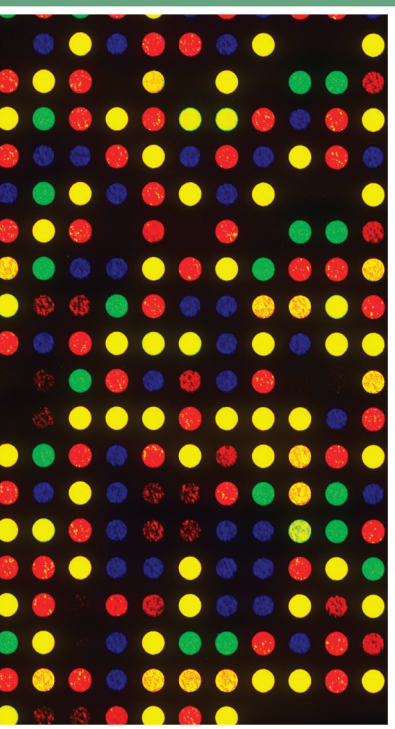
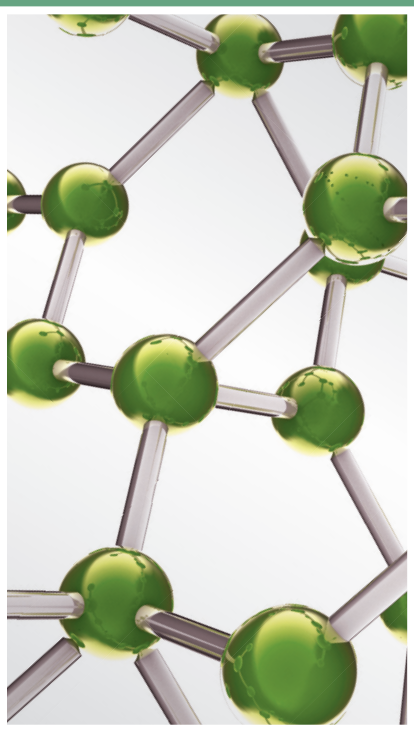
eCAM Evidence-Based Complementary and Alternative Medicine

Evidence Based Alternative Medicines in Pain Management 2016

Guest Editors: Haroon Khan, Vincenzo De Feo, Najeeb Ur Rehman, and Agnieszka Najda






Evidence Based Alternative Medicines in Pain Management 2016

Evidence Based Alternative Medicines in Pain Management 2016

Guest Editors: Haroon Khan, Vincenzo De Feo, Najeeb Ur Rehman, and Agnieszka Najda

Editorial Board

Contents

Evidence Based Alternative Medicines in Pain Management 2016
Haroon Khan, Vincenzo De Feo, Najeeb Ur Rehman, and Agnieszka Najda
Volume 2016, Article ID 7078351, 2 pages

Antinociceptive Activity of *Zanthoxylum piperitum* DC. Essential Oil
Graciela Rocha Donald, Patrícia Dias Fernandes, and Fabio Boylan
Volume 2016, Article ID 3840398, 8 pages

TCM Pattern Questionnaire for Lateral Elbow Pain: Development of an Instrument via a Delphi Process
Marcus Gadau, Shi-Ping Zhang, Wing-Fai Yeung, Zhao-Xiang Bian, and Ai-Ping Lu
Volume 2016, Article ID 7034759, 12 pages

MAPK Pathways Are Involved in Neuropathic Pain in Rats with Chronic Compression of the Dorsal Root Ganglion
Yu-Juan Qu, Lei Jia, Xiao Zhang, Hui Wei, and Shou-Wei Yue
Volume 2016, Article ID 6153215, 8 pages

Methanolic Extract of *Clinacanthus nutans* Exerts Antinociceptive Activity via the Opioid/Nitric Oxide-Mediated, but cGMP-Independent, Pathways
Mohammad Hafiz Abdul Rahim, Zainul Amiruddin Zakaria, Mohd Hijaz Mohd Sani, Maizatul Hasyima Omar, Yusnita Yakob, Manraj Singh Cheema, Siew Mooi Ching, Zuraini Ahmad, and Arifah Abdul Kadir
Volume 2016, Article ID 1494981, 11 pages

The Efficacy and Safety of the Combination of Total Glucosides of Peony and Leflunomide for the Treatment of Rheumatoid Arthritis: A Systemic Review and Meta-Analysis
Zhitao Feng, Juan Xu, Guochao He, Meiqun Cao, Lihong Duan, Liguo Chen, and Zhengzhi Wu
Volume 2016, Article ID 9852793, 8 pages

Uliginosin B, a Possible New Analgesic Drug, Acts by Modulating the Adenosinergic System
Eveline Dischkaln Stolz, Paola Fontoura da Costa, Liciane Fernandes Medeiros, Andressa Souza, Ana Maria Oliveira Battastini, Gilsane Lino von Poser, Carla Bonan, Iraci L. S. Torres, and Stela Maris Kuze Rates
Volume 2016, Article ID 5890590, 8 pages

The Effectiveness of Cupping Therapy on Relieving Chronic Neck and Shoulder Pain: A Randomized Controlled Trial
Lee-Mei Chi, Li-Mei Lin, Chien-Lin Chen, Shu-Fang Wang, Hui-Ling Lai, and Tai-Chu Peng
Volume 2016, Article ID 7358918, 7 pages

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 7078351, 2 pages
http://dx.doi.org/10.1155/2016/7078351

Editorial

Evidence Based Alternative Medicines in Pain Management 2016

Haroon Khan,[1] Vincenzo De Feo,[2] Najeeb Ur Rehman,[3] and Agnieszka Najda[4]

[1]*Department of Pharmacy, Abdul Wali Khan University, Mardan 23200, Pakistan*
[2]*Department of Pharmacy, University of Salerno, Fisciano, 84084 Salerno, Italy*
[3]*UoN Chair of Oman's Medicinal Plants and Marine Natural Products, University of Nizwa, Oman*
[4]*Quality Laboratory of Vegetable and Medicinal Materials, Department of Vegetable Crops and Medicinal Plants, University of Life Sciences in Lublin, 58 K. St. Leszczyńskiego Street, 20-068 Lublin, Poland*

Correspondence should be addressed to Haroon Khan; hkdr2006@gmail.com

Received 2 August 2016; Accepted 2 August 2016

Pain is an unpleasant sensation but indeed it is one of the vital alarm systems in human body. It helps in the recognition of various stimuli of intensities that could be potentially harmful to the tissue. In comparison to short-lasting acute pain, chronic pain persists even after the resolution of the initial cause and often loses its protective value, rather producing unwanted effects. Pain of any origin, if it persists, may become resistant to standard treatments, greatly affecting the patients' quality of life. Therefore, advancing our understanding of the pathogenesis of chronic pain is crucial to identify novel therapeutic approaches. There are several therapeutic approaches which are used for the effective management of pain including use of drugs and alternate measures.

The use of medicinal plants in the management of pain is as old as man himself. In this connection, G. R. Donald et al. investigated the antinociceptive activity of *Zanthoxylum piperitum* DC essential oil in various animal models. The oil showed marked antinociceptive effect in formalin-induced acute pain model and glutamate-induced nociception. However, capsaicin-induced nociception and carrageenan induced paw edema were not antagonized by the *Z. piperitum* oil. Similarly, a Malaysian research group studied the antinociceptive effect of methanolic extract of *Clinacanthus nutans* leaves *in vivo*. After producing significant pain reliving effects in acetic acid induced abdominal constriction, formalin-induced paw licking, and hot-plate tests, extract was subjected to the determination of possible mechanism. This antinociceptive activity was fully antagonized by naloxone (a nonselective opioid antagonist) but was partially reversed by L-arginine (L-arg; a nitric oxide [NO] precursor), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; NO synthase inhibitor), or their combinations thereof.

E. D. Stolz et al. evaluated the uliginosin B (ULI) a natural acylphloroglucinol that has been proposed as a new molecular scaffold for developing analgesic and antidepressant drugs. Its effect is generally attributed to its ability to increase monoamines in the synaptic cleft by inhibiting their neuronal uptake without binding to their respective transporters. Additionally, the selective adenosine A receptor antagonist DPCPX and the selective A 2A antagonist ZM-241385 prevented its effect in the hot-plate test in mice. Pretreatment with inhibitors of adenosine reuptake (dipyridamole) or adenosine deaminase (EHNA) did not affect the ULI effect. On the other hand, its effect was completely prevented by an inhibitor of ecto-5′-nucleotidase (AMPCP). This finding was confirmed *ex vivo*, whereby ULI treatment increased AMP and ATP hydrolysis in spinal cord and cerebral cortex synaptosomes, respectively. Thus, the activation of A_1 and A 2A receptors and the modulation of ecto-5′-nucleotidase activity contributed to the antinociceptive to its effect. Y.-J. Qu et al. showed mitogen-activated protein kinases (MAPKs); pathways were involved in neuropathic pain in rats with chronic compression of the dorsal root ganglion. The specific inhibitors of MAPKs contributed to the attenuation of mechanical allodynia CCD rats and the large size MAPKs positive neurons in dorsal root-ganglia were crucial.

The Traditional Chinese medicine (TCM) has developed and used a sophisticated system of individualized medicine in the form of pattern diagnosis and classification for hundreds years. Acupuncture is a centuries old practice in the traditional Chine's system of treatment for pain management. The study used acupuncture treatment for lateral elbow pain (LEP) as an example to study the diagnostic practice of individualized acupuncture treatment. A provisional version of LEP pattern questionnaire was developed based on a recent systematic review on TCM pattern diagnosis for LEP. A Delphi panel of 33 clinical experts from seven different countries was formed in Chinese and English language. Consensus was found on four TCM patterns that could underlie LEP, namely, the *wind-cold-dampness pattern*, the *qi stagnation and blood stasis pattern*, the *dual deficiency of qi and blood pattern*, and the *retained dampness-heat pattern*. A list of signs and symptoms indicating one of the four TCM patterns and a list of preferred treatment modalities for each pattern were also generated.

The systemic review and meta-analysis of Z. Feng et al. on the efficacy and safety of the combination of total glucosides of peony and leflunomide for the treatment of rheumatoid arthritis concluded that the combination of total glucosides of peony and leflunomide in treatment of RA presented the characteristics of notably decreasing the levels of laboratory indexes and higher safety in terms of liver function for the treatment of rheumatoid arthritis. However, this conclusion should be further investigated by increasing the sample size.

Cupping therapy (CT) is a traditional Chinese medical (TCM) treatment which has been practiced for thousands of years. Researchers from Taiwan investigated the effectiveness of cupping therapy (CT) in changes on skin surface temperature (SST) for relieving chronic neck and shoulder pain (NSP) among community residents. A quasi experimental design consists of sixty subjects with self-perceived NSP. The results showed that no participants experienced localized skin burns or adverse reactions in the treatment regions. Two participants in the cupping group reported mild low back pain related to the seated position. In this study, one treatment of CT is shown to increase SST and reduce SBP. In conjunction with the physiological effects, the subjective experience of NSP is reduced. CT mimics an analgesic effect which has no known negative side effects and may be considered safe. However, further studies are required to improve the understanding and potential long term effects of CT.

In conclusion, pain management is still a major clinical problem worldwide. The different articles of this special issue indicated that various researchers around the world are working on both chemical and nonchemical interference for effective pain management in order to get patient compliance. We can also suggest that the combination of drugs with other techniques (without use of drugs) could provide better results in painful conditions.

Haroon Khan
Vincenzo De Feo
Najeeb Ur Rehman
Agnieszka Najda

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 3840398, 8 pages
http://dx.doi.org/10.1155/2016/3840398

Research Article

Antinociceptive Activity of *Zanthoxylum piperitum* DC. Essential Oil

Graciela Rocha Donald,[1] Patrícia Dias Fernandes,[1] and Fabio Boylan[2]

[1]*Federal University of Rio de Janeiro, Institute of Biomedical Sciences, Avenida Carlos Chagas Filho 373, CCS, Laboratory J10, 21941-902 Rio de Janeiro, RJ, Brazil*
[2]*School of Pharmacy and Pharmaceutical Sciences, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland*

Correspondence should be addressed to Fabio Boylan; fabio.boylan@tcd.ie

Received 12 February 2016; Accepted 26 June 2016

Academic Editor: Haroon Khan

Zanthoxylum piperitum DC. (ZP) is a traditional medicinal plant used mainly in countries from Asia such as Japan. This study aimed to investigate the antinociceptive effect of ZP essential oil (ZPEO). The major component present in the essential oil was beta-phellandrene (29.39%). Its antinociceptive activity was tested through animal models (formalin-, capsaicin-, and glutamate-induced paw licking and hot plate). The anti-inflammatory effect was evaluated through the carrageenan-induced leukocyte migration into the subcutaneous air pouch (SAP), with measurement of cytokines. The results showed antinociceptive effect for ZPEO for the first phase of the formalin-induced licking, glutamate, and hot plate tests. However, ZPEO had no effect on reducing paw licking induced by capsaicin. Finally, ZPEO had no effect against inflammation induced by carrageenan.

1. Introduction

Essential oils are naturally occurring complex molecules composed mainly of monoterpenes. They have been used in several industries around the world, especially for cosmetics including beauty creams and perfumes due to their pleasant scents. Essential oils have also been used to treat several diseases and some of them have been tested for medicinal purposes, such as treating pain and inflammation [1].

Known in Japan as Asakura sansho, *Zanthoxylum piperitum* (ZP) first attracted the attention of researchers due to its aroma [2, 3]. Later, the research became more focused on its antioxidative effect resulting in very positive outcomes contributing to the cosmetic industry. In 2001, Hashimoto et al. [4] reported the ability of an aliphatic acid from ZP in inducing relaxation in the circular muscle of the gastric body.

Perhaps due to its promising results as an antioxidant, ZP was also tested for anti-inflammatory activities targeting nitric oxide and cytokines production. This treatment was made from fresh and dried fruits of ZP and showed an inhibitory effect on cytokines (TNF-α and IL-1β) production from mouse macrophage cells [5]. The anti-inflammatory effect correlated with the production of nitric oxide was also described after testing ZPEO. The essential oil also had an effect on reduction of cyclooxygenase-2 expression and activity. Later, a glycoprotein (24 kDa) was isolated from ZP fruits to investigate its anti-inflammatory potential. It was shown to suppress cytokines IL-1β, IL-6, and TNF-α production and expression of inducible nitric oxide synthase (iNOS), COX-2, and myeloperoxidase 9 (MMP-9) [6]. This glycoprotein was reported to prevent inflammatory gastrointestinal diseases [7], and a larger glycoprotein (115 kDa) was also effective in blocking proinflammatory signals [8].

The strong correlation between antioxidant compounds also having anti-inflammatory activities was later reported by Diaz et al. [9]. Furthermore, substances that are antioxidant and anti-inflammatory have been reported to be likely to have an anticancer effect [10]. This anticancer effect was first described in relation to ZP by the Japanese group of Hirokawa et al. [11], suggesting that ZP extract could potentially be useful against breast cancer.

ZP was also tested as part of a herbal formulation for periodontitis showing substantial improvement especially in recovery of collagen gingival tissue [12]. In Korea ZP is used in traditional medicine as a diuretic and to treat digestive disorders. It is also used to help the cardiovascular system [12]. Some ZP compounds have been described to inhibit cholesterol acetyltransferase, thus contributing to helping the cardiovascular system, which validates the traditional use since cholesterol ester plays an important role in cardiovascular diseases [13]. The fact that ZP is used for digestive disorders could indicate an effect on stomach pain that might suggest pain relief properties for this species.

As part of our continuous interest in search for pharmacological effect of natural products and because ZP is widely used to treat several disorders, in this work we focused our efforts on the evaluation of the possible antinociceptive effect of essential oil obtained from *Zanthoxylum piperitum*.

2. Material and Methods

2.1. Plant Material. Plant material (aerial parts) of *Z. piperitum* was collected from the Glasnevin Botanic Gardens, Dublin, and dried at room temperature for two weeks. Dr. Colin Kelleher from the Glasnevin Botanic Gardens identified the species and a herbarium sample is kept in the Botanic Garden under the collection number 1984.1920.

2.2. Isolation of the Essential Oil. Air-dried, to constant weight, plant material (3 batches of 250 g of aerial parts) was subjected to hydrodistillation with circa one liter of distilled H_2O for 2.5 h using the original Clevenger-type apparatus. The yield was 0.015% (w/w, dried weight basis) for the oil isolated from *Z. piperitum* (ZPEO). The obtained oil was separated by extraction with Et_2O (Merck, Germany), dried over anhydrous Na_2SO_4 (Aldrich, USA), and immediately analysed.

2.3. Chemical Analysis of ZPEO. Qualitative analyses were carried out on a GC-QP2010 PLUS Shimadzu with a ZB-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness) under the experimental conditions reported for GC-FID analysis. The essential oil components were identified by comparing their retention indices and mass spectra to published data and computer matching with WILEY 275 and the National Institute of Standards and Technology (NIST 3.0) libraries provided by a computer-controlled GC-MS system. The results were also confirmed by comparing the compounds' elution order with their relative retention indices reported in the literature [14]. The retention indices were calculated for all the volatile constituents using the retention data of linear *n*-alkanes C8–C24.

2.4. Animals. Swiss Webster mice (20–25 g, two months old), donated by Instituto Vital Brazil (Niterói, Rio de Janeiro, Brazil), were used in this study. The animals were maintained in standard conditions (room with light-dark cycle of 12 h, 22 ± 2°C to 70% to 80% humidity, and with food and water *ad libitum*). Twelve hours before assays the animals were maintained in fasting in order to avoid food interference with the absorption of the tested substances. Animals were acclimatized to the laboratory conditions for at least 1 h before each test on set and were used only once throughout the experiments. All protocols were conducted in accordance with the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals and followed the principles and guidelines adopted by the National Council for the Control of Animal Experimentation (CONCEA), approved by the Biomedical Science Institute/UFRJ, Ethical Committee for Animal Research, and received the number DFBCICB015-04/16. All experimental protocols were performed during the light phase. Animal numbers per group were kept at a minimum and according to rules from CONCEA. At the end of each experiment mice were killed by ketamine/xylazine overdose.

2.5. Formalin-Induced Acute Pain. Twenty microliters of 2.5% formalin (37% formaldehyde) was injected in the plantar region of the right hind paw of mice 30 min after oral treatment with ZPEO (10, 30 or 100 μL/kg) or vehicle (oil) or 1 hour after oral treatment with morphine (2.5 mg/kg) or acetylsalicylic acid (200 mg/kg). The animals were placed individually in a transparent glass chamber and the duration of time (in seconds) that they spent licking their paw after injection of formalin was recorded and analysed over two separate periods: 0–5 minutes after injection (named early phase or neurogenic pain) and 15–30 minutes after injection (named late phase or inflammatory pain).

2.6. Capsaicin-Induced Nociception. This test was based on the method described by Sakurada et al. [15] with some modifications. Capsaicin (1.6 μg/paw) was injected into the plantar region of the right hind paw of the mice one hour after treatment. The animals were placed individually in a transparent glass chamber and paw licking duration (seconds) was recorded (0–5 minutes after capsaicin injection) and analysed.

2.7. Glutamate-Induced Nociception. This method was first described by Beirith et al. [16]. One hour after oral treatment of ZPEO, the plantar region of the right hind paw of the mice was injected with 20 μL of glutamate solution in PBS (3.7 ng/paw). The animals were placed individually in a transparent glass chamber and paw licking duration (seconds) was recorded (0–15 minutes after glutamate injection) and analysed.

2.8. Central Nociception: Hot Plate Test. This test was based on the method described by Ohlsson [17]. Mice were treated with ZPEO (10, 30, or 100 μL/kg, p.o.), vehicle (oil), or morphine (2.5 mg/kg, p.o.). They were placed on the hot plate apparatus (Insight, Brazil), kept at a constant temperature of 55 ± 0.5°C. The latency time until the animal began jumping, licking, or shaking the paw was recorded. The measurements occurred before treatment (baseline, mean of 60 and 30 minutes before treatment) and 30, 60, 90, 120, and 180 minutes after treatment. In order to prevent tissue damage to paw, a maximum exposure time (cut-off) of the animal's paws to the heated plate was established.

Aiming to investigate the antinociceptive mechanism involved, the animals were treated intraperitoneally 15 minutes before the oral treatment with ZPEO, with naloxone (a nonselective antagonist of the opioid receptor, 1 mg/kg) or atropine (a nonselective antagonist of the muscarinic receptor, 1 mg/kg).

The results for the hot plate test were expressed as a percentage increase compared to baseline (% ICB), calculated by the formula latency × 100/baseline − 100 and area under the curve.

2.9. Acute Toxicity. To exclude a possible toxicity in bone marrow and circulating leukocytes 24 hours after treatment with 100 μL/kg of ZPEO mice were anesthetized with ketamine/xylazine, blood was collected by orbital plexus into a heparinized tube, and after that mice were euthanized. The bone marrow was collected from the animal's femur by washing with 1 mL of PBS into the cavity. Haemogram analysis was performed in a CellPocH-100iV Diff (Sysmex) hematology analyser.

2.10. Inflammation Model: Subcutaneous Air Pouch (SAP) Model. This model was described by Sedgwick et al. [18] with modifications done by Raymundo and colleagues [19]. The animals received a dorsal subcutaneous injection of sterile air (10 mL) and an addition of 7 mL of air on the third day. On the sixth day, animals received a subcutaneous injection of sterile carrageenan solution (1%; 1 mL). Mice were pretreated with vehicle or the ZPEO (10, 30, or 100 μL/kg) 1 h before carrageenan injection into the SAP. The control group received an injection of sterile PBS (1 mL) into the SAP. Animals were sacrificed 24 h after carrageenan injection. The cavity was washed with 1 mL of PBS and the exudates were collected. The total cell counts were carried out from the exudates using a CellPocH-100iV Diff (Sysmex) hematology analyser. The exudates were centrifuged at 12,000 rpm for 8 min at 4°C and aliquots of the supernatants were stored at −20°C for dosages of tumour necrosis factor-α (TNF-α) and extravasated protein. TNF-α dosage was carried out by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (B&D, USA). Extravasated protein was determined using the BCA method (BCA™ Protein Assay Kit, Pierce). The results are expressed as pg/mL of TNF-α or mg/mL of protein.

2.11. Statistical Analysis. All experimental groups consisted of 6–10 mice. The results are presented as the mean ± SD. Statistical significance between groups was performed by applying analysis of one-way variance (ANOVA) followed by Dunnett's and Bonferroni's test using *GraphPad Prism 5.0* software. p values less than 0.05 ($p < 0.05$) were considered significant.

3. Results

GC-FID and GC/MS Analyses. Analysis of the essential oil was carried out by GC and GC/MS. The GC/MS analyses (three repetitions) were performed on a GC-QP2010 PLUS Shimadzu with a ZB-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm film thickness) and coupled with a 5975B mass-selective detector from the same company. The injector and interface were operated at 260° and 200°, respectively. The oven temperature was raised from 60 to 240° at a heating rate of 3° min^{-1} and then isothermally held for 10 min. As a carrier gas, He at 1.0 mL min^{-1} was used. The sample, 1 mL of the solutions, in Et_2O (10 mg in 1 mL of Et_2O), was injected in a pulsed split mode (the flow was 1.5 mL/min for the first 0.5 min and was then set to 1.0 mL/min throughout the remainder of the analysis; split ratio 40 : 1). The mass-selective detector was operated at the ionization energy of 70 eV, in the 35–650 amu range and a scanning speed of 3 scans/sec. GC (FID) analysis was carried out under the same experimental conditions using the same column and the same gas chromatograph type as described for the GC/MS. The percentage composition was computed from the total ion chromatogram peak areas without the use of correction factors. Qualitative analysis was based on the comparison of their linear retention indices relative to retention times of C8–C24 *n*-alkanes on the DB-5MS column with those reported in the literature [14] and by comparison of their mass spectra with those from Wiley 6, NIST07, MassFinder 2.3 (Figure 1 and Table 1).

3.1. Effect of Z. piperitum on Formalin-Induced Acute Pain. In the formalin-induced acute pain test, all doses (10, 30, and 100 μL/kg) were able to decrease paw licking in the first phase of the test. The doses reduce by 28%, 34%, and 43.3% the licking time, respectively. However, ZPEO did not decrease licking in the second phase of the response to formalin injection (Figure 2).

3.2. Effect of Z. piperitum on Glutamate-Induced Nociception. ZPEO decreased paw licking induced by glutamate at a dose of 10, 30, and 100 μL/kg (25%, 39%, and 64%, resp.). The standard drug morphine presented a 79% reduction of the glutamate paw licking (Figure 3).

3.3. Effect of Z. piperitum on Capsaicin-Induced Nociception. In order to verify if ZPEO would interfere with TRPV1 receptors, it was tested in a model of pain induced by capsaicin. The result of this evaluation showed that oral administration of ZPEO (100 μL/kg) was unable to decrease licking induced by capsaicin (Figure 4).

3.4. Effect of Z. piperitum on Thermal Nociception. Through the hot plate test, which measures central antinociception, ZPEO was only able to increase animal paw withdrawal threshold when treated with a higher dose of 100 μL/kg. The lower dose of 30 μL/kg did not show activities. The treatment of the animals with either naloxone, a nonselective antagonist of the opioid receptor, or atropine, a nonselective antagonist of the muscarinic receptor, did not have any effect on that antinociception (Figure 5).

3.5. Effect of Z. piperitum on Leukocytes Migration into Subcutaneous Air Pouch (SAP). ZPEO did not reduce the number of leukocytes that migrate to the SAP after carrageenan injection (Figure 6) nor did it have an effect on TNF-α production by leukocytes and on extravasation of protein (Figures 7 and 8).

Table 1: Chemical constituents identified in ZPEO. See Figure 1.

ZPEO	RT	%	RI Lit	RI
(1) Alpha-pinene	5.33	9.75%	933	930
(2) Sabinene	6.23	0.12%	976	971
(3) Beta-myrcene	6.75	3.34%	991	987
(4) Limonene	8.02	5.48%	1031	1028
(5) β-Phellandrene	8.08	29.39%	1031	1030
(6) *cis*-β-ocimene	8.54	0.79%	1040	1044
(7) Terpinolene	9.91	0.30%	1088	1084
(8) β-Linalool	10.43	0.33%	1097	1099
(9) β-Citronellal	12.43	6.83%	1153	1151
(10) β-Terpineol	13.52	0.14%	1159	1179
(11) α-Terpineol	14.10	0.72%	1189	1194
(12) β-Citronellol	15.39	10.32%	1228	1226
(13) *cis*-Geraniol	16.31	0.64%	1252	1249
(14) Piperitone	16.41	0.24%	1255	1251
(15) 2-Decanone	18.04	0.33%	1292	1291
(16) Citronellyl acetate	20.32	0.57%	1354	1348
(17) Neryl acetate	21.45	0.57%	1365	1377
(18) Caryophyllene	22.89	5.56%	1419	1413
(19) α-Bergamotene	23.49	0.22%	1436	1429
(20) α-Humulene	24.27	0.51%	1455	1449
(21) Germacrene D	25.25	1.38%	1480	1475
(24) E,E-α-farnesene	26.29	0.16%	1508	1502
(25) *trans*-Nerolidol	28.38	2.95%	1563	1520
(28) (E,E)-Farnesol	33.83	3.98%	1722	1711
(29) (E,E)-Farnesyl acetate	37.78	14.55%	1843	1830

Total % of components identified: 99.17%.

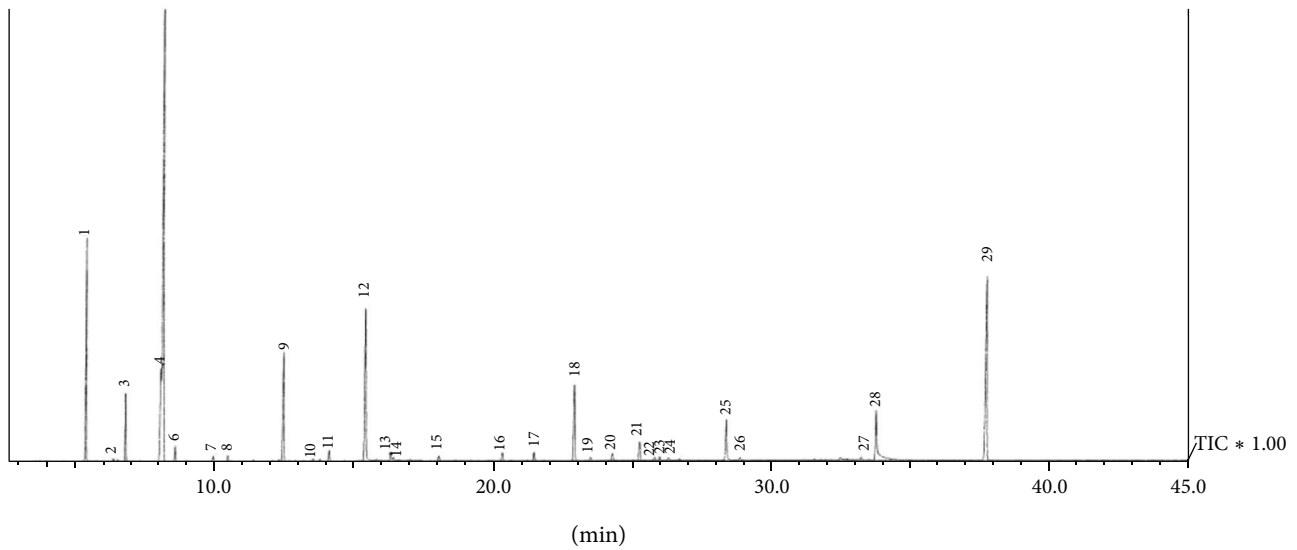


Figure 1: Gas chromatogram of *Z. piperitum* essential oil.

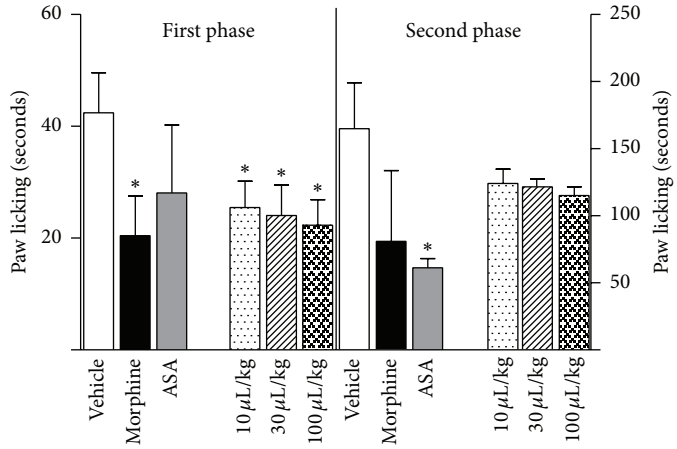


Figure 2: Effect of essential oil from *Zanthoxylum piperitum* DC. on formalin-induced licking in mice (first and second phases). Animals were pretreated with ZPEO (10, 30, or 100 μL/kg, p.o.), acetylsalicylic acid (ASA, 200 mg/kg, p.o.), morphine (2.5 mg/kg, p.o.), or vehicle (oil). Results are presented as mean ± SD (n = 6–10). Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group ($^*p < 0.05$).

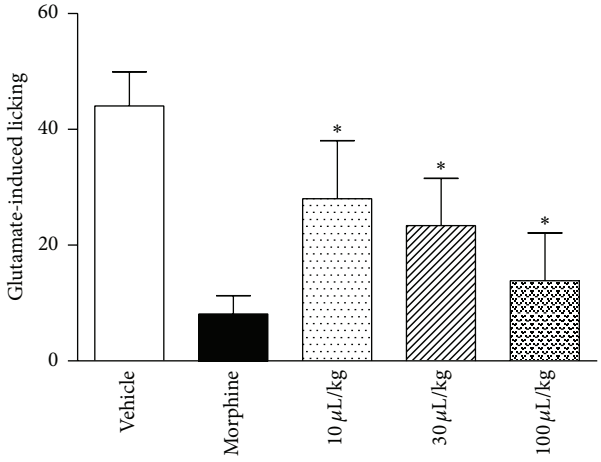


Figure 3: Effect of essential oil from *Zanthoxylum piperitum* DC. on glutamate-induced licking in mice. Animals were pretreated with ZPEO (10, 30, or 100 μL/kg, p.o.), morphine (2.5 mg/kg, p.o.), or vehicle (oil). Results are presented as mean ± SD (n = 6–10). Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group ($^*p < 0.05$).

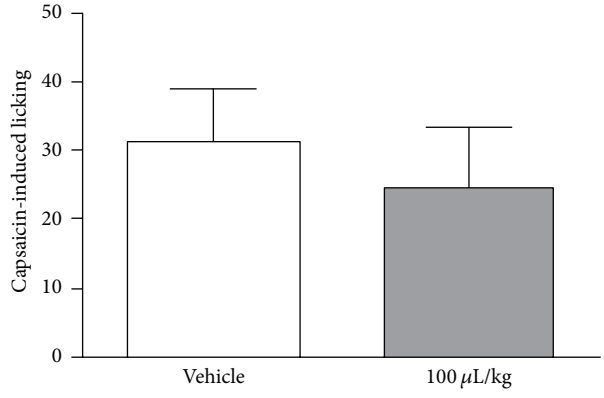


Figure 4: Effect of essential oil from *Zanthoxylum piperitum* DC. on capsaicin-induced licking in mice. Animals were pretreated with ZPEO (100 μL/kg, p.o.) or vehicle (oil). Results are presented as mean ± SD (n = 6–10). Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group.

3.6. Acute Toxicity. Twenty-four hours after oral administration of ZPEO (100 μL/kg) aliquots of blood and femoral fluxes were obtained and leukocyte counts were performed. The results indicated that the essential oil did not affect leukocyte number either in bone marrow or in blood (data not shown).

4. Discussion

The formalin test is believed to be a test that closely simulates clinical pain because it generates injured tissue. The test is known to induce phases and predominately involves C fibres. The early phase of this test has participation of substance P and bradykinin while the later phase also known as inflammatory pain phase has involvement of histamine, serotonin, prostaglandin, and again bradykinin [20]. In this study, ZPEO was able to decrease paw licking in the first phase but not in the second phase. The fact that bradykinin appears in both phases could indicate that the effect observed is not through bradykinin interference.

More recent publications indicate the participation of glutamate in both phases of the formalin test and a special participation of adenosine A_2 NMDA glutamate receptor binding in the first phase of the formalin test [21]. Interestingly, in the present research, ZPEO was effective in reducing nociception in the glutamate-induced licking test even in the lower dose of 10 μL/kg. This result suggests that the ZPEO could act on glutamatergic pathways and could involve the same NMDA receptor. An antagonist of this receptor could

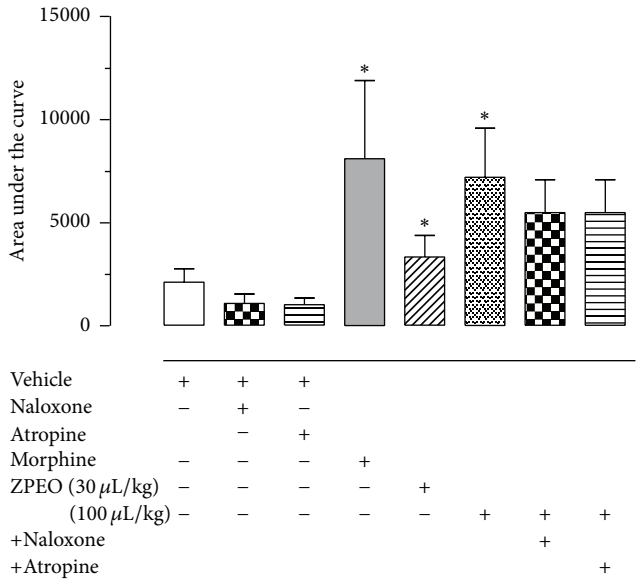


Figure 5: Effect of essential oil from *Zanthoxylum piperitum* DC. on thermal nociception (hot plate test). Animals were orally pretreated with ZPEO (30 or 100 μL/kg), morphine (2.5 mg/kg), or vehicle (oil). Results are presented as mean ± SD (n = 6–10) of area under the curve. Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group ($^*p < 0.05$).

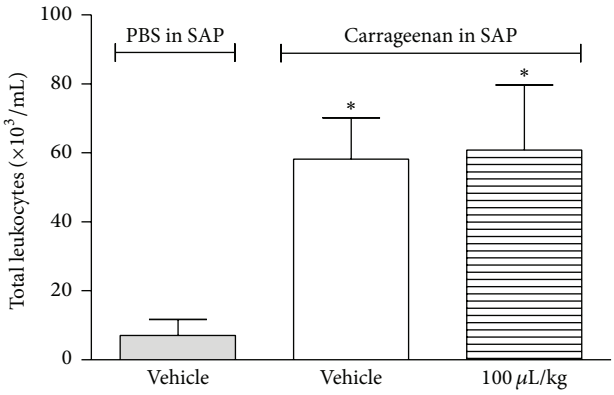


Figure 6: Effect of ZPEO on leukocytes migration through SAP model. Animals were pretreated with ZP (100 μL/kg, p.o.) 1 h prior to carrageenan (1%) injection into the SAP. The group vehicle received either carrageenan (1%) or PBS injected into the SAP. Results are presented as mean ± SD (n = 6–10) of total leukocytes (×10^3/mL). Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group. $^*p < 0.05$ when comparing to vehicle-treated group.

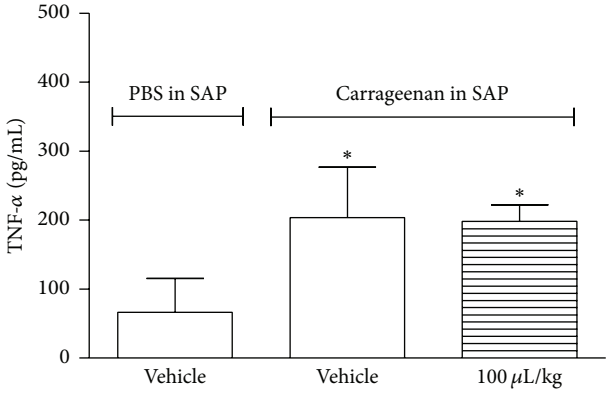


Figure 7: Effect of ZPEO on TNF-α production in the subcutaneous air pouch (SAP) model. Animals were pretreated with ZPEO (100 μL/kg, p.o.) 1 h prior to carrageenan (1%) injection into the SAP. The group vehicle received either carrageenan (1%) or PBS injected into the SAP. Results are presented as mean ± SD (n = 6–10) of TNF-α (pg/mL). Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group. $^*p < 0.05$ when comparing to vehicle-treated group.

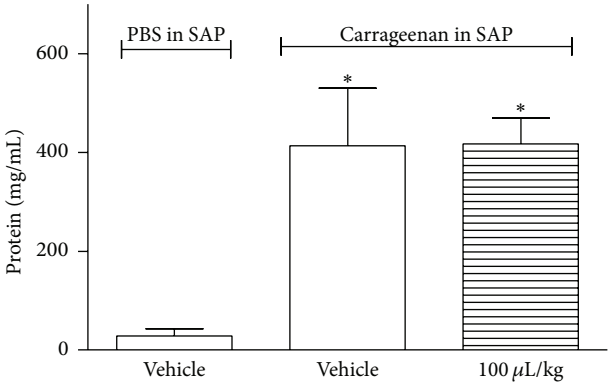


Figure 8: Effect of ZPEO on protein extravasation in the subcutaneous air pouch (SAP) model. Animals were orally pretreated with ZPEO (100 μL/kg) 1 h prior to carrageenan (1%) injection into the SAP. The group vehicle received either carrageenan (1%) or PBS injected into the SAP. Results are presented as mean ± SD (n = 6–10) of protein (mg/mL). Statistical analyses were performed using GraphPad Prism version 5.1 software, by one-way ANOVA with Dunnett's posttest with multiple comparisons against vehicle-treated group. $^*p < 0.05$ when comparing to vehicle-treated group.

be used in future studies to check this. On the other hand, only the higher dose 100 μL/kg had an effect in the hot plate test, which measures central nociception activity and which mechanism involves mainly Aδ fibres but also has involvement of glutamate receptors. Drugs such as morphine and oxotremorine are, respectively, opioid and muscarinic receptor agonists, and they also have an antinociceptive effect in the hot plate test [22]. The use of the opioid and cholinergic antagonists did not decrease the antinociception observed for ZPEO. Therefore, this suggests that the effect of ZPEO observed in the hot plate test is mediated through neither the opioid nor the muscarinic receptors. For this reason, it is very likely that the effect of ZPEO observed in both models is due to the ability of ZPEO compound(s) to inhibit the excitatory transmission induced by glutamate.

This effect is also observed using morphine and as shown in the results this drug has an effect in the hot plate test and a potent effect in the first phase of the formalin- and glutamate-induced licking tests. Nevertheless, the fact that ZPEO antinociception is not mediated through the opioid receptor makes it an ideal candidate as a potential analgesic, since medicines that have an effect through this mechanism have been shown to be more likely to cause side effects such as tolerance, hyperalgesia, and drug dependence [23].

ZPEO did not reduce capsaicin-induced licking. This result indicates that the compounds in ZPEO are not having an effect on TRPV1 receptors, although this receptor is also playing an important role in the hot plate models and its connection with glutamatergic pathway [24].

In this present study ZPEO had no effect on inflammation inducing mediators such as TNF-α. The anti-inflammatory effect of ZPEO has been reported by several research groups. Reference [25] showed ZPEO effect on cyclooxygenase, an enzyme involved in prostaglandin production and which is an inflammatory mediator. Regarding mediators, Yang et al. [26] reported ZP effect on TNF-α using ZP compounds isolated from pericarps; these compounds were ZP amides (A, B, D, and F), bungeanumamide A, tumuramide C, hypericin, sesamin, and quercitrin. Also, the ability of suppressing TNF-α, interleukin- (IL-) 1β, and interleukin-6 was reported earlier by Kim et al. [27]. The major compounds described in that research were octanoic acid (13.4%), *n*-heptanol (9.8%), and 1-octanol (8.1%). None of these compounds were present in the ZPEO; perhaps this could explain the inability of ZPEO as an anti-inflammatory agent.

The toxicity of ZP has been looked into by other researchers and it was found that it is phytotoxic due to the presence of the compound eucarvone [28]. The cell death occurred due to overproduction of reactive oxygen species (ROS). Eucarvone was not present in ZPEO analysed in the present study. On the other hand, Lee et al. [6] reported the antioxidant and protective effect against hepatotoxicity for glycoproteins from ZP. This protective effect is obtained by inducing apoptosis and enhancing the activity of natural killer cells [29]. Therefore, the importance of chemical identities when testing ZP essential oils is emphasised.

5. Conclusions

The essential oil of *Z. piperitum* has a significant antinociceptive activity. This effect is not mediated through opioid or muscarinic receptors. However, it seems to have interference on the glutamatergic pathway. These findings suggest that *Z. piperitum* essential oil containing the chemical profile presented in this study has potential as an analgesic medicine and the fact that it is not mediated through the opioid mechanism suggests that it is less likely to cause serious side effects.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors would like to thank Mr. Alan Minho for the technical assistance and Instituto Vital Brazil (Niterói, Rio de Janeiro, Brazil) for donation of animals used in this study. This work was supported by the National Council for Scientific and Technological Development (CNPq, fellowship to P. D. Fernandes and G. R. Donald and grant support to P. D. Fernandes) and Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ, fellowship to P. D. Fernandes and grant support to P. D. Fernandes). Fabio Boylan wishes to acknowledge the High Education Authority's Programme for Research in Third-Level Institutions Cycle 5's funding support for TBSI and to reinforce the importance of SFI programme ISCA-Brazil (Grant no. SFI/13/ISCA/2843), which contributed to the collaborative work between Brazil and Ireland.

References

[1] B. Adorjan and G. Buchbauer, "Biological properties of essential oils: an updated review," *Flavour and Fragrance Journal*, vol. 25, no. 6, pp. 407–426, 2010.

[2] K. Imaizumi, Y. Totsuka, S. Oda, and Y. Yamada, "Production of shoot-tip culture lines of asakura-sanshoo (*Zanthoxylum-piperitum* DC f inerme makino) with high aroma emanation," *Bioscience Biotechnology and Biochemistry*, vol. 57, pp. 591–593, 1993.

[3] Y. Wu, M. Shimoda, and Y. Osajima, "Volatile aroma compounds in young leaves and green fruits of Japanese pepper," *Nippon Nogeikagaku Kaishi*, vol. 70, no. 9, pp. 1001–1005, 1996.

[4] K. Hashimoto, K. Satoh, Y. Kase et al., "Modulatory effect of aliphatic acid amides from Zanthoxylum piperitum on isolated gastrointestinal tract," *Planta Medica*, vol. 67, no. 2, pp. 179–181, 2001.

[5] Y.-K. Park, "Inhibitory effects of Zanthoxylum piperitum on the LPS-induced production of nitric oxide and proinflammatory cytokines in RAW264.7 cells," *The Korea Journal of Herbology*, vol. 21, no. 4, pp. 69–76, 2006.

[6] J. Lee, S.-J. Lee, and K.-T. Lim, "Preventive effects of ZPDC glycoprotein (24 kDa) on hepatotoxicity induced by mercury chloride in vitro and in vivo," *Cell Biochemistry and Function*, vol. 32, no. 6, pp. 520–529, 2014.

[7] S.-J. Lee and K.-T. Lim, "Inhibitory effect of ZPDC glycoprotein on the expression of inflammation-related cytokines through p38 MAP kinase and JNK in lipopolysaccharide-stimulated RAW 264.7 cells," *Inflammation Research*, vol. 58, no. 4, pp. 184–191, 2009.

[8] C.-H. Park, P.-S. Oh, and K.-T. Lim, "Inhibitory effect of phytoglycoprotein (115 kDa) on the expression of TNF-α and interleukin-1β via inhibition of MAP kinase in primary cultured mouse thymocytes," *Bioscience, Biotechnology and Biochemistry*, vol. 73, no. 10, pp. 2196–2202, 2009.

[9] P. Diaz, S. C. Jeong, S. Lee, C. Khoo, and S. R. Koyyalamudi, "Antioxidant and anti-inflammatory activities of selected medicinal plants and fungi containing phenolic and flavonoid compounds," *Chinese Medicine*, vol. 7, no. 26, pp. 1–9, 2012.

[10] S. Crawford, "Anti-inflammatory/antioxidant use in long-term maintenance cancer therapy: a new therapeutic approach to disease progression and recurrence," *Therapeutic Advances in Medical Oncology*, vol. 6, no. 2, pp. 52–68, 2014.

[11] Y. Hirokawa, T. Nheu, K. Grimm et al., "Sichuan pepper extracts block the PAK1/cyclin D1 pathway and the growth of NF1-deficient cancer xenograft in mice," *Cancer Biology and Therapy*, vol. 5, no. 3, pp. 305–309, 2006.

[12] M. H. Kim, Y. Y. Choi, H. J. Lee, H. Lee, J.-C. Park, and W. M. Yang, "Topical application of herbal formula for the treatment of ligature-induced periodontitis," *Journal of Periodontal and Implant Science*, vol. 45, no. 4, pp. 145–151, 2015.

[13] Y.-D. Park, W. S. Lee, S. An, and T.-S. Jeong, "Human acyl-CoA: cholesterol acyltransferase inhibitory activities of aliphatic acid amides from *Zanthoxylum piperitum* DC," *Biological and Pharmaceutical Bulletin*, vol. 30, no. 1, pp. 205–207, 2007.

[14] R. P. Adams, *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectrometry*, Allured Publishing Corporation, Carol Stream, Ill, USA, 2001.

[15] T. Sakurada, K. Katsumata, K. Tan-No, S. Sakurada, and K. Kisara, "The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord," *Neuropharmacology*, vol. 31, no. 12, pp. 1279–1285, 1992.

[16] A. Beirith, A. R. S. Santos, and J. B. Calixto, "Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw," *Brain Research*, vol. 924, no. 2, pp. 219–228, 2002.

[17] L. Ohlsson, "Algesimetric studies by the hot plate method in mice," *Acta Pharmacologica et Toxicologica*, vol. 9, no. 4, pp. 322–331, 1953.

[18] A. D. Sedgwick, Y. M. Sin, A. R. Mackay, A. Al-Duaij, and D. A. Willoughby, "Studies into the mode of action of non-steroidal anti-inflammatory drugs using a model of facsimile synovium," *Journal of Pharmacy and Pharmacology*, vol. 36, no. 3, pp. 171–174, 1984.

[19] L. J. R. P. Raymundo, C. C. Guilhon, D. S. Alviano et al., "Characterisation of the anti-inflammatory and antinociceptive activities of the *Hyptis pectinata* (L.) Poit essential oil," *Journal of Ethnopharmacology*, vol. 134, no. 3, pp. 725–732, 2011.

[20] A. Tjølsen, O.-G. Berge, S. Hunskaar, J. H. Rosland, and K. Hole, "The formalin test: an evaluation of the method," *Pain*, vol. 51, no. 1, pp. 5–17, 1992.

[21] M. J. Hussey, G. D. Clarke, C. Ledent, S. M. O. Hourani, and I. Kitchen, "Reduced response to the formalin test and lowered spinal NMDA glutamate receptor binding in adenosine A2A receptor knockout mice," *Pain*, vol. 129, no. 3, pp. 287–294, 2007.

[22] T. D. Dulu, T. I. Kanui, P. K. Towett, G. M. Maloiy, and K. S. P. Abelson, "The effects of oxotremorine, epibatidine, atropine, mecamylamine and naloxone in the tail-flick, hot-plate, and formalin tests in the naked mole-rat (*Heterocephalus glaber*)," *In Vivo*, vol. 28, no. 1, pp. 39–48, 2014.

[23] T. Trang, R. Al-Hasani, D. Salvemini, M. W. Salter, H. Gutstein, and C. M. Cahill, "Pain and poppies: the good, the bad, and the ugly of opioid analgesics," *The Journal of Neuroscience*, vol. 35, no. 41, pp. 13879–13888, 2015.

[24] Y. A. Andreev, S. A. Kozlov, Y. V. Korolkova et al., "Polypeptide modulators of TRPV1 produce analgesia without hyperthermia," *Marine Drugs*, vol. 11, no. 12, pp. 5100–5115, 2013.

[25] S.-J. Lee and K.-T. Lim, "ZPDC glycoprotein inhibits inflammation-related cytokine and protein via nuclear factor-kappa B in dextran sulfate sodium-stimulated ICR mouse," *Biomedicine & Pharmacotherapy*, vol. 63, no. 7, pp. 528–536, 2009.

[26] S. Y. Yang, B. H. Tai, S. B. Song et al., "NF-κB activation and ppar transactivational effects of a new aliphatic acid amide from pericarps of zanthoxylum piperitum," *Bulletin of the Korean Chemical Society*, vol. 35, no. 8, pp. 2361–2366, 2014.

[27] C. Kim, S. J. Lee, C.-G. Hyun, and N. H. Lee, "Chemical constituents of supercritical extracts from *Prunus yedoensis*, *Saururus chinensis*, *Zanthoxylum piperitum* and their anti-inflammatory activities," *International Journal of Pharmacology*, vol. 9, no. 4, pp. 258–264, 2013.

[28] Y. Sunohara, Y. Baba, S. Matsuyama, K. Fujimura, and H. Matsumoto, "Screening and identification of phytotoxic volatile compounds in medicinal plants and characterizations of a selected compound, eucarvone," *Protoplasma*, vol. 252, no. 4, pp. 1047–1059, 2015.

[29] J. Lee, S.-J. Lee, and K.-T. Lim, "ZPDC glycoprotein (24kDa) induces apoptosis and enhances activity of NK cells in N-nitrosodiethylamine-injected Balb/c," *Cellular Immunology*, vol. 289, no. 1-2, pp. 1–6, 2014.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 7034759, 12 pages
http://dx.doi.org/10.1155/2016/7034759

Research Article

TCM Pattern Questionnaire for Lateral Elbow Pain: Development of an Instrument via a Delphi Process

Marcus Gadau,[1] Shi-Ping Zhang,[1] Wing-Fai Yeung,[2] Zhao-Xiang Bian,[1] and Ai-Ping Lu[1]

[1]*School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Hong Kong*
[2]*School of Nursing, The Hong Kong Polytechnic University, Hung Hom, Hong Kong*

Correspondence should be addressed to Shi-Ping Zhang; spzhang@hkbu.edu.hk

Received 12 February 2016; Accepted 29 May 2016

Academic Editor: Vincenzo De Feo

Individualized acupuncture treatment has been practiced for pain therapy. This study used acupuncture treatment for lateral elbow pain (LEP) as an example to study the diagnostic practice of individualized acupuncture treatment. A provisional version of LEP pattern questionnaire was developed based on a recent systematic review on TCM pattern diagnosis for LEP. A Delphi panel of 33 clinical experts from seven different countries was formed, and the Delphi survey was conducted in Chinese and English language for two rounds. Consensus was achieved from all 26 panelists who responded to the second round on 243 items of the instrument, which included a 72-question-long questionnaire. The mean level of expert consensus on the items of the final questionnaire was 85%. Consensus was found on four TCM patterns that could underlie LEP, namely, the *wind-cold-dampness pattern*, the *qi stagnation and blood stasis pattern*, the *dual deficiency of qi and blood pattern,* and the *retained dampness-heat pattern.* A list of signs and symptoms indicating one of the four TCM patterns and a list of preferred treatment modalities for each pattern were also generated. Our instrument shows considerable content validity. Further validity and reliability studies are under way.

1. Background

Personalized medicine has become the new trend in modern medical care [1–3]. Traditional Chinese medicine (TCM) has developed and used a sophisticated system of individualized medicine in the form of pattern diagnosis and classification for hundreds if not thousands of years already. However, there has been much variation in clinical practices even guided by the same TCM theory [4–6]. These variations are problematic when one tries to validate or replicate the effectiveness of the practice. Methods adopted from the current biomedical research to evaluate the efficacy of TCM interventions are often conceptually incompatible with the theory and clinical practice of TCM [7, 8]. Therefore, there is much need for standardized, validated instruments which can facilitate Chinese medicine diagnosis and which can be used by practitioners and researchers alike in their diagnostic process.

Tennis elbow or lateral elbow pain (LEP) is a common musculoskeletal pain condition with a prevalence of at least 1–3%. Incidence rates increase up to 10% for people between 40 and 50 years of age and symptoms are often prevailing for 1.5–2 years, therefore causing considerable loss of life quality for sufferers as well as accounting for substantial economic loss [9]. Acupuncture is frequently used to treat LEP [10].

In a previous systematic review, we have identified major TCM patterns associated with LEP. In this Delphi study, we wanted to investigate whether there is agreement between the literature and actual clinical practice. The overall aim of this study was to develop a practical instrument that will facilitate acupuncture practitioners with an easily applicable questionnaire to readily assess the underlying TCM pattern of LEP. We planned to achieve this aim through the following processes: first, we wanted to generate an initial questionnaire based on a systematic review and discussions within the research team. This preliminary questionnaire would then be presented to a Delphi panel and would undergo a Delphi survey with the following primary objectives: (1) to find consensus on which TCM patterns are the most common patterns underlying LEP; (2) to design and validate a questionnaire that would help diagnose a TCM pattern for LEP; and (3) to generate and find consensus on a list of signs and symptoms that would

be indicative of one of the TCM patterns. We also used this survey to gather information for a basic list of recommended acupuncture and moxibustion treatment modalities for each pattern, as the ultimate purpose of pattern diagnosis is to guide clinical practice. This list of acupuncture/moxibustion treatment recommendations for LEP may serve as the basis for future studies.

2. Methods

2.1. Formation of Research Team. A research team, consisting of all the authors, was formed to conduct the Delphi study. The team met regularly to initially determine the aim of the pattern questionnaire and then to generate its items, to define appropriate criteria for the selection of the Delphi expert panel, to analyze and discuss quantitative and qualitative answers after both rounds, to provide appropriate feedback to the expert panel after each round, and to monitor the progress of the study.

2.2. Selection of Participants. Before the commencement of this study, ethical approval was obtained from the Committee on the Use of Human and Animal Subjects in Teaching and Research at the Hong Kong Baptist University, Hong Kong (reference number HASC/Student/12-13/007). Purposive sampling was used for the selection of experts. Experts were chosen with the purpose that they have knowledge and experience about acupuncture treatment for LEP, with an assumption that their knowledge about LEP signs can be used to readily determine the items in our questionnaire. We aimed to identify panelists who have a broad range of knowledge in the treatment of LEP with acupuncture and ideally previous experience of having undertaken or currently undertaking clinical research on acupuncture, including RCTs. To qualify as panelists, possible candidates were screened before entering the study for a minimum acupuncture experience of five years, had to be frequently treating LEP with acupuncture, and had to be regularly using pattern diagnosis in their clinical practice. Candidates were also asked if they had previous experience with acupuncture clinical research. Recommendations from candidates meeting these criteria for inviting additional potential panelists were taken into consideration.

National and international experts were recruited from disciplines involved in the diagnosis and treatment of LEP with acupuncture including acupuncture practitioners, acupuncture researcher, and acupuncture educators.

Prospective panelists were sent an information package via email or mail to inform them of the study goals as well as the format of a Delphi study prior to sending out the questionnaires. Immediately after the prospective panelist had agreed to participate in the study, the initial questionnaire was sent to the panelist.

Names of participating panelists are mentioned in the Acknowledgments unless they indicated otherwise. The identity of the expert panelists was disclosed to the participants before the publication of this study.

2.3. Generation of Items. In order to generate an initial questionnaire which was presented to the expert panelists in round 1 of the Delphi survey, information was compiled from the following sources by the research team: (a) a systematic review on TCM pattern diagnosis for LEP [11], which consisted of (i) a journal review, (ii) a textbook literature review, and (iii) a data-mining process, as well as (b) meetings of the research team, which also consisted of specialist acupuncture clinicians to acquire expert opinion and to identify relevant criteria as well as to further discuss particular questionnaire items.

Findings from these sources were collected and reviewed. The research team removed nonrelevant items and composed a preliminary questionnaire, which was divided into four sections: the 1st section stated initial possible TCM patterns that could underlie LEP. In the 2nd section, signs and symptoms, which could be clinically relevant to determine the pattern underlying LEP, were reformulated into colloquial Yes/No questions that a practitioner could address to a patient. This list of questions was divided into (i) symptoms at the local elbow area, (ii) other symptoms, and (iii) physical signs. Other symptoms were subdivided into body/limbs, digestive/stools, mind, upper body, physical signs, tongue features, and pulse features. In the 3rd section, the expert needed to decide which sign or symptom would be indicative of which pattern. And, finally, in the 4th and final section of the questionnaire the expert was asked which acupuncture/moxibustion treatment modality he or she would recommend for each pattern.

In Sections 1 and 2, the experts could choose to either agree or disagree on an item to be included in the final questionnaire. Items in Sections 3 and 4 were rated in a multiple-choice format, with multiple responses allowed (which signs and symptoms indicate which pattern and which treatment modality would be recommended for which pattern). For all four sections of the questionnaire, the experts had the chance also to choose "Other" or "Alternative method" and then could clarify their choice, in case an expert wanted to make a choice that was not provided as a default choice. At the end of each of the four sections, we included the opportunity for the experts to comment on their responses as well as to leave additional comments. The English translations of TCM patterns, pulse, and tongue features were based on the WHO standard terminologies on TCM [11].

2.4. Delphi Process. The Delphi method is a structured process in which consensus of opinions from a group of experts is obtained using a series of questionnaires in quasi-anonymity and with controlled opinion feedback [12]. McKenna [13] suggested that consensus in a Delphi study should be equated with 51% agreement among experts; Sumsion [14] recommended 70%, yet Green et al. [15] proposed 80% while Crisp et al. [16] challenged the idea that consensus should be equated to a percentage number and concluded that stability of the response throughout the rounds is a better index for consensus. Thus, there are no standard guidelines on an appropriate level of consensus and no apparent scientific rationale on how to decide on the degree of agreement that would amount to a consensus [17]. Based on our research on previously conducted Delphi studies, especially in the

field of TCM [18–22], and due to the fact that our initial questionnaire was generated on the basis of a thorough, systematic review, the research team decided to set a content validity index (CVI) of ≥0.51. This CVI represents a minimal level of 51% of consensus among the experts and therefore a de facto majority to determinate whether or not an agreement was found between the experts and whether or not an item should remain in the next round. This consensus level was set before the start of round 1. The CVI was calculated by the number of experts who declared an item suitable divided by the total number of experts, who rated the item. We preset the amount of Delphi rounds to two, due to reasons that are further elaborated in the Discussion.

2.5. Round 1. In this first round, the panelists were to decide whether or not to retain an item for the final questionnaire as well as to suggest new items (i.e., a pattern, a sign or symptom, or a treatment modality that was not a default option), as previously described.

2.6. Round 2. Based on the results of round 1, all items with a CVI ≥ 0.51, as well as all potential new items, were presented to the experts. If an expert suggested including an additional item, it was evaluated for relevance by the research team and if it was deemed relevant, it was included in round 2. The expert panel was informed of the following: "For any item of the questionnaire to appear in round 2 at least 51% ('the majority') of all experts had to decide to include it." In the light of this information, the expert panel rerated all items of the four sections and was asked to either agree or disagree to retain an item for the final instrument. Experts were also informed that the second round was the final round of the Delphi survey. The final instrument would then only contain items, which consensus was reached upon after two rounds of the Delphi process.

2.7. Data Analysis. Data was collected and analyzed by Marcus Gadau and independently reviewed by Shi-Ping Zhang and Wing-Fai Yeung. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 21.

3. Results

3.1. Description of Expert Panel. Figure 1 shows the Delphi process. Of the 34 experts initially invited to participate in the study, 33 (97%) agreed to participate and were sent the provisional questionnaire for evaluation. One expert did not want to participate in the study, because he stopped using pattern diagnosis in his practice recently. Of the 33 experts who agreed to participate, 28 completed round 1. All 28 experts had a minimum experience of five years in the treatment of lateral elbow pain with acupuncture and had frequently been using pattern differentiation in their acupuncture practice. Finally, 26 experts completed the second round, after which the Delphi process was terminated. The reason why seven of the 33 initial panelists did not complete round 1 or round 2 is unknown to the authors, as no explanation had been given. The characteristics of the 26 panelists who completed both rounds of the Delphi study and therefore represented the expert panel are presented in Table 1.

Table 1: Profile of Delphi experts.

Characteristics (n = 26)	
Gender	
Male	15
Female	9
Acupuncture experience	
5–10 years	14
11–20 years	6
+21 years	5
Region	
Asia-Pacific (incl. Australia)	11
Europe	12
North America	2
Profession (multiple responses allowed)	
Acupuncture practitioner	23
Acupuncture researcher	13
Acupuncture educator	17

3.2. Delphi Round 1. Of the original 679 items provided in the provisional questionnaire, 244 items (35.9%) remained after round 1 (Figure 1).

One additional item was added after round 1 in Section 2 of the questionnaire (see Table 3): *(Item 22) "How severely does your elbow pain affect your ability to carry out routine tasks (e.g. driving, opening jars, carrying shopping bags)? - Not at all; Mildly; Medium; Severely (answering options)."* This question was proposed by one panelist and was accepted by the research team because a question assessing the severity of functional impairment in regard to performing daily tasks was not yet presented in the original set of items and was deemed relevant for the purpose of the instrument. One expert suggested adding blood stasis as an individual pattern to Section 1 of the questionnaire (see Table 2). However, the research team did not include this item for round 2, due to findings from the previous systematic review suggesting that blood stasis appeared rather frequently in association with qi stagnation in LEP and this pattern was already included. A second expert suggested subdividing or adding in different kinds of qi- and blood-deficiencies based on the organ system they were caused by, such as spleen/stomach weakness leading to qi- and blood-deficiency. Another expert suggested subdividing or adding different channel-specific qi-stasis and blood stasis pattern such as qi-stasis and blood stasis of the hand greater Yang meridian. The research team decided not to include these patterns because they are mere subpattern of a pattern that is already included.

One expert suggested adding fire needling and another expert suggested adding distal needling acupuncture (DNA) as a recommended treatment modality to Section 4 of the questionnaire (see Table 5). Fire needling was considered too

Table 2: Patterns associated with LEP.

Item	Chinese name	Experts that agree % ($n = 26$)
(1) Wind-cold-dampness pattern	风寒湿证	100
(2) Retained dampness-heat pattern	湿热内蕴证	77
(3) Dual deficiency of qi and blood pattern	气血两虚证	87
(4) Qi stagnation and blood stasis pattern	气滞血瘀证	100

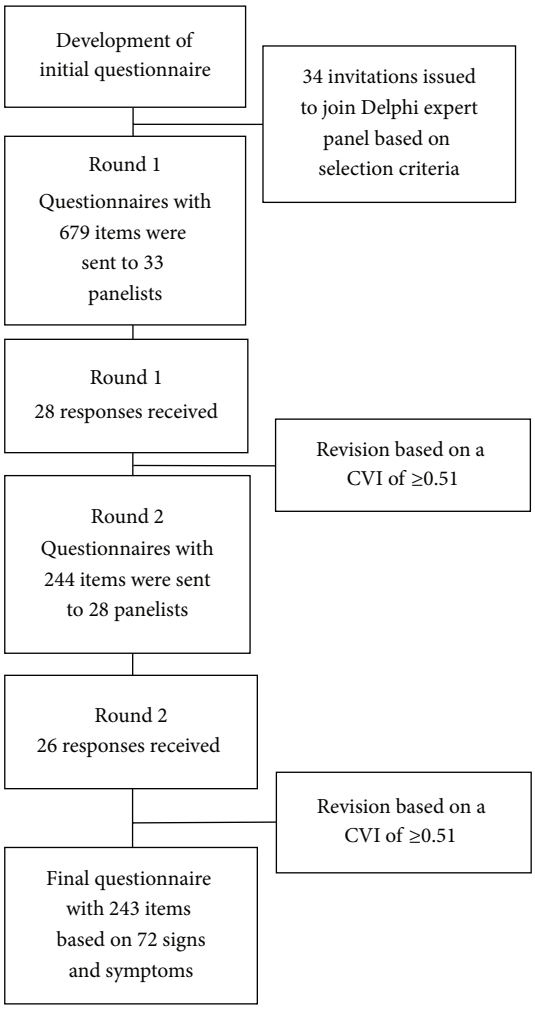


Figure 1: Flowchart of Delphi process.

invasive and is not very commonly practiced outside of China because it is a potentially dangerous treatment modality and was therefore not included. DNA is a style of manual needling, rather than a new treatment modality, and was also not included, because manual needling includes all styles and forms of manual stimulation acupuncture, including DNA.

Other experts suggested adding tuina-massage, herbal therapy (internal and external), electromagnetic stimulus, or osteopathic therapy as recommended treatment modalities. We did not include these as the spectrum of relevant therapies for the questionnaire was preset to acupuncture practice, which must involve the use of acupoint stimulation, such as acupuncture, moxibustion, acupressure, acupotomy (scalpel therapy), auricular acupuncture, or acupressure.

3.3. Delphi Round 2. Expert consensus was found on 243 of 244 items from round 1 (all four sections combined). A consensus was achieved from all 26 panelists who responded to the second round on all four TCM patterns in Section 1 that could underlie LEP, namely, the *wind-cold-dampness pattern*, the *qi stagnation and blood stasis pattern*, *the dual deficiency of qi and blood pattern,* and the *retained dampness-heat pattern* (see Table 2).

The following question from Section 2 was excluded, because it did not reach a CVI of ≥0.51 in round 2: *"Does eating cold foods (i.e. watermelon, salads, icy-cold drinks) decrease the pain?"*

The final instrument that derived from a systematic review, textbook-research, and finally a two-round Delphi process was generated. It has a list of four common patterns underlying LEP (Table 2), a main-questionnaire with 72 Yes/No questions (Table 3) that can help to differentiate one of the four most common TCM patterns underlying LEP and a list of common symptoms (Table 4) that are indicative of one of the four most common patterns. A list of preferred treatment modalities (Table 5) for each of the four patterns was established as well. All items of the instrument had a CVI of at least 0.51 and therefore show considerable content validity.

4. Discussion

In our final TCM pattern diagnosis for LEP instrument, we identified 25 local signs and symptoms and 45 systemic signs and symptoms, as well as 16 tongue and pulse features that may be associated with the four most commonly seen LEP patterns. Even though we laid emphasis on the four most commonly seen LEP patterns during the development of this questionnaire, it can also be used in the diagnostic process of identifying a mixed pattern presentation (e.g., dual deficiency of qi and blood coexisting with wind-cold-dampness).

The experts agreed on 99.6% of all items that were presented to them in the final Delphi round, and the mean CVI of all items in the final questionnaire was 0.88 (95% CI, 0.87 to 0.90), representing an 88% consensus level. We are therefore confident that our findings adequately represent a robust consensus of TCM expert opinions. Such high agreement might be because our initial questionnaire derived from a systematic review.

We chose clinical experts rather than academic experts for our Delphi panel and the experts came from many

Table 3: Final LEP pattern differentiation questionnaire.

Item	Experts agree % ($n = 26$)
Local symptoms:	
(1) Does your elbow feel cold?	88
(2) Does your elbow feel hot?	92
(3) Does cold exposure increase the pain?	100
(4) Does cold exposure relieve the pain?	80
(5) Does heat exposure increase the pain?	80
(6) Does heat exposure relieve the pain?	96
(7) Do you strongly dislike cold on your elbow?	92
(8) Do you strongly dislike wind on your elbow?	78
(9) Do you strongly dislike heat on your elbow?	71
(10) Does local pressure increase the pain?	100
(11) Does local pressure relieve the pain?	88
(12) Does movement increase the pain?	100
(13) Does movement relieve the pain?	88
(14) Does rest increase the pain?	84
(15) Does rest relieve the pain?	100
(16) Which term best describes your pain?	100
Dull, lingering	92
Cramping	100
Stabbing	96
Hot, burning	88
Numbness sensation	88
(17) How severe is your pain?	100
Mild	96
Medium	100
Severe	100
(18) Is there any uncomfortableness during movement?	96
None	95
Mild	95
Medium	96
Severe	95
(19) How was the onset of your elbow pain?	100
Slow, gradual onset	100
Sudden onset	100
(20) How long have you had the condition?	100
More than 3 months	100
1 week–3 months	100
Less than 1 week	100
(21) Is the pain intermittent or constant?	100
Intermittent	100
Constant	100
(22) How severely does your elbow pain affect your ability to carry out routine tasks (e.g. driving, opening jars, carrying shopping bags)?	100
Not at all	95
Mildly	96
Medium	100
Severely	100

Table 3: Continued.

Item	Experts agree % (n = 26)
Systemic symptoms:	
(23) Do your hands and feet usually feel cold?	92
(24) Do your entire arms and legs usually feel cold?	88
(25) Do your hands and feet usually feel too hot?	67
(26) Are your hands and feet usually sweaty?	74
(27) Do you often experience spontaneous sweating over your entire body?	78
(28) Does your entire body usually feel heavy?	80
(29) Do you usually have a feeling of fullness, especially in the epigastric region (upper area of the belly)?	67
(30) Do you usually feel full or bloated after eating?	75
(31) Do your arms and legs usually feel weak?	76
(32) Do you experience numbness in your arms and legs?	80
(33) Does eating cold foods (e.g. watermelon, salads, ice-cold drinks) increase the pain?	58
(34) Does eating warm food (e.g. chili, pepper, ginger, hot soups) increase the pain?	54
(35) Does eating warm food (e.g. chili, pepper, ginger, hot soups) decrease the pain?	68
(36) Do you usually have poor appetite?	61
(37) Do you usually have excessive appetite?	57
(38) Do you usually feel thirsty?	63
(39) Do you usually feel thirsty, but you do not want to drink?	67
(40) Are you usually not thirsty at all?	67
(41) Do you usually have loose stools?	67
(42) Do you usually have dry stools?	63
(43) Do you usually have copious, clear urine?	71
(44) Do you usually have scanty, dark-yellow urine?	63
(45) Do you have urinary difficulties?	52
(46) Do you usually feel tired and easily fatigued?	79
(47) Do you usually feel restless and/or agitated?	74
(48) Do you have difficulties sleeping and/or shallow sleep	78
(49) Do you frequently experience mood changes?	70
(50) Do you easily get angry?	83
(51) Are you usually worried and/or anxious?	70
(52) Does your head usually feel heavy?	67
(53) Do you usually feel dizzy?	61
(54) Do you usually have a lot of saliva in your mouth?	55
(55) Do you usually experience a bitter taste in your mouth, especially in the morning?	61
(56) Do you usually have a sticky taste in your mouth?	65
(57) Are you usually short of breath?	70
Physical signs:	
(58) The elbow feels cold to touch (to the practitioner)?	96
(59) The elbow feels warm to touch (to the practitioner)?	92
(60) The elbow is swollen and/or reddened?	92
(61) Does the face appear pale and/or lusterless?	87
(62) Does the complexion appear oily?	73
(63) Does the complexion appear reddened and/or dry?	74
(64) Does the complexion appear dark?	74
(65) Do the lips appear brittle?	70
(66) Do the lips appear purple?	83

Table 3: Continued.

Item	Experts agree % ($n = 26$)
(67) Do the lips appear excessively red?	65
(68) Do the nails appear brittle?	74
(69) Does the voice appear strong?	65
(70) Does the voice appear soft?	74
Tongue and pulse features:	
(71) Tongue features	91
Pale tongue	86
Red tip of the tongue	76
Red tongue	78
Scanty fur	77
Slimy fur	78
Thick fur	76
Thin fur	86
White fur	87
Yellow fur	77
(72) Pulse features	92
Fine pulse	91
Rapid pulse	82
Slippery pulse	77
String-like pulse	91
Sunken pulse	86
Replete (strong) pulse	82
Weak pulse	87

different countries across four continents. We, therefore, may assume that our Delphi findings represent the current international notion of TCM clinical practice in regard to LEP pattern diagnosis. The high level of agreement between the literature review (academic consensus) and Delphi experts (clinical consensus) then suggests that our findings have a high degree of generalizability of LEP pattern diagnosis in TCM theory and practice. The provisional instrument created via this Delphi study has achieved considerable content validity, yet requires further face-, criterion-, and construct-validity as well as test-retest and reliability testing before it may be clinically used. We are therefore currently conducting such studies for both the English as well as the Chinese language versions of the instrument.

Even though the primary aim of the study was to provide an instrument to assist with the pattern diagnosis of LEP, we also wanted to gather information for pattern-based treatments for future studies. Therefore, we asked the experts in the last section of the questionnaire which acupuncture/moxibustion treatment modalities they would recommend for which pattern (Table 5). The average consensus level of treatment recommendations that passed the cut-off criteria of 51% expert agreement and therefore remained in round 2 was a striking 91%. Surprising to the authors, the experts did not recommend using ginger-moxibustion for the qi stagnation and blood stasis pattern; however, they did see indirect moxibustion (the use of a moxa stick held about 3 cm away from the skin) or the combined use of acupuncture and moxibustion fit for use for this pattern. Another unexpected recommendation was the use of manual acupuncture but not electroacupuncture for the dual deficiency of qi and blood pattern. An explanation from TCM theory for these two unanticipated recommendations is perhaps that both methods involve a rather strong stimulus to the injured elbow, which would be deemed contraindicated for the deficiency pattern and too "hot" or proinflammatory for the stasis pattern, which is associated with signs of eminent inflammation.

In the literature [12–27] we found that most Delphi studies used between two and four rounds to achieve consensus. Before we started to collect the data, we chose to terminate the Delphi process after two instead of four rounds out of practical reasons and due to the fact that previous research teams [25] found that the chances for low response rates increase exponentially after two Delphi rounds. A phenomenon called "response fatigue" sets in, as clinical experts are usually extremely busy with little time to spare in their tight schedules. We informed all participating experts that the Delphi study would be terminated after two rounds; this was also done to motivate participants and reduce response attrition after round 1. We also sent out regular email reminders before the deadline of each round, as

Table 4: Patterns and their indicating signs and symptoms.

Item	Experts agree % (n = 26)
(1) Wind-cold-dampness pattern	
(1) Elbow feels cold to the patient	100
(2) Cold exposure increases the pain	100
(3) Heat exposure relieves the pain	96
(4) Patient strongly dislikes cold on the elbow	96
(5) Patient strongly dislikes wind on the elbow	83
(6) Local pressure increases the pain	63
(7) Movement increases the pain	79
(8) Movement relieves the pain	75
(9) Rest relieves the pain	68
(10) Nature of pain: Dull/lingering	87
(11) Nature of pain: Cramping	88
(12) Nature of pain: Numbness sensation	96
(13) Pain severity: Medium	88
(14) Pain severity: Severe	92
(15) Uncomfortableness during movement: Medium	79
(16) Onset of elbow pain: Slow, gradual onset	72
(17) Onset of elbow pain: Sudden onset	80
(18) Duration of condition: 1 week–3 months	88
(19) Duration of condition: Less than 1 week	76
(20) Intermittent or constant pain: Constant	92
(21) Hands and feet usually feel cold (to the patient)	79
(22) Entire body usually feels heavy	68
(23) Eating warm food (e.g. chili, pepper, ginger, hot soups) decreases the pain	71
(24) Loose stools	68
(25) Copious, clear urine	79
(26) Patient usually feels tired and is easily fatigued	82
(27) Elbow feels cold to touch (to the practitioner)	96
(28) Pale tongue	91
(29) White tongue fur	96
(30) Slippery pulse	86
(31) String-like pulse	83
(2) Retained dampness-heat pattern	
(1) Elbow feels hot to the patient	100
(2) Cold exposure relieves the pain	86
(3) Heat exposure increases the pain	91
(4) Heat exposure relieves the pain	52
(5) Patient strongly dislikes heat on the elbow	82
(6) Local pressure increases the pain	91
(7) Movement increases the pain	86
(8) Nature of pain: Hot/burning	100
(9) Pain severity: Medium	82
(10) Pain severity: Severe	95
(11) Uncomfortableness during movement: Medium	86
(12) Uncomfortableness during movement: Severe	90
(13) Duration of condition: 1 week–3 months	95
(14) Intermittent or constant pain: Constant	91
(15) Scanty, dark-yellow urine	82
(16) Patient usually feels restless and/or agitated	65
(17) Sensation of heaviness of the head	57
(18) Usually bitter taste in the mouth, especially in the morning	71

Table 4: Continued.

Item	Experts agree % (n = 26)
(19) Usually a sticky taste in the mouth	76
(20) Elbow is swollen and/or reddened	95
(21) Red tongue	91
(22) Thick tongue fur	77
(23) Yellow tongue fur	86
(24) Rapid pulse	91
(25) Slippery pulse	91
(26) Replete (strong) pulse	67
(3) Dual deficiency of qi and blood pattern	
(1) Elbow feels cold to the patient	90
(2) Cold exposure increases the pain	95
(3) Local pressure relieves the pain	90
(4) Movement increases the pain	86
(5) Rest relieves the pain	90
(6) Nature of pain: Dull/lingering	95
(7) Nature of pain: Numbness sensation	90
(8) Pain severity: Mild	95
(9) Pain severity: Medium	81
(10) Onset of elbow pain: Slow, gradual onset	95
(11) Duration of condition: More than 3 months	95
(12) Intermittent or constant pain: Constant	86
(13) Hands and feet usually feel cold (to the patient)	81
(14) Spontaneous sweating	70
(15) Limbs usually feel weak	81
(16) Numbness sensation in limbs	81
(17) Poor appetite	70
(18) Loose stools	75
(19) Patient usually feels tired and is easily fatigued	86
(20) Patient usually feels dizzy	60
(21) Elbow feels cold to touch (to the practitioner)	81
(22) Pale, lusterless face	90
(23) Soft voice	80
(24) Pale tongue	95
(25) Thin tongue fur	86
(26) White tongue fur	90
(27) Fine pulse	95
(28) Sunken pulse	75
(29) Weak pulse	95
(4) Qi stagnation and blood stasis pattern	
(1) Local pressure increases the pain	96
(2) Movement relieves the pain	83
(3) Nature of pain: Stabbing	100
(4) Nature of pain: Numbness sensation	77
(5) Pain severity: Medium	87
(6) Pain severity: Severe	100
(7) Uncomfortableness during movement: Mild	73
(8) Uncomfortableness during movement: Medium	96
(9) Onset of elbow pain: Slow, gradual onset	79
(10) Duration of condition: More than 3 months	79
(11) Duration of condition: 1 week–3 months	79

Table 4: Continued.

Item	Experts agree % ($n = 26$)
(12) Intermittent or constant pain: Intermittent	83
(13) Intermittent or constant pain: Constant	82
(14) String-like pulse	92

Table 5: Treatment recommendations.

Treatment intervention	Patterns and agreement of experts % ($n = 26$)			
	Wind-cold-dampness pattern	Retained dampness-heat pattern	Dual deficiency of qi and blood pattern	Qi stagnation and blood stasis pattern
Acupuncture	92	91	96	96
Manual acupuncture	92	91	91	83
Electro-acupuncture	88	—	—	96
Moxibustion	92	—	91	87
Ginger-moxibustion (direct moxibustion with a thin slice of ginger between skin and moxa cone)	88	—	81	—
Moxa stick (indirect moxibustion about 3 cm away from elbow)	96	—	91	88
Acupuncture and moxibustion	96	—	95	92

—, treatment modality CVI < 0.51: experts do not recommend this treatment modality.

well as extra reminders to experts, who have not responded after the deadline passed, also to enhance the response rate. We felt that two Delphi rounds would suffice to achieve a reliable level of consensus among experts because our initial set of items for the questionnaire derived from a systematic review and therefore would already possess some inherent level of consensus as it represents the concentrated opinions of authoritative texts.

There is a potential risk for bias in the expert selection, as the method is based on nonrandomized sampling. Therefore, representativeness cannot be assured [26]. However, we set distinct inclusion and exclusion criteria to ensure some degree of homogeneity among the experts. There are again no rules for the minimum or maximum amount of experts in a Delphi study and the choice of the size of the expert panel for most reviewed previous Delphi studies depended largely on common sense, resources and time available, and other practical reasons. Ten to fifteen subjects were deemed sufficient by Delbecq et al. [27]. We set out to have more than double the amount of experts on our panel, in case there would be a larger attrition quote. A potential risk for response bias was minimized due to the time spent educating the panel and by choosing only two Delphi rounds. Our relatively low attrition rate of 21% (7 out of 33) was probably due to these precautions taken. Experts were informed that the items presented to them in round one were generated from the literature. However, experts had ample opportunity to provide other patterns, signs, and symptoms and could comment on the items presented to them. This was done to avoid early closure on ideas and to prevent that experts alter their views due to perceived pressure to conform to the literature.

After having performed a systematic review as the basis for the initial questionnaire, we felt comfortable to choose an agreement of 51% among experts as having achieved consensus. However, we would also like to point out that the mean consensus level for all items that remained at the end of round 2 was 88% and that 85.5% of all items (208 out of 243 items) reached a consensus level of over 70%, thus making our expert consensus much more robust than a consensus just defined by a de facto majority of 51%. We attempted to address the issue of subjectivity with retained items, as another potential criticism of our study, by having chosen an expert panel with a broad range of backgrounds and geographical regions. We also believe that the very robust level of consensus that was achieved with the majority of final items has minimized the risk of such subjectivity. However, one should bear in mind that expert consensus does not automatically mean that the right answers were found. Another limitation might be that due to the selection of experts, there might be acupuncture styles practiced that were not adequately represented in our expert panel. Lastly, while interpreting the results of our Delphi study one should acknowledge the potential influence of biases and that the current preliminary instrument will have to undergo rigorous validity and reliability testing before its clinical use can be recommended.

5. Conclusion

While the TCM pattern diagnosis system has the potential to refine treatment by identifying subtle differences in etiology, pathogenesis, and body constitution, a lack of standardization in terminology and consensus on diagnostic criteria are

significant barriers. The provisional instrument derived from our study has obtained robust consensus and could be seen as a way of controlling information variance. It has been realized that similar sets of information must be collected and standardized terminology and diagnostic criteria must be used before consensus among practitioners in regard to the TCM pattern diagnosis can be reached [28, 29]. Only with the use of standardized instruments like ours may information variance be reduced, which in combination with using a standardized terminology and diagnostic criteria could improve diagnostic-reliability as well as interrater reliability and intertrial reproducibility. This would help to overcome some of the shortcomings in TCM research and practice and represents a significant advancement in clinical TCM research [30]. Our instrument may, therefore, contribute to the standardization of TCM pattern diagnosis.

Competing Interests

The authors declare that they have no competing interests, including financial or nonfinancial interests from the funders.

Authors' Contributions

Marcus Gadau, Shi-Ping Zhang, Wing-Fai Yeung, Zhao-Xiang Bian, and Ai-Ping Lu were responsible for the conception and the design of this study and the revision of the paper. Marcus Gadau, Shi-Ping Zhang, and Wing-Fai Yeung formed the research team and generated the initial questionnaire. Marcus Gadau conducted the Delphi survey and collected the questionnaires. Marcus Gadau, Shi-Ping Zhang, and Wing-Fai Yeung discussed survey results and provided feedback to the expert panel. Marcus Gadau performed the final analysis of the results. Marcus Gadau and Shi-Ping Zhang drafted the paper. All authors reviewed and approved the final paper.

Acknowledgments

The authors wish to thank the following members of the Delphi expert panel who agreed to be identified and participated in one round or both rounds of our survey: Sergio Bangrazi, Amerigo Boriglione, Edward Cheng, Shirley Cheng, Robert Doane, Dong Fang Hao, Lily Lai, Kyleen Lee, Ranic Leung, Hong Li, Wei Hong Li, Aldo Liguori, Stefano Liguori, Xin Lun, Carolyn Michaeil, Elmar Pestel, Filomena Petti, Rinaldo Rinaldi, Bernd Schleifer, Laura Silli, Yuan Sheng Tan, Robert Tronska, Florian Wagner, Sean Walsh, Jun Jun Yang, Xiao Fei Yang, Ann Yao, Chris Zaslawski, and Quan Ming Zhang. Marcus Gadau was supported by the UGC's Hong Kong Ph.D. fellowship scheme 2013/14 (PF12-16701). This study was partially supported by HKBU Faculty Research Grants to Shi-Ping Zhang (FRG1/14-15/056).

References

[1] W. Wang, A. Russell, and Y. Yan, "Traditional Chinese medicine and new concepts of predictive, preventive and personalized medicine in diagnosis and treatment of suboptimal health," *EPMA Journal*, vol. 5, no. 1, article 4, 2014.

[2] M. A. Bruce, M. Sims, S. Miller, V. Elliott, and M. Ladipo, "One size fits all? Race, gender and body mass index among U.S. adults," *Journal of the National Medical Association*, vol. 99, no. 10, pp. 1152–1158, 2007.

[3] A. Zhang, H. Sun, P. Wang, Y. Han, and X. Wang, "Future perspectives of personalized medicine in traditional Chinese medicine: a systems biology approach," *Complementary Therapies in Medicine*, vol. 20, no. 1-2, pp. 93–99, 2012.

[4] C. J. Hogeboom, K. J. Sherman, and D. C. Cherkin, "Variation in diagnosis and treatment of chronic low back pain by traditional Chinese medicine acupuncturists," *Complementary Therapies in Medicine*, vol. 9, no. 3, pp. 154–166, 2001.

[5] G. G. Zhang, B. Bausell, L. Lao, W.-L. Lee, B. Handwerger, and B. Berman, "The variability of TCM pattern diagnosis and herbal prescription on rheumatoid arthritis patients," *Alternative Therapies in Health and Medicine*, vol. 10, no. 1, pp. 58–63, 2004.

[6] G. G. Zhang, W. L. Lee, B. Bausell, L. Lao, B. Handwerger, and B. Berman, "Variability in the Traditional Chinese Medicine (TCM) diagnoses and herbal prescriptions provided by three TCM practitioners for 40 patients with rheumatoid arthritis," *Journal of Alternative and Complementary Medicine*, vol. 11, no. 3, pp. 415–421, 2005.

[7] V. Scheid and H. MacPherson, *Integrating East Asian Medicine Into Contemporary Healthcare*, Churchill Livingstone, London, UK, 2012.

[8] S. Pritzker and K. K. Hui, "Building an evidence-base for tcm and integrative east-west medicine: a review of recent developments in innovative research design," *Journal of Traditional and Complementary Medicine*, vol. 2, no. 3, pp. 158–163, 2012.

[9] E. Allander, "Prevalence, incidence, and remission rates of some common rheumatic diseases or syndromes," *Scandinavian Journal of Rheumatology*, vol. 3, no. 3, pp. 145–153, 1974.

[10] M. Gadau, W.-F. Yeung, H. Liu et al., "Acupuncture and moxibustion for lateral elbow pain: A systematic review of randomized controlled trials," *BMC Complementary and Alternative Medicine*, vol. 14, article 136, 2014.

[11] World Health Organization, *WHO International Standard Terminologies on Traditional Medicine in the Western Pacific Region*, World Health Organization, Geneva, Switzerland, 1st edition, 2007.

[12] M. Adler and E. Ziglio, *Gazing into the Oracle: The Delphi Method and Its Application to Social Policy and Public Health*, Jessica Kingsley, London, UK, 1996.

[13] H. P. McKenna, "The Delphi technique: a worthwhile research approach for nursing?" *Journal of Advanced Nursing*, vol. 19, no. 6, pp. 1221–1225, 1994.

[14] T. Sumsion, "The Delphi technique: an adaptive research tool," *British Journal of Occupational Therapy*, vol. 61, no. 4, pp. 153–156, 1998.

[15] B. Green, M. Jones, D. Hughes, and A. Williams, "Applying the Delphi technique in a study of GPs' information requirements," *Health and Social Care in the Community*, vol. 7, no. 3, pp. 198–205, 1999.

[16] J. Crisp, D. Pelletier, C. Duffield, A. Adams, and S. Nagy, "The Delphi method?" *Nursing Research*, vol. 46, no. 2, pp. 116–118, 1997.

[17] F. Hasson, S. Keeney, and H. McKenna, "Research guidelines for the Delphi survey technique," *Journal of Advanced Nursing*, vol. 32, no. 4, pp. 1008–1015, 2000.

[18] C. A. Smith, C. J. Zaslawski, Z. Zheng et al., "Development of an instrument to assess the quality of acupuncture: results from

a Delphi process," *Journal of Alternative and Complementary Medicine*, vol. 17, no. 5, pp. 441–452, 2011.

[19] J.-D. Lin, L.-L. Chen, J.-S. Lin, C.-H. Chang, Y.-C. Huang, and Y.-C. Su, "BCQ-: a body constitution questionnaire to assess Yin-Xu. Part I: establishment of a provisional version through a Delphi process," *Forschende Komplementarmedizin*, vol. 19, no. 5, pp. 234–241, 2012.

[20] C. A. Smith, S. Grant, J. Lyttleton, and S. Cochrane, "Using a Delphi consensus process to develop an acupuncture treatment protocol by consensus for women undergoing Assisted Reproductive Technology (ART) treatment," *BMC Complementary and Alternative Medicine*, vol. 12, article 88, 2012.

[21] T. Alraek, E. Borud, and A. White, "Selecting acupuncture treatment for hot flashes: a Delphi consensus compared with a clinical trial," *Journal of Alternative and Complementary Medicine*, vol. 17, no. 1, pp. 33–38, 2011.

[22] P. Webster-Harrison, A. White, and J. Rae, "Acupuncture for tennis elbow: an e-mail consensus study to define a standardised treatment in a GPs' surgery," *Acupuncture in Medicine*, vol. 20, no. 4, pp. 181–185, 2002.

[23] C.-C. Hsu and B. A. Sandford, "The Delphi technique: making sense of consensus," *Practical Assessment, Research and Evaluation*, vol. 12, no. 10, pp. 1–8, 2007.

[24] A. Habibi, A. Sarafrazi, and S. Izadyar, "Delphi technique theoretical framework in qualitative research," *The International Journal of Engineering and Science*, vol. 3, no. 4, pp. 8–13, 2014.

[25] H. P. McKenna, "The essential elements of a practitioners' nursing model: a survey of psychiatric nurse managers," *Journal of Advanced Nursing*, vol. 19, no. 5, pp. 870–877, 1994.

[26] S. Keeney, F. Hasson, and H. McKenna, "Consulting the oracle: ten lessons from using the Delphi technique in nursing research," *Journal of Advanced Nursing*, vol. 53, no. 2, pp. 205–212, 2006.

[27] A. L. Delbecq, A. H. Van de Ven, and D. H. Gustafson, *Group Techniques for Program Planning*, Scott Foresman, Glenview, Ill, USA, 1975.

[28] M. M.-K. Poon, K.-F. Chung, W.-F. Yeung, V. H.-K. Yau, and S.-P. Zhang, "Classification of insomnia using the traditional Chinese medicine system: a systematic review," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 735078, 9 pages, 2012.

[29] M. Kim, D. Cobbin, and C. Zaslawski, "Traditional Chinese medicine tongue inspection: an examination of the inter- and intrapractitioner reliability for specific tongue characteristics," *Journal of Alternative and Complementary Medicine*, vol. 14, no. 5, pp. 527–536, 2008.

[30] R. N. Schnyer, L. A. Conboy, E. Jacobson et al., "Development of a Chinese medicine assessment measure: an interdisciplinary approach using the Delphi method," *Journal of Alternative and Complementary Medicine*, vol. 11, no. 6, pp. 1005–1013, 2005.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 6153215, 8 pages
http://dx.doi.org/10.1155/2016/6153215

Research Article

MAPK Pathways Are Involved in Neuropathic Pain in Rats with Chronic Compression of the Dorsal Root Ganglion

Yu-Juan Qu, Lei Jia, Xiao Zhang, Hui Wei, and Shou-Wei Yue

Department of Physical Medicine & Rehabilitation, Qilu Hospital, Shandong University, Jinan, Shandong 250012, China

Correspondence should be addressed to Shou-Wei Yue; drg98@hotmail.com

Received 21 December 2015; Accepted 28 June 2016

Academic Editor: Haroon Khan

The aim of the present study was to investigate whether the MAPK pathways were involved in the mechanism of neuropathic pain in rats with chronic compression of the dorsal root ganglion. We determined the paw withdrawal mechanical threshold (PWMT) of rats before and after CCD surgery and then after p38, JNK, or ERK inhibitors administration. Western blotting, RT-PCR, and immunofluorescence of dorsal root ganglia were performed to investigate the protein and mRNA level of MAPKs and also the alternation in distributions of positive neurons in dorsal root ganglia. Intrathecal administration of MAPKs inhibitors, SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and U0126 (ERK inhibitor), resulted in a partial reduction in CCD-induced mechanical allodynia. The reduction of allodynia was associated with significant depression in the level of both MAPKs mRNA and protein expression in CCD rats and also associated with the decreased ratios of large size MAPKs positive neurons in dorsal root ganglia. In conclusion, the specific inhibitors of MAPKs contributed to the attenuation of mechanical allodynia in CCD rats and the large size MAPKs positive neurons in dorsal root ganglia were crucial.

1. Introduction

Neuropathic pain caused by lesion or inflammation results from the dysfunction and derangement in transmission and signal processing within the nervous system. It is characterized by the symptoms of allodynia, hyperalgesia, and spontaneous pain [1, 2] and it does not depend on the continued presence of tissue-damaging stimuli and is recognized as a disease in itself [3]. Chronic compression of the dorsal root ganglia (CCD) in rats is a typical model of neuropathic pain. During the formation and development of neuropathic pain, inflammation is inevitable. Pain and hyperalgesia that are produced by tissue damage or infection are common features of the inflammatory process [4]. Evidence demonstrates that a substantial proportion of mediators are involved in the symptoms of neuropathic pain, including cytokines, bradykinin, ATP and adenosine, serotonin, eicosanoids, and neurotrophins [1]. Kinds of drugs are used to alleviate neuropathic pain, but they exhibit limited efficacy and undesirable side effects, and neuropathic pain responds poorly to such drug treatments [5].

Mitogen-activated protein kinases (MAPKs), including p38 mitogen-activated protein kinase (p38), c-Jun N-terminal kinase (JNK), and extracellular-regulated kinase (ERK), are a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular posttranslational and transcriptional responses. A variety of extracellular stimuli activate intracellular MAPKs by phosphorylation, which modulates the intracellular responses that drive different downstream signaling [6]. It is well established that MAPK activation mechanisms are involved in the modulation of nociceptive information and the peripheral and central sensitization produced by intense noxious stimuli [7–12]. Several studies have demonstrated that MAPK pathways play essential roles in inflammation and tissue remodeling [13, 14], and the inhibition of MAPKs produces anti-inflammatory effects in various inflammatory diseases [13]. MAPKs belong to a highly conserved family of serine/threonine protein kinases and are well known to be involved in various aspects of cell signaling and gene expression in the central nervous system (CNS) [15]. MAPKs are thought to be involved in the modulation of inflammation-induced pain hyperalgesia in

DRGs and the spinal cord [16]. When the physiopathological mechanisms of inflammatory pain have been studied in patients with amputation neuroma, spinal cord injury, or other causes of neuropathic pain, the mitogen-activated protein kinases (MAPKs) have been found to play a critical role. The phosphorylated forms of these kinases maintain and increase pain signals from the peripheral nociceptors or DRGs by posttranslationally modifying proteins and regulating the transcription of critical genes.

It is demonstrated that specific members of the MAPK family might mediate pain-associated spatial and temporal plasticity in the HF; for example, the local injection of MAPK inhibitors significantly depresses thermal and mechanical hyperalgesia [10, 17–19]. Following peripheral nerve injury, ERK and p38 MAPK are activated and their expression levels are increased in the spinal dorsal horns [7, 10, 20]. There is also evidence supporting that p38 reduces pain by inhibiting p38 phosphorylation via decreasing TNF-α [21]. Additionally, JNK signaling plays a crucial role in mediating antinociception and chronic tolerance to the antinociceptive effects of morphine in acute, inflammatory, and neuropathic pain states [22]. The spinal activation mechanisms of MAPK signaling pathways in both neurons and microglia are involved in the antinociceptive effects of pregabalin in a zymosan-induced peripheral inflammatory pain model [23].

Notwithstanding these reports, the underlying role of MAPKs in CCD rats remains unexplored with modern techniques. In the present study, we thus assessed the effects of MAPKs inhibitors in gene and protein expressions and cellular distribution in DRGs and also their effects on allodynia in CCD rats.

2. Materials and Methods

2.1. Animals and Surgical Procedure. Adult male Wistar rats weighing 180–220 g were provided by the Experimental Animal Center of Shandong University and were housed in a pathogen-free air room at a temperature of 20 ± 2°C at two per cage on a 12 h light/dark cycle with water and food available ad libitum. The animals were allowed 7 days to habituate to the housing prior to manipulation and half an hour to habituate to the experimental environment before every behavioral study was performed. All experimental procedures were approved by the Animal Care and Use Committee of the Shandong University.

Rats were anesthetized by 10% chloral hydrate (300 mg/100 g i.p.), and then two stainless steel rods were implanted unilaterally into the intervertebral foramen at L4 and L5 [24, 25]. Rats in sham-operation group underwent the same operation but with no steel bar insertion. The rats with autophagy phenomenon, feeling deficiency, and disability were eliminated.

2.2. Behavioral Testing. Behavioral testing was performed using the ipsilateral hind paw of the animals prior to the operation, on postoperative day 4, and 2 hours after the injection of inhibitors. The paw withdrawal mechanical thresholds (PWMTs) were evaluated with a BME-404 Mechanical Analgesia Tester (CAMS-Chinese Academy of Medical Sciences, Beijing, China) [25]. The probe was pressed against the lateral plantar surface of the hind paw with sufficient force. A positive response was noted when the paw was immediately withdrawn. The rats were tested again at least five minutes later, the tests were repeated five times, and the average was calculated and used in the statistical analyses.

2.3. Western Blot Analysis. Four days after surgery, CCD rats were intrathecally injected with MAPKs inhibitors for 2 h. The L4 and L5 ganglia from the operated side were quickly and carefully harvested. The samples of total protein were separated by sequential 5% and 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The membranes were incubated in 5% milk for 2 h at room temperature. Next, the membranes were incubated with primary antibody at 4°C overnight and subsequently with horseradish peroxides- (HRP-) conjugated secondary antibodies for 1 h. The signals were detected with Immobilon™ Western Chemiluminescent HRP Substrate. The primary antibodies were rabbit anti-ERK polyclonal antibody (1 : 1,000, CST, USA), rabbit anti-p-ERK polyclonal antibody (1 : 2,000, CST, USA), rabbit anti-JNK polyclonal antibody (1 : 1,000, CST, USA), rabbit anti-p-JNK polyclonal antibody (1 : 1,000, CST, USA), rabbit anti-p38 polyclonal antibody (1 : 200, CST, USA), and rabbit anti-P-p38 polyclonal antibody (1 : 1,000, CST, USA). The second antibody was goat-anti-rabbit antibody (1 : 8,000, Zhongshan Golden Bridge, Beijing, China). The protein bands were developed with a FluoroChem 9900 imaging system (USA), and the quantifications of the intensities of the bands were performed with the Quantity One software and normalized to β-tubulin (1 : 1,000, CST, USA).

2.4. Immunolocalization of p38, ERK, and JNK in Dorsal Root Ganglia. Rats were deeply anesthetized with 5% isoflurane and perfused transcardially with cold normal saline followed by fixative containing 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 6.9). The ipsilateral lumbar L4-L5 DRGs were removed rapidly after perfusion, postfixed in the same fixative overnight at 4°C, and then dehydrated and paraffin-infused. A series of paraffin sections (4 μm) were cut using a rotary microtome. The sections were incubated separately in mixtures of the following primary antibodies at 4°C overnight: rabbit anti-ERK polyclonal antibody (1 : 200, CST, USA), rabbit anti-JNK polyclonal antibody (1 : 200, CST, USA), and rabbit anti-p38 polyclonal antibody (1 : 50, CST, USA). The primary antibodies were combined with mouse-anti-NF200 polyclonal antibody (1 : 1,000, Abcam, Cambridge, UK). Then the sections were incubated in Alexa Fluor 488-conjugated Affinipure Donkey Anti-Rabbit IgG (H+L) and Alexa Fluor 594-conjugated Donkey Anti-Mouse IgG (H+L) for 2 h at room temperature. DAPI was used to stain the cell nuclei.

Labeled sections were examined under an Olympus-u-rfl-t/dp 72 automatic fluorescence microscope using the image analysis system of the microscope (JA) and analyzed using the IPP.6 software. For the quantitative analyses of

the numbers of positive neurons, three immunofluorescent stained nonconsecutive sections were imaged per ganglion. The data were collected from three animals for each inhibitor (SB203580, U0126, and SP600125).

2.5. Real-Time Quantitative RT-PCR. L4 and L5 ganglions were harvested in the same manner as described above. Fragments of p38, JNK, ERK, or β-actin were amplified with the following primers: p38 (forward, 5′-CCTGCGAGGGCTGAAGTA-3′; reverse, 5′-ACGGACCAAATATCCACTGTCT-3′), JNK (forward, 5′-AGCCTTGTCCTTCGTGTC-3′; reverse, 5′-AAAGTGGTCAACAGAGCC-3′), ERK1 (forward, 5′-CCAGAGTGGCTATCAAGAAG-3′; reverse, 5′-TCCATGAGGTCCTGAACAA-3′), ERK2 (forward, 5′-TGCCGTGGAACAGGTTGT-3′; reverse, 5′-TGGGCTCATCACTTGGGT-3′), and β-actin (forward, 5′-AGACCTTCAACACCCCAG-3′; reverse, 5′-CACGATTTCCCTCTCAGC-3′). Instrument control, automated data collection, and data analysis were all performed using the Light Cycler software program, version 4.0. The $2^{-\Delta\Delta CT}$ method was used to analyze the data.

2.6. Chemicals and Reagents. The following chemicals were used in this study: SB203580 (p38 inhibitor, CST, USA, recommended concentration = 40 μmol/L), SP600125 (JNK inhibitor, CST, USA, recommended concentration = 50 μmol/L), and U0126 (ERK inhibitor, CST, USA, recommended concentration = 40 μmol/L). All of the chemicals were dissolved in DMSO, and the final experimental dilutions were made in normal saline on the day of the experiment.

2.7. Data Analysis and Statistics. All calculations and statistical analyses were performed using Prism 5.0 (Graph Pad Software, San Diego, CA, USA). A two-way repeated measures ANOVA was used to analyze the differences in the PWLs, the level of protein and gene expression, and the neurons distribution. Values in the test were expressed as means ± SDs. P values < 0.05 were considered significant.

3. Results

3.1. PWMT Changes after the CCD Operation. To detect whether the inhibitors of MAPKs attenuated CCD-induced neuropathic pain, PWMTs were examined before surgery, 4 days after surgery, and 2 h after inhibitors administration. As shown in Figure 1, the CCD group developed evident mechanical allodynia hyperalgesia in the ipsilateral hind paw compared with the control group. The PWMT significantly decreased at 4 days after the CCD operation ($n = 8$ in each group; $^{**}P < 0.01$). Furthermore, CCD-induced allodynia was attenuated by SB203580, SP00125, and U0126 ($n = 8$ in each group; $^{\#}P < 0.05$), while there was no significant difference between sham group and control group ($n = 8$ in each group).

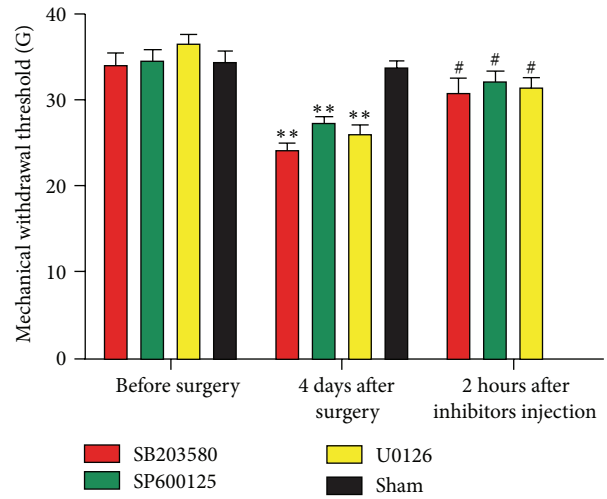


Figure 1: Alternations in PWMTs. $^{**}P < 0.01$ compared with control group; $n = 8$ in each group; $^{\#}P < 0.05$ compared with the CCD groups; $n = 8$ in each group.

3.2. Changes in Protein Expressions of p38, ERK, and JNK in the DRGs. To investigate whether p38, ERK, and JNK expression and phosphorylation were altered, pharmacological inhibitors were administered to the CCD rats. As demonstrated in Figure 2, the levels of p38, JNK, ERK, P-p38, P-JNK, and P-ERK protein expression in the CCD rats significantly increased ($n = 5$, $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with sham group). These CCD-induced increases in protein expression level were diminished significantly by inhibitors (SB203580, SP600125, and U0126) administration ($^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ compared with CCD groups).

3.3. Changes in Gene Expressions of p38, ERK, and JNK in the DRGs. Pharmacological inhibitors of MAPKs not only diminished the protein expression of p38, ERK, and JNK in DRGs of CCD rats but also affected the level of gene expressions. As demonstrated in Figure 3, the levels of p38, JNK, and ERK gene expression in the CCD rats significantly increased ($n = 6$ and $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group). These CCD-induced increases in gene expression level were diminished significantly by inhibitors (SB203580, SP600125, and U0126) administration ($n = 6$ and $^{\#\#}P < 0.01$ compared with CCD groups).

3.4. Changes in p38, ERK, and JNK Distributions in DRG Neurons. To quantify the proportions of positive cells within defined subsets of sensory neurons, we counted the numbers of positive neurons detected in the NF200-immunoreactive neuronal profiles.

As demonstrated in Figures 4(a)–4(c), p38, JNK, and ERK were expressed in both the nuclei and cytoplasm; the proportions of NF200 positive large size neurons among all of the neurons in the DRG tissues increased significantly ($n = 6$ and $^{*}P < 0.05$ and $^{**}P < 0.01$) after CCD surgery compared with control groups. After SB203580, SP600125, or U0126 administration, the proportions of NF200 positive neurons significantly decreased ($n = 6$ and $^{\#}P < 0.05$ and

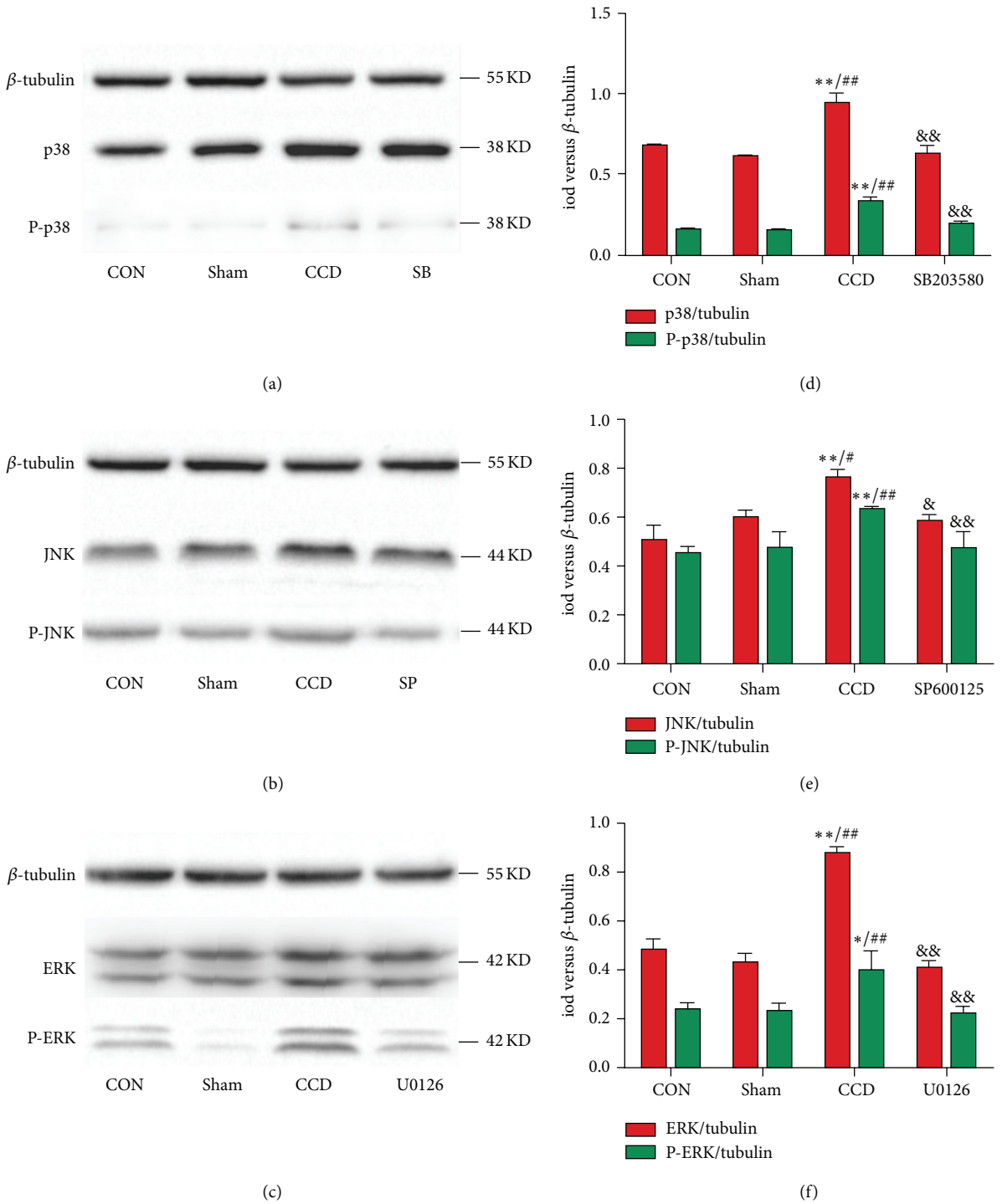


Figure 2: Effects of the inhibitors on protein expressions of p38, JNK, and ERK and their phosphorylation. (a)–(c) show the expressions of p38, P-p38, JNK, P-JNK, ERK, and P-ERK after SB203580, U0126, and SP600125 administration, and (d)–(f) show the iods compared with β-tubulin. $^{*}P < 0.05$, $^{**}P < 0.01$, and $n = 5$ for each group compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the sham group; $n = 5$ in each group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ compared with the CCD group; $n = 5$ in each group.

$^{\#\#}P < 0.01$ compared with CCD group). As to NF200 negative small size neurons, though there were some changes in proportion, we could not find any obvious regulation of these changes.

4. Discussion

This is the first study showing the role of MAPK pathways in neuropathic pain in DRGs of CCD rats. Intrathecal administration of the MAPKs inhibitors, SB203580,

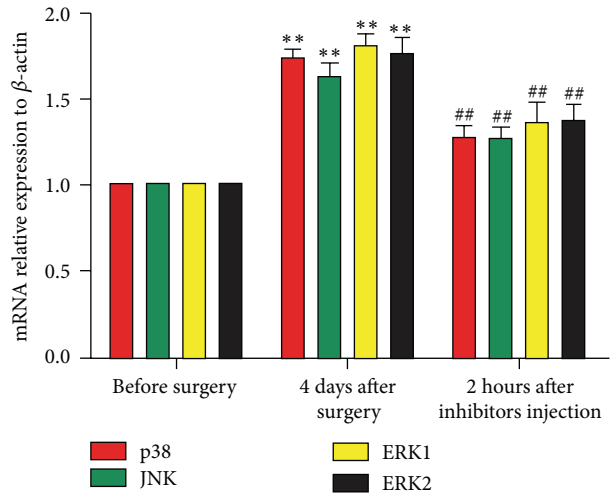


FIGURE 3: Relative expression levels of p38, JNK, and ERK mRNA in DRGs. $n = 6$ in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the normal rats. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with CCD rats.

SP600125, and U0126, resulted in a partial reduction in CCD-induced mechanical allodynia. The reduction of allodynia was associated with significant depression in the level of both MAPKs gene and protein expression in CCD rats, and the large size MAPKs positive neurons in dorsal root ganglia were crucial in maintaining the neuropathic pain.

The CCD model has been proven to be a typical model of neuropathic pain. In CCD rats, the direct mechanical compression of the DRG [26] and secondary inflammatory processes [27] induce hyperexcitability of the DRG neurons, and this hyperexcitability is associated with allodynia and changes in the lower paw withdrawal latency after CCD surgery. Using specific inhibitors of MAPKs contributed to the attenuation of mechanical allodynia in CCD rats. MAPK pathways may be new targets of neuropathic pain treatment.

In the CNS, the activation of the p38 MAPK pathway constitutes a key step in the development of neuroinflammation. Inflammatory stimuli bind to receptors on the cell surface to trigger intracellular signal transduction pathways, such as the p38 MAPK pathways [28, 29]. Subsequently, intracellular p38 MAPK is activated and profoundly modulates somatic inflammatory responses. The expression of ERK in the DRGs has been implicated in the induction of neuropathic pain behaviors in rat models of chronic constriction injury (CCI) and the normalization of those behaviors after decompression of the CCI reflects the reversal of the pain behaviors [30]. The expression of JNK is also activated in the spinal DRG after nerve injury, and this expression of p-JNK can maintain mechanical allodynia [31]. Inhibitors of MAPKs administration in recommendatory dose and time specifically decreased the upregulated protein expression of MAPKs (p38 and P-p38 by SB203580, JNK and P-JNK by SP600125, and ERK and P-ERK by U0126) in CCD rats. Similar changes were found in the gene expression of p38, JNK, and ERK. However, the dose/time dependence of these inhibitors may require further analysis.

Neurons in DRGs are divided into three types (large: >35 μm with Aβ fiber; middle: 20–35 μm with Aδ fiber; small: <20 μm with C fiber), mainly depending on their size, electrophysiological property, and neuronal processes [32]; generally, Aβ fiber conducts proprioception and tactile sense, C fiber conducts nociception signal, and Aδ fiber conducts both. The proportions of NF200 positive large size neurons among the p38, JNK, or ERK positive neurons in the DRG tissues increased significantly after CCD surgery; then the proportions were decreased by SB203580, SP600125, or U0126 administration, while the proportions of NF200 negative small size neurons change without explicable regulation. Therefore, the large size neurons with Aβ fiber contributed mainly to the MAPKs mediated neuropathic pain in CCD rats.

5. Conclusions

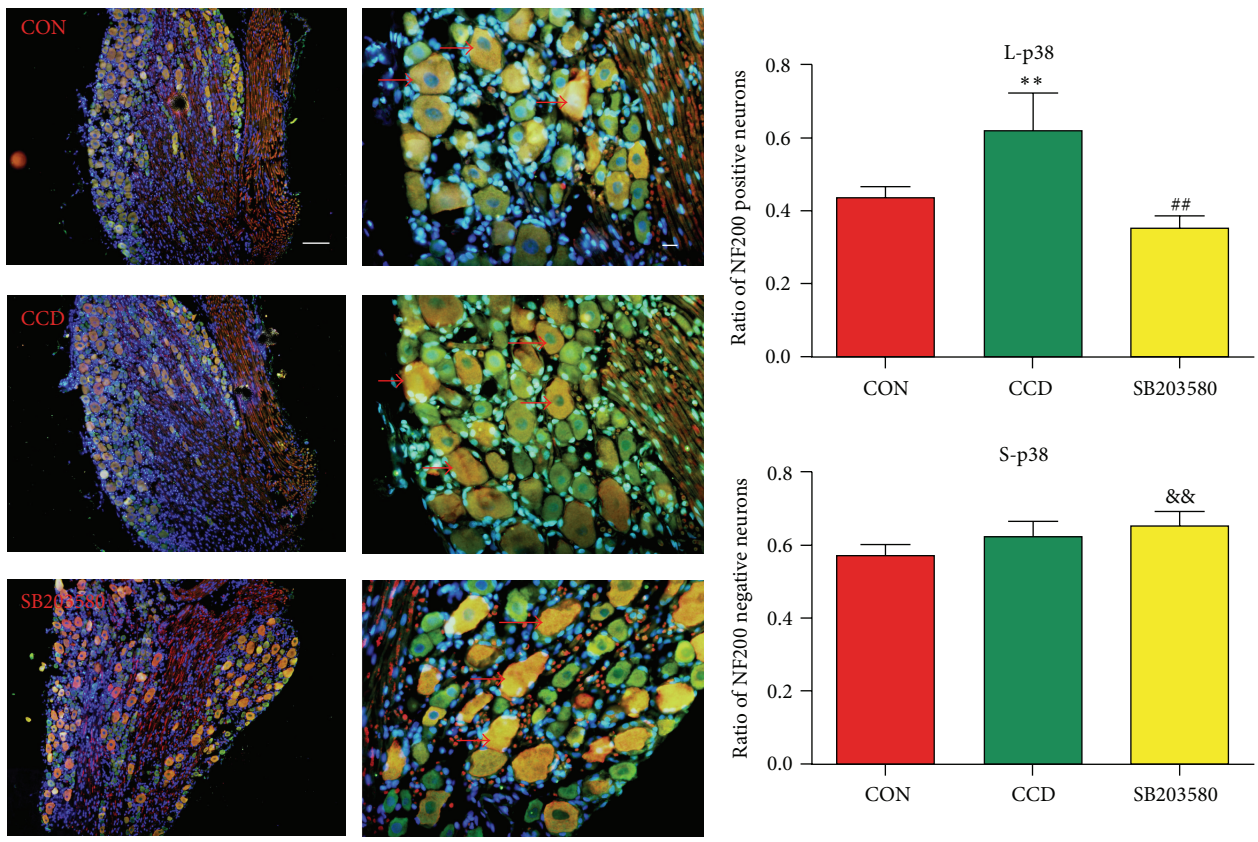
In conclusion, the present study demonstrated that specific inhibitors of MAPKs contributed to the attenuation of mechanical allodynia in CCD rats and the large size MAPKs positive neurons in dorsal root ganglia were crucial. Therefore, MAPK pathways are involved in the mechanism of neuropathic pain in CCD rats.

Competing Interests

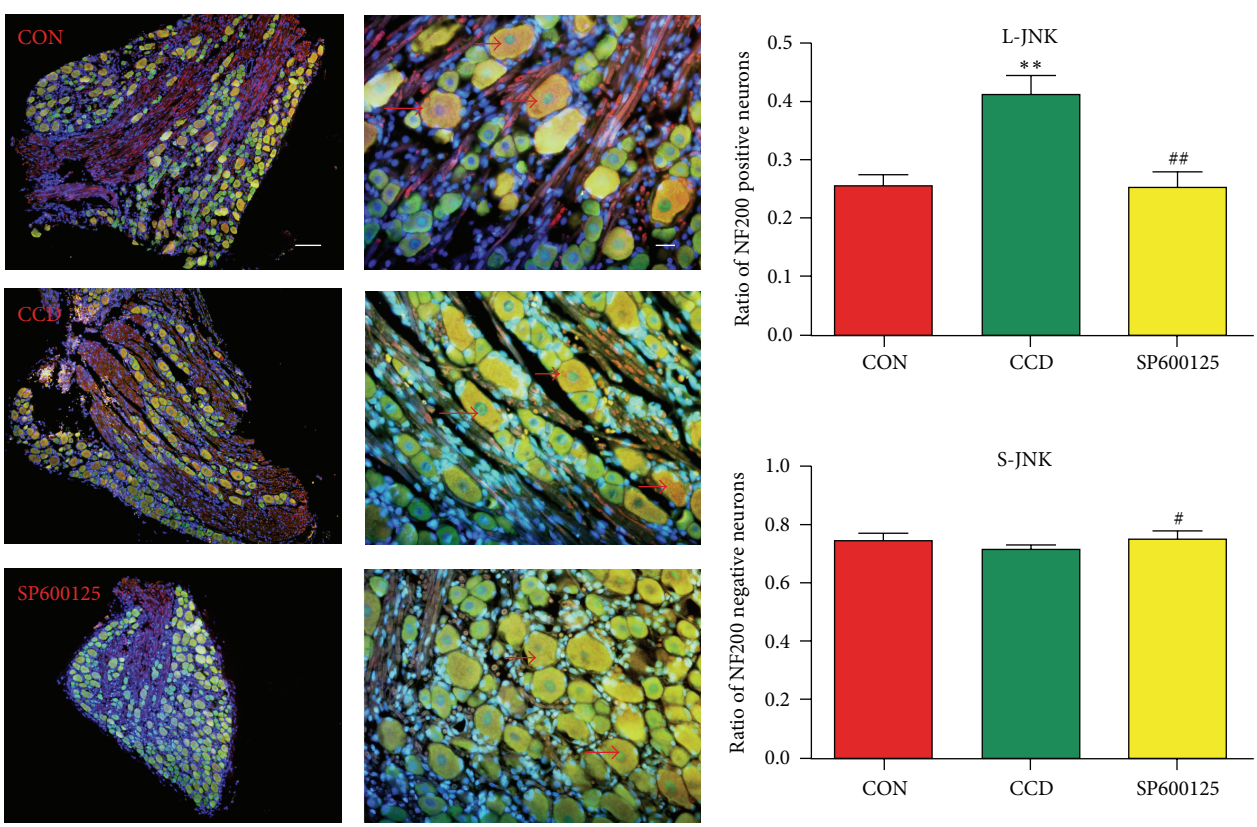
The authors declare no conflict of interests.

Authors' Contributions

Yu-Juan Qu and Shou-Wei Yue conceived and designed the experiments. Yu-Juan Qu, Lei Jia, and Xiao Zhang performed the experiments. Yu-Juan Qu and Lei Jia analyzed the data. Yu-Juan Qu, Xiao Zhang, and Hui Wei interpreted the results. Yu-Juan Qu and Shou-Wei Yue wrote the paper.

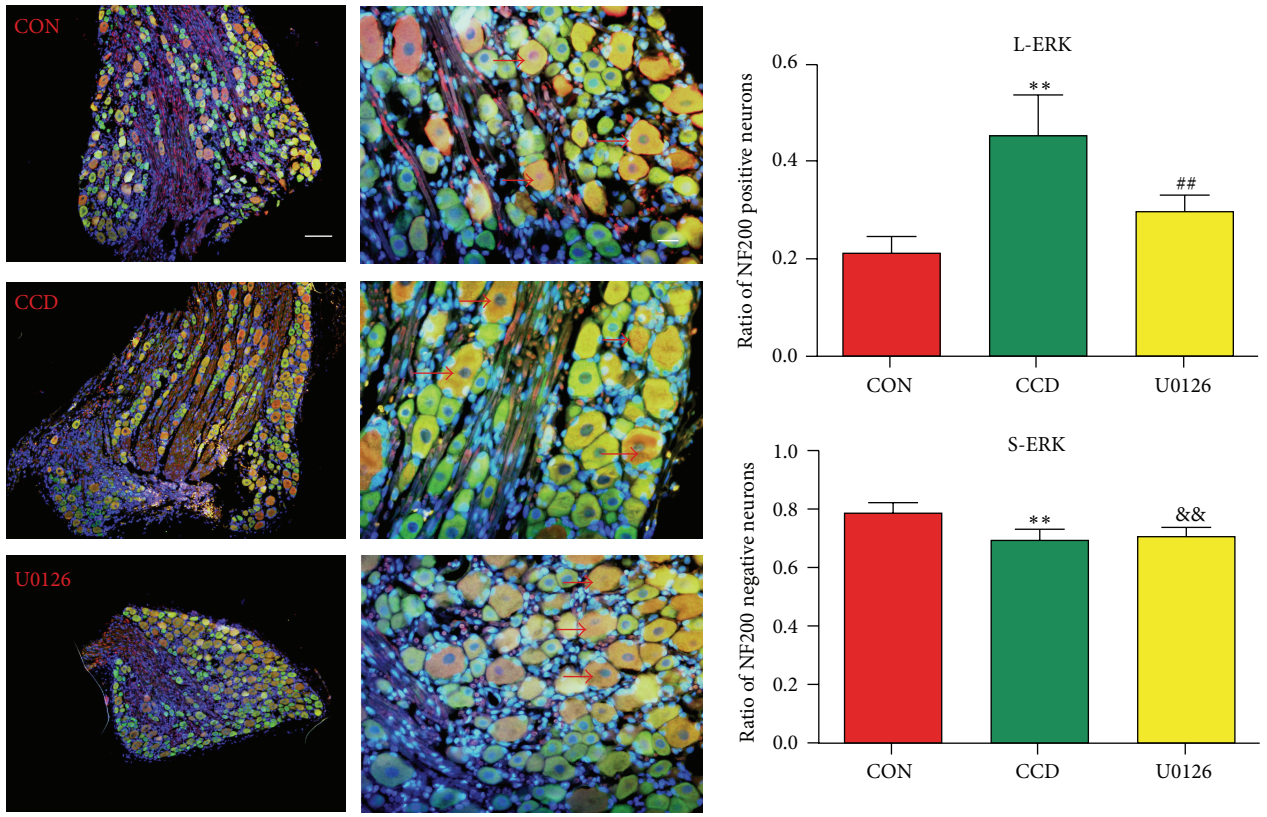


(a)



(b)

Figure 4: Continued.



(c)

FIGURE 4: Changes in the distributions of p38, JNK, or ERK/NF200 positive or negative neurons in the DRG tissue. (a)–(c) illustrate the neuronal distributions and ratios of the NF200 positive or negative neurons. $N = 6$; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the CCD group and $^{\&\&}P < 0.01$ compared with the control group. Scale bars: 100 μm for the low-power field and 20 μm for high-power field (→: NF200 positive neurons).

Acknowledgments

This study was supported by National Natural Science Foundation of China (Grant no. 81372123). All animal experiments were performed at the Animal Care Center of the Key Laboratory of Cardiovascular Remodeling and Functional Research of Shandong University (Jinan, Shandong, China).

References

[1] K. Hargreaves, R. Dubner, F. Brown, C. Flores, and J. Joris, "A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia," *Pain*, vol. 32, no. 1, pp. 77–88, 1988.

[2] I. Tegeder, E. Niederberger, R. Schmidt et al., "Specific inhibition of IκB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats," *The Journal of Neuroscience*, vol. 24, no. 7, pp. 1637–1645, 2004.

[3] M. Gangadhar, R. K. Mishra, D. Sriram, and P. Yogeeswari, "Future directions in the treatment of neuropathic pain: a review on various therapeutic targets," *CNS and Neurological Disorders—Drug Targets*, vol. 13, no. 1, pp. 63–81, 2014.

[4] F. V. Hassani, R. Rezaee, H. Sazegara, M. Hashemzaei, K. Shirani, and G. Karimi, "Effects of silymarin on neuropathic pain and formalin-induced nociception in mice," *Iranian Journal of Basic Medical Sciences*, vol. 18, no. 7, pp. 715–720, 2015.

[5] W. S. Kingery, "A critical review of controlled clinical trials for peripheral neuropathic pain and complex regional pain syndromes," *Pain*, vol. 73, no. 2, pp. 123–139, 1997.

[6] A. G. Turjanski, J. P. Vaqué, and J. S. Gutkind, "MAP kinases and the control of nuclear events," *Oncogene*, vol. 26, no. 22, pp. 3240–3253, 2007.

[7] R.-R. Ji, H. Baba, G. J. Brenner, and C. J. Woolf, "Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity," *Nature Neuroscience*, vol. 2, no. 12, pp. 1114–1119, 1999.

[8] S.-X. Jin, Z.-Y. Zhuang, C. J. Woolf, and R.-R. Ji, "p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain," *The Journal of Neuroscience*, vol. 23, no. 10, pp. 4017–4022, 2003.

[9] Y. Kawasaki, T. Kohno, Z.-Y. Zhuang et al., "Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization," *The Journal of Neuroscience*, vol. 24, no. 38, pp. 8310–8321, 2004.

[10] K. Obata and K. Noguchi, "MAPK activation in nociceptive neurons and pain hypersensitivity," *Life Sciences*, vol. 74, no. 21, pp. 2643–2653, 2004.

[11] X.-S. Song, J.-L. Cao, Y.-B. Xu, J.-H. He, L.-C. Zhang, and Y.-M. Zeng, "Activation of ERK/CREB pathway in spinal cord contributes to chronic constrictive injury-induced neuropathic pain in rats," *Acta Pharmacologica Sinica*, vol. 26, no. 7, pp. 789–798, 2005.

[12] R.-R. Ji, R. W. T. Gereau, M. Malcangio, and G. R. Strichartz, "MAP kinase and pain," *Brain Research Reviews*, vol. 60, no. 1, pp. 135–148, 2009.

[13] K. Newton and V. M. Dixit, "Signaling in innate immunity and inflammation," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 3, 2012.

[14] S. Khan, O. Shehzad, H.-G. Jin et al., "Anti-inflammatory mechanism of 15,16-epoxy-3α-hydroxylabda-8,13(16),14-trien-7-one via inhibition of LPS-induced multicellular signaling pathways," *Journal of Natural Products*, vol. 75, no. 1, pp. 67–71, 2012.

[15] M. Koistinaho and J. Koistinaho, "Role of p38 and p44/42 mitogen-activated protein kinases in microglia," *Glia*, vol. 40, no. 2, pp. 175–183, 2002.

[16] R.-R. Ji, K. Befort, G. J. Brenner, and C. J. Woolf, "ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity," *The Journal of Neuroscience*, vol. 22, no. 2, pp. 478–485, 2002.

[17] M.-G. Liu, R.-R. Wang, X.-F. Chen, F.-K. Zhang, X.-Y. Cui, and J. Chen, "Differential roles of ERK, JNK and p38 MAPK in pain-related spatial and temporal enhancement of synaptic responses in the hippocampal formation of rats: multi-electrode array recordings," *Brain Research*, vol. 1382, pp. 57–69, 2011.

[18] D. C. Choi, J. Y. Lee, E. J. Lim, H. H. Baik, T. H. Oh, and T. Y. Yune, "Inhibition of ROS-induced p38MAPK and ERK activation in microglia by acupuncture relieves neuropathic pain after spinal cord injury in rats," *Experimental Neurology*, vol. 236, no. 2, pp. 268–282, 2012.

[19] J. A. Black, L. Nikolajsen, K. Kroner, T. S. Jensen, and S. G. Waxman, "Multiple sodium channel isoforms and mitogen-activated protein kinases are present in painful human neuromas," *Annals of Neurology*, vol. 64, no. 6, pp. 644–653, 2008.

[20] L. D. Cesare Mannelli, C. Ghelardini, A. Toscano, A. Pacini, and A. Bartolini, "The neuropathy-protective agent acetyl-l-carnitine activates protein kinase C-γ and MAPKs in a rat model of neuropathic pain," *Neuroscience*, vol. 165, no. 4, pp. 1345–1352, 2010.

[21] C. K. F. Lima, R. M. Silva, R. B. Lacerda et al., "LASSBio-1135: a dual TRPV1 antagonist and anti-TNF-alpha compound orally effective in models of inflammatory and neuropathic pain," *PLoS ONE*, vol. 9, no. 6, Article ID e99510, 2014.

[22] D. J. Marcus, M. Zee, A. Hughes et al., "Tolerance to the antinociceptive effects of chronic morphine requires c-Jun N-terminal kinase," *Molecular Pain*, vol. 11, no. 1, article 34, 2015.

[23] C. J. Young, P. Kihyun, and B. K. Young, "Inhibition of mitogen-activated protein kinases phosphorylation plays an important role in the anti-nociceptive effect of pregabalin in zymosan-induced inflammatory pain model," *Biological and Pharmaceutical Bulletin*, vol. 37, no. 10, pp. 1694–1698, 2014.

[24] X.-L. Ding, Y.-H. Wang, L.-P. Ning et al., "Involvement of TRPV4-NO-cGMP-PKG pathways in the development of thermal hyperalgesia following chronic compression of the dorsal root ganglion in rats," *Behavioural Brain Research*, vol. 208, no. 1, pp. 194–201, 2010.

[25] Y. Zhang, Y.-H. Wang, H.-Y. Ge, L. Arendt-Nielsen, R. Wang, and S.-W. Yue, "A transient receptor potential vanilloid 4 contributes to mechanical allodynia following chronic compression of dorsal root ganglion in rats," *Neuroscience Letters*, vol. 432, no. 3, pp. 222–227, 2008.

[26] X.-J. Song, D.-S. Xu, C. Vizcarra, and R. L. Rupert, "Onset and recovery of hyperalgesia and hyperexcitability of sensory neurons following intervertebral foramen volume reduction and restoration," *Journal of Manipulative and Physiological Therapeutics*, vol. 26, no. 7, pp. 426–436, 2003.

[27] C. Ma, K. W. Greenquist, and R. H. LaMotte, "Inflammatory mediators enhance the excitability of chronically compressed dorsal root ganglion neurons," *Journal of Neurophysiology*, vol. 95, no. 4, pp. 2098–2107, 2006.

[28] K. Ono and J. Han, "The p38 signal transduction pathway: activation and function," *Cellular Signalling*, vol. 12, no. 1, pp. 1–13, 2000.

[29] S. Kumar, J. Boehm, and J. C. Lee, "p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases," *Nature Reviews Drug Discovery*, vol. 2, no. 9, pp. 717–726, 2003.

[30] T.-J. Tseng, Y.-L. Hsieh, and S.-T. Hsieh, "Reversal of ERK activation in the dorsal horn after decompression in chronic constriction injury," *Experimental Neurology*, vol. 206, no. 1, pp. 17–23, 2007.

[31] Z.-Y. Zhuang, Y.-R. Wen, D.-R. Zhang et al., "A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance," *The Journal of Neuroscience*, vol. 26, no. 13, pp. 3551–3560, 2006.

[32] R. D. Rose, H. R. Koerber, M. J. Sedivec, and L. M. Mendell, "Somal action potential duration differs in identified primary afferents," *Neuroscience Letters*, vol. 63, no. 3, pp. 259–264, 1986.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 1494981, 11 pages
http://dx.doi.org/10.1155/2016/1494981

Research Article

Methanolic Extract of *Clinacanthus nutans* Exerts Antinociceptive Activity via the Opioid/Nitric Oxide-Mediated, but cGMP-Independent, Pathways

Mohammad Hafiz Abdul Rahim,[1] Zainul Amiruddin Zakaria,[1,2] Mohd Hijaz Mohd Sani,[1] Maizatul Hasyima Omar,[3] Yusnita Yakob,[4] Manraj Singh Cheema,[1,2] Siew Mooi Ching,[5] Zuraini Ahmad,[1] and Arifah Abdul Kadir[6]

[1]*Department of Biomedical Science, Faculty of Medicine and Health Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Integrative Pharmacogenomics Institute (iPROMISE), Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Puncak Alam Campus, Level 7, FF3, 42300 Bandar Puncak Alam, Selangor, Malaysia*
[3]*Phytochemistry Unit, Herbal Medicine Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia*
[4]*Molecular Diagnostics and Protein Unit, Specialised Diagnostics Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia*
[5]*Department of Family Medicine, Faculty of Medicine and Health Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[6]*Department of Veterinary Pre-Clinical Sciences, Faculty of Veterinary Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

Correspondence should be addressed to Zainul Amiruddin Zakaria; dr_zaz@yahoo.com

Received 27 December 2015; Accepted 14 March 2016

Academic Editor: Haroon Khan

The objectives of the present study were to determine the mechanisms of antinociceptive effect of methanol extract of *Clinacanthus nutans* (Acanthaceae) leaves (MECN) using various animal nociceptive models. The antinociceptive activity of orally administered 10% DMSO, 100 mg/kg acetylsalicylic acid (ASA), 5 mg/kg morphine, or MECN (100, 250, and 500 mg/kg) was determined using the acetic acid-induced abdominal constriction (ACT), formalin-induced paw licking (FT), and hot plate tests (HPT). The role of opioid and nitric oxide/cyclic guanosine monophosphate (NO/cGMP) systems was also investigated. The results showed that MECN produced a significant ($p < 0.05$) antinociceptive response in all nociceptive models with the recorded ED_{50} value of 279.3 mg/kg for the ACT, while, for the early and late phases of the FT, the value was >500 mg/kg or 227.7 mg/kg, respectively. This antinociceptive activity was fully antagonized by naloxone (a nonselective opioid antagonist) but was partially reversed by L-arginine (L-arg; a nitric oxide [NO] precursor), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; an NO synthase inhibitor), or their combinations thereof. In contrast, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ; a soluble guanylyl cyclase inhibitor) enhanced the extract's antinociception. UHPLC analysis revealed the presence of several flavonoid-based compounds with antinociceptive action. In conclusion, MECN exerted the peripherally and centrally mediated antinociceptive activity via the modulation of the opioid/NO-mediated, but cGMP-independent, systems.

1. Introduction

Opioids, such as morphine, and nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid, are universally used for the treatment of pain. Although treatments for pain have seen rapid progression, particularly in the field of analgesic drug development, their clinical efficacy and tolerability are often surpassed by the accompanied unwanted adverse effects [1]. Therefore, there is a need to look for an alternative approach to treat pain that has fewer or, possibly,

no side effects. Drugs derived from natural sources, especially plants, are vital for the treatment of numerous diseases [2]. The exploration and investigation of plants utilized as pain-relieving agents in traditional ethnomedicine is one of the useful and reasonable strategies in the search for new drugs [3, 4].

Treatment of pain involved the usage of opioids and nonsteroidal anti-inflammatory drugs, and, despite their effectiveness in curing pain, prolonged usage of these classes of drugs has been associated with various unwanted side effects [5]. The risk from NSAID use involves increased GI bleeding and ulceration, increased potential for myocardial infarction, stroke, and Stevens-Johnson syndrome. Opioids, used for moderate-to-severe pain, provide excellent pain relief and are easier to metabolize but have the unwanted effects of sedation, nausea, confusion, and delirium [6]. Other than that, certain types of pain like cancer-related pain are not effectively treated with conventional drugs; thus, patients suffering from this type of pain will seek for alternative treatment [5]. Nowadays the number of patients that are using herbal remedies and complementary and alternative medicine for treatment of pain is growing rapidly [7]. Over the last 20 years, Americans have sought a more "natural" or "holistic" approach to treatment of medical problems in general and pain in particular. Americans spend billions of dollars annually to find a holistic treatment with effective pain relief and few side effects, on complementary and alternative medicine, including herbal therapies [8]. Such increase in popularity and use of CAM by the general public strongly demands that health care professionals have the knowledge to assess, intervene, and advise patients on effective and safe CAM practices [9, 10].

One of the medicinal plants that have gained attention among the scientists is *Clinacanthus nutans* (Burm. f.) Lindau, a plant belonging to the family Acanthaceae. Locally known as "*Belalai Gajah*," it is a shrub native to the tropical Southeast Asian countries. The fresh leaves are consumed raw as vegetables and mixed with juices and can be used to brew drinks; the dried leaves can be steeped in hot water and served as herbal tea [11]. In Indonesia, Malaysia, and Thailand in particular, the plant is traditionally used in the treatment of skins rashes, insect and snake bites, mental stress, diabetes, rheumatoid arthritis, fever, dysentery, burns, scalds, diarrhea, and herpes skins infections [11]. Scientifically, extracts of *C. nutans* have been shown to exert antibacterial [12], anti-inflammatory [13], antiherpes [14, 15], antioxidant [16], antiproliferative [17], cytotoxic, and antimutagenic [18] activities and demonstrated to affect the immune response when studied *in vivo* (mice) [19] or *in vitro* (human cells) [20]. Moreover, the plant has also been developed into oral-based agent for the treatment of recurrent aphthous stomatitis [21] while the oral toxicity study revealed that *C. nutans* is safe for consumption [22].

Various chemical constituents (i.e., stigmasterol, lupeol, β-sitosterol, betulin, vitexin, isovitexin, schaftoside, isomollupentin-7-O-β-glucopyranoside, orientin, isoorientin, sulfur-containing glucosides, glycoglycerolipids, and monoacylmonogalactosylglycerol) have been isolated and identified from *C. nutans* [11]. However, the bioactivity of some of these compounds still remains to be elucidated. Additionally the presence of *n*-pentadecanol, eicosane, 1-nonadecene, heptadecane, dibutyl phthalate, *n*-tetracosanol-1, heneicosane, behenic alcohol, 1-heptacosanol, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, nonadecyl heptafluorobutyrate, eicosanoyl trifluoroacetate, 1,2-benzenedicarboxylic acid, dinonyl ester, phthalic acid, and dodecyl nonyl ester was reported in the chloroform extract of *C. nutans* leaves [19]. Several other compounds have also been identified and further demonstrated to have some degrees of bioactivity. For example, three types of phaeophytins, namely, 13^2-hydroxy-(13^2-*R*)-phaeophytin b, 13^2-hydroxy-(13^2-*S*)-phaeophytin a, and 13^2-hydroxy-(13^2-*R*)-phaeophytin, have been identified from the chloroform extract of *C. nutans* leaves and were reported to exhibit anti-herpes simplex activity [23]. Despite the various reports on pharmacological activity of *C. nutans*, there has been no study on MECN's antinociceptive activity to date. The proposed antinociceptive study is attributed to finding that *C. nutans* exerts anti-inflammatory activity [14] and contains several classes of phytoconstituents (i.e., flavonoids, saponins, and triterpenes) that are strongly associated with antinociceptive activity [13]. Thus, the present study aimed at determining the antinociceptive activity of methanol extract of *C. nutans* (MECN) and to elucidate the possible mechanisms of antinociception involved.

2. Materials and Methods

2.1. Plant Material and Extraction. Fresh *C. nutans* leaves were obtained from Clinnthus Enterprise (Kuala Lumpur, Malaysia) in January 2013. Authentication of the plant was made by Dr. Shamsul Khamis, a botanist from the Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, and a voucher specimen (SK 2679/15) has been deposited at the herbarium of the institute. Extraction was carried out according to the method previously described [24]. To obtain the MECN, 250 g of *C. nutans* leaves, which were dried in an oven at 40°C for 1-2 days and ground into powder form using an electric grinder (RT-08; Rong Tsong Precision Technology, Taichung, Taiwan), was soaked in methanol (Fisher Scientific, Loughborough, England) in the ratio of 1 : 20 (w/v) for 72 hours at room temperature. The supernatant was filtered using a steel filter, cotton wool, and Whatman Number 1 filter paper. The residue underwent the same soaking procedures twice. The supernatant collected from each extraction was pooled and evaporated using a vacuum rotary evaporator (Hei-VAP Value; Heidolph, Schwabach, Germany) at 40°C under reduced pressure. These processes yielded approximately 53 g of dried MECN (yield was 21.2% (w/w)), which was then stored at 4°C until it was used.

2.2. Phytochemical Screening of MECN. The phytochemical screening of fractions was performed according to the conventional protocols as described by Ikhiri et al. [25].

2.3. Chemicals Used in the UHPLC Analysis of MECN. Formic acid, methanol, and LCMS grade acetonitrile were purchased

from Merck (Darmstadt, Germany). HPLC grade water was prepared from distilled water using a Milli-Q-system (Millipore, MA, USA) and was used during analytical HPLC analysis. Various pure flavonoid-based standards (HPLC grade) were purchased from Extrasynthese (Lyon, France). All of the other solvents and chemicals used in this study were of analytical grade. Stock and working standards were prepared by dissolving these analytes in 100% methanol. The standard solutions stored at 4°C were stable for at least 3 months.

2.4. UHPLC-ESI Profiling of MECN. The UHPLC system was performed on a Dionex 3000 UHPLC system acquired from Thermo Fisher Scientific (USA) that consists of an autosampler equipped with a column oven, a tray compartment cooler, and a binary pump with built-in solvent degasser. Samples (10 μL) were injected and the chromatographic separation was performed on a BEH C18 UHPLC column, 100 mm × 2.5 μm, 1.7 μm (WATERS) at a flow rate of 0.3 mL/min. The mobile phases used were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The separation was conducted using the following multistep gradient: initial conditions (t = 0 min) were 90% A and 10% B with a linear gradient reaching 15% B at t = 3 min. The gradient was then increased to 50% B in the next 7 min (t = 10 min) and further increased to 90% B for the next 2 min (t = 12 min). Finally, the programme was returned to the initial solvent composition at t = 17 min for the next analysis.

The UHPLC system was coupled to a Linear Ion Trap Orbitrap mass spectrometer (Q Exactive) from Thermo Fisher Scientific (USA) equipped with an electrospray ionization (ESI) source. The mass detection was performed in a range of 150–1500 *m/z*. The ESI source was operated in negative ion mode under the following specific conditions: source voltage: 3.2 kV; sheath gas: 35 arbitrary units; auxiliary gas: 15 arbitrary units; sweep gas: 10 arbitrary units; and capillary temperature: 320°C. Nitrogen (>99.98%) was employed as sheath, auxiliary, and sweep gas. Instrument control and data acquisition were performed with Chameleon 6.8 software and Xcalibur 2.2 software (Thermo Fisher Scientific).

2.5. GC-MS Analysis of MECN. GC-MS analysis of MECN was performed using Agilent 7890A (Agilent Technologies) coupled with MSD quadrupole detector 5975 C (Agilent Technologies). Separation of analytes by gas chromatography was carried out using a Hewlett Packard HP-5MS silica capillary column (30 m × 0.25 mm × 0.25 mm). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 mL/min and an injection volume of 1 μL was employed (split ratio of 1 : 10), injector temperature was 250°C, and ion-source temperature was 280°C. The oven temperature was programmed from 100°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C and then 12°C/min to 280°C, ending with a 17 min isothermal at 280°C. Mass spectra were taken at 70 eV, a scan interval of 0.5 sec, and fragments from 45 to 450 Da. Total GC running time was 35.50 min. The relative % amount of each component was calculated by comparing its average peak area to the total areas; software adopted to handle mass spectra and chromatograms was a Turbomass. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components were compared with the spectrum of the known components stored in the NIST library.

2.6. Experimental Animals. The antinociceptive studies were carried out using either the adult male ICR mice (25–30 g) or Sprague-Dawley rats (150–180 g), which were obtained from the Animal Source Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), Serdang, Malaysia. The animals were kept at room temperature (27 ± 2°C; 70–80% humidity; 12 h light/dark cycle) in the Animal Holding Unit, Faculty of Medicine and Health Science, UPM, for at least 48 h prior to the procedure. Commercial food pellets (Gold Coin Feedmills, Port Klang, Malaysia) and water were supplied *ad libitum*. The animal experimental protocols were in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as adopted from Zimmermann [26] and have been approved by the UPM Institutional Animal Care and Use Committee (Ref. Number UPM/IACUC/AUP-R032/2013). The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the treatments. Experiments were conducted between 0930 and 1830 h to minimize the effects of environmental changes.

2.7. Drugs and Chemicals. Acetylsalicylic acid (ASA), morphine hydrochloride, naloxone hydrochloride, L-arginine (L-arg), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formaldehyde was purchased from R & M Chemicals (Essex, England). Acetic acid, dimethyl sulfoxide (DMSO), and methanol were purchased from Fisher Scientific (England). Drugs were dissolved in physiological saline (0.9% (w/v) NaCl). Morphine and ASA were prepared by dissolving in distilled water; MECN was dissolved in 10% DMSO (v/v) in distilled water. Control animals received only solvent vehicle. All drugs, chemicals, and MECN solutions were administered in the volume of 10 mL/kg and were freshly prepared just before use. The MECN doses (100, 250, and 500 mg/kg) used were based on our recent acute and subchronic toxicity studies of MECN (personal communication), which were further supported by the previous oral toxicity studies that reported no toxic or sedative effects at the stated doses [22, 27].

2.8. Nociceptive Tests

2.8.1. Acetic Acid-Induced Abdominal Constriction Test. The procedure was conducted as previously described [28]. Mice (n = 6) were treated with vehicle (10% DMSO; 10 mL/kg; per os (p.o.); negative control), ASA (100 mg/kg; p.o.; positive control), or MECN (100, 250, and 500 mg/kg; p.o.) for 60 min

before the administration of phlogistic agent (0.6% acetic acid; 10 mL/kg; intraperitoneal (i.p.)). The animals were then immediately placed individually in glass cages and 5 min later abdominal constriction resulting from acetic acid injection involving contraction of the abdomen and stretching of at least one hind limb was measured. The number of abdominal constrictions produced was counted cumulatively for 25 min. Antinociceptive activity was expressed as the reduction of the mean number of abdominal constrictions in test groups compared to the control group, calculated as the percentage inhibition of abdominal constrictions (percentage of inhibition) using the following formula: (mean [(control − test group)/control group] × 100%).

2.9. Hot Plate Test. The hot plate test was carried out according to the method previously described [29]. Mice (n = 6) were placed on a hot plate (Model 7280; Ugo Basile, Milan, Italy) heated to 50 ± 0.2°C, and the latency to a discomfort reaction was recorded when the animals licked their forepaws or hind paws or jumped. Animals were selected a day prior to the test based on their reactivity: only animals with response latencies of 5–7 sec were used. The discomfort reaction time was recorded before and at 60, 90, 120, 150, 180, and 210 min following the administration of vehicle (10 mL/kg; p.o.; positive control), morphine (5 mg/kg; i.p.), or MECN (100, 250, and 500 mg/kg; p.o.) 60 min before the test. A cut-off time of 20 sec was set to prevent tissue injury. Prolongation of the latency times of the test groups compared with that of the controls, which indicates antinociceptive activity, was used for statistical comparison.

2.10. Formalin-Induced Paw Licking Test. The formalin-induced paw licking test was performed as previously described [30]. Rats (n = 6) received vehicle (10 mL/kg; p.o.), ASA (100 mg/kg; p.o.), morphine (5 mg/kg; i.p.), or MECN (100, 250, and 500 mg/kg; p.o.) 60 min before the formalin injection. Nociception was induced by injecting 50 μL formalin (5% v/v) in the intraplantar (i.pl.) region of the right hind paw. Following injection of the phlogistic agent formalin, the animals were immediately placed individually in a transparent observation glass chamber. The duration the animal spent licking the injected paw (considered an indicator of pain) was recorded. The nociceptive response develops in two phases: 0–5 min after formalin injection (early phase, neurogenic pain response) and 15–30 min after formalin injection (late phase, inflammatory pain response), which were recorded.

2.11. Involvement of Opioidergic System. The protocol used was similar to the method previously described [31]. To evaluate the involvement of opioidergic system in the antinociceptive properties of MECN, separate groups of animals (n = 6) were treated with the nonselective opioid receptor antagonist naloxone (5 mg/kg; i.p.) 15 min before the administration of vehicle (10 mL/kg; p.o.) or MECN (500 mg/kg; p.o.). The antinociceptive effect was evaluated using the acetic acid-induced abdominal writhing test, hot plate test, and formalin-induced paw licking test as described above.

2.12. Involvement of L-Arg/Nitric Oxide/Cyclic Guanosine Monophosphate Pathway. To investigate the possible contribution of L-arg/nitric oxide/cyclic guanosine monophosphate (L-arg/NO/cGMP) pathway to the antinociceptive effect of MECN, the previously described method was adopted [28]. Mice (n = 6) were pretreated with the NO precursor, L-arg (20 mg/kg; i.p.), the NO inhibitor, L-NAME (20 mg/kg; i.p.), the nonspecific guanylyl cyclase inhibitor, ODQ (2 mg/kg; i.p.), or combinations thereof (L-arg + L-NAME or L-arg + ODQ) 5 min before the administration of vehicle (10 mL/kg; p.o.) or MECN (500 mg/kg; p.o.). Sixty minutes after the administration of test solutions, mice were subjected to the acetic acid-induced abdominal writhing test as described earlier.

2.13. Statistical Analysis. Statistical analysis was performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard error of the mean (SEM). Mean differences between the control and treatment groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. In all cases, differences were considered significant if $p < 0.05$.

3. Results

3.1. Phytochemicals Constituents of MECN. Except for alkaloids and tannins, the phytochemicals screening of MECN showed the presence of flavonoids, saponins, steroids, and triterpenes.

3.2. UHPLC Profile of MECN

3.2.1. Identification of Phenolic Compounds in MECN. *C. nutans* extract was analyzed based on the accurate mass data of the molecular ions, in which ions detected were tentatively identified by their generated molecular formula using the data analysis software (Xcalibur) that provided list of possible elemental formulas. These findings were compared together with the standard flavonoids available in the laboratory and further supported by the thorough survey of the literature (Figure 1). The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm.

In the present study, major flavonoid compounds found in MECN belonged to the family of flavone *C*-glycoside. The UHPLC-ESI analysis of MECN revealed the presence of 16 phenolic compounds (Table 1). The compounds detected were gallic acid, 4-hydroxybenzoic acid, caffeic acid, coumaric acid, ferulic acid, schaftoside, vitexin, orientin, isoorientin, isovitexin, luteolin, apigenin, forsythosides H, forsythosides I, diosmetin glycoside, and diosmetin.

3.3. GC-MS Profile of MECN. The GC-MS profile of MECN is shown in Figure 2. A total of 39 peaks were identified from MECN with the major compounds constituted of (i) 2-ethyl-oxetane (16.6%), (ii) 9,12,15-octadecatrienoic

Table 1: Phenolic compounds tentatively identified in *C. nutans* extract.

Peak number	t_R (min)	$[M-H]^-$ (m/z)	Error (ppm)	Molecule formula	Proposed compound
1	0.63	169.01270	−2.661	$C_7H_5O_5$	Gallic acid
2	1.22	137.02347	1.091	$C_7H_5O_3$	4-Hydroxybenzoic acid
3	1.51	179.03314	−4.162	$C_9H_7O_4$	Caffeic acid
4	2.55	163.0387	−1.598	$C_9H_7O_3$	Coumaric acid
5	3.06	563.13776	−3.253	$C_{26}H_{27}O_{14}$	Schaftoside
6	3.51	193.04881	−3.757	$C_{10}H_9O_4$	Ferulic acid
7	4.04	431.09622	−2.443	$C_{21}H_{19}O_{10}$	Vitexin
8	4.52	447.09158	−1.359	$C_{21}H_{19}O_{11}$	Orientin
9	5.11	623.19568	−2.193	$C_{29}H_{35}O_{15}$	Forsythoside H
10	5.37	447.09152	−1.158	$C_{21}H_{19}O_{11}$	Isoorientin
11	5.50	623.19604	−2.193	$C_{29}H_{35}O_{15}$	Forsythoside I
12	5.55	431.09644	−1.933	$C_{21}H_{19}O_{10}$	Isovitexin
13	5.70	503.11783	−1.138	$C_{24}H_{24}O_{12}$	Diosmetin glycoside
14	6.85	285.03860	−2.682	$C_{15}H_9O_6$	Luteolin
15	7.66	269.04404	−1.523	$C_{15}H_9O_5$	Apigenin

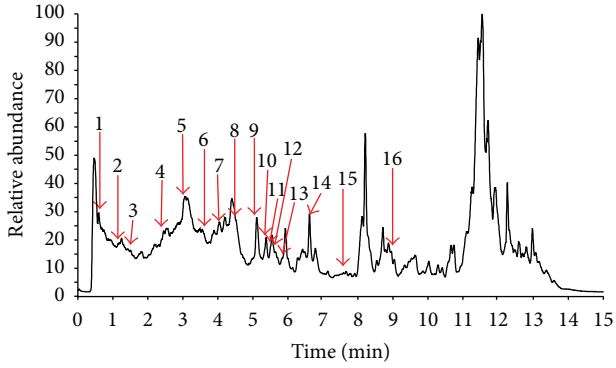


Figure 1: TIC (Total Ion Chromatography) profile of UHPLC-ESI of *C. nutans* extract. The numbering peaks correspond to those listed in Table 1.

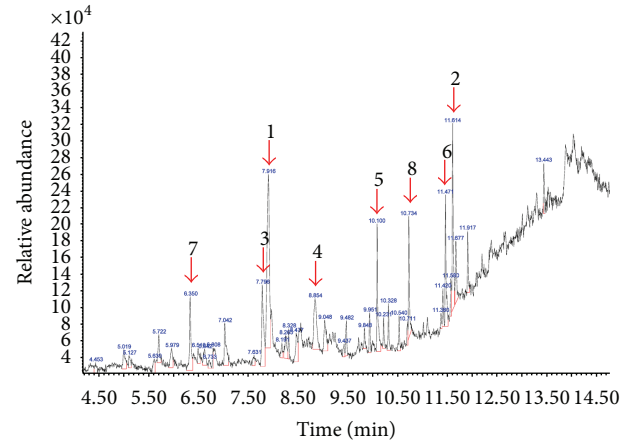


Figure 2: GC-MS profile of MECN showing approximately 39 detected peaks with major peaks representing (i) 2-ethyl-oxetane (16.6%), (ii) 9,12,15-octadecatrienoic acid (7.6%), (iii) 2,3-dimethylpyridine (6.4%), (iv) 3-deoxy-D-mannoic lactone (5.7%), (v) neophytadiene (5.4%), (vi) phytol (5.3%), (vii) 2,3-dihydrobenzofuran (4.5%), and (viii) *n*-hexadecanoic acid (4.6%).

acid (7.6%), (iii) 2,3-dimethylpyridine (6.4%), (iv) 3-deoxy-D-mannoic lactone (5.7%), (v) neophytadiene (5.4%), (vi) phytol (5.3%), (vii) 2,3-dihydrobenzofuran (4.5%), and (viii) *n*-hexadecanoic acid (4.6%).

3.4. Acetic Acid-Induced Abdominal Writhing Test. Figure 3 depicts the effect of MECN on acetic acid-induced abdominal writhing in mice. Administration of MECN (100, 250, and 500 mg/kg) *per os* produced significant ($p < 0.001$) and dose-related inhibition in the number of acetic acid-induced abdominal writhing responses. At the tested doses, MECN produced 32.43, 51.35, and 70.26% inhibition of constrictions, respectively, in comparison to the control group. The ED_{50} value recorded for the abdominal constriction test was 279.3 mg/kg. ASA, a standard nonsteroidal anti-inflammatory drug (NSAID), also caused a significant inhibition (46.78%) of acetic acid-induced abdominal writhing, which is equal in strength to the 250 mg/kg MECN.

3.5. Hot Plate Test. The antinociceptive effect of orally administered MECN against thermal-induced nociception is described in Table 2. At 100 and 250 mg/kg, MECN caused no significant changes in response latency to thermal-induced nociception when compared to the control group. In contrast, 500 mg/kg MECN significantly ($p < 0.05$) delayed response latency at the interval of 60 to 210 min after its administration as compared to the control group. Moreover, the opioid agonist, morphine, caused dose-dependent prolongation of latency response time at the interval of 60 to 210 min as compared to the control group (Table 2).

3.6. Formalin-Induced Paw Licking Test. Figure 4 shows the antinociceptive activity of orally administered MECN when assessed using the formalin-induced paw licking test. The extract, at 250 and 500 mg/kg, caused a significant ($p < 0.05$) decrease in the formalin-induced licking time in the first phase (neurogenic phase; 0–5 min; Figure 4(a)) of the

Table 2: Effects of MECN on the hot plate test in mice.

Group	Dose (mg/kg)	Latency of discomfort(s) at respective time interval (min)						
		0 min	60 min	90 min	120 min	150 min	180 min	210 min
10% DMSO		6.29 ± 0.15	6.88 ± 0.29	6.89 ± 0.31	6.28 ± 0.12	6.76 ± 0.43	6.67 ± 0.33	6.46 ± 0.12
Nalox	5	6.55 ± 0.33	6.02 ± 0.34	5.50 ± 0.29	5.53 ± 0.37	5.63 ± 0.09	5.35 ± 0.15	5.20 ± 0.39
MECN	100	6.52 ± 0.24	6.50 ± 0.33	6.23 ± 0.25	6.25 ± 0.21	6.32 ± 0.27	5.99 ± 0.26	6.17 ± 0.22
	250	6.08 ± 0.11	6.28 ± 0.28	6.68 ± 0.22	6.78 ± 0.19	6.59 ± 0.32	6.17 ± 0.18	6.39 ± 0.20
	500	6.65 ± 0.35	10.28 ± 0.81***	9.92 ± 0.55**	9.52 ± 1.08***	9.14 ± 0.51*	9.14 ± 0.36*	8.78 ± 0.81*
Nalox + MECN	5 + 500	6.60 ± 0.38	5.98 ± 0.38$^{\#}$	5.52 ± 0.57$^{\#}$	5.79 ± 0.27$^{\#}$	5.54 ± 0.30$^{\#}$	5.56 ± 0.32$^{\#}$	5.59 ± 0.32$^{\#}$
Morphine	5	6.02 ± 0.15	17.00 ± 0.90***	18.42 ± 0.47***	17.25 ± 0.93***	13.47 ± 1.31***	11.87 ± 1.04***	11.15 ± 0.71***
Morphine + Nalox	5 + 5	6.58 ± 0.24	7.08 ± 0.24$^{\#}$	7.40 ± 0.21$^{\#}$	7.58 ± 0.40$^{\#}$	7.03 ± 0.36$^{\#}$	7.33 ± 0.40$^{\#}$	7.23 ± 0.40$^{\#}$

Mice were treated with vehicle (10 mL/kg, p.o.), MECN (100, 250, and 500 mg/kg, p.o.), or morphine (5 mg/kg, p.o.) 60 min before the test. Naloxone (Nalox, 5 mg/kg, i.p.) was administered 15 min before MECN (500 mg/kg, p.o.), morphine (5 mg/kg, p.o.), or vehicle (10 mL/kg, p.o.). Data expressed are the mean ± SEM of reaction time (sec) of six mice. Statistical analysis was performed using 2-way ANOVA followed by Tukey's *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.001$, and $^{***}p < 0.0001$ compared to control; $^{\#}p < 0.0001$ compared to 500 mg/kg MECN or morphine-treated group.

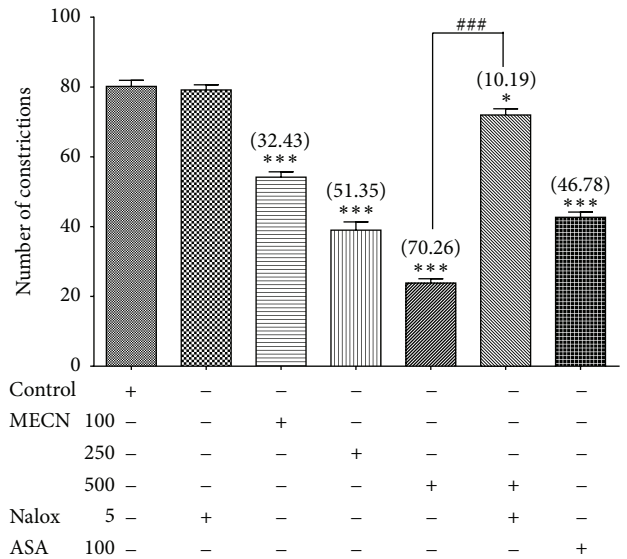


Figure 3: Effect of MECN on acetic acid-induced abdominal constriction in mice. Animals were treated with vehicle (10 mL/kg, p.o.), ASA (100 mg/kg, p.o.), or MECN (100, 250, and 500 mg/kg, p.o.) 60 min before acetic acid (0.6%, 10 mL/kg, i.p.) treatment. Naloxone (Nalox, 5 mg/kg, i.p.) was administered 15 min before MECN (500 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.). Each column represents the mean ± SEM of six mice. Statistical analyses were performed using 1-way ANOVA followed by Tukey's *post hoc* test. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to control group; $^{\#\#\#}p < 0.001$ compared to 500 mg/kg MECN-treated group. Values in parentheses denote percentage of inhibition.

test with the recorded percentage of nociceptive inhibition of 27.03% and 39.64%, respectively. In the second phase (inflammatory phase; 15–30 min; Figure 4(b)) of the test, all doses of MECN decreased the formalin-induced licking time significantly ($p < 0.05$) with the recorded percentage of antinociception ranging between 40 and 74% when compared to the control group. Thus, the recorded ED_{50} value for the early and late phases was >500 mg/kg or 227.7 mg/kg, respectively. Standard drugs, ASA, decreased the licking time significantly ($p < 0.05$) (60.75%) only in the second phase while morphine caused significant ($p < 0.05$) inhibition of the pain response in both phases of formalin test (77.46% and 96.47%, resp.).

3.7. Opioidergic System Involvement. Figure 3, Table 2, and Figures 4(a) and 4(b) depict the involvement of opioid receptors in the antinociceptive effect of MECN assessed using the abdominal constriction-, hot plate-, and formalin-induced paw licking test, respectively. The extract was prechallenged with a nonselective opioid antagonist, naloxone, prior to assessment using various nociceptive models. Used alone, naloxone did not affect acetic acid-induced nociception, whereas pretreatment with naloxone significantly reversed ($p < 0.001$) the antinociceptive effect of MECN.

In the hot plate test, naloxone alone also did not cause any significant changes in the response latency at 60, 90, 120, 150, 180, or 210 min whereas pretreatment with naloxone significantly ($p < 0.05$) blocked the antinociceptive effect of MECN at 60, 90, 120, 150, 180, and 210 min. Naloxone also reversed the antinociceptive effect of opioid agonist, morphine, significantly ($p < 0.05$) at 60, 90, 120, 150, 180, and 210 min.

Moreover, the antinociceptive effect of MECN and morphine in both phases of the formalin test was significantly antagonized at the early phase ($p < 0.01$) and late phase ($p < 0.001$) after pretreatment with naloxone.

3.8. L-*Arg/NO/cGMP Pathway Involvement.* Figures 5(a) and 5(b) show the effect of L-arg, L-NAME, ODQ, or combinations thereof on antinociceptive activity of MECN assessed using the acetic acid-induced abdominal constriction test. L-arg did not affect the acetic acid-induced nociception in 10% DMSO-treated group but significantly ($p < 0.05$) reversed the antinociceptive activity of MECN. Conversely, L-NAME caused significant ($p < 0.05$) reduction in the acetic acid-induced nociception in 10% DMSO-treated group and significantly ($p < 0.05$) reversed the antinociceptive activity of MECN. On the other hand, pretreatment of a combination between L-arg and L-NAME (as L-arg + L-NAME) exerted significant ($p < 0.05$) antinociceptive activity in the 10%

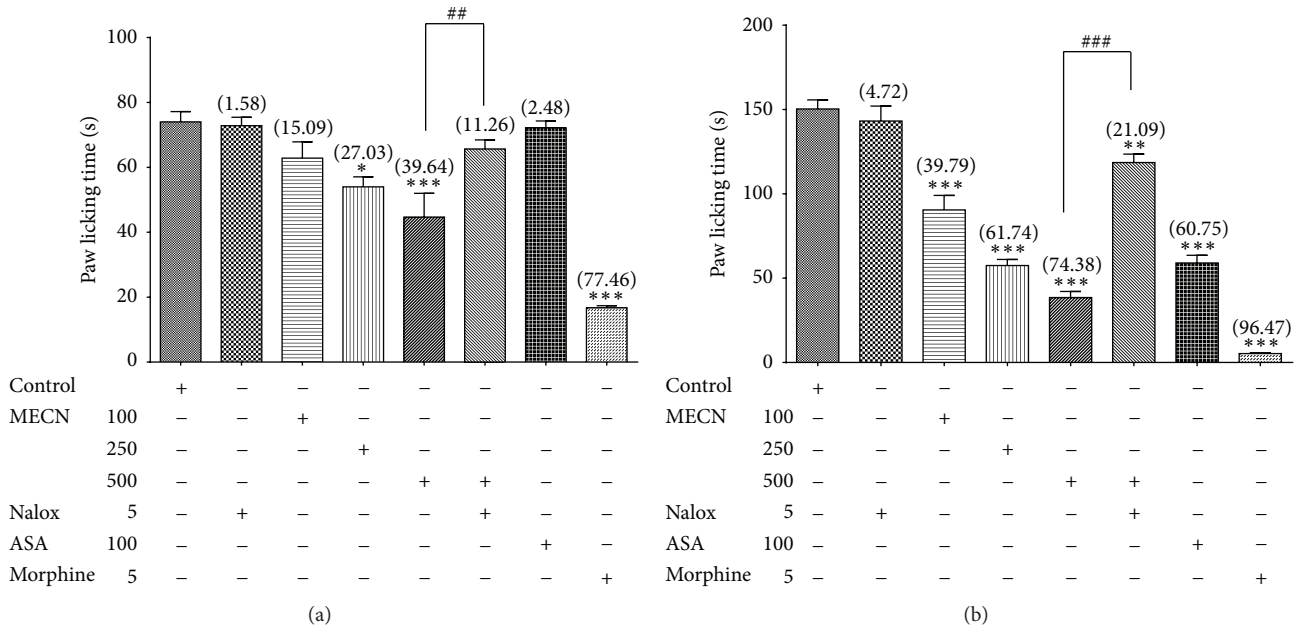


Figure 4: Effect of MECN on formalin-induced paw licking in rats. (a) Early phase; (b) late phase. Rats were treated with vehicle (10 mL/kg, p.o.), ASA (100 mg/kg, p.o.), MECN (100, 250, and 500 mg/kg, p.o.), or morphine (5 mg/kg, p.o.) 60 min before intraplantar administration of 5% formalin (50 μL in distilled water) into the right hind paw. Naloxone (Nalox, 5 mg/kg, i.p.) was administered 15 min before MECN (500 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.). Each column represents the mean ± SEM of six rats. Statistical analyses were performed using 1-way ANOVA followed by Tukey's *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to 500 mg/kg MECN-treated group. Values in parentheses denote percentage of inhibition.

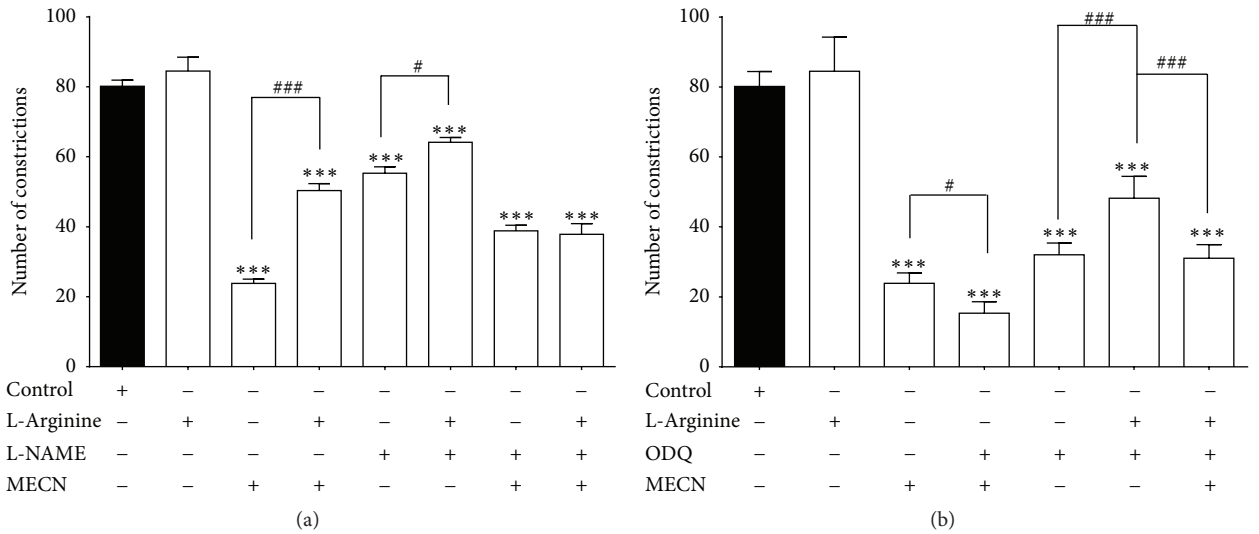


Figure 5: The involvement of L-arg/NO/cGMP pathway in the modulation of MECN antinociception as assessed using the abdominal constriction test. (a) Effects of pretreating animals with L-arg, L-NAME, or their combination on the antinociceptive activity of MECN. (b) Effects of L-arg, ODQ, or their combination on the antinociceptive activity of MECN. Animals were treated with MECN (500 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.) 60 min before acetic acid (0.6%, 10 mL/kg, i.p.) treatment. L-arg (20 mg/kg, i.p.), L-NAME (20 mg/kg, i.p.), ODQ (2 mg/kg, i.p.), or combinations thereof (L-arg + L-NAME or L-arg + ODQ) were administered 5 min before MECN (500 mg/kg, p.o.) or vehicle (10 mL/kg, p.o.). Each column represents the mean ± SEM of six mice. Statistical analyses were performed using 1-way ANOVA followed by Tukey's *post hoc* test. $^{***}p < 0.001$ compared to control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared to 500 mg/kg MECN, L-NAME, L-arg, L-arg + L-NAME, or L-arg + ODQ group.

DMSO-treated group but significantly ($p < 0.05$) reversed the antinociceptive activity of MECN.

Pretreatment with ODQ or a combination between L-arg and ODQ (as L-arg + ODQ) significantly ($p < 0.05$) attenuated the acetic acid-induced nociception in 10% DMSO-treated group but failed to significantly affect the antinociceptive activity of MECN.

4. Discussion

The extract (MECN) demonstrated a wide safety margin and is safe for oral consumption up to the dose of 5000 mg/kg while for the chronic oral consumption the dose is up to 2500 mg/kg, all of which did not cause any toxicity, mortality, or body weight changes. From the acute and subchronic toxicities study, the dose range (100, 250, and 500 mg/kg) for antinociceptive study was determined and decided to be 10-, 20-, and 50-fold reduction of the dose used in acute toxicity study (5000 mg/kg) [32].

Phytochemical screening of MECN revealed the presence of flavonoids, saponins, triterpenes, and steroids, which is in line with previous reports [11, 19, 23, 33]. The UHPLC profiling of MECN demonstrated the presence of several flavonoid-based compounds that belong to the family of flavone *C*-glycoside as reported previously by Chelyn et al. [34]. Sixteen compounds were detected in MECN, namely, gallic acid, 4-hydroxybenzoic acid, caffeic acid, coumaric acid, ferulic acid, schaftoside, vitexin, orientin, isoorientin, danisovitexin, luteolin, apigenin, forsythosides H, forsythosides I, diosmetinacetylglycoside, and diosmetin. Although MECN as a crude extract contains various types of bioactive compounds, flavonoid-based compounds, in part, have been reported to demonstrate antinociceptive activity. Of those detected compounds, at least gallic acid [35], caffeic acid [36], ferulic acid [37], vitexin [38], and apigenin [39] have been reported to exert antinociceptive activity when given orally. These compounds are suggested to work synergistically to exert the observed antinociceptive activity in MECN.

Following the antinociceptive studies, MECN attenuated the chemical-induced (i.e., acetic acid- and formalin-induced) and thermal-induced (i.e., hot plate model) nociceptive models suggesting that the antinociceptive profile of MECN includes peripheral and central mechanisms of action. This suggestion was based on previous claims that any substances that can attenuate the abdominal constriction and hot plate tests [40] or reversed the response latency to formalin-induced nociception in both the early and late phases of formalin test [41] possess peripheral and central antinociceptive activity.

Further postulations could also be made regarding the mechanisms of antinociception exerted by MECN based on the extract ability to attenuate the respective nociceptive model. The abdominal constriction test is a characteristic model for inflammatory pain and is frequently used to investigate the antinociceptive potential of any extracts or natural/synthetic compounds [31]. Positive results obtained from this model also, if not supported by other models, could suggest that the tested extract/compound possesses peripherally mediated antinociceptive activity [42]. According to Ikeda et al. [43], increased level of inflammatory mediators (i.e., cyclooxygenase (COX), prostaglandins (PGs), histamine, serotonin, bradykinin, etc.) upon the administration of acetic acid leads to the excitation of peripheral nociceptive neurons entering dorsal horn of the central nervous system. Therefore, the ability of MECN to attenuate acetic acid-induced nociception indicates the peripheral antinociceptive action partly via the attenuation of several inflammatory mediators' action that lead to impediment of pain transduction at the primary afferent nociceptors. Although considered a sensitive nociception model, this test is also believed to be a nonspecific test as muscle relaxants and other drugs might give false positive results [44].

To avoid misinterpretation of results obtained from the abdominal constriction model, additional experiments using other models of nociception are warranted. The hot plate test is aimed at studying the spinal antinociceptive potential of any tested substances by measuring the animal nociceptive response latencies to thermal stimulus following treatment with the substances. The principal response of thermal-induced nociception occurs predominantly at the supraspinal level [41]. The hot plate test is specifically used to investigate the central antinociceptive potential of any extract/compound and is specifically affected only by the centrally acting drugs (i.e., opioids) [44]. The ability of MECN to reverse the painful thermal stimulus suggests the involvement of central antinociceptive mechanism. However, the fact that highest dose of MECN is required to attenuate thermal-induced nociception indicates that MECN was not a strong agent at the central thermal-stimulated nociceptive pathway.

To further support the suggested involvement of peripheral and central mechanisms in the modulation of antinociceptive activity of MECN, the formalin-induced paw licking test (or formalin test) was adopted. This model can be used to investigate the ability of new extract/compound to affect the peripheral or central nociceptive pathways due to its characteristic biphasic nociception, known as early phase and late phase [24]. The former corresponds to neurogenic pain, is observed immediately after the administration of formalin, and persists for 5 min (0–5 min) as a response to the direct action of formalin on nociceptors in the subplantar region. The late phase corresponds to inflammatory-mediated pain resulting from a tonic response due to the release of inflammatory mediators [24]. The late phase occurs between 15 and 30 min after the administration of formalin. Moreover, the ability to reverse the early phase suggests the extract/compound ability to inhibit the non-inflammatory-mediated nociception while the ability to reverse the late phase suggests the extract potential to inhibit the inflammatory-mediated nociception. From the results obtained from the three models of nociception, MECN is suggested (i) to have peripheral and central antinociceptive action; (ii) to possess antinociceptive activity against both the non-inflammatory-mediated and inflammatory-mediated nociception; and (iii) to exert opioids' characteristic due to its ability to attenuate the peripheral and central models of nociception.

Being the standard drugs for the treatment of pain, opioids effectiveness has been overshadowed by various side

effects including dependence and tolerance. In an attempt to find better pain-relieving agents with possibly no or less side effects, the potential of MECN to exert its antinociceptive activity via the opioid receptors was also investigated using the three nociceptive models. From the results obtained, the peripheral and central antinociceptive activities of MECN were blocked by naloxone, a nonselective opioid antagonist, suggesting the involvement of opioid receptor system.

Further study on the involvement of L-arg/NO/cGMP pathway in the MECN-induced antinociceptive effect was also carried out based on previous reports that standard analgesics like morphine also utilized this pathway to exert its analgesic effect. To the best of our knowledge, there has been no attempt to determine the role of L-arg/NO/cGMP pathway in the modulation of antinociceptive activity of MECN. NO production in the body leads to the activation of soluble guanylate cyclase (sGC) and elevation in the cGMP level within the target cells [45]. Despite the various roles played by NO, its involvement in the mechanisms of pain modulation, either as an antinociceptive or as a pronociceptive agent, is well acknowledged and has been attributed to the NO capability to manipulate nociception processing in both the peripheral and central nervous systems [45, 46]. The L-arg/NO/cGMP pathway has been reported to play significant role in the modulation of antinociceptive activity of morphine [46, 47]. Since MECN was shown to possess characteristics of morphine, there is a need to also determine the role of L-arg/NO/cGMP pathway in the antinociceptive activity of MECN. From the results obtained, the presence of NO from the conversion of L-arg did not affect nociception threshold at the respective dose of L-arg used but reduced the antinociceptive intensity of MECN indicating the importance of NO presence. While reduction of NO level due to the administration of L-NAME alone, at the respective dose used, triggered antinociceptive action, it also reversed the antinociceptive activity of MECN. The observations following the administration of L-NAME as described above plausibly suggest that although decrease in NO level triggered antinociception as previously reported, reduced NO did not synergistically enhance or maintain, but reduced, the antinociceptive intensity of MECN. The reason for this observation was not clearly understood, but it is suggested that, at certain concentration of NO reduction, MECN tends to reduce, but not lose, its activity. The ability to maintain the antinociceptive activity also possibly suggested that MECN, which contains several bioactive compounds that exert antinociceptive activity, triggered several antinociceptive mechanisms other than the NO-mediated pathway. NO also increases cGMP levels by activating soluble guanylyl cyclase (sGC), which affects pain and analgesia. The ability of cGMP pathway to affect nociceptive process [48] can be seen when ODQ, which inhibits the cGMP pathway, induced antinociceptive activity when given alone. However, ODQ failed to affect the antinociceptive activity of MECN suggesting that MECN might have triggered an NO-mediated, cGMP-independent pathway. The role of NO-dependent, cGMP-independent pathway in the modulation of antinociceptive activity has been reported elsewhere [49] and might support the present observations. Overall, these observations suggest that the antinociceptive activity of MECN involves the modulation of, partly, L-arg/NO-mediated, but cGMP-independent, pathway. Moreover, based on these observations, the antinociceptive activity of MECN is suggested to involve modulation of different subsets of nociceptive primary sensory neurons.

5. Conclusions

This is the first demonstration that oral systemic administration of MECN has both central and peripheral antinociceptive activities, which occur via the activation of opioid receptors and modulation of the L-arg/NO-mediated, but cGMP-independent, pathway.

Competing Interests

The authors declare no potential competing interests with respect to the research, authorship, and/or publication of this paper.

Acknowledgments

This research was supported by the Fundamental Research Grant Scheme (FRGS; Reference no. R/FRGS/107.00/00290A/001/2014/000175) awarded by the Ministry of Higher Education (MOHE), Malaysia, and the Research Acculturation Collaborative Effort (RACE; Reference no. R/RACE/A07.00/00290A/002/2015/000234) from the Universiti Putra Malaysia, Malaysia. The authors thank the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia, for providing the facilities while doing this research.

References

[1] M. O. Sofidiya, E. Imeh, C. Ezeani, F. R. Aigbe, and A. J. Akindele, "Antinociceptive and anti-inflammatory activities of ethanolic extract of *Alafia barteri*," *Revista Brasileira de Farmacognosia*, vol. 24, no. 3, pp. 348–354, 2014.

[2] K. Pandima Devi, M. Sreepriya, T. Devaki, and K. Balakrishna, "Antinociceptive and hypnotic effects of *Premna tomentosa* L. (Verbenaceae) in experimental animals," *Pharmacology Biochemistry and Behavior*, vol. 75, no. 2, pp. 261–264, 2003.

[3] E. Elisabetsky, T. A. Amador, R. R. Albuquerque, D. S. Nunes, and A. D. C. T. Carvalho, "Analgesic activity of *Psychotria colorata* (Willd. ex R. & S.) Muell. Arg. alkaloids," *Journal of Ethnopharmacology*, vol. 48, no. 2, pp. 77–83, 1995.

[4] H. O. Vongtau, J. Abbah, O. Mosugu et al., "Antinociceptive profile of the methanolic extract of *Neorautanenia mitis* root in rats and mice," *Journal of Ethnopharmacology*, vol. 92, no. 2-3, pp. 317–324, 2004.

[5] M. Khatta, "A complementary approach to pain management," November 2015, http://www.medscape.com/viewarticle/556408.

[6] Y. D'Arcy, "Pain in the older adult: pain assessment," *The Nurse Practitioner*, vol. 33, no. 3, pp. 18–25, 2008.

[7] N. M. Dhanani, T. J. Caruso, and A. J. Carinci, "Complementary and alternative medicine for pain: an evidence-based review,"

Current Pain and Headache Reports, vol. 15, no. 1, pp. 39–46, 2011.

[8] J. H. Wirth, J. C. Hudgins, and J. A. Paice, "Use of herbal therapies to relieve pain: a review of efficacy and adverse effects," *Pain Management Nursing*, vol. 6, no. 4, pp. 145–167, 2005.

[9] N. G. Cuellar, B. Cahill, J. Ford, and T. Aycock, "The development of an educational workshop on complementary and alternative medicine: what every nurse should know," *Journal of Continuing Education in Nursing*, vol. 34, no. 3, pp. 128–135, 2003.

[10] J. M. Wilkinson and P. Tinley, "Knowledge, beliefs, and use of complementary and alternative medicine by Australian podiatric physicians," *Journal of the American Podiatric Medical Association*, vol. 99, no. 2, pp. 121–128, 2009.

[11] S. Y. Shim, I. Aziana, and B. Y. Khoo, "Perspective and insight on *Clinacanthus nutans* Lindau in traditional medicine," *International Journal of Integrative Biology*, vol. 14, no. 1, pp. 7–9, 2013.

[12] D. Huang, W. Guo, J. Gao, J. Chen, and J. Olatunji, "*Clinacanthus nutans* (Burm. f.) Lindau ethanol extract inhibits hepatoma in mice through upregulation of the immune response," *Molecules*, vol. 20, no. 9, pp. 17405–17428, 2015.

[13] S. Y. Ho, W. P. Tiew, M. Priya, S. A. S. Mohamed, and A. A. Gabriel, "Phytochemical analysis and antibacterial activity of methanolic extract of *Clinacanthus nutans* leaf," *International Journal of Drug Development and Research*, vol. 5, no. 3, pp. 349–355, 2013.

[14] P. Wanikiat, A. Panthong, P. Sujayanon, C. Yoosook, A. G. Rossi, and V. Reutrakul, "The anti-inflammatory effects and the inhibition of neutrophil responsiveness by *Barleria lupulina* and *Clinacanthus nutans* extracts," *Journal of Ethnopharmacology*, vol. 116, no. 2, pp. 234–244, 2008.

[15] P. Kunsorn, N. Ruangrungsi, V. Lipipun, A. Khanboon, and K. Rungsihirunrat, "The identities and anti-herpes simplex virus activity of *Clinacanthus nutans* and *Clinacanthus siamensis*," *Asian Pacific Journal of Tropical Biomedicine*, vol. 3, no. 4, pp. 284–290, 2013.

[16] S. Sangkitporn, S. Chaiwat, K. Balachandra, T. D. Na-Ayudhaya, M. Bunjob, and C. Jayavasu, "Treatment of herpes zoster with *Clinacanthus nutans* (bi phaya yaw) extract," *Journal of the Medical Association of Thailand*, vol. 78, no. 11, pp. 624–627, 1995.

[17] B. Sriwanthana, P. Chavalittumrong, and L. Chompuk, "Effect of *Clinacanthus nutans* on human cell-mediated immune response *in vitro*," *The Thai Journal of Pharmaceutical Sciences*, vol. 20, no. 4, pp. 261–267, 1996.

[18] P. Pannangpetch, P. Laupattarakasem, V. Kukongviriyapan, U. Kukongviriyapan, B. Kongyingyoes, and C. Aromdee, "Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans* (Burm.f) Lindau," *Songklanakarin Journal of Science and Technology*, vol. 29, no. 1, pp. 1–9, 2007.

[19] Y. K. Yong, J. J. Tan, S. S. Teh et al., "*Clinacanthus nutans* extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 462751, 8 pages, 2013.

[20] S. Rathnasamy, K. B. Mohamed, S. F. Sulaiman, and A. Akinboro, "Evaluation of cytotoxic, mutagenic and antimutagenic potential of leaf extracts of three medicinal plants using *Allium cepa* chromosome assay," *International Current Pharmaceutical Journal*, vol. 2, no. 8, pp. 131–140, 2013.

[21] S. Timpawat and L. Vijrabhava, "Clinical evaluation of *Clinacanthus nutans* Lindau in orabase in the treatment of recurrent *aphthous stomatitis*," *Mahidol Dental Journal*, vol. 14, no. 1, pp. 10–16, 1994.

[22] X. W. P'ng, G. A. Akowuah, and J. H. Chin, "Acute oral toxicity study of *Clinacanthus nutans* in mice," *International Journal of Pharmaceutical Sciences and Research*, vol. 3, no. 11, pp. 4202–4205, 2012.

[23] S. Sakdarat, A. Shuyprom, C. Pientong, T. Ekalaksananan, and S. Thongchai, "Bioactive constituents from the leaves of *Clinacanthus nutans* Lindau," *Bioorganic & Medicinal Chemistry*, vol. 17, no. 5, pp. 1857–1860, 2009.

[24] M. I. Mohamad Yusof, M. Z. Salleh, T. Lay Kek, N. Ahmat, N. F. Nik Azmin, and Z. A. Zakaria, "Activity-guided isolation of bioactive constituents with antinociceptive activity from *Muntingia calabura* L. leaves using the formalin test," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 715074, 9 pages, 2013.

[25] K. Ikhiri, D. Boureima, and D.-D. Dan-Koulodo, "Chemical screening of medicinal plants used in the traditional pharmacopoeia of Niger," *International Journal of Pharmacognosy*, vol. 30, no. 4, pp. 251–262, 1992.

[26] M. Zimmermann, "Ethical guidelines for investigations of experimental pain in conscious animals," *Pain*, vol. 16, no. 2, pp. 109–110, 1983.

[27] X. W. P'ng, G. A. Akowuah, and J. H. Chin, "Evaluation of the sub–acute oral toxic effect of methanol extract of *Clinacanthus nutans* leaves in rats," *Journal of Acute Disease*, vol. 2, no. 1, pp. 29–32, 2013.

[28] N. Abacioğlu, B. Tunçtan, E. Akbulut, and I. Çakici, "Participation of the components of l-arginine/nitric oxide/cGMP cascade by chemical-induced abdominal constriction in the mouse," *Life Sciences*, vol. 67, no. 10, pp. 1127–1137, 2000.

[29] S. G. Wilson, C. D. Bryant, W. R. Lariviere et al., "The heritability of antinociception II: pharmacogenetic mediation of three over-the-counter analgesics in mice," *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 2, pp. 755–764, 2003.

[30] M. H. M. Sani, Z. A. Zakaria, T. Balan, L. K. Teh, and M. Z. Salleh, "Antinociceptive activity of methanol extract of *Muntingia calabura* leaves and the mechanisms of action involved," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 890361, 10 pages, 2012.

[31] Z. A. Zakaria, S. Mustapha, M. R. Sulaiman, A. M. Mat Jais, M. N. Somchit, and F. C. Abdullah, "The antinociceptive action of aqueous extract from *Muntingia calabura* leaves: the role of opioid receptors," *Medical Principles and Practice*, vol. 16, no. 2, pp. 130–136, 2007.

[32] Z. A. Zakaria, M. H. Rahim, N. Mohtarrudin et al., "Acute and sub-chronic oral toxicity studies of methanol extract of *Clinacanthus nutans* in mice," *African Journal of Traditional, Complementary and Alternative Medicines*, vol. 13, no. 2, p. 210, 2016.

[33] P. Tuntiwachwuttikul, Y. Pootaeng-On, P. Phansa, and W. C. Taylor, "Cerebrosides and a monoacylmonogalactosylglycerol from *Clinacanthus nutans*," *Chemical and Pharmaceutical Bulletin*, vol. 52, no. 1, pp. 27–32, 2004.

[34] J. L. Chelyn, M. H. Omar, N. S. Mohd Yousof, R. Ranggasamy, M. I. Wasiman, and Z. Ismail, "Analysis of flavone *C*-glycosides in the leaves of *Clinacanthus nutans* (Burm. f.) Lindau by HPTLC and HPLC-UV/DAD," *The Scientific World Journal*, vol. 2014, Article ID 724267, 6 pages, 2014.

[35] G. Trevisan, M. F. Rossato, R. Tonello et al., "Gallic acid functions as a TRPA1 antagonist with relevant antinociceptive and antiedematogenic effects in mice," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 387, no. 7, pp. 679–689, 2014.

[36] A. Mehrotra, R. Shanbhag, M. R. Chamallamudi, V. P. Singh, and J. Mudgal, "Ameliorative effect of caffeic acid against inflammatory pain in rodents," *European Journal of Pharmacology*, vol. 666, no. 1–3, pp. 80–86, 2011.

[37] W.-H. Lv, L. Zhang, S.-J. Wu, S.-Z. Chen, X.-B. Zhu, and J.-C. Pan, "Analgesic effect of ferulic acid on CCI mice: behavior and neurobiological analysis," *Zhongguo Zhong Yao Za Zhi*, vol. 38, no. 21, pp. 3736–3741, 2013.

[38] S. M. Borghi, T. T. Carvalho, L. Staurengo-Ferrari et al., "Vitexin inhibits inflammatory pain in mice by targeting TRPV1, oxidative stress, and cytokines," *Journal of Natural Products*, vol. 76, no. 6, pp. 1141–1149, 2013.

[39] M. M. G. Pinheiro, F. Boylan, and P. D. Fernandes, "Antinociceptive effect of the *Orbignya speciosa* Mart. (Babassu) leaves: evidence for the involvement of apigenin," *Life Sciences*, vol. 91, no. 9-10, pp. 293–300, 2012.

[40] A. Khatun, M. Z. Imam, and M. S. Rana, "Antinociceptive effect of methanol extract of leaves of *Persicaria hydropiper* in mice," *BMC Complementary & Alternative Medicine*, vol. 15, article 63, 2015.

[41] M. Rezaee-Asl, M. Sabour, V. Nikoui, S. Ostadhadi, and A. Bakhtiarian, "The study of analgesic effects of *Leonurus cardiaca* L. in mice by formalin, tail flick and hot plate tests," *International Scholarly Research Notices*, vol. 2014, Article ID 687697, 5 pages, 2014.

[42] S. Trongsakul, A. Panthong, D. Kanjanapothi, and T. Taesotikul, "The analgesic, antipyretic and anti-inflammatory activity of *Diospyros variegata* Kruz.," *Journal of Ethnopharmacology*, vol. 85, no. 2-3, pp. 221–225, 2003.

[43] Y. Ikeda, A. Ueno, H. Naraba, and S. Oh-Ishi, "Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice," *Life Sciences*, vol. 69, no. 24, pp. 2911–2919, 2001.

[44] D. Le Bars, M. Gozariu, and S. W. Cadden, "Animal models of nociception," *Pharmacological Reviews*, vol. 53, no. 4, pp. 597–652, 2001.

[45] Z. A. Zakaria, M. R. Sulaiman, M. N. Somchit, A. M. M. Jais, and D. I. Ali, "The effects of l-arginine, d-arginine, l-name and methylene blue on *Channa striatus*-induced peripheral antinociception in mice," *Journal of Pharmacy and Pharmaceutical Sciences*, vol. 8, no. 2, pp. 199–206, 2005.

[46] Y. Cury, G. Picolo, V. P. Gutierrez, and S. H. Ferreira, "Pain and analgesia: the dual effect of nitric oxide in the nociceptive system," *Nitric Oxide*, vol. 25, no. 3, pp. 243–254, 2011.

[47] V. Granados-Soto, M. D. O. Rufino, L. D. Gomes Lopes, and S. H. Ferreira, "Evidence for the involvement of the nitric oxide-cGMP pathway in the antinociception of morphine in the formalin tests," *European Journal of Pharmacology*, vol. 340, no. 2-3, pp. 177–180, 1997.

[48] D. Sachs, F. Q. Cunha, and S. H. Ferreira, "Peripheral analgesic blockade of hypernociception: activation of arginine/NO/cGMP/protein kinase G/ATP-sensitive K^+ channel pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 10, pp. 3680–3685, 2004.

[49] N. Morioka, A. Inoue, T. Hanada et al., "Nitric oxide synergistically potentiates interleukin-1β-induced increase of cyclooxygenase-2 mRNA levels, resulting in the facilitation of substance P release from primary afferent neurons: involvement of cGMP-independent mechanisms," *Neuropharmacology*, vol. 43, no. 5, pp. 868–876, 2002.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 9852793, 8 pages
http://dx.doi.org/10.1155/2016/9852793

Review Article

The Efficacy and Safety of the Combination of Total Glucosides of Peony and Leflunomide for the Treatment of Rheumatoid Arthritis: A Systemic Review and Meta-Analysis

Zhitao Feng,[1,2,3,4] Juan Xu,[5,6] Guochao He,[7] Meiqun Cao,[3,4] Lihong Duan,[3,4] Liguo Chen,[1] and Zhengzhi Wu[2,3,4]

[1]*Postdoctoral Workstation of Chinese and Western Integrative Medicine, School of Medicine, Jinan University, Guangzhou, Guangdong, China*
[2]*The Futian Affiliated Hospital of Guangdong Medical Institute, Shenzhen, Guangdong, China*
[3]*The First Affiliated Hospital of Shenzhen University, Shenzhen, Guangdong, China*
[4]*Shenzhen Institute of Geriatrics, Shenzhen, Guangdong, China*
[5]*Department of Rheumatology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China*
[6]*Department of Traditional Chinese Internal Medicine, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou, Guangdong, China*
[7]*Department of Orthopedic Surgery, Hunan Provincial Hospital of Traditional Chinese Medicine, Zhuzhou, Hunan, China*

Correspondence should be addressed to Zhengzhi Wu; szwzz001@163.com

Received 25 November 2015; Accepted 29 December 2015

Academic Editor: Agnieszka Najda

Objective. To evaluate the efficacy and safety of the total glucosides of peony (TGP) and leflunomide (LEF) for the treatment of rheumatoid arthritis (RA). *Methods.* Randomized controlled trials (RCTs) on the efficacy and safety of the combination of TGP and LEF versus LEF alone for the treatment of RA were retrieved by searching PubMed, EMBASE, Cochrane Library, the China National Knowledge Infrastructure database, and Wanfang database. *Results.* Eight RCTs including 643 RA patients were included in the present meta-analysis. The quality of included studies was poor. The levels of ESR ($P < 0.0001$), CRP ($P < 0.0001$), and RF ($P < 0.0001$) in RA patients who received the combination of TGP and LEF were significantly lower than RA patients who received LEF therapy alone. The pooled results suggest that the combination of TGP and LEF caused less abnormal liver function than LEF alone ($P = 0.02$). No significant difference in the gastrointestinal discomfort was identified between the combination of TGP and LEF and LEF alone groups ($P = 0.18$). *Conclusion.* The combination of TGP and LEF in treatment of RA presented the characteristics of notably decreasing the levels of laboratory indexes and higher safety in terms of liver function. However, this conclusion should be further investigated based on a larger sample size.

1. Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disorder characterized by synovial inflammation and angiogenesis and cartilage and bone destruction [1, 2]. The estimated incidence of RA in the industrialized world is 1% [3]. RA may cause progressive disability and a number of systemic complications, such as pulmonary, cardiovascular, psychological, and skeletal diseases [4]. It has been reported that early and sufficient application of conventional disease modifying antirheumatic drugs (DMARDs), such as leflunomide (LEF), methotrexate, sulfasalazine, hydroxychloroquine, and glucocorticoids, can effectively inhibit inflammation and bone erosion in RA patients [5]. The European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) recommend the application of DMARDs as soon as the confirmation of RA diagnosis [6]. In addition, DMARDs-naïve patients should be treated

with either conventional DMARD monotherapy or DMARD combination therapy [7, 8]. Numerous studies reported that the combination of two or multiple DMARDs was more effective than single DMARD for the treatment of RA [9, 10].

Total glucoside of peony (TGP) is a biologically active compound extracted from traditional Chinese medicine of peony. Paeoniflorin (90%) is the major component in TGP. Previous studies have reported that paeoniflorin/TGP had both anti-inflammatory and immune-regulatory effects [11–13]. TGP has been widely used for the treatment of autoimmune diseases, especially RA, by alleviating inflammation [14]. In addition, TGP was able to relieve inflammation reactions, reduce joint pain and swelling, and delay bone erosion and destruction [15]. LEF is an efficient DMARD widely used for the treatment of RA [16]. LEF exhibits predominant functions including immunomodulation, immunosuppression, and antiproliferation [17]. LEF can prevent the progress of RA by inhibiting inflammatory reactions, protecting cartilage and bone from destruction, and delaying radiologic progression [16].

Currently, several clinical studies reported that the combination of TGP and LEF significantly improved the symptoms and prevented the progression of RA compared with LEF alone. However, most of these results were from uncontrolled clinical trials or retrospective studies. In addition, the safety of the combination of TGP and LEF for the treatment of RA is not clear. In the present study, we conducted a meta-analysis to evaluate the efficacy and safety of the combination of TGP and LEF for the treatment of RA. Our results provide evidence for the application of the combination of TGP and LEF for the treatment of RA patients.

2. Materials and Methods

2.1. Search Strategy. We searched the following databases to identify appropriate trials: PubMed (1865 to December 2015), EMBASE (1947 to December 2015), Cochrane Library (1993 to December 2015), the China National Knowledge Infrastructure database (1979 to December 2015), and the Wanfang database (1982 to December 2015). The search terms were (rheumatoid arthritis OR RA) AND (total glucosides of peony OR TGP) AND (leflunomide) AND (randomized controlled trial). Manual search in the references from original studies was performed to identify additional trials.

2.2. Study Selection. Studies meeting the following criteria were selected. (i) Patients were diagnosed with RA, according to the 1987 guidelines by the American Rheumatology Association. (ii) Studies were performed as a RCT describing a correct randomization procedure. Trials that used inappropriate methods of randomization (e.g., open alternation) were excluded. (iii) RA patients were treated with the combination of TGP and LEF, while controls were treated with LEF alone. (iv) Clinical outcomes included at least one of the following parameters: therapeutic effects, erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), C reactive protein (CRP), and side effects. (v) Intervention lasted for four weeks or longer.

2.3. Data Extraction. The relevant data was extracted by two independent reviewers (Zhitao Feng and Guochao He), including the study design, randomization, diagnostic criteria for RA, the first author's name, year of publication, sample size, treatment duration, dose, outcomes, and adverse events (AE). Disagreements were resolved by consensus or arbitrated by the third investigator (Zhengzhi Wu).

2.4. Data Synthesis and Analysis. Statistical analyses were performed using Review Manager 5.2 software (Cochrane Collaboration, Oxford, UK). Dichotomous data and continuous outcomes were presented as odds ratios (ORs) and mean difference (MD), respectively, both with 95% confidence interval (CI). The Cochrane's chi-square test and Higgins I^2 were used to assess heterogeneity [26]. A considerable level of heterogeneity was defined when the value was <0.10 or the I^2 value was >50%. A fixed-effect model was employed when no statistical heterogeneity was identified among studies; otherwise the random-effect model was used [27].

3. Results

3.1. Study Selection. A total of 74 studies were identified by searching in the databases mentioned above. Of these, 18 studies were deemed to be duplicated. 56 eligible studies were retrieved for detailed evaluation. After content review, 8 non-RCT studies, including one case report, 5 meeting abstracts, and 3 review articles, were excluded. In addition, 6 studies in which no RA patients were enrolled, 24 studies in which the combination of TGP and LEF or LEF alone was not applied, and one study without clinical outcomes of interest were also excluded from this meta-analysis. Finally, a total of 8 trials including 319 RA cases and 324 controls that meet our inclusion criteria were included in the present meta-analysis. The general procedure of study selection was detailed in Figure 1.

3.2. Study Characteristics. The included studies have been published between 2006 and 2015. All the eight RCTs were conducted in China and published in Chinese with randomization procedure and single center. The participant numbers in the individual studies varied from 38 to 100. The duration of the interventions (the combination of TGP and LEF or LEF alone) in the included studies varied from 4 to 24 weeks, except one study in which the treatment duration was not described [19]. Four studies described the therapeutic effects that were evaluated on the basis of four classes of outcomes including "cure," "significant effective," "effective," and "ineffective" [19, 21, 23, 25]. Six trials reported the AEs in detail [18, 19, 21, 22, 24, 25]. In addition, six trials mentioned the ESR [18–20, 22, 23, 25]; four trials referred to RF [18, 19, 23, 25]; and three trials analyzed CRP [18, 22, 25]. The characteristics of the included RCTs were shown in Table 1.

3.3. Risk of Bias Assessment. The risk of bias assessment was summarized in Figure 2. The quality of all included studies was poor. While all the eight studies reported randomization, none of them described the specific methods applied. Additionally, none of the eight studies mentioned the allocation

Table 1: The characteristics of the included studies.

Author	Participants		Interventions		Duration	Outcomes
	Experiment	Control	Experiment	Control		
Wu et al. [18]	50	50	TGP + LEF	LEF	12 weeks	ESR, CRP, RF, AE
Li [19]	48	48	TGP + LEF	LEF	NA	Therapeutic effects, ESR, RF, AE
Dong [20]	33	33	TGP + LEF	LEF	12 weeks	ESR
Tao et al. [21]	52	52	TGP + LEF	LEF	4 weeks	Therapeutic effects, AE
Yu and Zhang [22]	39	40	TGP + LEF	LEF	24 weeks	ESR, CRP, AE
Si [23]	17	21	TGP + LEF	LEF	24 weeks	Therapeutic effects, ESR, RF
Ma [24]	40	40	TGP + LEF	LEF	12 weeks	AE
Zhao and Liu [25]	40	40	TGP + LEF	LEF	12 weeks	Therapeutic effects, ESR, CRP, RF, AE

Note: TGP: total glucosides of peony; LEF: leflunomide; ESR: erythrocyte sedimentation rate; CRP: C reaction protein; RF: rheumatoid factor; AE: adverse event; NA: not available.

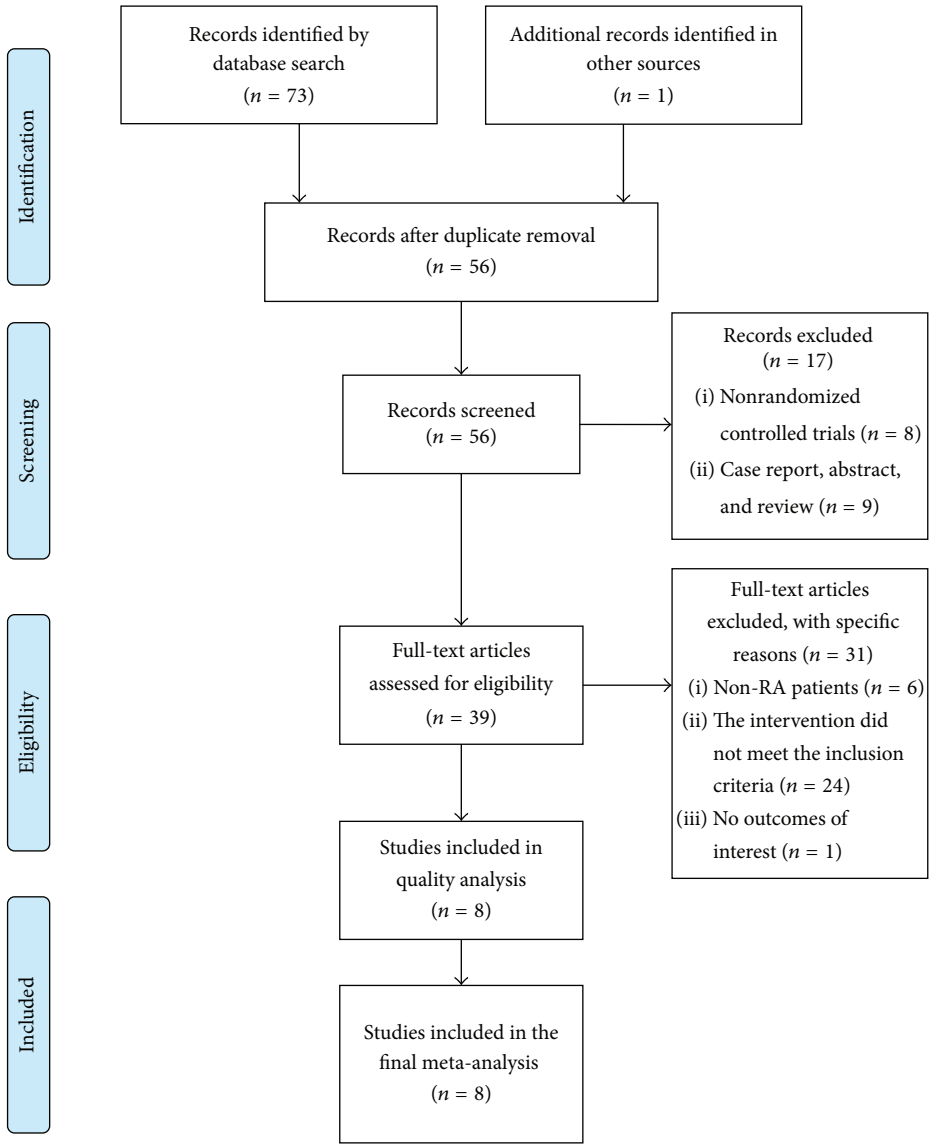


Figure 1: Flow diagram of the study selection procedure.

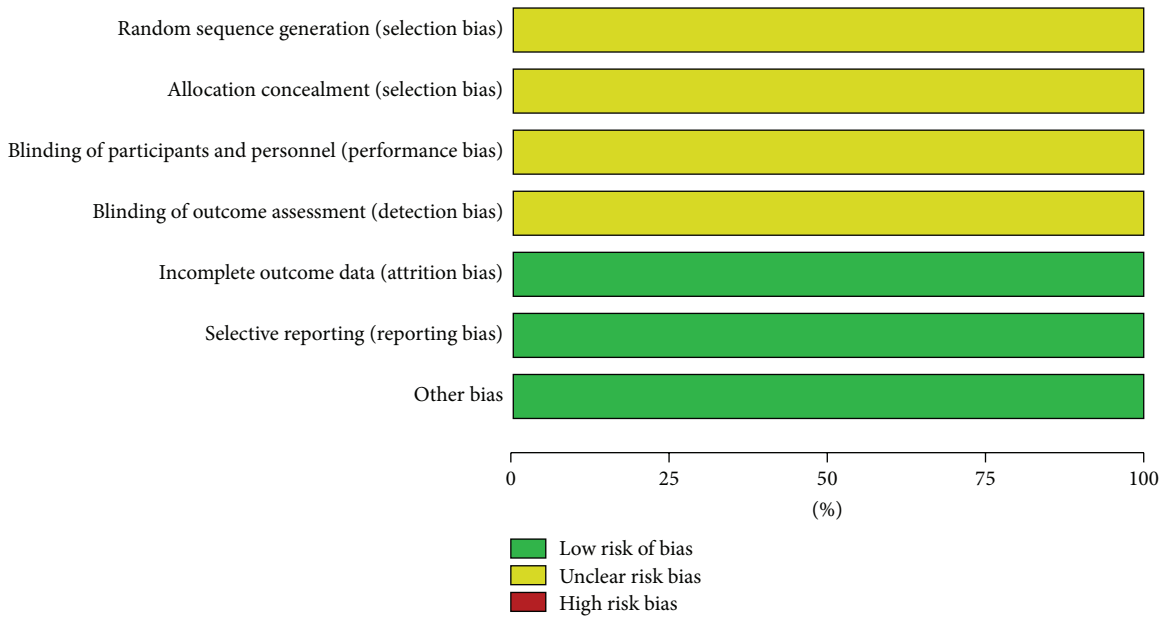


Figure 2: Risk of bias assessment.

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, fixed, 95% CI	Year
Zhao and Liu	39	40	34	40	11.4%	6.88 [0.79, 60.06]	2006
Si	14	17	12	21	25.3%	3.50 [0.77, 15.96]	2010
Tao et al.	48	52	41	52	42.2%	3.22 [0.95, 10.88]	2013
Li	46	48	38	48	21.2%	6.05 [1.25, 29.32]	2014
Total (95% CI)		**157**		**161**	**100.0%**	**4.31 [2.02, 9.16]**	
Total events	147		125				

Heterogeneity: $\chi^2 = 0.65$, df = 3 ($P = 0.88$); $I^2 = 0\%$
Test for overall effect: $Z = 3.79$ ($P = 0.0002$)

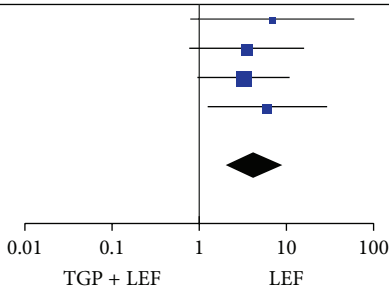


Figure 3: Meta-analysis of the therapeutic effects of the combination of TGP and LEF or LEF alone. TGP: total glucosides of peony; LEF: leflunomide.

concealment, blinding of participants and personnel, and blinding of outcome assessment. All of the eight studies addressed the incomplete outcomes as well as selective reporting. No other bias was identified.

3.4. The Therapeutic Effects of the Combination of TGP and LEF versus LEF Alone. To evaluate the therapeutic effects of the combination of TGP and LEF or LEF alone, data were extracted from four trials including 318 patients. A fixed-effect model was employed to pool the data because no significant heterogeneity was identified among the included trials ($P = 0.88$, $I^2 = 0\%$). As shown in Figure 3, a significantly higher effective rate was identified in the LEF group compared with the combination of TGP and LEF group (OR = 4.31, 95% CI = 2.02 to 9.16, and $P = 0.0002$).

3.5. The Effects of the Combination of TGP and LEF or LEF Alone on Serum Levels of ESR, CRP, and RF. Six, three, and four trials reported the effects of the combination of TGP and LEF or LEF alone on serum levels of ESR, CRP, and RF. Significant heterogeneity was found among these studies (all $P < 0.10$ or $I^2 > 50\%$). Therefore, a random-effect model was used to analyze the data. The pooled results revealed significant differences in serum levels of ESR (MD = −6.67, 95% CI = −9.88 to −3.479, and $P < 0.0001$), CRP (MD = −5.85, 95% CI = −8.66 to −3.05, and $P < 0.0001$), and RF (MD = −14.98, 95% CI = −21.82 to −8.14, and $P < 0.0001$) between the combination of TGP and LEF group and the LEF alone group (Figure 4).

3.6. Safety Profile and AEs. The safety profile was assessed for all included trials. The main AEs included abnormal liver function that was defined as follows: the serum level of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) was >1.5-fold above the upper limits of the normal value and gastrointestinal discomfort including nausea,

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI	Year
Zhao and Liu	25.64	6.41	40	28.48	7.58	40	27.7%	−2.84 [−5.92, 0.24]	2006
Si	31.1	8.6	17	42.1	15.6	21	11.5%	−11.00 [−18.82, −3.18]	2010
Yu and Zhang	21	15	39	30	21	40	11.1%	−9.00 [−17.03, −0.97]	2010
Dong	24.12	18.78	33	29	18.54	30	9.1%	−4.88 [−14.10, 4.34]	2014
Li	30.6	6.4	48	35.8	9.2	48	27.3%	−5.20 [−8.37, −2.03]	2014
Wu et al.	22.64	15.16	50	35.85	20.41	50	13.3%	−13.21 [−20.26, −6.16]	2015
Total (95% CI)			**227**			**229**	**100.0%**	**−6.67 [−9.88, −3.47]**	

Heterogeneity: $\tau^2 = 7.18$; $\chi^2 = 10.14$, df = 5 ($P = 0.07$); $I^2 = 51\%$
Test for overall effect: $Z = 4.09$ ($P < 0.0001$)

Mean difference IV, random, 95% CI
−50 −25 0 25 50
TGP + LEF LEF

(a)

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI	Year
Zhao and Liu	23.34	6.31	40	26.67	6.92	40	31.8%	−3.33 [−6.23, −0.43]	2006
Yu and Zhang	6	6	39	12	10	40	26.7%	−6.00 [−9.63, −2.37]	2010
Wu et al.	3.15	2.34	50	10.84	5.36	50	41.5%	−7.69 [−9.31, −6.07]	2015
Total (95% CI)			**129**			**130**	**100.0%**	**−5.85 [−8.66, −3.05]**	

Heterogeneity: $\tau^2 = 4.25$; $\chi^2 = 6.72$, df = 2 ($P = 0.03$); $I^2 = 70\%$
Test for overall effect: $Z = 4.09$ ($P < 0.0001$)

Mean difference IV, random, 95% CI
−20 −10 0 10 20
TGP + LEF LEF

(b)

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI	Year
Zhao and Liu	38.56	14.35	40	46.37	15.34	40	34.2%	−7.81 [−14.32, −1.30]	2006
Si	56.1	20.1	17	82.5	30.6	21	13.1%	−26.40 [−42.60, −10.20]	2010
Li	59.4	8.6	48	75.9	9.1	48	43.7%	−16.50 [−20.04, −12.96]	2014
Wu et al.	67.41	47.3	50	85.61	56.82	50	9.1%	−18.20 [−38.69, 2.29]	2015
Total (95% CI)			**155**			**159**	**100.0%**	**−14.98 [−21.82, −8.14]**	

Heterogeneity: $\tau^2 = 24.62$; $\chi^2 = 7.37$, df = 3 ($P = 0.06$); $I^2 = 59\%$
Test for overall effect: $Z = 4.29$ ($P < 0.0001$)

Mean difference IV, random, 95% CI
−100 −50 0 50 100
TGP + LEF LEF

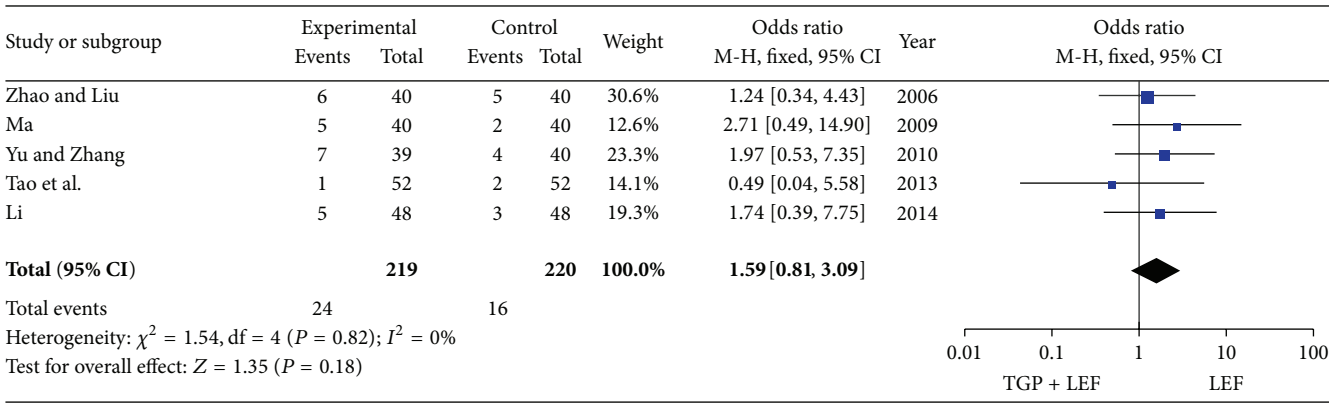
(c)

Figure 4: Meta-analysis of the effects of the combination of TGP and LEF or LEF alone on serum levels of ESR, CRP, and RF. (a) ESR: erythrocyte sedimentation rate; (b) CRP: C reaction protein; (c) RF: rheumatoid factor.

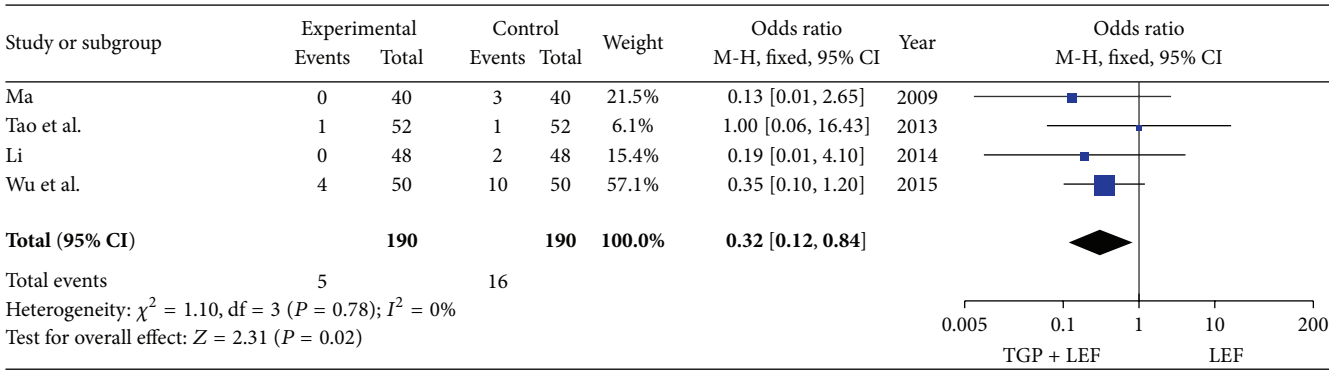
emesis, and diarrhea. A fixed-effect model was applied to pool the data because no heterogeneity was observed (all $P > 0.10$ or $I^2 < 50\%$). Our results revealed a higher rate of abnormal liver function (OR = 0.32, 95% CI = 0.12 to 0.84, and $P = 0.02$) in the LEF group compared with the combination of TGP and LEF group. However, no significant difference in gastrointestinal discomfort was identified between these two groups (OR = 1.59, 95% CI = 0.81 to 3.09, and $P = 0.18$) (Figure 5).

4. Discussion

4.1. Summary of Evidence. To the best of our knowledge, this is the first meta-analysis of the efficacy and safety of the combination of TGP and LEF for the treatment of RA. Eight RCTs including 319 patients in the treatment group and 324 individuals in the control group were included in the present meta-analysis. The pooled results suggest better therapeutic effects of the LEF alone compared to TGP plus LEF. The efficacy assessment system based on the improvement of signs and symptoms may lead to the heterogeneity of results. Only one study described the clinical outcomes defined by the American College of Rheumatology (ACR) criteria. The results in this study showed a higher response rate in the combination of TGP and LEF group compared with the LEF alone group [20]. In addition, one trial reported the clinical outcomes evaluated according to the European League Against Rheumatism (EULAR) response criteria [28]. This study also showed better treatment effects in the combination of TGP and LEF group than the LEF alone group [18]. Given the relatively small sampling size, we did not pool these results. Next, we should include internationally recognized standards such as the ACR or EULAR criteria and expand sampling size to further study the effects of the combination



(a)



(b)

Figure 5: Forest plot of the adverse events caused by the combination of TGP and LEF or LEF alone in RA patients. (a) Gastrointestinal discomfort; (b) abnormal liver function.

of TGP and LEF for the treatment of RA. However, the pooled data showed superior effects of the combination of TGP and LEF on reducing serum levels of ESR, CRP, and RF, compared with the LEF alone.

To evaluate the efficacy and safety of drugs, the AEs should also be fully considered. More RA patients receiving the LEF alone treatment had abnormal liver function compared with RA patients receiving the combination of TGP and LEF. However, no significant difference in gastrointestinal discomfort was identified between these two groups. The AEs mentioned above were relieved by relevant treatments.

4.2. Mechanisms of the Combination of TGP and LEF on RA. The mechanisms of TGP for the treatment of RA have been extensively investigated. A large number of studies have analyzed the function and effects of TGP in animal models and patients. It has been shown that TGP suppressed the proliferation of lymphocytes and neutrophils and induced the apoptosis of lymphocytes in animals with collagen-induce arthritis (CIA) or complete Freund's adjuvant-induced arthritis (AA) [29–33]. Several researchers suggest that TGP inhibited the production of proinflammatory mediators in synoviocytes [33–35]. Furthermore, both *in vitro* and *in vivo* studies suggest that TGP could balance the differentiation and function of Th1 and Th2 cells and inhibited the production of proinflammatory mediators, such as TNF-α, IL-1β, IL-6, and GM-CSF, in synoviocytes, macrophages, and lymphocytes [11, 36, 37]. A study in rabbits with antigen-induced arthritis (AIA) showed that TGP reduced the level of RANKL and improved OPG expression, suggesting that TGP inhibited juxta-articular osteoporosis and subchondral bone destruction [35, 38]. LEF, an isoxazole immunomodulatory agent, was approved by the U.S. Food and Drug Administration (FDA) for the treatment of RA in 1999 [17]. It has been proved that LEF inhibits mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), a key enzyme involved in *de novo* synthesis of pyrimidine ribonucleotide uridine monophosphate (rUMP) [39, 40], causing cell cycle arrest at the G1 phase and decrease in DNA and RNA syntheses. In addition, LEF can suppress the proliferation of autoimmune T-cell and the production of antibodies by B-cells and increase the synthesis of immunosuppressive cytokines such as transforming growth factor beta (TGF-β) [41]. Furthermore, LEF can inhibit the tyrosine kinase, which is critical for signal transduction and differentiation of activated cells and induction cell growth [42]. Taken together, these studies suggest that the combination of TGP and LEF is an effective therapy for the treatment of RA.

4.3. Limitations and Strengths of the Present Meta-Analysis. Nevertheless, some limitations of this meta-analysis should be discussed. First, the number of RCTs and the number of patients included in retrieved studies were limited. In the assessment of publication bias, the power of this meta-analysis was modest due to the limited number of trials and patients. Second, some included studies were of poor quality. Although all trials had a randomization design, very few studies reported the randomization procedure at length. The allocation concealment and blinding of participants or outcome assessment were not available, resulting in high risk of selection or detection bias. Third, heterogeneity was identified in included trials. We believe that differences in dose, treatment duration, detection methods, and evaluation criterion were the major sources of the heterogeneity. Fourth, all the RCTs included in the present meta-analysis were conducted in China and published in Chinese, causing high risk of selection bias. Therefore, the conclusion of the present meta-analysis should be further analyzed in the future.

5. Conclusion

While the therapeutic effects of the combination of TGP and LEF might not be better than that of LEF alone, the combination of TGP and LEF is superior to the LEF alone in reducing the levels of ESR, CRP, and RF. In addition, the combination of TGP and LEF is safer than the LEF alone regarding the abnormal liver functions caused by the treatment. Given the small sample size and heterogeneity of the included trials, multicenter and larger scale RCTs are needed to verify our conclusion.

Conflict of Interests

The authors have declared that they have no conflict of interests.

Authors' Contribution

Zhitao Feng and Zhengzhi Wu conceived the study. Zhitao Feng, Guochao He, and Juan Xu performed literature searches and study selection. Lihong Duan and Meiqun Cao performed data extraction and quality assessment. Zhitao Feng and Guochao He conducted analysis of the data. Zhitao Feng and Juan Xu wrote the paper. Liguo Chen and Zhengzhi Wu revised the paper.

Acknowledgments

This project was supported by grants from the National Natural Science Foundation of China (no. 81273665), National High Technology Research and Development Program (no. 2014AA020907), the Key Project of Shenzhen Science and Technology Program (no. JCYJ20130401115547212), the Guangdong Provincial Natural Science Foundation (2015A030310067), and Shenzhen Technology Innovation Foundation (no. JCYJ20150401171352927).

References

[1] H. A. Elshabrawy, Z. Chen, M. V. Volin, S. Ravella, S. Virupannavar, and S. Shahrara, "The pathogenic role of angiogenesis in rheumatoid arthritis," *Angiogenesis*, vol. 18, no. 4, pp. 433–448, 2015.

[2] Z. Feng, G. He, Z. Chen, Z. Wu, and J. Li, "Lack of association of matrix metalloproteinase-3 gene polymorphism with susceptibility to rheumatoid arthritis: a meta-analysis," *BMC Musculoskeletal Disorders*, vol. 15, article 376, 2014.

[3] T. K. Kvien, "Epidemiology and burden of illness of rheumatoid arthritis," *PharmacoEconomics*, vol. 22, no. 2, supplement 1, pp. 1–12, 2004.

[4] I. B. McInnes and G. Schett, "The pathogenesis of rheumatoid arthritis," *The New England Journal of Medicine*, vol. 365, no. 23, pp. 2205–2219, 2011.

[5] B. Svensson, A. Boonen, K. Albertsson, D. van der Heijde, C. Keller, and I. Hafström, "Low-dose prednisolone in addition to the initial disease-modifying antirheumatic drug in patients with early active rheumatoid arthritis reduces joint destruction and increases the remission rate: a two-year randomized trial," *Arthritis & Rheumatism*, vol. 52, no. 11, pp. 3360–3370, 2005.

[6] M. I. Koenders and W. B. van den Berg, "Novel therapeutic targets in rheumatoid arthritis," *Trends in Pharmacological Sciences*, vol. 36, no. 4, pp. 189–195, 2015.

[7] J. A. Singh, D. E. Furst, A. Bharat et al., "2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis," *Arthritis Care & Research*, vol. 64, no. 5, pp. 625–639, 2012.

[8] J. S. Smolen, R. Landewe, F. C. Breedveld et al., "EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update," *Annals of the Rheumatic Diseases*, vol. 73, no. 3, pp. 492–509, 2014.

[9] C. Grigor, H. Capell, A. Stirling et al., "Effect of a treatment strategy of tight control for rheumatoid arthritis (the TICORA study): a single-blind randomised controlled trial," *The Lancet*, vol. 364, no. 9430, pp. 263–269, 2004.

[10] V. Rantalaiho, M. Korpela, L. Laasonen et al., "Early combination disease-modifying antirheumatic drug therapy and tight disease control improve long-term radiologic outcome in patients with early rheumatoid arthritis: the 11-year results of the Finnish Rheumatoid Arthritis Combination Therapy trial," *Arthritis Research and Therapy*, vol. 12, no. 3, article R122, 2010.

[11] W. Cao, W. Zhang, J. Liu et al., "Paeoniflorin improves survival in LPS-challenged mice through the suppression of TNF-α and IL-1β release and augmentation of IL-10 production," *International Immunopharmacology*, vol. 11, no. 2, pp. 172–178, 2011.

[12] H. Huang, E. J. Chang, Y. Lee, J. S. Kim, S. S. Kang, and H. H. Kim, "A genome-wide microarray analysis reveals anti-inflammatory target genes of paeonol in macrophages," *Inflammation Research*, vol. 57, no. 4, pp. 189–198, 2008.

[13] L.-L. Zhang, W. Wei, N.-P. Wang et al., "Paeoniflorin suppresses inflammatory mediator production and regulates G protein-coupled signaling in fibroblast—like synoviocytes of collagen induced arthritic rats," *Inflammation Research*, vol. 57, no. 8, pp. 388–395, 2008.

[14] W. Zhang and S.-M. Dai, "Mechanisms involved in the therapeutic effects of *Paeonia lactiflora* Pallas in rheumatoid arthritis," *International Immunopharmacology*, vol. 14, no. 1, pp. 27–31, 2012.

[15] M. Zhao, G.-P. Liang, M.-N. Tang et al., "Total glucosides of paeony induces regulatory $CD4^+CD25^+$ T cells by increasing Foxp3 demethylation in lupus $CD4^+$ T cells," *Clinical Immunology*, vol. 143, no. 2, pp. 180–187, 2012.

[16] D. Golicki, M. Niewada, J. Lis, K. Pol, T. Hermanowski, and M. Tłustochowicz, "Leflunomide in monotherapy of rheumatoid arthritis: meta-analysis of randomized trials," *Polish Archives of Internal Medicine*, vol. 122, no. 1-2, pp. 22–32, 2012.

[17] K. E. Donahue, D. E. Jonas, R. A. Hansen et al., *Drug Therapy for Rheumatoid Arthritis in Adults: An Update*, Agency for Healthcare Research and Quality, Rockville, Md, USA, 2012.

[18] T. T. Wu, R. He, J. N. Liu, and Y. Li, "The efficacy observation on leflunomide combined with total glucosides of paeony capsules for rheumatoid arthritis," *Proceeding of Clinical Medicine*, vol. 24, no. 5, pp. 342–345, 2015.

[19] M. Li, "The clinical efficacy of leflunomide combined with TGP in the treatment of elderly rheumatoid arthritis and its effect on C-reactive protein and erythrocyte sedimentation rate," *China & Foreign Medical Treatment*, no. 33, pp. 45–47, 2014.

[20] W. H. Dong, "The clinical observation on total glucosides of paeony combined with leflunomide for the treatment of elderly rheumatoid arthritis," *Clinical Journal of Traditional Medicine*, vol. 26, no. 5, pp. 490–491, 2014.

[21] L. Tao, Y. Lian, X. H. Guan, and L. H. Lei, "Treating 52 cases of rheumatoid arthritis with leflunomide and total glucosides of paeony capsules. Guide of China medicine," *Guide of China Medicine*, vol. 11, no. 17, pp. 403–404, 2013.

[22] J. H. Yu and H. Zhang, "The clinical research on total glucosides of paeony combined with leflunomide for the treatment of active rheumatoid arthritis," *Forum on Tradtional Medicine*, vol. 25, no. 4, pp. 9–10, 2010.

[23] H. M. Si, "The clinical observation on leflunomide combined with total glucosides of paeony for the treatment of elderly rheumatoid arthritis," *Guide of China Medicine*, vol. 8, no. 31, pp. 108–109, 2010.

[24] F. Y. Ma, "The efficacy and satety of total glucosides of paeony combined with leflunomide in the treatment of rheumatoid arthritis," *Liaoning Journal of Traditional Chinese Medicine*, vol. 36, no. 6, pp. 1509–1510, 2009.

[25] Y.-X. Zhao and Y. Liu, "Clinical observation on effects of leflunomid and total glucosides of paeony on rheumatoid arthritis," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 26, no. 4, pp. 355–357, 2006.

[26] J. P. T. Higgins, S. G. Thompson, J. J. Deeks, and D. G. Altman, "Measuring inconsistency in meta-analyses," *The British Medical Journal*, vol. 327, no. 7414, pp. 557–560, 2003.

[27] R. DerSimonian and N. Laird, "Meta-analysis in clinical trials," *Controlled Clinical Trials*, vol. 7, no. 3, pp. 177–188, 1986.

[28] A. M. van Gestel, M. L. L. Prevoo, M. A. van't Hof, M. H. van Rijswijk, L. B. A. van de Putte, and P. L. C. M. van Riel, "Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria," *Arthritis & Rheumatism*, vol. 39, no. 1, pp. 34–40, 1996.

[29] Y. Chang, L. Zhang, C. Wang, X.-Y. Jia, and W. Wei, "Paeoniflorin inhibits function of synoviocytes pretreated by rIL-1α and regulates EP4 receptor expression," *Journal of Ethnopharmacology*, vol. 137, no. 3, pp. 1275–1282, 2011.

[30] D. Jiang, Y. Chen, X. Hou, J. Xu, X. Mu, and W. Chen, "Influence of *Paeonia lactiflora* roots extract on cAMP-phosphodiesterase activity and related anti-inflammatory action," *Journal of Ethnopharmacology*, vol. 137, no. 1, pp. 914–920, 2011.

[31] H. Tsuboi, K. Hossain, A. A. Akhand et al., "Paeoniflorin induces apoptosis of lymphocytes through a redox-linked mechanism," *Journal of Cellular Biochemistry*, vol. 93, no. 1, pp. 162–172, 2004.

[32] H.-M. Xu, W. Wei, X.-Y. Jia, Y. Chang, and L. Zhang, "Effects and mechanisms of total glucosides of paeony on adjuvant arthritis in rats," *Journal of Ethnopharmacology*, vol. 109, no. 3, pp. 442–448, 2007.

[33] Y.-Q. Zheng and W. Wei, "Total glucosides of paeony suppresses adjuvant arthritis in rats and intervenes cytokine-signaling between different types of synoviocytes," *International Immunopharmacology*, vol. 5, no. 10, pp. 1560–1573, 2005.

[34] Q.-T. Wang, L.-L. Zhang, H.-X. Wu, and W. Wei, "The expression change of β-arrestins in fibroblast-like synoviocytes from rats with collagen-induced arthritis and the effect of total glucosides of paeony," *Journal of Ethnopharmacology*, vol. 133, no. 2, pp. 511–516, 2011.

[35] L. Zhu, W. Wei, Y.-Q. Zheng, and X.-Y. Jia, "Effects and mechanisms of total glucosides of paeony on joint damage in rat collagen-induced arthritis," *Inflammation Research*, vol. 54, no. 5, pp. 211–220, 2005.

[36] W.-L. Jiang, X.-G. Chen, H.-B. Zhu, Y.-B. Gao, J.-W. Tian, and F.-H. Fu, "Paeoniflorin inhibits systemic inflammation and improves survival in experimental sepsis," *Basic and Clinical Pharmacology and Toxicology*, vol. 105, no. 1, pp. 64–71, 2009.

[37] Y.-Q. Zheng, W. Wei, L. Zhu, and J.-X. Liu, "Effects and mechanisms of Paeoniflorin, a bioactive glucoside from paeony root, on adjuvant arthritis in rats," *Inflammation Research*, vol. 56, no. 5, pp. 182–188, 2007.

[38] C. C. Wei, F. T. You, L. Y. Mei, S. Jian, and C. Y. Qiang, "Total glucosides of paeony prevents juxta-articular bone loss in experimental arthritis," *BMC Complementary and Alternative Medicine*, vol. 13, article 186, 2013.

[39] R. I. Fox, M. L. Herrmann, C. G. Frangou et al., "Mechanism of action for leflunomide in rheumatoid arthritis," *Clinical Immunology*, vol. 93, no. 3, pp. 198–208, 1999.

[40] F. M. Meier, M. Frerix, W. Hermann, and U. Müller-Ladner, "Current immunotherapy in rheumatoid arthritis," *Immunotherapy*, vol. 5, no. 9, pp. 955–974, 2013.

[41] W. W. Cao, P. N. Kao, Y. Aoki, J. C. Xu, R. A. Shorthouse, and R. E. Morris, "A novel mechanism of action of the immunomodulatory drug, leflunomide: augmentation of the immunosuppressive cytokine, TGF-β1, and suppression of the immunostimulatory cytokine, IL-2," *Transplantation Proceedings*, vol. 28, no. 6, pp. 3079–3080, 1996.

[42] F. Behrens, M. Koehm, and H. Burkhardt, "Update 2011: leflunomide in rheumatoid arthritis—strengths and weaknesses," *Current Opinion in Rheumatology*, vol. 23, no. 3, pp. 282–287, 2011.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 5890590, 8 pages
http://dx.doi.org/10.1155/2016/5890590

Research Article

Uliginosin B, a Possible New Analgesic Drug, Acts by Modulating the Adenosinergic System

Eveline Dischkaln Stolz,[1] Paola Fontoura da Costa,[1] Liciane Fernandes Medeiros,[2] Andressa Souza,[2] Ana Maria Oliveira Battastini,[3] Gilsane Lino von Poser,[1] Carla Bonan,[4] Iraci L. S. Torres,[2] and Stela Maris Kuze Rates[1]

[1] *Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, 90610-000 Porto Alegre, Brazil*
[2] *Laboratório de Farmacologia da Dor e Neuromodulação: Investigações Pré-Clínicas, Departamento de Farmacologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, 90046-900 Porto Alegre, Brazil*
[3] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, 90035-003 Porto Alegre, Brazil*
[4] *Laboratório de Neuroquímica e Psicofarmacologia, Faculdade de Biociências, Pontificia Universidade Católica do Rio Grande do Sul, 90619-900 Porto Alegre, Brazil*

Correspondence should be addressed to Stela Maris Kuze Rates; stela.rates@ufrgs.br

Received 28 January 2016; Accepted 21 February 2016

Academic Editor: Vincenzo De Feo

Uliginosin B (ULI) is a natural acylphloroglucinol that has been proposed as a new molecular scaffold for developing analgesic and antidepressant drugs. Its effects seem to be due to its ability to increase monoamines in the synaptic cleft by inhibiting their neuronal uptake without binding to their respective transporters, but its exact mode of action is still unknown. Considering the importance of the purinergic system to pain transmission and its modulation by monoamines availability, the aim of this study was to investigate the involvement of adenosinergic signaling in antinociceptive effect of uliginosin B. The selective adenosine A_1 receptor antagonist DPCPX and the selective A_{2A} antagonist ZM 241385 prevented the effect of ULI in the hot-plate test in mice. Pretreatment with inhibitors of adenosine reuptake (dipyridamole) or adenosine deaminase (EHNA) did not affect the ULI effect. On the other hand, its effect was completely prevented by an inhibitor of ecto-5′-nucleotidase (AMPCP). This finding was confirmed *ex vivo*, whereby ULI treatment increased AMP and ATP hydrolysis in spinal cord and cerebral cortex synaptosomes, respectively. Altogether, these data indicate that activation of A_1 and A_{2A} receptors and the modulation of ecto-5′-nucleotidase activity contribute to the antinociceptive effect of ULI.

1. Introduction

Uliginosin B (ULI) is a dimeric acylphloroglucinol consisting of filicinic acid and phloroglucinol moieties, which occurs in *Hypericum* species native to South America [1]. This molecular pattern has been proposed as a prototype to develop analgesic and antidepressant drugs [2–5].

Preclinical studies suggested that ULI has antidepressant properties, which seems to be due to its ability to increase monoamines availability in the synaptic cleft by inhibiting their neuronal uptake [2]. Nevertheless, ULI does not bind to the monoamine sites on neuronal transporters, which indicates that it acts differently from the classical antidepressants [2]. It is noteworthy that ULI deserves attention as a drug potentially useful to reduce the dose of morphine in clinical practice [6]. Its antinociceptive effect involves the activation of monoaminergic, glutamatergic, and opioid receptors, apparently without binding to these receptors [2, 3, 5]. Therefore, other molecular targets for ULI might be considered.

The relationship between purinergic system/nociceptive pathways has been reported [7]; numerous studies described the interaction between purinergic, monoaminergic, and opioid pathways [8–14].

Adenosine triphosphate (ATP) stimulates cellular excitability, augments the release of excitatory amino acids, initiates a nociceptive response, and can lead to apoptosis [15, 16]. ATP released from cells into the extracellular space has a short half-life in the extracellular milieu since it is rapidly degraded to adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine by ectonucleotidase pathway, which includes the E-NTPDase family (ectonucleoside triphosphate diphosphohydrolase) and ecto-5′-nucleotidase (for review see Robson et al. [17] and Zylka [18]). These enzymes control the availability of ligands (ATP, ADP, AMP, and adenosine) to activate purinoceptors, as well as the duration of receptor activation. In addition, these enzymes may provide a protective function by maintaining extracellular ATP/ADP and adenosine levels within physiological concentrations (for review see Burnstock [19]). Adenosine levels are also controlled by deamination to inosine through adenosine deaminase (ADA), cell release, and reuptake through nucleoside transporters (NTs) in bidirectional equilibrative processes driven by chemical gradients and unidirectional concentrative processes driven by sodium electrochemical gradients [20, 21]. The activation of adenosine receptors appears to be involved in the modulation of nociceptive and inflammatory pathways [7]. These effects depend on the availability of adenosine in the synaptic cleft, as well as intensity and modality of the stimulus [11].

Interestingly, several drugs that increase monoamine availability or act through the activation of opioid receptors present antinociceptive effect mediated by activation of adenosine receptors [12–14].

In view of these observations, the aim of this study was to investigate the involvement of purinergic pathway in the antinociceptive effect of ULI, including the effect of ULI on adenosine metabolism.

2. Material and Methods

2.1. Uliginosin B Obtention. ULI (Figure 1(a)) was obtained according to Stolz and coworkers [3] from *n*-hexane extract of the aerial parts (all sections above ground) of *Hypericum polyanthemum* Klotzsch ex Reichardt (Hypericaceae) (Figure 1(b)), harvested in Caçapava do Sul, Brazil (voucher specimen ICN 175915). Plant collection was authorized by the Conselho de Gestão do Patrimônio Genético and Instituto Brasileiro do Meio Ambiente (number 003/2008, Protocol 02000.001717/2008-60).

The purity (96%) of uliginosin B was confirmed through HPLC analysis coupled to an ultraviolet detector [1, 22] and its structure was characterized by ^{1}H and ^{13}C NMR spectra [23]. It was stored at −20°C, protected from light and moisture until use. Immediately before biological testing, it was suspended in saline containing 2% polysorbate 80. Unpublished studies by us demonstrated that, in these storage conditions, ULI has good stability and remains unaltered for approximately 2 years.

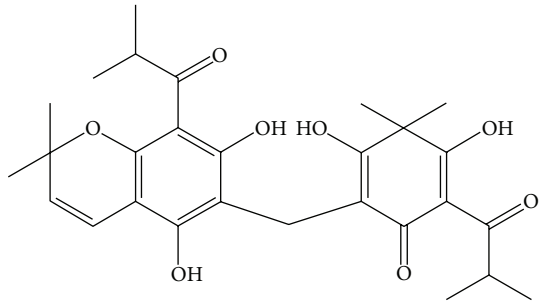
2.2. Animals. Adult male CF1 mice (25–35 g) were used for *in vivo* and *ex vivo* experiments. Animals were housed under a 12-hour light/dark cycle (lights on at 7:00 am) at constant temperature (23 ± 1°C) with free access to standard certified rodent diet and tap water. All experiments were approved by a local Ethics Committee of Animal Use (UFRGS: 21060/2011) and were in compliance with Brazilian law [24–26] and conformed to the Laboratory Guide for the Care and Use of Animals [27]. Animal handling and all experiments were performed in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort.

2.3. Behavioral Experiments. Pain sensitivity was assessed by the hot-plate test as described elsewhere [3]. First, each animal freely explored the nonfunctioning hot-plate apparatus for 60 s. Then, the animal returned to its home-cage and the apparatus was turned on and stabilized at 55 ± 1°C. Mice baseline responsiveness was determined by recording the time elapsed until the animal licked one of its hind paws or jumped. Mice that presented a baseline reaction of more than 20 s were not used. Immediately, the animals received one of the following compounds: adenosine A_1 receptor antagonist: 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) 0.1 mg/kg (0.01 mg/mL, i.p.); adenosine A_{2A}-receptors antagonists: 4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol (ZM 241385) 3 mg/kg (0.3 mg/mL, i.p.); inhibitor of adenosine deaminase: erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) 5 mg/kg (0.5 mg/mL, i.p.); adenosine reuptake inhibitor: dipyridamole (DIP) 30 mg/kg (3 mg/mL, i.p.); ecto-5′-nucleotidase inhibitor: alpha-beta-methylene adenosine 5′-diphosphate (AMPCP) 2 mg/kg (0.2 mg/mL, i.p.). The doses of each tested drug were chosen based on the literature, and lack of antinociceptive effect in the hot-plate test was confirmed in our laboratory [28–31]. After 15 min, the animals were treated with ULI 15 mg/kg (1.5 mg/mL, i.p.) or vehicle (saline plus 2% polysorbate 80; 1 mL/100 g, i.p.) and reexposed to the hot-plate (55 ± 1°C) 30 min later. A maximum latency time of 40 s was imposed (cut-off). The results are expressed as percentages of maximal possible analgesic effect (% MPE) using the following formula:

$$\%\ \text{MPE} = \frac{(\text{post-drug latency} - \text{pre-drug latency})}{(\text{cut-off latency} - \text{pre-drug latency})} \times 100. \quad (1)$$

2.4. NTPDase and Ecto-5′-nucleotidase Activity

2.4.1. Synaptosomal Preparation. The mice were divided into three groups: handled only (sham), treated with 15 mg/kg ULI (1.5 mg/mL, i.p.), or vehicle (saline plus 2% polysorbate 80, i.p.). After 30 min the animals were killed and the spinal cord and cerebral cortex were removed. The tissues were prepared according to Rozisky et al. [32] and synaptosomes were isolated as described by Nagy and Delgado-Escueta [33]. Protein concentration was determined by the Coomassie blue method [34] using bovine serum albumin as a standard.



(a)

(b)

Figure 1: Uliginosin B structure (a). *Hypericum polyanthemum*, plant used to obtain ULI (b).

2.4.2. Determination of NTPDases and Ecto-5′-nucleotidase Activity. The ATP, ADP, and AMP hydrolysis was performed as described previously [32, 35]. The synaptosomal fraction (10–20 μg protein) was preincubated for 10 min at 37°C in 100 μL of incubation medium containing 45 mM Tris-HCl buffer (pH 8), 0.1 mM EDTA, 1.5 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, and 225 mM sucrose for ATP and ADP hydrolysis. For AMP hydrolysis the samples were incubated in 80 μL ecto-5′-nucleotidase incubation medium containing 0.1 M Tris-HCl (pH 7), 10 mM $MgCl_2$, and 0.15 M sucrose. The reactions were initiated by the addition of 1 mM ATP, ADP, or AMP and stopped by the addition of 200 μL 10% trichloroacetic acid. Finally, 100 μL samples were taken for the assay of released inorganic phosphate (Pi) [36]. The enzyme activities were expressed as nmol of inorganic phosphate released per minute per milligram of protein (nmol $Pi \cdot min^{-1} \cdot mg^{-1}$ protein).

2.5. Statistical Analysis. The results were evaluated by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test using the Sigma Stat software, version 2.03 (Jandel Scientific Corporation). All results were expressed as mean ± standard error of the mean (SEM).

3. Results

The influence of selective A_1 and A_{2A} receptor antagonist pretreatments on the ULI effect in the hot-plate test is depicted in Figure 2. One-way ANOVA revealed a significant antinociceptive effect of ULI (Figure 2(a): $F_{(3,35)} = 25.611$, $p < 0.001$; Figure 2(b): $F_{(3,35)} = 19.555$, $p < 0.001$), which was prevented by pretreatment with the adenosine A_1 receptor antagonist DPCPX ($p < 0.001$) and the adenosine A_{2A} receptor antagonist ZM 241385 ($p < 0.001$).

The data depicted in Figure 3 show the effect of adenosine metabolism on the antinociceptive effect of ULI. Pretreatment with inhibitor of adenosine deaminase (EHNA) or nucleoside transporter inhibitor (dipyridamole) did not affect the ULI nociceptive response. One-way ANOVA revealed a significant effect in the group treated with ULI and ULI plus EHNA or ULI plus dipyridamole in relation to the control groups (Figure 3(a): $F_{(3,39)} = 17.819$, $p < 0.001$; Figure 3(b): $F_{(4,44)} = 19.248$, $p < 0.001$). Pretreatment with ecto-5′-nucleotidase inhibitor (AMPCP) prevented the ULI antinociceptive effect on the hot-plate test. One-way ANOVA revealed a significant effect only in the group treated with ULI (Figure 3(c): $F_{(3,35)} = 12.981$, $p < 0.001$), which was prevented by the AMPCP pretreatment ($p < 0.001$).

The activities of NTPDases and ecto-5′-nucleotidase were assessed *ex vivo* after acute treatment of mice with ULI (15 mg/kg, i.p.) (Figure 4). In spinal cord synaptosomal preparations, the results showed that the treatment with ULI increased AMP hydrolysis only; ATP and ADP hydrolysis remained unaltered (Figure 3(a), ATP: $F_{(2,17)} = 0.663$, $p = 0.530$; ADP: $F_{(2,17)} = 1.494$, $p = 0.256$; AMP: $F_{(2,17)} = 6.921$, $p < 0.01$). In cerebral cortex synaptosomes, treatment with ULI increased the ATP hydrolysis and there were no changes on ADP and AMP hydrolysis (Figure 3(b), ATP: $F_{(2,14)} = 5.579$, $p < 0.05$; ADP: $F_{(2,14)} = 3.327$, $p = 0.071$; AMP: $F_{(2,14)} = 0.934$, $p = 0.420$).

4. Discussion

Herein we demonstrated for the first time the involvement of the purinergic system in the antinociceptive effect of uliginosin B (ULI), a dimeric acylphloroglucinol from *Hypericum* species native to South America. Previous data showed that ULI (15 mg/kg, i.p.) produces antinociceptive effect in hot-plate test [3, 5, 6]. We now show that the pretreatment with DPCPX and ZM 241385, selective adenosine A_1 and A_{2A} receptor antagonists, respectively, completely prevented the antinociceptive effect of ULI in the mice hot-plate test. This finding indicates that the activation of these receptors mediates the effect of ULI. However, ULI does not have the classical structural requirements for binding to adenosine receptors, since it lacks nitrogen atoms and amine groups, which seem to be crucial for ligands of

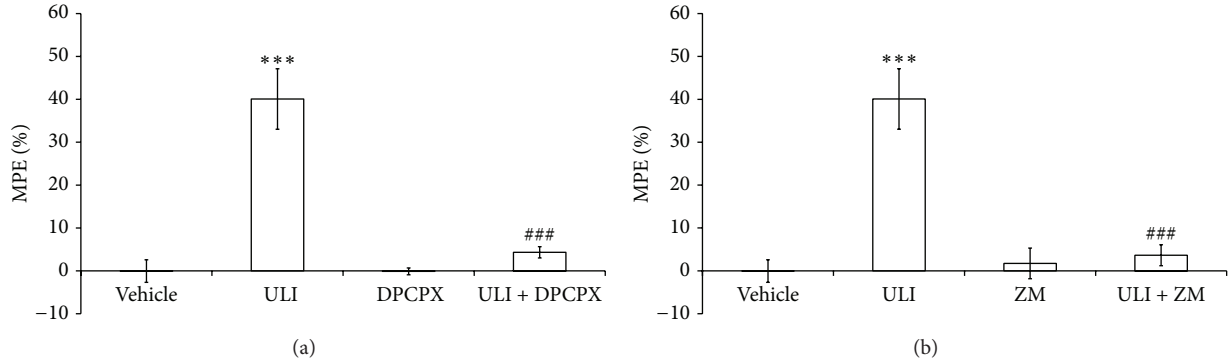


Figure 2: Effect of pretreatment with DPCPX (0.1 mg/kg, i.p. (a)) or ZM 241385 (ZM: 3 mg/kg, i.p. (b)) 15 min before the treatment with uliginosin B (ULI: 15 mg/kg, i.p.) in the hot-plate test. Percentages of maximal possible analgesic effect (% MPE) are presented as means ± SEM (n = 9 mice/group). $^{***}p < 0.001$ compared to vehicle group; $^{###}p < 0.001$ compared to uliginosin B (15 mg/kg, i.p.) group (ANOVA followed by Student-Newman-Keuls). Data are presented in mean ± SEM (n = 10 mice/group). Significantly different values were detected by one-way ANOVA followed by Student-Newman-Keuls: $^{***}p < 0.001$ compared to vehicle.

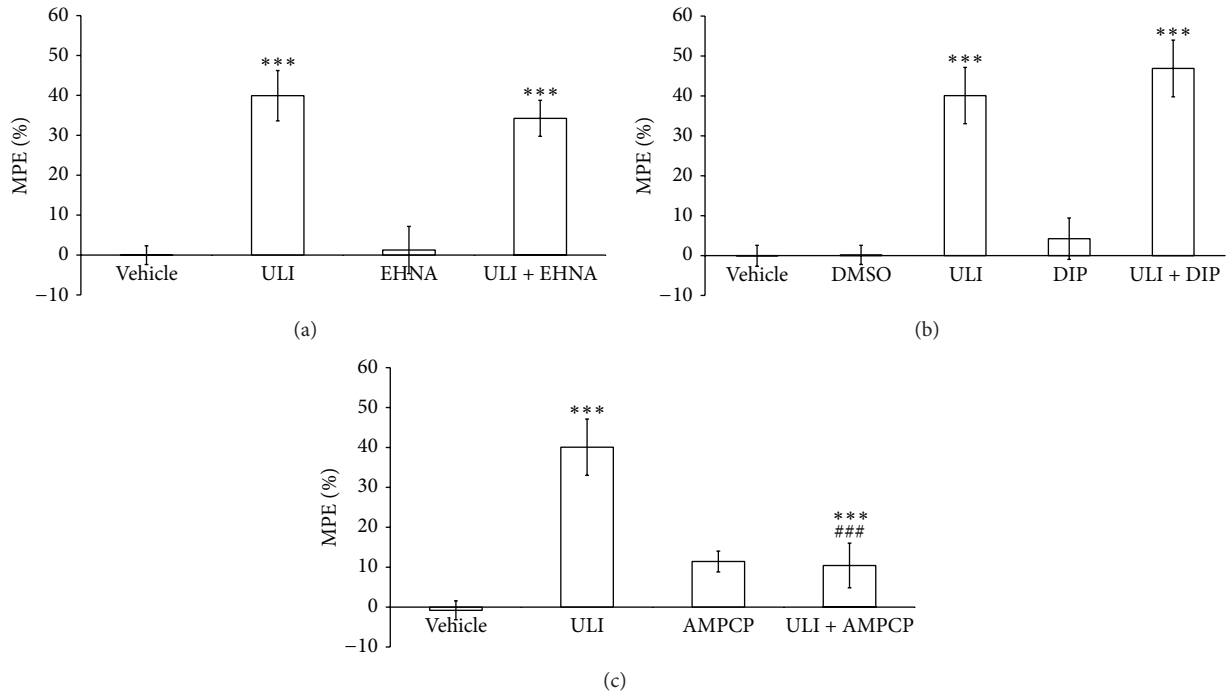


Figure 3: Effect of pretreatment with EHNA (5 mg/kg, i.p. (a)), dipyridamole (DIP: 30 mg/kg, i.p. (b)), or AMPCP (2 mg/kg, i.p. (c)), before treatment with uliginosin B (ULI: 15 mg/kg, i.p.) in the hot-plate test. Percentages of maximal possible analgesic effect (% MPE) are presented as means ± SEM (n = 9-10 mice/group). $^{***}p < 0.001$ compared to vehicle group; $^{###}p < 0.001$ compared to uliginosin B (15 mg/kg, i.p.) group (ANOVA followed by Student-Newman-Keuls). Data are presented in mean ± SEM (n = 10 mice/group). Significantly different values were detected by one-way ANOVA followed by Student-Newman-Keuls: $^{***}p < 0.001$ compared to vehicle.

these receptors [37–40]. Another possibility could be due to allosteric interactions. Nevertheless, although the structure activity relationship is still not completely established, the main allosteric modulators of adenosine receptors also contain nitrogen atoms or amine groups. In addition, compounds that act allosterically and/or orthosterically at the A_1 adenosine receptor have often close structural resemblance, which suggests that the allosteric site on the A_1 adenosine receptor is closer or very similar to the orthosteric site of this receptor [41]. Thus, we supposed that the activation of adenosine receptors following ULI treatment could result from increased adenosine availability.

As already mentioned, previous studies by some of us demonstrated that ULI has antidepressant-like effect by inhibiting synaptosomal monoamines reuptake and possibly enhancing the extracellular monoamine availability [2]. Amitriptyline and desipramine, which are antidepressants that increase the availability of monoamines, displayed

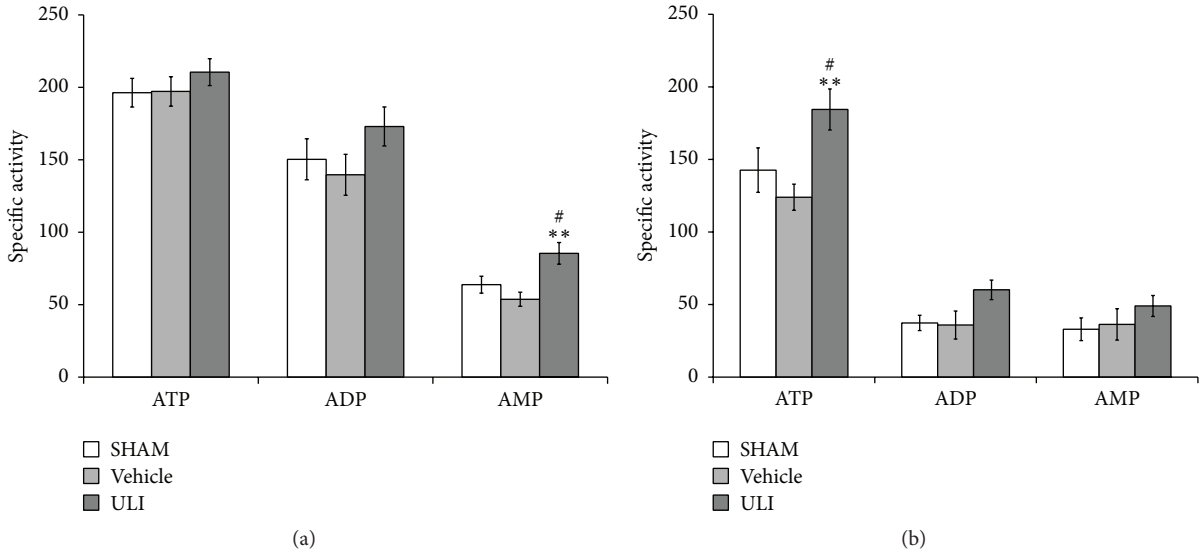


Figure 4: ATP, ADP, and AMP hydrolysis in synaptosomes from spinal cord (a) and cerebral cortex (b) of mice treated with uliginosin B (15 mg/kg, i.p.). Values are presented as means ± SEM (n = 5-6 mice/group). Specific enzyme activities were expressed as nmol Pi·min^{-1}·mg^{-1} protein. $^{**}p < 0.01$ compared to correspondent sham group; $^{\#}p < 0.05$ compared to correspondent vehicle group (ANOVA followed by Student-Newman-Keuls).

antinociceptive effects dependent of adenosine receptors activation [8–10, 42–44]. In addition, antidepressants that increase the extracellular availability of monoamines seem to modulate nucleotide hydrolysis in the central nervous system (CNS), presenting stimulatory or inhibitory effect depending on the treatment duration and brain structure [45–47]. The effects of antidepressants on adenosine system seem to reflect increased availability of adenosine following an effect on transport and not necessarily effects on amine transporters [48]. Therefore, we decided to investigate the effect of ULI on nucleotide hydrolysis and adenosine metabolism in order to observe whether adenosine availability has influence on the antinociceptive properties of this compound.

It is noteworthy that our data indicate that the antinociceptive effect of ULI could be, at least in part, dependent of adenosine availability, since it was prevented by pretreatment with AMPCP, an ecto-5′-nucleotidase inhibitor. This result was confirmed by the *ex vivo* assay in spinal cord synaptosomes which pointed to an increase in AMP hydrolysis induced by ULI. In cerebral cortex synaptosomes, treatment with ULI increased the ATP hydrolysis and there were no changes on ADP and AMP hydrolysis. These different effects on AMP hydrolysis could be due to an increased expression of ecto-5′-nucleotidase in the cerebral cortex [49] or to a different processing of the protein, which has been shown to be present in different isoforms in nerve terminals [50, 51], or instead to an abrogation of the negative allosteric modulation of this enzymatic activity by adenine nucleotides [52]. Spinal cord and cerebral cortex, which have been considered as important antinociceptive pathways, possess a high density of adenosine receptors [11, 53, 54].

The fact that ULI stimulates ATP hydrolysis without altering ADP hydrolysis in the cerebral cortex agrees with other studies. ATP, through activation of P_{2X3} receptors, generally facilitates nociceptive transmission while ADP (via P_{2Y} receptors) may decrease the excitatory effect of ATP [55, 56]. ATP can facilitate nociceptive sensitivity by the activation of both ATP-gated ion channels (P2X receptors) and G protein-coupled (P2Y) receptors contributing to nociceptive signaling in peripheral sensory neurons. On the other hand, Gi-coupled P2Y receptors activation can modulate pain neurotransmission [57].

An extensive review by Cunha [58] has pointed that the activation of adenosine A_1 and A_{2A} receptors is proven to be associated with the release of monoamines, glutamate, and other neuromodulators in different brain regions. In addition, the release of adenosine may be modulated by activation of these neurotransmitter pathways [11, 59–62], as well as opioids [63–66]. Quarta and coworkers [67] have postulated a circuit under physiological conditions of high adenosine release, where regulation of the activation of adenosine A_1 and A_{2A} receptors could induce glutamate and dopamine release. In this context, it is possible to hypothesize that an increase in adenosine levels could be responsible for an increase in the availability of monoamines and activation of glutamate and opioid receptors, previously described for ULI [2, 3, 5, 6]. Further studies are needed in order to substantiate this assumption.

As a final point, our results demonstrate that the ULI effects on adenosine metabolism involve mainly the modulation of adenosine levels by ecto-5′-nucleotidase activity, since the nociceptive response of this phloroglucinol derivative was not altered by pretreatment with dipyridamole and EHNA, which are nucleoside transporter and ADA inhibitors, respectively. In addition, considering that ATP plays a key role as a danger signal in the brain [68], the ULI ability to increase ATP hydrolysis, with consequent generation of adenosine, may indicate a neuroprotective effect. On the other hand, the

results so far do not rule out the possibility that ULI could be a trigger of ATP release. Further experiments are planned in order to investigate these outcomes.

5. Conclusion

In conclusion, the present results indicate that uliginosin B increases the availability of adenosine, via ecto-5′-nucleotidases, with consequent activation of adenosine receptors (particularly A_1 and A_{2A}), which play a role in the antinociceptive effect of this phloroglucinol. These findings opened a new avenue for searching the mode of action of this original neuroactive molecular pattern.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors are grateful to Sergio Bordignon, Ph.D., for collecting and identifying the plant material and to CAPES/Brazil and CNPq/Brazil for financial support.

References

[1] G. V. Ccana-Ccapatinta, F. M. C. de Barros, H. Bridi, and G. L. von Poser, "Dimeric acylphloroglucinols in *Hypericum* species from sections *Brathys* and *Trigynobrathys*," *Phytochemistry Reviews*, vol. 14, no. 1, pp. 25–50, 2015.

[2] A. C. Stein, A. F. Viana, L. G. Müller et al., "Uliginosin B, a phloroglucinol derivative from Hypericum polyanthemum: a promising new molecular pattern for the development of antidepressant drugs," *Behavioural Brain Research*, vol. 228, no. 1, pp. 66–73, 2012.

[3] E. D. Stolz, A. F. Viana, D. R. Hasse, G. L. von Poser, J.-C. do Rego, and S. M. K. Rates, "Uliginosin B presents antinociceptive effect mediated by dopaminergic and opioid systems in mice," *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 39, no. 1, pp. 80–87, 2012.

[4] C. Socolsky, S. M. K. Rates, A. C. Stein, Y. Asakawa, and A. Bardón, "Acylphloroglucinols from *Elaphoglossum crassipes*: antidepressant-like activity of crassipin A," *Journal of Natural Products*, vol. 75, no. 6, pp. 1007–1017, 2012.

[5] E. D. I. Stolz, D. R. A. Hasse, G. L. I. von Poser, and S. M. K. Rates, "Uliginosin B, a natural phloroglucinol derivative, presents a multimediated antinociceptive effect in mice," *The Journal of Pharmacy and Pharmacology*, vol. 66, no. 12, pp. 1774–1785, 2014.

[6] E. D. Stolz, L. G. Müller, C. B. Antonio et al., "Determination of pharmacological interactions of uliginosin B, a natural phloroglucinol derivative, with amitriptyline, clonidine and morphine by isobolographic analysis," *Phytomedicine*, vol. 21, no. 12, pp. 1684–1688, 2014.

[7] J. Sawynok, "Adenosine receptor targets for pain," *Neuroscience*, 2015.

[8] M. J. Esser, T. Chase, G. V. Allen, and J. Sawynok, "Chronic administration of amitriptyline and caffeine in a rat model of neuropathic pain: multiple interactions," *European Journal of Pharmacology*, vol. 430, no. 2-3, pp. 211–218, 2001.

[9] J. Sawynok, A. R. Reid, and M. J. Esser, "Peripheral antinociceptive action of amitriptyline in the rat formalin test: involvement of adenosine," *Pain*, vol. 80, no. 1-2, pp. 45–55, 1997.

[10] A. Ulugol, H. C. Karadag, M. Tamer, Z. Firat, A. Aslantas, and I. Dokmeci, "Involvement of adenosine in the anti-allodynic effect of amitriptyline in streptozotocin-induced diabetic rats," *Neuroscience Letters*, vol. 328, no. 2, pp. 129–132, 2002.

[11] J. Sawynok and X. J. Liu, "Adenosine in the spinal cord and periphery: release and regulation of pain," *Progress in Neurobiology*, vol. 69, no. 5, pp. 313–340, 2003.

[12] G. J. Keil and G. E. Delander, "Time-dependent antinociceptive interactions between opioids and nucleoside transport inhibitors," *Journal of Pharmacology and Experimental Therapeutics*, vol. 274, no. 3, pp. 1387–1392, 2014.

[13] T. W. Stone, "The effects of morphine and methionine-enkephalin on the release of purines from cerebral cortex slices of rats and mice," *British Journal of Pharmacology*, vol. 74, no. 1, pp. 171–176, 1981.

[14] K. O. Aley and J. D. Levine, "Multiple receptors involved in peripheral $\alpha 2$, μ, and A1 antinociception, tolerance, and withdrawal," *The Journal of Neuroscience*, vol. 17, no. 2, pp. 735–744, 1997.

[15] G. Burnstock and M. Williams, "P2 purinergic receptors: modulation of cell function and therapeutic potential," *Journal of Pharmacology and Experimental Therapeutics*, vol. 295, no. 3, pp. 862–869, 2000.

[16] G. Burnstock, "Physiology and pathophysiology of purinergic neurotransmission," *Physiological Reviews*, vol. 87, no. 2, pp. 659–797, 2007.

[17] S. C. Robson, J. Sévigny, and H. Zimmermann, "The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance," *Purinergic Signalling*, vol. 2, no. 2, pp. 409–430, 2006.

[18] M. J. Zylka, "Pain-relieving prospects for adenosine receptors and ectonucleotidases," *Trends in Molecular Medicine*, vol. 17, no. 4, pp. 188–196, 2011.

[19] G. Burnstock, "Introduction to purinergic signalling in the brain," *Advances in Experimental Medicine and Biology*, vol. 986, pp. 1–12, 2013.

[20] P. L. Ipata, "Origin, utilization, and recycling of nucleosides in the central nervous system," *Advances in Physiology Education*, vol. 35, no. 4, pp. 342–346, 2011.

[21] C. D. Bonan, "Ectonucleotidases and nucleotide/nucleoside transporters as pharmacological targets for neurological disorders," *CNS and Neurological Disorders—Drug Targets*, vol. 11, no. 6, pp. 739–750, 2012.

[22] J. D. M. Nunes, A. V. Pinhatti, G. L. von Poser, and S. B. Rech, "Promotive effects of long-term fertilization on growth of tissue culture-derived *Hypericum polyanthemum* plants during acclimatization," *Industrial Crops and Products*, vol. 30, no. 2, pp. 329–332, 2009.

[23] W. L. Parker and F. Johnson, "The structure determination of antibiotic compounds from Hypericum uliginosum. I," *Journal of the American Chemical Society*, vol. 90, no. 17, pp. 4716–4723, 1968.

[24] Ministério da Ciência e Tecnologia e Inovação (Brasil), "Lei procedimentos para o uso científico de animais," Lei n. 11794, Diário Oficial da União (DOU), 2008.

[25] Brasil: Ministério da Ciência Tecnologia e Inovação—CONCEA, *Diretriz Brasileira para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos—DBCA*, Ministério

da Ciência Tecnologia e Inovação, Brasilia, Brazil, 2013, http://www.mct.gov.br/upd_blob/0226/226494.pdf.

[26] Brasil, Ministério da Ciência Tecnologia e Inovação, CONCEA. Diretrizes da prática de eutanásia do CONCEA. Brasilia-DF, 2013, http://www.mct.gov.br/upd_blob/0226/226746.pdf.

[27] Committee for the Update of the Guide for the Care and Use of Laboratory Animals Institute for Laboratory Animal Research Division on Earth and Life Studies, *Laboratory Guide for the Care and Use of Animals*, The National Academies Press, 2011.

[28] F. P. Nascimento, S. M. Figueredo, R. Marcon et al., "Inosine reduces pain-related behavior in mice: involvement of adenosine A1 and A2A receptor subtypes and protein kinase C pathways," *Journal of Pharmacology and Experimental Therapeutics*, vol. 334, no. 2, pp. 590–598, 2010.

[29] D. F. Martins, L. Mazzardo-Martins, F. Soldi, J. Stramosk, A. P. Piovezan, and A. R. S. Santos, "High-intensity swimming exercise reduces neuropathic pain in an animal model of complex regional pain syndrome type I: evidence for a role of the adenosinergic system," *Neuroscience*, vol. 234, pp. 69–76, 2013.

[30] H. Hosseinzadeh and M. E. Shooshtari, "Antinociceptive interaction between adenosine and carbamazepine in mice," *Medical Journal of the Islamic Republic of Iran*, vol. 12, no. 4, pp. 381–386, 1999.

[31] A. Grenz, H. Zhang, J. Weingart et al., "Lack of effect of extracellular adenosine generation and signaling on renal erythropoietin secretion during hypoxia," *American Journal of Physiology—Renal Physiology*, vol. 293, no. 5, pp. 1501–1511, 2007.

[32] J. R. Rozisky, R. S. da Silva, L. S. Adachi et al., "Neonatal morphine exposure alters E-NTPDase activity and gene expression pattern in spinal cord and cerebral cortex of rats," *European Journal of Pharmacology*, vol. 642, no. 1–3, pp. 72–76, 2010.

[33] A. Nagy and A. V. Delgado-Escueta, "Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll)," *Journal of Neurochemistry*, vol. 43, no. 4, pp. 1114–1123, 1984.

[34] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[35] A. M. O. Battastini, J. B. T. da Rocha, C. K. Barcellos, R. D. Dias, and J. J. F. Sarkis, "Characterization of an ATP diphosphohydrolase (EC 3.6.1.5) in synaptosomes from cerebral cortex of adult rats," *Neurochemical Research*, vol. 16, no. 12, pp. 1303–1310, 1991.

[36] K.-M. Chan, D. Delfert, and K. D. Junger, "A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity," *Analytical Biochemistry*, vol. 157, no. 2, pp. 375–380, 1986.

[37] A. P. Ijzerman, P. J. M. Van Galen, and K. A. Jacobson, "Molecular modeling of adenosine receptors. I. The ligand binding site on the A1 receptor," *Drug Design and Discovery*, vol. 9, no. 1, pp. 49–67, 1992.

[38] J. Kim, J. Wess, A. M. Van Rhee, T. Schöneberg, and K. A. Jacobson, "Site-directed mutagenesis identifies residues involved in ligand recognition in the human A2a adenosine receptor," *Journal of Biological Chemistry*, vol. 270, no. 23, pp. 13987–13997, 1995.

[39] A. J. Kooistra, L. Roumen, R. Leurs, I. J. P. de Esch, and C. de Graaf, "From heptahelical bundle to hits from the haystack: structure-based virtual screening for GPCR ligands," *Methods in Enzymology*, vol. 522, pp. 279–336, 2013.

[40] K. A. Jacobson, "Structure-based approaches to ligands for G-protein-coupled adenosine and P2Y receptors, from small molecules to nanoconjugates," *Journal of Medicinal Chemistry*, vol. 56, no. 10, pp. 3749–3767, 2013.

[41] A. Göblyös and A. P. Ijzerman, "Allosteric modulation of adenosine receptors," *Biochimica et Biophysica Acta (BBA)—Biomembranes*, vol. 1808, no. 5, pp. 1309–1318, 2011.

[42] J. Sawynok and A. Reid, "Desipramine potentiates spinal antinociception by 5-hydroxytryptamine, morphine and adenosine," *Pain*, vol. 50, no. 1, pp. 113–118, 1992.

[43] J. Sawynok, A. R. Reid, and B. B. Fredholm, "Caffeine reverses antinociception by amitriptyline in wild type mice but not in those lacking adenosine A1 receptors," *Neuroscience Letters*, vol. 440, no. 2, pp. 181–184, 2008.

[44] M. J. Esser and J. Sawynok, "Caffeine blockade of the thermal antihyperalgesic effect of acute amitriptyline in a rat model of neuropathic pain," *European Journal of Pharmacology*, vol. 399, no. 2-3, pp. 131–139, 2000.

[45] E. L. Pedrazza, M. R. Senger, E. P. Rico et al., "Fluoxetine and nortriptyline affect NTPDase and 5′-nucleotidase activities in rat blood serum," *Life Sciences*, vol. 81, no. 15, pp. 1205–1210, 2007.

[46] E. L. Pedrazza, M. R. Senger, L. Pedrazza, F. F. Zimmermann, J. J. de Freitas Sarkis, and C. D. Bonan, "Sertraline and clomipramine inhibit nucleotide catabolism in rat brain synaptosomes," *Toxicology in Vitro*, vol. 21, no. 4, pp. 671–676, 2007.

[47] E. L. Pedrazza, E. P. Rico, M. R. Senger et al., "Ecto-nucleotidase pathway is altered by different treatments with fluoxetine and nortriptyline," *European Journal of Pharmacology*, vol. 583, no. 1, pp. 18–25, 2008.

[48] J. Sawynok, A. R. Reid, J. L. Xue, and F. E. Parkinson, "Amitriptyline enhances extracellular tissue levels of adenosine in the rat hindpaw and inhibits adenosine uptake," *European Journal of Pharmacology*, vol. 518, no. 2-3, pp. 116–122, 2005.

[49] K. Kohring and H. Zimmermann, "Upregulation of ecto-5′-nucleotidase in human neuroblastoma SH-SY5Y cells on differentiation by retinoic acid or phorbolester," *Neuroscience Letters*, vol. 258, no. 3, pp. 127–130, 1998.

[50] R. Napieralski, B. Kempkes, and W. Gutensohn, "Evidence for coordinated induction and repression of ecto-5′-nucleotidase (CD73) and the A2a adenosine receptor in a human B cell line," *Biological Chemistry*, vol. 384, no. 3, pp. 483–487, 2003.

[51] R. A. Cunha, P. Brendel, H. Zimmermann, and J. A. Ribeiro, "Immunologically distinct isoforms of ecto-5′-nucleotidase in nerve terminals of different areas of the rat hippocampus," *Journal of Neurochemistry*, vol. 74, no. 1, pp. 334–338, 2000.

[52] R. A. Cunha, "Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals," *Neurochemical Research*, vol. 26, no. 8-9, pp. 979–991, 2001.

[53] J. D. Geiger, F. S. LaBella, and J. I. Nagy, "Characterization and localization of adenosine receptors in rat spinal cord," *The Journal of Neuroscience*, vol. 4, no. 9, pp. 2303–2310, 1984.

[54] N. A. Sowa, B. Taylor-Blake, and M. J. Zylka, "Ecto-5′-nucleotidase (CD73) inhibits nociception by hydrolyzing AMP to adenosine in nociceptive circuits," *The Journal of Neuroscience*, vol. 30, no. 6, pp. 2235–2244, 2010.

[55] H. Z. Ruan and G. Burnstock, "Localisation of P2Y1 and P2Y4 receptors in dorsal root, nodose and trigeminal ganglia of the rat," *Histochemistry and Cell Biology*, vol. 120, no. 5, pp. 415–426, 2003.

[56] Z. Gerevich and P. Illes, "P2Y receptors and pain transmission," *Purinergic Signalling*, vol. 1, no. 1, pp. 3–10, 2004.

[57] S. A. Malin and D. C. Molliver, "Gi-and Gq-coupled ADP (P2Y) receptors act in opposition to modulate nociceptive signaling and inflammatory pain behavior," *Molecular Pain*, vol. 6, no. 21, pp. 1–12, 2010.

[58] R. A. Cunha, "Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors," *Neurochemistry International*, vol. 38, no. 2, pp. 107–125, 2001.

[59] M. Sweeney, T. White, and J. Sawynok, "5-Hydroxytryptamine releases adenosine from primary afferent nerve terminals in the spinal cord," *Brain Research*, vol. 462, no. 2, pp. 346–349, 1988.

[60] M. I. Sweeney, T. D. White, and J. Sawynok, "5-Hydroxytryptamine releases adenosine and cyclic AMP from primary afferent nerve terminals in the spinal cord in vivo," *Brain Research*, vol. 528, no. 1, pp. 55–61, 1990.

[61] M. A. da Silva Pereira, *Estudo da Atividade Antinociceptiva de Adenosina em Camundongos—Análise do Mecanismo de Ação*, Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade do Vale do Itajaí, Florianópolis, Brazil, 2005.

[62] M. J. Millan, "Descending control of pain," *Progress in Neurobiology*, vol. 66, no. 6, pp. 355–474, 2002.

[63] J. Sawynok, M. I. Sweeney, and T. D. White, "Adenosine release may mediate spinal analgesia by morphine," *Trends in Pharmacological Sciences*, vol. 10, no. 5, pp. 186–189, 1989.

[64] J. Sawynok, D. J. Nicholson, M. I. Sweeney, and T. D. White, "Adenosine release by morphine and spinal antinociception: role of G-proteins and cyclic AMP," *NIDA Research Monograph Series*, vol. 105, pp. 40–46, 1990.

[65] J. Sawynok, "Adenosine receptor activation and nociception," *European Journal of Pharmacology*, vol. 347, no. 1, pp. 1–11, 1998.

[66] J. Sawynok, A. Reid, and A. Poon, "Peripheral antinociceptive effect of an adenosine kinase inhibitor, with augmentation by an adenosine deaminase inhibitor, in the rat formalin test," *Pain*, vol. 74, no. 1, pp. 75–81, 1998.

[67] D. Quarta, J. Borycz, M. Solinas et al., "Adenosine receptor-mediated modulation of dopamine release in the nucleus accumbens depends on glutamate neurotransmission and N-methyl-D-aspartate receptor stimulation," *Journal of Neurochemistry*, vol. 91, no. 4, pp. 873–880, 2004.

[68] R. J. Rodrigues, A. R. Tomé, and R. A. Cunha, "ATP as a multitarget danger signal in the brain," *Frontiers in Neuroscience*, vol. 9, article 148, 2015.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 7358918, 7 pages
http://dx.doi.org/10.1155/2016/7358918

Research Article

The Effectiveness of Cupping Therapy on Relieving Chronic Neck and Shoulder Pain: A Randomized Controlled Trial

Lee-Mei Chi,[1,2] Li-Mei Lin,[3] Chien-Lin Chen,[4,5] Shu-Fang Wang,[6] Hui-Ling Lai,[7] and Tai-Chu Peng[1,7]

[1] *Institute of Medical Sciences, Tzu Chi University, Hualien 970, Taiwan*
[2] *Department of Nursing, Tzu Chi University of Science and Technology, Hualien 970, Taiwan*
[3] *Department of Nursing, Chang Gung University of Science and Technology, Taoyuan 333, Taiwan*
[4] *Department of Chinese Medicine, Taipei Tzu Chi Hospital, New Taipei City 23142, Taiwan*
[5] *School of Post-Baccalaureate Chinese Medicine, Tzu Chi University, Hualien 970, Taiwan*
[6] *Chinese Lactation Consultant Association, Hualien 970, Taiwan*
[7] *Department of Nursing, Tzu Chi University, Hualien 970, Taiwan*

Correspondence should be addressed to Tai-Chu Peng; ptc2008@mail.tcu.edu.tw

Received 15 October 2015; Accepted 4 January 2016

Academic Editor: Haroon Khan

The research aimed to investigate the effectiveness of cupping therapy (CT) in changes on skin surface temperature (SST) for relieving chronic neck and shoulder pain (NSP) among community residents. A single-blind experimental design constituted of sixty subjects with self-perceived NSP. The subjects were randomly allocated to two groups. The cupping group received CT at SI 15, GB 21, and LI 15 acupuncture points, and the control group received no intervention. Pain was assessed using the SST, visual analog scale (VAS), and blood pressure (BP). The main results were SST of GB 21 acupuncture point raised from 30.6°C to 32.7°C and from 30.7°C to 30.6°C in the control group. Neck pain intensity (NPI) severity scores were reduced from 9.7 to 3.6 in the cupping group and from 9.7 to 9.5 in the control group. The SST and NPI differences between the groups were statistically significant ($P < 0.001$). One treatment of CT is shown to increase SST. In conjunction with the physiological effect the subjective experience of NSP is reduced in intensity. Further studies are required to improve the understanding and potential long-term effects of CT.

1. Introduction

Chronic neck and shoulder pain (NSP) is a type of musculoskeletal pain typically occurring in middle- and older-aged people [1–3]. The prevalence of NSP is approximately 16% to 78% among the general population [2–4]. The impact of chronic pain on the family includes social activities, life changes, emotional impact, and alteration of future plans [5].

Cupping therapy (CT) is a traditional Chinese medical (TCM) treatment which has been practiced for thousands of years. The World Health Organization's (WHO) definition of cupping is a therapeutic method (Code 5.3.2) involving the application of suction by creating a vacuum. This is typically done using fire in a cup or jar (Code 5.3.7) on the dermis of the affected part of the body [6].

In Taiwan, approximately 12.8% of the participants reported the use of cupping therapies in the past year [7]. The cupping mechanism constitutes creating a vacuum on the skin, with the ensuing negative pressure resulting in capillary rupture. This method is known as retained or dry cupping [8]. The skin of the localized area becomes flushed and may show petechiae and ecchymosis [9] or bruising, in which the duration is therapeutically beneficial [10]. Cupping has multiple therapeutic functions which include (1) warming the channels to remove cold, (2) promoting qi and blood circulation, (3) relieving swelling, (4) accelerating healing, (5) adjusting body temperature, (6) fibromyalgia [11], (7) stroke rehabilitation, hypertension, musculoskeletal pain, herpes zoster [8, 12], (8) facial paralysis, acne, and cervical spondylosis [13], and (9) alleviating pain [14], including

chronic neck [15–17], shoulder pain [2], and low back pain [17, 18].

Traditional acupuncture points, *jianshongshu* (SI 15), *jianjing* (GB 21), and *jianju* (LI 15), have been suggested for improving NSP. The SI 15 point is positioned on the back, approximately 3 to 4 cm lateral to the lower border of the spinous process of the seventh cervical vertebra (dazhui). This point is associated with shoulder and back pain and coughing. The GB 21 is situated at the midpoint that connects the dazhui point (DU 14) and the acromion (the shoulder peak). It is primarily used to treat headaches, neck pain, stroke-induced speech impairment, and shoulder, back, and arm pain. The LI 15 point is located on the lateral side of the arm and on the deltoid muscle. It is the depressed area distal and anterior to the acromion when the arms are stretched outward or forward. This point is used to treat shoulder joint pain and hemiplegia [19].

The current literature remains sparse for studies on skin temperature differences at acupuncture points in relation to thermal effect of cupping therapy. Liu et al. showed that localized skin temperature increased [20, 21], while blood pressure decreased [22], after CT. It is suggested that these physiological responses to CT may be related to the positive therapeutic effect. Currently, due to the paucity of available research focusing on skin temperature changes due to CT, the potential effect and its relationship remains unclear. This study investigated the effectiveness of CT for relieving chronic NSP among community residents and the changes in skin surface temperature (SST).

2. Methods

2.1. Subjects. This study was a single-blind experimental design. Subjects with diagnosed and self-perceived chronic NSP were recruited in Hualien City, Taiwan, via advertising and e-mail from October 2012 to February 2013. This research was conducted in a nursing research laboratory at the Tzu Chi University of Science and Technology. The room temperature was controlled at 20 to 24°C and the humidity level was maintained at 60 to 70%. A Chinese traditional medicine nurse and traditional Chinese medical practitioner were also asked to verify the choice and location of the selected acupuncture points and the cupping treatment.

The inclusion criterion is as follows: (1) working at least 40 hours a week and (2) suffering work-related NSP continuously for at least 3 consecutive months with an intensity of at least 3 points on the visual analog scale (VAS, 0–10). Participants were excluded if the following exist: (1) infection, injury, or bleeding of the skin surrounding the area for cupping therapy, (2) neuropathy in the cervical spinal cord, (3) analgesic ingestion within 4 hrs preceding experiment, and (4) consumed coffee, tea, or any other caffeinated beverage within 4 hrs prior to the baseline measurement. Also, no tobacco products had been smoked for a minimum of 30 min before the baseline data were recorded.

2.2. Sample Size. In the pilot study ($n = 6$) for NSP a statistically significant result between group difference of 1.18 (effect size = 0.81) using the VAS was found. Employing the Wilcoxon Mann-Whitney test (G power v 3.1.3) [23] to achieve a power of 0.8, with Cronbach's α value = 0.05 and an effect size of 0.80, the required size for each group is minimum of 27 subjects.

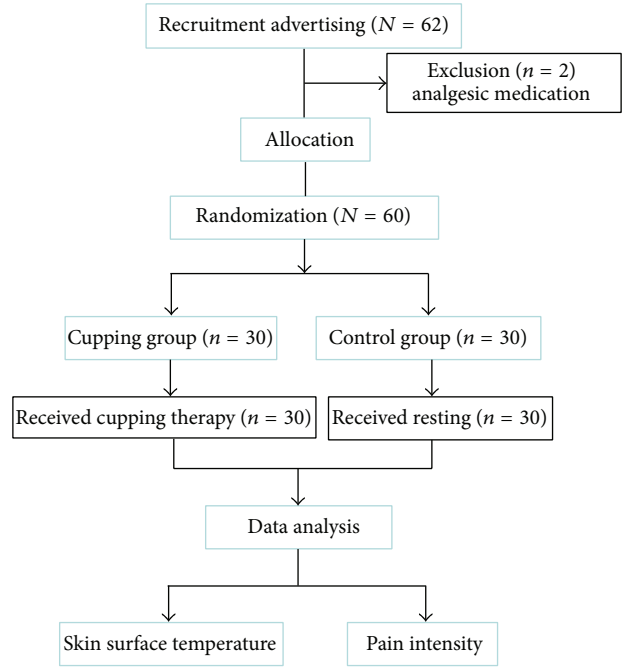


Figure 1: Flowchart of this study.

2.3. Randomization. Subjects were assigned "cupping group" or "control group" based on random selection from sealed envelopes which had been sequence coded prior to study commencement. Neither the researcher nor the participants were aware of which group the participants would be assigned to. Figure 1 displays the flowchart of the study.

This study was reviewed and approved by the Research Ethics Committee of the Buddhist Tzu Chi General Hospital (Registration number 101-60). Written consent was obtained from the participants prior to the start of the study. The objectives of the research were explained and the option to withdraw from the study at any time was made known.

2.4. Intervention. The cupping group received fire CT at three acupuncture points, SI 15, GB 21, and LI 15. The medium size glass cup with diameter of 4 cm and volume of 260 mL (Cosmos International Supplies Co., Ltd., Taiwan) was used. Participants were asked to sit comfortably in a chair with both feet flat on the floor and expose their neck and shoulder regions. The cupping procedure is as follows: (1) an alcohol swab is ignited, (2) the burning swab is quickly placed inside the cup and withdrawn, (3) the cups are placed over the three acupuncture points, (4) the cups were then removed after 10 min [24], and (5) the same process was repeated for the same amount of time on the subject's left side (Figure 2(a)). The entire treatment totaled 20 minutes to treat both sides of the body.

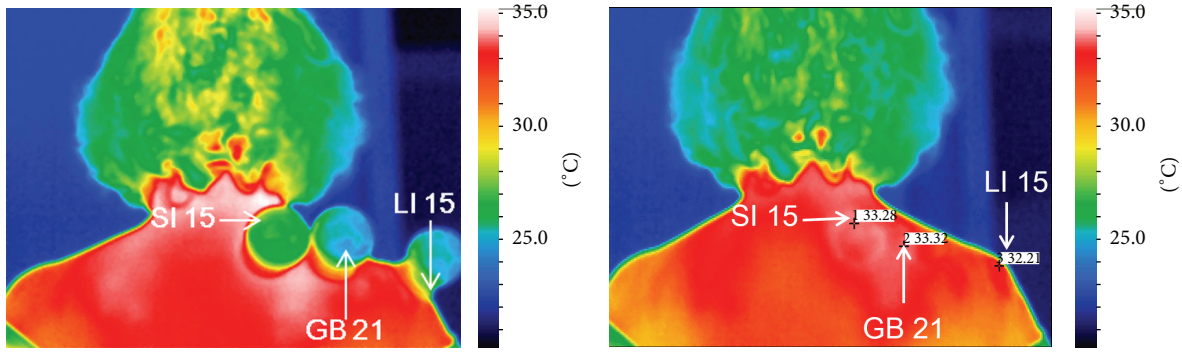


Figure 2: The skin surface temperature (°C) at SI 15, GB 21, and LI 15 acupuncture points displayed by infrared camera by cupping (a) and after cupping therapy (b).

Participants in the control group received resting for 20 min.

2.5. Outcomes. Participant characteristics included demographic data such as age, sex, and a brief medical history including past experience of cupping.

2.5.1. Skin Surface Temperature (SST) and Blood Pressure (BP). An infrared camera (FLIR ThermaCAM P25 HS system) was used to measure SST of the right SI 15, GB 21, and LI 15 acupuncture points (Figure 2(b)). Measurements were recorded for SST at 4 time points with a 5-minute interval between each measurement. The FLIR infrared camera is an infrared thermal detector, with 320 × 240 pixel geometric resolution of 76.800 pixels per picture. Measurements can be performed which range from 0 to 250°C ± 0.001°C. The data was transferred to a notebook computer using the ThermaCAM Researcher V.2.8 software (FLIR Systems Inc., Portland, Oregon, USA).

BP was measured using a mercury sphygmomanometer (Model S-300, standard sphygmomanometer, Taiwan) using the participants' right arm. BP was recorded both before and after intervention.

2.5.2. Neck and Shoulder Pain Intensity. Pain was scored using VAS; a Likert scale was used for evaluating the subjective experience of pain intensity [25, 26]. The neck pain intensity test involved (1) leaning forward and backward, (2) rotating to the left and right, and (3) inclining to the left and right [27]. The shoulder pain intensity assessment involved (1) raising both arms, stretching the chest, and extending the arms backward to touch the back of the neck and (2) raising both arms upward, placing them against the ears, and placing the palms together [28]. The subjects were asked to select a point on the scale that most accurately reflected their level of pain before and then after the pain inducing movement [29].

2.6. Statistical Analysis. Data were analyzed using SPSS V.18.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The univariate analysis of covariance (ANCOVA) was used to assess the level of NSP intensity. ANCOVA was used to assess the changes in the SST and BP, while adjusting the baseline for both groups. The Friedman test was conducted to evaluate the overall changes within each group. Wilcoxon test was used to compare the difference within groups. A P value of <0.05 was considered statistically significant.

Table 1: Group demographic characteristics. Categorical variables: Chi-square test. Continuous variables: Mann-Whitney U test.

Variables	Cupping $n = 30$	Resting $n = 30$	P
Gender (%)			0.640
Male	3 (10.0)	2 (6.7)	
Female	27 (90.0)	28 (93.3)	
Age (mean ± SD)	43.6 ± 8.0	42.5 ± 7.4	0.486

3. Results

The study recruited a total of sixty-two participants and excluded two cases due to analgesic ingestion prior to the experiment. The participant gender representation within the study was female (91.7%; $n = 55$) and 8.3% male ($n = 5$). The subjects aged from 24 to 61 years with a median age of 43.6 ± 8 years. There were no significant differences between the cupping and control groups for subjects' gender and age at baseline (Table 1).

3.1. Skin Surface Temperature (SST) Changes. The average temperatures at the SI 15 acupuncture point showed no significant differences between groups before CT. The SST at the SI 15 point increased to a peak of 32.8 ± 0.5°C at 5 minutes after CT. This temperature is significantly higher than the baseline (30.7 ± 0.5°C) ($P < 0.01$) (Table 2). The Friedman tests revealed that, from baseline to 5 minutes after cessation of treatment, CT acts to increase the SST of SI 15 ($P < 0.01$). During the resting period for the control group, the SI 15 temperature showed no significant difference from baseline ($P > 0.05$) (Figure 3(a)).

The average temperatures at the GB 21 acupuncture point showed no significant differences between groups before

Table 2: Changes in SST at three acupuncture points between groups at 5-minute intervals. p 5th min: the 5th min of rest after cupping therapy. Note: $^{+}$ANCOVA was used to compare groups' difference after adjustment of baseline differences. $^{++}$Friedman test was used to compare the difference within group. $^{*}P < 0.05$.

Measurement indices	Mean (SEM)				Friedman test	
	Baseline	5 min	10 min	p 5th min	χ^2	P^{++}
SI 15						
Cupping	30.68 (0.51)	31.33 (0.45)	32.18 (0.46)	32.82 (0.53)	14.040	0.003*
Resting	30.99 (0.57)	30.72 (0.58)	30.78 (0.57)	30.89 (0.59)	3.367	0.338
F	—	11.915	32.684	48.949		
P^{+}	—	0.011*	0.001*	0.001*		
GB 21						
Cupping	30.62 (0.50)	31.09 (0.61)	32.08 (0.71)	32.72 (0.62)	14.040	0.003*
Resting	30.71 (0.42)	30.57 (0.50)	30.61 (0.47)	30.60 (0.45)	1.653	0.647
F	—	16.930	8.548	22.729		
P^{+}	—	0.004*	0.022*	0.002*		
LI 15						
Cupping	29.39 (0.39)	29.78 (0.42)	30.70 (0.47)	31.12 (0.78)	11.880	0.008*
Resting	29.65 (0.37)	29.56 (0.40)	29.65 (0.43)	29.64 (0.46)	0.120	0.989
F	—	9.007	28.726	24.828		
P^{+}	—	0.020*	0.001*	0.002*		

CT. The SST of the GB 21 point gradually increased to a peak of 32.7 ± 0.6°C after 5-minute CT. This value is significantly higher than the baseline of 30.6 ± 0.5°C ($P < 0.01$). The Friedman tests revealed that, from baseline to 5 minutes after CT, the SST of GB 21 remained elevated ($P < 0.01$). The control group, during the resting period, showed a gradual decrease in temperature to 30.6 ± 0.5°C at 15 minutes at the GB 21 acupuncture point. There were no significant differences from baseline (30.7 ± 0.4°C) ($P > 0.05$) (Figure 3(b)) for GB 21 within the control group.

The SST of the LI 15 was 29.4 ± 0.4°C at baseline within the cupping group and 29.7 ± 0.4°C within the control group ($P > 0.05$). The SST of the LI 15 point increased to 31.1 ± 0.8°C at 5 minutes after CT, which is significantly higher than baseline ($P < 0.01$). The Friedman test supports the within group results, which show that, from baseline to 5 minutes after cessation of treatment, SST remains elevated at LI 15 ($P < 0.01$). The ANCOVA test indicates significant differences between the groups at each time point for GB 21, SI 15, and LI 15 acupuncture points ($P < 0.05$) (Figure 3(c)). It is important to note that the results for LI 15 show lower temperatures. This is due to the distance from the acupuncture point to the lens of the infrared camera.

3.2. BP Changes. The systemic blood pressure (SBP) decreased from 117.7 ± 2.9 mmHg to 111.8 ± 2.3 mmHg, in the cupping group ($P = 0.003$). The control group also showed slight reduction from 113.8 ± 3.0 mmHg to 109.7 ± 3.1 mmHg ($P = 0.117$). There was no significant difference between the two groups; however cupping appears to have some influence on the SBP.

3.3. Pain Intensity Changes. At baseline, the VAS of neck pain intensity (NPI) was 9.7 ± 1.6 in the cupping group and 9.7 ± 1.6 in the control group. The posttreatment NPI decreased by 6.1 in the cupping group and decreased by 0.2 in the control group (Figure 4(a)). The ANCOVA test demonstrated significant differences between the groups ($P < 0.001$).

The VAS of shoulder pain intensity (SPI) was 8.5 ± 0.9 for the cupping group at the baseline and 8.5 ± 0.9 in the control group. The posttreatment SPI decreased by 5.9 in the cupping group and decreased by 0.6 in the control group (Figure 4(b)). The difference between the groups was statistically significant ($P < 0.001$).

4. Discussion

The CT therapeutic method can cause vasodilatation and stimulate blood circulation to increase metabolism and accelerate the elimination of waste and toxins from the body. This effect acts to improve physical function [30] and affect BP [22]. Xu et al. demonstrated changes in skin temperature in the cupping area before and after cupping. When the cup was removed, 10 minutes after cupping, the skin temperature in the cupping area was elevated compared to the control area and showed significant difference [21]. Al-Rubaye also showed immediate clinical changes after cupping which included the sensation of increased warmth on the skin surface [22]. Similarly, Liu et al. showed that blood flow to the skin of the back in healthy humans on acupuncture points increased immediately following removal of the cup [20]. After CT, several other immediate signs of the therapeutic method may be observed and are dependent on the modality in use. Cupping increases blood flow to the cupped region (hyperemia); the subject experiences warmth as a result of vasodilatation. Due to vasodilatation and edema, histological changes are readily observable at the skin surface. After cupping effects often include erythema, edema, and ecchymosis in a variety of circular arrangements [31].

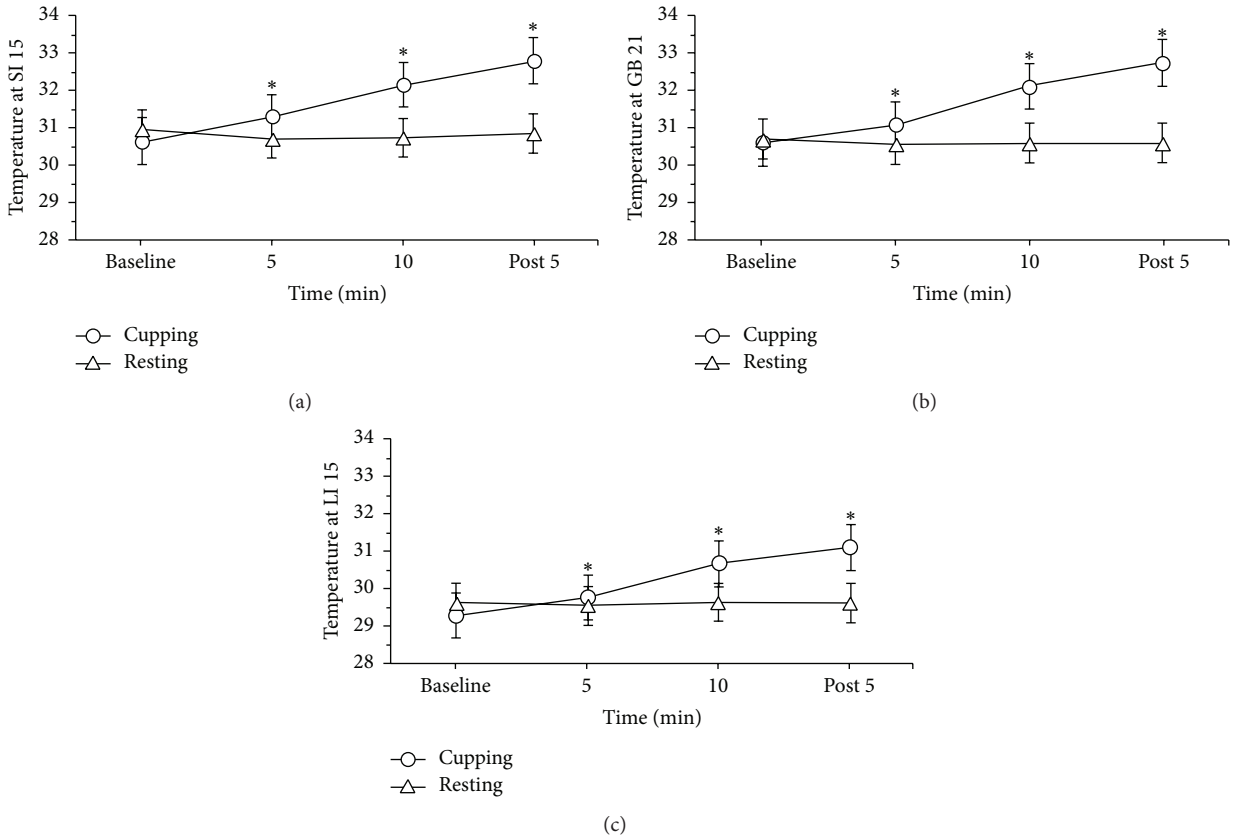


Figure 3: Change in SST (°C) at three acupuncture points during cupping therapy at 5-minute intervals. ∗: difference between groups at SI 15 (a), GB 21 (b), and LI 15 (c) acupuncture points ($P < 0.05$).

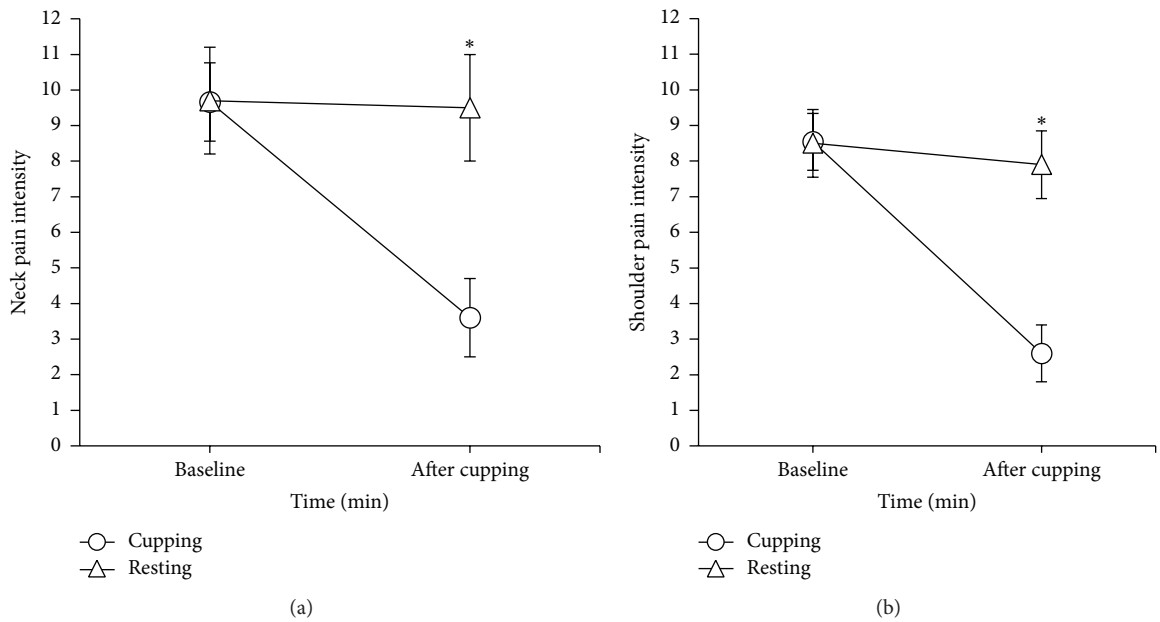


Figure 4: Visual analog scale (mean ± SEM) of subjects with chronic neck pain (a) and chronic shoulder pain (b). ∗: univariate analysis of covariance (ANCOVA) was used to compare groups' difference after adjustment of baseline differences ($P < 0.05$).

Cupping increased SST in this study. The results showed that SST at GB 21 was elevated from 30.6 to 32.1°C during the cupping period and increased after removal of the cup. Similarly, both SI 15 and GB 21 acupuncture points showed increased SST (2.1°C) after cup removal at the 5 min interval. At the LI 15 acupuncture point SST was elevated by 1.7°C.

The study outcome supports the efficacy of CT as a complementary therapy for treating NSP. The results indicate that CT provides significant and effective relief of NSP compared to the control.

Yuan et al. conducted a systematic review and meta-analysis of traditional Chinese medicine for neck pain and low back pain. It was suggested that cupping may be more effective than medications for treatment of chronic neck or lower back pain [17]. Lauche et al. targeted 50 participants with nonspecific neck pain and implemented 10 to 15 min of cupping therapy on the lower trapezius muscle. Their results showed that, at rest and during movement, the pain level on the VAS (0–10) decreased by 1.79 and 1.97, after cupping, respectively [16]. Kim et al. found that 6 sessions of cupping therapy (wet and dry) on neck pain acupuncture points in 40 patients were more effective than the use of a heating pad [15]. The German study of Lauche et al. found that home-based CT was more effective than progressive muscle relaxation in patients with chronic neck pain. The pain reduction effect remained evident at the one week after intervention interval [32].

Huang et al. employed cupping therapy around the neck and shoulder regions, combined with acupuncture and massage. This treatment was implemented once a day to comprise one session. A full course of treatment entails five sessions and a total of four courses were conducted for the experiment. Their results illustrated that this regimen could significantly reduce shoulder pain [33]. The current study used ANCOVA to assess the level of NPI and SPI. The baseline was adjusted in both groups to control for the potential bias when using VAS. This allows for a more reliable assessment of the CT effect.

In the current study, no participants experienced localized skin burns or adverse reactions in the treatment regions. Two participants in the cupping group reported mild low back pain related to the seated position. In the systematic review by Yuan et al. no serious or life-threatening side effects were noted [17]. The majority of adverse effects are related to wet cupping therapy, which results in (1) skin laceration, (2) whole body itching, (3) pain at the cupping sites, (4) generalized body ache [15], (5) factitious panniculitis, and (6) iron deficiency anemia [9]. Dry cupping, by comparison, is a safe and effective treatment modality for NPI and SPI.

CT is often used to treat pain, such as low back pain, fibromyalgia, shoulder pain, chronic nonspecific neck pain, cardiovascular diseases, angina, arthritis, and high blood pressure. The clinical evidence of CT is minimal [34]. Findings from this study strongly suggest that CT is effective for relieving pain, with no adverse effects. CT has the potential to eliminate reliance on analgesics and reduce health care costs.

Limitations of the study were primarily based on the limited availability of the participants. As participants were available for only one session, follow-up or multiple sessions were not possible. Improving the validity and reliability of this research requires (1) increasing the number of therapy sessions, (2) enlarging the sample size, (3) achieving equal representation of both sexes, and (4) age distribution.

5. Conclusion

Chronic NSP is a common problem in adults. CT is one of many effective treatments in traditional Chinese medicine. CT is used worldwide, as it is easy to learn and has few side effects. In this study, one treatment of CT is shown to increase SST and reduce SBP. In conjunction with the physiological effects, the subjective experience of NSP is reduced. CT mimics an analgesic effect which has no known negative side effects and may be considered safe. However, further studies are required to improve the understanding and potential long-term effects of CT.

Conflict of Interests

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

Acknowledgments

The authors thank the Office of Research and Development of Tzu Chi University of Science and Technology for funding this project (Grant no. TCCT-992A14). The support and cooperation of all the participants are appreciated.

References

[1] B. C. Anderson and R. J. Anderson, "Evaluation of the patient with shoulder complaints," UpToDate, 2011, http://www.uptodate.com.

[2] N. A. Broadhurst, C. A. Barton, L. A. Yelland, D. K. Martin, and J. J. Beilby, "Managing shoulder pain in general practice," *Australian Family Physician*, vol. 35, no. 9, pp. 751–752, 2006.

[3] E. Schell, T. Theorell, D. Hasson, B. Arnetz, and H. Saraste, "Impact of a web-based stress management and health promotion program on neck-shoulder-back pain in knowledge workers? 12 month prospective controlled follow-up," *Journal of Occupational and Environmental Medicine*, vol. 50, no. 6, pp. 667–676, 2008.

[4] K. H. Kim, Y. R. Kim, S. H. Noh et al., "Use of acupuncture for pain management in an academic Korean medicine hospital: a retrospective review of electronic medical records," *Acupuncture in Medicine*, vol. 31, no. 2, pp. 228–234, 2013.

[5] C. West, K. Usher, K. Foster, and L. Stewart, "Chronic pain and the family: the experience of the partners of people living with chronic pain," *Journal of Clinical Nursing*, vol. 21, no. 23-24, pp. 3352–3360, 2012.

[6] World Health Organization, *WHO International Standard Terminologies on Traditional Medicine in the Western Pacific Region, Aupuncture and Moxibustion: Cupping*, WHO Library Cataloguing in Publication Data, Geneva, Switzerland, 2007.

[7] K.-C. Lin, M.-L. Chen, M.-L. Yeh, C.-H. Hsu, Y.-L. Chen, and P. Chou, "Prevalence, pattern, and predictors of use of complementary and alternative medicine in Taiwan," *Taiwan Journal of Public Health*, vol. 28, no. 1, pp. 53–68, 2009.

[8] H. Cao, M. Han, X. Li et al., "Clinical research evidence of cupping therapy in China: a systematic literature review," *BMC Complementary and Alternative Medicine*, vol. 10, article 70, 10 pages, 2010.

[9] S.-B. Kim and Y.-H. Lee, "Numerical analysis of the change in skin color due to ecchymosis and petechiae generated by cupping: a pilot study," *Journal of Acupuncture and Meridian Studies*, vol. 7, no. 6, pp. 306–317, 2014.

[10] M. Pringle, "Some thoughts on fire cupping," *Journal of Chinese Medicine*, vol. 83, pp. 46–49, 2007.

[11] H. Cao, H. Hu, B. Colagiuri, and J. Liu, "Medicinal cupping therapy in 30 patients with fibromyalgia: a case series observation," *Forschende Komplementärmedizin*, vol. 18, no. 3, pp. 122–126, 2011.

[12] M. S. Lee, J.-I. Kim, and E. Ernst, "Is cupping an effective treatment? An overview of systematic reviews," *Journal of Acupuncture and Meridian Studies*, vol. 4, no. 1, pp. 1–4, 2011.

[13] H. Cao, X. Li, and J. Liu, "An updated review of the efficacy of cupping therapy," *PLoS ONE*, vol. 7, no. 2, Article ID e31793, 14 pages, 2012.

[14] S. Huang and Y. Cao, "Cupping therapy," *Journal of Chinese Medicine*, vol. 82, pp. 52–57, 2006.

[15] T.-H. Kim, J. W. Kang, K. H. Kim et al., "Cupping for treating neck pain in video display terminal (VDT) users: a randomized controlled pilot trial," *Journal of Occupational Health*, vol. 54, no. 6, pp. 416–426, 2012.

[16] R. Lauche, H. Cramer, C. Hohmann et al., "The effect of traditional cupping on pain and mechanical thresholds in patients with chronic nonspecific neck pain: a randomised controlled pilot study," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 429718, 10 pages, 2012.

[17] Q. L. Yuan, T. M. Guo, L. Liu et al., "Traditional Chinese medicine for neck pain and low back pain: a systematic review and meta-analysis," *PLoS ONE*, vol. 10, no. 2, Article ID e0117146, pp. 1–37, 2015.

[18] C.-Y. Huang, M.-Y. Choong, and T.-S. Li, "Effectiveness of cupping therapy for low back pain: a systematic review," *Acupuncture in Medicine*, vol. 31, no. 3, pp. 336–337, 2013.

[19] D. H. Chen, Ed., *Clinical Graphic Acupuncture Points*, Wenguang, Taipei, Taiwan, 1993.

[20] W. Liu, S. A. Piao, X. W. Meng, and L. Wei, "Effects of cupping on blood flow under skin of back in healthy human," *World Journal of Acupuncture—Moxibustion*, vol. 23, no. 3, pp. 50–52, 2013.

[21] P. C. Xu, S. L. Cui, A. C. W. Derrik et al., "Preliminary observation on effect of cupping on the skin surface temperature of patients with back pain," *World Journal of Acupuncture-Moxibustion*, vol. 24, no. 4, pp. 59–61, 2014.

[22] K. Q. A. Al-Rubaye, "The clinical and histological skin changes after the cupping therapy (Al-Hijamah)," *Journal of the Turkish Academy of Dermatology*, vol. 6, no. 1, pp. 1–7, 2012.

[23] F. Faul, E. Erdfelder, A. Buchner, and A.-G. Lang, "Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses," *Behavior Research Methods*, vol. 41, no. 4, pp. 1149–1160, 2009.

[24] D. Y. Lai and M. L. Chang, "The nursing of traditional Chinese medicine: theory and practice," in *Nursing of Cupping*, H. C. Chiang, Ed., pp. 69–81, Wagner, Taichung, Taiwan, 2005.

[25] N. Sahin, E. Ozcan, K. Sezen, O. Karatas, and H. Issever, "Efficacy of acupunture in patients with chronic neck pain—a randomised, sham controlled trial," *Acupuncture & Electro-Therapeutics Research*, vol. 35, no. 1-2, pp. 17–27, 2010.

[26] M. E. Wewers and N. K. Lowe, "A critical review of visual analogue scales in the measurement of clinical phenomena," *Research in Nursing & Health*, vol. 13, no. 4, pp. 227–236, 1990.

[27] M. Braun, M. Schwickert, A. Nielsen et al., "Effectiveness of traditional Chinese 'Gua sha' therapy in patients with chronic neck pain: a randomized controlled trial," *Pain Medicine*, vol. 12, no. 3, pp. 362–369, 2011.

[28] K. E. Roach, E. Budiman-Mak, N. Songsiridej, and Y. Lertratanakul, "Development of a shoulder pain and disability index," *Arthritis Care and Research*, vol. 4, no. 4, pp. 143–149, 1991.

[29] J. Treleaven, G. Jull, and N. LowChoy, "Smooth pursuit neck torsion test in whiplash-associated disorders: relationship to self-reports of neck pain and disability, dizziness and anxiety," *Journal of Rehabilitation Medicine*, vol. 37, no. 4, pp. 219–223, 2005.

[30] M. Arslan, N. Yeşilçam, D. Aydin, R. Yüksel, and S. Dane, "Wet cupping therapy restores sympathovagal imbalances in cardiac rhythm," *Journal of Alternative & Complementary Medicine*, vol. 20, no. 4, pp. 318–321, 2014.

[31] P. Mehta and V. Dhapte, "Cupping therapy: a prudent remedy for a plethora of medical ailments," *Journal of Traditional and Complementary Medicine*, vol. 5, no. 3, pp. 127–134, 2015.

[32] R. Lauche, S. Materdey, H. Cramer et al., "Effectiveness of home-based cupping massage compared to progressive muscle relaxation in patients with chronic neck pain: a randomized controlled trial," *PLoS ONE*, vol. 8, no. 6, Article ID e65378, pp. 1–9, 2013.

[33] J. J. Huang, S. Y. Su, L. M. Lei et al., "Treatment of shoulder pain after apoplectic hemiplegia by acupuncture, massage and cupping," *Shaanxi Journal of Traditional Chinese Medicine*, vol. 30, no. 5, pp. 591–593, 2009.

[34] B. Chen, M.-Y. Li, P.-D. Liu, Y. Guo, and Z.-L. Chen, "Alternative medicine: an update on cupping therapy," *The Quarterly Journal of Medicine*, vol. 108, no. 7, pp. 523–525, 2015.