a 6 : 4 : 4 : 4 : 3 ratio. N-GHJTY was prepared via water extraction at 100°C for 3 h and concentrated using a rotary vacuum evaporator (EYELA, Japan) after filtration followed by lyophilization using a vacuum freeze drier (Samwon Freezing Engineering Co., Korea). The yield was about 29.5%.

2.4. Reagents and High-Performance Liquid Chromatography (HPLC) Analysis. Chlorogenic acid (**1**) and berberine chloride (**2**) were purchased from Acros Organics (Pittsburgh, PA, USA) and Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), respectively. Nodakenin (**3**), decursin (**6**), and decursinol angelate (**7**) were purchased form NPC BioTechnology (Yeongi, Korea). Isoferulic acid (**4**) and oxypeucedanin hydrate (**5**) were purchased from ChemFaces Biochemical Co. Ltd. (Wuhan, China). The purities of the seven reference compounds were ≥98.0% by HPLC analysis and the chemical structures of the seven marker compounds are shown in Figure 1. HPLC-grade solvents, methanol, acetonitrile, and water were obtained from J.T.Baker (Phillipsburg, NJ, USA). Analytical grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

For quality assessment of the seven marker compounds in N-GHJTY, all experiments were conducted using a Shimadzu Prominence LC-20A Series (Shimadzu, Kyoto, Japan) equipped with a solvent delivery unit (LC-20AT), online degasser (DGU-20A3), column oven (CTO-20A), autosample injector (SIL-20AC), and photodiode array (PDA) detector (SPD-M20A). Lab solution software (version 5.54 SP3, Kyoto, Japan) was used for data acquisition and processing.

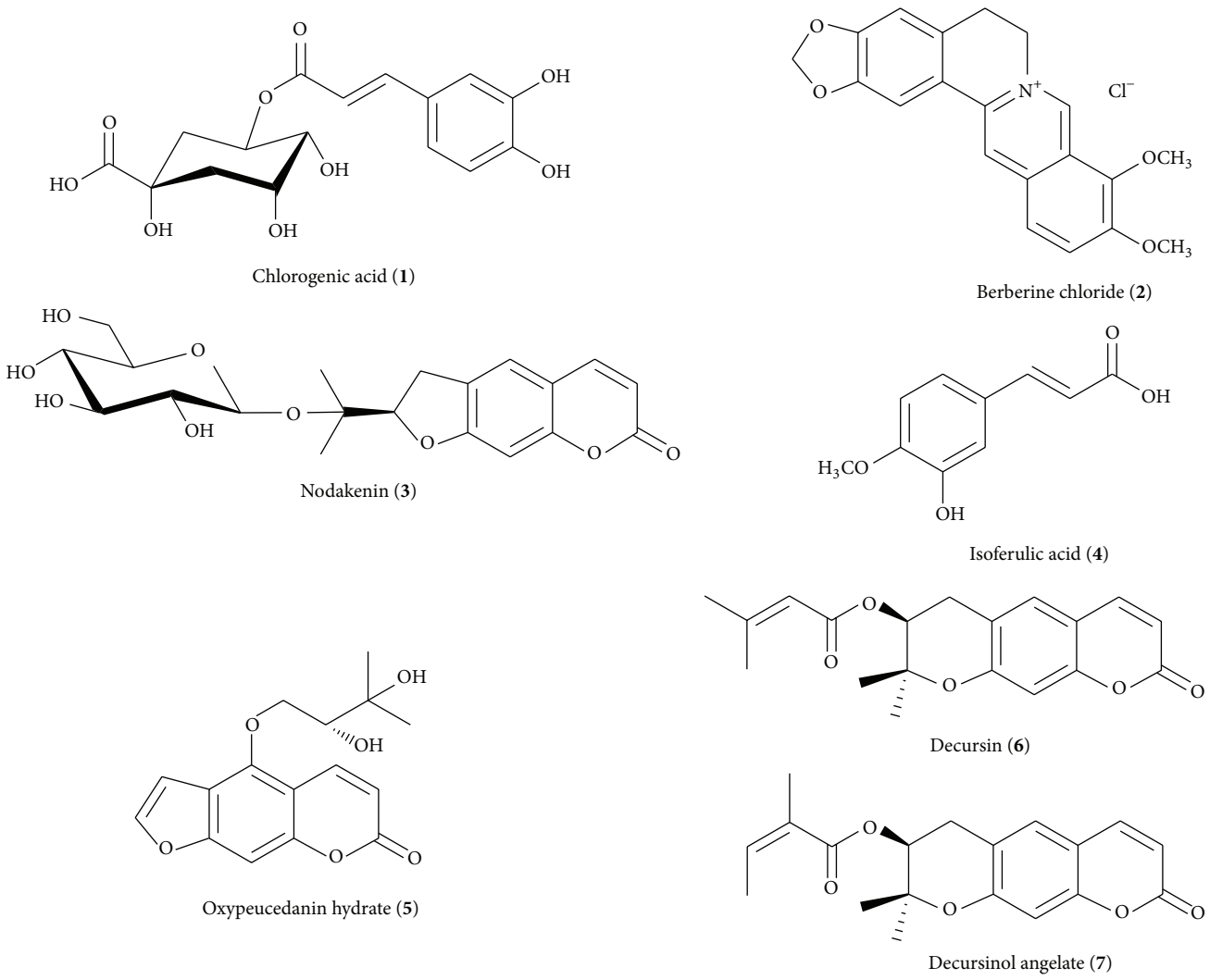


Figure 1: Chemical structure of the seven marker compounds.

Chromatographic separation of all analytes was performed using a Waters SunFire C18 column (4.6 × 250 mm; 5 μm, Milford, MA, USA). The mobile phases consisted of 0.1% (v/v) formic acid in distilled water (A) and 0.1% (v/v) formic acid in acetonitrile (B) and the gradation condition was optimized as follows: with range of 0–30 min, 10–100% B; 30–40 min, 100% B; 40–50 min, 100–10% B; 50–60 min, 10% B. The flow rate of mobile phase was maintained at 1.0 mL/min, and the injection volume was 10 mL. The flow rate was maintained at 1.0 mL/min, and the injection volume was 10 mL. For HPLC analysis, lyophilized N-GHJTY (200 mg) was dissolved in 20 mL of 70% methanol and extracted for 60 min by sonication. The N-GHJTY extract solution was passed through a 0.2-μm syringe filter (PALL Life Sciences, Ann Arbor, MI, USA) before HPLC analysis.

2.5. Blood and Serum Tests. Blood samples were collected, and 100 μL was used for a complete blood count (CBC) analysis via a Multispecies Hematology Analyzer (950, Hemavet, USA). Serum was separated from the rest of the blood using a high-speed centrifuge (VS-600CFi, Korea) at 3500 rpm (g = 27.391) for 20 min, and aspartate aminotransferase (AST) and alanine transaminase (ALT) levels were measured.

2.6. Measurement of TNF-α, IL-1β, and IL-6. TNF-α was measured using a Rat TNF-α kit (Invitrogen, USA), IL-1β was assessed using a Rat IL-1β kit (R&D Systems, USA), and IL-6 was evaluated using a Rat IL-6 kit (Invitrogen, USA). Optical densities (OD) of all samples were measured at 450 nm via Spectramax (M2, Molecular Devices, USA).

2.7. Hematoxylin and Eosin (HE) Staining. The right knee joint was removed and fixed in Bouin solution for over 24 h. Decalcification was conducted in a 2.5% nitric acid solution, which was changed once a day for 7 days. The removed tissue was dehydrated using Tissue Processor (Tissue-Tex II, Japan), deparaffinized, stained with HE (Muto, Japan), and observed under an optical microscope (Nikon, Japan).

2.8. Safranin O-Fast Stain. After deparaffinization, the right knee joint was reacted with Weigert's Iron Hematoxylin

Table 1: Changes in paw swelling after N-GHJTY administration in rats with CFA-induced arthritis (mL).

Group	Before	Days after CFA (days N-GHJTY administration)					
		10 (0)	12 (2)	14 (4)	16 (6)	18 (8)	20 (10)
Normal	1.36 ± 0.03	1.47 ± 0.04	1.49 ± 0.04	1.55 ± 0.05	1.57 ± 0.06	1.63 ± 0.05	1.66 ± 0.06
CFA-control	1.24 ± 0.01	3.77 ± 0.25$^{\#\#}$	3.78 ± 0.26$^{\#\#}$	3.91 ± 0.26$^{\#\#}$	3.91 ± 0.28$^{\#\#}$	3.96 ± 0.28$^{\#\#}$	4.00 ± 0.30$^{\#\#}$
N-GHJTY-62.5	1.26 ± 0.03	3.68 ± 0.33	3.72 ± 0.31	3.68 ± 0.26	3.53 ± 0.23	3.35 ± 0.21	3.23 ± 0.17*
N-GHJTY-125	1.24 ± 0.03	3.68 ± 0.15	3.67 ± 0.15	3.60 ± 0.17	3.36 ± 0.20	3.15 ± 0.20*	3.03 ± 0.20*
N-GHJTY-250	1.31 ± 0.04	3.63 ± 0.16	3.66 ± 0.16	3.49 ± 0.10	3.22 ± 0.08*	3.05 ± 0.05*	2.91 ± 0.09**

Mean ± SE.
$^{\#\#}P < 0.01$ versus normal group.
$^{**}P < 0.01$ and $^{*}P < 0.05$ versus CFA-control group.

(Sigma, USA) solution for 10 min and stained with 0.001% Fast Green (Sigma, USA) solution for 5 min. The knee joint tissue was then reacted with 1% acetate solution for 10 s and stained with 0.1% Safranin O (Sigma, USA) solution for 5 min; thereafter, the tissue was dehydrated and observed under an optical microscope (Nikon, Japan).

2.9. Statistical Analysis. Data were analyzed using SPSS 21.0 version for Windows by a nonparametric Mann-Whitney U test. A one-way analysis of variance was conducted on each group, and results are expressed as mean ± standard error (SE). Comparisons between groups were performed using the post hoc least squared differences (LSD) test. $P < 0.05$ and $P < 0.01$ were considered statistically significant.

3. Results

3.1. Quality Assessment of Seven Marker Components in N-GHJTY. HPLC was performed using the seven marker compounds in N-GHJTY for quality control. The selected compounds were as follows: compound **1** (Lonicerae Folium), compound **2** (Phellodendri Cortex), compounds **3**, **6**, and **7** (Angelicae Gigantis Radix), compound **4** (Clematis Radix), and compound **5** (Osterici Radix). All analytes were separated within 30 min and the typical three-dimensional chromatogram of the 70% methanol extract of N-GHJTY is shown in Figure 2. Quantitation was achieved by photodiode array (PDA) detection at 310 nm (**5**), 325 nm (**1** and **4**), 330 nm (**6** and **7**), 335 nm (**3**), and 340 nm (**2**) based on retention time and UV spectrum. The retention times of components **1**–**7** were 8.94, 10.80, 12.00, 12.86, 15.95, 26.02, and 26.24 min, respectively. Using a calibration curve, we determined that correlation coefficients (r^2) of all seven compounds were ≥0.9996. Under optimized chromatography conditions, concentrations of N-GHJTY marker compounds **1**–**7** were 5.46 ± 0.33, 1.87 ± 0.29, 1.70 ± 0.18, 1.64 ± 0.58, 2.03 ± 0.33, 1.09 ± 0.02, and 0.81 ± 0.01 mg/g, respectively.

3.2. Effect of N-GHJTY on Gross Lesions of the Paw and Knee Joint. Paw and knee joint swelling and rubefaction served as external objective indicators for evaluating the severity of the inflammatory arthritic model. The CFA-control group showed rubefaction and hind paw and knee joint swelling—both of which gradually decreased following N-GHJTY

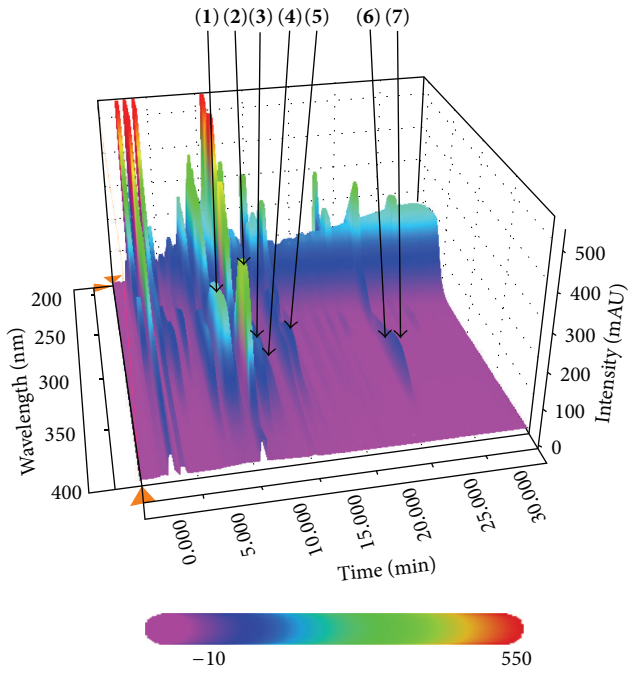
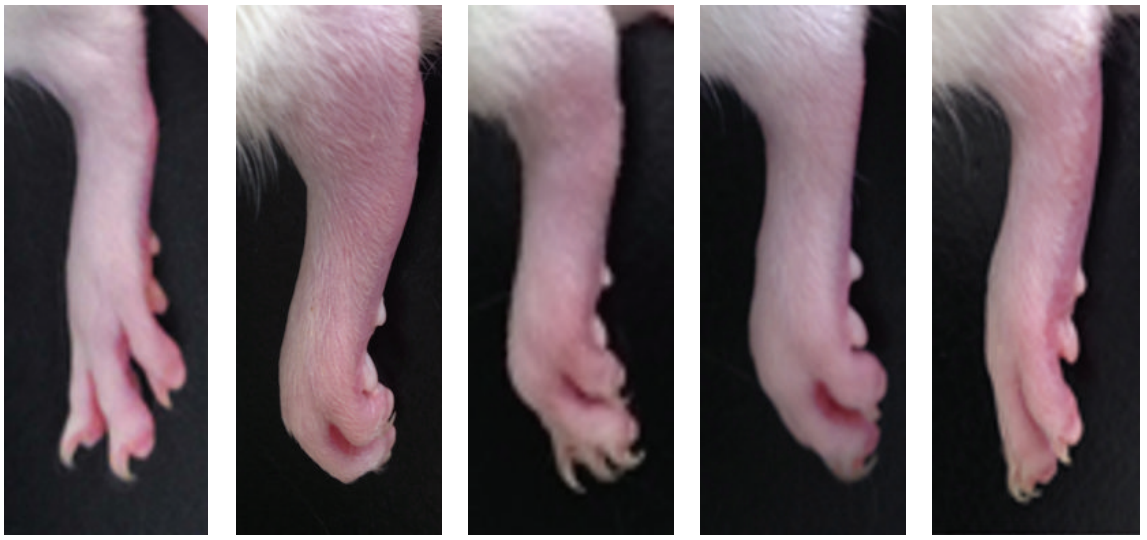


Figure 2: Three-dimensional chromatogram of N-GHJTY. Chlorogenic acid (**1**), berberine chloride (**2**), nodakenin (**3**), isoferulic acid (**4**), oxypeucedanin hydrate (**5**), decursin (**6**), and decursinol angelate (**7**).

treatment at all concentrations (62.5, 125, and 250 mg/kg) (Figure 3).

3.3. Effect of N-GHJTY on Paw and Knee Joint Swelling. Changes in paw and knee joint swelling are presented in Table 1. Approximately 3 days after the second immunization, the rat knee joint began to swell and the paw and knee joint were observed to increase in size. On the 10th day, the CFA-control group showed a significant increase in both paw and knee joint swellings compared to the normal group. This volume significantly decreased in the N-GHJTY-250 group on the 6th day, the N-GHJTY-125 group on the 8th day, and the N-GHJTY-62.5 group on the 10th day compared to the CFA-control group (Table 1).

3.4. Effect of N-GHJTY on Body Weight. Twelve days after CFA injection, statistically significant reductions in body



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

Figure 3: Effect on N-GHJTY in gross lesions in the hind paw and knee swelling in rats with CFA-induced arthritis. (a) Normal group, (b) CFA-control group, (c) N-GHJTY-62.5 group (62.5 mg/kg), (d) N-GHJTY-125 group (125 mg/kg), and (e) N-GHJTY-250 group (250 mg/kg).

Table 2: Changes in body weight after N-GHJTY administration in rats with CFA-induced arthritis (%).

Group	Before	Days after CFA (days N-GHJTY administration)					
		10 (0)	12 (2)	14 (4)	16 (6)	18 (8)	20 (10)
Normal	100.0 ± 0.0	145.2 ± 1.8	148.3 ± 2.4	153.6 ± 2.8	159.4 ± 3.2	163.4 ± 3.6	164.0 ± 3.7
CFA-control	100.0 ± 0.0	134.8 ± 1.7	139.5 ± 2.2$^{\#}$	144.7 ± 3.4	148.9 ± 4.3	153.4 ± 5.2	154.0 ± 5.1
N-GHJTY-62.5	100.0 ± 0.0	135.5 ± 2.2	142.9 ± 1.8	149.6 ± 1.9	157.4 ± 2.2	161.4 ± 1.6	162.1 ± 1.7
N-GHJTY-125	100.0 ± 0.0	136.0 ± 2.5	142.0 ± 2.3	148.6 ± 2.5	156.1 ± 3.2	159.9 ± 3.6	161.1 ± 3.6
N-GHJTY-250	100.0 ± 0.0	136.4 ± 3.5	143.2 ± 3.7	149.5 ± 4.1	157.6 ± 4.8	161.5 ± 5.1	162.8 ± 5.4

Mean ± SE.
$^{\#}P < 0.05$ versus normal group.

Table 3: Effect of N-GHJTY on aspartate and alanine aminotransferase levels in rats with CFA-induced arthritis.

Group	Aspartate aminotransferase (U/L)	Alanine aminotransferase (U/L)
Normal	82.0 ± 5.0	28.4 ± 1.6
CFA-control	93.7 ± 4.6$^{\#}$	38.2 ± 2.9$^{\#}$
N-GHJTY-62.5	86.0 ± 7.8	39.0 ± 2.1
N-GHJTY-125	85.4 ± 1.9	28.7 ± 3.4
N-GHJTY-250	80.0 ± 3.0*	28.8 ± 1.3*

Mean ± SE.
$^{\#}P < 0.05$ versus normal group.
$^{*}P < 0.05$ versus CFA-control group.

Table 4: Effect of N-GHJTY on TNF-α, IL-1β, and IL-6 levels in rats with CFA-induced arthritis.

Group	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
Normal	2.33 ± 0.12	22.9 ± 0.95	4.3 ± 0.41
CFA-control	4.85 ± 0.67$^{\#\#}$	36.7 ± 0.94$^{\#\#}$	18.0 ± 0.87$^{\#\#}$
N-GHJTY-62.5	3.24 ± 0.32*	26.8 ± 0.84*	11.0 ± 0.5*
N-GHJTY-125	3.01 ± 0.18*	26.2 ± 0.52**	10.4 ± 0.6*
N-GHJTY-250	2.84 ± 0.17*	25.4 ± 0.84**	9.7 ± 0.39*

Mean ± SE.
$^{\#\#}P < 0.01$ versus normal group.
$^{**}P < 0.01$ and $^{*}P < 0.05$ versus CFA-control group.

weight were observed in the CFA-control group when compared to the normal group. N-GHJTY treatment at all concentrations (62.5, 125, and 250 mg/kg) resulted in an increase in body weight; however, this change was not significant (Table 2).

3.5. Effect of N-GHJTY on Transaminase Levels. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels are indicated in Table 3. As shown in the results, only the N-GHJTY-250 group showed a significant decrease in AST and ALT when compared to CFA-control group rats.

3.6. Effect of N-GHJTY on Proinflammatory Cytokines. TNF-α, IL-1β, and IL-6 levels are indicated in Table 4. As shown in the results, TNF-α, IL-1β, and IL-6 in the CFA-control group showed a significant increase when compared to levels in the normal group rats. A significant decrease in TNF-α, IL-1β, and IL-6 levels was observed in all N-GHJTY treatment

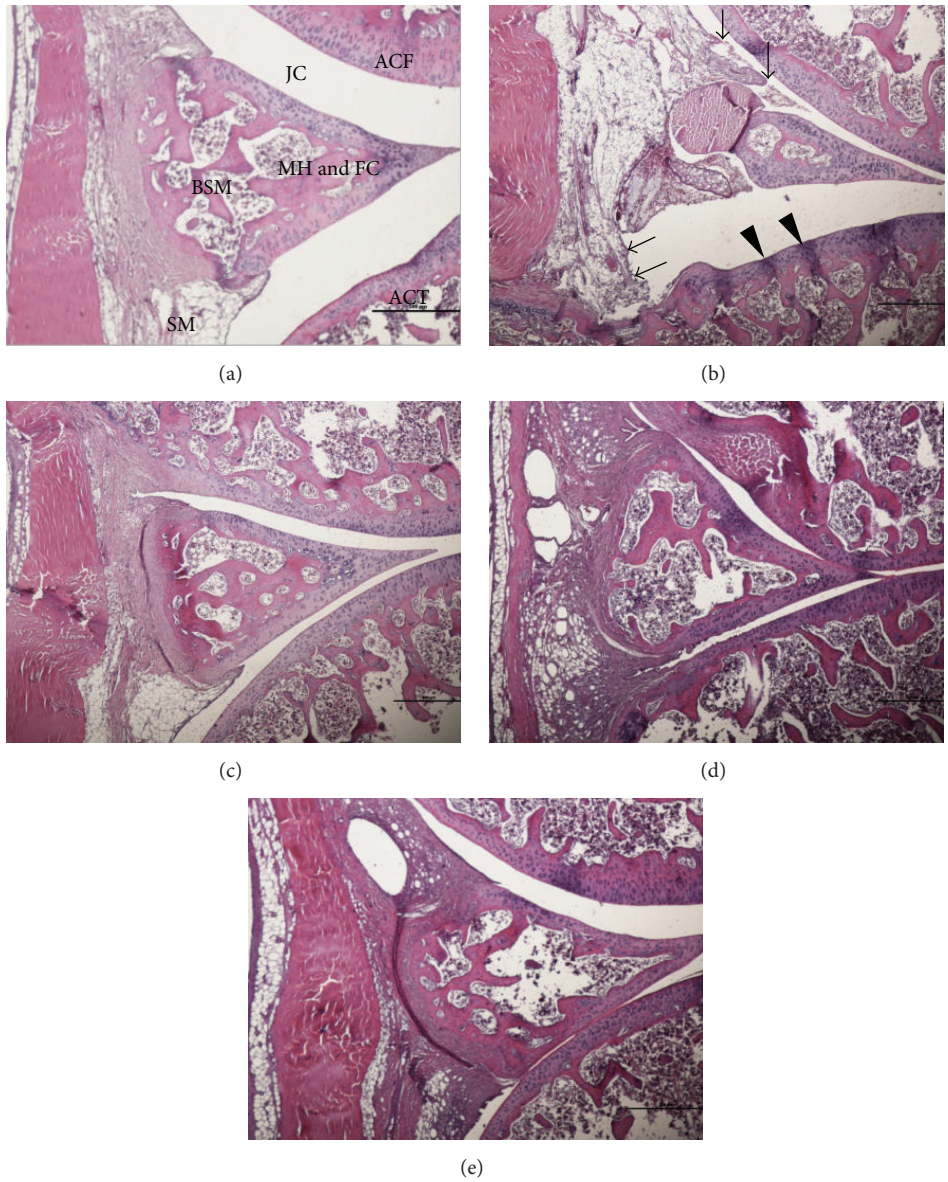


Figure 4: Influence of N-GHJTY on CFA-induced histopathological changes in the knee joints of CFA-induced arthritic rats. Arrows (↓) indicate a damaged synovial membrane. Arrow heads (▼) indicate compressed articular cartilage in the CFA control. (a) Normal group, (b) CFA-control group, (c) N-GHJTY-62.5 group (62.5 mg/kg), (d) N-GHJTY-125 group (125 mg/kg), and (e) N-GHJTY-250 group (250 mg/kg). JC: joint cavity. ACF: articular cartilage of the femur. ACT: articular cartilage of the tibia. BSM: bony spicule within the meniscus. SM: synovial membrane. MH&FC: meniscus of hyaline and fibrocartilage. HE stain, scale bars = 500 nd.

groups (62.5, 125, and 250 mg/kg) when compared to the CFA-control group rats (Table 4).

3.7. Effects of N-GHJTY on Histopathological Changes Assessed with HE Staining. Representative HE stained histopathological lesions in the hind knee joint of normal, CFA-control, N-GHJTY-62.5, N-GHJTY-125, and N-GHJTY-250 groups are shown in Figure 4. Loose synovial membrane, membrane destruction, disorganized cell arrangement, and compressed cartilage were observed in the CFA-control group (Figure 4(b)). Histopathological changes improved in all N-GHJTY groups (62.5, 125, and 250 mg/kg) compared to the CFA-control group. These groups presented close synovial membrane and regular cartilage; the surface of the cartilage in the tibia and femur was smooth and exhibited no noticeable damage (Figures 4(c), 4(d), and 4(e)).

3.8. Effects of N-GHJTY on Histopathological Changes Assessed with Safranin O-Fast Staining. Safranin O-fast stain was conducted to observe histopathological changes in the knee joint. In the normal group (Figure 5(a)), a positive reaction to proteoglycans in the calcified zone was observed and the cartilage was even. The CFA-control group exhibited little positive reactions, and chondrocyte nuclei appeared

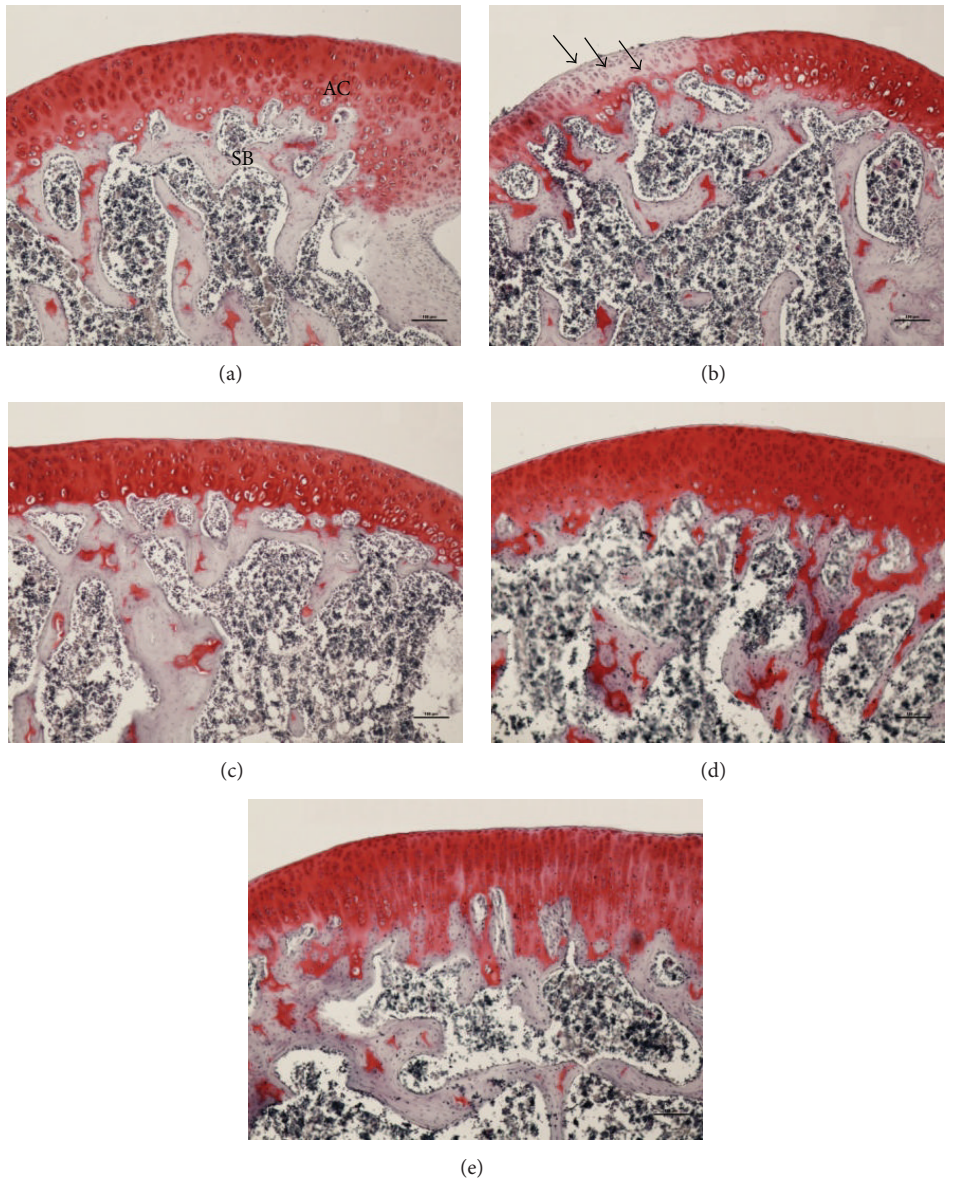


Figure 5: Influence of N-GHJTY on CFA-induced histopathological changes in the knee joints of CFA-induced arthritic rats. A number of shrunken nuclei (arrows ↓) were observed in the CFA control. (a) Normal group, (b) CFA-control group, (c) N-GHJTY-62.5 group (62.5 mg/kg), (d) N-GHJTY-125 group (125 mg/kg), and (e) N-GHJTY-250 group (250 mg/kg). AC: articular cartilage. SB: spongy bone. Safranin O-fast stain, scale bars = 100 μm.

contracted when compared to nuclei in the normal group (Figure 5(b)). N-GHJTY-62.5, N-GHJTY-125, and N-GHJTY-250 groups exhibited a greater number of positive reactions in the calcified zone of the cartilage when compared to the CFA-control group (Figures 5(c), 5(d), and 5(e)).

4. Discussion

RA is an abnormal autoimmune disease that causes synovial inflammation and damage to joint structure. One characteristic that is specific to RA is the network of new blood vessels that extensively develops in the synovial membrane. This destructive vascular tissue, which is called pannus, extends from the synovium to invade the junction between the cartilage and subchondral bone. With progression of the disease, joint inflammation and the resulting structural changes caused by pannus lead to reduced joint motion, possible ankyloses, joint instability, muscle atrophy from disuse, stretching of the ligaments, and involvement of the tendons and muscles [1].

The CFA approach developed by Pearson [19] is a widely used arthritic model, which is induced in susceptible strains of rats via injection of heat-killed mycobacterium tuberculosis [20]. After CFA injection, a rapid, reliable, robust, and easily measurable polyarthritis develops [21]. Importantly, the joint pathology seen in the rat model shares the synovial

hyperplasia and cartilage degradation observed in human arthritis, particularly RA [22–24]. CFA has recently become a popular tool to observe the efficacy of herbal medicines for treating arthritis. For example, Zhang et al. used the CFA approach to demonstrate the antiarthritic effect of an extract from *Dioscorea zingiberensis* C.H. Wright [25]. Additionally, Zhang et al. used CFA to demonstrate antioxidant effects of Genkwa flos flavonoids [26], while Obiri et al. demonstrated that *Xylopia aethiopica* (Annonaceae) fruit extract suppresses adjuvant-induced arthritis in rats [27]. Several studies have also shown that herbal medicines are effective in treating RA in humans. Shao Li et al. reported that Qing-Luo-Yin extract may have a protective effect against excessive tissue breakdown, angiogenesis, and degradation of extracellular matrix in RA [28]. Chi Zhang et al. also reported that some herbal medicines have beneficial effects on pain management and swollen joint relief in individuals with RA [29]. Additionally, Liu et al. reported that Xinfeng, a patent Chinese herbal medicine, is effective and safe in the treating RA [30].

In the current study, we found that treatment with the N-GHJTY resulted in a gradual decrease of CFA-induced paw and knee joint swelling, as well as rubefaction. This finding was present in all N-GHJTY-treated groups (62.5, 125, and 250 mg/kg) and exhibited a dose-dependent effect—with the N-GHJTY-250 group showing the greatest decrease in paw swelling (Figure 3). In terms of time course, the N-GHJTY-250 group showed significantly less paw and knee joint swelling on the 6th day after treatment, the N-GHJTY-125 group on the 8th day, and the N-GHJTY-62.5 group on the 10th day (Table 1). Moreover, rats in all N-GHJTY groups (62.5, 125, and 250 mg/kg) exhibited increases in body weight when compared to those in the CFA-control group.

Regarding proinflammatory cytokines, all N-GHJTY-treated groups (62.5, 125, and 250 mg/kg) displayed significant reductions in TNF-α, IL-1β, and IL-6 when compared to rats in the CFA-control group. RA is initiated by a T cell-mediated immune response that stimulates the release of cytokines and promotes antibody formation, which leads to destruction of the joint [1]. Specifically, TNF-α induces the production of IL-1β and IL-6 [31], with the former being a crucial mediator of the inflammatory response, causing various autoinflammatory syndromes [32, 33], and the latter being secreted by T cells and macrophages to stimulate the immune response [34, 35]. Thus, proinflammatory cytokines (particularly IL-1β, IL-6, and TNF-α) play an important role in arthritis onset [36], while inhibitors of these cytokines are effective in controlling chronic inflammation [37, 38].

The protective effects of N-GHJTY were confirmed by our histopathological investigations with HE and Safranin O-fast staining. Specifically, rats in the CFA-control group exhibited loose synovial membrane, membrane destruction, disorganized cell arrangement, and compressed cartilage—all of which were prevented in rats treated with N-GHJTY. As mentioned, release of enzymes and inflammatory mediators from damaged tissues perpetuates the inflammatory process [1]. It has also been shown that cytokines released by inflamed synovial tissue can reach systemic circulation and act on other organs [39]. Rheumatoid factor (RF), which is an autoantibody found in RA, forms an immune complex with immunoglobulin G that contributes to RA progression by further triggering inflammatory responses and attracting inflammatory cells. Thus, the ability of N-GHJTY to protect against cartilage and synovial membrane destruction could additionally prevent systemic inflammation by preventing damaged synovial tissue from releasing cytokines.

To investigate if repeated N-GHJTY treatment could induce toxicity, we observed transaminase levels (AST and ALT) in all N-GHJTY-treated groups. Our findings revealed that N-GHJTY treatment did not alter either AST or ALT, suggesting that N-GHJTY was not toxic to rats. Moreover, investigations of various diseases (including arthritis) have reported that AST and ALT leak into the blood stream in proportion to the extent of tissue damage [40–42]. Indeed, transaminase levels were slightly increased in the CFA-control group in the current study. Consistent with our findings on the anti-inflammatory effects of N-GHJTY, we observed that the highest dose of this new herbal combination (i.e., 250 mg/kg) resulted in a slight decrease of transaminase levels, suggesting improved liver function in these animals.

Taken together, our findings suggest that N-GHJTY administration could prevent inflammatory cells from infiltrating and destroying synovial tissue and could also suppress the release of proinflammatory cytokines. Moreover, N-GHJTY was effective in preventing the destruction of joint tissue and facilitated the repair of CFA-induced injury to the joint cartilage, resulting in reduced paw and knee swelling. Thus, the present study provides evidence supporting the clinical use of N-GHJTY for treating arthritis.

5. Conclusions

N-GHJTY, a new complex herbal medication, was effective in treating a rat model of inflammatory arthritis. Specifically, N-GHJTY significantly suppressed the progression of CFA-induced arthritis, as was evident from the decrease in paw and knee joint swelling, and was effective in preventing articular cartilage and synovial tissue degeneration. We also revealed that the protective mechanisms of N-GHJTY treatment could be partially explained by a decrease in the proinflammatory cytokines, TNF-α, IL-1β, and IL-6. Additional studies are required to determine other molecular mechanisms associated with N-GHJTY administration, as well as specific therapeutic effects.

Competing Interests

The authors declare no competing interests regarding the publication of this paper.

Authors' Contributions

Wangin Kim and Sangbin Park contributed equally to this work.

Acknowledgments

This study was supported by a Grant (no. HI13C2285) from the Korea Healthcare Technology R&D Project, Ministry for Health & Welfare Affairs, Republic of Korea.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 5836879, 9 pages
http://dx.doi.org/10.1155/2016/5836879

Research Article

Effects of Wutou Decoction on DNA Methylation and Histone Modifications in Rats with Collagen-Induced Arthritis

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Received 16 October 2015; Accepted 11 February 2016

Academic Editor: Musa T. Yakubu

Background. Wutou decoction (WTD) has been wildly applied in the treatment of rheumatoid arthritis and experimental arthritis in rats for many years. Epigenetic deregulation is associated with the aetiology of rheumatoid arthritis; however, the effects of WTD on epigenetic changes are unclear. This study is set to explore the effects of WTD on DNA methylation and histone modifications in rats with collagen-induced arthritis (CIA). *Methods*. The CIA model was established by the stimulation of collagen and adjuvant. The knee synovium was stained with hematoxylin and eosin. The DNA methyltransferase 1 (DNMT1) and methylated CpG binding domain 2 (MBD2) expression of peripheral blood mononuclear cells (PBMCs) were determined by Real-Time PCR. The global DNA histone H3-K4/H3-K27 methylation and total histones H3 and H4 acetylation of PBMCs were detected. *Results*. Our data demonstrated that the DNMT1 mRNA expression was significantly lowered in group WTD compared to that in group CIA ($P < 0.05$). The DNA methylation level was significantly reduced in group WTD compared to that in group CIA ($P < 0.05$). Moreover, H3 acetylation of PBMCs was overexpressed in WTD compared with CIA ($P < 0.05$). *Conclusions*. WTD may modulate DNA methylation and histone modifications, functioning as anti-inflammatory potential.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown aetiology which is characterized by swelling, pain, stiffness, and deformity of peripheral joints [1]. Environmental factors and epigenetic deregulation are associated with the etiopathology of RA [2]. Epigenetics is defined as stable and heritable changes in gene expression which occur without a change in DNA sequence [3]. The predominant epigenetic mechanisms are DNA methylation, histone modification, and chromatin remodeling. It has been extensively demonstrated that epigenetics plays an important role in the pathogenesis of RA [4–8].

Moreover, various TCM-based herbal formulas and the extracts, such as Wutou decoction (WTD) [9], Xinfeng capsule [10], and *Tripterygium wilfordii* Hook F [11], have been employed in ameliorating articular and extra-articular manifestations of RA. WTD is constituted of six individual herbs which are prepared as seen in Table 1. Clinical studies demonstrated that WTD which was described in a famous TCM monograph Synopsis of Prescriptions of the Golden Chamber in Han Dynasty of China has been wildly applied for the treatment of RA [12], sciatica [13], and scapulohumeral periarthritis [14]. Meanwhile, *in vivo* animal experiments also validated that WTD or its derivatives significantly alleviate swelling, arthritis index, and hyperaemia in rats with adjuvant-induced arthritis [15–18].

In traditional Chinese medicine (TCM), RA falls into the category of arthromyodynia caused by wind, cold, and dampness which was described in the Yellow Emperor's Classic of Internal Medicine. WTD is capable of dispelling dampness by warming the channels and alleviating pain by

Table 1: The composition of herbal formula WTD.

Crude herbs	Content	Main components
Ephedra (*Herbal Ephedrae*)	9	Ephedrine, D-pseudoephedrine
Red Peony Root (*Radix Paeoniae Rubra*)	4.5	Paeoniflorin
White Peony Root (*Radix Paeoniae Alba*)	4.5	Paeoniflorin
Root of Membranous Milkvetch (*Radix Astragali*)	9	Astragaloside
Prepared Liquorice Root (*Radix Glycyrrhizae Preparata*)	9	Glycyrrhizic acid
Prepared Monkshood Mother Root (*Radix Aconiti Preparata*)	6	Aconitine, mesaconitine

WTD: Wutou decoction.

dispersing coldness which is employed in the treatment of RA. And yet, the impacts of WTD on epigenetic changes are unknown. The study aims to explore the effect of WTD on DNA methylation and histone modifications in rats of collagen-induced arthritis (CIA).

2. Materials and Methods

2.1. Reagents and Main Devices. Bovine type II collagen (catalog: 20022) and incomplete Freund's adjuvant (catalog: 7002) were purchased from Amyjet Scientific Inc., China. The components of WTD except *Herbal Ephedrae* (Chinese Herbal Powder, Beijing Tcmages Pharmaceutical Co., Ltd., China) and *Herbal Ephedrae* were provided by Tongji Hospital. TRIzol Reagent (Invitrogen, 15596026), DNase I (Fermentas, EN0521), RevertAid Reverse Transcriptase (Fermentas, EP0442), dNTP (Fermentas, R0191), RiboLock RNase Inhibitor (Fermentas, E00381), All-in-One™ qPCR Master Mix (GeneCopoeiaTM, AOPR-1200), DNA Extraction Kit (Aidlab Biotechnologies Co., Ltd., China, catalog: DN07), and Lymphocyte Separation Medium (Tian Jin Hao Yang Biological Manufacture Co., Ltd.) were ordered from Wuhan Boster Company, China. MethylFlash™ Methylated DNA Quantification Kit (catalog: P-1034), EpiQuik™ Total Histone Extraction Kit (catalog: OP-0006), EpiQuik™ Global Pan-Methyl Histone H3-K4 Quantification Kit (catalog: P-3028), EpiQuik™ Global Pan-Methyl Histone H3-K27 Quantification Kit (catalog: P-3044), EpiQuik™ Total Histone H3 Acetylation Detection Fast Kit (catalog: P-4030), and EpiQuik™ Total Histone H4 Acetylation Detection Fast Kit (catalog: P-4032) were purchased from Amyjet Scientific Inc., China.

Electric Homogenizer (Glas-Col, USA); Ice Machine (AF-100AS, Scotsman, USA); Centrifuge (2-16K, Sigma, USA); Nucleic Acid/Protein Analyzer (DU730, Beckman Coulter, Inc., California, USA); Microplate Reader (BioTek Synergy2, Vermont, USA); Heated Circulating Bath (DKB-600B, Keelrein instrument Co., Ltd., China); MiliporeRiOs 5 Water Purification Systems (Millipore, USA); Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems, California, USA); and Esco Airstream Class II Biological Safety Cabinet (Beijing, China) were used.

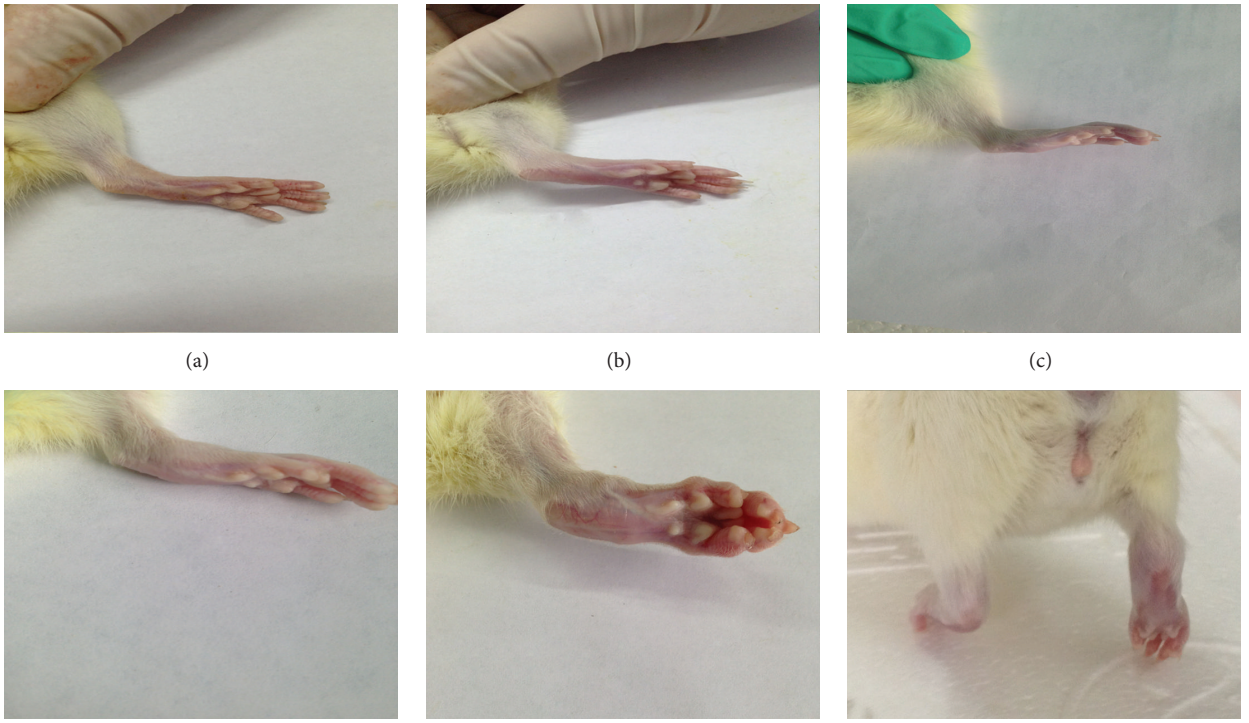
2.2. Animals and Rearing Conditions. Five-week-old, weighted 130–150 g female Wistar rats ($n = 45$), SPF grade, were provided by the Center for Disease Control and Prevention of Hubei province (the animal certificate SCXK no. 2008-0005) and fed in the barrier system according to The Guidelines for the Care and Use of Animals in Research enforced by Hubei Municipal Science and Technology Commission. All protocols were approved by the Institutional Animal Care and Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Food and water were given *ad libitum* throughout the experiment. The rats were caged in a standard barrier system with a 12 h light/dark cycle.

2.3. Grouping and Treatment. After 7 days of acclimation, the animals were randomly divided into three groups: normal group (N), CIA group, and WTD group (4.2 g/kg/d) with 15 rats in each group. The rat doses of treatment group were converted from human doses (Chinese Pharmacopeia, 2010) based on body surface areas. The final concentration of treatment group was 0.42 g/mL for WTD group. Oral gavage was performed once a day from day 28 after the initial immunization for four weeks. Rats in groups N and CIA were orally administered with the same volume of distilled water. The rats were fasted for 12 h but permitted water *ad libitum* before blood collection.

2.4. CIA Model Establishment and Arthritis Index Assessment. CIA model was established according to the manufacturer's standard protocols and previous research [19]. In addition to group N, CIA model was established in the other two groups. Bovine type II collagen was emulsified in an equal volume of incomplete Freund adjuvant, and 0.2 mL of the emulsion was injected subcutaneously about 3 cm distal from the base of the tail to induce arthritis. To ensure a high CIA incidence, a booster immunization (administer 0.1 mL of the emulsion subcutaneously in the tail) was performed two weeks after the initial immunization about 1.5 cm distal from the base of the tail.

As shown in Figure 1, the arthritis severity was evaluated by applying a previous published scoring system [20]. Once arthritis occurred, the arthritis index (AI) was evaluated twice a week for arthritis development. The arthritis score for each rat was the sum of the scores for two hind paws.

2.5. Sampling. At sixty minutes after the last intragastric administration, the rats were narcotized with 10% chloral hydrate by intraperitoneal injection. Blood was extracted from aorta abdominalis. The synovium isolated from knee joints was fixed in 4% paraformaldehyde for 12 h and embedded in paraffin. Paraffin-embedded synovial tissues were



(a) (b) (c)

(d) (e) (f)

Figure 1: Arthritis index assessment: (a) 0; (b) 1; (c) 2; (d) 3; (e) 4; (f) right hind paw which is unable to bear weight.

Table 2: Primer sequence.

Gene	Primer	Sequence
β-actin	Forward	5′-CGTTGACATCCGTAAAGACCTC-3′
	Reverse	5′-TAGGAGCCAGGGCAGTAATCT-3′
DNMT1	Forward	5′-CGCTCATTGGCTTTTCTACCG-3′
	Reverse	5′-AGAACTCGACCACAATCTT-3′
MBD2	Forward	5′-AATGATGAGACCCTTCTGTCTGCC-3′
	Reverse	5′-TCCTCTAGTTTCTTTCGGACTTGTTG-3′

DNMT1: DNA methyltransferase 1; MBD2: methyl CpG binding domain 2.

cut in 5 μm thick sections and stained with hematoxylin and eosin. The sections were evaluated by two independent pathologists. The peripheral blood mononuclear cells (PBMCs) were separated by Lymphocyte Separation Medium using density gradient centrifugation method.

2.6. Real-Time PCR for PBMCs DNA Methyltransferase 1 (DNMT1) and Methylated CpG Binding Domain 2 (MBD2) Expression. Total RNA was extracted from PBMCs with TRIzol Reagent consistent with the manufacturer's instructions. RNA purity and concentration were measured by 1.8% agarose gel electrophoresis and Nucleic Acid/Protein Analyzer. cDNA was synthesized in accordance with the manufacturer's instructions. The cDNA was stored at −20°C prior to PCR amplification. Real-Time PCR reactions were performed applying StepOne Real-Time PCR System by following the manufacturer's instructions. Thermal cycler protocol: stage 1, Reps 1 95°C 10 min; stage 2, Reps 40 95°C 10 s, 60°C 20 s, and 72°C 20 s. The data were analyzed by employing $2^{-\Delta\Delta ct}$ method. The primers were designed in accordance with published sequences (Table 2).

2.7. Determination of Global DNA Methylation of PBMCs. Total DNA was extracted from PBMCs with DNA Extraction Kit consistent with the manufacturer's instructions. DNA purity and concentration were measured by 1% agarose gel electrophoresis and Nucleic Acid/Protein Analyzer. The global DNA methylation of PBMCs was detected according to the manufacturer's instructions. The amount and percentage of methylated DNA in total DNA were calculated using the following formulas: 5-mC (ng) = (Sample OD − ME3 OD)/(Slope × 2) or 5-mC% = (5-mC Amount (ng)/S) × 100%.

2.8. Determination of Global Pan-Methyl Histones H3-K4 and H3-K27 of PBMCs. Total histones were extracted from

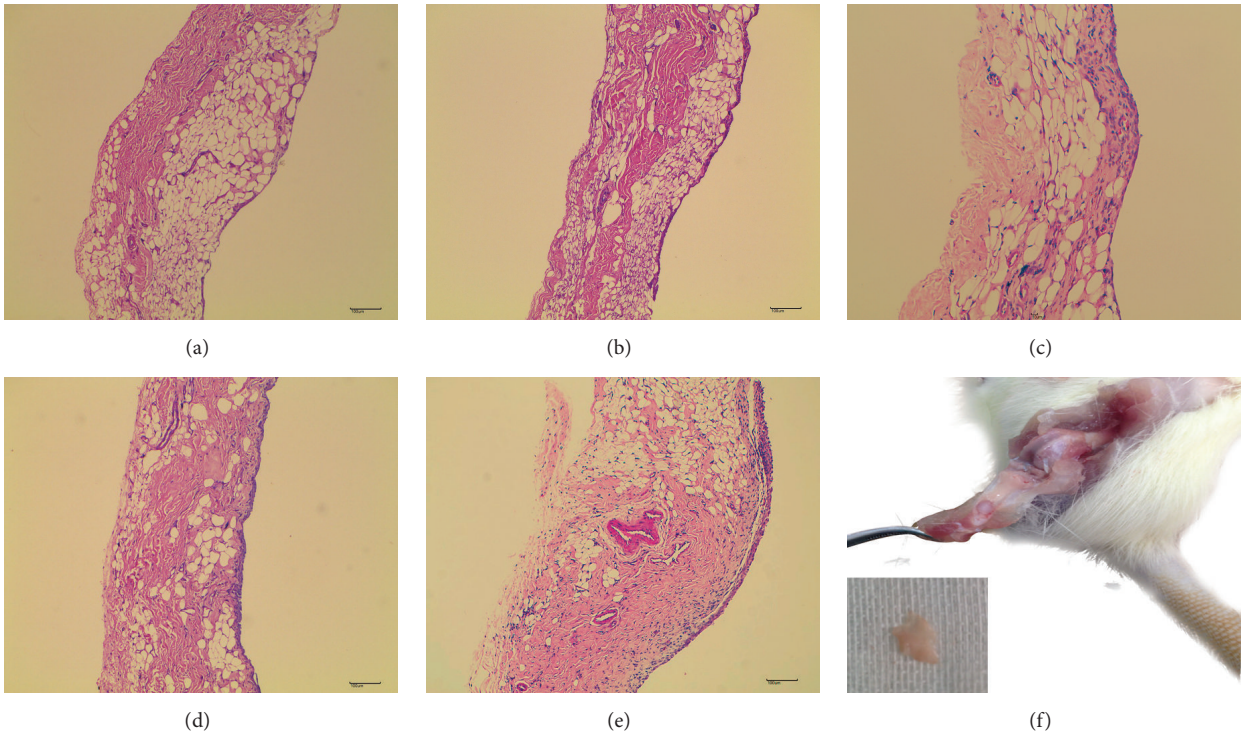


Figure 2: The hematoxylin and eosin staining of synovium corresponding to arthritis index score. (a), (b), (c), (d), and (e) represent the arthritis index scores 0, 1, 2, 3, and 4, respectively. (f) The process of isolating knee synovium.

PBMCs with Histone Extraction Kit according to the manufacturer's instructions. Histone concentration was measured by BCA protein assay kit. The global histones H3-K4 and H3-K27 methylation of PBMCs was detected according to the manufacturer's instructions. The amount and percentage of mono-, di-, and trimethylated H3-K4 and H3-K27 were calculated applying the following formulas: Methylation% = OD (treated (tested) sample − blank)/OD (untreated (control) sample − blank) × 100% or Amount (ng/mg protein) = (OD (sample − blank)/Protein (μg) × slope) × 1000.

2.9. Determination of Total Histones H3 and H4 Acetylation of PBMCs. Total histones were extracted from PBMCs with histone extraction kit in accordance with the manufacturer's instructions. Histone concentration was measured by BCA protein assay kit. The total histones H3 and H4 acetylation of PBMCs was detected according to the manufacturer's instructions. The amount and percentage of acetyls H3 and H4 were calculated using the following formulas: Acetylation% = OD (treated (tested) sample − blank)/OD (untreated (control) sample − blank) × 100% or Amount (ng/mg protein) = (OD (sample − blank)/Protein (μg) × slope) × 1000.

2.10. Statistical Analysis. All data with a normal distribution were presented as mean ± standard deviation and analyzed with aid of SPSS17.0 statistical software. Statistical significance was determined by one-way analysis of variance (ANOVA). For data with equal variances assumed, ANOVA followed by LSD test was applied. For data with equal variances not assumed, ANOVA followed by Dunnett's $T3$ test was adopted. A probability of less than 0.05 was considered to be statistically significant.

3. Results

3.1. The Hematoxylin and Eosin Staining of Synovium Corresponding to AI Score. The histopathological characteristics of synovial membrane corresponding to AI score were presented as follows: 0 (AI score): no alteration; 1 (AI score): the synovial lining cells form 2-3 layers; 2 (AI score): the synovial lining cells form 4-5 layers (multinucleated cells might occur); 3 (AI score): the synovial lining cells form 5–8 layers, hyperplastic synovial stroma, and pannus formation; 4 (AI score): the synovial lining cells form more than 8 layers, numerous inflammatory cell infiltration, and neovascularization (Figure 2).

3.2. Expression of PBMCs DNMT1 and MBD2 mRNA. Compared with group N, the DNMT1 mRNA expression was significantly elevated in group CIA ($P < 0.05$). The DNMT1 mRNA expression was significantly lowered in group WTD compared to that in group CIA ($P < 0.05$) (Figure 3(a)). Compared with group N, the MBD2 mRNA expression was increasing in group CIA; however, there was no significant difference between the two groups. Compared with group CIA, there was a reduction in group WTD; however, there was no significant difference between the two groups (Figure 3(b)).

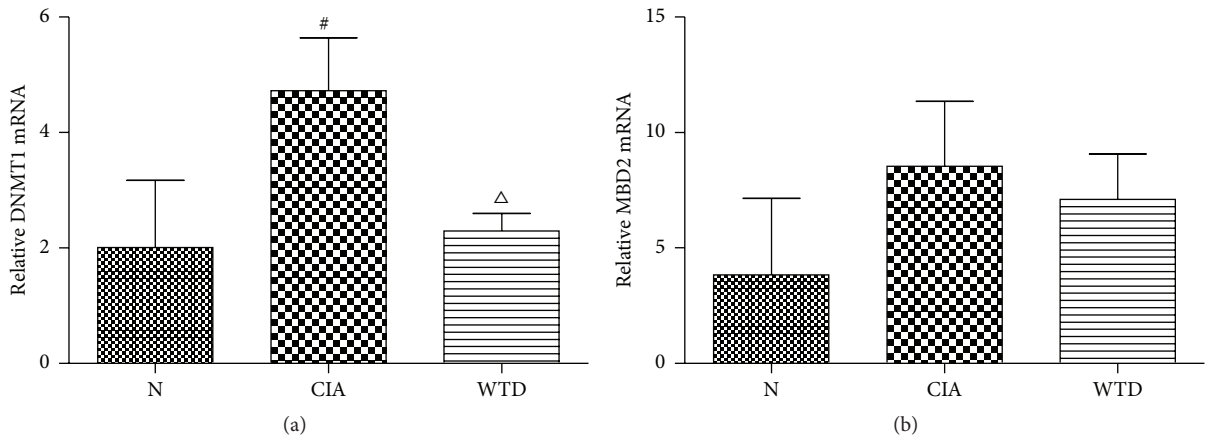


Figure 3: (a) Expression of PBMCs DNMT1 mRNA and (b) expression of PBMCs MBD2 mRNA. Values are mean ± SD. $^{\#}P < 0.05$ compared with group N and $^{\triangle}P < 0.05$ compared with group CIA. N: normal; CIA: collagen-induced arthritis; WTD: Wutou decoction; PBMCs: peripheral blood mononuclear cells; DNMT1: DNA methyltransferase 1; MBD2: methyl CpG binding domain 2.

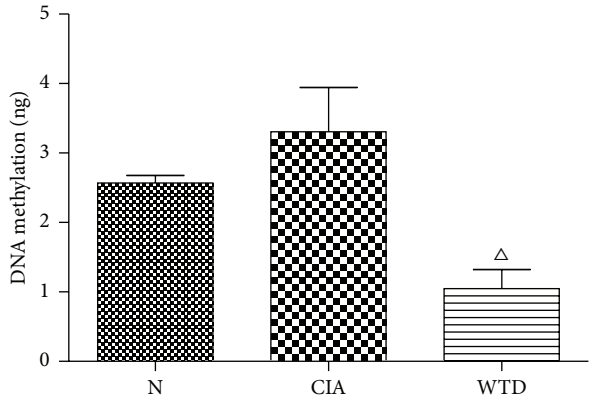


Figure 4: Expression of global DNA methylation level in PBMCs. Values are mean ± SD. $^{\triangle}P < 0.05$ compared with group CIA. N: normal; CIA: collagen-induced arthritis; WTD: Wutou decoction; PBMCs: peripheral blood mononuclear cells.

3.3. Expression of Global DNA Methylation Level in PBMCs. Compared with group N, the global DNA methylation level was enhancing in group CIA; however, there was no significant difference between the two groups. Compared with group CIA, the global DNA methylation level was significantly reduced in group WTD ($P < 0.05$) (Figure 4).

3.4. Expression of Global Pan-Methyl Histones H3-K4 and H3-K27 in PBMCs. Compared with group N, the mono-, di-, and trimethylated H3-K4 and H3-K27 were increasing in group CIA; however, there was no significant difference between the two groups. Compared with group CIA, the mono-, di-, and trimethylated H3-K4 and H3-K27 were enhancing in group WTD; however, there was no significant difference between the two groups (Figures 5(a) and 5(b)).

3.5. Expression of Total Histones H3 and H4 Acetylation in PBMCs. Compared with group N, the total histone H3 acetylation in group CIA was no significant alteration. The total histone H3 acetylation in group WTD was significantly elevated compared to that in group CIA ($P < 0.05$) (Figure 6(a)). Compared with group N, the total histone H4 acetylation level was enhancing in group CIA; however, there was no significant difference between the two groups. Compared with group CIA, the total histone H4 acetylation level was reduced in group WTD; however, there was no significant difference between the two groups (Figure 6(b)).

4. Discussion

Based on the character of herbs, WTD has been wildly employed in the treatment of RA. However, the mechanism by which WTD acts on RA is unclear. Systems biology-based investigation indicated that the predicted effect or molecules of WTD were significantly enhanced in neuroactive ligand-receptor interaction and calcium signaling pathway [9]. As the primary component of WTD, the methanol extracts of *Aconitum* roots have shown inhibition of hind paw edema produced by carrageenin in mice [21]. Seventy-four components of WTD have been identified by applying an ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry method [22]. The study focuses on investigating the potential epigenetic mechanisms of WTD in rats with CIA.

CIA is a typical experimental autoimmune disease that is widely used as a model of RA. CIA model was firstly established by Trentham and colleagues [23]. There are substantial differences in histopathological characteristics of synovium even in two knees of a rat with CIA. Consequently, it is difficult to compare the pathological difference among the groups. For the reasons outlined above we only elucidated the histopathological characteristics of synovial membrane corresponding to AI score.

DNA methylation, the most characterized epigenetic mark, occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by a family of DNMTs with

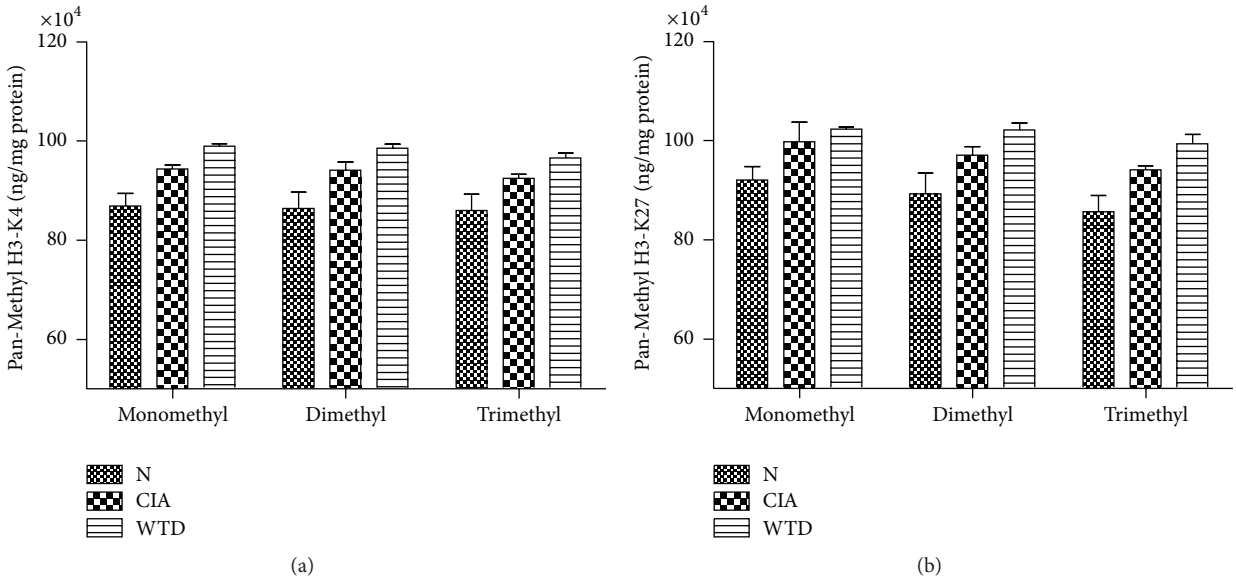


Figure 5: Expression of Global Pan-Methyl Histones H3-K4 and H3-K27 in PBMCs. Values are mean ± SD. N: normal; CIA: collagen-induced arthritis; WTD: Wutou decoction; PBMCs: peripheral blood mononuclear cells.

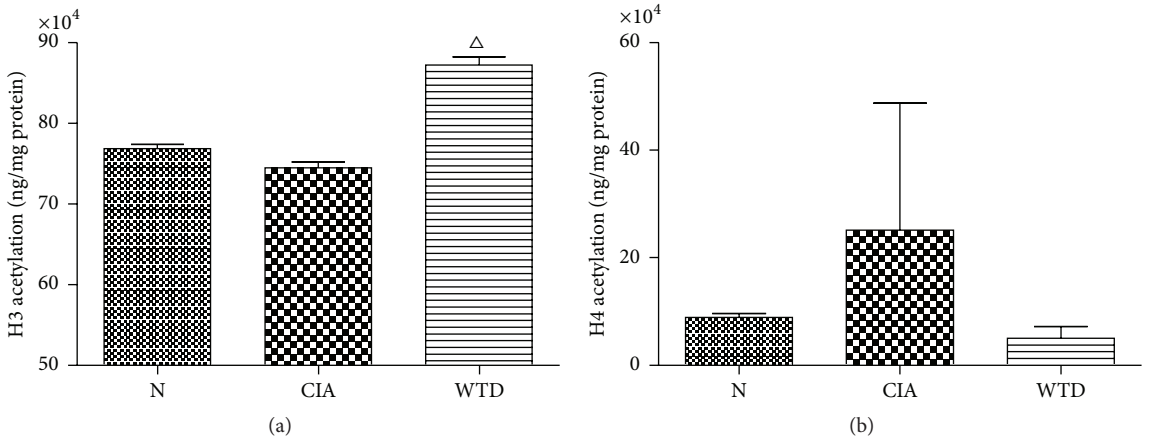


Figure 6: Expression of total histones H3 and H4 acetylation in PBMCs. Values are mean ± SD. $^{\triangle}P < 0.05$ compared with group CIA. N: normal; CIA: collagen-induced arthritis; WTD: Wutou decoction; PBMCs: peripheral blood mononuclear cells.

S-adenosyl-methionine as the methyl source, resulting in 5-methylcytosine. DNA methylation is catalyzed by DNMTs, predominantly including DNMT1, DNMT3a, and DNMT3b. DNMT1 is the most abundant DNMT in mammalian cells and is considered to be the key maintenance DNMT in mammals. DNMT1 principally methylates hemimethylated CpG dinucleotides in the mammalian genome. DNA methylation suppresses the expression of genes at transcriptional level [24].

Our results were consistent with the findings of Liu et al. concerning the higher expression of DNMT1 mRNA in group CIA than that in N [25]. Compared with group CIA, the DNMT1 mRNA expression was significantly lowered in group WTD. The global DNA methylation level was significantly reduced in group WTD compared to that in group CIA, which may result from the lower expression of DNMT1 mRNA in group WTD. However, tumor necrosis factor α (TNFα) blocker has no impact on DNMT1 mRNA expression in RA patients [25]. The different mechanisms of WTD and TNFα blocker in the treatment of RA may contribute to the differences.

Human MBDs are a family of methyl CpG binding domain proteins consisted of methyl CpG binding protein 2 (MECP2), MBD1, MBD2, MBD3, and MBD4. Apart from MBD3, each of these proteins is capable of binding specifically to methylated DNA. MECP2, MBD1, and MBD2 can also bind to histone deacetylases (HDACs) functioning as transcription repressors [24]. MBD2 selectively binds to methylated DNA and may function as a mediator of the biological consequences of the methylation signal [26].

The MBD2 mRNA expression was increasing in group CIA compared to that in N; however, there was no significant difference between the two groups. Clinical study also demonstrated that the MBD2 mRNA expression was higher in RA patients [25]. However, the MBD2 mRNA expression was decreasing in group WTD compared with group CIA, although there was no significant difference between the two groups. Anti-TNFα biological agents do not seem to affect mRNA expression of MBD2 in RA patients [25]. These suggest that anti-TNFα biologics and WTD could not influence MBD2 mRNA expression in treating RA.

Current researches also showed that DNA hypomethylation exists in synovial fibroblasts, T cell, and PBMCs in RA patients [25, 27, 28], indicating that DNA hypomethylation is associated with the pathogenesis of RA. MBD2 participates in silencing of methylated genes [29] and MBD2 activates gene demethylation [30]. It was assumed that group CIA had a higher expression of DNMT1 mRNA with the succeeding global hypermethylation of DNA and then led to a higher expression of MBD2 mRNA through a feedback mechanism.

Histone methylation and histone acetylation, two post-translational modifications of histones, are investigated intensively for their critical roles in modulating gene transcription. Histone methylation marks can be correlated with transcriptional activation or silencing, dependent on the position and the degree of methylation [31]. The trimethyl mark on H3-K4 (H3-K4me3) was often associated with active transcription [32]. Enhancer of zeste homologue 2 (EZH2) generated the trimethyl mark on H3-K27 (H3-K27me3) which correlated with gene silencing [32, 33].

Our experiments suggested that the mono-, di-, and trimethylated H3-K4 and H3-K27 had no significant increase in group WTD compared to that in group CIA. EZH2, a histone methyltransferase enhancer, was overexpressed in RA synovial fibroblasts (SF) compared with osteoarthritis (OA) SF [34]. However, the studies regarding histones H3-K4 and H3-K27 methylation are rare. Further studies are needed to clarify the role of histone methylation in RA.

Histone acetylation is regulated by the opposite activity of two enzyme families, histone acetyl transferases (HATs) and HDACs. Histone acetylation is catalyzed by HATs, enhancing the rate of gene transcription. On the other hand, histone deacetylation is catalyzed by HDACs, leading to transcriptional silence of certain genes. The imbalance between histone acetylation and deacetylation regulates the transcription rates of various genes and has been indicated to be related to disease states [35].

Our data indicated that the total histone H3 acetylation in group WTD was significantly elevated compared to that in group CIA. HDAC1 was overexpressed in RASF compared to OASF, supporting cell proliferation and survival of RASF [36]. Meanwhile, HDAC2 also played a crucial role in cell proliferation and apoptosis of RASF [36]. In addition, nuclear HDAC activity and expression of HDAC1 were significantly elevated in RA compared to those in OA synovial tissues [37]. A wide range of HDAC inhibitors (HDACis) showed protective effects in prophylactic and therapeutic models of RA [38]. Trichostatin A (TSA), a nonselective HDACi, could potently inhibit the lipopolysaccharide (LPS)-induced production of TNF and interleukin-6 (IL6) in both RA and healthy PBMCs [39]. The HDAC-3-selective inhibitor MI192 inhibited TNF production at high concentrations and dose-dependently reduced IL6 in RA PBMCs but not healthy PBMCs [39]. Class I/II HDACi TSA as well as class III HDAC nicotinamide blocked the TNFα-stimulated expression of IL6 and the LPS-induced expression of IL6 and TNFα in macrophages of RA patients [38]. TSA time-dependently increased the acetylation of H3 and H4 in macrophages [38]. However, incubation of macrophages with nicotinamide failed to induce detectable acetylation of H3 or H4 [38]. WTD may function as a nonselective HDACi, resulting in the acetylation of H3.

5. Conclusions

In recent years, DNA methylation and histone modifications were involved in the pathogenesis of RA. Moreover, numerous epigenetics-based drugs were employed in attenuating the inflammatory activity of RASF or macrophages, especially various selective or nonselective HDACis. WTD may serve as a traditional drug in alleviating disease activity of RA via DNA methylation and histone modifications. The methylated loci and other epigenetic changes of RA are needed to be investigated to confirm this.

Conflict of Interests

The authors have no competing interests.

Authors' Contribution

Ya-Fei Liu and Cai-Yu-Zhu Wen carried out all the assays and drafted the paper. Ya-Fei Liu and Cai-Yu-Zhu Wen contributed equally to this work and should be considered co-first authors. Ya-Fei Liu, Zhe Chen, Yu Wang, and Ying Huang participated in the design of the study and carried out the statistical analysis. Yong-Hong Hu and Sheng-Hao Tu conceived of the study and were responsible for its design and helped in revising the paper. All authors read and approved the final paper.

Acknowledgments

The authors thank Professor Ming Xiang for helpful suggestions in modeling. This work was supported by Grants from the National Natural Science Foundation of China (no. 81341085).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 4098686, 7 pages
http://dx.doi.org/10.1155/2016/4098686

Research Article

Biological Evaluation and Docking Analysis of Daturaolone as Potential Cyclooxygenase Inhibitor

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Received 1 October 2015; Revised 4 February 2016; Accepted 8 February 2016

Academic Editor: Bamidele Victor Owoyele

This study deals with the isolation of the active constituent(s) from a methanolic extract of *Pistacia integerrima* J. L. Stewart barks and it was also oriented to evaluate the *in vivo* and *in silico* anti-inflammatory activity. By NMR and crystallography techniques, we have isolated a triterpenoid identified as daturaolone (compound **1**). This compound showed *in vivo* a significant and dose dependent (1–30 mg/kg) anti-inflammatory activity on carrageenan-induced mouse paw oedema (ED_{50} = 10.1 mg/kg) and on acetic acid-induced writhing responses in mice (ED_{50} = 13.8 mg/kg). In the *in vivo* experiments, the effect of tested compound was also evaluated in presence of the reference drug diclofenac (1–30 mg/kg). Moreover, *in silico* analysis of receptor ligand complex shows that compound **1** interacts with cyclooxygenases (COXs) binding sites displaying an interesting interaction with COX-1. These findings suggest that compound **1** isolated from *P. integerrima* possesses *in vivo* anti-inflammatory and antinociceptive potentials, which are supported *in silico* by an interaction with COXs receptors.

1. Introduction

Pistacia integerrima J. L. Stewart is one of twenty species belonging to the genus *Pistacia* (Anacardiaceae). This moderate size tree widely grows in the subalpine regions of Himalaya as well as in various regions of Pakistan and India [1, 2]. Many species of genus *Pistacia* are used in traditional medicine against various ailments [3]. In fact their plant extracts have been studied for a variety of biological activities such as bronchodilator, antiemetic, diuretic, analgesic, anti-inflammatory, and antirheumatic effects [3, 4]. Different experimental evidences have suggested that the anti-inflammatory properties of some species of genus *Pistacia* are due to the enzymatic inhibition of cyclooxygenase and lipoxygenase. These effects seem to be related to the presence of terpenoids and flavonoids [5, 6]. On this basis, this study was focused on the isolation and characterization of the constituent(s) from a methanolic extract of *P. integerrima* barks and it was also oriented to evaluate its biological activity by a classical *in vivo* and *in silico* approach.

2. Materials and Methods

2.1. Plant Material. *P. integerrima* was collected from Murree Hills (Pakistan) and it was successively identified by Prof. Rashid A. (Department of Botany, University of Peshawar, Pakistan). A voucher specimen (number 20037) was deposited at Department of Botany, University of Peshawar, Pakistan.

2.2. Extraction and Isolation. The barks (8.9 kg) were shade-dried at room temperature, grounded into powder, and subsequently extracted thrice with methanol at room temperature, giving 387 g of residue. The methanol extract was dissolved in water and successively extracted with *n*-hexane (44.2 g), chloroform (98.3 g), ethyl acetate (49.4 g), and *n*-butanol (66.9 g). An aliquot (10.2 g) of the chloroform extract was chromatographed on a silica gel column, eluting with *n*-hexane and ethyl acetate mixtures of increasing polarity. Two hundred and fifty-five fractions were eluted and combined in 10 major fractions (RF-1 to RF-10), according to their TLC similarity. From RF-3 (61.2 mg; eluted with *n*-hexane-EtOAc, 82 : 18) compound **1** was obtained as colorless crystals which were separated from the solution by decantation. The crystals were recrystallized with appropriate solvents (*n*-hexane-acetone, 4 : 1). The structural elucidation of the isolated compound was performed by spectroscopic methods (^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC, NOESY, COSY, HREI-MS, and IR). Spectra were obtained on a Vector 22 (Bruker) Fourier transform infrared (FTIR) spectrometer, employing KBr windows with CH_2Cl_2 as the solvent against an air background. ^{1}H-NMR (600 MHz) and ^{13}C-NMR (125 MHz) spectra were registered on a Bruker Avance spectrometer. The 2D-NMR spectra were obtained on a Bruker Avance NMR spectrometer. Mass spectral information (EI and HR-EI-MS) was recorded on Jeol-JMS-HX-110 mass spectrometer and calculated in electron impact mode on Finnigan MAT-312 and MAT-95 XP; ions were given in m/z (%). Melting points of compound **1** were determined in glass capillaries tubes by Bicote melting point apparatus (Bibby Scientific limited, UK) and the UV spectra were measured in chloroform by using UV-visible recording spectrometer Model Hitachi-U-3200 (Japan). The IR spectra were recorded on FT-IR Nicolet 380 (Thermo Scientific, UK) and the single X-rays on Kappa APEXII CCD diffractometer (SADABS; Bruker, 2005).

2.3. In Vivo Procedures. Male BALB/c mice (25–30 g) were used in all the experiments. Animals were purchased from the Pharmacology Section of the Department of Pharmacy, University of Peshawar (Peshawar, Pakistan). The animals were maintained in standard conditions (22 ± 2°C and light/dark cycles, i.e., 12/12 h) and were fed with standard food and water *ad libitum*. The experimental protocols were approved by the Ethical Committee of the Department of Pharmacy, University of Peshawar (Pakistan). All the experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. All efforts were made to minimize animal suffering.

Successively, mice were randomly divided into 9 groups ($n = 6$) and lightly anaesthetized with enflurane 4% mixed in O_2/N_2O (1 : 1) atmosphere. A negative control group (group I) was injected with normal saline (10 mL/kg; i.p), whereas positive control groups (II, III, IV, and V) were treated with diclofenac sodium (1.0–30.0 mg/kg; i.p). Groups VI, VII, VIII, and IX received compound **1** at the doses of 1.0, 3.0, 10.0, and 30.0 mg/kg i.p., respectively. Thereafter, 50 μL of 1%γ-carrageenan (dissolved in saline) was injected subcutaneously into the subplantar tissue of the mice right paw, 30 min after administration of drugs. Oedema, measured with a hydropletismometer (Ugo Basile, Milan, Italy), was calculated by subtracting the initial paw volume to the paw volume measured at each time point (1–5 h) and successively reported as percentage (%) of decrease of paw volume, as previously described [7, 8].

In acetic acid-induced writhing test, animals were divided into 9 experimental groups ($n = 6$) and successively withdrawn from food 2 h before the experimental section. Group I (control group) was injected with saline (10 mL/kg, i.p.). Groups II, III, IV, and V received diclofenac sodium as standard drug (1.0–30.0 mg/kg, i.p.), while groups VI, VII, VIII, and IX were treated with 1.0, 3.0, 10.0, and 30.0 mg/kg i.p. of **1**, respectively. After 30 min, mice were treated intraperitoneally (i.p.) with 0.1 mL of a 1% solution of acetic acid. The number of abdominal constrictions (writhings) was counted 5 min after the acetic acid injection for 10 minutes of observation, as previously described [9]. Percentage (%) of anti-inflammatory effect was calculated using the following formula: % anti-inflammatory effect = number of writhings in tested animals/number of writhings in control animals × 100.

2.4. Computational Analysis. The COX-1 and COX-2 receptor amino acid sequence was obtained in the FASTA format from UniProt database (code number: P56476) followed by BLAST against PDB database for template selection [10]. The BLAST is commonly used to identify similar homologous structures which can provide template for homology model building [11]. The crystal structures of COX-1 and COX-2 receptor were obtained as the best hit according to their sequence identity. Obtaining the target and template sequence, successively the alignment was carried out by BioEdit sequence alignment editor software [12]. The 3D structures of COX receptors were generated by using MODELLER 9.12. The energy refinement process was carried out through Swiss PDB viewer v4.1.0 software [13]. Energy minimization was performed (500 steps of steepest descent followed by 1000 steps of conjugate gradient) without assigning any constraint [14]. All the residues adopt a stable conformation by avoiding steric hindrance. The refine 3D structures were validated by ProSA and Procheck online servers and the best model was selected for the docking studies [15]. The iGEMDOCK v2.1 software was used for the docking studies of COX-1 and COX-2 with compound **1**. The 2D structure of the ligands was drawn through ChemDraw software and saved in mol format. The 2D structures were converted into 3D followed by reduction and minimization through Avogadro software [16, 17]. The iGEMDOCK software was implemented with generic evolutionary algorithm (GA) to carry out automated molecular dockings. AutoDock Vina software was also used for the docking analysis. The software can work through AutoDock Tools (ADT) or Pyrex tools [18]. The macromolecules were cleaned from water residues and Gasteiger charges were calculated. The ligand molecules were prepared in ChemDraw software and Avogadro software and were saved in mol2 format. The docking procedure was calibrated by already cocrystallized ligand. The ligands

and macromolecules were uploaded in the Pyrex tool [19]. Finally, the receptor and ligand files were converted into pdbqt format.

2.5. Statistical Analysis. The results obtained were expressed as the mean ± S.E.M for the raw data or as the mean ± S.E.M of the percentage of the vehicle response, where each animal acted as its own control. For statistical analysis, one-way analysis of variance (ANOVA) was followed by Bonferroni correction for normally distributed data or by Dunnett's for nonparametric data in order to evaluate specific differences between individual groups. In some case, one sample t-test was used to evaluate significance against the hypothetical zero value. The ED_{50} value for the anti-inflammatory effect of compound **1** accompanied by its respective 95% confidence limits was determined by linear regression from individual experiments using GraphPad Prism 5.0 software (San Diego, CA, USA). Values were considered to be significant at $p \leq 0.05$.

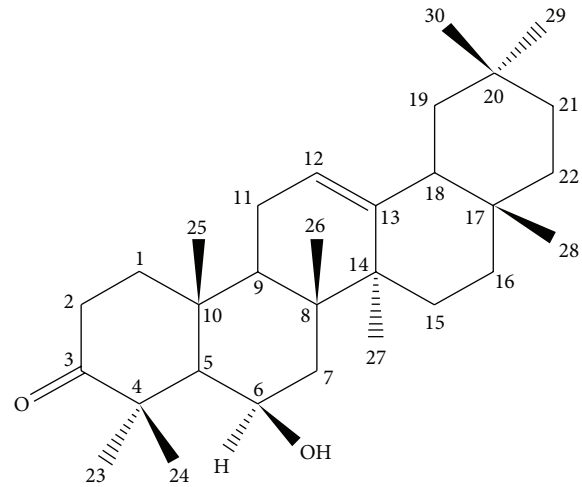

Figure 1: Structure of daturaolone (compound **1**).

3. Results and Discussion

The continuing search for new anti-inflammatory agents is due to the complexity of the inflammatory process and its role in host defense. However, the progress achieved in understanding the mechanisms involved in the inflammatory response has made the identification of new targets possible, paving the way for the search for new compounds with potential therapeutic effects on acute or chronic inflammatory diseases [20–23].

Most drug discovery is focused on the search for bioactive compounds obtained from natural sources and many drugs used today for the treatment of several diseases have been developed from natural products. In this context, terpenes, which comprise a very large family of natural products containing more than 50,000 structurally diverse compounds, have recently attracted the attention for their potential anti-inflammatory activities [24–26].

Here, for the first time, we have isolated from a methanol extract of *P. integerrima* barks a pentacyclic triterpenoid derivative identified as daturaolone (compound **1**). The molecular formula of compound **1** was determined as $C_{30}H_{48}O_2$ (Figure 1) by EIMS (m/z; 440.37; calcd. 440.3710) and NMR data. The assignment of protons and carbons was carried out by HMBC, HSQC, and ^{1}H-^{1}H-COSY experiments (Supplementary Material available online at http://dx.doi.org/10.1155/2016/4098686). Successively, the structure of compound **1** was confirmed by comparing its NMR and physical and X-ray crystallographic data with those already reported in literature [27].

In order to evaluate the potential anti-inflammatory activity of compound **1**, the carrageenan-induced mouse paw oedema model was employed. The intraplantar injection of carrageenan in mice leads to paw oedema, the first phase of which results from the early (1-2 hrs) release of histamine, serotonin, and kinins followed by a second phase (3-4 hrs) characterized by the production of prostaglandins and oxygen-derived free radicals and the production of inducible cyclooxygenase (COX-2). Only the presence of persistent stimuli or dysregulation of the resolution phase tempts the recruitment of local neutrophil activation and lymphocytes infiltration. For these reasons, carrageenan-induced paw oedema has been shown to be a useful model for the study of inflammation and for the evaluation of anti-inflammatory profiles of various drugs [28–30]. Results of the carrageenan-induced paw oedema showed the dose dependent anti-inflammatory profile of compound **1** in all time-course (1–5 hrs). The maximum anti-inflammatory effect was observed at 4 h, where compound **1** significantly ($p < 0.05$ and $p < 0.01$ for 10 and 30 mg/kg, resp.) displayed its anti-inflammatory profile (ED_{50} = 10.1 mg/kg) (Figure 2). More remarkable anti-inflammatory effects were observed after the administration of the reference drug, diclofenac (1.0–30.0 mg/kg; ED_{50} = 8.2 mg/kg) (Figure 2). The results observed at 4 h in the carrageenan-induced paw oedema confirmed the hypothesis that compound **1** could act by a mechanism related to the inhibition of COXs [31, 32].

To investigate the effect of compound **1** in another *in vivo* model of inflammation, the writhing test was employed. The acetic acid-induced abdominal writhing is a widely used model to detect the anti-inflammatory and analgesic potential of tested compound(s). In this test, the pain induction is due to the release of arachidonic acid and cyclooxygenase products. Drugs able to inhibit the writhing test (e.g., nonsteroidal anti-inflammatory drugs (NSAIDs)) possess analgesic and anti-inflammatory effects associated with the modulation of prostaglandins [32]. Anti-inflammatory effects of **1** on writhing test are shown in Figure 3. Compound **1** administered i.p. at different doses (1.0, 3.0, 10.0, and 30.0 mg/kg) exhibited significant ($^*p < 0.05$ and $^{**}p < 0.01$) inhibition of writhings with an extent of 31.00 ± 9.53% and 50.33 ± 8.95% at 10.0 and 30.0 mg/kg, respectively. No significant effects were observed at dose of 1.0 and 3.0 mg/kg (ED_{50} = 13.8 mg/kg). Maximal inhibition of the writhing response was slightly lower compared to inhibition evoked by diclofenac sodium at a dose of 30.0 mg/kg (75.33 ± 5.48%; $^{***}p < 0.001$). Moreover, the reference drug displayed a significant anti-inflammatory effect even at dose of 3.0 mg/kg

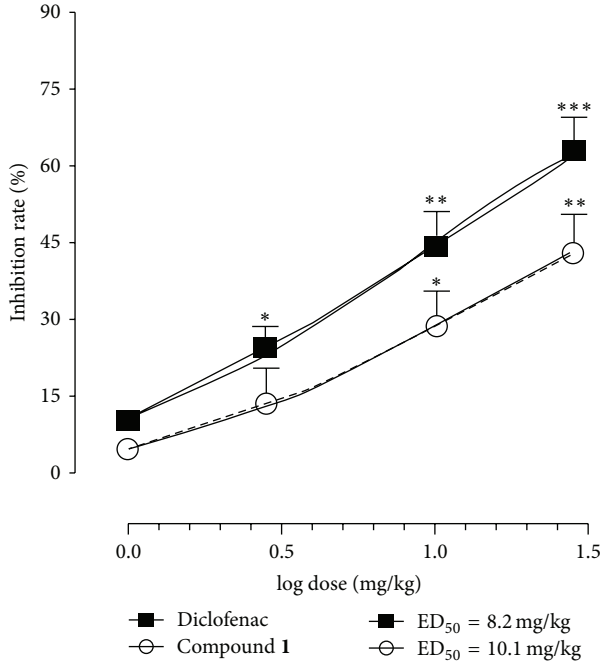

Figure 2: Effect of compound **1** on carrageenan-induced paw oedema. Compound **1** (1.0–30.0 mg/kg) or diclofenac (1.0–30.0 mg/kg) was administered intraperitoneally (i.p.) 30 min before the subcutaneous injection of 50 μL of 1% carrageenan and paw swelling measured at 4 h. Values reported as percentage (%) of inhibition of paw oedema are expressed as log dose (mg/kg) ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

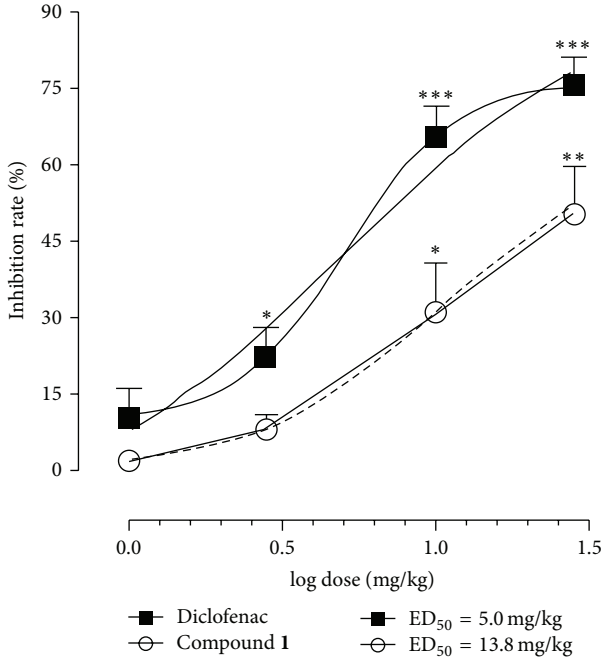

Figure 3: Effect of compound **1** and diclofenac on acetic acid-induced writhing responses in mice. Saline (10 mL/kg), compound **1** (1.0, 3.0, 10.0, and 30.0 mg/kg), or diclofenac (1.0, 3.0, 10.0, and 30.0 mg/kg) was administrated intraperitoneally (i.p.) 30 min before acetic acid injection (0.1 mL; i.p.). Values reported as percentage (%) of inhibition of writhings are expressed as log dose (mg/kg) ± SEM ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

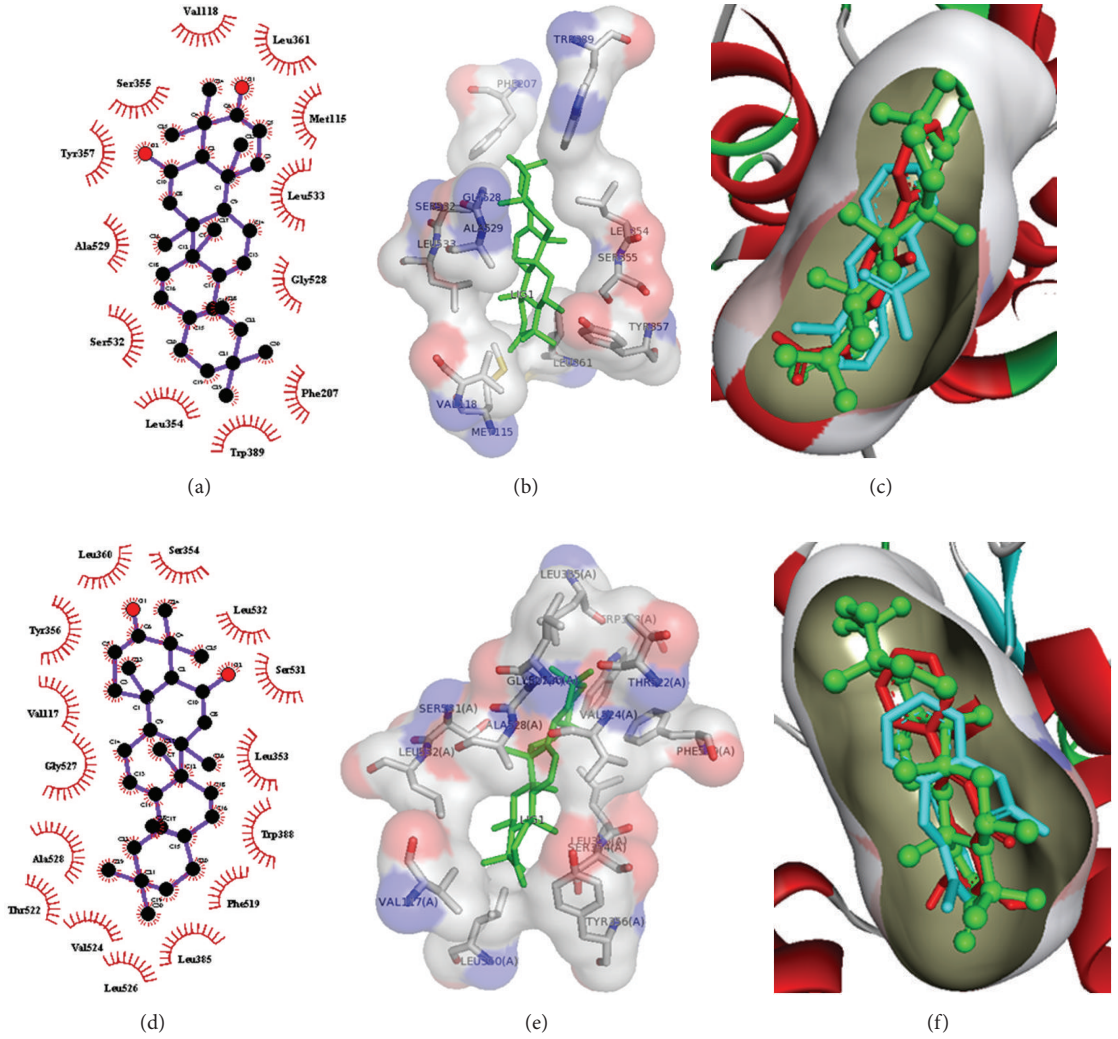

Figure 4: 2D and 3D model of compound **1** in the binding site of COX-1 ((a) and (d)) and COX-2 ((b) and (e)). Half-moon indicates the hydrophobic interactions. ((c) and (f)) Superimposition of compound **1** (colored by green) and diclofenac (colored by cyan) in the binding site of COX-1 (c) and COX-2 (f) enzyme (colored by red).

(21.67 ± 6.17%; $^{*}p < 0.05$) and 10.0 mg/kg (65.33 ± 7.78%; $^{***}p < 0.001$) (ED_{50} = 5.0 mg/kg).

Successively, in order to validate the *in vivo* anti-inflammatory activity of compound **1**, we investigated its mechanism of action from the viewpoint of *in silico* system. By docking studies, we have evaluated the interaction of compound **1** with the binding site of COXs enzymes. The analysis of receptor ligand complex based on the hydrogen bond interaction and hydrophobic interaction shows that compound **1** displayed a remarkable interaction with COX-1, whereas it interacts weakly with COX-2 binding site. Figure 4 shows that compound **1** establishes twelve hydrophobic contacts with COX-1 receptor formed by Meth115, Val118, Phe207, Leu354, Ser355, Tyr357, Leu361, Trp389, Gly528, Ala529, Ser532, and Leu533 (Figures 4(a) and 4(b)) and fifteen contacts with COX-2 receptor formed by Val117, Leu353, Tyr356, Leu360, Ser354, Leu385, Trp388, Phe519, Thr522, Val524, Leu526, Gly527, Ala528, Leu532, and Ser531 (Figures 4(d) and 4(e)). However, no hydrogen bonding was observed.

In order to rationalize the binding mode of compound **1**, we have used the crystal structure of COXs in complex with diclofenac, a nonspecific ligand of this receptor. In particular, the docking results of **1** on COX-1 showed a binding energy of −7.0 kcal/mol and a total energy of −90 kcal/mol, whereas these energy values were of −7.5 kcal/mol and −95 kcal/mol for COX-2 (Figures 4(c) and 4(f) for COX-1 and COX-2, resp.). The interaction energies of compound **1** with COX-1s receptor, compared to standard ligand, diclofenac (−5.7 kcal/mol and −80 kcal/mol in terms of binding and total energy, resp.), were most likely sufficient to justify the selective anti-inflammatory (COX-1 mediated) activity of the tested compound.

4. Conclusions

In our continuing program on the chemical and pharmacological characterization of natural compounds, a triterpenoid identified as daturaolone (compound **1**) was isolated from

Pistacia integerrima. Compound **1** displayed an interesting anti-inflammatory activity compared to diclofenac as demonstrated in classical *in vivo* models of inflammation. Moreover, by *in silico* studies, we have also established that this compound displayed a COX-1 inhibitory activity. However, further studies are needed to better clarify this anti-inflammatory activity and to discern its peripheral and/or central effect.

Conflict of Interests

The authors declare that there is no conflict of interests including any financial, personal, or other relationships with people or organizations.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 4359394, 10 pages
http://dx.doi.org/10.1155/2016/4359394

Research Article

Antibacterial and Anti-Inflammatory Activities of *Physalis Alkekengi* var. *franchetii* and Its Main Constituents

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Received 31 August 2015; Accepted 3 January 2016

Academic Editor: Musa T. Yakubu

This study was designed to determine whether the 50% EtOH fraction from AB-8 macroporous resin fractionation of a 70% EtOH extract of *P. Alkekengi* (50-EFP) has antibacterial and/or anti-inflammatory activity both *in vivo* and *in vitro* and to investigate the mechanism of 50-EFP anti-inflammatory activity. Additionally, this study sought to define the chemical composition of 50-EFP. Results indicated that 50-EFP showed significant antibacterial activity *in vitro* and efficacy *in vivo*. Moreover, 50-EFP significantly reduced nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6) production in lipopolysaccharide- (LPS-) stimulated THP-1 cells. Nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (examined at the protein level) in THP-1 cells were suppressed by 50-EFP, which inhibited nuclear translocation of p65. Consistent with this anti-inflammatory activity *in vitro*, 50-EFP reduced inflammation in both animal models. Finally, seventeen compounds (8 physalins and 9 flavones) were isolated as major components of 50-EFP. Our data demonstrate that 50-EFP has antibacterial and anti-inflammatory activities both *in vitro* and *in vivo*. The anti-inflammatory effect appears to occur, at least in part, through the inhibition of nuclear translocation of p65. Moreover, physalins and flavones are probably the active components in 50-EFP that exert antibacterial and anti-inflammatory activities.

1. Introduction

The continued evolution of dangerous multidrug resistant bacteria has led to significant increases in morbidity and mortality due to bacterial infections. Additionally, many of the currently prescribed antibacterial drugs have significant adverse side effects [1]. Consequently, the urgency for developing new highly effective and safe antibacterials is heightened [2]. To this end, increased attention is being given to the search for antibacterial drugs among natural products and specifically traditional Chinese medicines (TCMs).

One of the most common physiological responses to bacterial infection is the inflammatory response, which is often triggered by changes in humoral and cellular components after tissue injury [3]. The expression of inflammation mediators such as NO, PGE_2, and cytokines is regulated by the transcriptional regulator NF-κB [4], the expression of which is in turn regulated by a complex signaling cascade [5]. When monocytes are stimulated with LPS, inhibitory kappa Bα (IκB) is phosphorylated by the IκB kinase β (IKK) complex, ubiquitinated, and rapidly degraded, resulting in the release of NF-κB, which can translocate to the nucleus and bind to a host of NF-κB-binding promoter regions. Genes activated by NF-κB included diverse proinflammatory mediators such as iNOS, COX-2, TNF-α, IL-1β, and IL-6. Moreover, the MAPK signaling pathways stimulated with the LPS-TLR4 combination can activate various transcription factors, such as NF-κB and c-Jun, which also modulate the production of inflammatory mediators and cytokines [6]. In healthy tissues, this inflammatory response plays an essential role in host survival and tissue repair. However, these inflammatory mediators can be overexpressed by some stimuli leading to serious

inflammatory disorders [7, 8]. When this occurs, anti-inflammatory drugs are a common therapeutic approach for controlling the inflammatory process. Since bacterial infections can often elicit a problematic inflammatory response, medicines that can provide both an antibacterial and anti-inflammatory response would be of particular therapeutic interest.

P. alkekengi (Chinese name: Jindenglong) is a perennial herb taxonomically classified in the Solanaceae family and found widely throughout China. *P. alkekengi* fruits, calyces, roots, and whole plants have been used in traditional Chinese prescriptions, including clinical use of the fruits and calyces. Analysis of ancient medicinal research revealed that *P. alkekengi* has long been used as a traditional Chinese medicine (TCM) for a variety of ailments, including sore throat, cough, eczema, hepatitis, urinary problems, and tumors [9]. In a previous study, our research team identified a *P. alkekengi* extract fraction (50-EFP) effective for treating pharyngitis. Upon further investigation we have determined that the main components of that fraction included physalins and flavones. Because other studies have indicated that physalins and flavones have excellent antibacterial and/or anti-inflammatory activity [10–12], we set out to determine whether 50-EFP also has antibacterial and anti-inflammatory activity.

2. Materials and Methods

2.1. Plant Material. The calyces of *P. alkekengi* were collected from Maoer Mountain in Heilongjiang province in 2008 and the original plant was identified by Professor Zhenyue Wang of Heilongjiang University of Chinese Medicine. A voucher specimen (Number 20080602) was deposited at the Herbarium of Heilongjiang University of Chinese Medicine, China.

2.2. Strains and Reagents. Seven bacterial strains, including four Gram-positive bacteria, *Staphylococcus aureus* (ATCC 26112), *Staphylococcus epidermidis* (ATCC 27342), *Staphylococcus saprophyticus* (ATCC 24582), and *Enterococcus faecium* (ATCC 35667), and three Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pneumoniae* (NCTC 7465), and *Escherichia coli* (ATCC 87394), were obtained from Beijing ZK Kangtai Biological Co. (Beijing, China). These organisms were stored at −20°C supplemented with 10% glycerol. Beef extract, peptone, and agar powder were purchased from Aoboxing Bio-tech Co. (Beijing China). Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), LPS, penicillin, and streptomycin were obtained from Gibco BRL (NY, USA). Dimethyl sulfoxide (DMSO) was purchased from Beijing Chemical Works (Beijing, China). TNF-α, IL-1β, IL-6, and PGE2 ELISA detection kit were purchased from R&D Systems (Minneapolis, MN). All antibodies were purchased from Santa Cruz Biotechnology (CA, USA). All chemicals were purchased from Sigma (St. Louis, MO, USA).

2.3. Preparation of 50-EFP. The dried calyces of *P. alkekengi* (5 kg) were extracted with 70% ethanol (20 L) under reflux conditions for 2 h, for 2 times, to give a residue (1.4697 kg) after removal of solvent under reduced pressure. Then the extract solution (suspended in H_2O) flowed slowly through AB-8 macroporous resin chromatographic column (10 × 60 cm) with a flow rate of 2 BV/h. The remaining water extract (300.2 g) was fractioned with H_2O, 50% (104.6 g, 50-EFP), and 95% EtOH.

2.4. Isolation and Identification of Compounds from 50-EFP. The 50-EFP (105.0 g) was subjected to silica gel (200–300 mesh, Qingdao Marine Chemical Co., Qingdao, China) column chromatography with a stepwise CH_2Cl_2-MeOH gradient (30 : 1; 20 : 1; 10 : 1; 8 : 1; 5 : 1; 1 : 1, v/v) and finally with MeOH alone, to give eight fractions I–VIII. Fractions of II (19.8 g), III (15.6 g), IV (21.4 g), V (10.3 g), and VI (19.8 g) were further separated by octadecyl silica gel (ODS, 35–55 μm, Fuji) column chromatography with MeOH-H_2O gradient (10%, 30%, 50%, 70%, and 95%). Subfractions from ODS column chromatography were separated and purified by preparative HPLC (Waters 600) with MeOH-H_2O to afford 17 compounds (1–17). The structures of compounds 1–17 were determined by detailed NMR (Bruker DPX 400) data analyses, ESI-MS (Waters, Milford, MA, USA), and comparison of their spectral data with literature values.

2.5. Chromatographic Conditions. This analysis was performed using a Waters 2695 HPLC system and Symmetry C_{18} column (150 × 4.6 mm, Part Number WATO 45905). Before the analysis, 50-EFP was dissolved in methanol to a concentration of 5.0 mg/mL for the HPLC analysis. The mobile phase consisted of acetonitrile (A) and aqueous phosphoric acid (0.05% v/v) (B). The concentrations of solvent A in the linear gradient program were as follows: 5–8% at 0–15 min, 8–18% at 15–25 min, and 18–35% at 25–60 min. The mobile phase flow rate was 1.0 mL/min and the column temperature was controlled at 35°C. The UV wavelength was 230 nm. Ten microliters of samples was injected to the column.

2.6. Animals. Male ICR mice (6–8 weeks) were used throughout the experiments. The animals were housed under standard laboratory conditions (temperature at 25±1°C, humidity at 60%, and light from 6 a.m. to 6 p.m.), given standard rodent chow, and allowed free access to water. All procedures were approved by the Animal Care and Use Committee of China Pharmaceutical University and conform to the revised Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH) Publication Numbers 85-23 (1996).

2.7. Acute Toxicity. The acute toxicity test for 50-EFP evaluated any possible toxicity. ICR mice ($n = 10$ in each) were tested by orally administering different doses of 50-EFP by increasing or decreasing the dose according to the responses of animals [13]. The given maximum dose was 12.8 g/kg, while the control group only received distilled water. All animals were observed for any gross effect or mortality within 24 h.

2.8. Antibacterial Activity. The antibacterial activities of 50-EFP were evaluated by determining the minimum inhibitory concentration (MIC) and minimal bactericidal concentration

(MBC) *in vitro*. The MIC of 50-EFP for the isolated bacterial strains were determined by tube dilution method as previously described with as light modification [14]. Briefly, bacterial strains were grown on Mueller-Hinton (MH) agar plates and suspended in MH broth. The inoculum suspensions were prepared from 6 h broth cultures incubation and adjusted to obtain a 0.5 McFarland standard turbidity and were then diluted 1000-fold with the respective medium to the concentration of 1.5×10^5 CFU/mL. Twofold serial diluted concentrations of 50-EFP were added in MH broth ranging from 0.20625 to 26.4 mg/mL. To adjust the interference by plant pigments, a parallel series of mixtures with uninoculated broth was prepared. The bacterial suspensions were aerobically incubated for 18 h at 37°C. Triplicate samples were performed for each test concentration. MIC was defined as the lowest concentration inhibiting visible growth.

The MBC determination was carried out by spreading 0.1 mL of the cultures in each tube without visible growth onto sample free MH agar and incubated for 18 h. MBC was considered as the highest dilution at which almost bacterial inoculum was killed. The experiments were performed in triplicate.

2.9. Pseudomonas aeruginosa or Staphylococcus aureus-Induced Sepsis. Mice were randomly divided into six groups to receive 0.9% saline (normal and saline group, i.p.), amoxicillin (200 mg/kg), or 50-EFP (160, 320, and 640 mg/kg, i.p.). The mice were challenged intraperitoneally with *Pseudomonas aeruginosa* or *Staphylococcus aureus* (0.5 mL) containing 1.5×10^5 CFU/mL to induce sepsis model, respectively. Mice were treated with 50-EFP and amoxicillin, respectively, for one day (1, 6, and 12 h) before infection and 1, 6, and 12 h after infection. The mortality of the mice was observed for 24 h.

2.10. Cell Culture. The THP-1 cell, obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), was cultured in RPMI 1640 containing 10% heat-inactivated FBS supplemented with 1% penicillin/streptomycin under standard conditions. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2. The cells were seeded in 96-well (1×10^5 cells/mL) or 6-well (1×10^6 cells/mL) plates.

2.11. Cell Viability Assay. Cell viability was assessed by morphology and by reduction of MTT by mitochondrial dehydrogenases, according to the manufacturer's instruction (Sigma). THP-1 cells were treated with 50-EFP (0.2, 1, 5, 25, 100, and 500 μg/mL) and the plates were incubated for 24 or 48 h. The cells were then washed once before adding 100 μL of FBS-free medium containing MTT (5 mg/mL). After 4 hours of incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in dimethyl sulfoxide (DMSO). The optical density was measured at 570 nm.

2.12. NO Quantification. The accumulation of NO, a stable end product extensively used as an indicator of NO production, was assayed using the Griess reagent. THP-1 cells were seeded on 6-well tissue culture plates at 1×10^6 cells/mL containing the medium (2 mL), after which they were incubated with LPS (1 μg/mL) for 24 h. 50-EFP (25, 50, and 100 μg/mL) was pretreated for 6 h before LPS stimulation. The supernatants were mixed with equal amounts of Griess reagent. Samples were incubated at room temperature for 10 min. The absorbance was subsequently read at 540 nm using a microplate reader.

2.13. Determining TNF-α, IL-1β, IL-6, and PGE_2 Production. The amount of proinflammatory cytokines released in the culture medium was measured using TNF-α, IL-1β, IL-6, and PGE_2 ELISA kits based on the quantitative sandwich enzyme immunosorbent technique. THP-1 cells were cultured in six-well plates. 50-EFP (25, 50, and 100 μg/mL) was pretreated for 6 h before LPS stimulation. After treatment, THP-1 cells were incubated with LPS (1 μg/mL) for 24 h. Levels of TNF-α, IL-1β, IL-6, and PGE_2 in the culture media were quantified using ELISA detection kits. The absorbance was read at a wavelength of 450 nm using a microplate reader.

2.14. Nuclear Extract Protein Preparation. After treatment with 50-EFP, THP-1 cells were harvested, washed with PBS, centrifuged, and resuspended in ice-cold buffer A (10 mM HEPES (pH 7.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF). After 10 min of ice incubation, the cells were again centrifuged, resuspended in buffer C (20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), and incubated for 20 min at 0°C. After vortex mixing, the resulting suspension was centrifuged, and the supernatant (nuclear extract) was stored at −70°C. The protein concentration of the nuclear extract was determined by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.15. Western Blot Analysis. THP-1 cells in serum-free RPMI 1640 medium were incubated with 50-EFP for 6 h before LPS treatment. After treatment, cells were harvested, washed with PBS, and lysed with cell lysis buffer containing 1% phenylmethylsulfonyl fluoride. The lysate was centrifuged for 15 min at 12000 ×g and 4°C to remove insoluble materials. Supernates were then collected. Protein concentration was measured by bicinchoninic acid assay. Equal amounts of protein (20 μg) from each sample were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore Corporation, USA). Nonspecific sites were blocked by the incubating membranes (2 h at room temperature) in 5% (w/v) nonfat milk powder in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBS-T). Thereafter, the membranes were incubated overnight at 4°C with primary antibodies from Santa Cruz Biotechnology. The membranes were washed with TBS-T and incubated with the appropriate secondary HRP-conjugated antibodies at 1 : 1000 dilution. Following a 30 min wash, the membranes were visualized by enhanced chemiluminescence. Band intensity was measured and quantified.

Table 1: MIC and MBC of 50-EFP.

Strains	50-EFP (mg/mL)	Ceftriaxone sodium (μg/mL)
Staphylococcus aureus ATCC 26112 (MIC)	0.825	8.00
Staphylococcus aureus ATCC 26112 (MBC)	>3.30	16.00
Staphylococcus epidermidis ATCC 27342 (MIC)	0.825	8.00
Staphylococcus epidermidis ATCC 27342 (MBC)	6.60	16.00
Staphylococcus saprophyticus ATCC 24582 (MIC)	1.65	8.00
Staphylococcus saprophyticus ATCC 24582 (MBC)	3.30	16.00
Enterococcus faecium ATCC 35667 (MIC)	1.65	4.00
Enterococcus faecium ATCC 35667 (MBC)	>6.60	8.00
Pseudomonas aeruginosa ATCC 27853 (MIC)	0.825	0.06
Pseudomonas aeruginosa ATCC 27853 (MBC)	1.65	8.00
Streptococcus pneumoniae NCTC 7465 (MIC)	0.825	0.06
Streptococcus pneumoniae NCTC 7465 (MBC)	>13.20	8.00
Escherichia coli ATCC 87394 (MIC)	1.65	8.00
Escherichia coli ATCC 87394 (MBC)	3.30	16.00

2.16. Xylene-Induced Ear Edema and Cotton Pellet Implantation in Mice. The xylene-induced ear edema test was used to assess anti-inflammatory activity following the procedure described previously. ICR mice were randomized into five groups ($n = 10$), including a control group, a positive group (aspirin-treated, 80 mg/kg, i.g.), and 50-EFP treatment groups (50, 100, and 200 mg/kg, i.p.). Test groups of mice were given 50-EFP once every day for 3 consecutive days. Xylene (0.05 mL) was applied to the anterior and posterior surfaces of the right ear of each mouse 1 h after the last administration of 50-EFP. The left ear remained untreated and saved as a control. Ear disk of 7.0 mm in diameter was punched out and weighed. The weight difference between the left and the right ear disk of the same animal was evaluated as the extent of edema.

Two cotton pellets, weighing 10±1 mg each, sterilized in a hot air oven at 120°C for 2 h, were implanted subcutaneously through a skin incision, one on each side of the abdomen of the animal under light ether anesthesia and sterile technique [15]. Control groups received the vehicle (saline, 10 mL/kg), while the positive group was treated with 200 mg/kg of Qingkailing particles. Test groups of mice were given 50-EFP (50, 100, and 200 mg/kg, i.p.) once per day for 7 consecutive days simultaneously. On the 8th day after implantation, mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Both implanted cotton pellets were dissected and dried at 60°C for 18 h, and their dry weight was calculated.

2.17. Statistical Analysis. Data from at least three independent experiments were expressed as mean ± SD. Statistical comparisons between different groups were performed using one-way ANOVA, followed by Student-Newman-Keuls test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Compounds from 50-EFP. Seventeen known compounds (1–17) were isolated from 50-EFP. Eight physalins (1–8) were identified as physalin P (1), physalin G (2), physalin O (3), physalin N (4), physalin B (5), physalin E (6), physalin J (7), and physalin F (8), and nine flavones (9–17) were identified as apigenin-7-O-β-D-glucoside (9), apigenin-7, 4′O-β-D-di-glucoside (10), luteolin (11), chrysoeriol-7-O-β-D-glucoside (12), diosmetin-7-O-β-D-glucoside (13), luteolin-4′-O-β-D-glucoside (14), luteolin-3′-O-β-D-glucoside (15), luteolin-7-O-β-D-glucoside (16), and luteolin-7,3′-O-β-D-di-glucoside (17) by comparing their NMR spectroscopic and ESI-MS data with the literature values, respectively.

3.2. Acute Toxicity of 50-EFP in Mice. An acute toxicity study in mice indicated that the administration of graded doses of 50-EFP up to 12.8 g/kg produced no adverse effects on the general behavior or appearance of the mice and all the mice survived through the experimental evaluation period.

3.3. Antibacterial Activity

3.3.1. Antibacterial Activity In Vitro. The antibacterial activity of 50-EFP was quantitatively assessed by determining the MIC and MBC against seven bacterial strains, including four Gram-positive bacteria and three Gram-negative bacteria (Table 1). The results showed that the 50-EFP possessed antibacterial potential against each of the tested strains with MIC and MBC values ranging from 0.825 to 1.65 and 1.65 to 13.20 mg/mL, respectively.

3.3.2. Antibacterial Activity In Vivo. A *Staphylococcus aureus*- or *Pseudomonas aeruginosa*-induced sepsis model in mice was used to investigate the antibacterial efficacy of 50-EFP *in vivo* (Table 2). The mortality rate for untreated control groups infected with either *S. aureus* or *P. aeruginosa* was 100% within 24 h. At doses of 160, 320, and 640 mg/kg, 50-EFP significantly reduced mortality rates to 33.3%, 33.3%, and 58.3%, respectively, in *S. aureus*-infected mice. Likewise, at doses 160, 320, and 640 mg/kg, 50-EFP reduced mortality

Table 2: Protective effect of 50-EFP in *Staphylococcus aureus* and *Pseudomonas aeruginosa* infection-induced sepsis in mice.

Groups	Number	*Staphylococcus aureus* (24 h)		*Pseudomonas aeruginosa* (24 h)	
		Mortality	Mortality rate (%)	Mortality	Mortality rate (%)
Model	12	12	100	12	100
Amoxicillin (200 mg/kg)	12	2	16.7	3	25.0
50-EFP (640 mg/kg)	12	4	33.3	6	50.0
50-EFP (320 mg/kg)	12	4	33.3	7	58.3
50-EFP (160 mg/kg)	12	7	58.3	7	58.3

rates to 50%, 58.3%, and 58.3%, respectively, in *P. aeruginosa*-infected mice.

3.4. In Vitro Anti-Inflammatory Activity of 50-EFP. The anti-inflammatory effect of 50-EFP was assessed in LPS-stimulated THP-1 cells, a human monocytic cell line. First, the potential cytotoxicity of 50-EFT toward THP-1 cells was assessed in a standard viability assay using the colorimetric dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Viability was unaffected by 50-EFT concentrations up to 500 μg/mL (Figure 1(a)). Next, we investigated the 50-EFP effects on suppressing the levels of NO and PGE_2 in the LPS-stimulated promonocytic cells. Pretreatment with 50-EFP prior to LPS stimulation caused a significant reduction in NO and PGE_2 production in a 50-EFP concentration-dependent manner ($p < 0.01$; 50 and 100 μg/mL, Figure 1(b)). Additionally, Western blot analysis indicated that the expression of the genes that synthesize NO and PGE_2 (iNOS and COX-2, resp.) was markedly reduced in a dose-dependent fashion by 50-EFP pretreatment (Figure 1(c)). Notably, 50-EFP pretreatment did not affect the expression level of the β-actin control. These results suggest that 50-EFP suppresses both iNOS and COX-2 expression at the transcriptional level in addition to the protein level, resulting in the reduced expression of NO and PGE_2.

The expression of iNOS and COX-2 is normally induced by NF-κB activation. For this to occur the p65 protein NF-κB must translocate from the cytosol to the nucleus—a process that is mediated by phosphorylation and degradation of the I-$\kappa B\alpha$ subunit. Therefore, we assessed whether the impact of 50-EFP on iNOS and COX-2 expression occurs via an effect on p65 translocation by Western blot analysis (Figures 2(b) and 2(c)). Interestingly, 50-EFP pretreatment does repress LPS-induced nuclear translocation of p65 and I-$\kappa B\alpha$ phosphorylation almost completely, implying that 50-EFP prevents I-$\kappa B\alpha$ degradation and NF-κB activation. Next, ELISA analysis was used to investigate the effect of 50-EFP on the LPS-stimulated expression of other NF-κB-regulated genes: TNF-α, IL-1β, and IL-6. Whereas LPS stimulation significantly increased the production of the cytokines in untreated cells, 50-EFP pretreatment at 25, 50, and 100 μg/mL significantly suppressed the release of TNF-α, IL-1β, and IL-6 in a dose-dependent manner (Figure 2(a)).

3.5. Inhibition of Acute Ear Edema Induced by Xylene and Cotton Pellet-Induced Granulomatous Tissue Formation. A xylene-induced acute ear edema model in mice was used to investigate whether 50-EFP exerts anti-inflammatory effects *in vivo*. Whereas untreated animals show significant ear edema, treatment with 200 mg/kg 50-EFP significantly reduced ear edema (Figure 3(a)). These results indicate that 50-EFP does repress the inflammatory response induced by xylene. Additionally, 50-EFP significantly inhibited granulomatous tissue formation induced by cotton pellet in a dose-dependent manner ($p < 0.05$, 100 mg/kg; $p < 0.01$, 200 mg/kg).

3.6. HPLC Analysis of 50-EFP. The main components profile of 50-EFP was analyzed via HPLC. The representative chromatogram is shown in Figure 3(b). The identification of constituent of 50-EFP was based on the retention times and UV spectrum in comparison with authentic standards at a wavelength of 230 nm. Peak purity check and identification were conducted via a 210–400 nm UV scan through a diode array detector. Eight components (luteolin-7-O-β-D-glucoside (6), apigenin-7-O-β-D-glucoside (7), diosmetin-7-O-β-D-glucoside (8), physalin J (9), physalin F (10), physalin O (11), physalin B (12), and physalin P (13)) were identified in 50-EFP.

4. Discussion

Physalis alkekengi var. *franchetii* (Solanaceae) is a herb widely used in popular medicine for its antifebrile and detoxification effects and to treat sore throats. In a previous study, we found that 50-EFP can also be used to treat pharyngitis. Pathologies causing pharyngitis include a wide range of conditions with two primary underlying causes: inflammation and bacterial infection of pharyngeal mucosa [16–18]. Therefore, we investigated whether a key fraction of *P. alkekengi* extract known as 50-EFP has inherent antibacterial and anti-inflammatory activities.

Despite the advances in antibiotics in the past 70 years, infectious diseases still are an important cause of worldwide morbidity and mortality and account for approximately one-half of all deaths in tropical countries [19, 20]. Therefore, new antibacterial drugs with novel targets are necessary. The antibacterial activity of 50-EFP was assessed by broth dilution method *in vitro*. Bacterial infection was observed in both Gram-positive and Gram-negative strains. In the present study, we found that 50-EFP not only inhibited the growth of both strains (MIC), but also killed them (MBC) *in vitro*. In addition, we also assessed the antibacterial activity of 50-EFP toward *S. aureus* and *P. Aeruginosa in vivo*. *P. aeruginosa* is the most common pathogen that causes respiratory pneumonia, gastrointestinal disorders, bacteremia, and skin infections

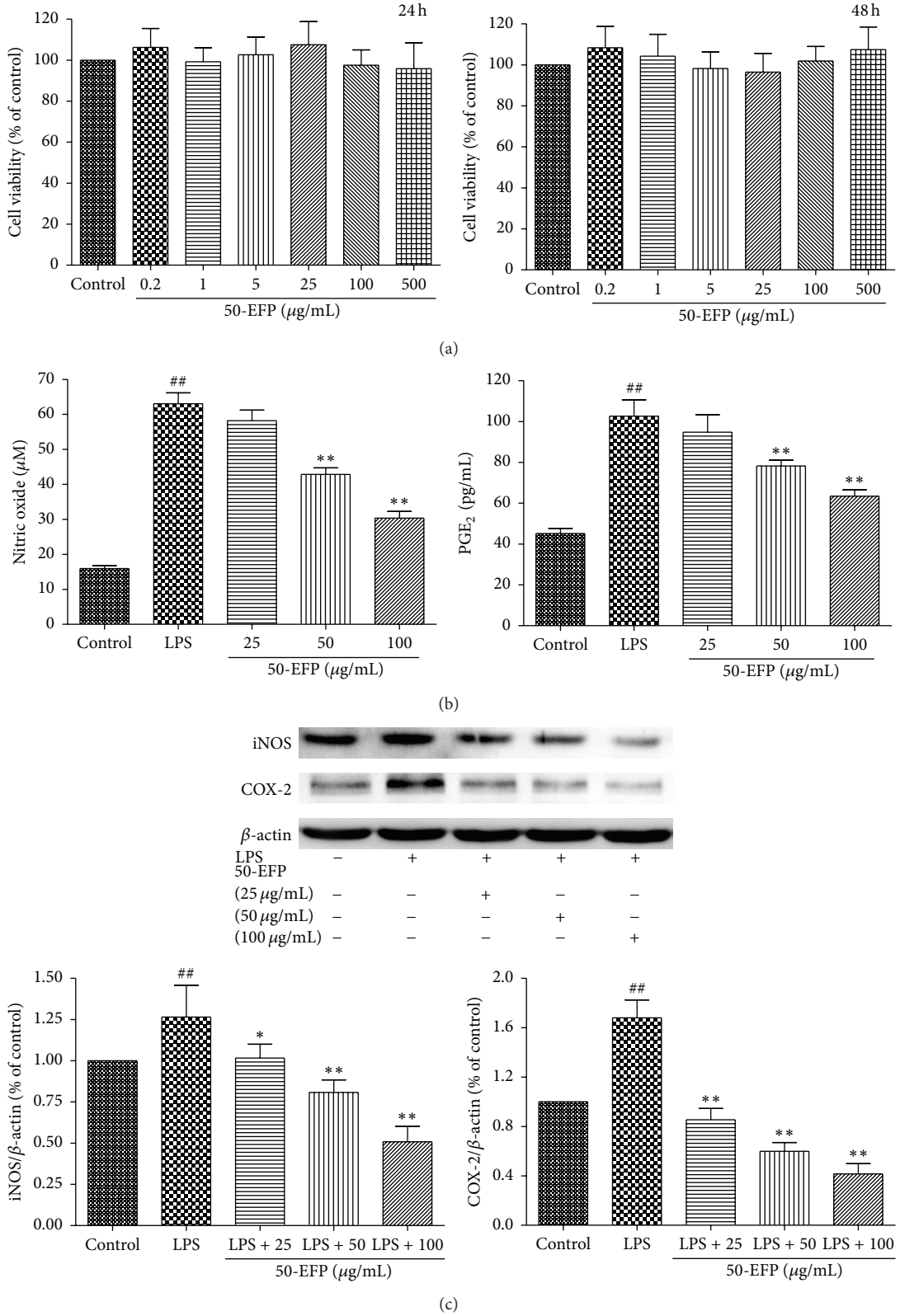

Figure 1: Effects of 50-EFP on THP-1 cell viability. (a) Cells were incubated with increasing concentrations of 50-EFP (0.2, 1, 5, 25, 100, and 500 μg/mL) for 12 h or 24 h. Cell viability was measured by the MTT assay. (b) Effect of 50-EFP on LPS-induced NO and PGE_2 production in THP-1 cells. THP-1 cells were pretreated with 50-EFP for 6 h before being incubated with LPS for 24 hours. The culture supernatant was analyzed for NO or PGE_2 production. (c) Total cellular protein was isolated and LPS-induced iNOS and COX-2 expression levels were measured using Western blotting analysis. $^{\#\#}p < 0.01$ versus control, $^{*}p < 0.05$; $^{**}p < 0.01$ versus LPS only group. Data are representative of three independent experiments.

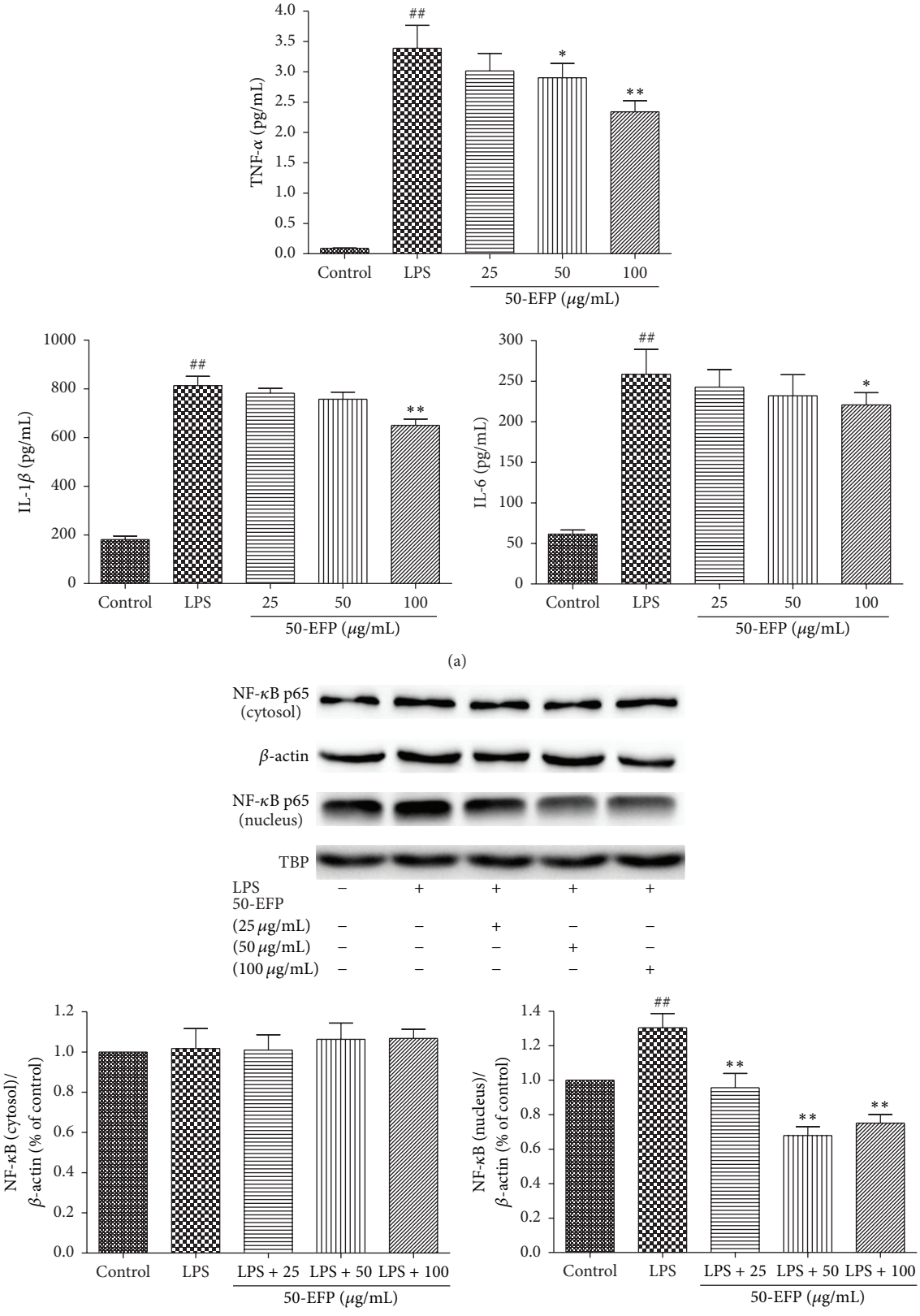

Figure 2: Continued.

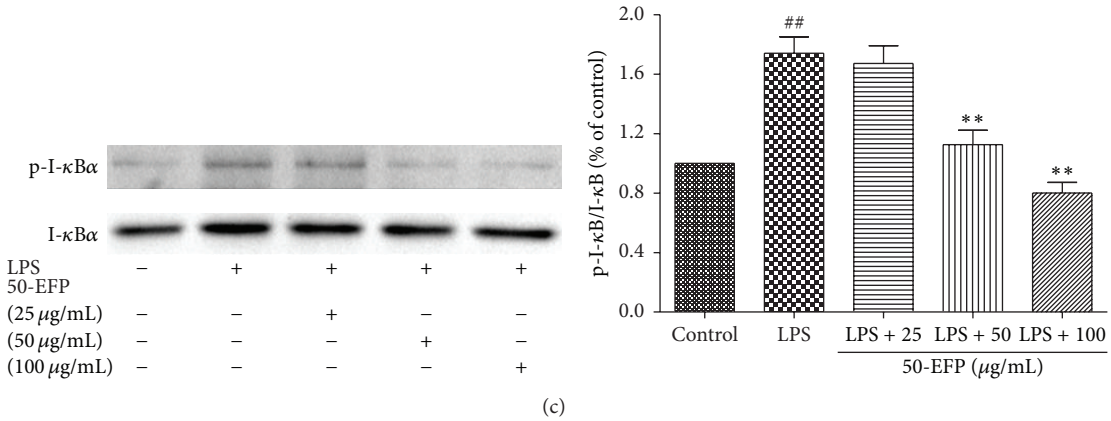

Figure 2: Effect of 50-EFP on LPS-induced TNF-α, IL-1β, and IL-6 cytokine production. (a) THP-1 cells were pretreated with 50-EFP for 6 h before being incubated with LPS for 24 hours. Production of TNF-α, IL-1β, and IL-6 cytokine was measured by ELISA. (b, c) Effect of 50-EFP on translocation of the NF-κB (p65) subunit into the nucleus and release of I-κBα into the cytosol upon LPS stimulation. The cells were treated with LPS alone or with LPS and 50-EFP for 6 hours. The level of I-κBα protein in the cytosol and NF-κB (p65) protein present in the cytosol and nucleus was determined by the Western blot analysis using anti-I-κBα or anti-NF-κB (p65) antibody. β-actin and TBP were used for cytosolic and nuclear control protein, respectively (in relative protein density units). $^{\#\#}p < 0.01$ versus control, $^{*}p < 0.05$; $^{**}p < 0.01$ versus LPS only group. Data are representative of three independent experiments.

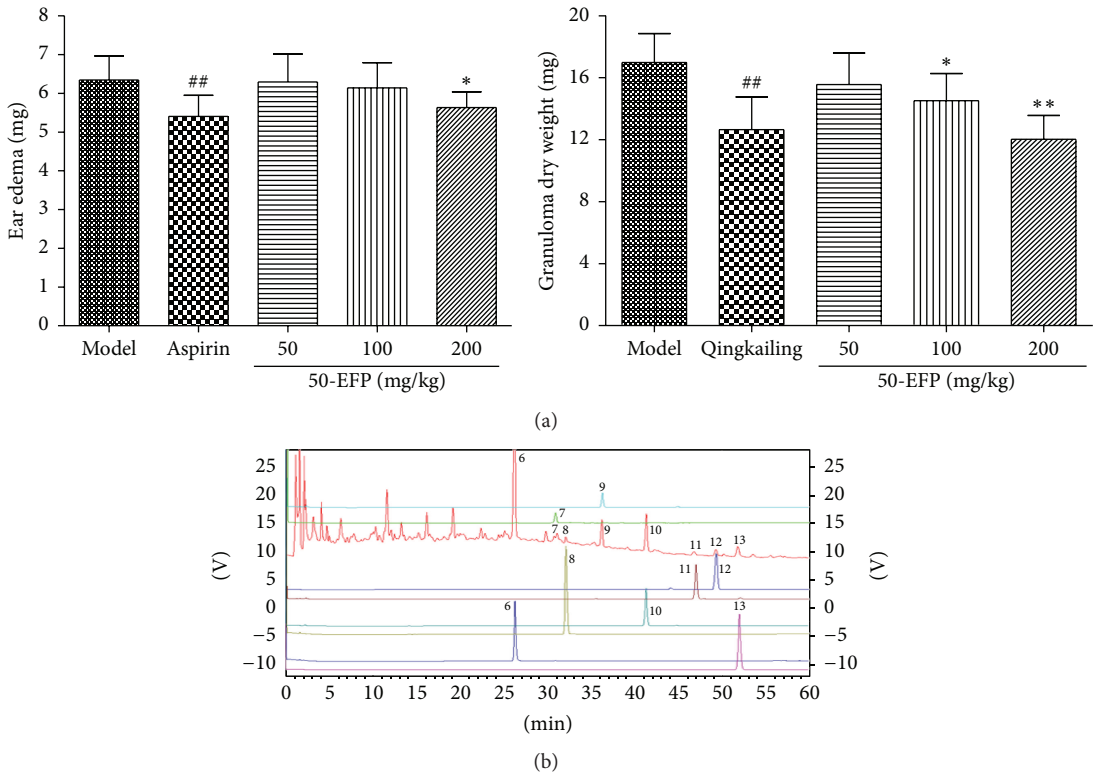

Figure 3: Anti-inflammatory effects of 50-EFP in mice. (a) Xylene-induced ear edema in mice and cotton pellet granuloma in mice. Data are presented as mean ± SD, $n = 10$. $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the model group. (b) HPLC chromatograms of 50-EFP, luteolin-7-O-β-D-glucoside (6), apigenin-7-O-β-D-glucoside (7), diosmetin-7-O-β-D-glucoside (8), physalin J (9), physalin F (10), physalin O (11), physalin B (12), and physalin P (13).

[21] and *S. aureus* is a major nosocomial pathogen that causes serious infections such as toxic shock syndrome and necrotizing pneumonia [22, 23]. We found that this antibacterial effect was also observed in an animal model of septic infection by either *S. aureus* or *P. aeruginosa*. These results confirm that 50-EFP does have broad-spectrum antibacterial activity and the potential for development as a systemic antibiotic.

In addition to its antibacterial activity, 50-EFP also has significant anti-inflammatory properties. In LPS-stimulated monocytes, 50-EFP inhibits the production and expression of a number of cytokines and inflammatory mediators, including NO, PGE_2, iNOS, COX-2, TNF-α, IL-1β, and IL-6, and the effect appears to be dependent on 50-EFP concentration. The basis of the 50-EFP anti-inflammatory activity appears to be inhibition of NF-κB activation and nuclear translocation. Activation of NF-κB requires phosphorylation and degradation of an NF-κB repressor, I-κBα [24, 25]. Once freed from I-κBα, NF-κB can translocate to the nucleus, where it is an inducer of a number of genes, including the inflammatory response genes repressed by 50-EFP [6]. Our results reveal that treatment with 50-EFP significantly represses I-κBα phosphorylation and NF-κB nuclear translocation, and consistent with this, we found that the expression of other NF-κB-regulated genes is also repressed by 50-EFP.

Importantly, the *in vitro* anti-inflammatory activity of 50-EFP is also manifested *in vivo*. In both an ear edema model and a cotton pellet granulomatous tissue model, 50-EFP showed a profound ability to reduce acute and chronic inflammatory reactions. These data provide a better understanding of the positive impact of *P. alkekengi* var. *franchetii* (Solanaceae) treatment of pharyngitis and indicate significant potential for the continued development of combined antibacterial/anti-inflammatory therapies based on the 50-EFP extract.

To further develop the potential therapies of 50-EFP, we had isolated and identified its components. We found that 50-EFP contains eight major components in two key medical classes: physalins and flavones. Previous studies have reported that physalins have suppressive activities on macrophage and lymphocyte cultures *in vitro* and also inhibit the production of proinflammatory mediators such as TNF-α [12]. Similarly, flavones have been reported to show significant antibacterial activity via the diffusion method and can suppress chemokine production in human monocyte THP-1 cells [10]. Thus, it is highly likely that the beneficial effects of 50-EFP, and thus *P. alkekengi*, are due to a unique combination of specific physalins and flavones found in the 50-EFP extract. Our mechanistic analysis and component identification should therefore provide a solid basis for more advanced evaluation and development of these components as potential therapeutic agents. This study also confirms the potential and underlying basis for treating inflammation-causing bacterial infections with traditional oriental medicines.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 7178105, 6 pages
http://dx.doi.org/10.1155/2016/7178105

Research Article

Anti-Inflammatory and Antioxidant Activities of *Salvia fruticosa*: An HPLC Determination of Phenolic Contents

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Received 19 September 2015; Revised 22 November 2015; Accepted 30 November 2015

Academic Editor: Roi Treister

Objectives. Salvia fruticosa Mill. (*S. fruticosa*) is widely used in folk medicine. Accordingly, the present study was designed to evaluate the antioxidant and anti-inflammatory activities of *S. fruticosa*, and to determine the phenolic constituents of its extracts. *Methods*. The antioxidant activity was determined using 2,2-diphenylpicrylhydrazyl assay. Total phenolic contents were estimated using Folin-Ciocalteu reagent, and high-performance liquid chromatography was performed to identify phenolic constituents. To evaluate the anti-inflammatory activity, carrageenan-induced mouse paw edema was determined plethysmographically. *Key Findings*. Different plant extracts demonstrated strong radical scavenging activity, where the ethyl acetate extract had the highest value in the roots and the lowest in the aerial parts. This antioxidant activity was correlated to the total phenolic content of different extracts, where rutin and luteolin were the most abundant constituents. Interestingly, both the roots and aerial parts revealed a significant anti-inflammatory activity comparable to diclofenac. *Conclusions*. This study is the first to demonstrate pharmacologic evidence of the potential anti-inflammatory activity of *S. fruticosa*. This activity may partly be due to the radical scavenging effects of its polyphenolic contents. These findings warrant the popular use of the East Mediterranean sage and highlight the potential of its active constituents in the development of new anti-inflammatory drugs.

1. Introduction

Oxygen is essential for the life of all aerobic organisms; nevertheless, oxidative stress may induce damage to cellular biomolecules and is implicated in many diseases [1]. On the other hand, phenolic compounds derived from plants have received high attention during recent years owing to their potential antioxidant effects and positive influence on human health. Plant phenolics also interact to prevent various diseases by quenching oxygen-reactive free radicals [2–4].

The use of plants as remedies is as ancient as human civilization, and plants still remain the main sources of useful drugs. Crude plant extracts have been recently documented worldwide as an important source of phytochemicals having several biological activities, including antioxidant and anti-inflammatory effects [2, 3, 5, 6]. The genus *Salvia*, as one of the largest genera of the family Lamiaceae (Labiatae), is represented by about 900 species throughout the world. Numerous species of the *Salvia* genus are economically important, have been used since ancient times in folk medicine, and have also been subjected to extensive pharmacognostic research to identify biologically active constituents [7]. Particularly, the species *Salvia fruticosa* Miller (*S. fruticosa* Mill.), also named as *S. libanotica* and formerly named as *S. triloba*, is native to the eastern Mediterranean and is known as East Mediterranean sage or Lebanese sage [8, 9]. It represents most of the imported sage in the United States rather than *S. officinalis*. In folk traditional medicine, *S. fruticosa* is widely used by people, herbalists, and pharmacists, either internally as infusions, inhaled in steam baths, or even applied externally. The plant is boiled as a tea for the relief of different pains, colds, influenza, and many other disorders. This endemic Lebanese plant has also been used for improving memory and as hypoglycemic agent

with an antioxidant potential [5, 8, 9]. Indeed, the Lamiaceae family includes a large number of plants that are well known for their antioxidant properties. In particular, the *genus Salvia* has been subject of intensive study in the past decades for its antioxidative and anti-inflammatory effects in relation to the active constituents, including the phenolic contents [6, 10, 11]. The anti-inflammatory activity of phenolics and flavonoids has also been shown to be a result of their antioxidant effect [11, 12]. Therefore, this research aimed to evaluate the anti-inflammatory and antioxidant activities of *S. fruticosa* root and aerial extracts, and to relate these activities to the plant extract phenolic constituents.

2. Materials and Methods

2.1. Plant Material. Fresh *S. fruticosa* Mill. (synonyms: *S. libanotica* and *S. triloba*) was collected during the flowering period in March and April 2012 from southern Mediterranean region at altitudes ranging from 200 to 400 m at the littoral of Beirut, Lebanon. The plant was identified by Dr. Georges Tohme, Professor of Taxonomy. A dried specimen was kept at the Faculty of Pharmacy, Beirut Arab University (herbarium number: ps-14-12). The plant was dried under shade at 25°C and the dried aerial parts and roots were ground separately with a blender.

2.2. Preparation of Plant Extract. The powdered parts of plant (80 g) each were extracted successively with chloroform, ethyl acetate, methanol, and butanol for 24 hours. The residues were removed by filtration. The extracts were concentrated (yield about 7%) in a rotary evaporator under reduced pressure at a temperature of 40–50°C and then lyophilized to get powders.

2.3. Determination of DPPH Scavenging Activity. In vitro 2,2-diphenyl picryl hydrazyl (DPPH) radical scavenging activity was carried out by adopting the method of Blois [13]. The hydrogen atoms or electrons donation ability of the corresponding extracts was measured from the bleaching of purple colored methanol solution of stable free radical DPPH [14]. The absorbance of different extracts was read against a blank at 517 nm at different time intervals for duration of 60-minute incubation period at room temperature. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Vitamin C was used as a positive control showing 100% scavenging activity.

DPPH radical scavenging activity % (SC%) = $(A_c - A_I/A_c) * 100$, where A_c is the absorbance of control and A_I is the absorbance of extract.

2.4. Determination of Phenolic Compounds. Total phenolic compounds were determined using Folin-Ciocalteu reagent (FCR) as described by Slinkard and Singleton [15]. In brief, serial dilutions of *S. fruticosa* roots and whole aerial parts were prepared. 0.2 mL of each solution was added to a glass test tube and 1 mL of FCR, 0.8 mL of Na_2CO_3 (7.5%) were pipetted to it. The preparations were stored at room temperature for 60 minutes and then their absorbances were read at 765 nm. Absorbance values were compared with standard solution of gallic acid equivalent [16]. Also, the quantities of HPLC-identified phenolic contents (gallic acid, rutin, and luteolin) were determined by carrying out column chromatography and determining the weight of corresponding fractions.

2.5. HPLC Analysis. A sample (200 μg/mL) was prepared in methanol. It was homogenized by using a vortex. Then, the plant extract was passed through a 0.45 μm filter before injection into a reverse phase NOVA-PAK C18 column at ambient temperature (20°C). The mobile phase was methanol and phosphate buffer (43 : 57). The flow rate was 1.0 mL/min and the wave length of detection was set at 254 nm [17].

2.6. Evaluation of the In Vivo Anti-Inflammatory Activity. The carrageenan-induced mouse paw edema model was adopted as recently described by our group [18]. Briefly, the tested methanolic extracts were administered intraperitoneally (i.p.) at a dose of 200 mg/kg body weight. The volume of the carrageenan-injected paw was determined plethysmographically immediately after injection and 4 hours later. The difference between the two readings gave the actual edema volume increase to calculate the percentage protection. The anti-inflammatory activity of the tested extracts relative to that of diclofenac was also calculated.

2.7. Animals. Male albino mice were kept for 1 week prior to the experimentation at the animal house of the Faculty of Pharmacy, Beirut Arab University. The environment consisted of a temperature of 25 ± 1°C, and standard mouse cages with a 12 h light/dark cycle. The animals had free access to water and standard laboratory chow (20% proteins, 5% fats, and 1% multivitamins) [2, 19]. Animal care and handling for the research were performed in accordance with the regulations and guidelines stipulated by the Institutional Animal Care and Use Guidelines (IACUG) at Beirut Arab University, Lebanon (IRB approval code: 2015A-020-P-P-0055).

2.8. Statistical Analysis. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey simultaneous comparison t-values. Statistical differences were considered to be significant at $P < 0.05$.

3. Results

The anti-inflammatory activity of the plant was carried out *in vivo* and the results were shown in Figure 1. Both the aerial parts and the roots of the crude extracts exhibited significant protection at 4 h against carrageenan-induced mouse paw edema by 50% and 44%, respectively. The promising anti-inflammatory activities of the aerial parts and the roots extracts relative to that of the standard drug diclofenac were 0.9 and 0.8, respectively.

Also, the *in vitro* antioxidant activity by using DPPH was investigated. The free radical scavenging activity of both aerial and root extracts is presented in Table 1. As can be seen, the ethyl acetate root extract of *S. fruticosa* showed highest scavenging activity at 60 min (42.15 ± 2.06) as compared to

Table 1: Free radical scavenging activity of *Salvia fruticosa* extracts at different time intervals (min).

	Sample	At 0′	At 20′	At 40′	At 60′
Aerial parts	Chloroform extract	0.01 ± 0.01	29.00 ± 12.60*	35.65 ± 4.40*	41.59 ± 6.10*
	Methanol extract	0.01 ± 0.01	29.96 ± 4.20*	36.69 ± 6.40*	41.50 ± 6.70*
	Ethyl acetate extract	0.01 ± 0.01	17.78 ± 6.60*	24.40 ± 2.05*	32.20± 4.50*
	Butanol extract	0.01 ± 0.01	19.81± 2.06*	30.70 ± 6.50*	38.62 ± 8.00*
Roots	Chloroform extract	0.01 ± 0.01	11.72 ± 2.04*	13.33 ± 2.10*	31.2 ± 2.09*
	Methanol extract	0.01 ± 0.01	13.42 ± 2.03*	19.33 ± 4.30*	32.16 ± 4.20*
	Ethyl acetate extract	0.01 ± 0.01	20.46 ± 4.04*	28.39 ± 2.03*	42.15 ± 2.06*
	Butanol extract	0.01 ± 0.01	13.05 ± 2.10*	20.47 ± 2.20*	25.43 ± 2.60*

Values are presented as % scavenging activity means ± SD. ∗ denotes significant difference from corresponding value at 0′. The level of significance was set at $P < 0.05$. Data were analyzed using one factor ANOVA followed by Tukey simultaneous comparison t-values.

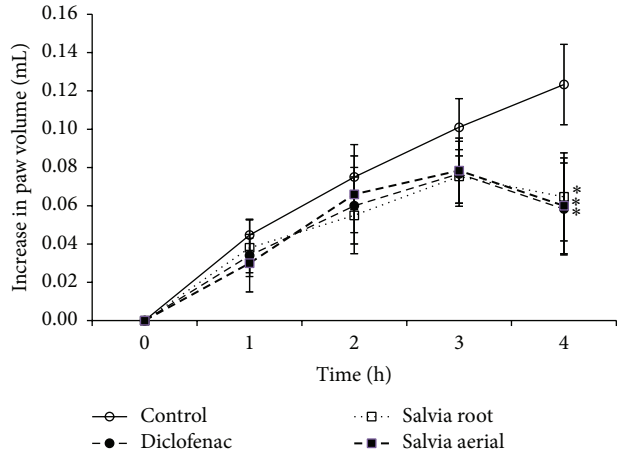

Figure 1: Effects of *S. fruticosa* extracts on mouse hind paw edema. The actual edema volume increase was measured relative to that of standard drug diclofenac 50 mg/kg. Values are presented as means ± SD, $n = 4$–7. ∗ denotes significant difference from control value at $P < 0.05$.

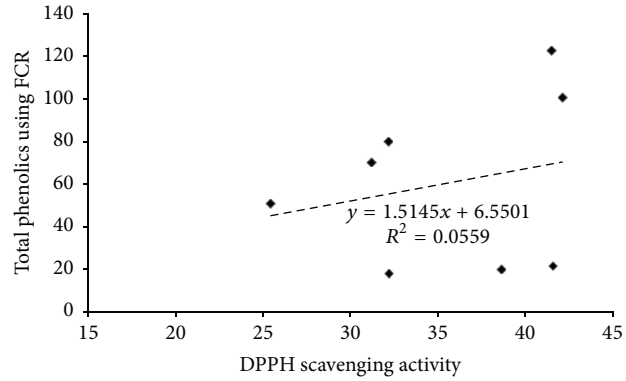

Figure 2: Relationship between antioxidant activity and total phenolic contents of different *S. fruticosa* extracts. Correlation coefficient $R^2 = 0.0559$ ($r = 0.24$).

that of aerial parts being the lowest (32.20 ± 4.50). The root extracts of methanol, chloroform, and butanol showed their scavenging activities in the decreasing order. However, the aerial parts extracts of butanol, methanol, and chloroform showed their scavenging activities in the increasing order (Table 1). The root extract showed the lowest scavenging activity at 60 min for butanol extract (25.43 ± 2.60), in comparison to that of aerial parts, where chloroform extract showed the highest one (41.59 ± 6.10).

In addition, the total phenolic contents of different extract solutions were determined by performing a reaction with FCR. Results were compared with the standard solutions of gallic acid equivalents and were presented in Table 2. The ethyl acetate extract showed highest FCR absorbance (100.66±3.34) in the roots as compared to highest absorbance of methanol extract in aerial parts (122.67 ± 0.44) as shown in Table 2. Regarding the corresponding antioxidant activity values at 60 min, in comparison with the phenolic contents, it could be noticed that the highest scavenging activity of ethyl acetate root extract (42.15±2.06) corresponds to highest phenolic content (100.66 ± 3.34). On the other hand, the lowest scavenging activity in the ethyl acetate extract of the aerial parts (32.20 ± 4.50) corresponds to lowest phenolic contents (17.89 ± 0.24). This observation demonstrated that there is certain correlation between the free radical scavenging activity and the total phenolic contents of the plant extracts. Indeed, an overall positive correlation between antioxidant activity and the total phenolic content of the extracts was illustrated in Figure 2. The relationship between the whole antioxidant activities and total phenolic contents of all the tested extracts based on the correlation (linear relationship) displayed a weak positive correlation ($R^2 = 0.0559$; $r = 0.24$).

Moreover, concerning the relationship with anti-inflammatory activity that both the aerial and root extracts showed promising effects (50% and 44%, resp.), the following was noticed. A similarity was evident in case of the significant radical scavenging activity ranging in the aerial parts from 32.20 to 41.59 and in the roots from 25.43 to 42.15. Nevertheless, this seemed somewhat different from the lower total phenolic content of all added aerial extracts (182.06 mg gallic acid/g extract) compared to that of twofold higher content in all added root extracts (301.76 mg gallic acid/g extract).

Furthermore, a column chromatography of the plant extracts isolated a high yield of phenolic contents, such as gallic acid (37 mg), rutin (23 mg), and luteolin (9.23 mg), as

Table 2: Free radical scavenging activity (SC%) of *Salvia fruticosa* extracts versus their total phenolic content.

Extract	Free radical scavenging activity at 60 min (mean SC% ± SD)	Total phenolics as mg gallic acid/g extract (mean ± SD)
Aerial parts		
Chloroform extract	41.59 ± 6.10	21.50 ± 0.38
Methanol extract	41.50 ± 6.70	122.67 ± 0.44
Ethyl acetate extract	32.20 ± 4.50	17.89 ± 0.24
Butanol extract	38.62 ± 8.00	20.0 ± 0.40
Roots		
Chloroform extract	31.2 ± 2.09	70.3 ± 0.9
Methanol extract	32.16 ± 4.20	80 ± 1.68
Ethyl acetate extract	42.15 ± 2.06	100.66 ± 3.34
Butanol extract	25.43 ± 2.60	50.80 ± 0.34

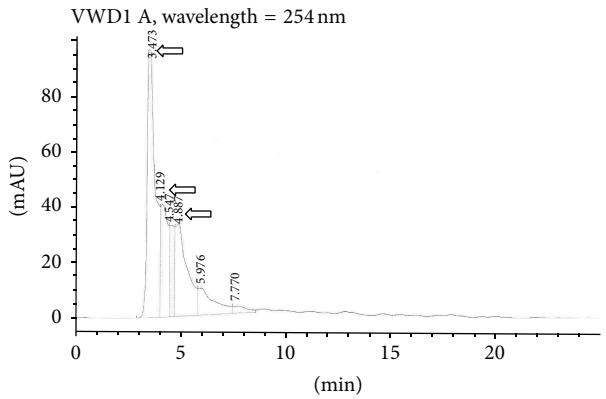

Figure 3: HPLC chromatogram for the determination of phenolic constituents of *S. fruticosa* extract. The methanolic plant extract showed several flavonoids. Major constituents, luteolin and rutin, are represented by arrows at the retention time values of 3.473 min and 4.887 min, respectively. Gallic acid also appeared at 4.129.

also identified in the HPLC analysis (Figure 3). The analysis of whole plant extracts showed that a large number of flavonoids were present. Luteolin and rutin were the most abundant phenolic constituents and were readily identified by comparison with authentic standards as shown in Figure 3. Luteolin appeared first at the retention time value of 3.473 and then rutin at 4.887. Gallic acid also appeared at 4.129.

4. Discussion

S. fruticosa was found to be a rich source of antioxidants, where the ethyl acetate root extract had the highest radical scavenging activity and the highest concentration of phenolics. In addition, the aerial parts of the plant showed the higher anti-inflammatory activity. Both the aerial parts and the roots exhibited significant protection against carrageenan-induced mouse paw edema with promising activities of the two crude extracts relative to that of the standard drug diclofenac reaching 90%. The anti-inflammatory activity of the extracts was in line with their antioxidant activity better than their phenolic contents. The observed anti-inflammatory activities of this extract may partly be attributed to the overall effects of the phenolics and other plant constituents having potent anti-inflammatory actions similar to diclofenac. The extent of decrease in the absorbance of DPPH in the presence of antioxidants correlates with their free radical scavenging potential. Accordingly, the ethyl acetate root extract of *S. fruticosa* showed highest scavenging activity as compared to that of aerial parts. The methanol, chloroform, and butanol root extracts showed lower activities in decreasing order. However, other studies carried out on several species such as *Salvia verticillata* showed the highest scavenging activity in methanol extract [20]. This activity was attributed to the presence of phenolic constituents present in this plant in different proportions and identified as gallic acid and rosmarinic acid which occurred in higher amount and was identified by TLC screening compared with references [20]. Concerning total phenolic content, the ethyl acetate root extract showed highest FCR absorbance as that of methanol extract of aerial parts meaning that they contained a high concentration of phenolic contents such as gallic acid (root), rutin, and luteolin (aerial) that were isolated by column chromatography and identified by HPLC. Although the ethyl acetate extract showed highest activity in the roots, it demonstrated lowest one in the aerial parts. In addition, this highest scavenging activity of ethyl acetate root extract corresponded to highest phenolic contents. In parallel, the lowest scavenging activity in the ethyl acetate extract of the aerial parts corresponded to lowest phenolic contents demonstrating that there was certain correlation between the free radical scavenging activity and the total phenolic contents of the different plant extracts. The overall positive correlation ($r = 0.24$) between antioxidant activity and the total phenolic content of the extracts might link the phenolic contents of this plant to its antioxidant action. This finding may warrant the popular use of *S. fruticosa* worldwide for treating several illnesses. Indeed, the antioxidant activity is considered one of the important bioactivities of *Salvia* plants, and it is attributed mainly to the major effective content of polyphenols and terpenes [6, 7]. Some polyphenolic extracts from *Salvia* were examined for antioxidant activity in correlation with their polyphenolic content. Some of the results revealed that polyphenolic extracts had strong free radical

scavenging activity against DPPH. Alternatively, other results showed that the total polyphenolic content is not correlated with antioxidant activity in other extracts [7]. Regarding the anti-inflammatory activity, both aerial and root extracts showed promising activities in line with that of free radical scavenging ones in the aerial parts and in the roots. However, it seemed somewhat different from the cumulative lower total phenolic content of different aerial extracts and that of higher by twofold total content in the root extracts. Therefore, total phenolic contents may at least in part be responsible for the radical scavenging and anti-inflammatory activities of *S. fruticosa*. Similarly, *S. miltiorrhiza* contains both hydrophobic and hydrophilic compounds. More than 30 diterpenes have been isolated and identified from the hydrophobic fraction. Investigations on hydrophilic compounds revealed major constituents including water-soluble phenolic acids [11]. Indeed, several researches on *Salvia* plant extracts displayed high antioxidant and anti-inflammatory activities but contained low levels of phenolics and flavonoids. Then, the potential anti-inflammatory activities of the plant extracts may at least in part be due to the radical scavenging activity of their polyphenolic content, in accordance with other previous researches and findings [6, 7, 21, 22]. The depiction of correlations (linear relationship) between extracts activity and polyphenolic contents helps to better understand the relationship between different activities and major extract constituents. A good agreement with literature reports was evident in that polyphenolics are the major antioxidant compounds in medicinal plants [6]. However, it should be noted that, sometimes, the antioxidant activities of some other plants did not show a good correlation with their polyphenol contents. These observations demonstrate that, in addition to polyphenols, other constituents may contribute to the antioxidant activities of medicinal plants, such as trace metal contents [6]. In many herbs, flavonoids containing multiple hydroxyl groups have strong antioxidant effect [3, 4, 23]. The HPLC of whole *S. fruticosa* extract also showed that a large number of flavonoids were present. Luteolin and rutin were the most abundant phenolic constituents. These phenolic compounds and others have also been successfully isolated from other *Salvia* species [10, 17, 20, 22, 23].

5. Conclusions

To our knowledge, the first evidence of powerful anti-inflammatory activity of *S. fruticosa* was demonstrated by this novel research. *S. fruticosa* was also found to be a rich source of antioxidants, where the root extracts had highest antioxidant activity and concentration of total phenolics. The potential anti-inflammatory activity of the plant extracts may hence be, at least in part, due to the radical scavenging activity of their polyphenolic content. Therefore, further evaluation of the anti-inflammatory activity of different *Salvia* extract fractions and isolated active constituents is warranted. Actually, the above-mentioned promising pharmacological activities highlight the plant's potential use in the development of new anti-inflammatory drugs. This investigation should be encouraged given the wide distribution, ease of cultivation, and the popular worldwide use of the East Mediterranean sage plant.

Disclosure

A part of this research was presented in abstract form at the 20th LAAS International Science Conference [Abstr.]: 516-17; March 27–29 (2014), Lebanese University, Hadath, Lebanon (available at http://www.biotech.ul.edu.lb/laas20/program.html).

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

The authors would like to thank Professor Aya Asaad and Professor Ahmed El-Mallah for valuable comments, as well as Sirine Zaatari for technical assistance. This work was supported by the Faculty of Pharmacy, Beirut Arab University.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 140247, 7 pages
http://dx.doi.org/10.1155/2015/140247

Research Article

Topical Anti-Inflammatory Activity of Oil from *Tropidurus hispidus* (Spix, 1825)

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Received 23 July 2015; Revised 23 September 2015; Accepted 28 October 2015

Academic Editor: Bamidele Owoyele

Tropidurus hispidus has been used in traditional medicine in several regions of Northeastern Region of Brazil. Its medicinal use involves the treatment of diseases such as warts, sore throat, tonsillitis, chicken pox, varicella, measles, asthma, alcoholism, and dermatomycosis. The present study evaluated the topical anti-inflammatory activity of *Tropidurus hispidus* fat in treating ear edema in an animal model. Oil from *T. hispidus* (OTH) was evaluated on its effect against experimental inflammation in mice. OTH was extracted from body fat located in the ventral region of *Tropidurus hispidus* using hexane as a solvent. We used the model of mouse ear edema induced by phlogistic agents, croton oil (single and multiple applications), arachidonic acid, phenol, capsaicin, and histamine, applied into the right ears of animals pretreated with acetone (control), dexamethasone, or OTH. OTH inhibited the dermatitis induced by all noxious agents, except capsaicin. This effect may be related to the fatty acids present in OTH.

1. Introduction

The term "Zootherapy" refers to the use of animals and products derived from animals for the treatment of diseases and health conditions [1]. In modern society, zootherapy constitutes an important alternative among many other practiced therapies in the whole world [2, 3]. The high biological and cultural diversity of Brazil translates into a richness of knowledge and traditional practices including the use of animals for medicinal purposes. According to Ferreira et al. [4], a high number of species are used for the treatment of diseases. Alves et al. [5] reported that at least 354 species of medicinal animals are used in Brazil, of which 96% are captured in the field and 21% are mentioned on one or more lists of animals at risk of extinction.

Despite the frequent use of zootherapeutic products in Brazilian traditional medicine, there are still few laboratory studies examining their efficacy [6, 7]. Body fat (lard) and skin

are the parts mostly used and they have been traditionally prescribed for the treatment of osteoporosis, rheumatism, arthritis, osteoarthritis, ulcers, gastritis, and inflammatory conditions. These diseases involve an inflammatory process, which suggests a potential use as anti-inflammatory agents, making the evaluation of zootherapeutics important for these purposes [8].

The lizards of the genus *Tropidurus* occur in continental South America, in east and west of the Andes and Galapagos Islands. They are found in savannas, cerrado, caatinga, forests, and plains habitats of South America [9] and inhabit 14 Brazilian states, mainly in the Northeast Region [10, 11].

Tropidurus hispidus is one of the species of lizards utilized in Brazilian popular medicine [12] in the treatment of warts [13], sore throat, tonsillitis, and pharyngitis [12]. Santos et al. [14] determined the biological activity of extracts of the skin and decoctions and found that *T. hispidus* showed antibacterial activity when combined with aminoglycosides. Additionally, prospecting studies revealed the presence of alkaloids [14]. On the other hand, besides being used against illnesses of bacterial origin, these lizards are also used in treatment of diseases of inflammatory character [12], although studies analyzing their efficacy have not yet been done. Therefore, the aim of the present work was to evaluate the topical anti-inflammatory activity of the fat of *T. hispidus* in mouse ear edema models.

2. Materials and Methods

2.1. Zoological Material. A total of 51 lizards of the species *Tropidurus hispidus* were collected (Permission of collection: 154/2007 number 23544-1 process number 17842812) in the municipality of Crato (S 7°14′ and W 39°24′), Ceara, Brazil, in April 2010. They were caught manually with slings and a 4-mm air gun and by active collections combing environments where these animals can occur. Once collected, the lizards were euthanized by freezing and dried at 70°C in drying oven to obtain decoctions and extracts. Voucher specimens were fixed in 70% alcohol and deposited in the collection of Zoology Department of Universidade Regional do Cariri (LZ-URCA-847).

2.2. Animals. We used *Swiss* mice (*Mus musculus*), 20–30 g, of both sexes, acclimated at mean temperature of 22°C (±3°C), kept in a 12-hour light/dark cycle, with feed and water *ad libitum*. Animals were donated by the Biotério da Faculdade de Medicina de Juazeiro do Norte (FMJ) (Protocol Number 2009-0319 CEP) and monitored in the Biotério Experimental of Universidade Regional do Cariri (URCA), in accordance with the guidelines and procedures for biosafety of animal houses [15, 16] and bioethics [17].

2.3. Chemicals. Arachidonic acid (AA), croton oil, capsaicin, histamine, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, USA). Dexamethasone (Decadron) was acquired from Aché (Brazil). Acetone, ethanol, and hexane of analytical grade were purchased from Dinâmica (Brazil). Ketamine and Xylazine were acquired from Syntec (Brazil).

2.4. Obtention of Oil of Tropidurus hispidus (OTH). The oil was extracted from fat concentrated in the ventral region. The extraction was done by a Soxhlet apparatus utilizing hexane as the solvent for a period of 4 hours. The oil was dried in a water bath at 70°C for 2 hours followed by cooling and storing in a freezer for future analyses.

2.5. Ear Edema Induced by a Single Application of Croton Oil. Groups of mice (n = 7/each) had their right ear treated topically with 20 μL of acetone, 100 mg/mL indomethacin (2 mg/ear), 4 mg/mL dexamethasone (0.08 mg/ear), 100, 200, and 400 mg/mL OTH in acetone (1, 2 and 4 mg/ear, resp.), or crude OTH (20 μL = 13 mg OTH). After 15 minutes, croton oil (5% v/v, 20 μL) in acetone was applied topically on the right ear and 20 μL of acetone (vehicle) on the left ear. After 6 hours, the animals were euthanized and discs with a thickness of 6 mm were obtained for evaluation [18].

2.6. Ear Edema Induced by Multiple Applications of Croton Oil. To evaluate the anti-inflammatory effect of OTH in a subacute inflammatory process, already established, we utilized a model with multiple applications of croton oil. An inflammatory process was induced in mice (n = 6/group) by the application of croton oil (5% v/v, 20 μL) in acetone on alternate days, for 9 days. OTH (13 mg/ear) and dexamethasone (0.1 mg/ear, positive control) were applied topically for 4 days (twice a day) 96 hours after the first application of croton oil. On the 9th day, animals were euthanized and discs with a thickness of 6 mm were obtained for evaluation [19]. Edema was evaluated daily.

2.7. Ear Edema Induced by Application of Phenol, Capsaicin, and Arachidonic Acid. The inflammatory process was induced in mice (n = 7/group) by applying on the outer and inner surfaces of the right ear 20 μL of the following phlogistic agents: 10% phenol (v/v); 0.01 mg/μL capsaicin or 0.1 mg/μL arachidonic acid (AA) diluted in acetone. The right ear was treated topically with, 15 minutes before the application of phlogistic agents, OTH (20 μL, pure), acetone (20 μL, negative control), indomethacin (100 mg/mL, positive control for AA), or dexamethasone (4 mg/mL, positive control for phenol and capsaicin). Edema was evaluated 1 h after application of AA and phenol and 30 minutes after application of capsaicin. [20–22].

2.8. Ear Edema Induced by Subcutaneous Injection of Histamine. Initially the animals (n = 7/group) were anesthetized with 10 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride. Next, the animals were topically pretreated with saline (20 μL), dexamethasone (4 mg/mL; 0.08 mg/ear), or pure OTH (13 mg/ear). After 30 minutes, histamine (5 μL, 100 mg/mL in saline) was administered subcutaneously using a 29 G hypodermic needle, in the ventral region of the right ear of the mice. The left ear received the same volume of saline (intradermal). 2 hours later, animals were euthanized and 6-mm discs were obtained from the ears for evaluation [21].

2.9. Quantification of Ear Edema. Edema was determined according to the weight obtained due to the inflammatory response. At the end of the period of exposure to each phlogistic agent, mice were euthanized by cervical dislocation and 6-mm discs were obtained from right and left ears with the use of a hole-punch and weighed on a precision balance (Mettler Toledo AB204). Ear edema was determined by the difference in weight (in mg) of the section removed from the right ear (treated with phlogistic agent) and the weight (in mg) of the section removed from the left ear (treated with vehicle used for dilution of irritant).

The mean percentage inhibition of edema (%) was calculated using the following formula: inhibition (%) = $[MPE_{cont} - MPE_{treat}/MPE_{cont}] \times 100$. MPE_{cont} is the mean percentage of edema of negative control group (treated with saline) and MPE_{treat} is the mean percentage of edema of group subjected to treatment with OTH or standard drug.

2.10. Statistical Analysis of Data. The values obtained were expressed as mean ± standard error (SEM). Differences obtained between groups were subjected to one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test or two-way ANOVA followed by the Bonferroni test (edema induced by multiple applications of croton oil). The accepted level of significance for the test was $p < 0.05$.

3. Results

Croton oil, arachidonic acid, phenol, capsaicin, and histamine elicited a significant inflammatory response, as seen by the increase in weight of the ears treated with the irritants in relation to the ears that received only the corresponding vehicle (acetone).

Single application of croton oil induced edema in the right ear of mice (Table 1). The antiinflammatory effect of pure and 100 mg/mL was significant compared to the control group ($p < 0.001$ and $p < 0.05$, resp.) (Figure 1 and Table 1).

In the subacute inflammatory model with multiple applications of croton oil, OTH and dexamethasone were applied twice a day for four days (Figure 2, arrows). Negative control showed edema of 11.6 ± 1.9 mg. Dexamethasone and OTH induced a significant decrease to 4.21 ± 0.40 mg ($^{**}p < 0.001$, 63.7% inhibition) and 4.21 ± 0.94 mg ($^{***}p < 0.001$, 63.7% inhibition), respectively (Figures 2 and 3).

Ear edema induced by AA (5.71 ± 0.26 mg) was also substantially reduced ($^{***}p < 0.001$) by pure OTH (3.13 ± 0.20 mg, 45.6% inhibition) and indomethacin (2.48 ± 0.47 mg, 57% inhibition) (Figure 4).

Dexamethasone and OTH inhibited ($^{*}p < 0.01$) the ear edema induced by phenol. The degree of edema of the negative control was of 9.70 ± 0.96 mg, while the groups treated with dexamethasone and OTH were both 6.0 ± 0.56 mg (38.1% inhibition), with the level of significance being $p < 0.05$ for dexamethasone and $p < 0.01$ for OTH (Figure 5).

Dexamethasone (2.00 ± 0.60 mg; 37.5% inhibition; $^{*}p < 0.05$), but not OTH, demonstrated an anti-inflammatory effect by reducing the capsaicin-induced ear edema in relation to the negative control (3.22 ± 0.38 mg) (Figure 6).

Table 1: Effect of OTH on ear edema induced by single application of croton oil.

Group	Concentration (mg/mL)	Edema (mg)	Inhibition (%)
Negative control	—	11.20 ± 0.39	—
Dexamethasone	4	1.43 ± 0.46***	87.5%
OTH	Pure	5.71 ± 0.68***	49.1%
OTH	100	8.37 ± 0.71*	25.0%
OTH	200	11.00 ± 0.73	1.78%
OTH	400	9.51 ± 0.74	15.0%

Values expressed as mean ± SEM ($^{*}p < 0.05$; $^{***}p < 0.001$ versus control); (ANOVA and Student-Newman-Keuls test).

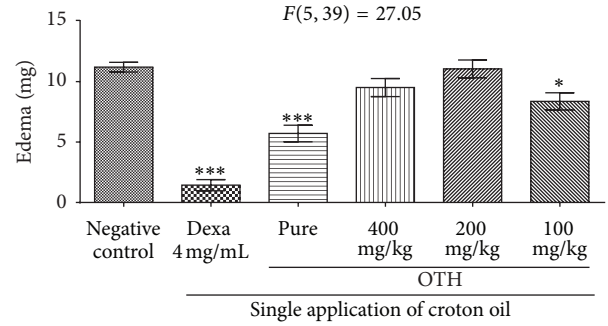

Figure 1: Effect of topical OTH on ear edema induced by single application of croton oil (OC) in Swiss mice. Animals were pretreated with saline in acetone, dexamethasone, OTH (pure, 100, 200, or 400 mg/ear). ANOVA followed by Student-Newman-Keuls test: $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to negative control.

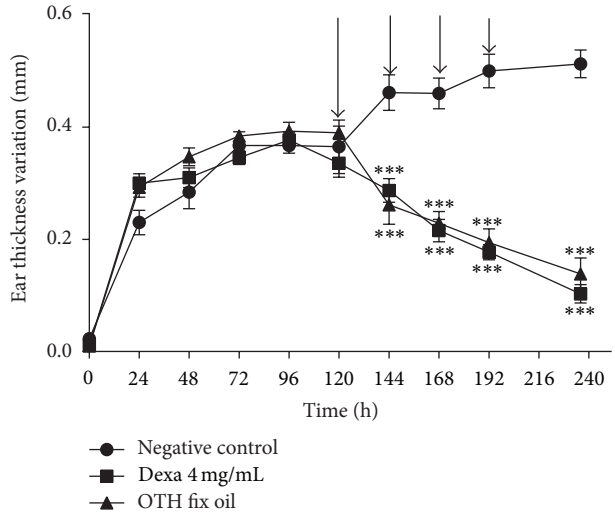

Figure 2: Dose-response curve of effect of OTH on ear edema induced by multiple applications of croton oil. $^{***}p < 0.001$ compared to negative control, two-way ANOVA followed by the Bonferroni test.

OTH (1.40 ± 0.37; 49.6% inhibition) and dexamethasone 1.55 ± 0.35 mg (44.2% inhibition) inhibited ($^{*}p < 0.05$) the ear edema induced by histamine when compared to the control group (2.78 ± 0.31 mg) (Figure 7).

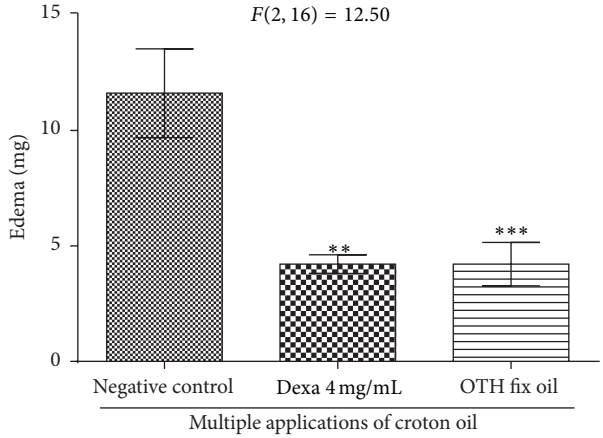

Figure 3: Effect of topical OTH on ear edema induced by multiple applications of croton oil. One-way ANOVA followed by Student-Newman-Keuls test. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to negative control.

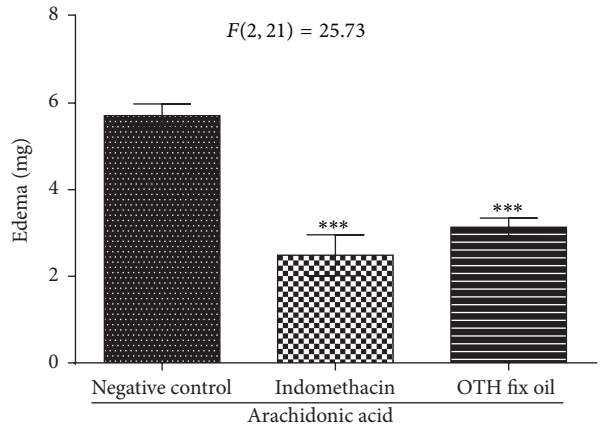

Figure 4: Effect of topical OTH on ear edema induced by arachidonic acid (AA). One-way ANOVA followed by Student-Newman-Keuls test. $^{***}p < 0.001$ compared to negative control.

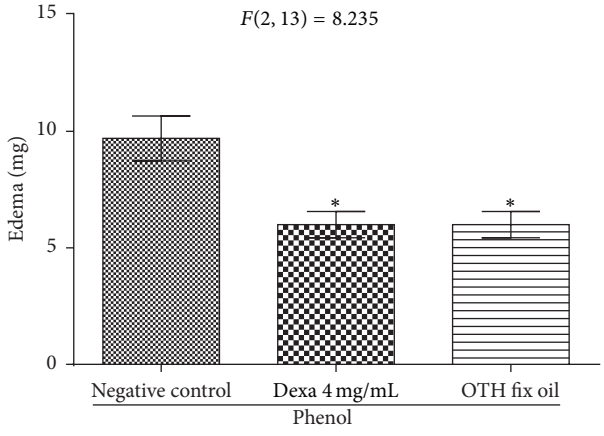

Figure 5: Effect of topical OTH on ear edema induced by phenol. One-way ANOVA followed by Student-Newman-Keuls test. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to negative control.

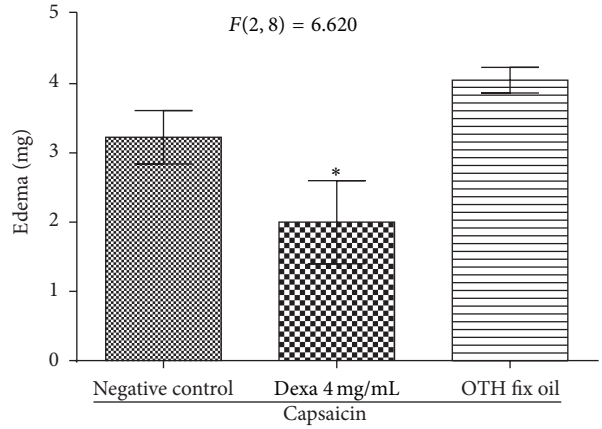

Figure 6: Effect of topical OTH on ear edema induced by capsaicin. One-way ANOVA followed by Student-Newman-Keuls test. $^{**}p < 0.01$ compared to negative control.

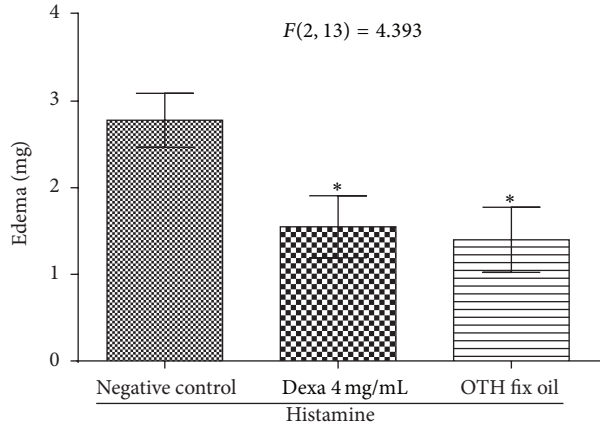

Figure 7: Effect topical of OTH on ear edema induced by the subcutaneous application of histamine. One-way ANOVA followed by the Student-Newman-Keuls test. $^{*}p < 0.05$ compared to negative control.

4. Discussion

Natural products of animal origin represent an important alternative for the treatment of diseases in people in various parts of the world [23]. According to Coutinho et al. [24], products of animal origin have been methodically tested by pharmaceutical companies as sources of new drugs. Ethnopharmacological studies focusing on remedies of animal origin are very important to indicate the possible therapeutic use of these natural products, as well as possibilities of their pharmaceutical development [25, 26].

Santos et al. [14] performed tests to evaluate microbiological activity of extracts from *T. hispidus* and found that, in combination with aminoglycosides, these extracts produced a significant reduction in the minimum inhibitory concentration (MIC) of the antibiotics, demonstrating the presence of bioactive substances in this species of lizard.

In the present work, we showed pharmacological activity of oil from *T. hispidus* (OTH) against experimental topical inflammation. Ferreira et al. [27], studying the medicinal effect of oil from the fat of the lizard *Tupinambis merianae*, showed no antibacterial effect but an effective reduction in topical inflammation induced by the same phlogistic agents used here.

Previous reports related that fatty acids have anti-inflammatory activity, supporting their utilization in the treatment of skin inflammation [28–30]. According to Das [30], fatty acids act directly in inflammatory processes.

We evaluated OTH at concentrations of 100, 200, and 400 mg/mL and the pure oil. Only the pure oil showed anti-inflammatory effect and it was used in the next tests. Subacute inflammation was induced by multiple applications of croton oil. This test simulates the chronic condition in humans and the drug test is administered after the establishment of the inflammatory process, which, according to Alford et al. [31], permits the evaluation of compounds capable of alleviating the subacute inflammation.

According to Green and Shuster [32], anti-inflammatory steroids (corticoids) and lipoxygenase (LOX) inhibitors are active in this model, while cyclooxygenase (COX) inhibitors appear to be inactive. OTH and dexamethasone inhibited the development of edema. When we compare the effect of pure OTH in the models of single and multiple applications of croton oil, we observe that the mean inhibition of edema was greater in multiple applications (63.7%) with a 4-day treatment. This can be explained by the bioavailability of substances that provide local anti-inflammatory action.

Arachidonic acid (AA) is metabolized into various mediators that induce the formation of edema, such as PGE_2, LTC_4, and LTD_4 [33]. According to Griswold et al. [34], the plasma membrane epidermal cells produce AA, which is oxidized to form prostaglandins, leukotrienes, and thromboxanes, responsible for inflammation, as part of the immune response elicited by antigens such as phospholipase A_2 (PLA_2). Thus, it is possible to identify, in this model, compounds that inhibit AA metabolism into prostaglandins (PG) and leukotrienes. However, Del Carmen Recio et al. [35] suggested that the edema induced by AA is preferentially a triage model for identifying LOX inhibitors. The nonsteroidal anti-inflammatory drugs (NSAID) inhibit the COX pathway, thereby impeding the synthesis of PG [36]. Thus, the reference drug utilized in this experimental model was indomethacin, an NSAID with anti-inflammatory action related to the nonselective inhibition of the COX isoforms (COX-1 and COX-2) and that effectively reverses edema induced by the topical application of AA, as described by Gabor [22]. Similarly, to indomethacin, OTH also appeared to be effective in inhibiting the formation of edema in this model, significantly decreasing the edema in ears sensitized by AA. According to Carlson et al. [37], models of edema induced by croton oil and AA are extremely useful in the detection of possible COX/LOX inhibitors in vivo. Thus, we believe that OTH is efficient in skin inflammatory disorders, and the use of *T. hispidus* in popular medicine for treating inflammatory diseases could be related to the reduction of the levels of AA metabolites in cutaneous tissue.

OTH did not inhibit the formation of edema induced by the topical application of capsaicin, suggesting that the compounds present in OTH do not appear to act directly on TRPV1 receptors. Studies have demonstrated that the edema induced by the topical application of capsaicin is inhibited by histamine and serotonin antagonists but not by COX inhibitors, such as indomethacin [38].

In the model of edema induced by histamine applied intradermally, the phlogistic agent causes vasodilation and an increase in vascular permeability, causing an edematogenic effect in a few minutes [39]. Prior topical application of OTH significantly inhibited edema induced by histamine.

Pure OTH also significantly reduced edema induced by phenol demonstrating the efficacy of this extract in the treatment of contact dermatitis caused by irritants. In response to exogenous stimuli, such as phenol, keratinocytes produce chemical mediators important in primary contact irritation, including cytokines associated with proinflammatory properties, as IL-1α, TNF-α, and IL-8 [40, 41]. One of the mechanisms by which phenol triggers cutaneous irritation is the rupture of the plasma membrane of keratinocytes through a direct effect, resulting in the release of preformed IL-1α, besides other inflammatory mediators such as the AA metabolites and reactive oxygen species (ROS).

However, despite the fact that the inflammatory response can be triggered by different routes, both models (histamine and phenol) share the involvement of AA metabolites and ROS. Therefore, we found that OTH could exert its anti-inflammatory effect by acting on these mediators, as demonstrated in the AA model. In view of the models of ear edema induced by croton oil, AA, capsaicin, histamine, and phenol, we found that OTH possibly has similar mechanism as drugs that reduce the production of AA metabolites. It is therefore suggested that the antiedematogenic action of OTH is linked to the factors that alter the production of inflammatory eicosanoids, where its action can be through the inhibition of the enzymes COX and LOX or the production of anti-inflammatory eicosanoids (PGE1, lipoxins, or others).

The anti-inflammatory activity of natural products obtained from animals has been proven in other studies. Yoganathan et al. [42] found that the oil from the bird *Dromaius novaehollandiae* promoted a 70% reduction of the ear inflammation in rats. Falodun et al. [43] observed the efficacy of fat from *Boa constrictor* against skin inflammation. These results showed the importance of evaluating more natural products obtained from animals in the treatment of inflammatory diseases.

Considering that animals represent an important source of medications used in traditional medicine, zootherapy has become extremely relevant within a conservationist perspective [44–46]. Alves and Rosa [47] emphasized that the great majority of animals traded for medicinal purposes are wild, making it necessary, most of the time, to kill them to obtain the zootherapeutic products, demonstrating that conservationist measures are needed regarding medicinal species threatened with extinction or that are widely used.

The substitution of threatened medicinal species by other animals with the same therapeutic indications, and not in extinction process, has become a strategy of sustainability

and conservation. Sodeinde and Soewu [48] commented that the pressure on threatened species utilized in traditional medicinal formulations could be reduced by their substitution by common species, when suitable, calling attention to sustainability of the substitute species aimed at assuring the viability of its exploitation. *Tropidurus hispidus* can be, in a certain context, considered a substitute species in relation to those threatened, since their existence does not run the risk of extinction and its use in traditional medicine is relatively low. As observed in this study, such species shows significant topical anti-inflammatory activity, and Santos et al. [14] have described its biological activity in combination with aminoglycosides against bacterial strains, thereby making it an alternative in management as a substitute species for those that show the same therapeutic indications and that are threatened.

5. Conclusion

The oil derived from the body fat of *T. hispidus* has anti-inflammatory activity against ear edema induced by croton oil, arachidonic acid, histamine, or phenol. The fatty acids present in OTH probably affect arachidonic acid and its metabolites, thereby reducing the production of proinflammatory mediators. In view of the great importance of animals in zootherapy, management and sustainability policies are needed in relation to threatened species.

Conflict of Interests

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

Acknowledgments

The authors are grateful to Fundação Cearense de Amparo a Pesquisa (FUNCAP) for providing financial support and to the postgraduation program in molecular bioprospecting of the Universidade Regional do Cariri-URCA. IBAMA provided them with a license to collect specimens, no. 23544-1, Process no. 17842812. Dr. A. Leyva helped with English translation and editing of the paper.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 425108, 13 pages
http://dx.doi.org/10.1155/2015/425108

Review Article

Effectiveness of Acupuncture for Treating Sciatica: A Systematic Review and Meta-Analysis

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Received 23 June 2015; Accepted 13 September 2015

Academic Editor: Roi Treister

This is a systematic review and meta-analysis, which aimed to assess the current evidence on the effects and safety of acupuncture for treating sciatica. In this review, a total of 11 randomized controlled trials were included. As a result, we found that the use of acupuncture may be more effective than drugs and may enhance the effect of drugs for patients with sciatica, but because of the insufficient number of relevant and rigorous studies, the evidence is limited. Future trials using rigorous methodology, appropriate comparisons, and clinically relevant outcomes should be conducted.

1. Introduction

Sciatica is a syndrome involving nerve root impingement or inflammation that has progressed sufficiently to cause neurological symptoms in the areas that are supplied by the affected nerve roots [1]. The most important symptoms include unilateral leg pain radiating to the foot or toes that is greater than low back pain and often associated with paresthesia, numbness, and weakness of the leg; it may involve increased pain on straight leg raising and neurological symptoms limited to one nerve root. Sciatica may be sudden in onset and may subsequently persist for days or weeks [2, 3].

Frymoyer reported that the prevalence of sciatica varies widely from 13% to 40% [4, 5]. According to the research of Konstantinou, most patients suffered sciatica in the fourth and fifth decades of their life [6]. The treatment for sciatica is primarily aimed at pain control by means of either conservative treatment or surgical techniques. According to the prior systematic review, nonopioid medication, epidural injections, and disc surgery are effective for pain reduction [7]; however, relevant side effects to epidural injections have been reported [8–10], and the effect of NSAIDs on sciatica is still uncertain, even though it is a common treatment to manage pain. Many patients report little relief [7], and the surgical procedures are invasive and expensive and may even cause neurological complications that may not be acceptable for all patients [11].

Acupuncture is a tried and tested system of traditional Chinese medicine, which has been used in China and other Eastern cultures for thousands of years. While acupuncture has been proposed for persistent sciatica, its efficacy has not been shown [7, 12]. To date, there has been substantial research into the anaesthetic and anti-inflammatory actions of acupuncture [13–15], and several randomized controlled trials (RCTs) have suggested that acupuncture can relieve the symptoms of sciatica [16, 17]. Despite these studies, an acupuncture-related systematic review has still fallen short of projected expectations.

This systematic review aimed to assess the current evidence on the effects and safety of acupuncture for sciatica.

2. Methods and Analysis

We conducted this systematic review according to a published protocol [18] and our review is reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [19].

2.1. Study Selection

2.1.1. Types of Studies. All randomized controlled trials (RCTs) in English, Chinese, and Japanese on acupuncture

treatment for sciatica were included for this review. Non-RCTs, quasi-RCTs, and randomized controlled trial protocol were excluded.

2.1.2. Types of Participants. Patients with sciatica were included, including those diagnosed with sciatica synonyms, such as radiculopathy, nerve root compromise, nerve root compression, nerve root pain, and pain radiating below the knee, with no restriction on gender and age. We excluded trials if they included lower back pain without sciatica.

2.1.3. Types of Interventions. Any type of invasive acupuncture were included, such as acupuncture, electroacupuncture, elongated needle acupuncture, auricular acupuncture, abdominal acupuncture, and warm acupuncture. Control interventions may include no treatment, sham acupuncture/placebo (e.g., acupuncture same acupuncture point without needle insertion or acupuncture the point close to it but it is not an acupuncture point), and Western medicine. As this review aims to assess the effectiveness and safety of acupuncture for treating sciatica, we excluded trials comparing two different types of acupuncture. Furthermore, the effectiveness of Chinese medicine is hard to assess, so we excluded trials comparing acupuncture with Chinese medicine.

2.1.4. Types of Outcome Assessments. The primary outcome of interest was pain intensity. Any validated measurement scales were included (e.g., Visual Analogue Scale (VAS), Numeric Rating Scale (NRS), and Short-Form McGill Pain Questionnaire (SF-MPQ)). Secondary outcomes were (1) global assessment (the proportion of patients improved or cured); (2) quality of life, for example, as assessed using the Medical Outcomes Study 36-Item Short Form health survey (SF-36); (3) physical examinations; (4) patient satisfaction; and (5) adverse effects.

2.1.5. Data Sources. A search strategy was used and conducted according to the Cochrane handbook guidelines [31]. The following nine databases were searched from their inception to May 2015: MEDLINE, EMBASE, CENTRAL, CBM, CMCC, VIP database, Wan-Fang Database, CNKI, and CiNii. The search strategy was based on the guidance of the Cochrane handbook.

The strategy for searching the PUBMED database is shown in Table 1. This search strategy was also applied to the other electronic databases.

2.1.6. Data Extraction. Two authors (Zongshi Qin and Xiaoxu Liu) extracted the data independently. Before beginning extraction, a small scope trial with one database was conducted to confirm that there were no differences between the two authors. After a common understanding was reached, standard extraction forms were used to collect data from included trials. Any disagreements were discussed and judged by an arbiter (Zhishun Liu).

2.1.7. Data Management. Two authors (Zongshi Qin and Xiaoxu Liu) used Endnote X7 (Thomson Reuters, New York, NY, USA) software to manage the trials that have been searched and remove duplicates. Data extracted were put into Revman V.5.3.3 software for analysis.

Table 1: Search strategy used in PubMed database.

Number	Search items
1	randomized controlled trial.pt
2	controlled clinical trial.pt
3	randomized.ti,ab
4	randomly.ti,ab
5	groups.ti,ab
6	trial.ti,ab
7	or 1–6
8	acupuncture.ti,ab
9	electro-acupuncture.ti,ab
10	elongated needle.ti,ab
11	three edged needle.ti,ab
12	(fire needle or warming needle).ti,ab
13	auricular acupuncture.ti,ab
14	abdominal acupuncture.ti,ab
15	warm acupuncture.ti,ab
16	pyonex.ti,ab
17	or 8–16
18	sciatica.ti,ab
19	sciatic neuralgia.ti,ab
20	ischialgia.ti,ab
21	ischioneuralgia.ti,ab
22	discogenic sciatica.ti,ab
23	bilateral sciatica.ti,ab
24	disc herniation-induced sciatica.ti,ab
25	or 18–24
26	7 and 17 and 25

This search strategy will be modified as required for other electronic databases.

2.1.8. Risk of Bias in Individual. The Cochrane Collaboration tool for assessing the risk of bias was used to facilitate the assessment of the risk of bias for trials included [32]. Two authors (Jiani Wu and Yanbing Zhai) independently evaluated methodological quality, which covers seven aspects: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other bias. Any disagreements were discussed and resolved by a third author (Zhishun Liu).

2.1.9. Measures of Treatment Effect. Dichotomous data were analysed using risk ratio (RR) and 95% confidence interval (CI). Continuous outcomes were analysed using mean differences (MD) with 95% CI or standardized mean differences (SMD) with 95% CI if different measurement scales are used.

2.1.10. Dealing with Missing Data. The listed corresponding author was contacted in an attempt to obtain any missing

information from their trial. We excluded 1 trial after 3 unsuccessful attempts to contact the authors to obtain missing data from the data synthesis [33].

2.1.11. Assessment of Heterogeneity. We used Higgins I^2 statistic to test clinical heterogeneity. Variability factors included in the trials were taken into consideration (e.g., type of intervention and duration of intervention). If $I^2 \geq 50\%$ or $P < 0.1$, there is substantial heterogeneity among the trials, and the design of trials and characteristics in the included trials were analysed.

2.1.12. Assessment of Reporting Biases. A funnel plot was used to assess the reporting biases when 10 or more trials were included in a meta-analysis. However, the number of studies included in our analysis may have been too small to test for funnel plot asymmetry [34].

2.1.13. Confidence in Cumulative Estimate. Details of acupuncture and control interventions were extracted on the basis of the revised Standard for Reporting Interventions in Clinical Trials of Acupuncture (STRICTA) [35], a checklist intended for use in conjunction with CONSORT that can estimate randomized controlled trials of acupuncture, including acupuncture rationale, needling details, treatment regimen, cointervention, control interventions, and treatment background. The acupuncture interventions in the included studies based on the STRICTA recommendations are presented in Table 2.

2.1.14. Data Synthesis. We used Revman V.5.3.3 software to perform meta-analysis of the trials included. Dichotomous data were determined by using RR with 95% CI, and continuous outcomes were analysed using WMD with 95% CI or SMD with 95% CI if different measurement scales are used. When statistical heterogeneity was observed, the random effects model was used; otherwise the fixed effect model was used to combine the data. When quantitative synthesis was not appropriate, we provided systematic narrative synthesis to describe the characteristics and findings of the included trials.

2.1.15. Subgroup Analysis and Sensitivity Analysis. We planned to conduct subgroup and sensitivity analyses in the published protocol as follows: we hypothesized a greater reduction in pain intensity and improvement in global assessment with acupuncture than with sham acupuncture; we also predicted that different types of sciatica or risks of bias in different trials would lead to moderate statistical heterogeneity.

3. Results

3.1. Selection of Studies. Our search strategy yielded a total of 1489 records. After 435 duplicate records were excluded, 1054 unique records were screened for eligibility. A total 1005 records were excluded based on review of the title and abstract. The remaining 49 records were deemed potentially relevant. After the full-text articles were reviewed, 7 studies were excluded because they were not true RCTs, 24 studies were excluded because they included inappropriate interventions, and 7 studies were excluded due to inappropriate design. One study was published in French and the full-text was unavailable; thus, we were unable to extract the data, and the study was therefore excluded from review [36]. In total, 11 studies met the criteria predesigned in our protocol and were therefore included in our review for systematic and meta-analysis [20–30]. All trials were published between 2004 and 2014; 9 studies were published in Chinese [22–30], and 2 were published in English [20, 21]. Two trials were multicentre trials while the others were single centre [20, 23].

Figure 1 uses a study flow diagram to summarize the results of the study searches.

3.2. Description of Studies

3.2.1. Patients. We included 11 trials that enrolled a total of 962 participants in our systematic review [20–30]. Ten trials were conducted in China (932 participants) [21–30] and 1 was conducted in Pakistan (40 participants) [20]. All patients had acute or chronic sciatica; 3 trials included 180 participants diagnosed with sciatica of the nerve trunk without lumbar disc herniation and low back pain [21, 26, 30] and 8 studies (782 participants) included patients with sciatica of the nerve roots [20, 22–25, 27–29], especially caused by lumbar disc herniation. All studies stated that patients with abnormal neuralgia such as compression pain from tumour or serious infection were excluded.

The characteristics of the included studies are summarized in Table 3.

3.2.2. Acupuncture Interventions. In general, all of the studies adopted a treatment theory based on traditional Chinese medicine theory and clinical experience. Many acupuncturists choose acupuncture points or corresponding acupuncture interventions based on their clinical experience during treatment. Electroacupuncture was used in most of the trials (6 studies) [20, 23–25, 28, 30], warming acupuncture was used in 3 studies [21, 27, 29], and manual needle stimulation was performed in 2 trials [22, 26]. The number of acupuncture points varied from 1 to more than 10; the most commonly used acupoints were Huantiao (GB 32), Weizhong (BL 40), and Yanglingquan (GB 34). The acupoints for each trial are shown in Table 4. The duration of interventions ranged from one to four weeks and only one trial mentioned 6 months of follow-up. The age of the patients ranged from 18 to 79 years. Eleven studies reported De-chi, a needle sensation of soreness and numbness.

3.2.3. Control Interventions. In 8 trials [20–27], acupuncture was compared to conventional medications; most of the medications were nonsteroidal anti-inflammatory drugs (NSAIDs). Two studies compared acupuncture plus conventional medication to the same conventional medication alone [28, 29]. One trial used sham acupuncture in the control group [30]; the needles in this trial were inserted in

Table 2: Acupuncture interventions in the included studies based on the STRICTA recommendation.

Author	Insertion depth	Response sought	Details of needling Stimulation method	Retention time	Needle type	Treatment regimen	Practitioner background
Wang and La 2004 [20]	NR	De-chi response manual	EA	25 min	NR	1 session once a day for 7 days	Physician
Chen et al. 2009 [21]	NR	De-chi response manual	WA	20–35 min	0.3 × 60 mm	3 sessions once daily for 10 days	NR
Zeng 2012 [22]	60 mm (GB 30/BL 54) others 25 mm	De-chi response manual	Manipulated every 10 min, pricking blood	30 min	0.3 × 75 mm	2 sessions once daily for 10 days	NR
Zhang et al. 2008 [23]	40–60 mm	De-chi response manual	Manipulated every 10 min and EA	20 min	0.3 × 40–75 mm	2 sessions once daily for 10 days	Professional acupuncturists
Hu et al. 2010 [24]	60 mm (GB 30) others 40 mm	De-chi response manual	Manipulated every 10 min and EA	30 min	0.3 × 50–75 mm	2 sessions once daily for 10 days	NR
Du et al. 2009 [25]	45–60 mm	De-chi response manual	EA	45 min	0.45 × 75 mm	4 sessions 3 times per week	NR
Chen 2010 [26]	40–75 mm	De-chi response manual	Manipulated every 10 min	30 min	0.3 × 25–40 mm	2 sessions 3 times per week	NR
Wang 2008 [27]	NR	De-chi response manual	WA	NR	0.4 × 75 mm	2 sessions once daily for 10 days	NR
Meng 2014 [28]	NR	NR	EA	30 min	NR	2 sessions once daily for 7 days	Qualified acupuncturist
Ren 2013 [29]	40–75 mm	De-chi response manual	WA	30 min	NR	1 session once a day for 10 days	NR
Zhao 2004 [30]	50–75 mm	De-chi response manual	EA	30 min	0.25 × 75 mm	2 sessions once a day for 10 days	NR

NR: not reported, De-chi: a needle sensation of soreness and numbness, EA: electroacupuncture, WA: warm acupuncture, and STRICTA: standards for reporting interventions in controlled trials of acupuncture.

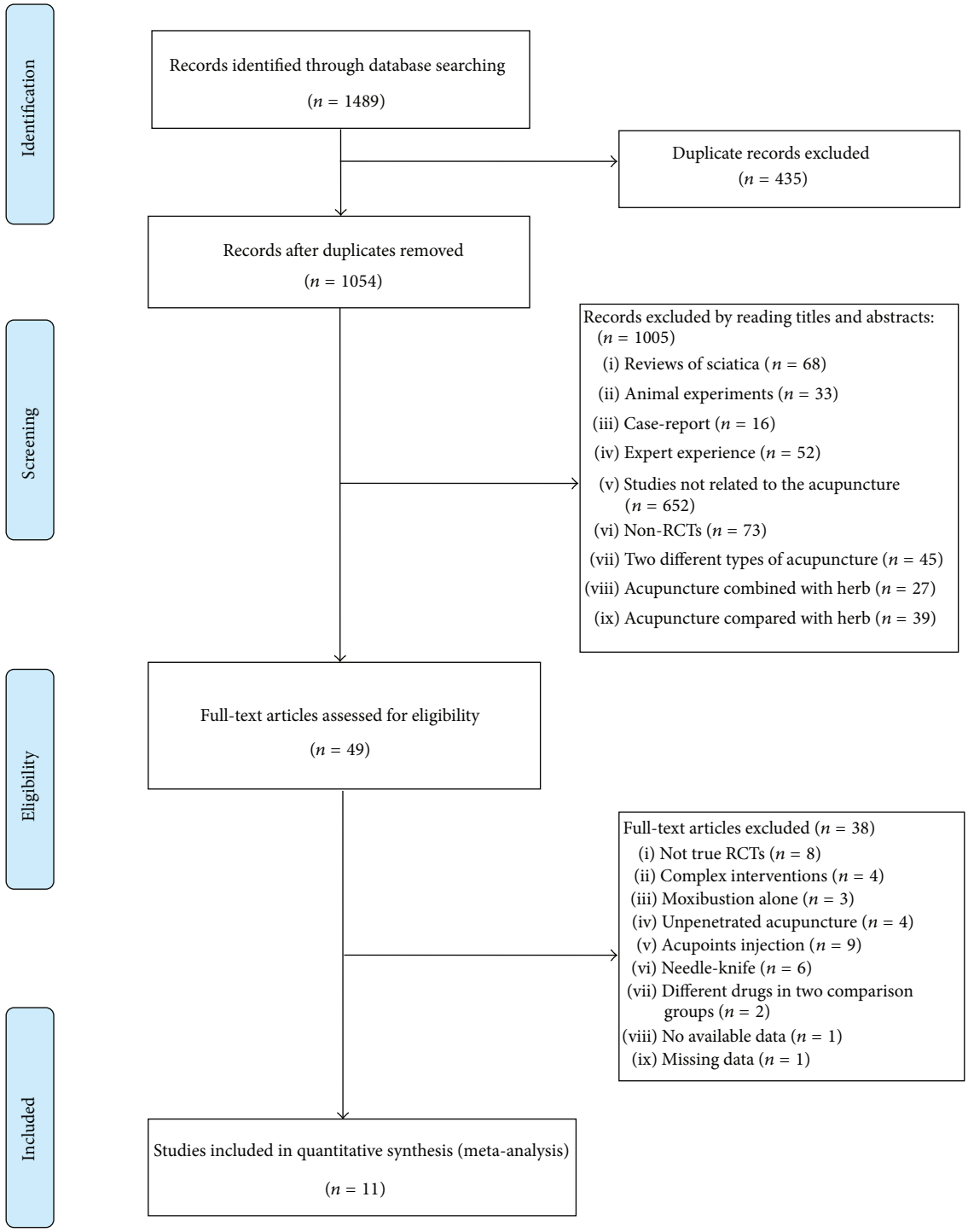

Figure 1: Study flow diagram.

nonacupuncture points (2 inches from the correct acupuncture points).

3.2.4. Outcome Measure. Five studies measured pain intensity using VAS [20, 22, 25, 26, 28], which is an important assessment scale for neuralgia pain; in addition, one study used JOA [25] and one study used BRS-6 to measure pain intensity [22]. For the outcome of global assessment, 9 studies compared the patients who were cured or improved with those who were not [21–24, 26–30]. For outcome measures in most studies, "cured" means that the sciatic neuralgia resolved and the limb function recovered, while "improved" was defined as decreased sciatic neuralgia and largely normal function, and "failed" meant no symptom improvement. One

Table 3: Summary of studies included in the review.

Author	Design	Number of subjects	Intervention type (A)	Control group (B)	Treatment regimen	Follow-up periods	Outcome measure	Results reported	Adverse events
Wang and La 2004 [20]	Parallel, 2 arms	40 (23/17)	EA	Diclofenac sodium 50 mg tid for 7 d	1 session once a day for 7 days	NR	(1) Laseque's sign angles (2) VAS	(1) (A) versus (B): 76.67 ± 1.63 versus 70.88 ± 2.11 (2) (A) versus (B): 25.71 ± 2.27 versus 35.33 ± 2.57	NR
Chen et al. 2009 [21]	Parallel, 3 arms	90 (30/30/30)	WA	(1) Nimesulide 0.1 g bid (2) Acupuncture points injection	3 sessions once daily for 10 days	NR	(1) Response rate (2) PT	(1) (A) significantly better than (B) ($P < 0.05$, 90% versus 73.33%) (2) 2.62 ± 0.59 versus 1.54 ± 0.39	NR
Zeng 2012 [22]	Parallel, 2 arms	60 (30/30)	MA Pricking blood	Ibuprofen 3 mg bid + Vb_2 10 mg tid	2 sessions once daily for 10 days	NR	(1) Response rate (2) BRS-6 (3) VAS	(A) significantly better than (B) (1) ($P < 0.05$, 83.3% versus 70%) (2) 4.87 ± 1.50 versus 5.87 ± 1.89 (3) 2.07 ± 1.05 versus 2.70 ± 1.34	NO
Zhang et al. 2008 [23]	Parallel, 2 arms	194 (98/96)	EA	Meloxicam 7.5 mg qd	2 sessions once daily for 10 days	NR	Response rate	(A) significantly better than (B) ($P < 0.05$, 86.53% versus 75%)	3 patients reported hypodermal bleeding in intervention group; 21 patients in control group reported GI problems
Hu et al. 2010 [24]	Parallel, 2 arms	100 (50/50)	EA	Meloxicam 7.5 mg qd	2 sessions once daily for 10 days	Six month	Response rate	(A) significantly better than (B) ($P < 0.05$, 94% versus 80%); after half year follow-up 83% versus 70%	5 patients in control group reported GI problems
Du et al. 2009 [25]	Parallel, 2 arms	62 (32/30)	EA	Diclofenac sodium 75 mg qd	4 sessions 3 times per week	NR	(1) JOA for total score (2) VAS	(1) 20.16 ± 3.54 versus 17.63 ± 3.23 (2) 2.12 ± 1.12 versus 2.10 ± 1.39	NR
Chen 2010 [26]	Parallel, 2 arms	60 (30/30)	MA	Ibuprofen 0.2 g tid + prednisone 30 mg qd	2 sessions 3 times per week	NR	(1) Response rate (2) VAS (3) MOS SF-36	(1) (A) significantly better than (B) ($P < 0.05$, 100% versus 83.3%) (2) VAS 2.78 ± 1.02 versus 4.64 ± 3.21 (3) MOS SF-36 for GH 57.76 ± 15.20 versus 59.07 ± 15.08	2 patients reported hypodermal bleeding in intervention group
Wang 2008 [27]	Parallel, 2 arms	104 (52/52)	WA	Ibuprofen 0.6 g bid + Vb_1 30 mg tid	2 sessions once daily for 10 days	NR	Response rate	(A) significantly better than (B) ($P < 0.05$, 96.2% versus 71.2%)	NR
Meng 2014 [28]	Parallel, 2 arms	60 (30/30)	EA + drugs (same as control group)	Ibuprofen 20 mg bid + Vb_1 30 mg tid	2 sessions once daily for 7 days	NR	(1) Response rate (2) VAS	(1) (A) significantly better than (B) ($P < 0.05$, 93.33% versus 43.33%) (2) 3.04 ± 0.53 versus 4.28 ± 0.62	NO

Table 3: Continued.

Author	Design	Number of subjects	Intervention type (A)	Control group (B)	Treatment regimen	Follow-up periods	Outcome measure	Results reported	Adverse events
Ren 2013 [29]	Parallel, 2 arms	60 (30/30)	WA + drugs (same as control group)	Mannitol 150 mL + dexamethasone 10 mg i.v.gtt and mecobalamin tablets 0.5 mg I.M.	1 session once a day for 10 days	NR	Response rate	(A) significantly better than (B) ($P < 0.05$, 93.3% versus 60%)	NR
Zhao 2004 [30]	Parallel, 2 arms	60 (30/30)	EA	Sham acupuncture	2 sessions once a day for 10 days	NR	Response rate	(A) significantly better than (B) ($P < 0.05$, 97.7% versus 73.3%)	NO

NR: not reported, EA: electroacupuncture, WA: warm acupuncture, i.v.gtt: intravenous drip, I.M.: intramuscular injection, VAS: Visual Analogue Scale, SF MPQ: Short-Form McGill Pain Questionnaire, PT: pain threshold, JOA: Japanese Orthopaedic Association score, BRS-6: 6-point behavior rating scale, MOS SF-36: the medical outcome study item short form health survey, GI: gastrointestinal, and GH: general health.

Table 4: Acupoints of each trial.

Wang and La 2004 [20]	Huantiao (GB 30), Weizhong (BL 40)
Chen et al. 2009 [21]	Shenshu (BL 23), Dachangshu (BL 25), Huantiao (GB 30), Weizhong (BL 40), and Kunlun (BL 60)
Zeng 2012 [22]	Huantiao (GB 30), Zhibian (BL 54), Chengfu (BL 36), Fengshi (GB 31), Weizhong (BL 40), Yanglingquan (BL 67), Chengshan (BL 57), Xuanzhong (GB 39), Kunlun (BL 60), and Zulinqi (GB 41)
Zhang et al. 2008 [23]	Jiaji (EX-B2), Yaoyangguan (DU 3), Huantiao (GB 30), and Yanglingquan (BL 67)
Hu et al. 2010 [24]	Yaoyangguan (DU 3), Shiqizhui (EX-B7), Huantiao (GB 30), Yanglingquan (BL 67), Weizhong (BL 40), and Chengshan (BL 57)
Du et al. 2009 [25]	Jiaji (EX-B2)
Chen 2010 [26]	Jiaji (EX-B2), Zhibian (BL 54), Huantiao (GB 30), Yinmen (BL 37), Weizhong (BL 40), Chengshan (BL 57), and Kunlun (BL 60)
Wang 2008 [27]	Jiaji (EX-B2), Zhibian (BL 54), Weizhong (BL 40), and Yanglingquan (BL 67)
Meng 2014 [28]	Jiaji (EX-B2), Huantiao (GB 30), Juegu (GB 39), Weizhong (BL 40), and Zhibian (BL 54)
Ren 2013 [29]	Dachangshu (BL 25), Shenshu (BL 23), Mingmen (DU 4), Guanyuanshu (BL 26), Qihaishu (BL 24), Zhibian (BL 54), Huantiao (GB 30), and Jiaji (EX-B2)
Zhao 2004 [30]	Huantiao (GB 30), Weizhong (BL 40)

study used Lasegue's sign to assess the effectiveness of the intervention [20]. The time frame of the outcome measures varied from immediately after the first treatment to 6 months after the completion of treatment.

3.2.5. Risk of Bias. All of the included RCTs mentioned randomization and 7 studies reported adequate sequence generation [21–24, 26–28]; 6 trials used a table of random numbers and 1 used SPSS software to create random numbers. Three studies provided details about appropriate allocation concealment [21, 22, 26], but the related details of the remaining RCTs were unclear even after contacting the authors. Only 3 trials in the review were considered to have a low risk of bias for outcome assessors blinding [23, 26, 30]. Because of the nature of acupuncture, none of the included RCTs blinded the acupuncturists and the patients. One RCT reported 6 drop-outs but did not provide any explanation of the reasons for this [23].

3.3. Effects of Acupuncture. The key results from the included trials are summarized in Figures 2–5.

3.3.1. Acupuncture versus Drugs

Visual Analogue Scale (VAS). In terms of pain intensity related to leg/lumbago pain, 4 studies involving 222 participants contributed VAS data for meta-analysis [20, 22, 25, 26]. Meta-analysis of 4 RCTs showed considerable heterogeneity (I^2 = 66%) between the results of the included trials; we explored this heterogeneity by excluding the trial with the longest acupuncture sessions (four weeks, which was twice as long as the others). With this trial excluded, the statistical heterogeneity was reduced (I^2 = 0%). After pooling, the data showed that acupuncture might have a better effect on pain relief than conventional medication (3 trials, 160 participants, MD −1.23, 95% CI −1.87 to −0.60, and I^2 = 0%) (Figure 2).

Assessment of the Straight Leg Raising Test. One study used the straight leg raising test to evaluate the effect of acupuncture and medication [20]; according to the trial, after one treatment session straight leg raising improved in both groups. While the acupuncture group improved more than the medication group, the researchers concluded that electroacupuncture was more effective than NSAIDs (diclofenac) for increasing Lasegue's sign angles (the angle of Lasegue's sign, 76.70 ± 1.63 versus 70.88 ± 2.11).

6-Point Behavioural Rating Scale (BRS-6). One study [22] found that the acupuncture arm might be more effective than medication in terms of the BRS-6 score (2.07 ± 1.05 versus 2.70 ± 1.34).

MOS Item Short Form Health Survey (MOS SF-36). One study used the MOS SF-36 [26]. There was a statistically significant difference between acupuncture and medication in reducing the SF-36 score (57.76 ± 15.20 versus 69.07 ± 15.08).

Japanese Orthopaedic Association (JOA) Score. One study used the JOA score [25]. There was a statistically significant difference between acupuncture and medication in increasing the JOA score (20.16 ± 3.55 versus 17.63 ± 3.23).

Global Assessment. In terms of global assessment, 6 studies involving 578 participants used global assessment as the outcome measure [21–24, 26, 27]. Data analysis showed that the patients in the acupuncture group improved more significantly after the end of the sessions than those in the medication group (6 trials, 578 participants, RR 1.21, 95% CI 1.12 to 1.30, and I^2 = 0%) (Figure 3). Although these 6 studies included sciatica of the nerve trunk and sciatica of the nerve roots and although the meta-analysis showed no heterogeneity, we still feel that the results may have been influenced by different types of sciatica. Thus, to evaluate the efficacy of acupuncture for different types of sciatica, a subgroup analysis was conducted according to our predesigned protocol; pooling the data of these studies showed that, for sciatica of the nerve roots [22–24, 27], the therapeutic effect of acupuncture was significantly better than drugs (4 trials, 474 participants, RR 1.08, 95% CI 1.02 to 1.14, and I^2 = 0%), and for sciatica of the nerve trunk [21, 26], acupuncture can provide symptom relief (2 trials,

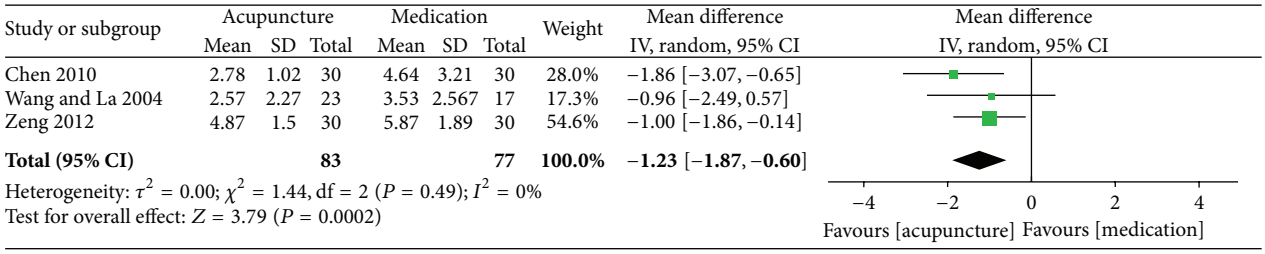

Figure 2

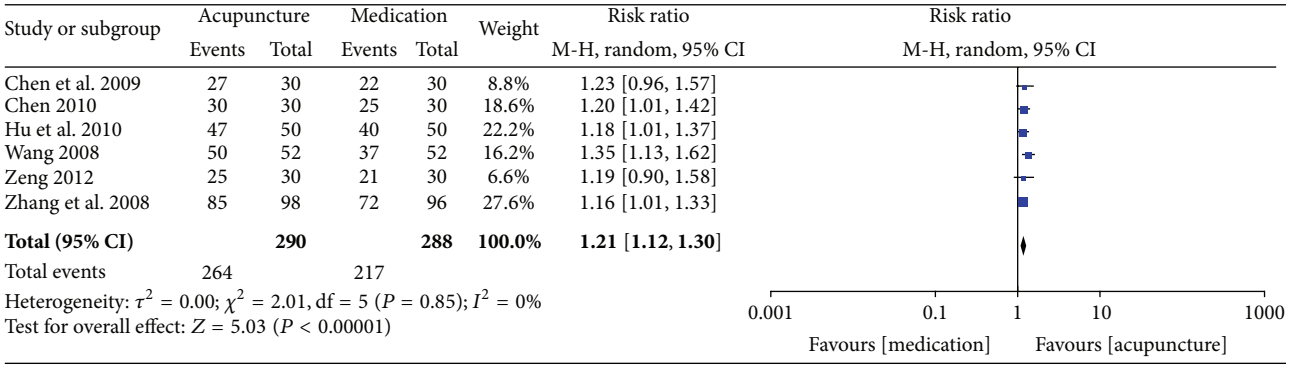

Figure 3

104 participants, RR 1.21, 95% CI 1.05 to 1.39, and $I^2 = 0\%$) (Figure 4).

3.3.2. Acupuncture versus Sham Acupuncture

Global Assessment. One study [30] reported that acupuncture provided more improvement in global assessment than sham acupuncture (29/30 versus 22/30).

3.3.3. Acupuncture Plus Drugs versus the Same Drugs

Pain Intensity. One study [28] involving 60 participants reported that acupuncture plus medication was significantly more effective than medication alone in providing pain relief (pain intensity on VAS; 3.04 ± 0.53 versus 4.82 ± 0.62) after two acupuncture treatment sessions.

Global Assessment. Two studies [28, 29] reported that acupuncture plus conventional medication provided significantly more improvement than conventional medication alone (2 studies, 87 participants, RR 1.77, 95% CI 1.29 to 2.45, and $I^2 = 37\%$) (Figure 5).

Adverse Effects. Three of 11 trials reported adverse effects [23, 24, 26]. In one trial, patients in the acupuncture group reported 3 cases of hypodermal bleeding while 21 patients in the medication group reported gastrointestinal problems including nausea, stomach ache, dyspepsia, and headache [23]. In another trial, no adverse events were reported in the acupuncture group while 5 patients in the medication group reported gastrointestinal problems [24]. One trial reported 2 adverse events in the acupuncture group and no adverse events in the control group [26]. Although acupuncture appears to be associated with few adverse effects, the evidence is limited.

4. Discussion

4.1. Summary of Main Results. Sciatica affects many people and is a common reason for seeking medical advice. It has considerable economic consequences in terms of health care resources and lost productivity [6, 37]. In this systematic review, although we made an extensive literature search, because of language barriers and the predefined inclusion criteria, only 11 studies of acupuncture for sciatica were eligible for our systematic review and meta-analysis. After combining 3 RCTs [20, 22, 26], the results of the meta-analysis showed that acupuncture may be more effective than NSAIDs (ibuprofen, meloxicam, and diclofenac) in decreasing the VAS for leg pain/lumbago, (3 trials, 160 participants, MD −1.23, 95% CI −1.87 to −0.60, and $I^2 = 0\%$) and 1 RCT concluded that acupuncture plus an NSAID (ibuprofen) was superior to the same NSAIDs alone (pain intensity on VAS; 3.04 ± 0.53 versus 4.82 ± 0.62) [28]. Although one prior systematic review reported that no evidence exists for NSAIDs being superior to placebo [38], NSAIDs were still suggested for pain control by the clinical guideline for diagnosis and treatment of sciatica from the Dutch College of General Practice [39]. In addition, VAS pain intensity score is the primary outcome of interest in sciatica; the score in the acupuncture group was significantly lower than that in the NSAIDs group (pooled MD = 1.23), but, considering that the sample sizes of the included trials were small, it is difficult

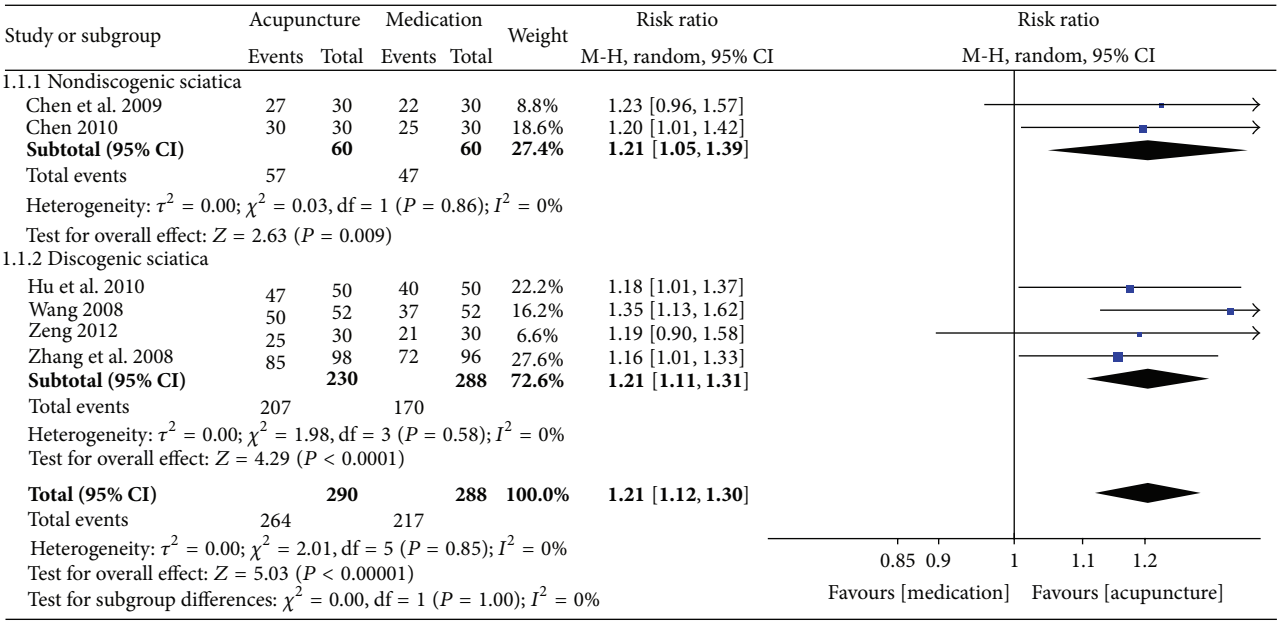

Figure 4

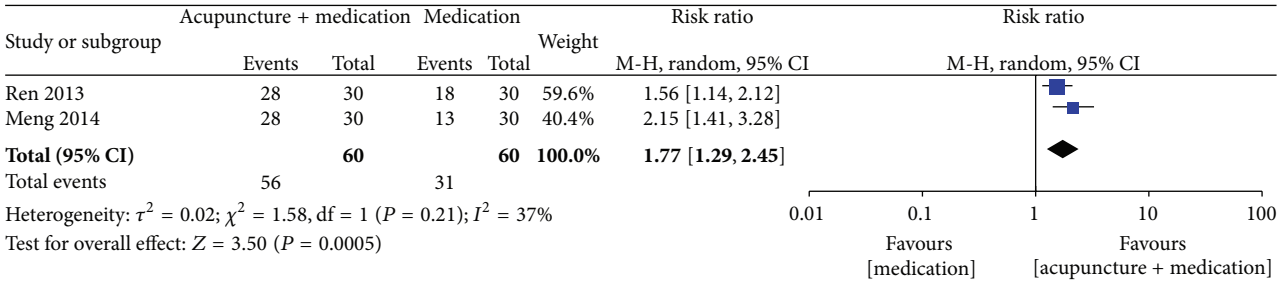

Figure 5

to draw conclusions. Moreover, there was sparse information in these RCTs regarding the processes of randomization and allocation concealment, and only 3 of the RCTs blinded the statisticians [23, 26, 30], which may have led to a considerable risk of bias. Therefore, the present findings suggest that acupuncture may be more effective than NSAIDs in relief of leg pain/lumbago, but the evidence is limited. In addition, compared with medication, acupuncture appears to be more effective regarding physical signs, motor function, or quality of life measured by other scales such as the JOA, BRS-6, SF-36, and Lasegue's sign. However, because these 4 trials reported the outcomes separately [20, 22, 25, 26] and meta-analysis was not possible for one trial, and taking into account the small sample sizes of the included trials, it was difficult to make robust conclusions.

In terms of global assessment, the combined results of 6 RCTs showed that acupuncture was superior to medication in improving global assessment (6 trials, 578 participants, RR 1.21, 95% CI 1.12 to 1.30, and $I^2 = 0\%$) [21–24, 26, 27], and acupuncture plus medication was better than the same medication alone (2 studies, 87 participants, RR 1.77, 95% CI 1.29 to 2.45, and $I^2 = 37\%$) in improving the global assessment [28, 29]. It is important to explain that we chose global assessment as the primary outcome of interest in our published protocol [18]; however, most of the included RCTs used "Criteria of diagnosis and therapeutic effect of diseases and syndromes in traditional Chinese medicine" to report outcomes on the basis of an ordinal assessment ("cured," "improved," and "failed"). This makes it difficult to evaluate and save global assessment as the primary outcome; hence we redesigned global assessment to be one of the secondary outcomes. Compared with sham acupuncture (2 inches from the real acupuncture point), 1 RCT suggested that real acupuncture may be more effective in global assessment (29/30 versus 22/30) [30]. Meta-analysis was impossible for a single trial with a small sample size; therefore, it is difficult to draw a conclusion without powerful evidence. However, the results may suggest that the treatment of acupuncture points may be relatively specific for sciatica.

Acupuncture appears to be associated with fewer adverse effects compared with NSAIDs. Six of the included 11 RCTs mentioned adverse events and only 2 of them reported

adverse events in the acupuncture group (5 cases of hypodermal bleeding) [23, 26]. Therefore acupuncture is safe for treating patients with sciatica. Even though acupuncture is associated with adverse effects such as hypodermal bleeding, in contrast to the gastrointestinal adverse effects associated with NSAIDs, acupuncture might be an option method for patients who cannot tolerate the adverse effects to the digestive system. More information is needed to better evaluate the adverse effects of the two interventions.

Given the characteristics of sciatica, the presence of inflammation and well-established nociceptive pathways may necessitate a threshold dose or duration of acupuncture treatment prior to clinical effect [38, 40]. This is supported by pathophysiologic and anatomic studies illustrating how the sustained nociceptive input caused by sciatica can have profound effects on the central nervous system, causing pathologic neuroplastic changes. The controlled stimulation of peripheral nociceptors with acupuncture may reverse such pathologic neuroplasticity in the central nervous system, especially when administered over a prolonged period [40].

The quality of trials is not sufficiently high and efforts to improve trial reporting are necessary; subsequent trials should comply with the CONSORT statement and STRICTA recommendations [32, 40]. Outcome measures should not be confined to global assessment. VAS, NRS, quality of life and mobility function, and follow-up should also be addressed in the future trials. As a prior published systematic review related to acupuncture reported that the cost effectiveness is another insufficiently researched aspect of acupuncture RCTs [41], the above issues should be taken into consideration to allow clinicians and patients to make evidence-based treatment decisions.

4.2. Applicability of Evidence. In this systematic review, 2 of the included trials were multicentre in nature [20, 23]; the other trials were of small sample size and most of the trials had poor methodological quality, lacking details regarding blinding and allocation concealment. The majority of the included trials used global assessment to measure outcomes and interventions varied greatly in terms of the acupuncture intervention methods, treatment periods, and the location of acupuncture points; the statistical results may have varied. In addition to the different acupuncture intervention methods, we also must take variations in the area of medications into consideration. Although most of the conventional medication was NSAIDs, variations in the effects of NSAIDs cannot be ignored.

4.3. Limitations of This Review. This review may be limited by the inherent methodological limitations of the included RCTs.

We chose to consider acupuncture treatment regardless of the frequency of administration, duration of each session, and number and location of acupoints in our published protocol. Any of these variables may have influenced the effects of acupuncture.

Because of the language barrier, we were unable to include other trials that may have met our inclusion criteria.

5. Conclusion

In conclusion, the results of this systematic review suggest that the use of acupuncture may more effectively relieve leg pain/lumbago and improve global assessment of sciatica when compared with NSAID (ibuprofen, meloxicam, and diclofenac) treatment. Moreover, adjuvant acupuncture may enhance the effect of medications in leg pain/lumbago relief. To patients, acupuncture points appear more effective than nonacupoints. Acupuncture is relatively safe and is rarely associated with serious adverse events in patients with sciatica. However, this meta-analysis was lacking in relevant and rigorous RCTs. Because the evidence was limited, higher quality and more rigorously designed clinical trials with larger sample sizes will be needed to further confirm our findings.

Disclosure

The data used in this systematic review was not individual data and there were no privacy issues to address.

Conflict of Interests

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

Authors' Contribution

Zhishun Liu and Zongshi Qin contributed to the conception of the study. The paper was drafted by Zongshi Qin and revised by Zhishun Liu. The search strategy was developed by Yanbing Zhai and Xiaoxu Liu and updated by Jiani Wu. Zongshi Qin and Xiaoxu Liu independently screened the potential studies, extracted data from the included studies, and completed the data synthesis. Jiani Wu and Yanbing Zhai assessed the risk of bias. Zhishun Liu arbitrated in cases of disagreement and ensured the absence of errors. All authors contributed to the interpretation of the results and gave their final approval for the version to be published.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 129103, 8 pages
http://dx.doi.org/10.1155/2015/129103

Research Article

Protective Effect of Tetrandrine on Sodium Taurocholate-Induced Severe Acute Pancreatitis

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Received 11 June 2015; Accepted 20 September 2015

Academic Editor: Musa T. Yakubu

Tet is a type of alkaloid extracted from *Stephania tetrandra*, and it has recently been demonstrated that Tet can protect against inflammation and free radical injury and inhibit the release of inflammatory mediators. The present study was designed to observe the protective effect of Tet on sodium taurocholate-induced severe acute pancreatitis (SAP). The rat model of SAP was induced by retrograde bile duct injection of sodium taurocholate and then treated with Verapamil and Tet. The results showed that Tet can reduce NF-κB activation in pancreas issue, inhibit the SAP cascade, and improve SAP through inducing pancreas acinar cell apoptosis and stabilizing intracellular calcium in the pancreas, thus mitigating the damage to the pancreas. Our study revealed that Tet may reduce systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS) to protect against damage, and these roles may be mediated through the NF-κB pathway to improve the proinflammatory/anti-inflammatory imbalance.

1. Introduction

Acute pancreatitis (AP) is an acute abdominal condition. There are two types of AP, mild and severe. Mild AP, which accounts for 80% of the cases of AP, is self-limiting. However, approximately 20% of the cases of AP are severe [1]. Severe acute pancreatitis (SAP) is a life-threatening condition with a high mortality rate that progresses rapidly and is associated with many complications. The pathological features of SAP include extensive pancreatic hemorrhage and large areas of necrotic tissue [2]. Clinically, SAP is often accompanied by systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), acute lung injury (ALI), acute renal insufficiency, and hepatic impairment. Multiple organ dysfunction syndromes (MODS) can develop in the later stages of SAP, and uncontrolled SAP can ultimately cause multiple organ failure (MOF) [3]. The mortality rate for SAP is as high as 20% to 30% [4].

Currently, there are no effective medications for treating SAP. The main therapeutic approaches for this disease are symptomatic treatments, which include gastric decompression, the provision of pain relief, and the correction of fluid, electrolyte, and pH balances [5]. Many researchers are screening active ingredients from traditional Chinese medicine to assess the potential of these ingredients to treat SAP [6–8]. Tetrandrine (Tet), a monomer extracted from Radix Stephaniae Tetrandrae, is a nonselective calcium channel antagonist [9]. The anti-inflammatory effects of Tet have gradually attracted increasing research attention [10]. Tet has particularly salutary effects with respect to preventing and treating acute and chronic inflammation, inhibiting the release of inflammatory mediators, regulating inflammatory cell function, and protecting against free radical damage [11].

Given the significant anti-inflammatory effects of Tet, we intend to utilize this drug for the treatment of SAP. In

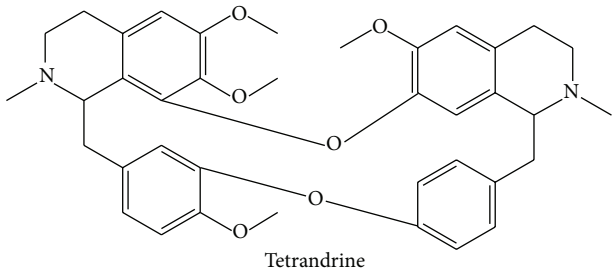

Figure 1: Chemical structure of Tetrandrine ($C_{38}H_{42}N_2O_6$).

this study, a stable animal model of SAP and SAP-associated organ damage was established. Tet intervention was then utilized to observe the protective effects of Tet treatment against SAP and SAP-related organ damage as well as the possible mechanisms of these effects.

2. Materials and Methods

2.1. Drugs. Tetrandrine and standard substance (molecular formula: $C_{38}H_{42}N_2O_6$) were purchased from Institute of Chinese Materia Medica, Nanjing (Figure 1).

2.2. Animal Groups. A total of 120 male specific pathogen-free (SPF) Sprague-Dawley (SD) rats were randomly divided into five groups: the normal group (the N group), the sham-operation group (the S group), the SAP model group (the M group), the Tet treatment group (the Tet group), and the Verapamil (Ver) treatment group (the Ver group). Random allocation was used to divide each group into three subgroups (for the 3 h, 6 h, and 12 h time points), with $n = 8$ for each subgroup.

2.3. Animal Modeling and Drug Dosing. The experimental rats were anesthetized with a 3 mL/kg intraperitoneal injection of 10% chloral hydrate. After the abdominal cavity was exposed, the intestine along the mesenteric border of the duodenum was punctured with a 3.5-gauge flat needle. This needle was retrogradely inserted approximately 0.5 cm into the ampullary opening of the biliopancreatic duct and used to inject a 1 mL/kg dose of 5% sodium taurocholate into this duct at a rate of 0.2 mL/min. Following this injection, a small artery clamp was used to occlude the duct for 2 minutes; subsequently, the punctures in the duodenal wall were sutured, and the surgical incision was closed with a double layer of stitching. The pancreas and duodenum treatments were switched in the S group. After the operation, the rats in each group were subcutaneously injected with 30 mL/kg of 0.9% NaCl. The rats were provided with water ad libitum after surgery [12].

In accordance with the results of preliminary experiments, after the SAP model had been induced, intraperitoneal injections of 40 mg/kg Tet and 1 mg/kg Ver were administered to rats in the Tel and Ver groups, respectively, and rats in the M group received an intraperitoneal injection of an equal volume of 0.9% NaCl. Rats in each group received a postoperative 30 mL/kg subcutaneous injection of 0.9% NaCl and were provided with water ad libitum after surgery. The three observation time points of 3 h, 6 h, and 12 h were established.

2.4. Detecting Wet/Dry Weight Ratios and Pathological Scores for Pancreatic Tissue. The wet weight of the body of the pancreas of each rat was determined. The dry weight of each of these pancreas samples was determined by incubating the samples in a 60°C oven for 72 hours until constant weights were attained. These two types of weights were then used to calculate wet/dry weight ratios for pancreatic tissue. Pancreatic tissue samples were also obtained for paraffin sectioning and hematoxylin-eosin (HE) staining. Appropriate histological scoring methods were adopted to determine pathological scores, with higher pathological scores indicating more severe tissue damage.

2.5. Detecting Serum Amylase Activity and the Myeloperoxidase (MPO) Activity of Pancreatic Tissue Homogenates. Using an automatic biochemical analyzer (Hitachi 7600), α-amylase (AMY) levels in the peripheral blood were measured in accordance with the procedures specified by an AMY activity assay kit. A colorimetric assay was used to determine MPO activity in pancreatic tissue homogenates. To eliminate edema-related effects, the MPO activity per gram of dry tissue was adopted as a measure that could relatively accurately represent the extent of neutrophil infiltration. In particular, the following equation was utilized: MPO units/g dry tissue = MPO units/g wet tissue × wet/dry weight ratio of the tissue.

2.6. Detecting Apoptosis and Necrosis in Pancreatic Acinar Cells. Single-cell suspensions of pancreatic acinar cells were prepared. To detect apoptosis in these suspensions, fluorescein isothiocyanate- (FITC-) labeled annexin V was used as a probe for flow cytometry and fluorescence microscopy observations; this approach relies on the fact that annexin V specifically binds to phosphatidylserine (PS), which translocates to the outer leaflet of the cell membrane during apoptosis. The nucleic acid dye propidium iodide (PI) can pass through the cell and nuclear membranes of cells in the middle and late stages of apoptosis, marking these cells with red fluorescence. The combined use of annexin V and PI can therefore successfully differentiate between early apoptotic cells, late apoptotic cells, and necrotic cells.

2.7. Detecting Calcium Ion Levels within Pancreatic Acinar Cells and the Transporter Activation of Nuclear Factor-Kappa B (NF-κB) in Pancreatic Tissue. Single-cell suspensions of pancreatic acinar cells were prepared, and the cell density of these suspensions was adjusted to 5×10^8 cells/L. A 2 mL sample of pancreatic acinar cell suspension was obtained, Fluo-2/AM was added to a final concentration of 5 μmol/L, and the resulting sample was placed in a 37°C water bath and incubated in the dark for 30 to 45 minutes. Flow cytometry was then used for the detection of fluorescence. To assess NF-κB activity, pancreatic tissue was immersed in a 4%

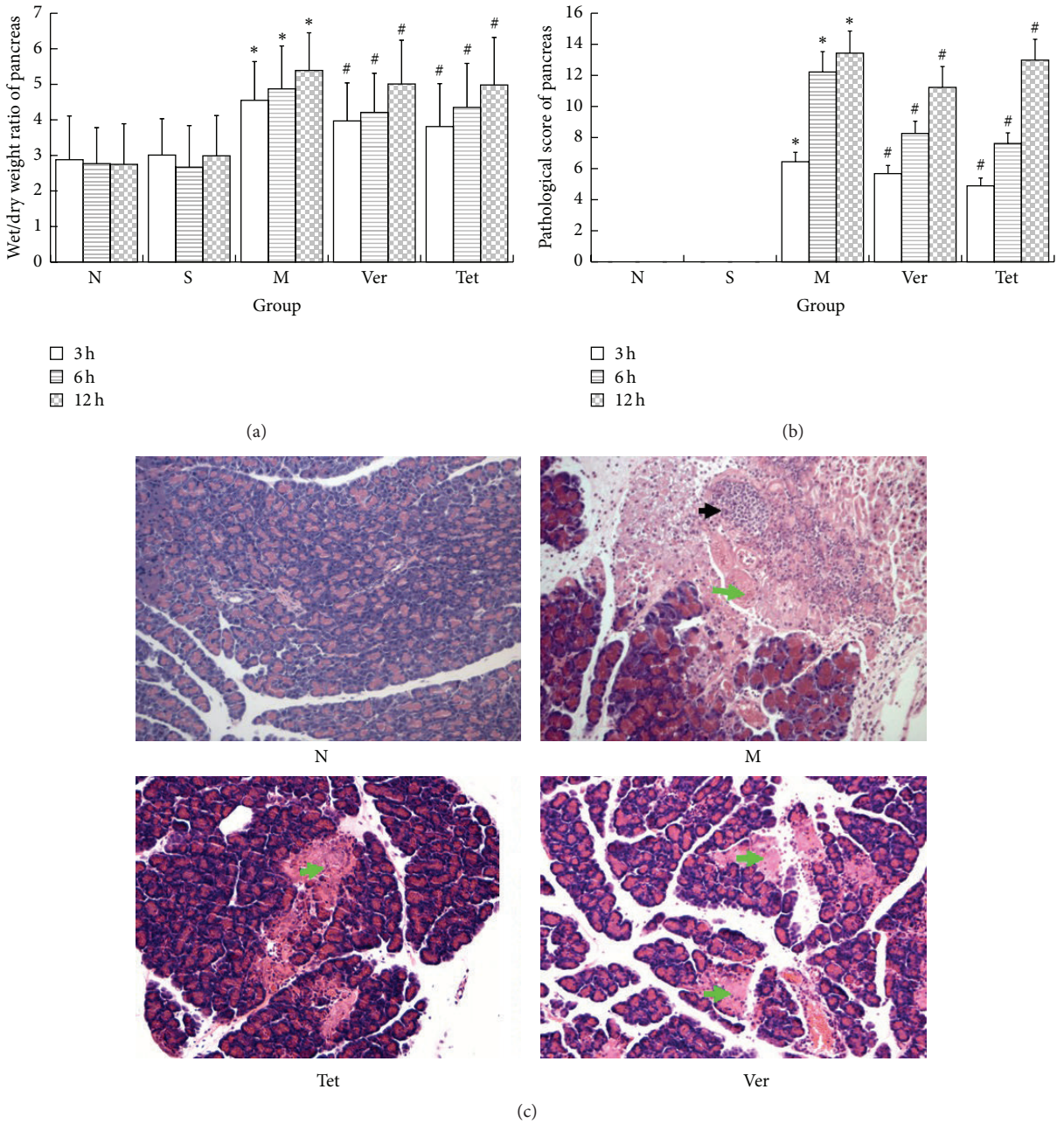

FIGURE 2: Pathological sections and scores of pancreatic tissues. (a) Chart comparing the wet/dry weight ratios of pancreatic tissue. The wet/dry ratios decreased significantly after therapeutic intervention; (b) pathological scores of pancreatic tissue samples. The pathological scores of the pancreas were significantly increased after model was established, whereas the pathological scores were significantly lower in Tet treatment group; (c) microscopic images of pathological sections of pancreatic tissue (100x). The structure of pancreatic cells was integrated in normal group, and the intercellular space was normal, and there were no signs of either hemorrhagic necrosis or the infiltration of inflammatory cells. In other groups, lobular spaces widened, acinar hemorrhage and edema were evident, large areas of necrosis were present, the parenchymal and interstitial infiltration of numerous inflammatory cells occurred, and fat necrosis lesions could be observed. However, the aforementioned phenomena were somewhat less severe in the Tet groups ($^{*}P < 0.05$ versus normal group; $^{\#}P < 0.05$ versus model group).

paraformaldehyde solution (at 4°C). Frozen sections were prepared after conventional embedding and fixing procedures were performed. NF-κB was measured in accordance with the procedures provided in an NF-κB activation assay kit. The following formula for the NF-κB activation rate was utilized for statistical analysis: NF-κB activation rate = (Total number of positive cells)/(Total number of cells) × 100%.

2.8. Statistics. All the data are expressed as the means ± SEM. The means of different groups (same times) were compared

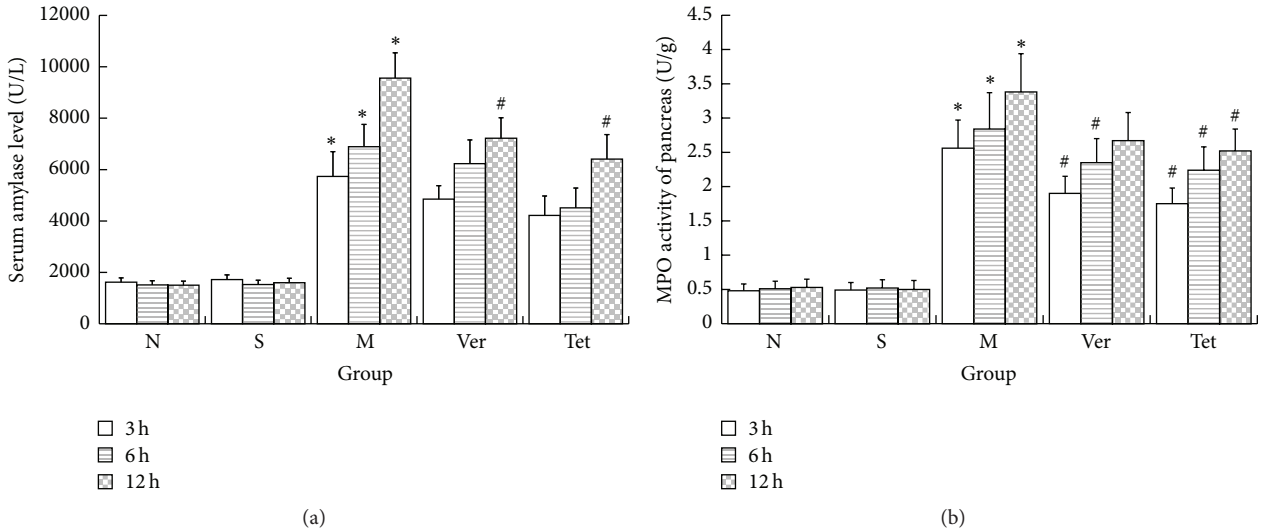

Figure 3: Serum amylase level and pancreatic MPO activity. (a) Serum amylase level. After the establishment of the SAP model, serum amylase was significantly elevated in the M group relative to the N and S groups but was lower in the Ver and Tet intervention groups than in the M group; (b) MPO activity of pancreatic tissue homogenates. After the establishment of the SAP model, the MPO activity of the pancreatic tissue homogenates was significantly elevated in the M group relative to the N and S groups but was lower in the Ver and Tet intervention groups than in the M group ($^{*}P < 0.05$ versus normal group; $^{\#}P < 0.05$ versus model group).

using a one-way analysis of variance (ANOVA). Differences were assumed to be significant at $P < 0.05$. The data were ranked using a nonparametric rank sum test, and the death rates of different groups were compared using fourfold table chi-square test.

3. Results

3.1. Pathological Changes in Pancreatic Tissue and the Pathological Scores of Each Examined Organ. In the S group, no significant changes in pancreatic tissue at any time point were observed. In the M group, edema, hemorrhage, inflammatory cell infiltration, and necrosis occurred in pancreatic tissue. These changes were accompanied by corresponding increases in the wet/dry weight ratios and pathological scores of the pancreatic tissue. Compared with the M group, the Ver and Tet groups exhibited reduced pathological changes and lower pathological scores (Figure 2).

3.2. Determinations of Serum Amylase and MPO Activity in Pancreatic Tissue Homogenates. In the S group, both serum amylase activity and MPO activity remained stable at each examined time point. Serum amylase activity was significantly higher in the M group than in the S group at each time point after the SAP model had been successfully induced. Serum amylase activity and MPO activity gradually increased over time. At each examined time point, serum amylase activity and MPO activity were lower in the Ver and Tet groups than in the M group. These results demonstrated that serum amylase activity and pancreatic MPO activity decreased after Tet or Ver treatments, suggesting that Tet and Ver can protect pancreatic tissue by alleviating pancreatic injury and reducing neutrophil infiltration (Figure 3).

3.3. The Detection of Pancreatic Acinar Cell Apoptosis and Necrosis. In the S group, apoptosis was observed to a certain extent among pancreatic acinar cells at each examined time point; however, the apoptosis rate was not high, and no significant necrosis was observed. In the M, Ver, and Tet groups, both apoptotic and necrotic pancreatic acinar cells were present at each time point. Moreover, at each time point, there were significantly higher numbers of apoptotic and necrotic cells in each of these three groups than in the S group. The number of apoptotic and necrotic cells increased over time in the M, Ver, and Tet groups. Relative to the M group, the Ver and Tet groups had significantly more apoptotic cells but significantly fewer necrotic cells at each time point. These findings suggested that, among pancreatic acinar cells in cases of SAP, the Tet and Ver treatments significantly reduced necrosis, resulting in an increased number of apoptotic cells and surviving cells. Thus, in the context of SAP, Tet and Ver can alleviate pancreatic necrosis and promote cellular apoptosis (Figure 4).

3.4. The Detection of Calcium Ion Levels in Pancreatic Acinar Cells. The fluorescence intensities of calcium ions within pancreatic acinar cells were greater in the Ver and Tet groups than in the S group at each time point, and these fluorescence intensities increased over time. The fluorescence intensities of calcium ions were lower in the Ver and Tet groups than in the M group at each time point. The differences in these intensities between the Ver group and the Tet group at each time point were not significant. These results demonstrated that, in the context of SAP, the fluorescence intensity of calcium ions within acinar and other pancreatic cells and pancreatic acinar cells was significantly reduced after receiving Tet or Ver treatment, suggesting that Tet and Ver could stabilize

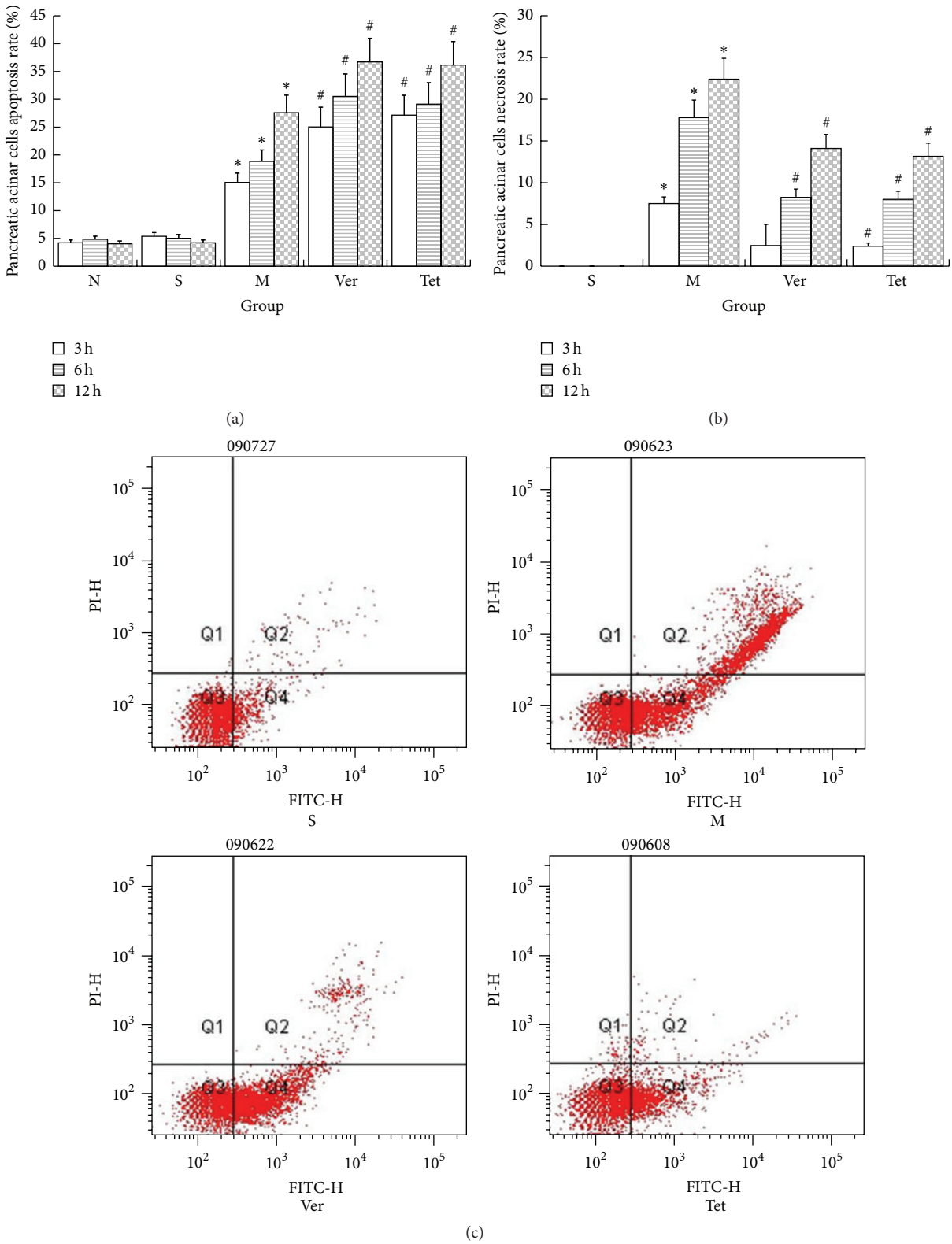

Figure 4: Apoptosis and necrosis of pancreatic acinar cells. (a) Apoptosis rates of pancreatic acinar cells. The apoptosis rates of pancreatic acinar cells were significantly higher in the M group than in the N and S groups. The groups treated with Ver or Tet exhibited higher apoptosis rates than the M group; (b) necrosis rates of pancreatic acinar cells. Necrosis of pancreatic cells was clearly evident after the induction of the SAP model, and necrosis rates were lower among cells treated with Ver or Tet than among untreated cells; (c) flow cytometry plots of acinar cell apoptosis ($^{*}P < 0.05$ versus normal group; $^{\#}P < 0.05$ versus model group).

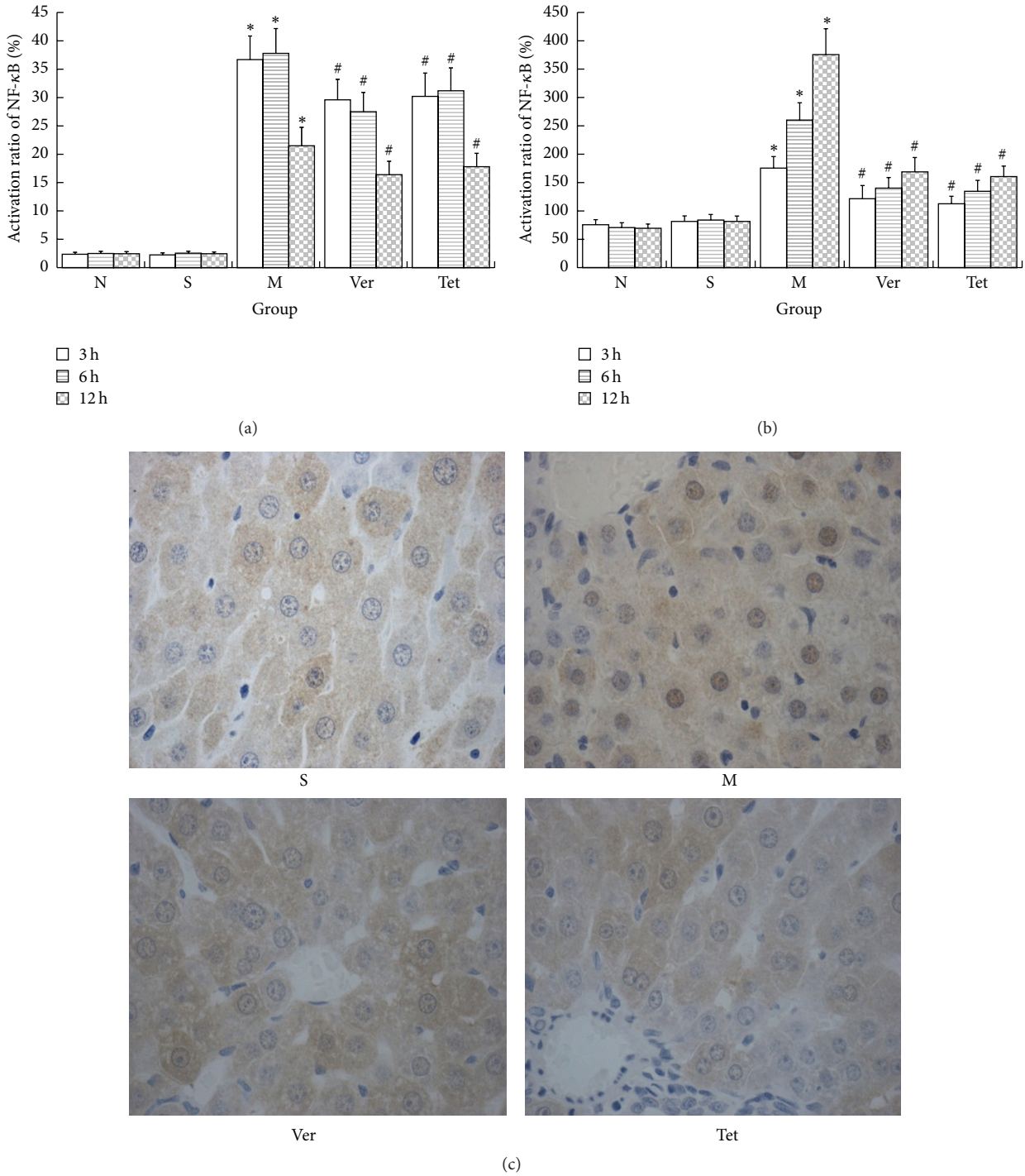

Figure 5: Calcium ion levels and NF-κB transporter activation rates in pancreatic tissue and liver. (a) NF-κB translocation rates in pancreatic tissue. TNF-κB transporter activation rates in pancreatic tissue were lower in SAP rats treated with Ver or Tet than in untreated SAP rats; (b) calcium ion levels in liver. Calcium ion levels were lower in SAP rats treated with Ver or Tet than in untreated SAP rats. (c) Histological images indicating NF-κB transporter activation in pancreatic tissue ($^{*}P < 0.05$ versus normal group; $^{\#}P < 0.05$ versus model group).

the intracellular calcium ion levels of acinar cells, reducing calcium overload and mitigating the damage to pancreatic cells.

3.5. The Detection of NF-κB Transporter Activation in Pancreatic Tissue. In the S group, NF-κB transporter activation was maintained at stable, low levels throughout the experiment, and activation rates were less than 3%. Within this group, the activation rate at each time point was not significantly different. NF-κB transporter activation rates were significantly higher in the M, Ver, and Tet groups than in the S group at each time point. This rate reached a peak during the 3–6 h

time period and subsequently decreased. NF-κB transporter activation rates were lower in the Ver and Tet groups than in the M group, suggesting that Tet or Ver treatment for SAP significantly decreased these rates in pancreatic tissue. This finding indicated that Tet and Ver can inhibit NF-κB transporter activation in pancreatic tissue in the context of SAP (Figure 5).

4. Discussion

Tet, which is also known as Tetrandrine hormone, has a wide range of pharmacological effects. (1) Ca^{2+} antagonism: Tet can act as a calcium antagonist by inhibiting extracellular Ca^{2+} influx, regulating the distribution of intracellular Ca^{2+}, and maintaining intracellular Ca^{2+} homeostasis [13]. (2) Immunosuppressive effects: Tet can inhibit IκB (inhibitor of kappa B) degradation, inhibiting NF-κB transporter activation and the expression of NF-κB-dependent genes. It can thereby significantly reduce the generation of the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Moreover, Tet inhibits the synthesis of antibodies by mitogen-stimulated B cells, promotes lymphocyte proliferation, and influences natural killer (NK) cell-mediated cytotoxicity [14]. (3) Tet acts as a free radical scavenger. Tet also inhibits the production of platelet activating factor, interleukin-1 (IL-1), prostaglandin (PG), and other inflammatory cytokines, thereby hampering the orientation, chemotaxis, and phagocytic activity of inflammatory cells and producing significant anti-inflammatory effects [15]. Therefore, in theory, Tet can interfere with many different aspects of the pathological process of AP; for example, Tet could affect initiating factors of AP, produce anti-inflammatory responses, and improve microcirculation, among other possibilities. Few studies have examined the use of Tet for the treatment of SAP. However, research has determined that, in rats, Tet can inhibit inducible nitric oxide synthase (iNOS) activity, reducing NO production and thereby significantly alleviating edema, hemorrhage, necrosis, and inflammatory responses in cases of acute hemorrhagic and necrotizing pancreatitis [16].

After the SAP model was induced, relative to the M group, the Tet group exhibited lower serum amylase levels, wet/dry weight ratios of pancreatic tissue, pathological scores for pancreatic tissue, and MPO activity levels in pancreatic tissue homogenates. These results indicated that, in the context of SAP, Tet can decrease amylase activity; mitigate pancreatic edema, neutrophil infiltration, and pancreatic injury; reduce the production and release of inflammatory mediators; inhibit the inflammatory response; improve inflammatory immune imbalances; and alleviate pancreatic injury. Thus, through these various effects, Tet can improve the condition of SAP patients. Furthermore, relative to the M group, the Tet group exhibited lower NF-κB transporter activation levels in pancreatic tissue, an increased number of apoptotic pancreatic acinar cells, fewer necrotic cells, and a greater number of surviving cells. These findings suggested that Tet can inhibit pancreatic NF-κB transporter activation, stabilize the intracellular calcium ion levels of pancreatic acinar cells, increase apoptosis among pancreatic cells, reduce necrosis among pancreatic cells, and increase cell survival. Thus, Tet could potentially intervene to ameliorate SAP-related organ damage by affecting multiple targets.

Conflict of Interests

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

Authors' Contribution

Xian-lin Wu and Jie-xing Li contributed equally to this paper.

Acknowledgments

This work was supported by the Fundamental Research Funds for the Central Universities (no. 21614306), National Natural Science Foundation of China (no. 81403302), and Natural Science Foundation of Guangdong province (no. 2014A030310477).

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