Evidence-Based Complementary and Alternative Medicine

Natural Products as a Source for New Leads in Gout Treatment

Lead Guest Editor: Samuel Martins Silvestre
Guest Editors: Paulo Jorge da Silva Almeida and Reda El-Shishtawy

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Editorial

Natural Products as a Source for New Leads in Gout Treatment

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Gout is a common inflammatory arthritis characterized by elevated plasmatic levels of uric acid. Several risk factors were identified, including diets rich in seafood and meat, alcohol consumption, and obesity. In this disease, when uric acid levels in the body increase due to increased formation and/or decreased excretion, the solubility limits of sodium urate are exceeded and precipitation occurs, particularly within joints, synovial fluid, and periarticular tissues. The deposits of urate crystals can initiate attacks of acute painful gouty arthritis which can evolve to chronic gout when permanent erosive joint deformity emerges. The estimated prevalence of this condition is increasing in many developed countries, particularly in men and postmenopausal women. Therefore, preventive and therapeutic strategies have been considered to avoid and treat this disease. In this context, the control of gout has been mainly performed by reducing the inflammation (e.g., with colchicine and nonsteroidal anti-inflammatory drugs), as well as by reducing the uric acid formation (e.g., using allopurinol and febuxostat) and by favouring its excretion (e.g., with probenecid and sulfinpyrazone). However, these approaches only have relative success and can originate several side effects, particularly in chronic use. For these reasons, over the years, researchers have been searching for alternative therapeutic strategies, particularly involving the use of natural products. Therefore, this Special Issue aimed to collect original research as well as review articles and meta-analysis addressing the use of natural products as well as extracts or isolated compounds in gout control. Accordingly, researchers were invited to contribute with manuscripts with *in vitro*, *in vivo*, and clinical studies focusing not only on the effects of natural products on the control of uric acid levels but also on potential treatments to reduce the gouty inflammatory arthritis. The topics of this Special Issue included: (1) sources of natural products used in the treatment of gout; (2) isolation and characterization of natural products useful in gout control; (3) natural products which increase uric acid excretion and/or reduce uric acid absorption; (4) natural products with antioxidant and anti-inflammatory effects in gout; (5) natural products which reduce uric acid biosynthesis or enhance its degradation; (6) comparison of natural and synthetic drugs in gout treatment; (7) safety of gout control with natural products; (8) chemical modifications of natural products useful in gout control to improve their efficacy and/or safety. For this Special Issue, eleven articles were published which are briefly described as follows.

The majority of the published works of this Special Issue focuses on plant extracts from different regions of the world, and their potential interest in gout control was explored by *in vitro* and/or *in vivo* studies, including xanthine oxidase inhibition and/or *in vivo* antihyperuricemic effect. In some cases, a partial phytochemical characterization was performed and potential mechanisms of action were explored. Of these, several works involve natural products from traditional Chinese medicine.

Abu Bakar et al. reported the optimization of conditions to extract phytochemical compounds from *Euphorbia hirta* L. (Ara Tanah) aiming to improve the antigout activity of extracts from the whole plant excluding roots. For this, response surface methodology and liquid-chromatography mass spectrometry analysis were performed. The authors established the best extraction conditions for total flavonoid

content, total phenolic content, and *in vitro* xanthine oxidase inhibitory activity as well as the optimized levels of these compounds and its bioactivity. In addition, the main phytochemical compounds in the optimized *E. hirta* extract were determined, also aiming to be useful for further development of antigout products.

The phytochemistry, antioxidant activity, antiproliferative effect, and acute toxicity of a methanolic extract of the roots of two Moroccan Aristolochia species (*Aristolochia baetica* and *Aristolochia paucinervis*) were explored by Bourhia et al. These plants have been largely used in folk medicine to treat several diseases, including rheumatologic conditions, namely, those caused by hyperuricemia. These authors found that the two studied plants contain different classes of secondary metabolites, including polyphenols and flavonoids. In addition, radical scavenging effects were observed as well as cytotoxicity in cancer cell lines. In an *in vivo* study, no signs of toxicities nor mortalities were observed on oral-treated mice with 2000 mg/kg of the two investigated exacts.

Tseuguem et al. demonstrated that aqueous and methanol leaf extracts of *Paullinia pinnata* (Sapindaceae), a plant used to treat various diseases including arthritis, improve monosodium urate-induced gouty arthritis in rats. In addition, they evidenced the analgesic, anti-inflammatory, and antioxidant effects of these extracts by *in vivo* studies. Specifically, these authors demonstrated that both extracts significantly reduced monosodium urate-induced inflammation and hyperalgesia in both ankle and paw. In addition, a significant decrease of synovial myeloperoxidase was observed as well as nitric oxide and malondialdehyde reductions in serum, spinal, and left and right hemispheres and an increase of superoxide dismutase activity.

The beneficial effects of a macroporous resin extract of *Dendrobium candidum* leaves in rats with hyperuricemia induced by a high-purine diet were evidenced by Lou et al. In fact, after administration of this extract during 9 weeks to model rats, liver and kidney function biochemical parameters, especially serum uric acid levels, were significantly improved. In addition, a reduction of xanthine oxidase and adenosine deaminase activities in liver was observed. Moreover, according to histological analysis, this extract significantly prevented kidney and liver from damage and intestinal injury. Furthermore, a reduction in inflammation in these hyperuricemic rats by inhibiting the expression of both NF-κB and TLR4 proteins was also reported.

Tu-Teng-Cao is a drug used in traditional Chinese medicine which has been widely applied in the clinical treatment of arthritis. In this context, Yao et al. evidenced that a Tu-Teng-Cao extract alleviates monosodium urate-induced acute gouty arthritis in rats by inhibiting uric acid and inflammation. Indeed, the treatment significantly attenuated the degree of ankle swelling, inflammation, and dysfunction index as well as the levels of proinflammatory cytokines in the joint fluid of the rat model of acute gouty arthritis. In addition, significant antihyperuricemia activity was also evidenced, and histological evaluation showed that the Tu-Teng-Cao extract relieved several signals of pathological damage. Moreover, this extract alleviated swelling, inflammation, and bleeding of the renal corpuscle and convoluted tubules of rats.

Huzhentongfeng is an extract from four Chinese medical herbs used in the treatment of gout. The suppressive effect of this extract on experimental gouty arthritis was explored by Wu et al. by means of *in vivo* and *in vitro* experiments. The investigators of this study observed that Huzhentongfeng could significantly suppress the paw swelling and neutrophil infiltration induced by monosodium urate intra-articular injection in rats compared with the control group. In addition, inhibition effects on the secretion of several inflammatory cytokines (IL-1β, IL-6, and TNF-α) were evidenced. Moreover, this product could prevent the oligomerization of ASC adapter proteins and had antioxidant effects in cell-free and cell-based tests.

Qu-Zhuo-Tong-Bi is an empirical traditional Chinese medicine prescription for the clinically treatment of acute gouty arthritis without serious adverse effects. In this context, Lv et al. studied the anti-inflammatory and analgesic effects of this product on acute gouty arthritis and the recurrent attack in model rats, as well as potential underlying mechanisms of action. These researchers observed that Qu-Zhuo-Tong-Bi extracts can suppress ankle swelling and synovial inflammation in the monosodium urate-induced gouty arthritis rat model, alleviate the acute attack, and prevent the recurrent attack of gouty arthritis. In addition, this treatment significantly decreased both mRNA and protein levels of NLRP3, as well as the production of IL-1 and TNF-α in the ankle joint. Therefore, it was concluded that Qu-Zhuo-Tong-Bi may be a promising herbal formula for the prevention and treatment of gouty arthritis in humans.

The use of isolated compounds from natural and semisynthetic origin is also presented in two articles of the Special Issue.

Wortmannin is a steroidal metabolite associated with several bioactivities, including anti-inflammatory effect. However, the mechanisms of action of this compound are not completely explored. Therefore, Mehran et al. presented multispectroscopic and molecular docking studies of the binding interaction between wortmannin and calf thymus DNA. Considering the observed results, these authors suggested that this molecule may exert its biological effects, at least in part, via interaction with DNA. In addition, it was demonstrated that wortmannin interacts with this biomolecule in the non-intercalating mode, which is considered as a new mechanism of action.

A review article, from Serrano et al., presented an integrated overview of recent studies with focus on isolated molecules with *in vitro* xanthine oxidase inhibition and *in vivo* hypouricemic effect on animal models. The analysis of data collected from the past six years demonstrated that a large number of compounds are being explored in *in vitro* studies and also in *in vivo* biological evaluations, and very promising molecules are being developed. In addition, it was verified that molecules from natural sources or their mimetics and semisynthetic derivatives constitute the majority of compounds being explored at the moment by means of *in vitro* and *in vivo* studies. Furthermore, as frequently the

observed *in vitro* XO inhibition results do not have a clear correspondence with the *in vivo* hypouricemic effects, it is necessary to perform other complementary studies to better establish the pharmacological profile of these molecules under development.

Finally, the analysis of existing clinical data on the use of natural products in gout control was also explored in two review articles.

The effectiveness of cherries in reducing uric acid and gout was studied by Chen et al. in a systematic review of the clinical evidence in this point. After application of the search strategy, six clinical studies reporting decreases in the incidence and severity of gout following the ingestion of cherries or products containing cherries were included in this review. Despite the described positive effects of cherry intake in gout, it was not possible to conduct a meta-analysis due to the lack of relevant studies and to the high degree of variation in the methodologies and metrics used in the studies included in this review. Therefore, additional more rigorous and larger clinical trials are needed, including long-term follow-up studies.

A systematic review and meta-analysis of randomized controlled trials on natural product dietary supplements on patients with gout was presented by Yang et al. After application of the search strategy and establishment of the clinical outcomes, nine randomized controlled trials were included in the review, and meta-analysis concerning the efficacy and safety of these supplements were performed. The authors observed that natural product dietary supplements appeared to be superior to control groups in affected joint pain, swelling, and activity limitation, while not in decreasing seric uric acid and C-reactive protein levels or the incidence of adverse effects. However, due to poor trial quality and the absence of standardized evaluation criteria, the current existing evidence is insufficient to allow a definitive statement about the efficacy and safety of these supplements. Hence, further larger and more rigorously designed randomized controlled trials are clearly needed in the future.

Thus, with contributions from research groups from diverse countries, this Special Issue presented recent experimental findings and reviews on natural and semisynthetic products with relevant potential for the prevention and treatment of gout.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of the Special Issue.

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Research Article

Tu-Teng-Cao Extract Alleviates Monosodium Urate-Induced Acute Gouty Arthritis in Rats by Inhibiting Uric Acid and Inflammation

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Gouty arthritis is an inflammatory joint disease closely related to hyperuricemia. It is characterized by deposition of monosodium urate crystals in the joints, resulting in an intense inflammatory process and pain. Control of hyperuricemia and anti-inflammation treatments are the main therapeutic approaches. However, the commonly used drugs for inhibiting uric acid and acute gouty arthritis have obvious gastrointestinal and renal toxicity; thus, there is an urgency to develop new alternative therapeutic drugs. An extract of *Tu-Teng-Cao* (TTC), a compound drug used in traditional Chinese medicine, has been widely applied to the clinical treatment of arthritis. In this study, we investigated the therapeutic effects of TTC on gouty arthritis. In this study, an animal model of acute gouty arthritis with hyperuricemia was established using potassium oxonate and monosodium urate crystals. After treatment with TTC, the results showed obvious therapeutic effects on the rat model of acute gouty arthritis. The treatment significantly attenuated the degree of ankle swelling, inflammation, and dysfunction index, and the levels of proinflammatory cytokines. In addition, TTC has significant antihyperuricemia activity in rats with hyperuricemia induced by potassium oxonate. Histological evaluation showed that TTC relieved pathological damage in rats with acute gouty arthritis induced by monosodium urate crystals. All the groups treated with TTC showed improvement in cartilage degeneration, cell degeneration, synovial hyperplasia, and inflammatory cell invasion in the ankle joint of rats. TTC significantly alleviated swelling, inflammation, and bleeding of the renal corpuscle and convoluted tubules of rats. The results of this study suggest that TTC is capable of treating gouty arthritis and decreasing ankle injury through the control of uric acid and inflammation.

1. Introduction

Gout is a metabolic disease characterized by hyperuricemia and deposition of urate crystals into the joints and accompanied by inflammatory reactions [1]. Acute arthritis is the most common initial symptom of primary gout. The patient presents with redness, swelling, and severe pain in the affected joints and surrounding tissues [2]. Epidemiological evidence suggests that this disease is more common in men aged >40 years and postmenopausal women [3]. The prevalence of gouty arthritis has been increased in recent decades, as a result of the increase in intake of high-fat and purine-rich foods, as well as genetic factors [4–6]. Notably, the incidence of gouty arthritis in younger individuals is gradually increasing, especially in young males [7].

In the pathogenesis of acute gouty arthritis, monosodium urate (MSU) crystals are formed due to supersaturation of uric acid concentration in the joint. This effect triggers the body's innate immune response, leading to an induction of inflammation cascades, causing severe inflammation in the joints [8]. In this process, MSU is recognized by the resident macrophages of tissue, thereby

initiating an inflammatory response and recruiting neutrophils. During this process, a large number of inflammatory factors and cellular contents are released, which is considered to be a key to the pathogenesis of acute gouty arthritis [9]. MSU crystals deposited in the tissue result in activation of the NLRP3 inflammasome [10, 11]. Activation of the NLRP3 inflammasome signaling pathway leads to the release of the proinflammatory cytokine interleukin-1β (IL-1β), which in turn induces the expression of a number of proinflammatory factors, such as tumour necrosis factor-alpha (TNF-α), IL-6, and IL-8. These cytokines can recruit monocytes and neutrophils flowing into the synovial membrane of the joint, causing local inflammatory reactions (e.g., increased vascular permeability, plasma exudation, fever, and swelling) and leading to severe joint pain, swelling, and dysfunction. This is the main pathological feature of gouty arthritis [12, 13]. Excessive and uninterrupted inflammation can cause damage to healthy tissue, ultimately leading to cartilage degeneration and joint damage [14].

Control of hyperuricemia and treatment that reduces inflammation are the major therapeutic approaches against gouty arthritis. Relevant drugs are mainly classified into uric acid-lowering drugs and acute-risk anti-inflammatory drugs [15]. The former group of drugs can effectively reduce the content of uric acid in the serum and are further divided into drugs that inhibit its production and promote its excretion [16, 17]. The drugs that inhibit the production of uric acid are represented by allopurinol, which acts by inhibiting the enzyme xanthine oxidase. The drugs that promote uric acid excretion are represented by benzbromarone, which acts by inhibiting the reabsorption of uric acid in the kidneys. However, these two types of drugs are associated with strong toxicity to the liver and kidneys and can easily lead to cardiovascular abnormalities [18]. More importantly, the currently available uric acid-lowering drugs do not exert an effect on the inflammatory pain of gout. Thus, anti-inflammatory treatment has become the first choice against acute episodes of gout. At present, commonly used drugs mainly include colchicine, nonsteroidal anti-inflammatory drugs, and glucocorticoids. Although these drugs are effective, adverse reactions (e.g., renal toxicity or gastrointestinal bleeding) are also prominent [19]. New biopharmaceuticals can be used for the treatment of gouty arthritis; however, the high cost and inherent drawbacks of biopharmaceuticals limit the application of these drugs [20]. The currently available first-line drugs for the treatment of gouty arthritis have been linked to several adverse effects. Thus, it is particularly urgent and important to identify alternative therapeutic strategies.

Traditional Chinese medicine has been using herbal drugs to treat gout for >2,000 years. Recently, studies have shown that numerous herbal drugs and their active ingredients can be used against MSU crystal-induced gouty arthritis, with multiple targets and low toxicity [21–23]. TTC is a crude extract from the traditional Chinese medicine compound *Tu-Teng-Cao*, which has been applied to the clinical treatment of acute-onset gout arthritis [24]. This study used the potassium oxonate- and MSU-induced acute gout arthritis rat model to evaluate the efficacy of TTC in treating hyperuricemia and gouty inflammatory arthritis, confirm the anti-inflammatory and analgesic effects, and preliminarily discuss the potential anti-inflammatory mechanisms of TTC. The aim of this investigation was to shed light on the future application of TTC as a drug for gouty arthritis offering easier storage and oral administration than traditional Chinese medicine compound decoctions.

2. Materials and Methods

2.1. Preparation Process and Quality Standard of TTC

2.1.1. Plant Materials. The main recipe of traditional Chinese medicine compound TTC was *Polygonum cuspidatum Sieb.et Zucc.*, *Sargentodoxa cuneate (Oliv.) Rehd. et Wils.*, *Smilax glabra Roxb.*, *Lonicera japonica Thunb.*, *Lysimachia christinae Hance.*, *Phellodendron chinense Schneid.*, *Commiphora myrrha Engl.*, and *Angelica dahurica (Fisch. ex Hoffm.) Benth. et Hook. f.* All herbal drugs were purchased from Anguo Herb Market (China). The material was identified by Dr. Xirong He and complied with the specification of Pharmacopoeia of the People's Republic of China (2015). A voucher specimen of the material is retained in the Centre of Chinese Medicine Preparations, Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences (Beijing, China).

2.1.2. Preparation of TTC. The herbal drugs were crushed using a blender, and distilled water was added in a liquid-solid ratio of 1 : 10 (g/mL). The herbs were extracted twice at 95°C for 1.5 h each time (R-501 rotary evaporator; Gaoke Instrument Factory, Gongyi, China). The extract solution was centrifuged, and the supernatant was concentrated in vacuum. The concentrated solution was precipitated with 95% (v/v) ethanol and incubated overnight. The precipitates were collected through centrifugation and dried by spray to obtain TTC [25].

2.1.3. Establishment of Quality Standards. The main pharmacological ingredients of this traditional Chinese medicine compound are *Lonicera japonica Thunb.* and *Polygonum cuspidatum Sieb. et Zucc.* The main active ingredients in *Lonicera japonica Thunb.* are chlorogenic acid and loganin. The main active ingredients in *Polygonum cuspidatum Sieb. et Zucc.* are polydatin and emodin. Chlorogenic acid and emodin are not specific components of *Lonicera japonica Thunb.* and *Polygonum cuspidatum Sieb. et Zucc.* Therefore, this preparation selects loganin and polydatin as the content measurement targets for the evaluation of quality. The content of the product was determined using high-performance liquid chromatography (e2695; Waters Corp., Massachusetts, USA), the content of loganin was 0.6 mg/g, and the content of polydatin was 1.0 mg/g [25].

2.2. Chemicals and Reagents. Potassium oxonate was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Uric acid sodium salt was obtained from Sigma-Aldrich (St. Louis, MO, USA). The uric acid assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-1β, and IL-6 were obtained from Cloud-Clone Corp. (Wuhan, China). All other reagents used were standard laboratory reagents of analytical grade and were purchased locally.

2.3. Preparation of MSU Crystals. MSU crystals were prepared by crystallizing the supersaturated solution of uric acid with some modifications as previously described [3]. Briefly, 5 g uric acid was dissolved and heated in 1,000 ml H_2O with sodium hydroxide (9 mL/0.5 N) and adjusted to pH 8.9 at 60°C. The solution was gradually cooled at room temperature and stored overnight at 4°C to allow crystallization. The crystals were sterilized by heating at 100°C for 2 h. The resulting MSU crystals (needle-shaped, length: 5–20 μm) were suspended in endotoxin-free phosphate-buffered saline (PBS) (25 mg/mL). The bacterial endotoxin contamination in MSU crystals was assessed using limulus amebocyte lysate (<0.01 EU/10 mg) and detection procedures in accordance with the Chinese Pharmacopoeia [26].

2.4. Animals. Sprague Dawley rats (male, specific pathogen free, weight: 230–270 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animals were maintained in a standard laboratory conditioned at a temperature of 25 ± 2°C with 50–55% relative humidity and a 12 h light/dark cycle. All the rats were fed with standard food and pure water. Animal experimentation and the corresponding protocol (no. 2018-0042) were approved by the Animal Ethics Committee of the Institute of Chinese Materia Medica China Academy of Chinese Medical Sciences. All the procedures were in strict accordance with the People's Republic of China legislation regarding the use and care of laboratory animals.

2.5. Drug Dosage. The adult conventional dosage of TTC was 0.1 g/kg/day. The high (1 g/kg/day), medium (0.5 g/kg/day), and low (0.25 g/kg/day) doses used in this study were converted based on the adult dose, which was equivalent to twice, equal to, and half the human dose, respectively. The standard drug used in this study was colchicine (0.3 mg/kg/day), which was equivalent to the adult dose [27].

2.6. Animal Model of Acute Gouty Arthritis with Hyperuricemia in Rats and Experimental Design. After a 7-day acclimatization period, an experimental animal model of hyperuricemia was induced using potassium oxonate (uricase inhibitor). Briefly, potassium oxonate (1.5 g/kg/day) dissolved in distilled water was orally administered to 70 rats; the dosing volume was 1 mL/100 g body weight, once daily for 21 consecutive days. In addition, 10 rats were used to form the normal control group (administration of distilled water only). Blood samples were collected from the eyelids of model rats at weeks 1, 2, and 3, and the levels of uric acid in the serum were measured. Rats with blood uric acid levels >110 μmol/L at week 3 indicated that the model was successfully induced. Animals with high or low levels of uric acid in the blood were removed, and 50 rats were selected for the subsequent experiment.

The successfully established hyperuricemia model rats were randomly divided into six groups ($n = 10$ per group): model control, normal control, colchicine, and TTC high dose (TTC-H), TTC medium dose (TTC-M), and TTC low dose (TTC-L). All rats were anesthetized with 2.5% isoflurane, followed by injection of 50 μL MSU crystals (25 mg/mL) or normal saline into the medial side of the right tibiotarsal joint (ankle) of each rat to further establish the model of acute gouty arthritis with hyperuricemia. Of note, the contralateral bulging of the joint capsule was the standard for drug injection [28]. After the administration of MSU, each group intragastrically received TTC or colchicine once daily for 7 days. The normal control group and the model control group were treated with PBS (Figure 1).

2.7. Assessment of Inflammation

2.7.1. Degree of Ankle Swelling. Following the injection of MSU crystals, the width of the right ankle joint at different time intervals was measured using a vernier scale. The left and right diameters (a) and the anteroposterior diameter (b) of the ankle joint were measured before establishing the arthritis model, and 12, 24, 48, 72, and 96 h after the administration of MSU crystals [29]. The ankle joint volume was calculated using the following formula: ankle joint volume $= 1/2 \times ab^2$.

2.7.2. Inflammation Index and Dysfunction Index. The progression of acute arthritis was evaluated by macroscopic scoring of the ankle joint. Data were recorded prior to establishing the arthritis model, and 12, 24, 48, 72, and 96 h after the administration of MSU crystals. The inflammation and dysfunction scores of the rats were visually determined by two independent observers.

The following criteria were used to score inflammation [30]:

Grade 0 (0 points): ankle joint is normal without any inflammatory reaction

Grade 1 (2 points): joints have erythema of skin, mild swelling, and visible bony marks

Grade 2 (4 points): joints are obviously red and swollen, bony landmarks disappear, and swelling is limited to the joints

Grade 3 (6 points): swelling outside the joint, degree of inflammatory reaction is more severe, ability of the foot is weakened, and the foot is often lifted off the ground

The following criteria were used to score dysfunction [31]:

Grade 0 (0 points): normal gait, and both feet are evenly grounded

Figure 1: Experimental design. A hyperuricemia model was established during the first 3 weeks through the daily administration of potassium oxonate. Subsequently, a model of acute gouty arthritis with hyperuricemia was established via injection of MSU crystals, as described in the Materials and Methods section. This was followed by the administration of TTC, colchicine, or PBS (control) once daily for 7 days. The degree of ankle swelling, inflammation index, and dysfunction index were assessed at 12, 24, 48, 72, and 96 h after the injection of MSU crystals. Rats were sacrificed to test for proinflammatory cytokines, renal and liver function, lipid profile, and histological analyses.

Grade 1 (2 points): toes are not unfolded, and foot is slightly limp

Grade 2 (4 points): foot is bent and clearly limping, and toes are on the ground

Grade 3 (6 points): foot completely lifted off the ground, three-legged gait

2.7.3. Proinflammatory Cytokines. After the end of the experimental period (day 8), the animals were sacrificed through euthanasia. The articular cavities were irrigated with 0.5 mL normal saline, the joint fluid was collected, and the samples were centrifuged at 3,000 rpm/min for 15 min. The levels of TNF-α, IL-1β, and IL-6 were measured using ELISA kits, according to the instructions provided by the manufacturer.

2.8. Determination of Uric Acid. On day 8 after treatment, blood samples were collected from the eye sockets of rats. The samples were maintained for 30 min at room temperature and subsequently centrifuged at 3,000 rpm/min for 15 min. The concentration of uric acid in the serum was determined through the phosphotungstic acid method [32] using a test kit, according to the instructions provided by the manufacturer.

2.9. Determination of Renal Function. The renal function (i.e., blood urea and creatinine) before and after administration was detected using a biochemical analyser (TBA-40FR; Toshiba Corp., Tokyo, Japan). The serum samples were the same as described in Section 2.8.

2.10. Histological Analysis. The ankle joints and kidneys of rats were fixed in 4% paraformaldehyde, and the ankles were decalcified using 10% ethylenediaminetetraacetic acid. Tissues were dehydrated by processing in different levels of alcohol, mixed with chloroform, immersed in paraffin, and cut into 5 μm thick slices. The tissue slices were stained with haematoxylin and eosin dye to show the extent of the sliding film and kidney damage. Images were independently evaluated using a light microscope (BX53; Olympus Corp., Tokyo, Japan) in a blinded manner.

Ankle histology scores were as follows (−~+++) [6]:

"−": normal
"+": mild infiltration of mononuclear cells
"++": moderate inflammatory cell immersion and cartilage destruction and mild oedema of intertissue
"+++": massive inflammatory cells into the synovial and joint space and significant oedema of intertissue and cartilage cells

Kidney histology scores were as follows (−~+++) [33]:

"−": normal
"+": minor infiltration of mononuclear cells
"++": mild swelling of glomerular and renal tubes and slight bleeding
"+++": significant swelling of glomerular and renal tubes and mild bleeding

2.11. Statistical Analysis. Measurement data were expressed as mean ± standard deviation and analysed using the GraphPad Prism 6.0 software. One-way analysis of variance was used, followed by Student's Newman–Keul's test. Histological results, as well as inflammation and dysfunction indices, were evaluated using the Statistical Package for the Social Sciences (SPSS) version 17.0 software (SPSS Inc., Chicago, IL, USA), using the rank-sum test. $^{*}P < 0.05$ denoted statistical significance.

3. Results

3.1. TTC Ameliorated Inflammation in Rats

3.1.1. TTC Mitigated the Degree of Ankle Swelling. MSU crystals were synthesized with uric acid and sodium hydroxide and diluted in endotoxin-free PBS prior to injection into the right ankle joint of rats. At 24 h after the injection of MSU crystals, significant redness, swelling, and deformity were observed at the ankle joints of the rats. In the model group, the ankle joint and toes were significantly red and swollen, bony landmarks disappeared, and the degree of

inflammation was severe (Figures 2(a) and 2(b)). There was no inflammatory reaction in the normal group, and bony landmarks were obvious (Figure 2(c)). The ankle joints in the TTC treatment groups were swollen; however, the swelling was confined to the joints without involving the toes, and the degree of inflammation was significantly lower than that noted in the model group (Figures 2(e)–2(g)). The severity of the inflammatory response was evaluated by the volume of ankle joint swelling. The volume of ankle joints was tested at 12, 24, 48, 72, and 96 h after the injection of MSU crystals. As shown in Table 1, there was no difference in the volume of ankle joints prior to establishing the model. The volume of the ankle joints in the model groups increased significantly after the injection of MSU crystals, indicating that the model was successfully established. The volume of the ankle joint at each time point was significantly mitigated after the administration of TTC, especially in the TTC-M group at 24, 48, and 72 h after administration. Notably, the volume in the TTC-L group at 24 h was significantly reduced compared with that reported in the model control group.

3.1.2. TTC Reduced the Inflammation and Dysfunction Indices. After the injection of MSU crystals into the ankle joint, the rats progressively developed a series of arthritis symptoms. In the preliminary experiment, we observed the acute phase of inflammation within 96 h after the administration of MSU. Hence, we measured the following index and cytokine levels at 0–96 h. The occurrence of arthritis was obvious after 12 h. The joints of rats in the model group gradually swelled and could not move freely, and the inflammation index scores were significantly higher than those recorded for the normal control group. The inflammatory scores of the three doses of TTC granules decreased after 24, 48, and 72 h. At 48 h after the administration of TTC, the TTC-M group showed a significant therapeutic effect, which was statistically different from that noted in the model group (Table 2). Inflammation can cause pain and dysfunction; thus, we performed behavioural testing of rats to evaluate the degree of inflammation. After the injection of MSU crystals, the index of joint dysfunction in the model group was significantly higher than that determined for the normal control group. At 24, 48, 72, and 96 h, the dysfunction scores of the three TTC groups were not statistically significant compared with those calculated for the model control group (Table 3).

3.1.3. TTC Decreased the Level of Proinflammatory Cytokines in the Joint Fluid. Acute gouty arthritis is characterized by the high expression of proinflammatory cytokines. The levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) were measured in the joint fluid of MSU crystal-induced rats using ELISA to ascertain whether TTC inhibited this characteristic. Treatment with TTC showed significantly lower levels of TNF-α and IL-6 in the MSU crystal-induced rats versus the model group. The level of IL-1β was not significantly different compared with that reported in the model control group (Figure 3). These results indicate that TTC can alleviate the symptoms of acute arthritis induced by MSU.

3.2. TTC Ameliorated Hyperuricemia in Rats. We evaluated the level of serum uric acid in the groups of treated rats to confirm whether TTC can also ameliorate hyperuricemia. Administration with potassium oxonate for 3 weeks caused hyperuricemia in rats, and the levels of uric acid in the serum were significantly increased compared with those measured in the normal control group. As expected, the increased level of uric acid was inhibited by treatment with TTC. A 7-day treatment with TTC (1, 0.5, and 0.25 g/kg) significantly reduced the levels of serum uric acid compared with those observed for the hyperuricemic control group (Figure 4).

3.3. TTC Improved the Renal Function of Rats Treated with MSU Crystals. The results of the renal function test showed after the injection of MSU crystals, the serum levels of blood urea and creatinine in the model group were significantly higher than those measured in the normal control group. After 7 days of administration, there was no significant difference in all TTC treatment groups compared with the model control group (Table 4).

3.4. TTC Improved Ankle Joint and Kidney Lesions in Rats Treated with MSU Crystals. Histopathological analysis was performed by evaluating the lesions of ankle joints (i.e., periosteum and cartilage) and kidneys (i.e., renal corpuscle and convoluted tubule) in rats treated with MSU crystals to assess whether TTC could improve the histological lesions in rats.

In the normal group, the cartilage and periosteum of the ankle joints in rats showed no degeneration and the structure was normal (Figures 5(a)A and 5(b)A). Obvious pathological changes were observed in the ankle joint of the rats in the model control group, showing chondrocyte vacuole degeneration, significant synovial thickening, increased inflammatory cells, swelling of interstitial tissue, and exudate (Figures 5(a)B and 5(b)B). All TTC treatment groups exhibited improved or alleviated cartilage degeneration, cell degeneration, synovial hyperplasia, and inflammatory cell invasion in the ankle joint of rats (Figures 5(a) D–F and 5(b)D–F). The results in Table 5 show that there was no lesion in the ankle joints of the normal control group. In addition, the pathological score of the model control group was greatly increased, which was significantly different from that reported in the normal group. After 7 days of TTC administration, the pathological scores of ankle joints in all TTC groups were not significantly different compared with those calculated in the model control group (Table 5).

In the normal group, the kidney structure of the rats was normal without obvious lesions (Figure 5(c)A). In the model control group, the renal corpuscle of rats was swollen, with blood stasis and increased mesangial cells. Simultaneously, the kidney convoluted tubules were swollen, the intimal cells were detached, and the interstitial tubules were inflamed

Figure 2: MSU crystals induced gouty arthritis in Sprague Dawley rats. These figures show the macroscopic signs observed in each group 24 h after the injection of MSU or PBS into the ankle joints. (a, b) Model control group (injection of MSU crystals). (c) Normal control group (injection of PBS). (d) Colchicine group. (e–g) TTC-H, TTC-M, and TTC-L (1, 0.5, and 0.25 g/kg, respectively) groups.

Table 1: Effects of TTC on the volume of ankle joints in rats with acute gouty arthritis.

Group	Dose	Unit	Before injection of MSU	After injection of MSU				
				12 h	24 h	48 h	72 h	96 h
Normal	—	mm^3	323.95 ± 23.48	324.18 ± 23.70	324.02 ± 23.39	325.07 ± 23.46	325.64 ± 23.50	327.02 ± 23.03
Model	—	mm^3	317.59 ± 22.49	707.45 ± 102.95$^{\#\#}$	922.78 ± 121.11$^{\#\#}$	746.59 ± 130.50$^{\#\#}$	489.88 ± 79.71$^{\#\#}$	428.44 ± 74.84$^{\#\#}$
Colchicine	0.3 mg/kg	mm^3	320.42 ± 23.83	635.78 ± 126.16	788.64 ± 119.54*	564.06 ± 66.87*	420.93 ± 54.30*	376.24 ± 45.33
TTC-H	1 g/kg	mm^3	318.56 ± 28.88	702.25 ± 89.91	864.86 ± 91.61	665.73 ± 88.41	446.62 ± 72.97	395.95 ± 74.66
TTC-M	0.5 g/kg	mm^3	314.06 ± 24.93	646.97 ± 102.36	809.73 ± 141.09*	625.73 ± 116.46*	426.27 ± 79.66*	385.67 ± 70.19
TTC-L	0.25 g/kg	mm^3	319.05 ± 26.65	671.16 ± 122.82	817.61 ± 150.29*	653.92 ± 164.91	504.37 ± 91.02	424.15 ± 83.42

Measurement data represent the mean ± standard deviation of 10 animals. One-way ANOVA followed by Student's Newman–Keul's test was used for statistical analysis. $^{\#\#}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$ compared with the model control group.

with blood stasis (Figures 5(c)B and 5(d)B). The pathological damage in the three TTC groups was significantly lower than that noted in the model control group. In the normal control group, the kidneys of rats did not have visible lesions (Figures 5(c)D–F and 5(d)D–F). The results in Table 6 show that there was no lesion in the normal control group, and the pathological scores of renal corpuscles and convoluted tubules in the model control group were significantly increased compared with those recorded in the normal group. After 7 days of TTC administration, the pathological score was significantly reduced. The evaluation of convoluted tubules showed that there were significant differences between the three TTC groups and the model control group. The evaluation of renal corpuscles showed that there were significant differences between the TTC-H (1 g/kg) group and the model control group (Table 6).

4. Discussion

Gouty arthritis is an inflammatory response triggered by the abnormal metabolism of uric acid. It is characterized by intense pain, redness, and swelling around the joints and connective tissues. The management of acute flares of gout is extremely difficult for clinicians. Currently, gouty arthritis is typically treated with nonsteroidal anti-inflammatory drugs, colchicine, adrenocortical hormones, or biological agents. However, these drugs are linked to serious side effects and lack of safety in patients with comorbidities [34, 35]. Existing evidence suggests that traditional Chinese medicines, as one of the most popular drugs of complementary and alternative medicine, possess a low toxicity profile and exhibit beneficial efficacy in the treatment of gouty arthritis [22–24, 36]. In this study, the effects of an extract (TTC) derived from a novel

Table 2: Effects of TTC on the inflammation index in rats with acute gouty arthritis.

Group	Unit	After injection of MSU									
		12 h					24 h				
		Grade 0	Grade 1	Grade 2	Grade 3	P value	Grade 0	Grade 1	Grade 2	Grade 3	P value
Normal	pcs	10	0	0	0		10	0	0	0	
Model	pcs	0	4	5	1	##	0	0	4	6	##
Colchicine	pcs	0	6	3	1		0	4	5	1	*
TTC-H	pcs	0	4	5	1		0	2	6	2	
TTC-M	pcs	0	5	4	1		0	3	5	2	
TTC-L	pcs	0	4	5	1		0	1	7	2	
Group	Unit	48 h					72 h				
		Grade 0	Grade 1	Grade 2	Grade 3	P value	Grade 0	Grade 1	Grade 2	Grade 3	P value
Normal	pcs	10	0	0	0		10	0	0	0	
Model	pcs	0	0	3	7	##	0	6	2	2	##
Colchicine	pcs	0	4	6	0	**	4	5	1	0	*
TTC-H	pcs	0	0	7	3		2	5	2	1	
TTC-M	pcs	0	3	5	2	*	2	6	2	0	
TTC-L	pcs	0	1	6	3		2	4	4	0	
Group	Unit	96 h									
		Grade 0	Grade 1	Grade 2	Grade 3	P value					
Normal	pcs	10	0	0	0						
Model	pcs	3	5	2	0	##					
Colchicine	pcs	6	4	0	0						
TTC-H	pcs	4	4	2	0						
TTC-M	pcs	5	5	0	0						
TTC-L	pcs	3	6	1	0						

Table 3: Effects of TTC on the dysfunction index in rats with acute gouty arthritis.

Group	Unit	After injection of MSU									
		12 h					24 h				
		Grade 0	Grade 1	Grade 2	Grade 3	P value	Grade 0	Grade 1	Grade 2	Grade 3	P value
Normal	pcs	10	0	0	0		10	0	0	0	
Model	pcs	0	7	3	0	##	0	3	3	4	##
Colchicine	pcs	0	8	2	0		0	6	3	1	
TTC-H	pcs	0	8	2	0		0	4	4	2	
TTC-M	pcs	0	8	2	0		0	5	3	2	
TTC-L	pcs	0	8	2	0		0	4	4	2	
Group	Unit	48 h					72 h				
		Grade 0	Grade 1	Grade 2	Grade 3	P value	Grade 0	Grade 1	Grade 2	Grade 3	P value
Normal	pcs	10	0	0	0		10	0	0	0	
Model	pcs	0	3	3	4	##	1	5	2	2	##
Colchicine	pcs	0	5	2	3		4	5	1	0	
TTC-H	pcs	0	4	3	3		1	6	2	1	
TTC-M	pcs	0	5	2	3		3	6	1	0	
TTC-L	pcs	0	3	4	3		2	4	4	0	
Group	Unit	96 h									
		Grade 0	Grade 1	Grade 2	Grade 3	P value					
Normal	pcs	10	0	0	0						
Model	pcs	3	6	1	0	##					
Colchicine	pcs	6	4	0	0						
TTC-H	pcs	4	5	1	0						
TTC-M	pcs	5	5	0	0						
TTC-L	pcs	3	6	1	0						

Measurement data represent the interquartile range of 10 animals. The rank-sum test for multiple sets of independent samples was used for statistical analysis. ##$P < 0.01$ compared with the normal control group; *$P < 0.05$ and **$P < 0.01$ compared with the model control group.

herbal formula on gouty arthritis were evaluated in rats injected with MSU crystals.

Potassium oxonate is a uric acid enzyme inhibitor, which can cause hyperuricemia in animals through intragastric or intraperitoneal injection. This model has been widely used in the screening of uric acid-lowering drugs [37, 38]. Recent studies indicate that MSU crystals are closely related to the pathology of gouty arthritis and are among the most effective

Figure 3: Effects of TTC on proinflammatory cytokines in MSU crystal-induced rats. The samples were ankle lavage fluid of rats. The evaluation time was 7 days after the administration of TTC. Data represent the mean ± standard deviation of values obtained from six animals. $^{\#\#}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the hyperuricemic control group (ANOVA followed by Student's Newman–Keul's test).

Figure 4: Effects of TTC on serum uric acid in hyperuricemic rats. Data represent the mean ± standard deviation of data obtained from 10 animals. $^{\#\#}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$ compared with the hyperuricemic control group (ANOVA followed by Student's Newman–Keul's test).

Table 4: Effects of TTC on the renal function of rats with acute gouty arthritis.

Group	Dose	Blood urea (mmol/L)	Creatinine (μmol/L)
Normal	—	6.42 ± 0.73	24.67 ± 4.36
Model	—	7.79 ± 0.94$^{\#\#}$	28.60 ± 0.74$^{\#}$
Colchicine	0.3 mg	7.32 ± 1.21	27.93 ± 5.13
TTC-H	1 g/kg	7.42 ± 1.89	27.63 ± 4.64
TTC-M	0.5 g/kg	7.41 ± 1.53	28.90 ± 7.87
TTC-L	0.25 g/kg	7.40 ± 1.01	26.36 ± 2.83

Measurement data represent the mean ± standard deviation of data obtained from 10 animals. One-way ANOVA and Student's Newman–Keul's test were used for statistical analysis. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$ compared with the model control group.

proinflammatory stimuli [39]. The formation of MSU crystals into the joint cavity caused acute inflammation, demonstrating equivalent symptoms to those found in clinical gout. This model has good predictive value for the clinical efficacy against gout arthritis at the research level [28]. Based on previous methods, we have made improvements and attempted to construct a new animal model. We successfully established a rat model of hyperuricemia with acute gouty arthritis through intragastric administration of potassium oxonate for 3 weeks and injection of MSU crystals into the ankle joint.

The phagocytosis of MSU crystals by macrophages activated the formation of the inflammasome. Subsequently, it induced high expression of a variety of proinflammatory cytokines, which are directly responsible for the influx of neutrophils and monocytes into the synovium, resulting in the enhancement of the inflammatory response [40]. Arthritis causes pain, and movement of the affected joint is typically reduced, which ultimately affects the ability to perform activities. Twenty-four hours after injection, significant swelling of the ankle joint was observed,

(a)

(b)

Figure 5: Continued.

Figure 5: Effect of TTC on MSU crystal-induced rat ankle joints and kidney lesions ($n = 10$). (A) Normal control group (injection of PBS). (B) Model control group (injection of MSU crystals). (C) Colchicine group. (D–F) TTC-H, TTC-M, and TTC-L (1, 0.5, and 0.25 g/kg, respectively) groups. (a) Cartilage: arrows indicating chondrocyte degeneration, and the nucleus disappears (B–C); enlargement of chondrocytes, and the nucleus becomes smaller or lysed (E–F). (b) Periosteum: arrows indicating thickening of synovia, myxedema, and increased number of cells (B–F). (c) Renal corpuscles: arrows indicating hypertrophy of glomerular mesangial cells, congestion in the glomeruli, and segmental increase in the number of parietal cells (B–F). (d) Convoluted tubules: arrows indicating swelling of renal tubules, shedding of endometrial cells, interstitial inflammation, stasis, and other lesions (B–F). Arrows indicating typical lesions; scale bar = 100 μm; original magnification ×400.

accompanied by joint dysfunction. Of note, after the administration of TTC, the ankle swelling in rats was obviously decreased. The inflammatory and dysfunction indices were also lower than those determined in the model group. The continuous observations in this experiment showed that the ankle joint volume at 24, 48, and 72 h and the inflammation index at 48 h in the TTC-M group were statistically different from those calculated in the model group. The TTC-L group showed a statistically significant difference in ankle joint volume at 24 h versus the model group. The TTC-H group did not show a significant therapeutic effect in terms of reducing the ankle joint volume, inflammation and dysfunction indices, and pathological damage. Combining the above results, we could observe that there was a dose-effect

Table 5: Effects of TTC on ankle joint lesions in rats with acute gouty arthritis.

Group	Unit	Tissue	Grade of lesions				P value
			−	+	++	+++	
Normal	pcs	Periosteum	10				
		Cartilage	10				
Model	pcs	Periosteum			9	1	##
		Cartilage			7	3	##
Colchicine	pcs	Periosteum		2	6	2	
		Cartilage		3	6	1	
TTC-H	pcs	Periosteum		1	9		
		Cartilage		2	6	2	
TTC-M	pcs	Periosteum		2	8		
		Cartilage		1	9		
TTC-L	pcs	Periosteum		5	4	1	
		Cartilage		2	8		

Table 6: Effects of TTC on kidney lesions in rats with acute gouty arthritis.

Group	Unit	Tissue	Grade of lesions				P value
			−	+	++	+++	
Normal	pcs	Renal corpuscles	10				
		Convoluted tubules	10				
Model	pcs	Renal corpuscles			8	2	##
		Convoluted tubules			4	6	##
Colchicine	pcs	Renal corpuscles		1	9		
		Convoluted tubules		2	8		**
TTC-H	pcs	Renal corpuscles		5	5		*
		Convoluted tubules		1	8	1	**
TTC-M	pcs	Renal corpuscles		2	8		
		Convoluted tubules		5	5		**
TTC-L	pcs	Renal corpuscles		2	8		
		Convoluted tubules		3	7		**

Measurement data represent the interquartile range obtained from 10 animals. The rank-sum test for multiple sets of independent samples was used for statistical analysis. $^{##}P < 0.01$ compared with the normal control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the model control group.

relationship between the TTC-M and TTC-L groups. Such observations are often noted in pharmacological experiments of traditional Chinese medicine. The reasons may be related to the complex dose-effect relationship of traditional Chinese medicine compound preparations. The TTC-M group represents the dose routinely used in the clinical setting. In the experiment, the concentration of the drug in the TTC-H group is twice the recommended dosage. The preparation of Chinese medicine compounds is complicated, and the excessive concentration limits the absorption of the active ingredients by the body, so pharmacodynamics effect is often not ideal.

Furthermore, MSU crystal-stimulated neutrophils release oxygen radicals, proteolytic enzymes, and proinflammatory cytokines, leading to damage of the articulation [41]. After the administration of TTC with three doses for 1 week, histopathological assessment revealed a treatment effect. TTC reduced synovial hyperplasia, cartilage damage, and bone erosion. Histopathological examination showed that treatment with TTC in all three groups alleviated pathological damage in the ankle joints of rats. However, the difference versus the model group was not statistically significant. The results were attributed to the rapid self-healing ability of rats [42]. The rat ankle joint is sensitive to inflammatory response and commonly used for drug development against arthritis. Nevertheless, the recovery ability of in rats with inflammatory injury was also strong. The continuous observations in this experiment showed that the model group of rats without any drug intervention exhibited the most severe ankle swelling at 48 and 72 h after the administration of MSU crystals into the ankle joint, which was gradually reduced after 96 h. By the end of the experiment (i.e., 7 days), most of the rats did not show obvious redness and swelling.

MSU crystals are one of the most effective proinflammatory stimuli, which can trigger, amplify, and sustain a strong inflammatory reaction in the joint cavity [3]. A previous study demonstrated that MSU crystals stimulate the synthesis and release of IL-1β [43]. IL-1β is the pivotal inflammatory mediator that regulates the differentiation, proliferation, and apoptosis of cells in gouty arthritis. In addition, IL-1β can induce the expression of a wide range of cytokines and chemokines, such as TNF-α, IL-6, IL-17, and monocyte chemoattractant protein-1. This leads to a large influx of neutrophils into the synovium, which is the main pathological feature of gout arthritis. Furthermore, MSU crystals can also stimulate the secretion of TNF-α and IL-6 by macrophages, neutrophils, and monocyte-macrophages, which induce acute inflammation. These persistent inflammatory reactions cause subsequent tissue damage [44]. Therefore, blocking the secretion of inflammatory mediators from MSU crystal-activated macrophages is beneficial to the control and management of acute gout arthritis. In this study, our results indicated that TTC significantly reduced the secretion of cytokines TNF-α and IL-6 in the synovial fluid of rats treated with MSU crystals. Moreover, the secretion of IL-1β tended to decrease compared with that reported in the model group, although there was no statistically significant difference.

Hyperuricemia is a disease in which the level of uric acid in the blood is elevated due to a decrease in purine metabolism or excretion of uric acid from the body. In the present study, a hyperuricemic rat model was generated using potassium oxonate to investigate the pharmacological effect of TTC on the reduction of uric acid function. After 3 weeks of intragastric administration of potassium oxonate, the levels of serum uric acid were significantly higher than those observed in normal rats (reached $\geq 110\,\mu$mol/L), which was in accordance with the expected model requirements. Potassium oxonate is an inhibitor of the uric acid enzyme and has demonstrated strong activity both *in vivo* and *in vitro*. It can effectively inhibit the activity of uricase and increase the levels of uric acid in the experimental animals [45]. In the present study, the levels of uric acid in the serum were measured before and after the administration of TTC. TTC significantly downregulated the increased levels of serum uric acid. These results suggested that TTC plays a role in the amelioration of hyperuricemia.

Uric acid is closely related to kidney disease. It can be handled by the kidneys and the process is complicated, requiring glomerular filtration, reabsorption of filtered urate, tubular secretion, and finally secretion and reabsorption. In the presence of abnormal metabolism of uric acid, the kidney is the most likely organ to be damaged. The deposition of uric acid crystals in the renal corpuscle and tubulointerstitium may cause an inflammatory reaction along with the release of cytokines. In this situation, intrarenal precipitation of uric acid also contributes to renal insufficiency [46]. In the present study, renal function in each group was measured after the administration of TTC. The analysis found that TTC exerted a protective effect on the kidneys. At the end of the experiment, pathological examination of the kidneys showed that TTC significantly alleviated pathological symptoms, such as swelling, cell degeneration, inflammation, and haemorrhage of renal corpuscles and convoluted tubules in rats.

5. Conclusions

We established an optimized rat model of acute gouty arthritis with hyperuricemia through administration of potassium oxonate and MSU crystals and clarified the overall effects of TTC against gouty arthritis. The results showed that TTC could inhibit the inflammatory cascade and regulate the metabolism of uric acid. Therefore, this extract may be an effective drug for the treatment of hyperuricemia and gouty arthritis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed to this study. R.M. Yao, X.L. Cui, and F.X. Meng conceived and designed the experiments. R.M. Yao, X. Mao, J. Sun, Y.J. Gao, Y.L. Xu, and B. Guo performed the experiments. S.S. Guo and L. Bao performed the histopathological examination. R.M. Yao, Z.H. Geng, and Y.Y. Bao contributed to the interpretation and statistical analysis of the data. R.M. Yao wrote the manuscript. All authors read and approved the final manuscript for submission.

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Review Article

From Xanthine Oxidase Inhibition to *In Vivo* Hypouricemic Effect: An Integrated Overview of *In Vitro* and *In Vivo* Studies with Focus on Natural Molecules and Analogues

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Hyperuricemia is characterized by elevated uric acid (UA) levels on blood, which can lead to gout, a common pathology. These high UA levels are associated with increased purine ingestion and metabolization and/or its decreased excretion. In this field, xanthine oxidase (XO), by converting hypoxanthine and xanthine to UA, plays an important role in hyperuricemia control. Based on limitations and adverse effects associated with the use of allopurinol and febuxostat, the most known approved drugs with XO inhibitory effect, the search for new molecules with XO activity is growing. However, despite the high number of studies, it was found that the majority of tested products with relevant XO inhibition were left out, and no further pharmacological evaluation was performed. Thus, in the present review, available information published in the past six years concerning isolated molecules with *in vitro* XO inhibition complemented with cytotoxicity evaluation as well as other relevant studies, including *in vivo* hypouricemic effect, and pharmacokinetic/pharmacodynamic profile was compiled. Interestingly, the analysis of data collected demonstrated that molecules from natural sources or their mimetics and semisynthetic derivatives constitute the majority of compounds being explored at the moment by means of *in vitro* and *in vivo* animal studies. Therefore, several of these molecules can be useful as lead compounds and some of them can even have the potential to be considered in the future clinical candidates for the treatment of hyperuricemia.

1. Introduction

Xanthine oxidase (XO) is a key enzyme in purine catabolism and, physiologically, catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid (UA) with concomitant reactive oxygen species (ROS) production [1, 2]. Nonetheless, enzymes such as hypoxanthine-guanine phosphoribosyl transferase (HGPRT), phosphoribosyl pyrophosphate synthetase (PRPS), and phosphoribosyl pyrophosphate aminotransferase (PRPPAT) also participate in purine metabolic pathway [3]. The overproduction or low excretion of urate can lead to hyperuricemia and subsequently to gout [3, 4]. In the field of low excretion, anion-exchanging uptake transporters (OAT1, OCT2, URAT1, and GLUT9) have also been reported to play important roles in the regulation of serum UA [3].

The most relevant target conditioning serum UA levels is XO, and excessive activity of this enzyme can lead to a pathological UA overproduction [5]. Therefore, its inhibition is of high interest, mainly in the treatment of gout, being allopurinol, febuxostat, and topiroxostat (Figure 1), the most known XO inhibitors. In addition to these clinically used drugs, over the years, several other molecules with XO inhibitory activity have been described [6–9]. Despite the existence of some reviews in this topic [6, 7, 9–11], it is necessary to complement these works with a study focusing on the *hit to lead* evolution in the development of new XO inhibitors with improved potency and safety when

Figure 1: Structures of the clinically used XO inhibitors.

compared with the clinically used drugs. Interestingly, as can be seen in this review, natural molecules and semisynthetic analogues and derivatives constitute a large group of compounds being explored at the moment in this context.

2. Materials and Methods

The present review covers the literature published in the last 6 years and develops the most relevant studies that focus not only in XO inhibition but also in the integration of these data with other relevant information for the *hit to lead* evolution in the development of new XO inhibitors. In this view, cytotoxicity studies, *in vivo* hypouricemic effect and/or pharmacokinetic/pharmacodynamic profile, are also discussed. For this, a research on scientific databases Web of Science, Pubmed, Scopus, and others was carried out using associations of the following key terms and synonyms: XO, cytotoxicity, hyperuricemia, hypouricemic, or pharmacokinetic profile.

The review is organized according to the origin of the compounds (synthetic and natural/semisynthetics/mimetics of natural structures) and then according to their chemical structures. The structures of the scaffolds and/or the specific compounds with the most promising results are presented in figures.

3. Results

3.1. Synthetic Derivatives

3.1.1. Purine Nucleus Analogues. Based on allopurinol structure, Rangappa and coworkers reported four 5H-thiadiazolopyrimidin-5-one analogues (Figure 2) as potent XO inhibitors with IC_{50} values in the range from 269 to 634 nM (XO from bovine milk) [12]. These compounds were later investigated on oxonate-treated rats, and it was observed that serum UA and creatinine levels significantly dropped at doses of 50 and 100 mg/kg. On the other hand, these pyrimidine analogues could significantly attenuate liver and kidney necrosis in oxonate-treated rats [13].

A series of pyrazolo[4,3-d]pyrimidine analogues (Figure 2) was prepared and evaluated by Yin and coworkers as XO inhibitors and was determined an IC_{50} value of 33.77 μg/mL for the most potent inhibitor. Furthermore, most of these pyrimidines presented IC_{50} values higher than 64 μg/mL against two human hepatocellular carcinoma cells [14]. More recently, this research group described novel purine derivatives with antitumoral effects and attenuated *in vitro* XO inhibition [15].

Saïd et al. performed an *in vitro* screening of novel pyranotriazolopyrimidines (Figure 2) at 100 μM for the inhibition of XO activity and determined their cytotoxic

Figure 2: Structures of analogues of the purine nucleus.

effect against four cancer cell lines. According to the results, the XO% of inhibition at 100 μM ranged between 4.4 and 25.5%, and relevant antiproliferative effects were observed [16].

3.1.2. Febuxostat Analogues. Based on the success of the drug febuxostat, two series of 1-phenyl-pyrazole-4-carboxylic acid derivatives (Figure 3) have been designed and synthesized by Li et al. They reported a high *in vitro* XO inhibition, with IC_{50} values in the nanomolar range, acting by a mixed-type inhibition mechanism. The most potent inhibitors (IC_{50} of 4.2 and 5.7 nM) were further *in vivo* tested in mice with induced acute hyperuricemia, in comparison with the standard inhibitors febuxostat and Y-700 (Figure 3). Both studied compounds decreased the UA levels from approximately 1000 to 200 μM after 5 hours at the dose of 5 mg/kg. Interestingly, the compound with $IC_{50} = 5.7$ nM (Figure 3, $R = 1$-piperidin-1-yl) presented the most promising hypouricemic effect, which was similar to that observed with febuxostat. However, the pharmacokinetic studies demonstrated that further strategy to improve the plasma concentration of this compound is required because the C_{max} and $AUC_{0-\infty}$ were remarkably lower than that determined for Y-700 [17].

In a study aiming to develop other febuxostat analogues with improved pharmacological properties, Xu et al. prepared a series of compounds structurally similar to this drug mainly exploring different 5-membered heterocyclic rings. Considering these data from *in vitro* XO inhibition, it was demonstrated that 2-phenylthiazole-4-carboxylic acid can be considered a new scaffold for this activity. Of the compounds studied, the best result was observed with 2-(4-isobutoxy-3-nitrophenyl)thiazole-4-carboxylic acid ($IC_{50} = 48.6$ μM; IC_{50} for febuxostat = 4.8 μM). Interestingly, a hypouricemic effect of this compound (Figure 4) in potassium oxonate hypoxanthine-induced hyperuricemic mice was observed, however, inferior to that observed with febuxostat [18].

Also considering the febuxostat structure, Song et al. described a series of thiazole derivatives bearing the 2-(indol-5-yl) or 2-(indol-2-yl) moiety (Figure 4) [19, 20]. In the first study, they explored the structure-activity relationship for 2-(indol-5-yl)thiazoles, which presented XO inhibition levels similar to febuxostat, with IC_{50} values between 3.0 and 16 nM in most of the cases. However, not all of these compounds have this activity confirmed in an oxonic acid–induced high-uric acid *in vivo* model. In this context, 2-(3-cyano-2-isopropylindol-5-yl)-4-methylthiazole-5-carboxylic acid

Figure 3: Structures of pyrazole febuxostat analogues.

Figure 4: Structures of thiazole febuxostat analogues.

(Figure 4) exhibits the best compromise between *in vitro* XO inhibitory activity (IC_{50} of 3.5 nM) and plasma UA-lowering activity (60% at 1 h and 10 mg/kg). The pharmacokinetics profile study of this compound showed excellent oral bioavailability and long half-life. Therefore, according to the authors, these results evidenced the interest in performing clinical studies with this compound [19]. More recently, the same research group presented structure-activity relationship data for analogous 2-(indol-2-yl)thiazoles (Figure 4). From the observed results, although not being the most promising *in vitro* (IC_{50} of 5.1 nM) compound, 2-(7-nitro-5-isopropoxy-indol-2-yl)-4-methylthiazole-5-carboxylic acid (Figure 4) exhibited the most potent UA-lowering activity in the potassium oxonate–induced hyperuricemic rat model (43% at 1 h and 10 mg/kg). Similarly to which was observed with the previously referred 2-(indol-5-yl)thiazole derivative, this 2-(indol-2-yl)thiazole has excellent oral bioavailability in pharmacokinetic studies [20].

Ali et al. synthesized a different series of substituted 2-benzamido-4-methylthiazole-5-carboxylic acid derivatives (Figure 4) as potential XO inhibitors and free radical scavengers. *In vitro* studies revealed that the presence of a fluoro or chloro group at the *para* position of the benzamide led to an excellent XO inhibitory activity, with IC_{50} values of 0.57 (in a mixed-type inhibition) and 0.91 μM, respectively. These results were confirmed in a potassium oxonate–induced hyperuricemic *in vivo* rat model, with 62 and 53% of UA inhibition being observed after 1 h at a dose of 10 mg/kg [21].

Mao et al. reported a series of 2-phenyl-1,6-dihydropyrimidine-5-carboxylic acid derivatives (Figure 5) as excellent XO inhibitors with calculated IC_{50} values between 0.0181 and 0.5677 μM. These IC_{50} values were substantially

Figure 5: Structures of dihydropyrimidine febuxostat analogues.

better than that reported for allopurinol (IC_{50} = 7.5902 μM) and comparable to that of febuxostat (IC_{50} = 0.0236 μM). The authors chose 2-(3-cyano-4-isopentyloxyphenyl)-6-imino-1,6-dihydropyrimidine-5-carboxylic acid (Figure 5), with an IC_{50} value of 0.0240 μM, as a representative compound to continue the study. This derivative act as a mixed-type inhibitor, with a K_i value of 0.0042 μM. Also, this compound was able to significantly reduce the serum concentration of UA at a single oral dose of 5 mg/kg on potassium oxonate-induced hyperuricemic mice, with a hypouricemic potency similar to allopurinol (dose = 10 mg/kg). In an acute oral toxicity study, this derivative did not lead to behavioral abnormality 24 h after the administration of a single dose of 2000 mg/kg, which suggested that the LD_{50} value might be higher than 2000 mg/kg, being near 400 times over the effective dose (5 mg/kg) [22].

Zhang et al. designed and prepared a group of 2-phenyl-6-oxo-1,6-dihydropyrimidine-5-carboxylic acid derivatives (Figure 5) bearing a tetrazol-1-yl and alkoxyl groups bounded to positions 3 and 4 of the phenyl ring, respectively, aiming to develop febuxostat analogues with higher selectivity for XO. Interestingly, all compounds had *in vitro* XO inhibitory properties (IC_{50} values ranging from 28.8 to 629 nM), and the most potent of these, 2-{4-[(3-chlorobenzyl)oxy]-3-(1H-tetrazol-1-yl)phenyl}-6-oxo-1,6-dihydropyrimidine-5-carboxylic acid (Figure 5), had an activity comparable to that observed with febuxostat (IC_{50} = 23.6 nM) and acted as a mixed-type inhibitor. The *in vivo* hypouricemic activity evaluation in the potassium oxonate–induced hyperuricemic rat model revealed that this compound (at an oral dose of 5 mg/kg) effectively reduced serum UA levels. However, its hypouricemic action was slightly lower than using febuxostat (5 mg/kg) and allopurinol (10 mg/kg). Furthermore, an acute oral toxicity study revealed that this dihydropyrimidine-5-carboxylic acid derivative was nontoxic to mice and could be tolerated at a dose up to 2000 mg/kg [23].

3.1.3. Dihydropyrimidinone Derivatives. Taking into account their structural resemblance with the pyrimidine ring of xanthine, a series of dihydropyrimidinones (Figure 6) were prepared and evaluated by Zafar et al. as potential XO inhibitors. Interestingly, from a set of 25 derivatives, 22 were found to be good to weak XO inhibitors (IC_{50} values ranging from 14.4 to 418 μM). These compounds were found to act by a competitive, noncompetitive, or mixed manner for XO inhibition. The best IC_{50} value was described for ethyl 4-ethyl-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-

Figure 6: Structures of dihydropyrimidinones with XO inhibitory activity.

carboxylate (Figure 6), being a noncompetitive inhibitor, with a $K_i = 20.5\,\mu$M. Additionally, no cytotoxic effects on mouse fibroblasts 3T3 at the concentration of $100\,\mu$M were observed for the most promising dihydropyrimidinones [24].

Also bearing the pyrimidine scaffold in their structure, several barbiturate and thiobarbiturate derivatives (Figure 6) were described as moderate XO inhibitors [25–27]. The XO inhibition of 3-substituted-2,1-benzisoxazoles (Figure 6) was demonstrated by Serrano et al. with an IC_{50} value of $22.10\,\mu$M being determined for the best compound. In addition, a marked reduction in proliferation at a $30\,\mu$M concentration induced by this barbiturate in the MCF-7 cell line was observed [25]. Moreover, 5-benzylidene [26] and phenylhydrazinylethylidene [26, 27] 1,3-disubstituted barbiturates (Figure 6) were described by Figueiredo et al. as moderate XO inhibitors, with IC_{50} values between 24.3 and $31.5\,\mu$M [26, 27]. The authors also demonstrated that these barbiturates have low cytotoxicity in normal human dermal fibroblasts. In fact, in a screening at $30\,\mu$M, a relative cellular proliferation ranging from 65 to 79% was observed, and an IC_{50} value of $82.02\,\mu$M was determined for the most cytotoxic barbiturate [26, 27].

3.1.4. Other Organic Synthetic Derivatives. 3H-Quinazolin-4-one derivatives (Figure 7) were synthesized by El-Sayed et al., and *in vitro* studies for XO inhibition demonstrated that none of these compounds evidenced better activity than allopurinol. For the best compound (Figure 7), an IC_{50} of $3.0\,\mu$g/mL was calculated, which is a higher value than that observed for allopurinol ($0.6\,\mu$g/mL). Cytotoxicity studies on colorectal cancer HT-29 and SW620 cell lines showed cellular viability nearly 40% at the concentration of $30\,\mu$g/mL [28].

Benzylidene nicotino/isonicotinohydrazide derivatives (Figure 8) were reported by Zafar et al. as being good *in vitro* XO inhibitors, with IC_{50} values of 0.96, 10.0, and $12.4\,\mu$M being determined for the three most potent compounds. In addition, it was demonstrated that these hydrazides act by a competitive mode of inhibition. These compounds were also found to be noncytotoxic against a mouse fibroblast cell line and thus were selected for *in vivo* studies. Interestingly, these authors demonstrated that two isonicotinohydrazides (Figure 8) were able to *in vivo* inhibit XO by 28 and 44% (at

Figure 7: Structure of a 3H-quinazolin-4-one derivatives with XO inhibitory activity.

Figure 8: Structures of pyridine synthetic derivatives with XO inhibitory activity.

50 mg/kg) against 100% observed for allopurinol at the same concentration [29].

A series of compounds incorporating the thieno[2,3-b] thiophene moiety (Figure 8) were synthesized and tested against several enzymes by Mabkhot et al. Three of these compounds demonstrated to be selective XO inhibitors, being determined for the most potent one (Figure 8) an IC_{50} value of $14.4\,\mu$M. This compound was also found to be noncytotoxic in a human prostate cancer cell line [30].

Under the paradigm "old drug, new indication," Niu et al. described olsalazine sodium (Figure 9), a commercial anti-ulcerative-colitis drug, as a serum UA levels reducer. For this, the author performed molecular docking to virtually screen potential XO inhibitors from a small approved drugs library. The *in vitro* studies showed olsalazine sodium as a promising compound in reducing XO activity, with an IC_{50} value of 3.4 mg/L (approximately $9.5\,\mu$M), acting by a hybrid-type inhibition mode. Finally, it was observed in *in vivo* studies that this drug decreased serum UA levels and serum/hepatic XO activities after intraperitoneal administration. Nevertheless, as the onset of hypouricemic action for olsalazine occurred 4 h after administration, the authors suggested that this drug might be particularly suitable for gout prevention and long-term treatment [31].

2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid (HMS) (Figure 9), a popular ultraviolet filter used in sunscreens, was studied as a potential hypouricemic agent by Zuo and coworkers. After an *in silico* molecular docking study, the XO inhibition by HMS was partially confirmed *in vitro*, and an IC_{50} of $36.1\,\mu$M was obtained, in comparison with $11.4\,\mu$M determined for allopurinol. In *in vivo* studies, it was observed that a 20 mg/kg dose of HMS administered to hyperuricemic mice leads to a significant reduction of serum UA levels. Possibly, this result was not only due to XO inhibition but also due to an upregulation of OAT1 and

Figure 9: Structures of olsalazine sodium, 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid, and Knoevenagel/tandem Knoevenagel and Michael adducts of cyclohexane-1,3-dione and aryl aldehydes.

downregulation of GLUT9 mRNA and protein expression. Additionally, toxicity studies showed no negative impact in mice body weight growth and in kidney function [32].

Arora et al. designed and prepared Knoevenagel/tandem Knoevenagel and Michael adducts of cyclohexane-1,3-dione and aryl aldehydes (Figure 9) as XO inhibitors with a slight structural similarity to flavonoid (IC_{50} range from 4.93 to 15.28 μM), xanthone (IC_{50} range from 4.08 to 13.14 μM), and chalcone ($IC_{50} = 3.66\,\mu$M) scaffolds. Most of the described compounds presented better IC_{50} values than allopurinol ($IC_{50} = 7.08\,\mu$M). 2,2-((2-chloro-6-fluorophenyl)methylene)-bis(3-hydroxycyclohex-2-en-1-one) and 2-((1H-indol-3-yl)methylene)cyclohexane-1,3-dione (Figure 9) acted as noncompetitive and competitive-type inhibitors, respectively. Additionally, no significant cytotoxicity at 10 μM on normal cells (HEK293) was observed for these compounds [33].

3.1.5. Metal Complexes. A dinuclear cyclam complex (Figure 10) was described as a XO inhibitor ($IC_{50} = 3.70\,\mu$M) by Zafar et al. This complex acted by a noncompetitive type of inhibition and was also found to be *in vitro* noncytotoxic and led to a marked inhibition of *in vivo* XO activity [34]. Özerkan et al. reported the synthesis and evaluation of novel palladium (II) complexes with tetradentate thiosemicarbazones (Figure 10). Two compounds are interesting XO inhibitors (uncompetitive inhibitory mode), with IC_{50} of 0.4 and 0.7 μg/mL, better values than the control allopurinol ($IC_{50} = 1.1\,\mu$g/mL). In addition, *in vitro* cytotoxicity studies on healthy 3T3 fibroblast cells demonstrated IC_{50} values of 7.1 and 6.2 μg/mL for these two compounds, respectively [35].

Complexes resulting from the combination of ferulic acid, a known XO inhibitor, and 3-aminopyrazole, a molecule with anti-inflammatory effects, and copper or zinc (Figure 10) were prepared by Li et al. These two compounds exhibited inhibitory effects of XO activity in mouse liver homogenates, with better results being observed for the copper complex. In addition, studies in hyperuricemia model mice revealed that the elevated levels of blood UA could be decreased by both complexes (10 mg/kg,

Figure 10: Structures of metal complexes with XO inhibitory activity and decreasing effect of serum UA levels.

intraperitoneally injected) and that their effect is similar to the observed with febuxostat. Furthermore, it was evidenced that these complexes had no relevant effect on serum creatinine values, indicative of potential low renal side effects [36].

3.2. Natural and Semisynthetic Compounds and Mimetics of Natural Structures

3.2.1. Phenolic Compounds and Analogues

(1) Simple Phenolic Compounds. Aiming to develop a novel XO inhibitor with a potent activity and low toxicity, Lü et al. described a series of natural catechols and analogues. Of these, 3,4-dihydroxy-5-nitrobenzaldehyde (DHNB) (Figure 11), structurally similar to protocatechuic aldehyde, demonstrated to be the most potent XO inhibitor, with an IC_{50} value of 3 μM, acting by a mixed-type inhibition. In addition, DHNB effectively reduced serum UA levels after oral administration in allantoxanamide-induced hyperuricemic mice at a dose of 100 mg/kg. Nevertheless, *in vivo* toxicity studies demonstrated that a large oral dose of 500 mg/kg of DHNB did not lead to any evident side effects, in contrast with 42% of death in mice treated with the same dose of allopurinol [37]. The hypouricemic effect of 2,5-dihydroxyacetophenone (Figure 11), a compound computationally screened from *Ganoderma applanatum* was explored by Liang et al. For this, the *in vitro* and *in vivo* XO inhibitory activity was studied, evidence that this compound is an inhibitor of this enzyme (*in vitro* $IC_{50} = 8.12\,\mu$M). In hyperuricemic mice, it was also demonstrated that after oral administration of this phenolic compound (20, 40, and 80 mg/kg) serum UA was markedly reduced and that blood nitrogen and creatinine levels were lower than that observed in hyperuricemic and allopurinol controls. By means of RT-PCR and Western blot, further studies on the mechanism of action were performed, and it was verified that RNA and protein expressions of OAT1 (organic anion transporter 1) were upregulated and that the expressions of GLUT9 (glucose transporter 9), URAT1 (uric acid transporter 1), and CNT2 (gastrointestinal concentrative nucleoside transporter 2) were downregulated [38].

Hydroxytyrosol (Figure 11) is another natural catecholic compound that was studied by Wan et al. against XO by *in vivo* animal model and *in vitro* inhibition assay. Interestingly, hydroxytyrosol has XO inhibitory activity, with a determined IC_{50} value of 8.75 mM. This molecule can also

Figure 11: Structures of simple phenolic compounds with XO inhibitory activity and decreasing effect of serum UA levels.

Figure 12: Structures of phenolic acids with XO inhibitory activity and decreasing effect of serum UA levels.

decrease serum UA levels and adjust the mRNA transcription levels of UA transporter genes. In fact, this compound reduced reabsorption transporter genes mRNA and increased secretion transporter genes mRNA to the normal [39].

(2) Phenolic Acids and Derivatives, Including Salvianolic Acids. Several phenolic acids and derivatives were studied as XO and cyclooxygenase-2 inhibitors by Nile et al. According to these authors, all compounds showed good inhibition of XO activity. The most interesting IC_{50} values were found for sinapic acid and propyl and stearyl gallate (Figure 12). In addition, generally, they did not show significant *in vitro* cytotoxicity at 10 μM except stearyl gallate; however, many of these ferulic acid derivatives revealed relevant toxicity at higher concentrations (50 μM). Furthermore, interesting anti-inflammatory action was also observed for some of these compounds [40]. Caffeic acid (Figure 12), a known antioxidant belonging to the family of cinnamic acids and being present in numerous plants, also has XO inhibitory properties. Recently, Wan et al. evaluated its reducing effects on plasmatic UA levels in hyperuricemia rats and explored potential mechanisms of action in this context. Interestingly, it was demonstrated that serum UA levels were reduced after intragastric administration of caffeic acid (100 mg/kg) to hyperuricemia rats. In addition, blood urea nitrogen and serum creatinine levels decreased as well as *in vivo* XO and adenosine deaminase activities. Moreover, *in vitro* assays confirmed the XO inhibitory properties of this phenolic acid ($IC_{50} = 53.45\,\mu$M) and evidenced that it is a competitive inhibitor of this enzyme, which suffers structural changes (e.g. reduction of α-helix content) in the presence of this compound. Furthermore, it also acts by regulating the mRNA transcription of the renal uric acid transporters. In fact, the transcription levels of URAT1 and GLUT9 mRNA significantly increased, and the transcription levels of OAT1, UAT, and ABCG2 mRNA were significantly reduced than that observed in the blank group [41].

Verbascoside, a cinnamate ester glycoside (Figure 13), also has *in vitro* inhibitory effect on XO ($IC_{50} = 81.11$ mg/mL). Additionally, a dose of 54 mg/kg of verbascoside in a hyperuricemic *in vivo* model could reduce serum UA to normal levels [42]. 1,2,3,4,6-Penta-O-galloyl-β-D-glucopyranose, another glycoside derivative, demonstrated *in vitro* inhibition of XO activity ($IC_{50} = 2.8\,\mu$M) in a non-competitive manner ($K_i = 3.1\,\mu$M) with a potency closely to the observed with allopurinol ($IC_{50} = 2.3\,\mu$M). Serum UA levels on hyperuricemic mice at a dose of 40 mg/kg were also lowered [43].

Figure 13: Structures of verbascoside and 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose.

A combination of *in silico*, *in vitro*, and *in vivo* studies aiming to explore the interaction between ten representative chemicals identified in *Salvia miltiorrhiza* and XO were applied by Tang et al. Of these compounds, salvianolic acid C (Figure 14) proved to be the most potent *in vitro* XO inhibitor ($IC_{50} = 8.79\,\mu$M), acting by a mix-competitive manner. *In vivo* studies demonstrated a dose-dependent hypouricemic action of this compound in potassium oxonate–induced mice. Interestingly, it was observed that salvianolic acid C and allopurinol exhibit comparable *in vitro* XO inhibitory activity, but the hypouricemic effect of salvianolic acid C was lower [44]. Although with lower potency, salvianolic acid A (Figure 14) was also described as *in vitro* XO inhibitor, with an IC_{50} value of 73.17 μM, and also led to a decrease UA levels in acute myocardial infarction rats [45, 46].

Thang et al. designed and synthesized a new series of 2-arylbenzo[b]furan derivatives (Figure 14), considering the structure and known bioactivity of salvianolic acid C. Most of these compounds exhibited potent *in vitro* XO inhibitory effect, particularly (E)-3-(2-(3,4-dihydroxyphenyl)-7-hydroxybenzofuran-4-yl)-N-methylacrylamide ($IC_{50} = 4.45\,\mu$M; IC_{50} of allopurinol = 10.61 μM), which induced a mixed-type XO inhibition with a K_i of 3.5 μM. Additionally, this compound (Figure 14) exhibit a dose-dependent hypouricemic action in potassium oxonate–induced hyperuricemic mice [47].

(3) Hydroxylated Chalcones, 6-Shogaol, Curcumin, and Analogues. The chalcone scaffold, found in many naturally occurring compounds, have a wide range of bioactive properties and have also been prepared by synthesis [48, 49]. As an example, Li and co-workers [50–52] prepared several chalcones and evaluated their XO inhibitory activity. Of these, 3,5,2′,4′-tetrahydroxychalcone (Figure 15) displayed

Figure 14: Structures of salvianolic acids C A and C analogues.

Figure 15: Structures of hydroxychalcones with XO inhibitory activity and decreasing effect of serum UA levels.

an interesting XO inhibition in a competitive manner, and IC_{50} and K_i values of 22.5 and 17.4 μM, respectively, were determined [52]. In addition, *in vivo* intragastric administration of this chalcone significantly reduced serum UA levels in hyperuricemic mice in a dose-dependent manner. The best effect occurred with doses between 2 and 4 mg/kg [50–52]. Studies performed by the authors suggested that this chalcone act by a dual mechanism [50]. In fact, this *in vivo* hypouricemic action can be associated not only with the inhibition of key enzymes (XO, PRPS, PRPPAT, and HGPRT) in the purine metabolism but also with an enhancement in UA excretion by inhibiting the expression of GLUT9 in the kidney [50, 51]. Finally, an acute toxicity study in mice showed that 3,5,2′,4′-tetrahydroxychalcone was safe at a dose up to 5 g/kg, making it suitable for future studies [52]. Other hydroxylated chalcones prepared by a Claisen–Schmidt condensation were described by Hofmann et al., and IC_{50} values between 1.2 and 93 μM as XO inhibitors were determined. Cytotoxicity tests revealed that the most active of these chalcones is noncytotoxic. However, further structure modifications on the chalcone scaffold are needed to optimize the XO inhibitory activity [53]. Later, Xie et al. described a synthetic series of hydroxychalcones (Figure 15) with moderate XO inhibition, and *in vitro* IC_{50} values of 47.3 and 56.8 μM were calculated for the most potent ones. These hydroxychalcones, which act by a mixed XO inhibition type, were tested *in vivo* in potassium oxonate–induced hyperuricemic mice (10 and 50 mg/kg, intragastrically administered). The results demonstrated that they led to a significant serum UA and XO activity reduction in both cases, with the highest significance at 50 mg/kg. Acute toxicity *in vivo* studies revealed no evident toxicity at doses up to 5 g/kg [54].

The XO inhibition in a mixed manner by isoliquiritigenin (Figure 15) was initially described by Cheng and coworkers (IC_{50} and K_i values of 55.8 and 17.4 μM, respectively) [55]. However, its poor absolute *in vivo* bioavailability was later reported [56]. In order to change that, Zang et al. developed an isoliquiritigenin-loaded self-microemulsifying drug delivery system. In fact, the *in vivo* oral bioavailability was enhanced in about 4.7 times, with an improvement in plasmatic concentrations. Additionally, the use of self-microemulsification proved to significantly improve the antihyperuricemic effect of isoliquiritigenin in rats. At the administered doses of 50–150 mg/kg, a reduction in serum UA levels from 25.2–51.9 to 41.7–78.8% for free drug and its drug delivery system was, respectively, demonstrated [57].

A study conducted by Peng et al. demonstrated that phenolic compounds isolated from ginger inhibit the XO activity at the concentration of 125 μM. 6-Shogaol (Figure 16), one of these phenols, also presented IC_{50} values of relative cell proliferation between 7.4 and 100.0 μM in the different tested cancer cell lines [58]. Later, Nile and Park reported again the XO inhibition by 6-shogaol, with an IC_{50} value of 15.2 μM *versus* 8.4 μM of allopurinol [59]. Due to the poor water solubility of 6-shogaol, Yu, Xu, and coworkers developed 6-shogaol-loaded solid lipid nanoparticles [60] and a 6-shogaol-loaded self-microemulsifying drug delivery system [61] to enhance its oral bioavailability. In fact, the nanoencapsulation led to a significant improvement of *in vivo* oral absorption, bioavailability, and acting time of 6-shogaol in healthy rats. 6-Shogaol–loaded solid lipid nanoparticles also could lower the serum UA levels in a higher extension than free drug in hyperuricemia/gouty arthritis rats at a 120 mg/kg dose. In addition, loaded solid lipid nanoparticles allowed an improvement in the organ protection effects of 6-shogaol [60]. The use of a 6-shogaol–loaded self-microemulsifying drug delivery system led to an increment of 571.18% in the oral bioavailability when compared with the administration of the free drug. In addition, an increment in the reduction of serum UA levels in hyperuricemic rats treated with 25, 50, and 100 mg/kg of dose was observed. As example, the highest dose (10 mg/Kg) led to a reduction in UA levels by 71.1% by the microemulsification and 60.0% by free drug. The 6-shogaol–loaded self-microemulsifying drug delivery system was also pointed as promising to reduce the kidney damage caused by hyperuricemia model [61].

Curcumin (Figure 17) was described as a XO inhibitor, with IC_{50} of 117.3 μM (IC_{50} of allopurinol = 28.9 μM) by Peng et al. [58]. Later, Chen et al. reported its *in vivo* activity on potassium oxonate–induced hyperuricemia mice. Oral administration in a dose of 20 or 40 mg/kg leads to a decrease of serum UA and an effective inhibition of serum and liver XO levels and to a reduction of kidney inflammation by NLRP3 inflammasome suppression [62]. α,β-Unsaturated cyclohexanone and cyclopentanone analogues of curcumin (Figure 17) were described as potential antihyperuricemia agents, acting by dual inhibition of XO and URAT1 by Ao et al. The most promising compound (Figure 17) with *in vitro* dual inhibition also demonstrated to reduce serum UA

6-shogaol

Figure 16: Structure of 6-shogaol.

Curcumin

Curcumin derivatives, by Ao et al. $n = 1$, $R = 4$-Br

Figure 17: Structure of curcumin and analogues with XO inhibitory activity and decreasing effect of serum UA levels.

levels in a hyperuricemic *in vivo* model. Interestingly, at the doses of 10 and 20 mg/kg, this curcumin derivative increased the urinary UA excretion and decreased the serum and hepatic UA levels as well as XO activity and URAT1 protein levels. According to these authors, this compound is a first-in-class of dual inhibitors and may serve as a reference compound for further design of antihyperuricemic drugs [63].

(4) Flavonoids and Analogues. Natural plant flavonoids have always been molecules of great interest [64]. Their hypouricemic action in mice was also evidenced [65] as well as of some semisynthesized derivatives [66]. In fact, the flavonoids luteolin, apigenin, diosmetin, chrysin, $O^{3'}$,O^{7}-dihexyl diosmetin, $O^{4'}$,O^{7}-dihexyl apigenin, and O^{7}-hexyl chrysin (Figure 18) showed XO inhibition, with IC_{50} values between 4.5 and 8.1 μg/mL. In addition, anti-inflammatory action and a relatively low cytotoxicity were found for these flavonoids [66]. Other series of apigenin flavonoid derivatives have been prepared by Su et al. by a coupling of the carboxyl alkyl group to 4′-, 5- or 7-hydroxyl groups of apigenin. The 4′-modified derivatives (Figure 18) were demonstrated to be more potent than apigenin in XO inhibition, with IC_{50} values between 0.098 and 0.82 μM, versus 3.2 μM. The insertion of a carboxyl *n*-hexyl group in this position (Figure 18) led to the best *in vitro* results. This molecule was evaluated for its hypouricemic effects on the potassium oxonate–induced hyperuricemic mouse model. Interestingly, the serum UA levels were significantly decreased by an intraperitoneal dose of 10 mg/kg, with no significant effects being observed with apigenin. Nevertheless, the *in vitro* results presented higher XO inhibition for apigenin derivatives than allopurinol, but *in vivo* tests did not confirm these results. The authors pointed to the poor bioavailability or short half-life of the compound as the cause of this reduced activity [67].

Four flavones with XO inhibitory activity were described by Metoui et al. from the Tunisian *Artemisia campestris* leaves. 2′,4′,5,7-Tetrahydroxy-5′,6-dimethoxyflavone, eupatilin, dimethoxycentaureidin, and cirsiliol (Figure 18) exhibited higher potency for XO inhibition than allopurinol ($IC_{50} = 8.2\,\mu$M), with IC_{50} values of 5.5, 3.3, 6.8, and 5.5 μM, respectively. In addition, these four flavones were *in vitro* tested against four cancer cell lines at a concentration of 15 μM to verify their cytotoxic activity. A proliferation value below or close to 50% was found for all tested flavones at this single concentration [68].

Luteolin (Figure 18) has been reported to have a significant XO inhibitory activity in comparison with allopurinol, with an IC_{50} value of 4.8 μM [69]. An inhibition effect assay performed by Dong et al. evidenced that luteolin and a luteolin–manganese(II) complex (Figure 18) reversibly inhibited XO in a competitive manner. Interestingly, the complex had a more remarkable hypouricemic effect than luteolin and both compounds proved to be noncytotoxic to a human liver cancer cell line [70]. A glycosylated derivative, luteolin-4′-glucoside (Figure 18), also inhibited XO, with an *in vitro* IC_{50} value of 0.26 μg/mL [71]. In addition, this molecule had potent uricosuric effects in hyperuricemic mice with renal mURAT1 and decreasing XO activity, as well as anti-inflammatory effects. Furthermore, it was noted that, at the same concentration of luteolin and luteolin-4′-O-glucoside, a better therapeutic effect was observed for luteolin [72]. 6-Hydroxyluteolin (Figure 18) was also described as an inhibitor of XO activity, with an IC_{50} value of 7.52 μM [73]. *In vivo* studies evidenced that this flavonol led to a significant dose-dependent reduction on the serum UA level of hyperuricemic rats at doses of 0.05, 0.1, and 0.3 mmol/kg. It was also demonstrated significant inhibition of rat liver XO (about 80%) in the highest dose [74].

de Araújo et al. described the improvement of *in vitro* XO inhibitory activity after enzymatic deglycosylation of rutin (Figure 18). However, this hydrolysis also leads to an increase in the cytotoxicity of this flavonoid against several tested cell lines. The total deglycosylated derivative, quercetin (Figure 18), was demonstrated to be the most potent XO inhibitor and had intermediary cytotoxicity [75]. In this context, it was previously demonstrated that quercetin has high XO inhibitory effects, with an IC_{50} value of 1.9 μM [76]. Glycosylated anthocyanins from purple sweet potato [77–80] as well as other glycosylated flavonoids and coumaroylspermidines from rape bee pollen [81] were described by Zhang research group as good XO inhibitors. In fact, these authors demonstrated that these compounds, particularly highly acylated anthocyanins, can reduce the kidney inflammation in hyperuricemic mice in addition to their hypouricemic effect [77, 80].

The flavonoids hesperetin (Figure 18), hesperidin, and G-hesperidin were also tested as XO inhibitors by De Souza et al. These authors demonstrated that hesperetin has more potent *in vitro* XO inhibitory activity than the glycosylated derivatives (hesperidin and G-hesperidin). This compound acted by a competitive inhibition mode, and IC_{50} and K_i values of 53 and 17 μM, respectively, were determined. In addition, *in vivo* studies demonstrated that monoglucuronides of hesperetin were the major forms present in plasma after the ingestion of this flavonoid [82].

(−)-Epigallocatechin-3-gallate (EGCG) (Figure 18), important catechin of green tea, was evaluated as XO inhibitor, with a determined IC_{50} of 12.5 μM by Lin et al. These

Figure 18: Structures of flavonoids with XO inhibitory activity and decreasing effect of serum UA levels.

authors described that EGCG may act as "suicide substrate," similarly to allopurinol [83]. More recently, Zhu et al. demonstrated that EGCG displayed inhibitory effects on hepatic XO at the dose of 50 mg/kg. In addition, this compound had a significant dose-dependent effect on lowering serum UA levels and in the regulation of GLUT9 and URAT1 mRNA expression levels in hyperuricemic mice, when compared with the model group. Furthermore, this molecule had effective renal protective effects in the prevention of glomeruli and kidney tubules damages, as evidenced by the analysis of histopathologic sections of hyperuricemic mice [84].

Two series of benzoflavone derivatives (Figure 19) were designed, synthesized, and evaluated for their XO inhibitory potential by Singh et al. Some of these derivatives had significant *in vitro* XO inhibition, with IC_{50} values lower than 10 μM. Among the series, the most potent compounds on *in vitro* studies were 7,8- and 5,6-benzoflavones

7,8- and 5,6-benzoflavone derivatives,
by Singh et al.
R_1 = 4-F or 2-F
R_2 = 4-NO_2, 4-Br or 3,5-F

Figure 19: Benzoflavone derivatives with XO inhibitory activity and decreasing effect of serum UA levels.

(E)-2-(4-bromophenyl)-1-(2,4-dihydroxyphenyl)ethanone oxime (BDEO)

Benzoxazole deoxybenzoin oxime derivatives, by Huang et al.
R_1 = 6-OCH_3, R_2 = 4-OCH_3

Figure 20: Structures of oxime analogues of flavonoids with XO inhibitory activity and decreasing effect of serum UA levels.

(Figure 19), which demonstrated IC_{50} values of 0.6 and 5.2 μM, respectively. These derivatives act as mixed-type inhibitors and has a higher potency than allopurinol (IC_{50} = 8.7 μM). Of these two compounds, *in vivo* studies demonstrated that only the 5,6-benzoflavone derivative presented the ability to reduce the serum UA to its normal level at a dose of 10 mg/kg. Interestingly, the authors explored six other derivatives on *in vivo* studies that evidenced the potential of at least three compounds as clinical candidates for the treatment of hyperuricemia. These compounds, which presented *in vitro* IC_{50} values of 4.9, 8.9, and 7.7 μM, showed to be able to reduce *in vivo* serum UA to values at a level comparable to that observed with febuxostat (dose of 5 mg/kg). Additionally, two of these benzoflavones did not led to behavioral abnormality on *in vivo* acute toxicity study [85].

(E)-2-(4-Bromophenyl)-1-(2,4-dihydroxyphenyl)ethanone oxime (BDEO) (Figure 20), a novel compound with a chemical structure similar to flavonoids, was described as an inhibitor of XO activity, with an IC_{50} value of 3.33 μM. Its UA uptake via URAT1 was evaluated *in vitro*, and the results demonstrated that this absorption was effectively inhibited. According to this study, BDEO effectively decreased hepatic XO activity and downregulated renal URAT1 protein expression in hyperuricemic mice, especially at doses of 10 and 20 mg/kg. Nevertheless, those indicators were not changed by BDEO in normal mice when compared with allopurinol and benzbromarone, reflecting its marked advantages in treating hyperuricemia and predicting its safety. Therefore, the authors suggested that BDEO may serve as a dual XO and URAT1 inhibitor for the treatment of hyperuricemia [86]. Considering the structure of flavonoids and synthetic intermediates for their preparation with antihyperuricemia effects, XO inhibition and also immune-regulating actions, several benzoxazole analogues of these compounds (Figure 20) were developed aiming to improve these activities. Specifically, two series of benzoxazole deoxybenzoin oxime derivatives were prepared as dual inhibitors of innate immune sensors and XO. *In vitro* studies evidenced that the majority of compounds suppressed XO activity, and the best result was observed with compound (E)-1-(6-methoxybenzo[d]oxazol-2-yl)-2-(4-methoxyphenyl) ethanone oxime (IC_{50} = 3.7 μM; IC_{50} for allopurinol = 2.9 μM). Enzyme kinetics studies demonstrated that this compound (Figure 20) acted as a competitive-type XO inhibitor. Using mice with oxonate-induced hyperuricemia, this compound reduced serum UA levels in a dose-dependent manner, consistent with *in vitro* studies. In addition, it was demonstrated that this benzoxazole has higher safety than allopurinol in normal mice and that it has antiacute gouty arthritis effect *in vivo* [87].

From a set of compounds with a chemical structure similar to flavonoids, 4-(2-(4-chlorophenyl)-1-((4-chlorophenyl)amino)ethyl)benzene-1,3-diol (CBED) (Figure 21) was found to be a dual XO and NLRP3 inhibitor after molecular docking studies by Liu and coworkers. *In vitro* results for XO inhibitory activity demonstrated an IC_{50} value of 3.9 μM, close to that obtained for allopurinol (IC_{50} = 2.1 μM). On the other hand, *in vivo* results demonstrated a reduction in serum UA levels at the dose of 20 mg/kg. Additionally, a remarkably suppression of NLRP3 inflammasome activation and regulation on hepatic XO activity were observed. Thus and according to the authors, CBED can lead not only to a hypouricemic effect but also to a reduction in kidney inflammation caused by high UA levels [88].

(5) Phenolic Coumarin and Xanthone Derivatives. To understand structure-activity relationships on a series of coumarin derivatives, Fais et al. prepared a series of twenty 3-arylcoumarins and eight 3-heteroarylcoumarins. Of these, 5,7-dihydroxy-3-(3′-hydroxyphenyl)coumarin (Figure 22) was proved to be the best XO inhibitor with a determined IC_{50} value 7-fold better than that observed for allopurinol (IC_{50} of 2.1 and 14.7 μM, respectively). *In vitro* kinetics studies revealed an uncompetitive inhibition mode and a K_i value of 0.4 μM. Finally, studies on 3T3 normal fibroblasts revealed no cytotoxic effect caused by this compound [89].

Norathyriol (Figure 22), a xanthone analogue, was described by Lin et al. as an uncompetitive concentration-dependent XO inhibitor, with IC_{50} of 7.8 μM, close to that observed for allopurinol (IC_{50} = 76.3 μM). The uncompetitive inhibition was appointed by the authors as a beneficial point in comparison with the competitive or mixed-type inhibition of allopurinol and febuxostat, respectively. In addition, an intragastric dose of 2.0 mg/kg of norathyriol was enough to reduce the serum UA levels in hyperuricemic mice to the normal values of healthy mice. *In vivo* results demonstrated also that norathyriol acted not only by XO inhibition but also by OAT1 activation [90]. Qin et al. demonstrated for the first time that the norathyriol 3-benzyloxy derivative J99745 (Figure 22) presented an *in vitro* XO inhibition, with a determined IC_{50} value of 3.297 μM. Experiments involving hyperuricemia mice showed that

4-(2-(4-chlorophenyl)-1-((4-chlorophenyl)amino)ethyl)benzene-1,3-diol (CBED)

Figure 21: Structure of 4-(2-(4-chlorophenyl)-1-((4-chlorophenyl) amino)ethyl)benzene-1,3-diol (CBED).

Figure 22: Structure of phenolic coumarin and of xanthone derivatives with XO inhibitory activity.

J99745 at doses of 10 and 30 mg/kg significantly reduced serum urate levels, enhanced UA excretion, and provided higher nephroprotective effects than allopurinol. These authors suggested that this molecule exerts a urate-lowering effect by the inhibition of XO activity and a decrease in URAT1 expression [91].

3.2.2. Terpenes and Dioscin. Lin et al. isolated a triterpenoid and an alkamide from *Ganoderma tsuga* and prepared four terpenoid derivatives by semisynthesis from 3-oxo-5α-lanosta-8,24-dien-21-oic acid (Figure 23). In addition, these compounds were evaluated as XO inhibitors, antioxidants, and antiproliferative agents against human prostatic cancer cells. Of these, two compounds with interesting XO inhibition (IC_{50} values of 313.3 and 43.9 μM) in a concentration-dependent manner were identified. The compound with the best activity (3-oxo-5α-lanosta-8-en-21-oic acid) (Figure 23), prepared by semisynthesis, was as a mixed inhibitor of XO, with a K_i value of 3.2 μM. The authors also reported the cytotoxicity of this compound against prostatic cancer cells, with an IC_{50} value of 23.9 μM being determined [92]. In previous works of this research group, the lanostanoid used as starting material had already been isolated from *Ganoderma tsugae* [92, 93]. In addition, five another lanostanoids were isolated and tested as XO inhibitors and as DPPH scavengers, and their cytotoxicity was evaluated against prostatic cancer cells and keratinocytes. Of these, three compounds inhibited the XO activity in a concentration-dependent manner with IC_{50} values of 90.2, 116.1, and 181.8 μM. The compound with the highest XO inhibition (3α-acetoxy-22-oxo-5α-lanosta-8,24-dien-21-oic acid) (Figure 23) is a mixed inhibitor with Ki of 0.6 μM. However, this compound also presented the most interesting result in human prostatic cancer cells, also enhancing the cytotoxicity induced by cisplatin [93]. Since a long time, the glycoside dioscin (Figure 23) is known as a weak XO inhibitor with an IC_{50} value of 115 μM [94]. Recently, it was evidenced that it has pronounced anti-hyperuricemic effects in mice by reducing serum UA levels over 4 h at 25 and 50 mg/kg of oral dose [95, 96]. This activity can be explained by the regulation of levels of mOAT1, mURAT1, mOCT2, and GLUT-9 expressions in the kidney [95, 96].

Ursolic acid (Figure 23), a bioactive pentacyclic triterpene extracted from the *Tribulus arabicus* hexane fraction, was found to be a potent XO inhibitor. The authors described an *in vitro* IC_{50} value of 10.3 μg/mL for the inhibition of XO activity (IC_{50} = 6.5 μg/mL for allopurinol). In addition, this *in vitro* activity was confirmed *in vivo* on mice with potassium oxonate–induced hyperuricemia. Results demonstrated an effective hypouricemic effect at a dose of 5 and 10 mg/kg with the reduction of UA levels in 56.1 and 79.9%, respectively [97]. Given its potential as a hypouricemic agent, ursolic acid is already patented in China for gout treatment [98]. In a recent study on triterpenic acids from apple pomace by Kai and coworkers, an *in vitro* cytotoxicity with IC_{50} values between 5.6 and 20.8 μg/mL in four cancer cell lines was described for ursolic acid. From this natural source, other compounds were isolated and tested against XO. In fact, beyond the most active betulinic acid (IC_{50} = 12.6 μg/mL), oleanolic acid (IC_{50} = 17.8 μg/mL), maslinic acid (IC_{50} = 21.5 μg/mL), erythrodiol (IC_{50} = 25.8 μg/mL), and uvaol (IC_{50} = 30.2 μg/mL) were also described (Figure 23). Nevertheless, all the described triterpenic acids did not show higher activity than allopurinol (IC_{50} = 9.6 μg/mL). By means of kinetic studies, a non-competitive type of inhibition for betulinic acid was revealed. Additionally, *in vitro* cytotoxic effects on four cancer cell lines and IC_{50} values from 6.2 to 40.1 μg/mL were obtained [99].

3.2.3. Other Natural and Semisynthetic Compounds. α-Lipoic acid (Figure 24) was found by Hameed and Ramadhan to be a potent XO inhibitor, with an IC_{50} value of 2.9 μg/mL when compared with 1.7 μg/mL of allopurinol. *In vivo* results on potassium oxonate–induced hyperuricemic mice demonstrated that a 10 mg/kg dose of α-lipoic acid and allopurinol led to a serum UA reduction from 4.4 mg/dL to 2.3 and 1.7 mg/dL, respectively [100].

The alkaloids from *Alphonsea elliptica* barks atherospermidine (IC_{50} = 46.3 μg/mL), liriodenine (IC_{50} = 7.7 μg/mL), *N*-methylouregidione (IC_{50} = 42.1 μg/mL), and kinabaline (IC_{50} = 50.7 μg/mL) (Figure 25) were described as possessing moderate inhibition of XO activity by Aldulaimi et al. In addition, the cytotoxicity on MCF-7 human breast cancer cells was evaluated, with determined IC_{50} values greater than 62.0 μg/mL [101].

The effect of lobetyolin (Figure 26) on XO inhibition was presented by Yoon and Cho. Even though a weak *in vitro* mixed XO inhibition (IC_{50} = 2985 μM), an *in vivo* significant hepatic XO activity reduction at a dose of 50 mg/kg was observed [102].

Figure 23: Terpenes and dioscin with XO inhibitory activity and decreasing effect of serum UA levels.

Figure 24: Structure of α-lipoic acid.

Figure 25: Structures of alkaloids with XO inhibitory activity.

Lobetyolin

Figure 26: Structure of lobetyolin.

Taurin

Peptide RDP-1

Figure 27: Structures of the amino acid taurine and of the peptide RDP-1.

In the field of small natural molecules, Feng et al. demonstrated that the administration of 1% taurine (Figure 27), a sulfur-containing semiessential amino acid, in drinking water could efficiently ameliorate kidney injury and decrease uric acid levels by regulating uric acid formation and excretion in hyperuricemic rats [103]. The significant inhibition of XO activity by taurine was already been evidenced [104].

A new peptide, RDP-1 (Figure 27), was isolated from the extract of shelled fruits of *Oryza sativa* and structurally characterized. *In vivo* biological evaluation evidenced that after intragastric administration, this compound (10 μg/Kg) reduced hyperuricemia induced by potassium oxonate in rats. In addition, a reduction of creatinine levels and an ameliorating effect on hyperuricemic nephropathy was observed. It was also demonstrated that this peptide inhibited XO *in vitro* and *in vivo*, probably by occupying the binding site to xanthine (docking studies). Furthermore, no acute toxicity in rats was observed, and authors also evidenced that RDP-1 was stable in several temperatures [105].

4. Conclusions

Given the relevance of XO as a validated target to reduce increased serum UA levels and also due to the fact that the commercialized drugs with XO inhibitory effects can have serious side effects, in recent years a large number of new alternative XO inhibitors were described. Despite the fact that the majority of tested products with relevant *in vitro* XO inhibition do not advance for further pharmacological evaluation, it is clear in this review that a large number of compounds are being explored not only in *in vitro* studies but also in *in vivo* biological evaluation, and very promising molecules can be recognized and used in future studies. It is also possible to conclude that the majority of compounds being explored in more advanced biological evaluation studies are natural structures or their mimetics and semi-synthetic derivatives. In addition, it is clear that frequently the observed *in vitro* XO inhibition results do not have a clear correspondence with the *in vivo* hypouricemic effects. This fact evidences the relevance of performing *in vivo* biological evaluation of the efficacy of the molecules in addition to the *in vitro* XO inhibition studies and access, as far as possible, other possible mechanisms of action, as well as the pharmacokinetics and toxicity profile of the molecules under study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Beneficial Effects of Macroporous Resin Extract of *Dendrobium candidum* Leaves in Rats with Hyperuricemia Induced by a High-Purine Diet

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Objectives. The incidence of hyperuricemia (HUA) is increasing year by year, and there are no ideal drugs for the treatment; the existing ones can cause serious liver and kidney damage. We have confirmed that the water extract of *Dendrobium candidum* leaves could reduce the level of uric acid in rats, but the active ingredients remain unknown, and the mechanism is not well understood. This research investigated the therapeutic effect of the macroporous resin extract of the *Dendrobium candidum* leaf (DLE) on hyperuricemia. In this study, hyperuricemia was induced in rats by a 5-week high-purine diet. After that, DLE was administered continuously for 9 weeks. The result showed that biochemical parameters of liver and kidney function, especially serum uric acid (UA) levels, were significantly improved with DLE, which may relate to the reduction of xanthine oxidase (XOD) and adenosine deaminase (ADA) in the liver. Moreover, DLE could significantly prevent kidney and liver from damage, and intestinal injury and reduce inflammation in hyperuricemic rats by inhibiting the expression of both NF-κB and TLR4 proteins. These results showed that the macroporous resin extract of the *Dendrobium candidum* leaves may be effective for the treatment of hyperuricemia in rats by inhibiting uric acid production and decreasing inflammation.

1. Introduction

Hyperuricemia (HUA) is a congenital or acquired disease that reduces uric acid excretion, causing an increase in serum uric acid (UA) [1]. The international diagnostic criteria for HUA is that on a normal purine diet after fasting for 2 days, the value of serum UA in men is above 420 μmol/L (7.0 mg/dL) and higher than 357 μmol/L (6.0 mg/dL) in women [2]. A large number of epidemiological and clinical studies have shown that HUA is not only a single metabolic disease but also closely related to many diseases such as hypertension [3], hyperlipidemia, diabetes [4], coronary heart disease [5], arteriosclerosis [6], and obesity. In recent years, with the improvement of living standards, irregular diet, and excessive intake of fat, the prevalence of HUA in China has increased by about 10 times, 7.3% to 58.4% for men and 1.3% to 23.8% for women [7].

The pathogenesis of HUA is complicated. Any factor causing uricogenesis or reduced excretion could lead to HUA [8]. The liver is the main organ for de novo purine synthesis and production of uric acid [9]. Uric acid is the final product of purine catabolism, and a large amount of purine catabolic enzymes is needed during the process. Generally, these enzymes fall into two categories: one is enzymes for uric acid synthesis promotion, including xanthine oxidase (XOD), adenosine deaminase (ADA),

guanine deaminase (GDA), and 5-phosphate ribose-1-pyrophosphate synthase (PRS); the other is enzymes inhibiting uric acid synthesis, such as hypoxanthine guanine phosphoribosyl transferase (HGPRT) [10, 11]. Among them, the XOD/ADA system produces a large amount of reactive oxygen species (ROS) while producing uric acid, causing further inflammation of liver and kidney [12]. Uric acid produces a large amount of active oxygen during the synthesis process. Reactive oxygen species not only cause liver and kidney damage but also can be activated by the toll-like receptor 4 (TLR4)/NF-κB signaling pathway, altering the microenvironment of membrane receptors, proteases, and ion channels, leading to increased uric acid production [13].

Drugs such as allopurinol and benzbromarone have long been used to treat hyperuricemia [14]. However, these drugs have long-term adverse effects. Hepatitis and renal function deterioration can occur after long-term use of allopurinol [15].

Dendrobium officinale Kimura et Migo belongs to Dendrobium Sw, Orchidaceae and was first described in the Shennong Bencao, a Chinese book on agricultural and medicinal plants published around the 2nd century CE [16]. The Chinese Pharmacopoeia stipulates that the medicinal part of *Dendrobium candidum* is the stem, and the stem is usually processed into a spiral product. But its leaves are often overlooked, and a large number of *Dendrobium candidum* leaves are wasted every year [17]. At present, the research on *Dendrobium candidum* is concentrated on the stem, and little research on the leaf is being carried out. In fact, *Dendrobium candidum* leaves have broad potential for therapeutic use. For example, *Dendrobium candidum* leaves have a good adjuvant effect on hypertension [18], hyperlipidemia [19], inflammation [20], and the like. In our previous studies, we have confirmed that the water extract of *Dendrobium candidum* leaves could reduce the level of uric acid in rats. Although we have studied some of its components [21, 22], its active ingredients are still unknown, and the mechanism is not yet fully understood. To the best of our knowledge, there are no reports in the literature on the treatment of hyperuricemia with extracts of *Dendrobium candidum* leaves.

This study aimed to establish a model of hyperuricemia through a modified purine feed to study the pharmacodynamics of *Dendrobium candidum* leaves and explore the mechanism of antihyperuricemia through the XOD/ADA system and the TLR4/NF-κB inflammatory signaling pathway. The study may lay the foundation for the development of new drugs for hyperuricemia.

2. Materials and Methods

2.1. Materials and Reagents. Dendrobium candidum leaves was purchased from Yunnan Alpine Agriculture Co., Ltd (Yunnan, China). ADS-17 macroporous adsorption resin was purchased from Ganzhou Baoen Adsorption Material Technology Co., Ltd (Hebei, China). Reagent kits such as serum uric acid (UA), creatinine (Cr), urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and adenosine deaminase (ADA) were purchased from Ningbo Meikang Biotechnology Co., Ltd. (Zhejiang, China). Hematoxylin-eosin (HE) dye solution and 3,3′-Diaminobenzidine (DAB) chromogenic agent were purchased from Nanjing Jiancheng Technology Co., Ltd. (Jiangsu, China). Modified Masson and periodic acid-schiff (PAS) dye solution were purchased from Shanghai Yuanye Technology Co., Ltd. (Shanghai, China). Rabbit polyclonal antibodies nuclear factor-kappaB (NF-κB), and toll-like receptor 4 (TLR4) were purchased from Huaan Biotechnology Co., Ltd. (Zhejiang, China). HRP-conjugated goat antirabbit IgG was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China).

2.2. Macroporous Resin Extract of Dendrobium Candidum Leaves (DLE). Dendrobium candidum (DC) leaves are provided by Yunnan Alpine Agriculture Co., Ltd. The preparation of macroporous resin extract of *Dendrobium candidum* leaves (DLE) was done as we described previously (Figure 1) [23]. Briefly, dried *Dendrobium candidum* leaves were extracted three times with 75% ethanol aqueous solution (*v*/*v*), and then, all the leachates were collected and concentrated under vacuum to acquire the ethanol extract. The petroleum ether and *n*-butanol extraction were successively adopted for the ethanol extract redissolved in aqueous solution. The *n*-butanol fraction was redissolved in aqueous solution for macroporous resin purification. The impurities were first rinsed by deionized water and 10% ethanol aqueous solution (*v*/*v*); the 50% ethanol aqueous solution (*v*/*v*) eluent was collected and concentrated under vacuum to prepare the DLE for further animal experiments.

2.3. Animals and Experimental Design. Male Sprague Dawley (SD) rats (license number: SCXK 2014-0001) were purchased from Zhejiang Academy of Medical Sciences (Hangzhou China). The housing facility is keeping with the national standards principles of GB14925-2010 (Laboratory Animal-Requirement of Environment and Housing Facilities) for laboratory. The care and experimental operation were conforming to the rules of the "Zhejiang province Administration Rule of Laboratory Aminal."

SD rats in the model group were given modified high-purine diet (0.15% adenine, 10% yeast extract, and 89.85% standard diet), whereas the normal group was fed with fundamental feed on a daily basis for five weeks. The model group rats were randomly divided into 3 groups ($n = 10$), including model group, low-dose group (4.375 mg/kg body weight, DLE-L), high-dose group (17.5 mg/kg body weight, DLE-H), based on UA (data were not shown), and the high-purine diet was continued during the drug treatment, and potassium oxazinate was given by gavage for 1h before blood sample collection. Then, the corresponding drugs and dosage were given to rats by oral administration. All rats were permitted to drink regular water freely.

2.4. Determination of Liver and Kidney Function Biochemical Parameters. Blood samples were taken from the ophthalmic venous plexus during the experiment and were water bathed for 2 hours at 37°C and centrifuged at 3500 rpm for 10 min.

Figure 1: Preparation of macroporous resin extract of *Dendrobium candidum* leaves (DLE). (a) Flowchart for determination of macroporous resin extract. (b) The wavelength of maximum absorption of flavonoids. (c) The rutin standard curve.

The serum was separated to detect the biochemical indexes of UA, Cr, and BUN by an automated biochemical analyzer (Hitachi 7020). At the end of the experiment, the serum was also separated to detect the biochemical indexes of ALT and AST.

2.5. Determination of the Fractional Excretion of Uric Acid (FEUA%). At the 9th week of administration, each group were placed in a metabolic cage fasting for solids and liquids for 24 h, and the urines of each group were collected for FEUA determination; this method was described by Pang et al. [24]. Formula for calculating uric acid excretion score is as follows: FEUA% = uUA ∗ sCr/(sUA ∗ uCr) ∗ 100%. uUA and uCr, uric UA and Cr; sUA and sCr, serum UA and Cr.

2.6. Determination of XOD and ADA Activities in Serum and Liver. At the end of the experiment, the rats were fasted overnight and anesthetized with pentobarbital. The liver tissue was weighed, and a volume of saline solution was added and mixed at a ratio (*w/v*) of 1 : 9. Then, the liver samples were homogenized and centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were prepared for the assay. The levels of XOD activities in the liver and serum were estimated by rats enzyme-linked immunosorbent assay (ELISA) kits according to operating instructions. The levels of ADA activities in the liver and serum were detected by an automated biochemical analyzer (Hitachi 7020).

2.7. Histopathology Observation of Kidney, Liver, and Intestine. Meanwhile, kidney, liver, and intestine were put into 4% formalin for fixation. Then, the organs were cut into applicable slice and went through washing, dehydration, and embedded to make of tissue wax chunks (MEIKO EC360 Tissue Embedder, Germany). All the specimens were cut into 4 μm thickness (LEICARM2245 slicing machine, Germany) and stained by hematoxylin-eosin (H&E). In addition, the specimens of kidney thickness were also used for Masson's trichrome and periodic acid-schiff (PAS) staining. All staining was photographed with the biological microscope (BX43, Olympus, Japan) and analysed with the Image-Pro-Plus 6.0 image analysis software to estimate glycogen deposition, inflammation, and fibrosis lesion.

Semiquantitative analysis was conducted on the proliferation rate of mesangial matrix by PAS staining; kidney tissue of five rats was randomly selected from each group. The complete glomerulus of each slices was observed with a microscope. Statistical analysis was performed on the glomerular mesangial proliferation area/total glomerular area using Image-Pro Plus 6.0 image analysis software.

For the semiquantitative analysis on intestinal villus height (V) and crypt depth (C), kidney tissue of five rats were randomly selected from the ileum H & E-stained slices, and six flattened villi were randomly selected under microscope. Statistical analysis was performed on each group of V, C, and V/C using Image-Pro Plus 6.0 image analysis software.

2.8. Immunohistochemistry (IHC) Observation. The immunohistochemistry (IHC) staining was similar as we described previously. The kidney and liver tissues were dewaxed and dehydrated before blocking endogenous peroxidase activity with 0.5% H_2O_2 for 10 min. Nonspecific binding was blocked with 10% normal goat serum in PBS for 1 h at room temperature. Subsequently, the sections were incubated with the anti-TLR4 and anti-NF-κB antibody, respectively (1 : 100), in PBS at 4°C overnight. A secondary antibody HRP conjugated goat antirabbit IgG was added. The signals were visualized by DAB staining, and the nuclei were

counterstained with hematoxylin. Positive staining presented yellow or brown color under the biological microscope. The data of protein expressions were present by semiquantitative analysis as integrated option density (IOD) in positive area of the microphotograph with the Image-Pro-Plus 6.0 image analysis software.

2.9. Statistical Analysis. All the experiment data were expressed as mean ± standard deviation. The data were subjected to one-way analysis of variance (ANOVA). Differences were considered statistically significant if the p value was less than 0.05. Diagrams were performed by Graph Prims 7.0.

3. Results

3.1. Preparation of Macroporous Resin Extract of Dendrobium candidum Leaves (DLE). The content of total flavonoids in dried leaves of *Dendrobium candidum* was 1.16 ± 0.19% as measured by sodium nitrite-aluminum nitrate colorimetry. The purity of the total flavonoids in the *n*-butanol extract obtained by the extraction with the organic reagent was 3.70 ± 0.17%. The purity of total flavonoids in the extract of the total flavonoids purified by ADS-17 macroporous adsorption resin was 18.07 ± 0.03%, and the purity improved by nearly 17 times.

3.2. DLE Decreased Serum UA and Prevented Liver and Kidney from Damage in the Hyperuricemic Rats. As shown in Figure 2(a)–2(c), at the 8th and 9th week of administration, the DLE of both high- and low-dose groups could stably reduce serum UA and Cr levels compared with the model group ($p < 0.01$). There was a significant difference of serum BUN between the DLE groups and the model group from the 4th week of administration ($p < 0.05$, $p < 0.01$).

AST and ALT are reliable indicators for the assessment of liver function, which is involved in the production of UA [25]. The results were shown in Figures 2(d) and 2(e), the DLE-L could significantly reduce the ALT and AST levels in comparison with the model group ($p < 0.01$), and the DLE-H could significantly reduce the AST level. These results demonstrated that the DLE could prevent the liver from damage in hyperuricemic rats. There was no significant difference in weight gain rate between the normal and model groups. The DLE exerted little effect on weight of the rats (Figure 2(f)).

3.3. Effects of DLE on Histopathology of Kidney and Intestine Tissues in Hyperuricemic Rats. Upon H&E staining, compared with the normal group, glomerular cell proliferation and inflammatory cells infiltration were mainly found in the model group, and glomerular extracellular matrix (ECM) accumulation, glomerular mesangial cell proliferation, and basal membrane thickness were observed as well. Both DLE-L and DLE-H could prevent renal injury through alleviating ECM accumulation and inflammatory cell infiltration (Figure 3(a)).

In Masson staining, blue indicates fibrosis. Compared with the normal group, although large areas of blue area were observed in the model group, in addition to the yellow-brown urate crystals (as shown by ↑), the mesangial proliferation is severe, and a large amount of blue collagen fibers are aggregated. DLE can prevent renal interstitial fibrosis and mesangial proliferation of glomeruli (Figure 3(b)).

Upon PAS staining, the glomerular basement membrane of the model group showed remarkable thick ending (as shown by ↑), and a large amount of glycogen deposition and mesangial matrix proliferation were found. We found that the ratio of mesangial matrix proliferation in the model group was significantly increased compared with that in the normal group ($p < 0.01$). The DLE reversed these parameters ($p < 0.01$) (Figures 3(c)–3(e)).

The intestine is involved in uric acid excretion. As shown in Figure 3(d), the villus height (V) of the model group was shorter, the depth of the crypts (C) was larger ($p < 0.01$), and the value of V/C was significantly decreased ($p < 0.05$) compared with the normal group. The DLE could largely decrease C level and increase V/C level compared with the model group ($p < 0.05$, $p < 0.01$) (Figures 3(f)–3(h)).

3.4. DLE Decreased Liver XOD and ADA in the Hyperuricemic Rats. Liver is the main organ for the de novo purine synthesis and the production of uric acid in vivo. XOD and ADA, purine catabolism enzymes, not only in blood but also in liver, are needed during the process. The effect of DLE lowering uric acid may be related to inhibiting uric acid production by acting on ADA and XOD. As shown in Figure 4(a) and 4(b), serum XOD levels in the model group were dramatically higher than that in the normal group ($p < 0.01$), and serum XOD levels in the DLE-L group decreased ($p < 0.05$) compared with the model group. In addition, the DLE-L significantly lowered serum XOD levels ($p < 0.05$). Also, there were no significant differences on ADA in the DLE, the normal, and the model groups. The results of XOD and ADA levels in liver tissue were basically higher than that in the normal group ($p < 0.01$), and the liver XOD and ADA levels in the DLE -H group decreased compared with the model group ($p < 0.05$, 0.01) (Figures 4(c) and 4(d)).

The FEUA% of the model group showed a tendency to decrease in comparison with the normal group. The DLE only slightly increased FEUA% level compared with the model group (Figure 4(e)). It indicated that the DLE has a notable effect on lowering serum UA in hyperuricemic rats induced by modified high-purine diet, but it has no obvious effect on excretion of uric acid. These results demonstrated that the DLE may inhibit the uric acid production in hyperuricemic rats by lowering ADA and XOD in liver.

3.5. Effect of DLE on TLR4/NF-κB Expression in Kidney and Liver of the Hyperuricemic Rats. Uric acid can alter the protein expression level of TLR4/NF-κB signaling pathway in kidney. The TLR4 and NF-κB expression was strongly expressed in renal tubular epithelial cells of the model group compared with the normal group ($p < 0.05$)

Figure 2: DLE decreased serum UA and prevents liver and kidney damage. (a) Serum uric acid (UA). (b) Serum creatinine (Cr). (c) Serum urea nitrogen (BUN). (d) Serum alanine aminotransferase (ALT). (e) Serum aspartate aminotransferase (AST). (f) Body weight change over time. Normal, the normal control group; Model, the model group; DLE-L, low dose of the macroporous resin extract of *Dendrobium candidum* leaves; DLE-H, high dose of the macroporous resin extract of *Dendrobium candidum* leaves. The data were expressed as mean ± SD (n = 9-10). $^{**}p < 0.01$ and $^{*}p < 0.05$ versus the normal control group; $^{\Delta\Delta}p < 0.01$ and $^{\Delta}p < 0.05$ versus the model group.

(Figures 5(a) and 5(b)). The DLE-H significantly decreased the TLR4 and NF-κB expression ($p < 0.05$), and this effect was positively related with the dosage, compared with the model group. In Figures 5(c) and 5(d), the TLR4 and NF-κB expression in liver of the model group were strikingly higher than that of the normal group ($p < 0.01$). Compared with the model group, the TLR4 and NF-κB expression in liver of the DLE group was significantly reduced ($p < 0.05$, 0.01).

4. Discussion

With people's lifestyle and diet changes in recent years, more seafood and alcohol are being consumed, particularly in economically developed and coastal regions. These foods are rich in purine, protein, nucleotides, and B vitamins [26]. A large amount of yeast and purine could interfere with normal purine metabolism in body, resulting in metabolic disorders such as increasing XOD activity and uric acid production [27].

The average uric acid pool in the human body is 1200 mg; about 750 mg of uric acid is produced per day and approximately 500 to 1000 mg excreted daily (about two-thirds of which is excreted by the kidneys, and the remaining one-third is excreted in the intestine) [28]. The kidney plays an indispensable role in uric acid excretion [29]. The proximal tubule is the site of uric acid reabsorption and secretion, and approximately 90% is reabsorbed into blood [30]. Reabsorption depends on the uric acid transporter on the proximal tubule [31].

Studies have shown that elevated renal uric acid levels can cause histological kidney damage. Elevated UA causes mild tubular interstitial fibrosis, tubular damage, macrophage infiltration, and the appearance of markers of collagen III deposition [32]. UA may also be involved in the progression of liver disease and may eventually contribute to the development of cirrhosis and amplify liver inflammation [33]. Modern pharmacological studies have shown that *Dendrobium candidum* has a certain protective effect against liver and kidney damage [34, 35]. A preliminary study in our laboratory found that *Dendrobium candidum* extract reduces uric acid levels. In fact, the chemical composition of stems and leaves is similar, so it is speculated that the leaves of *Dendrobium candidum* may have corresponding liver and kidney protection and a uric acid–lowering effect. We successfully established a model of hyperuricemia in rats through purine plus yeast feeding and found that the macroporous resin extract of *Dendrobium candidum* (DLE) could effectively reduce UA levels and improve renal function, which was assessed through kidney (CR, BUN) (Figures 2(b) and 2(c)) and liver function indicators (ALT, AST) (Figures 2(d) and

Figure 3: DLE prevents the kidney and intestine histopathology in hyperuricemic rats at the 9th week of administration. (a) Kidney damages directly reflected by hematoxylin-eosin (H & E) (x400). (b) Kidney damages directly reflected by Masson (x400). (c) Kidney damages directly reflected by periodic acid-schiff (PAS) (x 400). (d) Intestinal damages directly reflected by H&E (x200). (e) The data of kidney histopathology was semiquantitatively analysed. Normal, the normal control group; model, the model group; DLE-L, low dose of the macroporous resin extract of *Dendrobium candidum* leaves; DLE-H, high dose of the macroporous resin extract of *Dendrobium candidum* leaves. (f, g, and h) Villus height, depth of crypt, and ratio of villus height to depth of crypt of intestine. Normal, the normal control group; Model, the model group; DLE-L, low dose of the macroporous resin extract of *Dendrobium candidum* leaves; DLE-H, high dose of the macroporous resin extract of *Dendrobium candidum* leaves. The data were expressed as mean ± SD ($n = 5$). $^{**}p < 0.01$ and $^{*}p < 0.05$ versus the normal control group; $^{\Delta\Delta}p < 0.01$ and $^{\Delta}p < 0.05$ versus the model group.

2(e)). Histopathological investigation also shows that DLE can ameliorate liver and kidney damage. In fact, we also found that DLE can prevent pathology of the intestine in rats with hyperuricemia by decreasing the C level and increasing the V/C ratio. These results proved that the DLE could lower uric acid and prevent kidney, liver, and intestinal from damage, which are also associated with an increase in uric acid.

Figure 4: DLE decreased serum and liver XOD and ADA in hyperuricemic rats at the 9th week of administration. (a and b) Serum xanthine oxidase (XOD) and adenosine deaminase (ADA). (c and d) Liver xanthine oxidase (XOD) and adenosine deaminase (ADA). (e) The fractional excretion of uric acid (FEUA%). Normal, the normal control group; Model, the model group; DLE-L, low dose of the macroporous resin extract of *Dendrobium candidum* leaves; DLE-H, high dose of the macroporous resin extract of *Dendrobium candidum* leaves. The data were expressed as mean ± SD ($n = 9$-10). $^{**}p < 0.01$ and $^{*}p < 0.05$ versus the normal control group; $^{\Delta\Delta}p < 0.01$ and $^{\Delta}p < 0.05$ versus the model group.

Figure 5: Continued.

Figure 5: DLE improved TLR4/NF-κB pathway in kidney and liver of the hyperuricemic rats at the 9th week of administration. (a and b) Expression of TLR4 and NF-κB in kidney by immunohistochemistry (ICH) (x400) and semiquantitatively analysed as integrated option density (IOD) in positive area of the microphotograph. (c and d) Expression of TLR4 and NF-κB in kidney by immunohistochemistry (ICH) (x400) and semiquantitatively analysed as integrated option density (IOD) in positive area of the microphotograph. TLR4, toll-like receptor 4; NF-κB, nuclear factor-kappaB; Normal, the normal control group; Model, the model group; DLE-L, low dose of the macroporous resin extract of *Dendrobium candidum* leaves; DLE-H, high dose of the macroporous resin extract of *Dendrobium candidum* leaves. The data were expressed as mean ± SD ($n = 5$). $^{**}p < 0.01$ and $^{*}p < 0.05$ versus the normal control group; $^{\Delta\Delta}p < 0.01$ and $^{\Delta}p < 0.05$ versus the model group.

Many enzymes are involved in the process of converting adenine and guanine into uric acid. First, adenosine monophosphate (AMP) is converted to inosine by two different mechanisms. The amino group is first removed by adenine deaminase to form inosine monophosphate (IMP), then dephosphorylated with a nucleotidase to form inosine; the phosphate group is first removed by a nucleotidase to form adenosine, and then adenosine is deaminated by ADA to form inosine. Guanine monophosphate (GMP) is converted to guanosine by nucleotidase. The nucleosides, inosine and guanosine are further converted to the purine bases hypoxanthine and guanine by purine nucleoside phosphorylase (PNP). The hypoxanthine is then oxidized to form xanthine by xanthine oxidase (XOD) and xanthine dehydrogenase (XDH), and the guanine is deaminated by guanine deaminase to form xanthine. Xanthine is again oxidized by xanthine oxidase to form the final product, uric acid [30]. Our previous studies have found that *Dendrobium candidum* Simiao prescription (DSP) has a good uric acid–lowering effect in rats with adenine- and ethambutol-induced hyperuricemia. The study found that DSP can reduce the content of XOD, increase the uric acid excretion index, and increase the level of uric acid transporter [36]. This study found that the DLE could effectively reduce serum XOD levels. In addition, the XOD and ADA levels in the liver of the DLE group were significantly lower than those in the model group. These results indicate that DLE can inhibit uric acid production in hyperuricemic rats by reducing ADA and XOD.

When the enzyme XOD activity is increased, it directly promotes uric acid synthesis and then stimulates oxidative stress and inflammatory injury, which can damage the liver and kidney by triggering the toll-like receptor 4 (TLR4)/NF-κB signaling pathway [37, 38]. NF-κB is located in the TLR4 downstream signaling pathway [39]. The NF-κB signaling cascade is activated in proximal tubular epithelial cells of hypertensive mice with kidney injury [40]. Other studies have also found that TLRs/NF-κB signaling pathway and NLRP3 inflammatory body activation may lead to IL-1β–driven inflammatory response, causing hyperuricemia in kidney injury [41]. Through this study, it was found that DLE can significantly reduce the expression levels of TLR4 and NF-kB in liver and kidney and effectively inhibit the inflammatory reaction (Figure 5). DLE may reduce the level of uric acid by reducing the inflammation and pathology of liver and kidney by affecting the TLRs/NF-κB pathway.

In conclusion, it was found in this study that the macroporous resin extract (DLE) of *Dendrobium candidum* leaves is effective for the treatment of hyperuricemia. The DLE decreased serum UA and prevented liver, kidney, and intestinal from damage by suppressing the XOD/ADA system and the TLR4/NF-κB inflammatory signaling pathway. Our findings have potential value in the clinical treatment of hyperuricemia. However, there are some limitations in our study. For example, LPS is a lipopolysaccharide located in the cell wall of Gram-negative bacteria [42], which are produced because of the imbalance of the gut microbiota. As the permeability of the intestinal mucosa increases and the intestinal barrier is impaired, a large number of intracellular LPS are released into the blood [43], which then activate TLRs/NF-κB in the liver and kidney. In

this study, DLE was found to prevent the pathology in the intestine, but it is still unknown whether the prevention is related to the inhibition of the gut microbiota-LPS- TLRs/NF-κB axis. Additional study on the active constituents of *Dendrobium candidum* is required, especially including description of the protein and gene level of some of the indicators in the experiment.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Xiao-Jing Lou and Yu-Zhi Wang are co-first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Xiao-Jing Lou and Yu-Zhi Wang contributed equally to this work.

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Research Article

Optimization of Extraction Conditions of Phytochemical Compounds and Anti-Gout Activity of *Euphorbia hirta* L. (Ara Tanah) Using Response Surface Methodology and Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

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Gout is a common disease affected most of the people due to the elevation of uric acid in the blood. Flavonoid and phenolic compounds are reported to exert the anti-gout activity of medicinal plants. Hence, this study aimed at optimizing the extraction conditions of phenolic and flavonoid compounds as well as the anti-gout (xanthine oxidase inhibitory activity) *in vitro* of *Euphorbia hirta* using response surface methodology (RSM). The plant part used was the whole plant excluding roots. The effects of three independent variables (extraction time, X_1; extraction temperature, X_2; and solid-to-liquid ratio, X_3) on three response variables (total flavonoid content, Y_1; total phenolic content, Y_2; and xanthine oxidase inhibitory activity, Y_3) were determined using central composite design (CCD) while phytochemical profiling of the extracts was determined by liquid chromatography-mass spectrometry (LC-MS). Quadratic models produced a satisfactory fitting of the experimental data with regard to total flavonoid content ($r^2 = 0.9407$, $p < 0.0001$), total phenolic content ($r^2 = 0.9383$, $p < 0.0001$), and xanthine oxidase inhibitory activity ($r^2 = 0.9794$, $p < 0.0001$). The best extraction conditions observed for total flavonoid content, total phenolic content, and xanthine oxidase inhibitory activity were at a temperature of 79.07°C for 17.42 min with solid-to-liquid ratio of 1 : 20 g/ml. The optimum values for total flavonoid, total phenolic, and xanthine oxidase inhibitory activity were 67.56 mg RE/g, 155.21 mg GAE/g, and 91.42%, respectively. The main phytochemical compounds in the optimized *E. hirta* extract are neochlorogenic acid, quercetin-3β-D-glucoside, syringic acid, caffeic acid, ellagic acid, astragalin, afzelin, and quercetin. As conclusion, this study clearly demonstrated the best conditions to obtain higher xanthine oxidase inhibitory activity and phytochemical compounds which can be further used for the development of anti-gout agents.

1. Introduction

Euphorbia hirta L. or locally known as Ara tanah or Gelang susu in Malaysia is from the family of Euphorbiaceae. The genus of *Euphorbia* is considered to be the largest in the Euphorbiaceae family with about 1600 species and it is native to tropical and subtropical regions of Asia, Africa, and Central as well as South America [1]. This plant was found to possess potent antimicrobial [2], antidiarrhoeic [3], anti-hyperglycemic [4], anti-inflammatory [5], and anticancer [6] activities. In fact, in Malay traditional medicine, it has been used widely to treat various diseases including skin problems, gastrointestinal disorders, diarrhoea, and ulcer [2]. However, in Ayurvedic treatment, this plant has been used

traditionally to treat worm infestations in children, dysentery, jaundice, pimples, digestive problems, female disorders, respiratory ailments (cough, coryza, bronchitis, and asthma), and tumors [1]. In addition, the decoction or infusion of this plant has been used widely by practitioners of traditional medicine in treating asthma, kidney stones, heartburn, diarrhea, coughs, and menstrual problems [7]. Interestingly, many phytochemical compounds such as saponin, sterol, terpene, alkaloids, polyphenols, tannins, terpenoids, steroids, and flavonoids have been found in the aerial parts of this plant [4, 7]. This plant is also rich in minerals such as zinc, potassium, calcium, and magnesium [4]. In 2007, three compounds from the methanolic extract of *E. hirta*, namely, afzelin, quercetin, and myricetin which showed potent inhibition against malaria have been successfully isolated [8].

Extraction is considered to be one of the important steps in discovering potential bioactive compounds from plant materials, considering their quantity and type of bioactive compounds as they are widely used in nutraceutical, pharmaceutical, and cosmetic products as well as functional food ingredients and food additives [9, 10]. The type and concentration of solvent, temperature, time, pH, solid-liquid ratios, pressure, and the particle size of the plant have been identified from the previous studies as important factors affecting the extraction efficiency of the plant bioactive compounds [11–13]. Traditional method, which is known as the one-factor-at-a-time, has been used widely by many researchers in optimizing specific parameters in which only one factor plays a role as a variable at a time while keeping all other factors constant [14]. However, this method is considered to be less reliable as it does not include interactive effects among factors, time-consuming, and expensive. Misleading conclusions and false conditions also might be occurred at the end of this experiment method, ignoring the true significant factors during the extraction process [9]. To overcome this problem, response surface methodology (RSM) has been introduced and widely used nowadays for optimizing the extraction conditions. RSM is considered to be a powerful mathematical technique used widely in many industries for technological operations in order to optimize certain experimental conditions. Moreover, it evaluates the effects between the multiple factors and their interactions towards one or more response variables simultaneously, producing lesser number of experimental measurements [15]. It also helps to locate the region where the extraction is optimized by using clearer visual aid [16]. Based on the previous literature, the phenolic and flavonoid contents of guava puree [17], *Curcuma zedoaria* leaves [16], apples [18], *Feronia limonia* fruit [15], and *Mangifera pajang* [9] have been successfully optimized using RSM.

Xanthine oxidase inhibitory activity (XO) is an enzyme assay used widely in determining the anti-gout activity of the plant extracts. XO is known as the main enzyme causing the accumulation of uric acid in the blood which eventually leads to gout. The mechanism involves where it catalyses the oxidation of hypoxanthine into xanthine and then uric acid [19]. Optimization of extraction method to obtain higher total flavonoids and phenolics as well as XO inhibitory activity from *E. hirta* is essential for future nutraceutical product development especially for the gout treatment. To the best of our knowledge, this is the first report describing optimization of extraction process of phytochemical compounds and XO inhibitory activity from *E. hirta*. Therefore, this study aimed at optimizing the extraction temperature, time, and solid-to-liquid ratio for the extraction of total flavonoid content, total phenolic content, and anti-gout activity from *E. hirta* whole plant excluding roots using RSM.

2. Materials and Methods

2.1. Chemicals and Reagents. Gallic acid, xanthine, xanthine oxidase (buttermilk), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Folin–Ciocalteu reagents were obtained from Merck (Germany). Allopurinol, rutin, aluminum chloride hexahydrate, sodium nitrite, sodium hydroxide, and dipotassium hydrogen phosphate (K_2HPO_4) were of the highest purity (95–99%).

2.2. Plant Materials. The plant part used was the whole plant excluding roots. *E. hirta* was collected from the Department of Agriculture, Serdang, Selangor, Malaysia, and authenticated by Dr. Mohd Firdaus Bin Ismail (Institute of Bioscience, Universiti Putra Malaysia), and the voucher specimen was deposited in the Herbarium of the same institute under the number MFI0084/19.

2.3. Sample Extraction. The whole plant excluding root was soaked in water, washed, and oven-dried at 40°C for 72 hours. Then, it was ground and stored in a −20°C freezer prior to the extraction process. In order to mimic the human consumption, the plant was prepared using hot boiling water extraction procedure whereby each 1.0 g of dried plant was infused in 10–20 ml of distilled water at temperature between 50 and 100°C and continuously stirred for 5–60 min (Table 1) using a magnetic stirrer under 300 rpm. The infusion was left to cool to 15 min. Then, the extract was filtered using Whatman No. 1 filter paper and subjected to the xanthine oxidase inhibitory activity assay spectrophotometrically at 295 nm, for determination of total flavonoid and total phenolic contents.

2.4. Experimental Design. Central composite design (CCD) was developed in order to obtain optimized extraction conditions for higher total flavonoid content, total phenolic content, and xanthine oxidase inhibitory activity of *E. hirta*. CCD was chosen in this study as it provides more design points on each variable. Three selected independent variables in this study were extraction time (X_1: 5–60 minutes), extraction temperature (X_2: 50–100°C), and solid-to-liquid ratio (X_3: 1 : 10–1 : 20 g/ml), while the dependent variables chosen were total flavonoid content (Y_1), total phenolic content (Y_2), and xanthine oxidase inhibitory activity (Y_3). The responses of total flavonoid content and total phenolic

Table 1: Experimental design for the extraction process from central composite design.

Run order	X_1 (extraction time) (min)	X_2 (extraction temperature) (°C)	X_3 (solid-to-liquid ratio) (g/ml)
1[a]	32.5	75	15
2[a]	32.5	75	15
3[a]	32.5	75	15
4	32.5	50	15
5[a]	32.5	75	15
6	32.5	75	20
7[a]	32.5	75	15
8	5	50	20
9	32.5	75	10
10	60	50	10
11	60	50	20
12	60	100	10
13	60	75	15
14	32.5	100	15
15	5	100	20
16	5	75	15
17	5	100	10
18	5	50	10
19	60	100	20
20[a]	32.5	75	15

[a]Center point.

content were critical to be studied as the presence of both of them in the plants have been reported to be the reasons for treating the gout disease while xanthine oxidase inhibitory activity was the *in vitro* enzyme assay which has been used widely for determining the antigout activity of the plants. The complete design was generated by a statistical software package (Design Expert, version 7.0, Minneapolis, USA) which consists of 20 combinations including six replicates at the central point (Table 1).

2.5. Total Flavonoid Content (TFC) Determination. TFC of the plant extract was determined using aluminium chloride method [20]. The absorbance of the mixture was measured spectrophotometrically at 510 nm, and the results obtained were expressed as milligram rutin equivalent per gram (mg RE/g) based on the standard curve of various concentrations of rutin (0–100 μg/ml).

2.6. Total Phenolic Content (TPC) Determination. TPC of the plant extract was determined using Folin–Ciocalteu's colorimetric method with slight modification [21]. The absorbance was spectrophotometrically measured at 725 nm, and the results obtained were expressed as milligram gallic acid equivalent per gram (mg GAE/g) based on the standard curve with various concentrations of gallic acid (0 – 100 μg/ml).

2.7. Xanthine Oxidase (XO) Inhibitory Activity Assay. The inhibitory effect on XO was measured spectrophotometrically at 295 nm under aerobic condition following the existing method with some modifications [22, 23] where allopurinol (0–100 μg/ml) was used as a positive control. The degree of XO inhibitory activity was calculated by using the following equation:

$$\%\,\text{XO inhibition} = \left(1 - \frac{\beta}{\alpha}\right) \times 100\%, \quad (1)$$

where α is the activity of XO without test extract and β is the activity of XO with test extract.

2.8. Liquid Chromatography-Mass Spectroscopy (LC-MS) Analysis. Chromatography was performed according to Zakaria et al. [24] where 20 μL of sample was injected onto a Agilent C18 reverse-phase column (4.0 × 250 mm, 1.8 μm) and held at 50°C with a constant flow rate of 0.4 mL/min and total LC run time of 30 min. Sample elution was performed in a gradient manner using mobile phase comprising of water containing 0.1% acetic acid (solvent A) and HPLC grade acetonitrile containing 0.1% acetic acid (solvent B). The mobile phase composition (A : B) was gradually increased from 5.95 to 15.85 over 25 min and returned to the initial condition (95%) for 5 min for solvent A and 5% to 85% for 25 min and then decreased to the initial condition (5 : 95) over the next 5 min. For the mass analysis, the source conditions were as follows: nebulizer pressure was 40 psi, drying gas flow was set at 12 L/min, and drying gas temperature was 350°C and the MS/MS acquisitions were performed in the negative and positive electrospray ionization mode, for the mass range of 150 to 1500 m/z. Data acquisition was performed by Agilent MassHunter workstation Data Acquisition, while data processing was carried out with MassHunter Qualitative Analysis software. In addition, MS/MS experiments were carried out in the automatic and multiple reaction monitoring (MRM) mode where automatic MS/MS low-energy collision dissociation (CID) was performed at 5–8 eV collision energy. Peak identification was carried out based on comparison with literature values and online databases.

2.9. Statistical Analysis. Design Expert® Software (version 7, Stat-Ease Inc., Minneapolis, MN) was used in this study. A response surface analysis was employed to determine the regression coefficients and statistical significance of the model terms and to fit the mathematical models of the experimental data. The adequacy of the model was predicted through the regression analysis (r^2) and the analysis of variance (ANOVA) ($p < 0.05$).

3. Results and Discussion

3.1. Fitting the Response Surface Models. Fitting the models are crucial in interpreting the accuracy of the RSM mathematical models for prediction of the TFC, TPC, and XO inhibitory activity of *E. hirta* extract. In this present study, the relationship between the response functions (TFC, TPC, and XO inhibitory activity) and the independent variables (extraction time, extraction temperature, and solid-to-liquid ratio) was successfully identified by CCD. The results of the responses for the 20 runs following the experimental design are shown in Table 2. The yield of the TFC ranged from 51.11 to 65.55 mg RE/g while TPC ranged from 120.34 to 152.95 mg GAE/g. In terms of XO inhibitory activity, the highest activity was 97.57% whereas the lowest was 72.96%. As suggested by the Design Expert software, a quadratic polynomial model was selected and fitted well for all three independent variables and responses. In terms of coded values, the predicted responses for the TFC, TPC, and XO inhibitory activity could be expressed by the second-order polynomial equation via multiple regression analysis:

$$Y_{\text{TFC}} = 60.46 + 1.54X_1 + 1.11X_2 + 2.64X_3 - 2.28X_1X_2 + 1.28X_1X_3 + 1.28X_2X_3 - 1.72X_1^2 - 5.73X_2^2 + 2.84X_3^2, \quad (2)$$

$$Y_{\text{TPC}} = 142.28 + 2.47X_1 + 2.50X_2 + 4.84X_3 - 5.72X_1X_2 + 3.08X_1X_3 + 1.71X_2X_3 - 2.48X_1^2 - 17.24X_2^2 + 8.88X_3^2, \quad (3)$$

$$Y_{\text{XO inhibitory activity}} = 95.03 - 3.30X_1 - 1.12X_2 - 6.27X_3 - 0.63X_1X_2 3.48X_1X_3 - 0.74X_2X_3 - 0.16X_1^2 - 2.91X_2^2 - 3.32X_3^2, \quad (4)$$

where X_1, X_2, and X_3 are the coded variables for extraction time, extraction temperature, and solid-to-liquid ratio, respectively. A negative sign in each equation represents an antagonistic effect of the variables, and a positive sign represents a synergistic effect of the variables [16].

Positive coefficients for X_1, X_2, and X_3 were observed in equation (2), indicating that TFC was increased with the increase of extraction time (5–60 min), extraction temperature (50–100°C), and solid-to-liquid ratio (1 : 10–1:20). The same observation was also found for TPC where positive coefficients for X_1, X_2, and X_3 were observed in equation (3), indicating that TPC was increased with the increase of extraction time (5–60 min), extraction temperature (50–100°C), and solid-to-liquid ratio (1 : 10–1 : 20). In contrast, negative coefficients for X_1, X_2, and X_3 were observed in equation (4), indicating that XO inhibitory activity was decreased with the increase of extraction time (5–60 min), extraction temperature (50–100°C), and solid-to-liquid ratio (1 : 10–1 : 20). The RSM model coefficients were validated by analysis of variance (ANOVA) of the response variables for the quadratic polynomial model summarized in Supplementary Materials (Tables S1, S2, and S3). For the responses of TFC, TPC, and XO inhibitory activity, the models were highly significant when the computed F-values were greater than the tabulated F-value and the probability values were low ($p < 0.001$), indicating that the individual terms in each response model were significant on the interaction effect. In addition, the coefficient of determination (r^2) of the models was 0.9407, 0.9383, and 0.9794, respectively, indicating that 94.07, 93.83, and 97.94% match between the values of the predicted model and the values that were attained in the experimental data. Besides, the r^2 values were comparable to adjusted r^2, demonstrating good statistical model. The p values for the lack of fit were identified as 0.2462, 0.4015, and 0.5001, highlighting that the lack of fit of the models was significant at $p > 0.05$. Hence, the results indicate that the models could predict the TFC, TPC, and XO inhibitory activity of *E. hirta* extract efficiently when independent variables were within the ranges depicted here.

3.2. Effect of Extraction Parameters on Total Flavonoid Content (TFC). Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants [25]. They are categorized mainly into flavone, flavanol, and flavanone compounds. Table S1 (Supplementary data) shows that all independent variables (extraction time, temperature, and solid-to-liquid ratio) had a significant effect on TFC of *E. hirta* extract. Among these, solid-to-liquid ratio (X_3) was found to be the major effect on TFC in comparison with other variables, which may be due to the high F-value of 33.99. Interestingly, the analysis of the results also showed that all the interactions between time and temperature (X_1X_2), time and solid-to-liquid ratio (X_1X_3), and temperature and solid-to-liquid ratio (X_2X_3) were highly significant factors affecting the yield of TFC ($p < 0.05$). Based on the previous studies, extraction temperature, time, and solid-to-liquid ratio are considered to be the important parameters for the extraction efficiency of flavonoid compounds [26, 27]. Figures 1(a)–1(c) show the three-dimensional response surface plots for the influences of extraction temperature, time, and solid-to-liquid ratio on TFC. The response surface plots are very useful to see the interaction effects of the factors on the responses. As in Figure 1(a), the TFC extraction yields significantly increased as the extraction temperature and time increased from 50 to 75°C and 5 to 60 min, respectively, but started to decrease towards 100°C while keeping the solid-to-liquid ratio as constant. This was supported by Sheng et al. [28] where the yield of total flavonoids

Table 2: Response surface central composite design (uncoded) and results for total flavonoid content, total phenolic content, and xanthine oxidase inhibitory activity.

Run order	X_1 (extraction time) (min)	X_2 (extraction temperature) (°C)	X_3 (solid-to-liquid ratio) (g/ml)	Y_1/total flavonoid content (mg RE/g)[b]	Y_2 (total phenolic content (mg GAE/g)[c]	Y_3 (xanthine oxidase inhibitory activity) (%)
1[a]	32.5	75	15	50.92	130.53	95.13
2[a]	32.5	75	15	60.62	140.76	97.66
3[a]	32.5	75	15	60.54	140.63	97.28
4	32.5	50	15	50.78	120.76	90.07
5[a]	32.5	75	15	60.38	140.28	96.60
6	32.5	75	20	60.55	150.95	84.95
7[a]	32.5	75	15	60.44	130.66	96.44
8	5	50	20	60.34	150.87	89.75
9	32.5	75	10	50.27	120.49	94.57
10	60	50	10	40.98	110.38	95.31
11	60	50	20	60.99	150.97	73.32
12	60	100	10	50.11	110.69	94.94
13	60	75	15	50.44	120.43	87.39
14	32.5	100	15	50.89	130.45	89.26
15	5	100	20	70.41	160.92	88.93
16	5	75	15	60.26	140.30	97.44
17	5	100	10	50.17	110.81	94.95
18	5	50	10	40.96	110.34	94.83
19	60	100	20	60.75	150.42	72.96
20[a]	32.5	75	15	60.42	140.66	94.19

[a]Center point. [b]Total flavonoid content was expressed as mg rutin equivalents in 1 g of dried sample (mg RE/g). [c]Total phenolic content was expressed as mg gallic acid equivalents in 1 g of dried sample (mg GAE/g).

in the plant extract rose gradually with the increase of temperature from 50° to 90°C. The increase of extraction temperature promotes the solvent extraction process of the plant extract due to multiple effects of temperature on the mass transfer process by enhancing both diffusion coefficients and the solubility of flavonoid compounds as well as promoting the degradation of the plant matrix [26, 29].

Figures 1(b) and 1(c) display the effect of solid-to-liquid ratio-extraction time and solid-to-liquid ratio-extraction temperature on TFC of *E. hirta*, respectively. The yield of TFC increased when the solid-to-liquid ratio and extraction time increased (Figure 1(b)). As shown in Figure 1(c), as the ratio of solid-to-liquid increased from 1 : 15 to 1 : 20, the yield of TFC increased significantly from 59.82 mg/g to 64.06 mg/g with an increase in extraction temperature. This was supported by the previous study where the TFC of *Gynura medica* leaves significantly increased from 24.48 to 35.39 mg kaempferol/g DM as the liquid-to-solid ratio increased from 10 to 40 (v/w) [26]. The same trend was also observed by Sheng et al. [28] where the TFC increased gradually with the increase of liquid-to-solid ratio from 10 : 1 to 18 : 1. This might be due to the increase of the driving force for the mass transfer of the TFC, resulting in the high TFC obtained at the end of the study [26].

3.3. Effect of Extraction Parameters on Total Phenolic Content (TPC). One of the most important groups of secondary metabolites produced by all plants is phenolic compounds. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants [30]. Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins, acting mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against ultraviolet (UV) light [31]. Table S2 (Supplementary data) shows that all independent variables (extraction time, temperature, and solid-to-liquid ratio) had the significant effect on TPC of *E. hirta* extract. Among these, solid-to-liquid ratio (X_3) was found to be the major effect on TPC in comparison with other variables, which may due to the high F-value of 19.42. Interestingly, the analysis of the results also showed that the interactions between time and temperature (X_1X_2) and time and solid-to-liquid ratio (X_1X_3) were highly significant factors affecting the yield of TFC ($p < 0.05$) except for temperature and solid-to-liquid ratio (X_2X_3). Figures 2(a)–2(c) show the response surface plots for the influences of extraction temperature, extraction time, and solid-to-liquid ratio on TPC. As shown in Figure 2(a), TPC was increased as the extraction temperature increased and reached the maximum at 75°C but started to decrease towards 100°C especially at high levels of extraction time, implying that beyond a certain value of temperature, phenolic compounds can be denatured [32, 33]. Previous study has demonstrated that the yield of phenolic compounds was increased significantly over the extraction temperature range (25–75°C) where the maximum of TPC was at 65°C [34]. In fact, heat has been found to enhance the recovery of phenolic compounds [14, 35]. It was believed that high temperature increased and enhanced the diffusion and solubility rate of phenolic compounds by softening the plant tissue as well as disrupting the interactions between the phenolic compounds and protein or polysaccharides [36]. It is interesting to note that in the present study, increasing

Figure 1: Response surface plots of *E. hirta* showing the effect of (a) extraction time and extraction temperature, (b) solid-to-liquid ratio and extraction time, (c) solid-to-liquid ratio and extraction temperature on TFC. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

extraction temperature had a positive effect to TPC. From this scenario, it is believed that the phenolic compounds present in *E. hirta* are thermally stable up to 75°C. Figure 2(b) shows that the TPC increased significantly as the solid-to-liquid ratio increased from 1 : 15 to 1 : 20 with the increase of extraction time. Figure 2(c) also shows that the TPC increased significantly as the solid-to-liquid ratio increased from 1 : 15 to 1 : 20 with the increase of extraction temperature up to 75°C. Hence, from these figures, the solid-to-liquid ratio could drastically affect the yield of phenolic compounds during the extraction process. The similar result was observed from the previous study where the yield of phenolic compounds from date seeds water extract increased from 2.5 to 5.5 g/100 g as solvent ratio increased to the 60 : 1 level [37]. The same results were reported for the milled berries extraction where higher solid-to-liquid ratio resulted in higher yield of phenolic compounds [38]. This was in line with the mass transfer principle [39].

3.4. Effect of Extraction Parameters on XO Inhibitory Activity. XO inhibition assay is a common assay used for determining the anti-gout activity of the plant extracts [40]. XO catalyses the oxidation of hypoxanthine to xanthine and then to uric acid, which plays a crucial role in gout [41]. As reviewed by Ling and Bochu [42], phenolic and flavonoid compounds have been reported to be the potent plant-based XO inhibitors which make them crucial as anti-gout agents. Few studies from the previous years have successfully optimized the extraction conditions for higher XO inhibitory activity of the plants [43–45]. Figures 3(a)–3(c) illustrate the response surface plots for the influences of extraction temperature, extraction time, and solid-to-liquid ratio on XO inhibitory activity. Table S3 (Supplementary data) shows that all independent variables (extraction time, temperature, and solid-to-liquid ratio) had the significant effect on XO inhibitory activity of *E. hirta* extract. Among these, extraction time (X_1) and solid-to-liquid ratio (X_3) were found to be the major effects on XO inhibitory activity in comparison with extraction temperature (X_2), which may due to the high *F*-values of 66.27 and 239.46, respectively. Interestingly, the analysis of the results also showed that the interactions between time and solid-to-liquid ratio (X_1X_3) was highly significant factor affecting the XO inhibitory activity ($p < 0.05$). As shown in Figure 3(a), XO inhibitory activity

Figure 2: Response surface plots of *E. hirta* showing the effect of (a) extraction time and extraction temperature, (b) solid-to-liquid ratio and extraction time, and (c) solid-to-liquid ratio and extraction temperature on total phenolic contents. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

increased when the temperature increased from 50 to 75°C and started to decrease towards 100°C as the extraction time decreased. This was in agreement with Edirs et al. [46] where the antidiabetic activity of Kursi Wufarikun Ziyabit using protein tyrosine phosphatase-1B and α-glucosidase inhibition assays increased as the extraction temperature increased from 70 to 75°C and 65 to 70°C, respectively. This explains that an increase in the extraction temperature beyond certain value will denature or decompose some of the valuable compounds responsible for inhibiting XO activity [47]. In fact, the high temperature may cause the enzymes to be denatured as they are heat sensitive [48].

Meanwhile, Figure 3(b) clearly shows that the XO inhibitory activity decreased when the extraction time and solvent-to-liquid ratio increased. The XO inhibitory activity reached at maximum level (95.37%) when the extraction time and solvent to liquid ratio were 32.5 min and 1 : 15 g/ml, respectively. This implied that excessive extraction time (more than 32.5 min) was no longer useful to extract more active compounds from *E. hirta* which contributed to antigout activity. In fact, oxidation of the active compounds during the prolonged time of extraction process could also reduce the XO activity. However, lesser contact time during the extraction process of the plants would not allow good mixing between the samples and solvent, resulting in low XO inhibitory activity [43]. In addition, the interaction effects between the extraction temperature and solid-to-liquid ratio on XO inhibitory activity with a constant value of the extraction time at 32.5 min are shown in Figure 3(c). It can be seen that solid-to-liquid ratio gave greater impact on the XO inhibitory activity of *E. hirta* regardless of the temperature used. This was in agreement with Mehmood et al. [49] who also showed that the solute solvent ratio gave a significant effect on XO inhibition.

3.5. Optimization of Extraction Conditions. In this study, the desirability function approach suggested that the optimal extraction conditions for *E. hirta* water extract in obtaining maximum XO inhibitory activity (91.42%), TFC (67.56 mg RE/g), and TPC (155.21 mg GAE/g) were at 79.07°C with 17.42 min and 1 : 20 of solid-to-liquid ratio. The predicted values of all the responses were close to the experimental values with less than 10% error, which is relatively desirable

Figure 3: Response surface plots of *E. hirta* showing the effect of (a) extraction time and extraction temperature, (b) solid-to-liquid ratio and extraction time, and (c) solid-to-liquid ratio and extraction temperature on xanthine oxidase inhibitory activity. Color gradients indicate the level of optimization (red = high, green = intermediate, and blue = low).

and all the experimental data were within ±95% prediction intervals. Besides, the accuracy of our model was confirmed as no significant difference was observed ($p > 0.05$) between the experimental values and the predicted values (Table 3). Therefore, it can be concluded that the model from CCD was accurate and reliable enough for predicting the TFC and TPC as well as anti-gout activity of *E. hirta*.

3.6. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) Metabolite Profile of E. hirta Extract. The *E. hirta* optimized extracts were analyzed and profiled by LC-MS/MS analysis in positive and negative ionization modes in order to perform a qualitative characterization of chemical constituents in *E. hirta*. To our knowledge, this is the first validated method on the detection of active compounds in *E. hirta* whole plant excluding roots using LC-MS/MS analysis. The base peak chromatogram is depicted in Figures 4 (negative ionization mode) and 5 (positive ionization mode). Compounds were characterized for their retention times, the absorption spectrum in the UV-vis region, the mass spectrum obtained by MS-ESI, and the fragmentation profile as well as compared with the previous literature reports [50–53]. The results obtained from the LC-MS/MS analysis allowed the tentative assignment of nine chemical constituents using negative ionization mode comprising four phenolic acids: neochlorogenic acid ($[M-H]^-$ ion at m/z 353), caffeic acid ($[M-H]^-$ ion at m/z 179.034), syringic acid ($[M-H]^-$ ion at m/z 197.045), and ellagic acid ($[M-H]^-$ ion at m/z 300.999) and four flavonoids: quercetin-3β-D-glucoside ($[M-H]^-$ ion at m/z 463.087), astragalin ($[M-H]^-$ ion at m/z 447.094), afzelin ($[M+FA-H]^-$ ion at m/z 477.104), quercetin ($[M-H]^-$ ion at m/z 301.035) as well as one fatty acid: corchorifatty acid F ($[M-H]^-$ ion at m/z 346.081). In addition, LC-MS/MS analysis also allowed the tentative assignment of two chemical constituents using positive ionization mode: 2-undecanone 2,4-dinitrophenylhydrazone ($[M+H]^+$ ion at m/z 351.4) and demexiptiline ($[M+H]^+$ ion at m/z 279.3). In this study, the negative mode ESI was more sensitive for the detection of the phenolics and flavonoids in the extract. The same observations were also shown by Zakaria et al. [24] and Keskes et al. [54].

Table 3: Differences between the predicted value and the experimental value.

Parameters	Predicted value	Experimental value	Percentage of error (%)
Total flavonoid content (mg RE/g)	64.31 ± 0.00^{a}	67.56 ± 0.83^{a}	4.81
Total phenolic content (mg GAE/g)	152.95 ± 0.00^{b}	155.21 ± 0.67^{b}	1.46
Xanthine oxidase inhibitory activity (%)	88.78 ± 0.00^{c}	91.42 ± 0.74^{c}	2.89

Values were presented as mean ± standard deviation. Same superscripts within the row indicate no significant difference ($p > 0.05$) between predicted and experimental values.

Figure 4: LC-MS chromatogram, acquired in the negative ion mode, from an extract of *E. hirta* where peak labelling represents the compounds identified and listed in Table 4.

Table 4: Tentative identification of chemical constituents in the *E. hirta* water extract.

No	Tentative assignment	R_T (min)	Molecular weight	Precursor type	Precursor m/z	Molecular formula	MS/MS fragment ions	References
1	Neochlorogenic acid	2.2	354.09	$[M-H]^-$	353	$C_{16}H_{18}O_9$	191, 179	Massbank: PM013904
2	Caffeic acid	3.46	180.04	$[M-H]^-$	179.0341	$C_9H_8O_4$	135, 179, 134	Massbank: FiehnHILIC001102
3	Syringic acid	5.48	198.052	$[M-H]^-$	197.045	$C_9H_{10}O_5$	182, 166, 197	Massbank: FiehnHILIC001522
4	Ellagic acid	6.47	302.006	$[M-H]^-$	300.999	$C_{14}H_6O_8$	303, 300	Massbank: FiehnHILIC001170
5	Quercetin-3β-D-glucoside	6.89	464.09	$[M-H]^-$	463.0879	$C_{21}H_{20}O_{12}$	300, 301, 271	Pubchem (CID5280804)
6	Astragalin	8.53	448.1	$[M-H]^-$	447.094	$C_{21}H_{20}O_{11}$	447, 285, 284	Massbank: CCMSLIB00000845312
7	Afzelin	9.34	432.1	$[M+FA-H]^-$	477.104	$C_{21}H_{20}O_{10}$	285, 431, 284	Massbank: CCMSLIB00000845703
8	Quercetin	11.25	302.04	$[M-H]^-$	301.0359	$C_{15}H_{10}O_7$	107, 121, 151, 178, 301	Massbank: FiehnHILIC002955
9	Corchorifatty acid F	15.06	328.224	$[M-H]^-$	346.081	$C_{18}H_{32}O_5$	235, 311, 219	Pubchem (CID44559173)
10	2-Undecanone 2,4-dinitrophenylhydrazone	15.08	350.4	$[M+H]^+$	351.4	$C_{17}H_{26}N_4O_4$	351, 352	Pubchem (CID 5717665)
11	Demexiptiline	27.16	278.3	$[M+H]^+$	279.3	$C_{18}H_{18}N_2O$	204, 203, 149	Massbank: WA002119

Figure 5: LC-MS chromatogram, acquired in the positive ion mode, from an extract of *E. hirta* where peak labelling represents the compounds identified and listed in Table 4.

Previous studies have demonstrated that flavonoids and phenolic acids possess high biological and pharmacological activities [55]. In the LC chromatogram, the peak at retention time (R_T) 2.20 min was assigned to the presence of neochlorogenic acid which was also reported by the previous studies [56, 57]. In fact, neochlorogenic acid showed potent anti-inflammatory activity [58]. In this study, astragalin (kaempferol-3-O-glucoside), a natural flavonoid, was the main compound obtained in *E. hirta* water extract. Previous study also reported the presence of astragalin in other *Euphorbia* species [59]. As reviewed by Riaz et al. [60], astragalin showed potent anti-inflammatory activity by inhibiting the production of inflammatory mediators such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in macrophages [61, 62]. Moreover, Jia et al. [63] demonstrated for the first time that astragalin effectively inhibited the worsening of synovial inflammation and joint destruction in the animal model. In fact, flavonoid compounds from Malaysian medicinal plants showed potent anti-gout and anti-inflammatory activities from the previous studies [64, 65]. Hence, in this present study, we speculated that the anti-gout effects of *E. hirta* might be due to the presence of high concentration of astragalin in the extract.

4. Conclusion

RSM was successfully employed to optimize the phytochemical compounds and anti-gout activity of *E. hirta* where CCD proved to be an efficient tool for the optimization of these parameters. The second-order polynomial models for predicting responses were obtained, and the best combination of extraction temperature, time, and solid-to-liquid ratio were found to be 79.07°C with 17.42 min and 1 : 20, respectively. Further investigation on the secondary metabolites present in the optimized extract of *E. hirta* using LC-MS analysis revealed the presence of neochlorogenic acid, quercetin-3β-D-glucoside, syringic acid, caffeic acid, ellagic acid, astragalin, afzelin, quercetin, corchorifatty acid F, 2-undecanone 2,4-dinitrophenylhydrazone, and demexiptiline. Hence, *E. hirta* could be a natural source of polyphenols compounds which serve as ingredients of functional foods and be used in pharmaceuticals for the treatment of gout disease.

Abbreviations

CCD: Central composite design
LC-MS: Liquid chromatography-mass spectrometry
RSM: Response surface methodology
TFC: Total flavonoid content
TPC: Total phenolic content
XO: Xanthine oxidase.

Data Availability

The datasets generated and/or analyzed during the current study are available from the first author on reasonable request.

Conflicts of Interest

The authors do not have any conflicts of interest regarding the content of the present work.

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Supplementary Materials

Table S1: the regression coefficients and results of ANOVA for response surface quadratic model of total flavonoid content. Table S2: the regression coefficients and results of ANOVA for response surface quadratic model of total phenolic content. Table S3: the regression coefficients and results of ANOVA for xanthine oxidase inhibitory activity. (*Supplementary Materials*)

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Review Article

The Impact of Natural Product Dietary Supplements on Patients with Gout: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Natural product dietary supplements (NPDS) are frequently used for the treatment of gout, but reliable efficacy and safety data are generally lacking or not well organized to guide clinical decision making. This review aims to explore the impacts of NPDS for patients with gout. An electronic literature search was conducted to retrieve data published in English language from databases from inception to August 14, 2019. Randomized controlled trials (RCTs) that compared NPDS with or without placebo, diet modification, conventional pharmaceutics, or the other Chinese medicine treatment for gout patients were included. Two authors screened the articles, extracted the data, and assessed the risk of bias of each included trial independently. Meta-analysis was performed using Review Manager version 5.3.5. *Results*. Nine RCTS were enrolled in this review. The methodological quality of the nine RCTs was poor. The study results showed that in the majority of trials, NPDS demonstrated some degree of therapeutic efficacy for joint swelling, pain, and activity limitation. In contradistinction, serum uric acid (SUA) level (SMD −1.80, 95% CI: −4.45 to 0.86) ($p > 0.05$) and CRP levels ($N = 232$; SMD, −0.26; 95% CI, −0.55 to 0.04) ($p > 0.05$) did not improve significantly. The incidence of adverse events (AEs) was not lower in the participants treated with NPDS ($N = 750$; RR, 0.47; 95% CI, 0.20–1.11) ($p > 0.05$). *Conclusion*. Current existing evidence is not sufficient to provide clinical guidance regarding the efficacy and safety of NPDS as a treatment for gout due to poor trial quality and lack of standardized evaluation criteria. Larger and more rigorously designed RCTs are needed in the future.

1. Introduction

Gout is a form of inflammatory arthritis resulting from the deposition of monosodium urate crystals in the synovial joints and soft tissues in the setting of hyperuricemia, i.e., elevated serum uric acid level (SUA >6.8 mg/dL) [1]. It is a chronic and often debilitating disease featured by recurrent swelling, redness and pain in one or multiple joints, which, if untreated or poorly controlled, can lead to limited musculoskeletal function, work-related disability, and significant morbidities such as hypertension, cardiovascular disease, chronic kidney disease, and poor health-related quality of life [2, 3]. The prevalence of gout, as well as its social and economic burden, has been rising globally, especially for middle-aged and elderly men. In 2007-2008, the prevalence of gout among US adults (8.3 million individuals) was 3.9% [4], and the incidence has continued to rise, due in part to economic development, dietary habit changes, aging population, *l*, and increasing-associated comorbidities [1, 5].

Both American and European rheumatology society guidelines for gout management focus on controlling gout flares and long-term reduction in total uric acid levels [6, 7].

Conventional medicines are limited in patients with gout because of interactions, inherent drug toxicity, and polypharmacy for patients with multiple comorbidities. Dietary therapy can lower uric acid levels, delay gout complications, and, in many cases, reduce or elimilate the need for conventional medications. Strong evidence suggests that diet is the most modifiable factor in gout management [8–10].

Natural product dietary supplements (NPDS), also named natural dietary supplements (NPS), are defined as systemically ingested, nonmineral, nonvitamin, and natural product-derived substances [11]. Previous studies have suggested that natural products including vegetables, nuts, legumes, fruits, and whole grains play an important role in the development of hyperuricemia and gout [12]. To date, no specific guidelines have recommended NPDS for gouty arthritis. Many publications have discussed the importance of natural compounds derived from animals, plants, and microbe sources for the treatment of human diseases [13]. Natural products are often regarded by consumers and patients as gentle and safe to human body because of their natural properties. In reality, the active ingredients of these natural products are compounds, which, if powerful enough to provide a beneficial effect, may also be powerful enough to cause adverse effects [14]. Natural products can regulate both the production and the excretion of uric acid, which may benefit the treatments of hyperuricemia, as reported in a previous review [15]. Several herbal compounds have been found to have antihyperuricemic effects in vitro and in vivo, suggesting they may play a role in the treatment of gout arthritis, while further research studies are needed to explore their potential action [16].

Standard epidemiology and treatment are required to further evaluate and verify the clinical efficacy and safety of the NPDS in gout treatment; therefore, providing valid and reliable data for accurate application of NPDS therapy are needed in the future [17].

Hence, our review aims to reveal some insights from previously published, randomized, controlled trials (RCTs) exploring the impacts of NPDS on gout patients and presents the evidence for natural dietary.

2. Materials and Methods

2.1. Search Strategy. A comprehensive electronic literature search was performed in the following databases from the database inception to August 14, 2019, limited to English language only, and excluding animal studies: Ovid MEDLINE (R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations and Daily, Ovid Cochrane Central Register of Controlled Trials, Ovid Embase, Ovid Cochrane Database of Systematic Reviews, and Scopus. Search terms used were (natural product OR diet OR supplement OR remedy OR remedies OR medicine OR prescriptions OR preparations OR extract OR indigenous OR traditional OR alternative OR complementary OR primitive OR Chinese OR China OR Japan OR Kampo OR oriental OR Asian OR Korean OR native American OR Indian OR Hindu OR siddha OR Tibet OR Africa OR Brazil OR rongoa OR Ayurveda OR herb OR natural OR plant OR flower OR fruit OR leaf OR leaves OR tea) AND (antigout OR anti-gout OR gout OR gouty OR hyperuricemia). All retrieved papers were manually scanned to identify further possible articles missed by electronic searching. Two reviewers screened the publications independently; any discrepancy between the two reviewers was resolved by consensus or by a discussion with a third reviewer if needed. The quality of RCTs was assessed using the Cochrane Collaboration Risk of Bias Assessment Tool [18]. The PRISMA flow chart is shown in Figure 1.

2.2. Participants. All subjects were aged 18 years or above diagnosed with gout based on the diagnostic criteria [6].

2.3. Interventions. All trials evaluating any natural product or natural compounds derived from animals, plants and microbe sources alone or in combination were included.

2.4. Comparators. All trials with any control groups such as no treatment/waiting list, sham therapy/placebo, non-pharmacological therapy (e.g., diet modification), and pharmacological therapy (e.g., allopurinol), alone or in combined therapies, were enrolled.

2.5. Outcomes. Outcome measures should include at least one or more of the following measurements: (1) pain relief evaluated by the visual analogue scale (VAS) or numeric rating scale (NRS); (2) joint function improvement by the NRS; (3) health-related quality of life by 36-Item Short-Form Health Survey (SF-36) and the Gout Assessment Questionnaire; (4) clinical efficacy identified by reduction in SUA levels; (5) inflammation markers by white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) level; and (6) safety was monitored by the reporting of adverse events (AEs).

2.6. Selected Trials. RCTs involving human participants were included in this review. Any study focused on the efficacy and safety of NPDS (single or compound) on gout was included. The abstract publication languages were restricted to English language only.

2.7. Data Extraction. Two review authors extracted relevant information independently from the retrieved trials. An Excel spreadsheet was designed to record descriptive data, the methodological quality of original studies, treatment regimen and duration, outcomes, efficacy, and AEs. The raw data such as means and standard deviations (SD) for continuous outcomes and event numbers or participants for dichotomous outcomes were extracted. Any disagreement between the two reviewers was resolved by a discussion between the two reviewers or referring to the original author.

2.8. Assessment of Risk of Bias in Included Studies. The potential bias of each trial was assessed using Cochrane Risk of Bias Tool for Randomized Controlled Trials [19] by two

Figure 1: PRISMA flow chart.

reviewers independently in the following domains: risk of selection, reporting, performance, detection, attrition, and other sources of. Each domain was assessed as either "high," "low," or "unclear" bias. Any discrepancy was resolved by consensus or resort to a third review author.

2.9. Data Synthesis. Treatment efficacy was analyzed with the Cochrane Collaboration's statistical software, RevMan version 5.3.5 (Cochrane Collaboration, London, UK). Outcomes of continuous variables were presented as mean differences (MDs) between the observation and control groups with the corresponding 95% confidence intervals (CIs). A meta-analysis was performed only if the studies were sufficiently homogeneous. Results of dichotomous data were presented as risk ratios (RRs) with the corresponding 95% CIs. The random-effect model was applied, if $I^2 > 50\%$ indicated significant heterogeneity among the studies. Subgroup analysis was performed according to the different NPDS interventions. The study number of each outcome was insufficient, so publication bias could not be analyzed with a funnel plot.

3. Results

3.1. Characteristics of the Trials. The literature search retrieved 2021 references, of which 20 were duplicates; 1856 articles were rejected on reading their titles and abstracts, and 145 articles were identified for detailed review. Finally, 9 articles were included (Figure 2).

A total of nine RCTs with 1156 participants were included in this review. Seven trials were conducted in China [20–26], 1 in the USA [27], and 1 in New Zealand [28]. All the studies were published from 2008 to 2019. Seven studies evaluated herbal therapies, with the remaining two evaluating skim milk and cherry extract. Six trials had two arms;

Figure 2: Risk of bias summary.

two trials had three arms; one trial had four arms. Five trials reported natural product formulation in decoction, two in capsules, and one in powder. Five trials recorded gout flare-up; four trials did not record gout flare-up. Five trials reported no AEs; four trials reported AEs. Two trials assessed the compliance of patients (Table 1).

3.2. Methodological Quality. Seven of the nine RCTs had lower risk regarding randomization due to reported methods of sequence generation. Three trials used a random number table [22, 24, 28], two trials referred to SAS software for setting the random variable seeds [20, 25], one adopted the random envelope method [21], and the other used online computer random number generator [27]. The other two trials had an unclear risk for random sequence generation due to lack of detail [23, 26]. Two trials had high-risk bias for allocation concealment [20, 25]. Two trials had unclear randomization without description in sufficient detail [23, 26]. Five trials had lower risk bias for allocation concealment [21, 22, 24, 27, 28]. Three trials were double-blinded trials with a lower risk for blinding participants and study personnel [21, 22, 28]. Two trials did not use a blinding method to participants and study personnel during the study with a high risk due to the great appearance difference between Chinese medical formulation pills and decoction with conventional pharmaceutics such as indomethacin, benzbromarone, and allopurinol [20, 25]. The other four trials had insufficient information to permit a judgment [23, 24, 26, 27]. Three trials could be regarded as lower risk for blinding of outcome assessors [20–22]. One trial had high risk in outcome assessment due to a lack of blinding [25]. Five trials had unclear risk in outcome assessment without description in sufficient detail [23, 24, 26–28]. Two trials reported cases with poor compliance which were dropped from the study [22, 24], two trials reported cases lost to follow-up [21, 28], and two trials had missing values in the results [20, 27], which might affect the results. Four trials were considered as a high risk of attrition bias with selective data reporting [21, 23, 27, 28]. The other five trials were classified as low risk [20, 22, 24–26]. For poor blinding and allocation design, one trial was assessed as a high risk of bias in the other resource [25]. Two trials were estimated at a lower risk because of their study design [23, 24]. The other

Table 1: Characteristics of the included studies.

Author	Year	Country	Condition	Arms	Population	NPDS	Formulation	Control group	Evaluation criteria	Therapeutic efficacy
Shi et al. [25]	2008	China	Gout	4	107	Modified prescriptions of simiao pill	Pill	Indomethacin and benzbromarone	Guiding principle of clinical research on new drugs of traditional Chinese medicine	Joint arthralgia, erythema, and swelling, blood uric acid and gout recurrence↓
Renbin et al. [26]	2008	China	Gouty arthritis	2	120	Modified simiao tang	Decoction	Allopurinol tablet	An assemblage of guiding principles of clinical and preclinical research on new drugs	The index of swelling and pain in the joints, BUA, and CRP ↓
Song et al. [24]	2008	China	Gout	2	200	Weicao capsule	Capsule	Tong Feng Ding capsule	Diagnosis and curative effect standard of traditional Chinese medicine disease	Joint swelling and pain, laboratory indices↓
Zhang et al. [23]	2010	China	Acute gouty arthritis	2	67	Blood-letting cupping plus herbal medicine	Decoction	Diclofenac sodium enteric-coated tablets	The Budzyuski index of pain	Joint pain and swelling, BUA↓
Dalbeth et al. [28]	2012	New Zealand	Gout	3	120	SMP/GMP/G600	Powder	GMP, G600	The 10-point Likert scale	Gout flare, the severity of joint pain↓
Wang et al. [22]	2014	China	Acute gouty arthritis	2	176	Chuanhu antigout mixture	Decoction	Colchicine	—	Gout recurrence, CRP, AEs↓
Xie et al. [21]	2017	China	Intercritical and chronic gout	2	210	Compound tufuling oral liquid	Decoction	Placebo solution	—	The frequency of recurrent joint swelling and pain, BUA↓
Yu et al. [20]	2018	China	Gout with hyperuricemia	3	72	Yellow-dragon Wonderful-seed Formula	Decoction	Allopurinol group	SF-36	Joint pain, BUA, and CRP↓
Singh et al. [27]	2019	USA	Gout	2	84	Cherry extract	Capsule	Diet modification	—	Joint pain intensity↓, average pain in last 24 h↓

NPDS: natural product dietary supplement; CRP: C-reactive protein; BUA: blood uric acid; SMP: skim milk powder; GMP: glycomacropeptide; G600: G600 milk fat extract.

six were regarded as an unclear risk of other sources of bias [20–22, 26–28]. The methodological quality of the nine RCTs was poor because of two or more criteria listed as a high or unclear risk of bias. The study methodology was summarized in Figure 2.

3.3. Measurable Outcomes

3.3.1. Joint Swelling, Pain, and Limitation Relief. Study results suggested that NPDS treatments were superior to control interventions in joint swelling, pain, and activity limitation, except one trial which reported improvement just in joint pain-relieving, not in swelling and activity limitation [22]. In the double-blind RCT, 176 participants with acute gouty arthritis were divided to either the chuanhu antigout mixture 250 ml orally daily plus placebo (mimetic agent of colchicine) or colchicine 1 pill (0.5 mg/pill) orally, twice daily, for 3 days and later once daily. All the participants orally administered pain-reliving medication etoricoxib 60 mg once for 10 days. Results indicated that the chuanhu antigout mixture had a favorable effect in decreasing of joint pain score, instead of joint swelling and limitation compared with colchicine [22].

The evaluation criteria for therapeutic effect of joint swelling, pain, and activity limitation varied in the enrolled trials. Besides three trials [21, 22, 27], the other six trials have different criteria to assess the therapeutic effect [20, 23–26, 28]. To investigate the compatibility of modified prescriptions of simiao pill on patients with acute gouty arthritis, Shi et al. [25] evaluated the clinical efficacy with "guiding principles of clinical research on new drugs of traditional Chinese medicine," which included 4 grades (none, mild, moderate, and severe), with scores 0, 1, 2, and 3, respectively. Affected joint arthralgia, erythema, and swelling, as well as blood uric acid level and gout recurrence were evaluated comprehensively. In the other trial to observe the efficacy of the modified simiao tang for gouty arthritis and blood uric acid, Renbin et al. [26] used "an assemblage of guiding principles of clinical and preclinical research on new drugs (western drugs)," which included "clinical cure, markedly improved, improved, and ineffective." The index of swelling and pain in the joints were scored according to the swelling and pain in the joints before and after treatment. Song et al. [24] classified the therapeutic effect in three grades: "markedly effective, effective, and ineffective" on the basis of the criteria "diagnosis and curative effect standards of traditional Chinese medicine disease." These criteria covered joint symptoms and signs as well as laboratory indices. Zhang et al. [23] used the criteria to assess efficacy effects with three categories of "cured, improved, and failed," based on both joint swelling and pain. The affected joint pain was assessed with the assessment of the Budzyuski index, which graded in 0, 1, 2, 3, 4, and 5 with increasing pain severity. Dalbeth et al. [28] recorded gout flare and severity of pain with the 10-point Likert scale. Yu et al. [20] adopted the SF-36 scale to evaluate the change in affected joint swelling, pain and activity. The levels of SUA and urine urate were measured at week 0 and week 4. A meta-analysis of joint swelling, pain, and limitation relief could not be employed due to the inconsistency and heterogeneity of interventions, controls, and outcome measures.

3.3.2. Serum Uric Acid (SUA) Level. Four RCTs included 420 patients (211 patients in the observation groups and 209 in the control groups) provided SUA changes before and after treatment. The study result showed that there was no significant difference between the NPDS interventions and the control groups in terms of SUA reduction (SMD −1.80, 95% CI: −4.45 to 0.86) ($p > 0.05$). Because $I^2 = 99\%$, a random-effect model was used for the analysis. Subgroups were divided by treatment; the subgroup meta-analysis showed that modified simiao tang was better in reducing SUA than the allopurinol tablet [26]. Due to the lack of detail in the reports, no meta-analysis was conducted in one-third of the trials [21–23, 28]. In addition, the other study reported serum urate value instead of SUA level [27] and was temporarily omitted from the meta-analysis. The effects of NPDS on the serum uric acid (SUA) levels of patients with gout are shown in Figure 3.

3.3.3. CRP Value. Four studies [20, 22, 26, 28] reported CRP value changes pre- and posttreatment, and the meta-analysis of three trials [20, 22, 26] showed that the NPDS therapies were not more effective than treatment with conventional pharmaceutics such as allopurinol and colchicine ($N = 232$; SMD, −0.26; 95% CI, −0.55 to 0.04) ($p > 0.05$). In the subgroup meta-analysis, modified simiao tang was superior to allopurinol tablets in decreasing CRP level [26]. The other study [28] which reported CRP change roughly was just temporarily omitted from the meta-analysis. The effects of NPDS therapies on CRP levels of patients with gout are shown in Figure 4.

3.3.4. Adverse Events. Five RCTs including 750 patients with 408 in the experimental groups and 342 in the control groups provided safety evaluation data [21, 22, 24, 27, 28]. The meta-analysis showed that NPDS therapies had not fewer adverse events than the control groups ($N = 750$; RR, 0.47; 95% CI, 0.20–1.11) ($p > 0.05$). In total, 112 patients experienced side effects. In the subgroup meta-analysis, chuanhu antigout mixture and compound tufuling oral-liquid had fewer AEs than colchicine and placebo solution separately [21, 22] ($p < 0.05$). The AEs included bitter taste, poor appetite, and leucopenia, as well as gastrointestinal reactions such as nausea, vomiting, diarrhea, and flatulence, which could be relieved by temporary dosage reducing or medication pause [22, 24, 28]. Xie and his colleagues [21] found fewer leucopenia incidence (2.16%) in the treatment group of the compound tufuling oral liquid compared with that of the placebo solution (9.86%), and more detailed information was not further reported. No other serious adverse events were recorded. Adverse events caused by NPDS interventions in patients with gout are shown in Figure 5.

Figure 3: Effects of NPDS on the serum uric acid (SUA) levels of patients with gout.

Figure 4: Effects of NPDS therapies on the CRP levels of patients with gout.

4. Discussion

Nine RCTs were included in our review, which compared Chinese herbs, cherry extract, and compound skim milk powder with a placebo control, conventional pharmaceutics, diet modification, and other Chinese medications for the treatment of gouty arthritis. Outcome measurements cover affected joint swelling, pain, and activity relieving, SUA and CRP value change, and AEs incidence.

The methodological quality of the included trials was poor with two or more assessments of high or unclear risk of bias. Despite mostly positive results in the therapeutic efficacy evaluation, the evidence data evaluating clinical efficacy were not convincing and robust, which could be attributed to the variety of evaluation criteria. Some studies comprehensively evaluated joint arthralgia, swelling, and activity limitation as well as laboratory indices [24, 25], some focused on joint swelling and pain [23, 26, 28], and one

Figure 5: Adverse events caused by NPDS interventions in patients with gout.

utilized SF-36, SUA and urine urate for assessment [20]. Poor methodological quality and the small number of the included trials were the other contributors. Therefore, to fully assess the clinical role of NPDS in the treatment of gout, high-quality RCTs of standardized evaluation criteria with international recognition are required in the future.

Until now, nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, corticosteroids, and prophylaxis are guideline-recommended pharmaceuticals with high-strength evidence for individuals with gout [2], but the use of these drugs is often associated with unwanted side effects and adverse events [29]. NPDS interventions attract a great deal of public attention due to their perception as being nontoxic, natural, and economical properties [16]. Our review suggests that NPDS interventions may have some clinical efficacy in terms of symptom improvement, reduction in SUA and CRP levels, all with a lower level of adverse events compared with standard pharmacological treatment. They may possibly be associated with potential toxicity due to their multiherbal components and different formulations (e.g., decoctions and powders) with may be associated with gastrointestinal reactions.

Numerous articles have been written describing the important role and safety of NPDS therapies in patients with gout [15, 16, 30–34]; but until now, no review has quantitatively synthesized the relevant RCTs. To our knowledge, this review is the first overview of available RCTs investigating the efficacy and safety of NPDS approaches in gout management. This review highlights the impact of natural products on gout management and permits the identification of current evidence gaps, thus informing clinical decision making and guiding future research.

This review does have limitations. First, due to the study design limitation, we only searched RCTs published in English, thus possibly omitting important studies which appeared only in non-English journals. In particular, Chinese language journals may be an additional resource worthy of systematic review. Second, the included RCTs are of low quality. This makes the comparative analysis difficult to perform and reduces the confidence in the meta-analysis.

Finally, the heterogeneity of the included studies was significant. More high-quality trials with large sample size RCTs are required in the future.

5. Conclusion

This review provides insight into the contemporary treatment of gout with NPDS. NPDS appeared to be superior to control groups in affected joint pain, swelling, and activity limitation, while not in decreasing SUA and CRP levels or the incidence of AEs. Current existing evidence is insufficient to permit a definitive statement about the efficacy and safety for gout patients due to poor trial quality and standardized evaluation criteria. Further larger and more rigorously designed RCTs are needed in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Study of the Binding Interaction between Wortmannin and Calf Thymus DNA: Multispectroscopic and Molecular Docking Studies

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Introduction. Wortmannin (WTN) is a steroid metabolite that inhibits phosphatidylinositol 3-kinase and other signaling pathways. Structurally, the WTN consists of a cyclopentanophenanthrene-like structure with several oxygen-rich moieties which have the potential to interact with deoxyribonucleic acid (DNA) molecules. *Methods*. We aim to evaluate the WTN and calf thymus DNA (ct-DNA) interaction with molecular docking using the AutoDock 4.2 software. UV and fluorescence spectroscopy and viscosity techniques were performed to confirm the *in silico* analysis. *Results*. Molecular docking showed that the WTN interacted with ct-DNA via hydrogen bonds at guanine-rich sequences. The number of hydrogen bonds between the WTN and DNA was 1-2 bonds (average 1.2) per WTN molecule. The *in silico* binding constant was $2 \times 10^3\,M^{-1}$. UV spectroscopy showed that the WTN induced a hyperchromic feature without wavelength shifting. The WTN and DNA interaction led to quenching of DNA-emitted fluorescence. The different concentrations of WTN had no effect on DNA viscosity. Taken together, our results demonstrated WTN interacts with DNA in the nonintercalating mode, which is considered as a new mechanism of action. *Conclusion*. These results suggest that the WTN may exert its biological effects, at least in part, via interaction with DNA.

1. Introduction

In 1957, Brian et al. isolated the wortmannin (WTN) from filtrates of *Penicillium wortmannii* Klocker, and Millan et al. proposed the molecular structure based on chemical and spectroscopic evidence, which is illustrated in Figure 1 [1, 2]. This steroid metabolite contains a cyclopentanophenanthrene-like structure, in which A ring is modified to lactone ring and a heterocyclic furan is added to cyclopentanophenanthrene as the fifth ring. WTN belongs to furanosteroids which originate from lanosterol [3].

The subsequent studies confirmed this structure and reported several biologic functions of WTN including antifungal, cell arresting, proapoptotic, and anti-inflammatory activities [5–8]. Due to anti-inflammatory effects of WTN, it may be a therapeutic option to treat of inflammatory disorders such as gout and rheumatoid arthritis.

In 1993, Yano et al. reported that WTN interacts with phosphatidylinositol 3-kinase and inhibits its signaling pathway [9]. The inhibitory effects of WTN on NF-kB and MAPK signaling pathways were also reported [10–12].

Here, we hypothesized that WTN interacts with deoxyribonucleic acid (DNA) molecules that mediate some of its biological function. This hypothesis is based on the evidence indicating that most steroid metabolites modulate the DNA function such as gene expression by direct interaction with DNA and histones [13, 14]. A pioneer study by Cohen and Kidson demonstrated that steroids interact with guanine homoribopolynucleotides predominantly via hydrogen (H) bonds. Polyguanine has maximum hydrogen atoms for H bond formation, and progesterone presents maximum affinity to polynucleotides due to the oxygen-rich structure [15]. On the other hand, WTN contains eight oxygen atoms, which can contribute to the formation of H bonds. In

Figure 1: WTN structure (obtained from PubChem [4]).

addition, the WTN contains a bay region at the cyclopentanophenanthrene-like structure that may mediate the possible WTN-DNA interaction via the hydrophobic interaction [16].

Therefore, here we propose that WTN may exert its biological effects, at least in part, via interaction with DNA besides the putative inhibitory effects on signaling pathways. Nevertheless, to the best of our knowledge, there is no report regarding *in silico* and/or *in vitro* experiments about the WTN and DNA interaction. In this study, we evaluated WTN and calf thymus DNA (ct-DNA) interaction by molecular docking, viscosity, UV spectroscopy, and fluorescence spectroscopy.

2. Materials

Highly polymerized ct-DNA and WTN *Penicillium funiculosum* were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tris (hydroxymethyl) aminomethane was obtained from Merck (Darmstadt, Germany). All experiments were performed in Tris buffer solution (10 mM‘ pH 7.4). The stock solution of ct-DNA was prepared by dissolving an appropriate amount of ct-DNA in buffer solution by shaking gently. The stock solution was stored for 24 h at 4°C and utilized not more than 4 days. The concentration of ct-DNA in stock solution was 4.4×10^{-4} M that determined by spectrophotometry at 260 nm using an extinction coefficient (ε) of 6600 $M^{-1} \cdot cm^{-1}$. DNA purity was checked using absorption of DNA in 260 nm (A_{260}) and 280 nm (A_{280}). The A_{260}/A_{280} ratio was 1.85, indicating that the ct-DNA was sufficiently pure and protein free [17, 18]. Stock solution of WTN (5×10^{-3} M in ddH_2O) was prepared immediately before experiments due to instability.

3. UV Absorption Spectra Analysis

UV spectra analysis is a simple technique for ligand-DNA interaction. Absorbance in 230–300 nm wavelength range was measured, using a spectrophotometer (Analytikjena spekol 2000) equipped with a 1.0 cm quartz cell. For quantification of the WTN-DNA binding constants, the absorption spectra measurements were performed at different concentrations of DNA ((0, 0.66, 1.1, 1.54, 2.2) × 10^{-5} M) and constant WTN concentration (1×10^{-5} M).

4. Fluorescence Emission Spectra Analysis

Fluorometric assay was performed, using a Shimadzu Spectrophotometer (RF-5301 PC) equipped with a quartz cell of 1.0 cm path length. WTN fluorescence emission spectra were recorded at 280–320 nm by excitation in 260 nm. Analysis was performed at constant concentration of WTN (7.5×10^{-6} M) in presence of different ct-DNA concentrations ((0, 0.22‘ 0.66‘1.1, 1.54, 1.98, 2.42‘ 2.86) × 10^{-5} M).

5. Viscosity Measurements

The effect of WTN on the viscosity of DNA was measured using a Lovis 2000 M digital micro viscometer. The temperature was kept constant at 25°C. The DNA concentration was 6.6×10^{-6} M while varying the WTN concentrations ((0, 0.25, 0.5, 0.75, 1) × 10^{-5} M). The data were presented as (η/η_0) 1/3 vs [WTN]/[DNA], where η and η_0 are the viscosity of ct-DNA in the presence and absence of WTN, respectively [19].

6. Molecular Docking Study

Docking study was performed, using the AutoDock Tools 4.2 software. Structure of B-DNA dodecamer (PDB ID: 1BNA) was obtained from the protein data bank (http://www.rcsb.org) [20]. The WTN molecular structure was drawn, and energy was minimized using the HyperChem 8.0.6 software. The DNA file was prepared by deleting water molecules followed by addition of polar H atoms and Gasteiger charge. Grid box dimensions at grid points in $x \times y \times z$ directions were set to 56‘ 59‘ and 49 with a grid spacing of 0.375 Å. The coordinate centers of grid box were set to $x = 1.389$‘ $y = 2.417$‘ and $z = 13.472$. Lamarckian genetic algorithms were used to carry out molecular docking calculations. The number of runs was set to 100. All other parameters were assigned the default values [21, 22].

7. Results and Discussion

7.1. UV Absorption Spectroscopy. Figure 2(a) shows the UV spectra of the WTN-DNA complex. WTN showed an absorbance at 260 nm, and the peak intensities were enhanced with increasing the concentration of ct-DNA. A hyperchromic effect without red or blue shift was observed for all molar ratios, which demonstrated there is a nonintercalative binding interaction [23, 24]. The observed spectral changes proposed that WTN interacts with DNA at the groove-binding model [23]. The binding constant (K_b) was calculated using equation (1) from UV data [25].

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} \times \frac{1}{K_b\,[\mathrm{DNA}]}, \quad (1)$$

Figure 2: (a) UV spectra of WTN in the presence of different concentrations of ct-DNA (pH 7.4 and room temperature). (b) Plot $A_0/A - A_0$ versus 1/[DNA] for K_b calculation.

where A_0 and A are the absorbance of WTN in the absence and presence of ct-DNA, respectively. The ε_G and ε_{H-G} are the absorption coefficients of WTN and WTN-DNA complex, respectively. The K_b is $2 \times 10^3\ M^{-1}$ obtained by plotting $A_0/A - A_0$ versus 1/[DNA] (Figure 2(b)). This value for K_b suggested the groove binding model that is similar to previous studied agents (e.g., Cu–Sn_2 complex, $1.67 \times 10^4\ M^{-1}$ [26], metformin complex, $8.3 \times 10^4\ M^{-1}$ [27], Ho(phen) $^2Cl^3$_H_2O ($1.36 \times 10^4\ M^{-1}$ [28], adefovir dipivoxil, $3.33 \pm 0.2 \times 10^4\ M^{-1}$ [20], and sorafenib complex, $5.6 \times 10^3\ M^{-1}$ [29]).

7.2. Fluorescence Spectra. The fluorescence emission spectra of WTN alone and in the presence of ct-DNA are shown in Figure 3(a). WTN has emission spectra with maximum emission at about 291 nm when excited at 260 nm. The fluorescence of WTN was quenched by ct-DNA without any shift in maximum emission in a concentration-dependent manner. Reduced emission intensities (quenching) confirmed WTN-DNA interaction. Fluorescence quenching is described by the Stern–Volmer equation (equation (2)) [30, 31]:

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q], \quad (2)$$

where F_0 and F denote the steady state fluorescence intensities in the absence and presence of the quencher Q, respectively, K_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the molecule without the quencher, $[Q]$ is the quencher concentration, and K_{sv} is the Stern–Volmer quenching constant. Figure 3(b) shows the Stern–Volmer plots of F_0/F versus $[Q]$.

The calculated K_{sv} value was 3.5×10^5, which is higher than the classical groove binding small molecule [32–34]. This severe quenching phenomenon indicated that WTN interacts with DNA strongly and probably via the intercalation model.

Apparent binding constant (K_b) and the binding stoichiometry (n) were calculated according to Zhang et al.'s equation (3) [35]:

$$\log\left[\frac{(F_0 - F)}{F}\right] = \log K_b + n \log [Q], \quad (3)$$

where F_0 and F are the fluorescence intensity in the absence and presence of a quencher at various concentrations $[Q]$, respectively. Figure 3(c) shows the plot of log $[(F_0 - F)/F]$ versus log [DNA]. The intercept is about 1.47, which indicates WTN binds to ct-DNA in more than one position. This observation is consistent with *in silico* docking analysis, which indicates in some positions WTN binds to ct-DNA via two H bonds.

7.3. Viscosity Study. Viscosity experiment is an effective tool to determine the binding mode between small molecules and ct-DNA [36]. In the intercalative binding mode, the DNA double helix separation and conformational change result in an increase in DNA viscosity while groove binding interaction has no significant effect on the DNA viscosity [37, 38]. The effect of WTN on the viscosity of ct-DNA at 25°C is shown in Figure 4. There is no significant change in the ct-DNA viscosity by increasing concentration of WTN. This observation suggests that the groove binding is the mode of interaction between WTN and ct-DNA.

7.4. Molecular Docking Study. Molecular docking study is an appealing method to understand the interaction between ligands and DNA [39]. Molecular models were made to discuss the binding modes using the AutoDock software for the interactions of WTN with ct-DNA (PDB ID: 1BNA). The structure of WTN was drawn and subjected to energy optimization [40].100 docking runs were successfully carried out' and the obtained runs data for the conformers are listed in Table 1. The first conformation was taken from one of the lowest binding energy docking conformation. Our results showed WTN incorporated to DNA and the H bond formed between amino groups at C-2 on guanine and oxygen atom of the acetoxy group on C-11. In some conformations, the oxygen atom of furan and lactone rings was involved in H bond formation. There are 63 groups in which H bonds formed, including 39 groups with single H bond with

Figure 3: (a) Fluorescence emission spectra of WTN in the presence of different concentrations of ct-DNA. (b) Stern–Volmer plot of F_0/F versus [Q]. (c) Plot of log $[(F_0 - F)/F]$ versus log [DNA].

Figure 4: Viscosity of ct-DNA in constant concentration (6.6×10^{-6}) at 25°C in presence of WTN at different concentrations ((0, 2.5, 5, 7.5, 10) $\times 10^{-6}$).

Table 1: The data on DNA and WTN docking obtained from the AutoDock software.

Rank	Run	Lowest binding energy (kcalM^{-1})	Mean binding energy (kcalM^{-1})	Number of cluster	Ki (μM)	Cluster RMSD	Reference RMSD
1	8	−6.83	−6.61	28	9.84	0.00	30.84
2	63	−6.28	−6.23	29	24.88	0.00	32.52
3	37	−6.20	−6.08	13	28.35	0.00	31.86
4	31	−6.19	−6.04	4	29.09	0.00	29.51
5	23	−6.11	−6.01	12	33.43	0.00	31.40
6	4	−5.99	−5.96	3	40.75	0.00	34.94
7	80	−5.91	−5.86	11	46.82	0.00	31.71

(a) (b)

Figure 5: (a) *In silico* groove binding of WTN and DNA. (b) H bond (green) between WTN and guanine.

guanine, 12 groups with two H bonds simultaneously with guanine and cytosine‘ 11 groups with cytosine, and 1 group with two H bonds between one WNT and two guanines. These results proposed that WTN have interaction predominantly with guanine. The energetically most desirable conformation of the docked pose is shown in Figure 5.

8. Conclusion

The results of this study indicated that WTN interacts with DNA molecules. Our results did not provide strong evidence about the mode of interaction but based on *in silico* molecular docking, UV absorption spectroscopy, fluorescence emission spectroscopy, and viscosity measurement, a fluctuation between intercalation and groove binding model. The H bond may be an important force in this interaction where DNA is a hydrogen donor and the WTN oxygen is considered as a H acceptor. To the best of our knowledge, this is the first report that revealed a new and nontraditional mechanism for the biologic effects of WTN.

Taken together lack of a red-blue shift in UV spectra and the value of obtained K_b, it is suggested the groove-binding mode. The UV spectra results and groove-binding mode were confirmed by *in silico* analysis. However, the high K_{sv} value suggests the intercalation mode of interaction.

As a third mechanism, it is possible that the interaction may occur through both groove binding and intercalation, which was reported for some molecules previously [41, 42].

Circular dichroism, isothermal titration calorimetry, and ionic strength and melting temperature experiments could be performed to elucidate the precise interaction mode. However, they were not carried out due to WNT instability. In addition, it should be determined whether the WTN-DNA interaction influences the gene expression and other biologic functions. Taken together, these results suggest that the WTN may exert its biological effects, at least partly, by interaction with DNA besides the putative signaling pathway inhibitory properties.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

A Traditional Clinic Chinese Medicine Prescription Qu-Zhuo-Tong-Bi (*QZTB*) Alleviates Gouty Arthritis in Model Rats

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Qu-Zhuo-Tong-Bi (*QZTB*) is an empirical traditional Chinese medicine prescription for treating acute gouty arthritis clinically without serious adverse effects in mainland China. However, the biochemical mechanism underlying the therapeutic action produced by *QZTB* treatment against acute gouty arthritis and the effect on recurrent attack remain unknown. In this study, we investigated the anti-inflammatory and analgesic effects of *QZTB* on acute gouty arthritis and the recurrent attack in rats, as well as the underlying mechanisms. The gouty arthritis model was established by intra-articular injection of monosodium urate (MSU) crystal suspension (2 mg/50 μL) into the right ankle joint of Sprague Dawley (SD) male rats. *QZTB* (500 mg/kg) and the positive control drug meloxicam were administrated by gavages twice a day for 7 days before, or 3 days after, first MSU injection in different experiments, respectively. The analgesic effects were evaluated by pain-like behaviors and hind paw mechanical withdrawal threshold testing. The anti-inflammatory activities were evaluated by ankle swelling measurement, histologic examination, NLRP3 inflammasome, and inflammatory cytokine expression. Western blot and quantitative real-time PCR were used to detect the protein and mRNA expressions of NLRP3. IL-1β and TNF-α level in the blood serum were detected by enzyme-linked immunosorbent assay (ELISA). *QZTB* can suppress ankle swelling and synovial inflammation in the MSU-induced gouty arthritis rat model. *QZTB* alleviated the acute attack and prevented the recurrent attack of gouty arthritis. In addition, *QZTB* treatment significantly decreased both mRNA and protein levels of NLRP3, as well as the production of IL-1 and TNF-α in the ankle joint of model rats. Taken together, these results suggest that *QZTB* may be a promising herbal formula for the prevention and treatment of gouty arthritis in humans.

1. Introduction

Gouty arthritis is an inflammatory disease caused by the deposition of monosodium urate (MSU) crystals in the joints, associated with purine metabolic disorder [1, 2]. Characterized by high serum uric acid level, acute inflammation, swelling of one or more synovial joints, and severe pain, it is commonly the first clinical manifestation of gout [3]. Recurrent attacks of gouty arthritis can lead to the formation of tophi and dense crystal deposits surrounded by fibrotic tissue, which cause disfigurement, bone destruction, and disability [4]. The management of gout, especially the recurrent acute attacks of chronic gouty arthritis, is still a problem to be resolved [5]. NLRP3 inflammasome, a member of nucleotide-binding oligomerization domain-(NOD-) like receptor (NLR) family, plays critical roles in gouty arthritis and many pathological inflammatory conditions [6, 7]. Current research studies show that MSU crystals trigger an inflammatory response through the activation of the NLRP3 inflammasome, which promotes IL-

1β production. IL-1β can activate other proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) which is critical for the initiation and propagation of the inflammatory response in gouty arthritis [8, 9]. Chemical compounds, such as nonsteroidal anti-inflammatory drugs, including naproxen and indomethacin, colchicine, corticosteroids, or a combination of them, are recommended as first-line systemic treatment for acute gouty arthritis [1, 10]. However, these drugs are associated with serious side effects, including chronic renal toxicity, gastrointestinal bleeding, and hepatic damage [11].

Traditional Chinese medicine (TCM) is an important part of complementary and alternative medicine, which has been widely applied to treat gouty arthritis and to cope with the complicated pathologic states of gout in different stages [12]. Our previous study has revealed that Qu-Zhuo-Tong-Bi (*QZTB*), an empirical traditional Chinese medicine prescription, has definite effects on treating hyperuricemia and gouty arthritis with less adverse reactions [13]. Furthermore, *QZTB* is a clinical experienced prescription, which has been widely prescribed to rheumatoid patients in China. Other functions of *QZTB*, such as reinforcing renal function, promoting blood circulation, and relieving pain, also have been clinically demonstrated [13–15]. However, the underlying mechanism of its anti-gouty arthritis and analgesic properties is still obscure.

Here, to investigate the acute anti-gouty arthritis and analgesic effect of *QZTB*, we applied the MSU crystal-induced animal model. And we further studied its effects on the recurrence of the second gouty attack. Our results demonstrated that *QZTB* had the potential of strong anti-inflammatory and analgesic effects and prevented the recurrent attack of gouty arthritis. The mechanism in anti-inflammation of *QZTB* could be contributed to the inhibition of the activation of the NLRP3 inflammasome.

2. Methods

2.1. Animals. Sprague Dawley male rats (180 ± 20 g body weight) were purchased from the experimental animal center of Zhejiang Chinese Medical University (SCXK (Yu)-2005-3001, Zhejiang Province, P.R. China). They were acclimatized for a week in a standard light- and temperature-controlled room at 22 ± 2°C, in 55 ± 10% relative humidity with a 12 h dark-light cycle, and they were freely fed with plentiful food and water. The animals were treated and cared for in accordance with the guidelines of experimental animal administration issued by the State Committee of Science and Technology of the People's Republic of China. The experimental protocol was approved by our departmental ethics committee.

2.2. Preparations of QZTB and Reagents. As reported in our previous papers [13, 16, 17], *QZTB* was composed of *Glabrous Greenbrier Rhizome* (Tu Fu Ling, 60 g), *Dioscorea septemloba Thunb.* (Bi Xie, 30 g), *Maydis stigma* (Yu Mi Xu, 15 g), *coix seed* (Mi Ren, 30 g), *Alismatis rhizome* (Ze Xie, 15 g), *Humulus scandens* (15 g), *Parasitc loranthus* (Sang Ji Sheng, 15 g), *Herba Siegesbeckiae* (Xi Qian Cao, 18 g), *Corydalis Rhizoma* (Yan Hu Suo, 18 g), *turmeric* (Jiang Huang, 12 g), and *Citrus medica* (12 g). All herbs were firstly soaked in 10 times distilled waters of their total weight for 1 h and then extracted twice with distilled water under reflux for 2 h. The filtered extracts were concentrated using a rotary evaporator at 50°C and freeze-dried into powder (*QZTB*). All the herbs were procured from Medical Pieces Co., Ltd., of Zhejiang Chinese Medical University (Hangzhou, Zhejiang, China). Meloxicam was purchased from Huadong Pharmaceuticals Ltd., Hangzhou, China. The *QZTB* powder and meloxicam were dissolved in 0.5% carboxymethyl cellulose in phosphate-buffered saline to the required concentration. Fresh solution was prepared before each experiment. The deionized water was purified by a Milli-Q system (Millipore, Bedford, USA). All other reagents used were of analytical grade and were purchased locally. The antibody against NLRP3 (cat# 15101) and β-actin (cat# 4970) was from Cell Signaling Technology (Danvers, MA). Levels of the cytokines IL-1β (cat# 583311, Cayman Chemicals Company, USA) and TNF-α (cat# 500850, Cayman Chemicals Company, USA) were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. RNA isolation kit was from Qiagen (cat# 300112, Invitrogen, CA, USA).

2.3. Induction of Gouty Arthritis with MSU Crystals and Medicine Treatment

2.3.1. Synthesis of MSU Crystals. The preparation of MSU crystals was reported previously [18–20]. Briefly, about 4 g of uric acid was dissolved and heated in 800 ml H_2O with NaOH (9 ml/0.5 N), adjusted to pH 8.9 at 60°C, cooled overnight in a cold room, washed, and dried. Needle-like crystals were recovered and were suspended in sterile saline.

2.3.2. MSU Crystal-Induced Gouty Arthritis in Animals. Adult male SD male rats were randomly divided into the following five groups, which consisted of 6–8 animals each: (1) the sham group (Sham), (2) gouty arthritis model group with MSU crystals injection (MSU + vehicle), (3) *QZTB*-treated group (500 mg/kg body weight) with MSU crystal injection (MSU + *QZTB*), and (4) the meloxicam-treated group (3 mg/kg body weight) with MSU crystal injection (MSU + meloxicam). 0.2 ml (4 mg) of MSU crystal suspension was injected into the right ankle joints [19, 21]. In order to evaluate the preventing efficacy, *QZTB* (500 mg/kg) and meloxicam (3 mg/kg) were orally administrated twice a day for 7 days before MSU injection. In the second experiment, *QZTB* (500 mg/kg) and meloxicam (3 mg/kg) were orally administrated for 3 days after the first injection of MSU crystal suspension. Following the treatment of *QZTB*, no evidence of systemic adverse effects was observed in any study group. The dosages of *QZTB* used in this study were based on those used in previous studies, and the dosages of meloxicam drugs were calculated based on the weight of rats [14, 15, 22]. *QZTB* and meloxicam were suspended in 0.5%

carboxymethylcellulose in phosphate-buffered saline and administered.

2.4. Measurement of Ankle Swelling. The inflammation was quantified by measuring the ankle thickness (in millimeter) using a digital caliper at different time points for each rat before and 5 h, 24 h, 30 h, and 48 h after MSU crystal injections in the first experiment. The results were expressed as the test value of the ankle thickness. At the end of the experimental period (48 h) after the first or second MSU crystal injection, respectively, the rats were euthanized by isoflurane overdose. Blood samples were collected from the eye socket vein 24 h after MSU crystal injection and centrifuged at 3000 rpm for 10 min at 4°C, and the serum was collected and stored at −80°C until further tests.

2.5. Animals Were Assessed for Nociception (Touch Allodynia and Overt Pain-Like Behaviors) Post-MSU Injection. Measurements of touch allodynia (significant decrease in paw withdrawal threshold (PWT) compared with baseline values) were carried out by using von Frey monofilaments as previously reported [23]. Behavioral tests ($n = 6$–8 animals/group) were carried out 16 h after injection of MSU by blinded examiners. Animals were placed in a chamber with a mesh metal floor (20 × 30 cm), covered by an opaque plastic dome 10 cm high, and were always allowed to habituate for 1 h before any test. Withdrawal threshold to tactile stimulation was measured with a set of von Frey hairs with a bending force ranging from 2.0 to 26 g for the rats. Stimulation was applied to the plantar surface of the ipsilateral hind paw. Each hair was indented in the midplantar skin until it just bent. Clear paw withdrawal, shaking, or licking was considered as a nociception-like response. The filament of 8 g was used first. The stimulation was applied five times (several seconds for each trial) with an interval of at least 5 min. The strength of the next filament was decreased if the animal responded or increased if the animal did not respond. The minimum strength that evoked nociceptive responses at least three times out of the five trials was considered as the mechanical withdrawal threshold. Animals that did not respond to all filaments were given a maximal strength of 26.0 g. Overt pain-like behaviors induced by MSU injection were assessed using a standing scale from 0 to 3, as previously described by Coderre and Wall and dos Santos et al. [24, 25] (0: equal weight on both hind paws; 1: completely on the floor, but toes are not spread; 2: foot curled with only some parts of the foot lightly touching the floor; 3: foot elevated completely).

2.6. Histologic Examination. At the end of the experimental period (48 h) after first or second MSU crystal injection, respectively, the rats were deeply anesthetized using sodium pentobarbital (60 mg/kg, i.p.). The ankle joints of rats were isolated, fixed with neutral-buffered 10% formalin, and then decalcified with ethylenediaminetetraacetic acid (EDTA) for 3-4 weeks, as described previously [26]. Sections were cut at 12 μm from the sagittal plane and then embedded in paraffin wax for histological analysis. The paraffin sections were stained with hematoxylin and eosin (H&E). The sections were evaluated via analyzing cell infiltration, pannus formation, and cartilage damage.

2.7. Measurement of Inflammatory Cytokines. IL-1β, a key cytokine in gout, is mediated by MSU crystals triggering the NLRP3 inflammasome [27]. To evaluate the anti-inflammatory effects of *QZTB* treatment in rats, we measured the inflammatory cytokines. Blood samples were incubated at room temperature for 30 min to clot and then centrifuged at 3000 rpm for 10 min at 4°C. The upper serum was transferred into a 1.5 ml microcentrifuge tube and stored at −80°C until further applications. The levels of TNF-α and IL-1β in serum were measured with enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemicals Company, USA) according to the manufacturer's protocol. The absorbance was read at 450 nm using a microplate reader.

2.8. Expression of NLRP3 Inflammasome in Ankle Joints

2.8.1. Quantitative Real-Time PCR Analysis. Total RNA was isolated from treated ankle joints using TRIzol reagent (Invitrogen, CA, USA), and the concentration of RNA was determined using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). 0.5 μg of RNAs was reverse-transcribed into cDNA with cDNA Reverse Transcription Kit (Bio-Rad, CA, USA). Quantitative PCR amplification was performed using a SYBR Green PCR master mix (Bio-Rad, CA, USA). Primers for rat NLRP3 and β-actin were synthesized by Sangon Biotech (Shanghai, China). The primer sequences are shown in Table 1. All real-time PCR experiments were run in quadruple. The mRNA expression levels of NLRP3 were analyzed using the $\Delta\Delta$Ct comparative quantification method following normalization to β-actin.

2.8.2. Western Blot Analysis. The ankle joints were collected and ground into powders using liquid nitrogen. The joint powders were transferred into 1.5 mL Eppendorf (EP) tubes and lysed in cold lysis buffer containing 1 mM phenylmethylsulfonylfluoride (PMSF). The samples were then vortexed at high speed for 15 s, incubated on ice for 15 min, and vortexed again at high speed for 15 s. After centrifugation (15,000 g for 15 min at 4°C), the total proteins obtained in the supernatant were quantified using BCA protein assay kit (Tiangen Biotech Co., Ltd., China), according to the manufacturer's instructions. The proteins were mixed with a 5x loading buffer and heated at 100°C for 3 min to denature. Western blot was then performed using 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Merk, Germany) (83 mm × 75 mm). After 1 h blocking with 5% dried skim milk dissolved in PBST (0.05% Tween 20), the membranes were individually incubated with primary antibodies overnight at 4°C and then incubated with secondary antibody for 1 h. The data were analyzed via densitometry using Molecular Analyst software (Bio-Rad Laboratories,

Table 1: Real-time PCR primer sequences.

Gene	Forward	Reverse
NLRP3	5′-GATGAACACTTGGAGCCCGT-3′	5′-GACTGGTGGGTTTGGGTCAG-3′
β-Actin	5′-ACAGGATGCAGAAGGAGATTAC-3′	5′-ACAGTGAGGCCAGGATAGA-3′

Hercules, California, USA), and quantitated levels were normalized to their respective blotting from β-actin.

2.9. Statistical Analysis. The results were expressed as the mean ± sem and then analyzed by GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used followed by Dunnett's tests for multiple comparisons or unpaired Student's *t*-tests for two-group comparisons, and *P* values <0.05 were considered statistically significant.

3. Results

3.1. Effect of Pretreatment of QZTB on MSU Crystal-Induced Ankle Swelling. To assess the effect of *QZTB* on the model of acute gout, we administered *QZTB* orally at 500 mg/kg twice a day for 7 days before MSU crystal injection (Figure 1). The dosage of *QZTB* is sufficient to reduce ankle swelling significantly in the preliminary experiment. On 0 h, 5 h, 24 h, 30 h, and 48 h after the first MSU crystal injection, we measured the ankle thickness of MSU crystal-induced rats. As shown in Figure 1, there was no difference in ankle diameters among all the experimental rats before model establishment. MSU crystals injection caused an increase in ankle diameter; the swelling reached a maximum at 24 h but gradually decreased at 30 h and 48 h, whereas *QZTB* (500 mg/kg) and meloxicam (3 mg/kg) treatment decreases the ankle swelling significantly. *QZTB* was able to inhibit the edema and presented inhibitory activity ($P < 0.001$) at 5 h, 24 h, 30 h, and 48 h after MSU injection with 24.5, 28.5, 16.5, and 22.7% of inhibition, respectively. Our study demonstrated that pretreatment of *QZTB* was able to prevent the ankle swelling produced by injection of MSU crystals in rats, which confirms its clinical use.

3.2. Pretreatment of QZTB Attenuated MSU Crystal-Induced Arthritis Pain-Like Behavior and Mechanical Allodynia. To determine whether pretreatment of *QZTB* prevents arthritis pain-like behavior and the development of mechanical allodynia, pain-like behavior and allodynia tests were performed 4 hours or 16 hours after the first MSU injection, respectively. We found that the MSU injection resulted in an increase of pain-like behavior scores ($P < 0.05$) and prominent mechanical allodynia ($P < 0.05$), as shown in MSU-vehicle group in Figure 2. Pretreatment of *QZTB* and meloxicam decreased the pain-like behavior scores and increased the paw withdrawal thresholds, as shown in Figure 2.

3.3. QZTB Attenuated Second Injections of MSU Crystal-Induced Recurrence of Arthritis Pain-Like Behavior and Mechanical Allodynia. Recurrent attack of gouty arthritis is a prominent problem in clinic. To determine the effects on recurrent attacks, *QZTB* was administered for 3 days after the first injection of MSU crystals. Ankle thickness was measured 24 h after every MSU injection. The pain-like behavior and mechanical allodynia were tested 4 hours or 16 hours after every injection of MSU crystal, respectively (Figure 3(a)). We found that *QZTB* suppressed the joint edema ($P < 0.05$) and the pain-like behavior scores ($P < 0.05$) and increased the paw withdrawal thresholds ($P < 0.01$) after the second injection of MSU crystal, as shown in Figures 3(b)–3(d). The inhibition of *QZTB* was also compared and normalized to the first injection of MSU and is shown in Figures 3(e)–3(g).

Figure 1: Effects of pretreatment of *QZTB* and meloxicam on MSU-induced gouty arthritis right ankle swelling in rats. *QZTB* (500 mg/kg·bw) and meloxicam (3 mg/kg·bw) decreased the ankle swelling. Values are expressed as mean ± sem of animals ($n = 6$–8). Comparisons were made as follows: $^{***}P < 0.001$, MSU + vehicle vs. Sham; $^{\#\#}P < 0.01$, MSU + *QZTB* vs. MSU + vehicle; $^{\&\&}P < 0.01$, MSU + meloxicam vs. MSU + vehicle. One-way analysis of variance (ANOVA) was used followed by Dunnett's tests.

3.4. Effect of QZTB on MSU Crystal-Induced Ankle Histological Manifestations. To further assess anti-gouty arthritic activity of *QZTB*, joints synovial and surrounding tissues were removed from the ankle joint and analyzed using H&E staining. As shown in Figure 4, histological assay showed that there was no inflammation and infiltration of inflammatory cells in ankle joints control group. With a high-dose injection of MSU crystals (2 mg), gouty arthritis rats showed apparent joint inflammation, a number of inflammatory cells infiltration, and hyperplasia synovial. These pathological states were ameliorated to some degree by

Figure 2: Effects of pretreatment of *QZTB* (500 mg/kg·bw) and meloxicam (3 mg/kg·bw) on pain-like behaviors and mechanical allodynia. Diagram of the experiment (a). *QZTB* (500 mg/kg.bw) and meloxicam (3 mg/kg·bw) decreased the pain-like behavior scores (b) and increased the paw withdraw thresholds (c) significantly. Comparisons were made as follows: $^{*}P < 0.05$, MSU + vehicle vs. Sham; $^{\#}P < 0.05$, MSU + *QZTB* vs. MSU + vehicle; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, MSU + meloxicam vs. MSU + vehicle. One-way analysis of variance (ANOVA) was used followed by Dunnett's tests ($n = 6$–8).

treating with *QZTB* and meloxicam. Although slight hyperplasia was observed in the synovial tissues of *QZTB* group, pretreatment with *QZTB* could suppress the infiltration of inflammatory cells and relieve the symptoms of MSU crystal-induced acute arthritis compared with the model group.

3.5. Effects of QZTB on Expressions of NLRP3 in Ankle Joints. NLRP3 inflammasome has been suggested as an important target for inflammatory disease control. To investigate whether *QZTB* regulates the expression of NLRP3, we detected both mRNA and protein levels of NLRP3 by qPCR and western blot, respectively.

As shown in Figure 5, compared with the normal group, MSU crystals induced an upregulation of the NLRP3 expressions at both mRNA and protein levels in rat ankles ($P < 0.001$). *QZTB* could inhibit the mRNA ($P < 0.001$) and protein expression ($P < 0.001$) levels of NLRP3 compared to the MSU-induced model group. Meloxicam could also decrease the mRNA and protein expression levels of NLRP3 in ankle joints in MSU-induced gouty arthritis rats.

3.6. Effects of QZTB on Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) Production. It has been reported that NLRP3 activation could lead to the increased release of IL-1β and TNF-α. As shown in Figure 6, elevation in serum levels of IL-1β ($P < 0.001$) and TNF-α ($P < 0.001$) was observed simultaneously in the MSU-induced rats. Compared to the MSU-induced group, meloxicam treatment (3 mg/kg) led to decrease in serum IL-1β ($P < 0.01$) and TNF-α ($P < 0.01$) level. *QZTB* suppressed the serum IL-1β ($P < 0.01$) and TNF-α ($P < 0.001$) levels (Figure 6) in the MSU crystal-induced rats, suggesting that *QZTB* can inhibit NLRP3 pathways contributing to arthritis in this acute gout model. These data suggested that *QZTB* can attenuate MSU-induced inflammation by suppressing the productions of IL-1β and TNF-α level.

4. Discussion

QZTB is an empirical traditional Chinese medicine prescription for clinical therapy of gouty arthritis. In the current study, the analgesic and anti-inflammatory effects of *QZTB* were examined in the MSU crystal-induced rats, and its potential mechanism was further investigated. Our results showed that *QZTB* could inhibit joint swelling, pain-like behavior, and allodynia caused by MSU crystal-induced inflammation, as well as joint inflammation, inflammatory cell infiltration, and hyperplasia synovial. The efficacy delivered by *QZTB* is comparable to meloxicam, which is the most frequently used drug for gouty arthritis clinical treatments. Moreover, *QZTB* could alleviate the recurrence of gouty arthritis, which is a prominent problem in clinic. The inhibition of NLRP3 by *QZTB*, evidenced by the decreased mRNA and protein expressions of NLRP3 in the

Figure 3: *QZTB* attenuated second injections of MSU crystal-induced recurrence of arthritis pain-like behavior and mechanical allodynia. Diagram of the experiment (a). Effects of pretreatment of *QZTB* (500 mg/kg·bw) and meloxicam (3 mg/kg·bw) on ankle swelling (b), pain-like behavior scores (c), and paw withdrawal thresholds (d) after the second injection of MSU crystal; the normalized inhibition ratio of *QZTB* and meloxicam (behavior scores after the second injection/first injection of MSU) (e–g). $^{*}P < 0.05$, $^{**}P < 0.01$, MSU + *QZTB* vs. MSU + vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, MSU + meloxicam vs. MSU + vehicle. One-way analysis of variance (ANOVA) was used followed by Dunnett's tests ($n = 6$–8).

Figure 4: Effect of *QZTB* on MSU crystal-induced ankle histological manifestations. Photographs of hind paws of vehicle and experimental animals (a). Effects of pretreatment of *QZTB* (500 mg/kg·bw) and meloxicam (3 mg/kg·bw) on H&E-stained histological manifestations of the right ankle in rats 48 h after injection of 2.0 mg MSU crystal-induced gouty arthritis (b). Original magnification: ×20 (Sham: control rat; MSU + vehicle: MSU-induced rat; MSU + *QZTB*: *QZTB*-treated MSU-induced rat; MSU + meloxicam: meloxicam-treated MSU-induced rat.

ankle joint, and the lower production of serum IL-1β and TNF-α level, further indicates the efficacy of *QZTB* on gouty arthritis in clinic.

During acute gouty attacks, the affected joint exhibits the classic symptoms of inflammation, especially the intense pain in the affected joint. The acute gouty attack can cause severe pain that some patients even had the desire to amputate the affected limb [28]. Recurrent gouty arthritis attack remains a challenging problem. With the interval shortening over time, gouty arthritis, commonly known as the first clinical manifestation of gout, may progress to chronic arthritis and ultimately results in a decreased quality of life [3, 29]. Moreover, in the early period of urate-lowering therapy (ULT), it is always associated with acute and recurrent gouty attacks and the prevention of attacks is necessary [30, 31]. Therefore, the goals of gouty arthritis treatment are not only to decrease the serum uric acid level and terminate acute attacks but also to prevent recurrent gouty arthritis attacks and complications [32]. However, nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, or corticosteroids are usually recommended for a period of 6 months and even longer to prevent the recurrent [33]. Patients should be carefully monitored for adverse effects, especially for patients with renal dysfunction. Colchicine often causes gastrointestinal discomfort, kidney damage, bone marrow suppression, and other adverse reactions. NSAIDs can cause gastrointestinal bleeding, renal impairment, and cardiovascular events [11]. Long-term use of hormones can lead to endocrine metabolic disorders and digestive and cardiovascular complications. These side effects affected patient compliance to a certain extent; thus, the complementary and alternative medicines are desirable. Here, we found that *QZTB* could alleviate the pain-like behavior and the allodynia caused by intra-articular

Figure 5: Effects of *QZTB* (500 mg/kg. bw) and meloxicam (3 mg/kg·bw) on mRNA and protein expressions of NLRP3 in ankle joints in MSU crystal-induced rats. Expression of NLRP3 protein and mRNA was determined by western blot analysis (a-b) and real-time PCR (c), respectively. The protein and mRNA expressions were normalized to β-actin. Values are expressed as mean ± sem. $^{***}P < 0.001$, MSU + vehicle vs. Sham, $^{###}P < 0.001$ MSU + *QZTB* vs. MSU + vehicle, $^{\&\&\&}P < 0.001$ MSU + meloxicam vs. MSU + vehicle. One-way analysis of variance (ANOVA) was used followed by Dunnett's tests ($n = 3$-4).

injection of MSU. Notably, *QZTB* prevented the swelling, pain-like behavior, and allodynia induced by the second injection of MSU, which means *QZTB* also could affect the recurrence of gouty arthritis.

Gouty arthritis is an inflammatory disease caused by deposition of MSU crystal in the joints as the serum uric acid level increases. Both uric acid and MSU released from tissue or cell damage can activate the NOD-like receptor family (pyrin domain containing 3, NLRP3). The NLRP3 inflammasome, a protein complex, has been reported playing an important role in the MSU-induced gouty arthritis [6, 34]. Here, we found that *QZTB* decreased both mRNA and protein levels of NLRP3, which may attribute to its effects on lowering uric acid as reported in our

Figure 6: Effects of *QZTB* (500 mg/kg·bw) and meloxicam (3 mg/kg·bw) on IL-1β (a) and TNF-α (b) production in MSU crystal-induced rats. Values are expressed as mean ± sem (n = 3-4). The asterisks denote significance levels. $^{***}P < 0.001$, MSU + vehicle vs. Sham; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, MSU + *QZTB* vs. MSU + vehicle; $^{\&\&}P < 0.01$ MSU + meloxicam vs. MSU + vehicle. One-way analysis of variance (ANOVA) was used followed by Dunnett's tests.

previous study [13]. Activated NLRP3 induces the conversion of procaspase-1 to active caspase-1, which in turn cleaves the inactive precursor cytokine pro-IL-1β into proinflammatory IL-1β [27]. The activation of IL-1β signaling pathway causes the transcription of proinflammatory cytokines such as TNF-α, which ultimately leads to inflammatory responses. Consistent with these results, we further demonstrated that *QZTB* decreases TNF-α and IL-1β levels, which greatly prove the alleviation of *QZTB* on pain and ankle swelling. Thus, combined with our previous study that *QZTB* exhibits efficacy in the treatment of hyperuricemia [13], it further suggests that *QZTB* would have high potential to treat the acute pain and recurrence of gouty arthritis in clinic.

The main problems of an empirical traditional Chinese medicine prescription for the treatment of health problems are chemical composition of the prescription and its quality control. In our previous reports, we identified four main components from *QZTB*, which are astilbin, diosgenin, curcumin, and quercetin, respectively. Although it is still unclear that which chemical components of *QZTB* contribute to its anti-inflammatory and anti-gouty arthritis effects in the present study. According to our previous studies [13, 16], astilbin could be the main functional composition of *QZTB*. The identification of the effective ingredients in *QZTB*, the related molecular biological mechanism, and the clinical research requires further in-depth investigation.

5. Conclusion

The present study demonstrated for the first time that *QZTB*, an empirical traditional Chinese medicine prescription in clinic, suppressed MSU crystal-induced swelling and pain in rats and exerted anti-inflammatory effects. The potential anti-gouty arthritis effect of *QZTB* may be attributed to the inhibition of NLRP3 inflammasome and downstream proinflammatory cytokines (IL-1β and TNF-α) levels. These findings indicate that *QZTB* may be a promising therapeutic formula for the prevention and treatment of gouty arthritis, especially of recurrent attacks, in a clinical setting.

Abbreviations

MSU: Monosodium urate
QZTB: Qu-Zhuo-Tong-Bi formula
IL-1β: Interleukin-1-beta
TNF-α: Tumor necrosis factor-alpha
NLRP3: NOD-like receptor family pyrin domain-containing 3
NSAIDs: Nonsteroidal anti-inflammatory drugs
TCM: Traditional Chinese medicine.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

H. L. and J. Y. were responsible for the design of the work. J.C, F.L, Z. X, and Y. J were responsible for the acquisition and analysis of data. H. L. and J.Y. drafted the manuscript. C.W and J.Y. approved the final version to be published.

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Research Article

Aqueous and Methanol Extracts of *Paullinia pinnata* (Sapindaceae) Improve Monosodium Urate-Induced Gouty Arthritis in Rat: Analgesic, Anti-Inflammatory, and Antioxidant Effects

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The profound modification of lifestyle and food habits has led to an important increase in the prevalence of gout. Unfortunately, there are current unmet needs for the treatment of this disease, prompting the search for new alternatives. *Paullinia pinnata* is a plant used to treat various diseases including arthritis. The present work aimed to investigate the antigouty activities of the aqueous (AEPP) and methanolic (MEPP) extracts of *P. pinnata* as well as their in vivo antioxidant properties. The gouty arthritis was induced by injecting 50 μl of monosodium urate (MSU, 100 mg/ml) in the left hind ankle of rats. *P. pinnata* extracts were administered orally at the doses of 100 and 200 mg/kg/day for 6 days, starting 24 h after MSU injection. Allopurinol 5 mg/kg/day was used as reference drug. Inflammation and hyperalgesia were daily monitored from 24 hours after treatment initiation and for the 6 consecutive days. Myeloperoxidase (MPO) quantification was done in collected synovial fluid. Nitrite oxide (NO), malondialdehyde (MDA), and superoxide dismutase (SOD) were evaluated in the spinal cord and the brain. The serum content of SOD was additionally quantified. AEPP and MEPP significantly ($p < 0.001$) reduce MSU-induced inflammation (22.41% to 93.65%) and hyperalgesia (33.33% to 64.44%) in both ankle and paw. AEPP and MEPP significantly ($p < 0.001$) reduce synovial MPO production with the percentage ranging from 76.30% to 85.19%. AEPP and MEPP significantly ($p < 0.001$) reduce serum, spinal, left and right hemispheres NO, and MDA and increase the SOD activity ($p < 0.001$). *P. pinnata* leaf extracts possess potent curative effects against MSU-induced gouty arthritis that combines analgesic, anti-inflammatory, and antioxidant activities. These findings support the use of *P. pinnata* leaves extracts in the treatment of gouty arthritis and further present the plant as a potent source of efficient antigouty medicine.

1. Introduction

Gouty arthritis is a painful inflammatory disease, characterized by a swollen and painful joint which usually extends to the surrounding tissue. Gouty arthritis usually occurs following hyperuricemia, identified as the major risk factor [1, 2]. This joint disorder is characterized by the precipitation, deposition, and crystallization of monosodium urate in the joints, leading to intense inflammation and painful process [3]. The gout prevalence ranges between 4 and 6.8% [4, 5]. Its incidence is constantly rising, and it is associated with many comorbidities. In the development of the pathology, the deleterious effects of urate are primarily due to its capacity to trigger the production of reactive oxygen species (ROS), thus promoting the oxidative status in gouty arthritis patients. Concordantly, oxidative stress status has been demonstrated in gouty arthritis [6].

The timeline treatment against gout focuses on the treatment of inflammation and the reduction of hyperuricemia. Many agents, including allopurinol, febuxostat,

colchicine, nonsteroidal anti-inflammatory drugs, corticosteroids, and more recently interleukin (IL)-1 inhibitors, have been developed and have demonstrated efficiency in gout treatment. Nevertheless, they have some limitations among which are little effect on acute gout attacks, contraindications with the comorbidities, and drugs' side effects, which include fever, skin rashes, allergic reactions, hepatitis, nephropathy, and gastrointestinal toxicity/bleeding [7–9]. There is, therefore, an important need for more effective new therapeutics with low side effects.

One of the most reliable sources of new compounds is made of medicinal plants. They have gained more interest these last decades and are now intensively used by industries for the production of phytopharmaceuticals and nutraceuticals.

The search for new natural drugs with antigouty effects, combining anti-inflammatory, analgesic, and antioxidant properties, is the focus of this manuscript. *Paullinia pinnata* Linn is a creeper plant (Sapindaceae) widely distributed in the west and the center regions of Cameroon. It is empirically used for the treatment of infection and painful and inflammatory diseases. The leaves decoction is used in the East Africa region, for the treatment of snake bites [10]. Phytochemical studies of this plant extract revealed the presence of phenols [11], flavonoids, triterpene, saponins and tannins [12], steroids, steroidal glycosides, and cerebroside and ceramide [13]. Compounds such as 2-*O*-methyl-L-chiro-inositol, β-sitosterol, and friedelin have been isolated from *P. pinnata* [14]. Previous pharmacological studies demonstrated the antimicrobial activities and protective effects against CCl4-induced hepatic damage and oxidative stress [14, 15]. Our recent studies showed that the aqueous and methanol extracts from the leaves of *P. pinnata* possess potent preventive effects on rheumatoid monoarthritis [16]. But whether these plant extracts can cure gouty arthritis is unknown. The present study was undertaken to evaluate the curative effect of the aqueous and methanol extracts of *P. pinnata* on the MSU-induced gouty arthritis in Wistar rats.

2. Materials and Methods

2.1. Plant Material and Preparation of Extracts. The fresh leaves of *P. pinnata* were harvested at Poumougne in the Koung-khi Division (West Region, Cameroon) in March 2017. The identification of plant specimens was done at the Cameroon National Herbarium in Yaoundé by Mr. Tadjouteu Fulbert in comparison with a voucher specimen under the reference number 10701/SRF. Cam.

The leaves were shade dried and ground into a fine powder. The aqueous extract was prepared by decoction. The powder (500 g) was boiled in 3.5 L of distilled water for 15 minutes. The mixture was filtrated on Whatman paper No. 1. The residue was re-extracted as previously described in 2 L of distilled water for 15 minutes. The two filtrates obtained were pooled together and evaporated in a ventilated steam room for 72 hours at 40°C to yield 89.54 g (17.91%).

The methanol extract was prepared as maceration. The powder (500 g) was soaked in 3.5 L of methanol for 72 hours and filtrated using Whatman paper No. 1. The plant material residue was re-extracted in 2 L of methanol for 24 hours. The two filtrates obtained were mixed and concentrated in a rotary evaporator under low pressure at 40°C and yielded 111.04 g (22.21%) of methanol extract.

Both aqueous and methanol extracts were prepared in 4% DMSO for the experiment prior to administration to animals.

2.2. Chemicals and Drugs. Uric acid, hexadecyltrimethylammonium bromide, epinephrine, O-dianisidin, and sodium azide were purchased from Sigma-Aldrich Chemical Co. (Taufkirchen, Germany). Sodium hydroxide, hydrochloric acid, bovine serum albumin, sodium chloride, and Dimethylsufoxide (DMSO) were purchased from Carl-Roth (Kalshur, Germany). Nitric acid was purchased from Qualikems. Allopurinol was obtained from Aspen-Pharma (Ireland). Ether was purchased from a local pharmacy (Dschang, Cameroon). Griess reagent, Biuret reagent, and carbonate buffer were prepared in the laboratory with ingredients from Sigma-Aldrich Chemical Co. (Taufkirchen, Germany). Thiobarbituric acid was purchased from Shanghai Zhanyun Chemical Co. Ltd (China). Trichloroacetic acid was purchased from Guangdon Guanghua Sci-Tech Co. Ltd (China).

2.3. Monosodium Urate (MSU) Crystal Synthesis. MSU crystals were synthesized according to a modified protocol of Tank et al. [17]. Two-hundred milliliters of an aqueous solution of NaOH (1 M) was stirred well and heated up to 70°C. After obtaining the required temperature, 1 g of uric acid was added into the NaOH solution and HCl (1N) was then used to maintain the mixture at pH 7.2. The solution was stirred at a constant temperature (70°C) for 4 hours and then stirred slowly at room temperature and stored at 4°C for 24 h. Fine particles of MSU crystals were found at the bottom of the glass. MSU particles were recovered by filtration, washed with distilled water; air-dried, and suspended in sterile saline (100 mg/ml).

2.4. Animals. Albino Wistar rats (150–200 g) were bred onsite in the Laboratory of Physiology and Phytopharmacology of the University of Dschang, Cameroon. Forty-two animals (10 to 12 weeks old) of either sex were divided into 7 experimental groups of 6 animals each (3 males and 3 females). Rats were habituated to pain threshold recording and paw volume measurement before the experiment. Rats with baseline pain threshold below or equal to 7.5 g were discarded.

2.5. MSU-Induced Gouty Arthritis. The experiment was set following the method of Zhang et al. [18], with some modifications, and performed as it is described below. Briefly, rats were anesthetized with ether, the left hind leg skin was sterilized with 75% ethanol, and the lateral malleolus located by palpation. Then, an insulin syringe needle was inserted vertically to penetrate the skin and turned distally for insertion into the articular cavity at the gap

between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. Fifty microliters of MSU was injected into the articulation to induce gouty arthritis (day 0). The same procedure was followed for all the groups except for the sham group in which only sterile saline was injected in the articulation. One day after MSU injection, the second baselines of inflammation and pain threshold were taken. Then, the 6 MSU-injected groups were treated as follows: negative control treated orally with DMSO (4% in distilled water), positive control treated with Allopurinol (5 mg/kg/day; dissolved in distilled water), and the 4 remaining groups treated orally with AEPP or MEPP at the doses of 100 and 200 mg/kg/day. The MSU-injected rats were treated daily for 6 consecutive days. The pain threshold at the ankle and paw was recorded using an analgesimeter (Ugo Basile, type 37215) on days 1, 2, 3, 4, 5, and 6 after the initiation of treatments. Briefly, awake rats were scruffed and placed in the analgesimeter. For each measuring time point, two trials were completed on the ipsilateral ankle and the values averaged. The same measurement procedure was conducted on the ipsilateral paw.

Immediately after pain threshold measurements, the paw and the ankle volumes were recorded using an electronic caliper (Fine Science, Heidelberg, Germany). Rats were slightly restrained, and the diameter of the paw was measured at the space between all footpads from the back (dorsal surface) to the front (plantar surface) of the paw and even the ankle.

On day 7 after MSU injection, animals were anesthetized by intraperitoneal injection of 15% ethyl carbamate solution (1.5 g/kg). Blood, spinal cord, and the left and right hemispheres were collected for antioxidant assays. The synovial liquid of the injected ankle was collected by washing the joint with 100 μl of NaCl 9‰ for the evaluation of myeloperoxidase level.

Blood collected in nonheparinized tubes was centrifuged at 3000 rpm, and the serum was kept at −20°C until use. The entire spinal cord and the left and right hemisphere were homogenized in 10% and 15% solution of ice-cold 0.1 M Tris buffer (pH 7.4) at 4°C, respectively, and centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was collected and keep at −20°C. Serum, spinal cord, left and right hemisphere supernatants were used for superoxide dismutase (SOD), nitrite oxide (NO), and malondialdehyde (MDA) estimation.

2.6. Nitric Oxide, Superoxide Dismutase, and Malondialdehyde Assays. The Griess reagent was used to estimate the nitric oxide content. To 250 μl of the tissue sample was added 250 μl of 1% sulfaniladide prepared in 5% orthophosphoric acid. The mixture was incubated for 5 minutes in the dark, then 250 μl of 0.1% naphthyl ethylenediamine was added, and then all incubated in the dark for additional 5 minutes. The optical density was read at 530 nm. The quantity of nitric oxide was calculated from the sodium nitrite's standard curve.

The SOD activity assessment was conducted according to the protocol of Wandji et al. [19]. To 70 μl of the sample was added 830 μl of carbonate buffer (pH = 10.2) and 100 μl of epinephrine (0.3 mM). The mixture was homogenized, and the absorbance read 60 and 120 seconds after epinephrine was introduced, at 480 nm using a spectrophotometer (Helios Epsilon). The inhibitory percentage of oxidation was calculated according to the following formula:

$$I\% = 100 - \left[\frac{\text{Ab sample}}{\text{Ab blank}}\right] \times 100, \quad (1)$$

where $I\%$ = inhibitory percentage and Ab = absorbance.

The SOD activity was calculated as:

$$A = \frac{I\%}{50 \times \text{protein quantity}}, \text{ with AA = the SOD activity,}$$

$$50 = \text{one unit.} \quad (2)$$

For the MDA assay, 500 μl of orthophosphoric acid (1%) and 500 μl of thiobarbituric acid (1%) were added to 100 μl of serum/supernatant. The mixture was homogenized and placed in boiling water bath for 15 minutes. Thereafter, the tubes were cooled on ice bath and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected, and the absorbance was read at 532 nm using a spectrophotometer (Helios Epsilon). The concentration of MDA was calculated based on the absorbance coefficient according to the following formula: D. O. = ε. C. L (where D. O. = optical density, ε = molar extinction coefficient of the MDA ($1.56 \times 10^5\ \mathrm{M}^{-1}.\mathrm{Cm}^{-1}$), C = concentration in MDA, and L = length of the optic journey (1 cm)).

2.7. Myeloperoxidase Assay. This assay was conducted as previously described by Garça et al. [20], with some modifications. In each well of a 96-well plate, 25 μl of the synovial liquid, 25 μl of potassium phosphate buffer, and 25 μl of 0.5% hexadecyltrimethylammonium bromide buffer were introduced. The mixture was incubated for 30 minutes at room temperature. Then, 10 μl of sodium azide (0.1 mM) and 150 μl of potassium phosphate buffer (50 mM; pH 5.5) containing 0.026% ortho-dianisidine dihydrochloride and 0.018% hydrogen peroxide were added to the reaction medium. The entire plate was once again incubated at room temperature for 30 minutes. Finally, the optical density of each well of the 96-well plate was read using an ELIZA microplate reader at 450 nm. MPO level was expressed using an arbitrary unit.

2.8. Statistical Analysis. Results are expressed as mean ± SEM. Time-dependent hyperalgesia and inflammation were analyzed using two-way ANOVA followed by Bonferroni's posttest while time point data were analyzed with one-way ANOVA followed by Tukey's posttest. All the analyses were performed with GraphPad Prism 5.01 software package. A p value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Anti-Inflammatory Effects of P. pinnata Extracts on MSU-Induced Gouty. Intra-ankle injection of MSU resulted in a significant inflammation that was maintained for at least 6 days. Daily oral administration of *P. pinnata* extracts significantly reduced the inflammation induced by MSU both at the level of the ankle and the paw. Two-way ANOVA (main effects time, treatment, and interaction) demonstrated significant effects at all the doses used ($p < 0.05$; $p < 0.01$; $p < 0.001$) (Figures 1(a)–1(d)). Roughly, both AEPP and MEPP showed dose-dependent effects. At the dose of 200 mg/kg, AEPP and MEPP exhibited maximal inhibitory percentages of 99.23 and 98.40%, respectively. The inhibitory percentage induced by allopurinol (5 mg/kg/day) ranged from 26.76 to 90.81% and was, in general, less efficient than *P. pinnata* extracts (Figure 1).

3.2. Antihyperalgesic Effects of P. pinnata Extracts on MSU-Induced Gouty. The intra-articular administration of MSU resulted in hyperalgesia both at the level of the ankle and the paw. The oral administration of *P. pinnata* extracts starting one day after MSU injection significantly reduced the mechanical hyperalgesia as measured with the Randall Sellito method (two-way ANOVA effects time, treatment, and interaction $p < 0.01$; $p < 0.001$). As demonstrated by Bonferroni posttest, AEPP, and MEPP induced time- and dose-dependent antihyperalgesic effects (Figures 2(a)–2(d)). MEPP at the dose of 200 mg/kg almost completely abolished the mechanical hyperalgesia, both at the level of the paw and the ankle (Figures 2(b) and 2(d)). The effect of allopurinol was equal to that of MEPP at the dose of 100 mg/kg.

3.3. Inhibitory Effects of P. pinnata Extracts on the MPO Activity in Synovial Liquid in Gouty Rats. In the process of inflammation, neutrophils through their oxidative burst produce MPO. As shown in Figure 3, the intra-articular injection on MSU drastically increased ($p < 0.001$) the MPO activity in the synovial fluid as compared to the sham group. AEPP and MEPP significantly ($p < 0.01$; $p < 0.001$) reduced the activity of MPO, with the highest effect observed with AEPP at the dose of 200 mg/kg (Figure 3(a)). The positive control allopurinol-treated group (5 mg/kg) (Figures 3(a) and 3(b)) also significantly ($p < 0.001$) reduces the MPO level as compared to untreated MSU control and more efficiently than both AEPP and MEPP at both doses (100 and 200 mg/kg).

3.4. P. pinnata Extracts Inhibit the NO Production in the Spinal Cord and the Right and Left Hemispheres of Gouty Rat. During the inflammatory process, immune cells play an essential role while defending the organism by producing nitrite oxide. For more insight on the anti-inflammatory activities of *P. pinnata* extracts, we evaluated the effect of AEPP and MEPP on the nitric oxide production in the spinal cord and the right and left hemispheres. The control group (MSU-DMSO) showed a marked increase ($p < 0.001$) in the NO content in all the sample regions collected. This increase was significantly and completely reversed ($p < 0.001$) by *P. pinnata* extracts and allopurinol in all the collected organs and at all the doses used, as compared to the control group (Figures 4(a)–4(f)).

3.5. Effects of P. pinnata on the SOD Activity in the Serum, Spinal Cord, and Right and Left Hemispheres of Gouty Rat. The impact of *P. pinnata* extracts on oxidative stress was evaluated through the SOD activity measured in the serum, spinal cord, and right and left hemispheres. MSU ankle injection did not significantly affect the SOD activity in all the tissues used, as compared to the sham group. However, the daily administration of AEPP and MEPP induced a tissue-dependent increase in the SOD activity. In fact, *P. pinnata* extracts did not affect the evaluated parameter in the right hemisphere. Besides, MEPP showed no effect on the SOD activity in the left hemisphere (Figure 5(h)). Allopurinol was able to significantly increase the SOD activity only in the serum and the left hemisphere, while both extracts did so in the serum and the spinal cord. More, AEPP increases the parameter in the left hemisphere in a dose-dependent manner (Figure 5(g)).

3.6. P. pinnata Extracts Reduce the MDA Content in the Spinal Cord and Right and Left Hemispheres of Gouty Rat. MDA is the end-product of lipid peroxidation and can indicate the status of oxidative stress. To evaluate the protective effect of *P. pinnata* extracts against the oxidative damages in the pain modulating/integrative structures, we quantified the MDA content in the spinal cord and cerebral hemispheres. As expected, we found a significant ($p < 0.001$) increase of MDA content in MSU-injected rats as compared to the sham group (Figures 6(a)–6(f)). AEPP and MEPP significantly ($p < 0.001$) reduced the MDA content in the spinal cord and right and left hemispheres as compared to the MSU-control group. The positive control allopurinol also produces a significant ($p < 0.001$) effect, but this inhibitory activity was lower than that of both extracts independently of the tissue.

4. Discussion

The present study was undertaken to investigate the ability of the aqueous (AEPP) and methanol (MEPP) extracts of *P. pinnata* against pain, inflammation, and oxidative stress in gouty arthritis, a disease of the musculoskeletal system characterized by painful inflammatory joints and oxidatives. Gout is the result of the accumulation of monosodium urate (MSU) crystals in the joint. Thus, experimental MSU-induced gouty arthritis is a trustworthy model of gout that mimics the clinical human condition. In the present study, the intra-ankle injection of MSU resulted in inflammation and hyperalgesia in both the ipsilateral ankle and paw, accompanied by an increase in MPO activity in synovial fluid and oxidative stress in the spinal cord and the brain. These results largely corroborate the previous findings [21–23].

Figure 1: Effects of the (a, c) aqueous (AEPP 100, 200 mg/kg/day) and (b, d) methanolic (MEPP 100, 200 mg/kg/day) extracts of *Paullinia pinnata* on the primary (ankle (a, b)) and secondary (paw (c, d)) inflammatory gout induced by MSU ankle injection in rat. (a) AEPP induces significant decreases in ankle swelling compared to rats treated with MSU and vehicle (3%DMSO). (b) MEPP induces significant decreases in ankle swelling compared to rats treated with MSU and vehicle. (c) AEPP induces significant decreases in paw swelling compared to rats treated with MSU and vehicle. (d) MEPP induces significant decreases in paw swelling compared to rats treated with MSU and vehicle. Allopurinol (5 mg/kg/day) is included as a positive control for anti-inflammatory effects. Each point represents the mean ± SEM of 6 individual rats. $^{**}p < 0.01$, $^{***}p < 0.001$; significant difference compared to the negative control group (MSU + DMSO) using two-way ANOVA with Bonferroni posttest. The black arrow indicates the beginning of treatments.

Oral administration of the AEPP and MEPP significantly reduced inflammation and hyperalgesia in a dose- and time-dependent manner, reduced the myeloperoxidase activity in synovial fluid, and exhibited significant in vivo antioxidant effects on NO, MDA, and SOD.

Cumulative literature is concordant on the physiopathological process of MSU-induced gout. In fact, at the first step, phagocytes in the joint such as macrophages, monocytes, or neutrophils will activate upon detection of MSU crystals, to produce active IL-1β which will subsequently activate NF-KB to trigger the transcription of cytokines and chemokines as well as the release of inflammatory mediators. The released mediators will finally activate nociceptor endings, opening TRPV1 and TRPA1 channels either directly or through intracellular signaling cascades. Furthermore, the antidromic firing of articular nociceptors releases neuropeptides from peripheral nerve terminals, thereby potentiating the local inflammatory response known as neurogenic inflammation [24, 25]. As a consequence of these intricate pathways, there will be hypersensitization of nociceptors ending for hypernociception and allodynia, as well as inflammation in the joint and surrounding tissues. AEPP and MEPP showed time-dependent analgesic and anti-inflammatory effects, suggesting that these extracts possess bioactive molecules that may interfere with the different pathways described above. *P. pinnata* extracts may, therefore, act on phagocytes and directly on the nervous system. Previous studies with the same

Figure 2: Effects of the aqueous (AEPP; 100, 200 mg/kg/day) and methanol (MEPP; 100, 200 mg/kg/day) extracts of *Paullinia pinnata* on the primary (ankle) and secondary (paw) mechanical hypersensitivity induced by ankle injection of MSU in rat. (a) AEPP induces significant decreases in mechanical hypersensitivity (i.e., decreased change in threshold from baseline) compared to rats treated with MSU and vehicle (3%DMSO). (b) MEPP induces significant decreases in mechanical hypersensitivity compared to rats treated with MSU and vehicle. (c) AEPP induces significant decreases in mechanical hypersensitivity compared to rats treated with MSU and vehicle. (d) MEPP induces significant decreases in mechanical hypersensitivity compared to rats treated with MSU and vehicle. Allopurinol (5 mg/kg/day) is included as a positive control for the antihyperalgesic effect. Each point represents the mean ± SEM of 6 individual rats. $^{**}p < 0.01$, $^{***}p < 0.001$; significant difference compared to the negative control group (MSU + DMSO) using two-way ANOVA with Bonferroni posttest. The black arrow indicates the beginning of treatments.

extracts of *P. pinnata* demonstrated their analgesic and anti-inflammatory effects in Complete Freund's Adjuvant- (CFA-) induced monoarthritis and suggested that the extracts may have effect both at the central and peripheral levels [16]. Besides, it was also demonstrated that these extracts inhibit the production of IL-1β and TNF-α by macrophages in inflammatory conditions. As IL-1β plays a pivotal role in the development of pain and inflammation in the context of hyperuricemia or MSU accumulation in the joint, it can be suggested that the analgesic and anti-inflammatory effects of *P. pinnata* extracts are related to their IL-1β inhibition capacity. As such, the analgesic effect of the plant extracts will be highly dependent on its anti-inflammatory properties.

Nevertheless, AEPP and MEPP were able to reduce the secondary analgesia at the level of the paw. This result suggests that *P. pinnata* extracts may act directly on the central nervous system, as secondary pain depends mostly on the sensitization of the central nervous system [26].

It has clearly been shown that neutrophil recruitment and activation are the cornerstones of the inflammatory response to monosodium urate (MSU) crystals [27]. Neutrophils infiltrated and accumulated in the synovial joint will undergo degranulation and release their secretion products

Figure 3: Effects of the aqueous (AEPP) and methanol (MEPP) extracts of *Paullinia pinnata* on myeloperoxidase (MPO) activity in the synovial liquid of MSU-injected animals. Allopurinol (5 mg/kg/day) was used as a positive control. (a) AEPP treatment at 100 mg/kg and 200 mg/kg reversed MSU-induced increases in MPO activity in synovial liquid. (b) MEPP treatment at 100 and 200 mg/kg reversed MSU-induced increases in MPO activity in synovial liquid. Each bar represents the mean ± SEM of 6 individual rats. $^{**}p < 0.01$, $^{***}p < 0.001$; significant difference compared to the negative control group (MSU + DMSO) using one-way ANOVA with Tukey's posttest. $^{£££}p < 0.001$ significant difference compared to the sham group using one-way ANOVA with Tukey's posttest.

Figure 4: Continued.

Figure 4: Effects of the aqueous (AEPP) and methanolic (MEPP) extracts of *Paullinia pinnata* on the nitrite oxide level in the spinal cord (a, b), the right hemisphere (c, d), and the left hemisphere (e, f). Plant extract treatment in all the organs used significantly reversed the overproduction of nitric oxide (NO) induced by the intra-ankle injection of MSU. Each bar represents the mean ± SEM of five repetitions. $^{***}p < 0.001$ significant difference compared to the negative control group (MSU + DMSO) using one-way ANOVA with Tukey's posttest. $^{£££}p < 0.001$ significant difference compared to the sham group using one-way ANOVA with Tukey's posttest.

Figure 5: Continued.

Figure 5: Effects of the aqueous (AEPP) and methanol (MEPP) extracts of *Paullinia pinnata* on the superoxide dismutase (SOD) activity in the serum (Figures 4(a) and 4(b)), spinal cord (Figures 4(c) and 4(d)), right hemisphere (Figures 5(e and f)), and left hemisphere (Figures 5(g and h)) of MSU-injected rats. Allopurinol (5 mg/kg/day) serves as a positive control for antigouty effects. SOD activity significantly increased after treatment with allopurinol, AEPP (100 and 200), and MEPP (100 and 200) in the serum (a and b). Only the AEPP 100 and the MEPP (100 and 200) significantly increase SOD activity in the spinal cord (c and d). Both the AEPP (100 and 200) and the MEPP (100 and 200) fail to increase SOD activity in the right hemisphere (e and f). The AEPP 200 and allopurinol significantly reverse MSU-induced SOD exhaustion in the left hemisphere (g and h). Each bar represents the mean ± SEM of 6 individual rats, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; significant difference compared to the negative control group (MSU + DMSO) using one-way ANOVA with Tukey's posttest. $^{£}p < 0.05$, $^{££}p < 0.01$, $^{£££}p < 0.001$; significant difference compared to the sham group using one-way ANOVA with Tukey's posttest.

among which are myeloperoxidases (MPO). To determine the effects of *P. pinnata* extract on neutrophils infiltration and activation, we evaluated the MPO activity in the synovial fluid. Both AEPP and MEPP potently reduced the MPO activity, confirming that these extracts reduce cell infiltration as previously demonstrated [16]. Also, the possibility that *P.*

Figure 6: Effects of the aqueous (AEPP) and methanol (MEPP) extracts of *Paullinia pinnata* on the malondialdehyde (MDA) content in the spinal cord of MSU-injected animals. Allopurinol (5 mg/kg/day) is used as a positive control for antigouty effects. (a) AEPP treatment at 200 mg/kg and 300 mg/kg reversed MSU-induced increases in MDA spinal cord content (one-way ANOVA significant main effect $p < 0.001$). (b) MEPP treatment at 100, 200, and 300 mg/kg reversed MSU-induced increase in MDA spinal cord content (one-way ANOVA significant main effect $p < 0.001$). Each bar represents the mean ± SEM of 6 individual rats. $^{***}p < 0.001$; significant difference compared to the negative control group (MSU + DMSO) using one-way ANOVA with Tukey's posttest. $^{£}p < 0.05$, $^{££}p < 0.01$, $^{£££}p < 0.001$; significant difference compared to sham using one-way ANOVA with Tukey's posttest.

pinnata extracts could inhibit the release of inflammatory mediators from leucocytes cannot be rolled out, as previously discussed. It has been shown that the methanol extract of the leaves of *P. pinnata* possesses β-sitosterol and friedelin [14]. The analgesic and the anti-inflammatory properties of these compounds have been demonstrated [28, 29]. Therefore, it can be assumed that the analgesic and anti-inflammatory effects of the methanol extract of *P. pinnata* is related to the presence of β-sitosterol and friedelin.

One of the early events associated with gout is the oxidative stress [30] at the level of the affected joint. Besides, the constant transmission of noxious stimulus in the spinal cord and the brain, promotes the release of reactive oxygen species (ROS), leading to oxidative stress in these tissues. It has been demonstrated that ROS mediate the development and maintenance of pain and further boost the peripheral and central sensitization in joint pain [30]. Concordantly, it was observed in the present study that MSU-induced gout was accompanied by substantial production of NO and malondialdehyde in the spinal cord and the brain while the superoxide dismutase content was not significantly affected. These data confirm the oxidative status in the brain and spinal cord of MSU gouty animals. AEPP and MEPP significantly reduced both NO and MDA contents and increased the SOD activity in the tissues, further confirming the in vivo antioxidant effects of *P. pinnata* extracts as previously suggested [16].

5. Conclusion

The present study shows that the aqueous and methanol extracts from the leaves of *P. pinnata* possess anti-hyperalgesic and anti-inflammatory effects against MSU-induced gouty arthritis. The analgesic effect is at least partially related to its anti-inflammatory activity. The anti-inflammatory effect seems to be due to the inhibition of the inflammatory cells infiltration and/or the inactivation of infiltrated cells. The antioxidant effect of these extracts also highly contributes to their antigouty activity. These results support the use of the leaves of *P. pinnata* extracts in Cameroonian folk medicine for the management of gouty arthritis.

Abbreviation

MSU: Monosodium urate
AEPP: Aqueous extract *Paullinia pinnata*
MEPP: Methanol extract *Paullinia pinnata*
NaOH: Sodium hydroxide
HCl: Hydrogen chloride
DMSO: Dimethyl-sulfoxide
MDA: Malondialdehyde
NO: Nitrite oxide
SOD: Superoxide dismutase
MPO: Myeloperoxidase
ROS: Reactive oxygen species
CFA: Complete Freud's Adjuvant.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All procedures used in the present study were approved by the local Ethical Committee (FS, University of Dschang) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain (protocol number PP00032).

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

TBN planned the work. PPT, BNP, and SMN collected the data. TBN and PPT carried out the statistical analysis. PPT and TBN drafted the manuscript. All the authors corrected and approved the final copy of the manuscript.

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Research Article

Suppressive Effect of Huzhentongfeng on Experimental Gouty Arthritis: An *In Vivo* and *In Vitro* Study

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Background. Huzhentongfeng (HZTF) is an extract from four Chinese medical herbs for treating gout. This study aims to evaluate its antigout activity and preliminary explore its mechanism *in vivo* and *in vitro*. *Methods*. The rats were intragastrically administered with HZTF for 5 days and then injected 0.1 ml (10 mg) of MSU crystals to their joints for generating a gout model to analyze the paw volume and histopathology of joint synovial tissues of rats with different doses. We also investigated the antioxidant capacity of HZTF *in vitro* using indication including lipid peroxidation, $DPPH^{\cdot}$, and $ABTS^{+}$ radical-scavenging capacity; besides, we used qRT-PCR to measure the effect of HZTF on interleukin (IL)-1β, caspase-1, NLRP3, and NQO1 expression in hydrogen peroxide-stimulated RAW264.7 macrophages and IL-1β, IL-6, and tumor necrosis factor (TNF)-α in MSU crystal-induced THP-1 monocytes. Confocal microscopy analysis was used to observe the dimerization of ASC adapter proteins. In addition, we also established quality standard of HZTF by using the high-performance liquid chromatography (HPLC) method. *Results*. HZTF could significantly suppress the paw swelling and neutrophil infiltration induced by MSU intra-articular injection in rats compared with the control group. HZTF also showed inhibition effects of inflammatory cytokines (IL-1β, IL-6, and TNF-α) secretion at 25.00 and 50.00 μg/ml in MSU-induced THP-1 cells but showed no effects of IL-1β, IL-6, and TNF-α mRNA expression in MSU-induced THP-1 cells. Furthermore, confocal microscopy analysis showed that HZTF could prevent the oligomerization of ASC. Moreover, HZTF also showed effects in cell-free and cell-base tests of antioxidant capacity. *Conclusion*. The results prove that HZTF possessed the potential preventive effect against gout arthritis, and the effect may be attributed to its preventing effect on neutrophil infiltration and proinflammatory cytokines secretion such as IL-1β, IL-6, and TNF-α which were caused by the activation of inflammasome.

1. Introduction

Gout is a common type of arthritis caused by hyperuricaemia and subsequent accumulation of monosodium urate crystal deposition in the joints, tendons, and surrounding tissues. The signs and symptoms of gout include sudden and intense pain, redness, swelling, and stiffness in a joint of the big toe or in another joint, such as the ankle, knee, elbow, wrist, and finger [1]. At present, there are many medications in clinic, such as nonsteroidal anti-inflammatory drugs (NSAIDs), colchicine, or corticosteroids, used to treat the symptoms of gout attacks, prevent future flares, and reduce the risk of gout complications. But they all have some limitations. Over 80% of patients treated with colchicine have experienced abdominal pain before full clinical improvement. In addition, the side effects of NSAIDs are more common in the elderly.

Therefore, it is critical to develop more effective agents with less adverse reactions.

HZTF is extracted from four Chinese medical herbs which are widely used in China: *Polygonum cuspidatum* Sieb.et Zucc, *Plantago depressa* Willd, *Ligustrum lucidum* Ait, and Vespae nidus. These four medicinal materials have been used for treating gout for centuries [2–11].

The dried root of *Polygonum cuspidatum* Sieb.et Zucc was known as "Huzhang" in China, as a traditional Chinese medicine, which was officially listed in the Chinese Pharmacopoeia. The compounds from *Plantago depressa* Willd have been demonstrated to have tremendous amount of pharmacological activities, including antioxidant, antibacterial, and anti-inflammatory activities [12, 13]. *Plantago depressa* Willd contains large amounts of polyphenols such as resveratrol, and it is considered one of the best sources of resveratrol because it contains higher amounts of this compound than other plants or fruits [14]. *Plantago depressa* Willd or Cheqiancao in Chinese is widely distributed and utilised around world, which has no significant toxicities in a regular dosage and has been used as medicinal plants for centuries [15, 16]. Currently, researches have shown that extracts from Plantain leaves exhibited antioxidant and antimicrobial activities. The above-mentioned bioactivities of *Plantain* leaves extracts are thought to be related to phenolic compounds and flavonoids [17]. *Ligustrum lucidum* Ait is a kind of evergreen shrub plant which can be found in Hu'nan, Jiangsu, Sichuan, and Zhejiang provinces in China. The mature fruits of this plant have a cardiac and roborant effect and known as a traditional herb medicine in China, which is also prescribed in Chinese Pharmacopoeia. Previous phytochemical investigations on *Ligustrum lucidum* Ait reported the isolation of secoiridoid glycosides, phenolic glycosides, and lignans [18, 19]. Beehive is the hive of *Parapolybia varia* Fabricius, a widely used traditional Chinese medicine. It is widely distributed in China and be harvested in autumn and winter. After removing the dead wasps, the hive is manufactured into a kind of traditional Chinese medicine by open-air drying. Because of its multiple pharmacological activities, including antivirus and anti-inflammatory beehive has been used in traditional Chinese medicine over thousands of years for treating various diseases, including malignant tumors, rheumatoid arthritis, lung diseases, skin disease, digestive and urinary disorders, and dental diseases [11, 20].

Huzhentongfeng (HZTF) is an extract developed by GuangZhou (Jinan) Biomedical Research and Development Center for treating gout. In order to obtain a good antigout effect, we combined the traditional Chinese medicine theory system with modern medical theory to extract these four medicinal materials to produce HZTF. According to the theory of traditional Chinese Medicine, *Polygonum Cuspidatum* is a monarch drug, *Plantain* is a minister drug, *Ligustrum* is an assistant drug, and Nidus Vespae is the guide drug. And, its main function is to treat acute gouty arthritis.

Firstly, in order to ensure the stability of product quality of HZTF, we also established a standard for the content of medicinal materials by using the HPLC method.

Synovial cells, monocytes-macrophages, and neutrophils will produce multiple cytokines, such as IL-1β, IL-6, and TNF-α, when they were stimulated by MSU crystals in the joints. Our study demonstrated that HZTF showed prominent effect on neutrophil infiltration and paw swelling in rats induced by intra-articular MSU injection. However, the concrete mechanism of HZTF preventing gouty arthritis remains unclear. So, in this study, we investigated the inhibition effects on expression levels of IL-1β, caspase-1, NLRP3, and NQO1 in hydrogen peroxide (H_2O_2)-induced RAW264.7 macrophages and expression and secretion of IL-1β, IL-6, and TNF-α from MSU crystal-induced THP-1 cells to investigate the mechanism of HZTF in treating gouty arthritis.

Moreover, to investigate whether the antigout effect of HZTF was induced by its antioxidation activity, we tested the antioxidant effects of HZTF using lipid peroxidation, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH)-scavenging activity, and 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical-scavenging activity *in vitro*.

2. Materials and Methods

2.1. Equipment. Stainless steel tape gauge, produced by Southwest China Tool General Plant of Guizhou Aviation Industry Group, China. The model of UV-visible spectrophotometer used in this experiment is UV2600 (Shimadzu, Japan), the model of the centrifugal machine is HC-2518 (Anhui USTC Zonkia, China), the real-time thermal cycler is CFX96 Touch (BioRad, USA), and the microplate reader is ELx808 (BioTek, USA).

2.2. Reagents. HZTF was produced by Yili Pharmaceutical Co., Ltd., Guangdong, China. Tongfengshu was produced by Qinghai Green Pharmaceutical Co., Ltd, Qinghai, China. Dexamethasone (DEX) and MCC950 were produced by Selleck, Shanghai, China. Phorbol-12-myristate-13-acetate (PMA), ascorbic acid (VC), ABTS, DPPH, AAPH, tris, ferrozine, ammonium thiocyanate, methanol, ethyl alcohol, hydrochloric acid, potassium peroxydisulfate, ferric chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium phosphate, hydrogen peroxide, uric acid, sodium hydroxide, MTT, and potassium persulfate were all in analytical grade and obtained from Aladdin Industrial Corporation, Shanghai, China. PCR Mastermix and cDNA reverse transcriptase kit were obtained from Takara, Beijing China. TRIzol reagent was from Tiangen, Beijing, China. Gene specific primers were from Sangon, Shanghai, China. Elisa kits were purchased from neobioscience, Guangdong, China. Anti-ASC antibody was from Proteintech, Illinois, USA.

2.3. Quality Standard of HZTF by HPLC. Quality standard of HZTF was performed as described in an earlier report with slight modifications [21]. Briefly, HZTF was dissolved in 70% ethanol, and HPLC analysis was conducted using Poroshell 120 SB-C18 (4.6×100 mm, 2.7 μm, Agilent, US). The chromatogram was monitored at wavelength 280 nm. The mobile phase consisted of A (0.1% formic acid) and B (acetonitrile) with an isometric elution as follows: A 16% and

B 84%. The flow rate of the mobile phase was 1.0 ml/min, and the injection volume was 10 μl.

2.4. Synthesis of MSU Crystals. MSU crystals were synthesized as described [22]. Briefly, 1.68 g of uric acid in 0.01 M NaOH was in a water bath heated to 70°C. Then, 0.5 M NaOH was added to maintain pH between 7.1 and 7.2, and the solution was incubated at room temperature with slow stirring and continuously for 24 h. The crystals were washed with absolute ethyl alcohol, freeze dried, and autoclaved.

2.5. Animals. Male Sprague-Dawley rats (200 ± 20 g) were purchased from the Experimental Animal Center of Henan Province, China. The animals were allowed to adapt to their environment for at least one week before initiation of the experiments. The animals were housed five per cage under a normal 12 h/12 h light/dark schedule. The animals were housed at room temperature (20 ± 2°C) with relative humidity (60 ± 5%) and were free access to a standard diet in the duration of the study. All studies were operated in accordance with the Institutional Animal Care Committee at the Experimental Animal Center of Henan Province and the China Council on Animal Care at the Experimental Animal Center of Henan Province.

2.6. MSU Crystal-Induced Inflammation in Rats

2.6.1. Animal Gout Model and Drug Administration. The rats were randomly divided into six groups. Each group contained ten rats. Group I served as controls and were intra-articular injected in the right posterior foot with 0.1 ml physiological saline. In group II, gouty inflammation was induced by intra-articular injection of 0.1 ml (10 mg) of MSU crystal suspension into the right posterior foot. Groups III, IV, and V comprised MSU crystal-induced rats treated with HZTF (0.25, 0.50, and 1.00 g/kg body weight, respectively). Group VI comprised MSU crystal-injected rats treated with positive control Tongfengshu (a commercially available Chinese patent medicine for gout, 1.50 g/kg body weight). HZTF and Tongfengshu were fully dispersed in distilled water. All doses of the respective drugs were expressed as grams per kilogram body weight. Drugs were intragastrically administered for 5 d once daily and also administration at the 5th day 1 h before the MSU crystal injection. Isoflurane was used to anesthetize the rats, and then the MSU suspension injection was given.

2.6.2. Assessment of Paw Swelling. The pathological degree was quantified by the swelling value of paw measured at 1, 2, 3, 4, 5, 6, and 7 h after MSU crystal injections. The degree of edema was expressed as paw swelling which was calculated by the following equation:

$$\text{foot swelling value} = b - a. \tag{1}$$

Here, a is the foot volume before MSU crystal injection and b is the foot volume after MSU crystal injection.

2.6.3. Histopathological Analysis. Rats were killed by cervical dislocation (7 h), and then the ankle joints of the right posterior foot of the rats were dissected and fixed in 10% neutral formaldehyde fixative for synovial tissue collection. After decalcification, the tissues were paraffin-embedded, sectioned, and stained with haematoxylin and eosin (H&E).

2.7. Cell-Free In Vitro Studies

2.7.1. Lipid Peroxidation. The thiobarbituric acid (TBA) method was carried out according to the method described by Costa et al. [23–25], and modified according to our situation. Briefly, 1 mL of the different concentrations of HZTF or VC was mixed with 1 mL of 10% yolk homogenate in tris buffer (pH 8.0). Then, 100 μl of 70 mM AAPH, 2.9 ml 20% acetic acid, and 3.0 ml 0.8% TBA solution were added. And, the mixture was boiled 60 min for full reaction; after cooling, adding 4.0 ml butanol, and fully extracting, the mixture was centrifuged at 2000 g for 10 min, extracting the organic layer. The final concentrations of HZTF or VC in the reaction system are 30.00, 60.00, 90.00, 120.00, and 150.00 μg/ml. The absorbance was measured at 532 nm by the ultraviolet-visible spectrophotometer. The decrease of absorbance indicates the increase of antioxidant activity of the samples. The antioxidant activity value of the samples was expressed as the percentage of lipid peroxidation inhibition rate which was calculated by the following equation:

$$\text{inhibition effect (\%)} = \left(1 - \frac{A_S}{A_C}\right) \times 100, \tag{2}$$

where A_S is the absorbance of test sample and A_C is the absorbance of blank control.

2.7.2. DPPH$^{\cdot}$ Radical-Scavenging Ability. DPPH$^{\cdot}$ radical-scavenging capacity was determined according to the modified method by Luo et al. [26]. HZTF and VC samples were prepared in 70% ethanol at the difference concentrations, then 1 ml of each sample mixed with 1 ml DPPH solution (0.1 M in 50% ethanol) and 3 ml distilled water. The final concentrations of HZTF are 4.00, 12.00, 20.00, 28.00 and 36.00 μg/ml, and VC are 2.00, 4.00, 6.00, 8.00 and 10.00 μg/ml. The mixtures were vortexed and allowed to equilibrate for 20 min at room temperature in the dark, and then measured the absorbance at 517 nm. [27, 28]. Scavenging activity was calculated using the formula:

$$\text{scavenging effect (\%)} = \left(1 - \frac{A_S}{A_C}\right) \times 100, \tag{3}$$

where A_S is the absorbance of the samples at different concentration and A_C is the absorbance of control.

2.7.3. ABTS$^+$ Radical-Scavenging Capacity. ABTS radical cation (ABTS$^+$ radical)-scavenging capacity was carried out according to the method of Re et al. with modification according to the situation in our laboratory [29–31]. ABTS$^+$ stock solution (3.0 mg/ml) was prepared by dissolving ABTS in 2.5 mM potassium persulfate solution and incubated the

mixture in dark overnight at room temperature. The $ABTS^+$ solution was diluted with sodium phosphate buffer (pH 7.4) to obtain absorbance of 0.7 ± 0.1 at 734 nm. 4 ml of HZTF or VC in different concentrations were mixed with 4 ml of $ABTS^+$ radical working solution and reacted for 300 s. Then, the absorbance was measured at 734 nm. The final concentrations of HZTF were 5.00, 7.50, 10.00, 12.50, and 15.00 μg/ml, and VC were 1.00, 2.00, 3.00, 4.00, and 5.00 μg/ml.

$$ABTS^+ \text{ radical scavenging } (\%) = \left(1 - \frac{A_S}{A_C}\right) \times 100, \quad (4)$$

where A_S is the absorbance of sample and A_C is the absorbance of the black control.

2.8. Cell-Base In Vitro Studies

2.8.1. Cell Culture. RAW murine macrophage 264.7 (RAW264.7) and THP-1 cell lines were purchased from the Institute of Biochemistry and Cell Biology, CAS, Shanghai, China. The RAW264.7 were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1x antibiotic solution (streptomycin (100 U/ml) and penicillin (100 U/ml)) in a humidified atmosphere supplied with 5% CO_2 and maintained at a temperature of 37°C. Cells were allowed to grow till they reached a confluency of 80–90% and washed with phosphate buffered saline (PBS) with regular replacement of culture medium.

THP-1 cells were maintained in 1640 medium supplemented with 10% FBS in a humidified atmosphere supplied with 5% CO_2 and maintained at temperature of 37°C. Cells were allowed to grow till they reached a density of 2×10^6 and then added the same volume of culture medium.

2.8.2. Cell Viability. Cell viability was performed by MTT assay. RAW264.7 macrophage cells were seeded in a 96-well culture plate. THP-1 cells were seeded in another 96-well culture plate for 3 hours with 30.82 μg/ml PMA, the day before drug treatment. After 24 h incubation, the cells were stimulated with varying concentrations of HZTF (3.12, 6.25, 12.50, 25.00, 50.00, 100.00, and 200.00 μg/ml) in FBS-free medium for further 24 h. After incubation, 10.0 μL MTT DMSO solutions (5 mg/ml) were added to each well. Then, the plates were incubated for 4 h at 37°C in dark. After incubation, 90 μL medium was removed from each well, 100 μL of DMSO was added to the wells, and then the plates were incubated for 15 min at 37°C under gentle shaking to dissolve the tetrazolium dye. The cell viability was calculated according to the results of absorbance at 570 nm and expressed as relative percentage viability. The relative cell percentage viability was calculated by the following equation:

$$\text{cell viability } (\%) = \left[\frac{A_S - A_b}{A_C - A_b}\right] \times 100, \quad (5)$$

where A_S is the absorbance of HZTF, A_C is the absorbance of the control, and A_b is the absorbance of cell blank.

2.8.3. Cell Treatment. Cells reaching a concentration of 5×10^6 cells/well in 12-well culture plate were considered for drug treatment. RAW264.7 macrophages were treated with varying concentrations of HZTF (100.00 and 200.00 μg/ml) and H_2O_2 (882.09 nM) for 24 h and prepared HZTF DMSO working solution in different concentrations. THP-1 cells were seeded with 30.82 μg/ml PMA in a 24-well culture plate for 3 hours the day before drug treatment. After 24 h of incubation, the cells were stimulated with varying concentrations of HZTF (6.25, 12.50, 25.00 and 50.00 μg/ml) and MSU in FBS-free medium for further 8 h, and then the cell culture medium of each well was collected for Elisa analysis.

2.8.4. Quantification of mRNA Levels by Quantitative Real-Time PCR. Total RNA was isolated from RAW264.7 macrophages treated with various concentrations of DEX (25 μg/ml) and HZTF (100.00 and 200.00 μg/ml) with H_2O_2 (0.15%) stimulation for 24 h and THP-1 cells pretreated with HZTF (25.00 and 50.00 μg/ml) and stimulated by MSU (1 mg/ml) for 8 h using the TRIzol reagent according to manufacturer's instructions. The total RNA was reverse transcribed by using cDNA reverse transcriptase kit, and then mRNA expression was amplified by PCR mastermix. Gene specific primers were designed manually using online NCBI primer-BLAST tool. Primer sequences of forward and reverse are shown in Table 1. Quantitative real-time PCR (qRT-PCR) was used to analyse the IL-1β, caspase-1, NLRP3, NQO1, and GAPDH gene expression of RAW 264.7 and IL-1β of THP-1, respectively. Transcription levels were assessed to utilize the step on real-time thermal cycler with PCR mastermix. Thermal cycling conditions were as follows: denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 65°C for 5 s. The levels of GAPDH gene expression served as an internal control.

2.8.5. ELISA Analysis. THP-1 cells were pretreated with MCC950 (4.26 μg/ml) or HZTF (6.25, 12.50, 25.00 and 50.00 μg/ml), stimulated by MSU (1 mg/ml) for 8 h, and collected the supernatant. Secretion of inflammatory cytokine (IL-1β, IL-6 and TNF-α) was measured by ELISA kits according to the assay procedure.

2.8.6. Western Blotting Analysis. Protein samples were obtained from the culture medium as same as in the ELISA assay and concentrated by the ultrafiltration device. Protein sample was denatured by mixing with 1/4 volume of 5x loading buffer, and metal bath at 100°C for 10 min. Samples were resolved by electrophoresis with 10% SDS-PAGE and transferred onto a PVDF membrane (Merk, Germany). After blocking nonspecific binding sites for 2 h with 5% dried skim milk dissolved in TBST, the membranes were individually incubated for overnight with anti-IL-1β, anti-IL-6, anti-caspase-1, anti- PGE2, and ASC. Develop the color of the blot rocking in 3, 3′-diaminobenzidine (DAB) substrate solution. Stop the reaction by pouring out the substrate after the expected band appears and then well rinsed

Table 1: Sequences of PCR primers used for qRT-PCR.

Species	Gene	Primer	Sequence
Mice	IL-1β	Forward primer	ACCTGGGCTGTCCTGATGAGAG
		Reverse primer	TGTTGATGTGCTGCTGCGAGAT
	Caspase-1	Forward primer	GGGACCCTCAAGTTTTGCC
		Reverse primer	CAACTTGAGCTCCAACCCTC
	NLRP3	Forward primer	TCTGTGTGGACCTAAGCCCC
		Reverse primer	GGGATACAGCCTTTCTCGGG
	NQO1	Forward primer	GAGAGGATGGGAGGTACTC
		Reverse primer	AATATCTGGGCTCAGGCGTC
	GAPDH	Forward primer	GTCATTGAGAGCAATGCCAG
		Reverse primer	GTGTTCCTACCCCCAATGTG
Human	IL-1β	Forward primer	TGATGGCTTATTACAGTGGCAATG
		Reverse primer	GTAGTGGTGGTCGGAGATTCG
	IL-6	Forward primer	CACTGGTCTTTTGGAGTTTGAG
		Reverse primer	GGACTTTTGTACTCATCTGCAC
	TNF-α	Forward primer	CAGAGGGAAGAGTTCCCCAG
		Reverse primer	CCTTGGTCTGGTAGGAGACG
	GAPDH	Forward primer	AGCCTCAAGATCAGCAATG
		Reverse primer	CACGATACCAAAGTTGTCATGGAT

with distilled water repeatedly. Dry the membrane, and store it in the dark place.

2.8.7. Confocal Microscopic Analysis. THP-1 cells were pretreated with MCC950 (4.26 μg/ml) or HZTF (50.00 μg/ml) and stimulated by MSU (1 mg/ml) for 8 h. Briefly, THP-1 cells were plated on coverslips and incubated with an anti-ASC antibody and then incubated with an anti-rabbit IgG-FITC antibody. Recording with a laser scanning confocal microscope (LSM710, Carl Zeiss, Oberkochen, Germany), the ASC speck formation was analyzed using Zen2010 software.

2.9. Statistical Analysis. All results were expressed as mean ± standard deviation ($n = 3$). Statistical analysis was carried out by SPSS 21 statistical software (IBM Inc., USA). A statistical difference was considered significant when P-values are less than 0.05.

3. Results

3.1. Quality Standard of HZTF by HPLC. As shown in Figure 1, four substances in HZTF were selected as content indicators: (1) plantamajoside, (2) polygonum cuspidin, (3) specnuezhenide, and (4) resveratrol glucoside, and all of them had distinct peaks under 280 nm ultraviolet absorption.

3.2. Effect of HZTF and Tongfengshu in In Vivo Studies. To determine the degree of edema, the swelling value of the right posterior foot ankle joints of rats were measured (Table 2). The foot-swelling values of MSU crystal-induced rats were significantly higher than that of the control rats at 1 h to 7 h ($P < 0.05$). Paw edema decreased significantly in MSU crystal-induced rats treated with HZTF (0.50 and 1.00 g/kg body weight) at 1 h to 7 h and Tongfengshu (1.50 g/kg body weight) at 1 h to 7 h ($P < 0.05$).

To compare the histological changes in the joints between different groups, joint tissues were sectioned and examined via H&E staining. The coverage of synovial cells on synovial cavity of the joints was fragmentary, and there was edema in loose connective tissue, as well as local infiltration of leukocytes (Figure 2). However, rats treated with HZTF (0.50 and 1.00 g/kg body weight) and Tongfengshu (1.50 g/kg body weight) decreased edema and leukocyte infiltration after 7 h. This change correlated well with the decrease in paw swelling induced by HZTF and Tongfengshu treatment.

3.3. In Vitro Cell-Free Antioxidation Experiment of HZTF. To investigate whether the antigout effect of HZTF is due to its antioxidant activity, we measured the antioxidant activity of HZTF using multiple methods. As shown in Figure 3(a), we can know that HZTF can significantly inhibit lipid peroxidation when the concentration was higher than 60.00 μg/ml, and the IC_{50} values of VC and HZTF are 49.94 ± 3.67 and 114.62 ± 3.43 μg/ml, respectively, which mean the lipid peroxidation inhibition ability of HZTF is significantly weaker than VC ($P < 0.05$). It is observed that the $DPPH^{\cdot}$ radical-scavenging activity of HZTF is shown in Figure 3(b). The IC_{50} values of VC and HZTF are 2.81 ± 0.62 and 13.04 ± 2.80 μg/ml, respectively. Though it can inhibit the $DPPH^{\cdot}$ radical, the IC_{50} value of HZTF is significantly higher than VC ($P < 0.05$). As shown in Figure 3(c), the $ABTS^{+}$ radical-scavenging activity of both VC and HZTF are in dose-effect relationships. HZTF can scavenge the $ABTS^{+}$ radical significantly when the concentration was higher than 5.00 μg/ml and the IC_{50} values of VC and HZTF are 1.48 ± 0.70 and 7.52 ± 0.50 μg/ml, respectively, which means the $ABTS^{+}$ radical-scavenging activity of HZTF is significantly weaker than VC ($P < 0.05$).

3.4. Cell Viability. MTT assay was utilized to measure the cytotoxicity of HZTF on RAW264.7 macrophages and THP-

Figure 1: The quality standard of HZTF was determined using an HPLC system, plantamajoside (1), polygonum cuspidin (2), specnuezhenide (3) and resveratrol glucoside (4).

1 cells. Figure 4 shows that the cell viability was not significantly altered after treating with HZTF for 24 h up to 200.00 μg/ml concentration, suggesting no cytotoxicity of HZTF up to 200.00 μg/ml.

3.5. Experiment with RAW264.7 Macrophages

3.5.1. RT-qPCR Analysis. As shown in Figure 5, changes of IL-1β, caspase-1, NLRP3, and NQO1 expression in H_2O_2-induced RAW264.7 macrophages were compared with the control group without H_2O_2, and the expression of IL-1β, caspase-1, and NLRP3 in RAW264.7 macrophages was increased by treatment with 0.15% H_2O_2 for 24 h. Interestingly, the expression of IL-1β, caspase-1, and NLRP3 in RAW264.7 macrophages treated with HZTF at 100.00 and 200.00 μg/ml were significantly reduced ($P < 0.05$). Moreover, an obvious difference ($P < 0.05$) was found between the mRNA expression of NQO1 with or without HZTF (100.00 and 200.00 μg/ml), and the expression of NQO1 was increased with the increase of HZTF concentrations, which may indicate a dose-dependent relationship between NQO1 expression and HZTF concentrations. In conclusion, the results showed that H_2O_2 upregulated the expression of IL-1β, caspase-1, and NLRP3 and downregulated the expression of NQO1. Treatment with HZTF inhibits the expression of IL-1β, caspase-1, and NLRP3 and enhanced the expression of NQO1 in H_2O_2-induced RAW264.7 macrophages at the same time.

3.6. Experiment with THP-1 Cells

3.6.1. ELISA Analysis. To investigate the mechanism of HZTF against gout, qPCR was used to measure the effect of HZTF on IL-1β, IL-6, and TNF-α expression and ELISA was used to measure the effect of HZTF on IL-1β, IL-6, and TNF-α secretion. As shown in Figures 6(a)–6(c) MSU or HZTF does not significantly affect the expression of IL-1β, IL-6, and TNF-α. As shown in Figures 6(d)–6(f), HZTF significantly reduced the secretion of IL-1β, IL-6, and TNF-α in THP-1 cells stimulated by MSU ($P < 0.05$). The IC_{50} of IL-1β, IL-6, and TNF-α secretion in THP-1 cells stimulated by MSU were 20.94 ± 2.13, 26.04 ± 1.28, and 24.87 ± 0.68 μg/ml, respectively.

3.7. Western Blotting Analysis. As shown in Figure 7 HZTF inhibited the procaspase-1, cleaved-caspase-1, pro-IL-1β, cleaved-IL-1β, ASC dimer, IL-6, and $PGEs_2$ in the culture medium which were secreted by THP-1 cells in a dose-dependent manner.

3.8. Confocal Microscopic Analysis. As a symbol of inflammasome activation, ASC forms oligomers in response to NLRP3 activators. Confocal microscopy analysis intuitively showed that HZTF suppressed MSU crystal-induced formation of ASC speckles in THP-1 cells (Figure 8).

4. Discussion

Inflammatory response to MSU crystals triggered the acute symptoms of gout and mainly mediated by macrophages and neutrophils. Intra-articular injection of MSU crystal can simulate gouty arthritis in humans and produces a painful response which is similar to the acute gouty attacks occurrence spontaneously [32]. Significant swelling accompanied by an extensive inflammatory response after MSU injection in the ankle joint is an important symbol in determining the degree of inflammation and therapeutic efficacy. The primary pathological symbol of gout is the neutrophils accumulated and infiltrated in the joint tissues, which actively phagocytosed MSU crystals. Then, the membrane lysed and released inflammatory cytokines and free radicals amplified the inflammatory response [33, 34]. We observed similar changes in the joint MSU injection rat model, so this model is suitable for studying the pathological mechanism of gout arthritis.

Plants which give antioxidant activities should be highly potent in the management of gout because they often share xanthine oxidase (XO) inhibitory effects [35, 36]. We measured the antioxidant activities to study whether the antigout effect of HZTF is related to its antioxidant capacity. Lipid peroxidation occurs in the process of reactive oxygen

Table 2: Effect of HZTF and Tongfengshu on the ankle joint edema of MSU crystal-induced rats.

Treatment	Dose (g/kg)	n	Foot-swelling values (cm, mean ± SD)						
			1 h	2 h	3 h	4 h	5 h	6 h	7 h
Ctrl	—	10	$0.009 \pm 0.002^{**}$	$0.010 \pm 0.004^{**}$	$0.008 \pm 0.003^{**}$	$0.0010 \pm 0.003^{**}$	$0.004 \pm 0.002^{**}$	$0.005 \pm 0.001^{**}$	$0.003 \pm 0.000^{**}$
MSU alone	—	10	0.169 ± 0.055	0.185 ± 0.056	0.208 ± 0.065	0.254 ± 0.059	0.289 ± 0.058	0.323 ± 0.051	0.304 ± 0.061
Tongfengshu	1.50	10	$0.107 \pm 0.023^{*}$	$0.142 \pm 0.046^{*}$	$0.161 \pm 0.042^{*}$	$0.193 \pm 0.049^{*}$	$0.202 \pm 0.048^{*}$	$0.253 \pm 0.053^{*}$	$0.239 \pm 0.055^{*}$
HZTF	0.25	10	0.139 ± 0.035	0.178 ± 0.050	0.197 ± 0.051	$0.206 \pm 0.056^{*}$	$0.226 \pm 0.055^{*}$	0.257 ± 0.028	$0.247 \pm 0.065^{*}$
HZTF	0.50	10	$0.123 \pm 0.042^{*}$	$0.150 \pm 0.056^{*}$	$0.168 \pm 0.056^{*}$	$0.204 \pm 0.060^{*}$	$0.218 \pm 0.041^{*}$	$0.245 \pm 0.025^{*}$	$0.237 \pm 0.062^{*}$
HZTF	1.00	10	$0.121 \pm 0.048^{*}$	$0.135 \pm 0.044^{*}$	$0.150 \pm 0.042^{*}$	$0.179 \pm 0.037^{*}$	$0.197 \pm 0.038^{**}$	$0.217 \pm 0.034^{**}$	$0.189 \pm 0.050^{**}$

$^{*}P < 0.05$ when compared with MSU groups, $^{**}P < 0.01$ when compared with MSU groups.

Figure 2: Histological examination of rat joints injected with MSU crystals (magnification, ×200). In the control group, synovial membrane was normal and no inflammatory cell infiltration was found. (a) Joints injected with MSU crystals demonstrated leukocytes infiltrations and edema. Synovial tissue proliferation of nodularity was observed in the synovial lining of the joint, as well as synovial membrane cells with some zones of degeneration and necrosis (MSU alone). (b) Acute inflammation with polymorphonuclear infiltration in the synovial lining of MSU crystal-injected rats was attenuated by Tongfengshu and HZTF. (c) The Tongfengshu (1.50 g/kg) group has no obvious edema in loose connective tissue, but there were still demonstrated inflammatory cells infiltration. (d–f) HZTF (0.25, 0.5 and 1.00 g/kg) groups demonstrated only mild infiltration of scattered leukocytes. The synovial of HZTF group (MSU and HZTF 1.00 g/kg) appeared normal.

Figure 3: Continued.

(c)

Figure 3: Lipid peroxidation inhibition ability. (a) DPPH· free radical-scavenging activity and (b) ABTS+ radical-scavenging capacity (c) of HZTF at different concentrations.

(a) (b)

Figure 4: Cell viability of RAW264.7 (a) and THP-1 (b) cells treated with HZTF at different concentrations.

(a) (b)

Figure 5: Continued.

Figure 5: Effect of HZTF on IL-1β (a), caspase-1 (b), NLRP3 (c), and NQO1 (d) expression in H_2O_2-stimulated RAW264.7 macrophages. The values are expressed as mean ± SD. Comparisons are made with: control or HZTF (100, 200 μg/ml) treated versus H2O2-stimulated RAW264.7 macrophages. *means significantly different ($P < 0.05$) and **means very significantly different ($P < 0.01$).

Figure 6: Continued.

Figure 6: Effect of HZTF on IL-1β (a), IL-6 (b), and TNF-α (c) expression and IL-1β (d), IL-6 (e), and TNF-α (f) secretion in THP-1 stimulated by MSU. *means significant different ($P < 0.05$) and **means very significant different ($P < 0.01$).

Figure 7: Effect of HZTF on procaspase-1, cleaved-caspase-1, pro-IL-1β, cleaved-IL-1β, ASC dimer, IL-6, and PGE_2 protein expression secreted into culture medium.

species (ROS) oxidizing the biological membranes, which ROS reacts with membrane phospholipids, enzyme and membrane receptor-associated polyunsaturated side chain, and nucleic acid fatty acid and produces lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal, and then the fluidity and permeability of cell membrane changed, resulting in the change of structure and function of cells [37, 38]. The free radical-scavenging capacity of HZTF was measured using commercially available stable free radical $DPPH^{\cdot}$. HZTF presented $DPPH^{\cdot}$ radical-scavenging capacity to some extent. It appears that antioxidant activity and reduction of $DPPH^{\cdot}$ both require the presence of a free OH group on the ellipticine ring [39], and if HZTF can reduce $DPPH^{\cdot}$, it may suggest that HZTF contains compounds of similar structure. Based on the special chemical properties of formed free radicals, ABTS assay is frequently used to measure the radical-scavenging capacity of compounds [40]. The $ABTS^{+}$ radical elimination is a common antioxidant method as well as the $DPPH^{\cdot}$ method to evaluate the antioxidant capacity of the chemical compound. Based on previous descriptions, *Polygonum cuspidatum*, *Plantain*, and *Ligustrum*, which were used to produce HZTF, can extract compounds with good antioxidant activity, and similar to HZTF, these compounds were also extracted from ethanol, which suggest that the main antioxidant compounds containing HZTF may be phenolic compounds too [41–43]. According to the results, the lipid peroxidation inhibition ability, $DPPH^{\cdot}$ radical-scavenging ability and $ABTS^{+}$ radical-scavenging capacity of HZTF were significantly weaker than VC ($P < 0.05$). VC is a strong antioxidant [44], but seldom used in acute gout treatment. HZTF has weaker antioxidant capacity than vitamin C, but has a strong antigout capacity, so we believed that the antioxidant capacity of HZTF may not be the main mechanism of its antigout effect.

IL-1β is a proinflammatory cytokine that induces local and systemic inflammation aimed to eliminate foreign matters and microorganisms. However, exorbitant levels of inappropriate IL-1β production have been shown to be a key process in the etiology of the disease. In these conditions,

Figure 8: THP-1 cells were fixed, permeabilized, and stained for ASC (green), and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). The arrows indicate ASC speckles (Magnification, ×600).

blocking IL-1β has proven very effective in clinical studies [45]. Caspase-1 and NLRP3 are closely linked to the pathogenesis of various metabolic diseases including gouty arthritis [22, 46]. At the same time, the expression increase of IL-1β, caspase-1, and NLRP3 is closely related to oxidative stress, and if we reduce the level of oxidative stress, we may suppress the expression of IL-1β, caspase-1, and NLRP3 [47]. NQO1 is an important cellular antioxidant enzyme, and NQO1 is an Nrf2-regulated downstream enzyme, and the mRNA expression of NQO1 was observed after treatment. In the present study, we investigated whether HZTF has any regulatory effect on the gene expression of IL-1β, caspase-1, NLRP3, and NQO1 in H_2O_2 stimulated RAW264.7 macrophages. Changes of IL-1β, caspase-1, and NLRP3 expression in H_2O_2-induced RAW264.7 macrophages were compared with the control group without

H_2O_2; the expression of IL-1β, caspase-1, and NLRP3 in RAW264.7 macrophages was increased by treatment with 0.15% H_2O_2 for 24 h. Interestingly, the expression of IL-1β, caspase-1, and NLRP3 in RAW264.7 macrophages treated with HZTF at 100.00 and 200.00 μg/ml were significantly reduced ($P < 0.05$). Furthermore, an obvious difference ($P < 0.05$) was found between the mRNA expression of NQO1 with or without HZTF (100.00 and 200.00 μg/ml), the expression of NQO1 was increased with the increase of HZTF concentrations, which may indicate a dose-dependent relationship between NQO1 expression and HZTF concentrations. In conclusion, the results showed that H_2O_2 upregulated the expression of IL-1β, caspase-1, and NLRP3, and downregulated the expression of NQO1. Treatments with HZTF inhibit the expression of IL-1β, caspase-1, and NLRP3, and enhanced the expression of NQO1 in H_2O_2-induced RAW264.7 macrophages at the same time.

Development of the acute and chronic inflammatory responses known as gout and pseudogout are associated with the deposition of MSU crystals, in joints and periarticular tissues. MSU engage the caspase-1-activating NALP3 (also called cryopyrin) inflammasome, resulting in the production of active IL-1β [22]. Macrophages from mice deficient in components of the inflammasome such as ASC (RAW264.7 macrophages) are defective in crystal-induced IL-1β activation [48]. THP-1 cells were chosen for this experiment. According to the result, MSU crystals did not increase IL-1β mRNA expression, but increased IL-1β secretion, and HZTF also did not affect the mRNA expression of IL-1β, IL-6, and TNF-α, but significantly reduced the secretion of IL-1β, IL-6, and TNF-α in THP-1 cells stimulated by MSU. In the western blotting test, we can know that HZTF inhibited procaspase-1, cleaved-caspase-1, pro-IL-1, cleaved-IL-1β, and IL-6 at the same time, suggesting that HZTF may inhibit not only the inflammasomes but also the NF-κb signaling. Also, in the western blotting result, HZTF showed inhibition effect in PGE_2 secretion, which suggests that the mechanism of antigout effect of HZTF may be varied. At the same time, confocal microscopy analysis intuitively demonstrated the inhibitory effect of HZTF on ASC dimerization. These results suggested that HZTF may prevent gout by inhibiting NALP3 inflammasome as well as blocking the NF-κb signaling pathway, which will increase the expression of IL-1β, IL-6, and TNF-α [49].

Earlier reports indicated that four flavours of traditional Chinese medicinal materials which were used to produce HZTF contained several components, of which plantamajoside, *Polygonum cuspidatum*, specnuezhenide and resveratrol glucoside were found to be the main active constituent [2–11]. In present study, HPLC fingerprint analysis of HZTF showed distinct peaks of all four active constituents under 280 nm ultraviolet absorption.

5. Conclusion

The present study confirmed the antigout effects of HZTF in *in vivo* experiments and showed its reduction effects on the secretion of proinflammatory cytokines IL-1β, IL-6, and TNF-α. At the same time, whether its antigout effect is related to its antioxidant capacity needs further study. This study provides a new promising therapeutic TCM for gout, and the precise mechanism of action is worth further to be investigated.

Abbreviations

TCM:	Traditional chinese medicine
MSU:	Monosodium urate
HZTF:	Huzhentongfeng
VC:	Ascorbic acid
DEX:	Dexamethasone
DMEM:	Dulbecco's modified eagle's medium
FBS:	Fetal bovine serum
PBS:	Phosphate-buffered saline
MTT:	Methylthiazolyldiphenyl-tetrazolium bromide
PMA:	Phorbol-12-myristate-13-acetat
DPPH:	1,1-Diphenyl-2-picryl-hydrazyl
ABTS:	3-Ethylbenzthiazoline-6-sulfonic acid
AAPH:	2,2′-Azobis (2-methylpropionamidine) dihydrochloride
Caspase-1:	Cysteine-aspartic proteases-1
NLRP3:	NACHT, LRR, and PYD domains-containing protein 3
ASC:	Apoptosis-associated speck-like protein containing
Caspase-1:	Cysteinyl aspartate specific proteinase-1
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
TNF-α:	Tumor necrosis factor-α
PGE_2:	Prostaglandin E2
NF-κB:	Nuclear factor-κb
NQO1:	NADPH dehydrogenase, quinone-1
Nrf2:	Nuclear factor (erythroid-derived 2)-like 2
HPLC:	High-performance liquid chromatography
Elisa:	Enzyme-linked immunosorbent assay
IC_{50}:	Half maximal inhibitory concentration
H&E:	Hematoxylin and eosin
SD:	Standard deviation
ANOVA:	One-way analysis of variance
LPS:	Lipopolysaccharide
PCR:	Polymerase chain reaction
RT-qPCR:	Real-time quantitative polymerase chain reaction.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All studies were operated in accordance with the Institutional Animal Care Committee at the Experimental Animal Center of Henan Province and the China Council on Animal Care at Experimental Animal Center of Henan Province.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Z. C. W., Z. L. Z., and P. J. Z. investigated the effectiveness of HZTF as an antigouty inflammation medicine in MSU crystal-induced gouty rat *in vivo* and performed the cell-base experimental study; Q. Z., Z. Z., and J. P. D. performed the cell-base antioxidation experiment; K. Y. and H. F. performed the cell-free antioxidation experiment; and Y. F. W. and Z. P. W. were major contributors in designing the whole experimental and writing the manuscript. All authors read and approved the final manuscript.

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Review Article

Effectiveness of Cherries in Reducing Uric Acid and Gout: A Systematic Review

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Background. Previous studies have reported the use of complementary therapies to reduce the risk of gout attacks. In this study, we assessed the effectiveness of cherries in reducing uric acid levels associated with gout. *Methods*. We searched for relevant studies on PubMed, Embase, and the Cochrane Library without restrictions on language from inception until August 15, 2019. The risk of bias was evaluated using the PRISMA statement and checklist, and the methodological quality was assessed using the Cochrane Collaboration tool. *Results*. The six studies included in this systematic review reported decreases in the incidence and severity of gout following the ingestion of cherries. Gout patients regularly ingesting cherry extract/juice reported fewer gout flare ups than those patients who did not supplement their diets with cherry products. Overall, we observed a positive correlation between the consumption of tart cherry juice and a decrease in serum uric acid concentration. *Conclusions*. Current evidence supports an association between cherry intake and a reduced risk of gout attacks. Note however that we were unable to conduct effective meta-analysis due to a lack of relevant studies and a high degree of variation in the methodologies and metrics used in previous studies. Further comprehensive trials or long-term follow-up studies will be required to evaluate the efficacy of cherry intake in treating patients with gout or hyperuricemia.

1. Introduction

Gout refers to the crystallization of uric acid in or around the joints. It is a prevalent consequence of inflammatory arthritis [1], commonly causing discomfort and intense pain [2, 3]. Patients diagnosed with gout and/or hyperuricemia must undergo continuous pharmacological therapies [4]. Allopurinol and febuxostat are the drugs most commonly prescribed to lower urate levels by inhibiting the activity of xanthine oxidase [5, 6].

Cherry is a nutritious fruit containing a variety of chemical compounds, which have proven beneficial to patients with gout, insomnia, and sore muscles. It has also proven beneficial to patients with chronic diseases, such as cardiovascular disease, diabetes, and cancer [7]. From the perspective of alternative medicine, diet is seen as a complementary factor associated with gout, based on the fact that the incidence of gout is higher among obese patients and those who ingest large quantities of alcohol, sugar, and/or purine products [1, 2]. A variety of foods, such as various fruits, are considered beneficial in reducing uric acid levels [8, 9]. However, further evidence is required to verify the effectiveness of nonpharmacological methods. Several studies have reported that cherry extract and/or cherry juice could be taken as a supplement to reduce uric acid levels in patients suffering from gout [9–11].

There is evidence indicating that the consumption of cherry can reduce uric acid concentrations; however, more substantial and integrative results are required. Our objective in the current systematic review was to assess the effectiveness of cherry products in reducing uric acid levels and mediating the incidence of gout attack.

2. Materials and Methods

2.1. Literature Review. We searched through PubMed, Embase, and the Cochrane Library for relevant studies (without language limitations) from inception until August 15, 2019. These databases cover most of the research articles pertaining to this topic. Eligible studies were identified by scanning electronic databases using various combinations of Medical Subject Headings (MeSH) and non-MeSH terms.

2.2. Data Sources and Search Methods. The search process was extended by (i) perusing the reference section of all relevant studies and (ii) manually searching through the abstracts of key journals and papers published at major annual meetings. The search terms included the following: (gout OR hyperuricemia) AND (cherry or cherry juice or cherry extract) AND (efficacy or effectiveness). We also checked the reference list of screening studies to identify other similar studies. The search strategy excluding manual search methods is shown in Table 1. This study was based on guidelines outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Figure 1).

2.3. Data Extraction and Quality Assessment. A data extraction form was used to obtain the following data from the included studies: first author, publication year, country, database used, study duration, study design, study subjects, mean age of study subjects, assigned groups, and outcomes. An assessment of methodological quality was performed independently by the authors Pei-En Chen and Tao-Hsin Tung. The quality assessment included the following items: allocation generation and concealment, blinding, follow-up duration, loss follow-up (%), and data-analysis method (intention-to-treat or per protocol). Discrepancies were resolved through discussion and consensus. Table 2 lists the results of a methodological quality assessment performed on all papers included in this study. We found that the most common sources of potential bias were inadequate allocation concealment and sequence generation. Due to the small number of papers and the degree of heterogeneity in the study design, interventions, and outcome indices, meta-analysis was deemed impractical.

Two authors also independently reviewed the titles and abstracts after all of the references from relevant studies had been imported to EndNote. After a thorough appraisal of selected publications, we indexed the full text and subsequently assessed the risk of bias using the Cochrane Handbook for Systematic Reviews of Interventions [15]. The handbook includes seven domains of bias risk: (1) random sequence generation, (2) allocation concealment, (3) blinding of participants and personnel, (4) blinding of outcome assessment, (5) incomplete outcome data, (6) selective reporting, and (7) other sources of bias. The Cochrane Collaboration Tool was used to assess the risk of bias by the Review Manager version 5.3.5 in the included trials [16]. Any disagreement was resolved through discussion with third author Ching-Wen Chien.

Table 1: Search strategy in PubMed up till August 15, 2019 (similar search conducted in other databases).

1	Gout [MeSH]
2	Hyperuricemia [Me]
3	Gout [title/abstract]
4	Hyperuricemia [title/abstract]
5	1 OR 2 OR 3 OR 4
6	Cherry [title/abstract]
7	Cherry juice [title/abstract]
8	Cherry extract [title/abstract]
9	6 OR 7 OR 8
10	Efficacy [title/abstract]
11	Effectiveness [title/abstract]
12	10 OR 11
13	5 AND 9 AND 12

Figure 1: PRISMA study flow chart.

Figure 2 presents a summary assessment of bias risk. Bell et al. [13] did not clearly describe how research populations are selected [13]. Jacob et al. [9] did not clearly illustrate whether the participants were blinded [9]. Schlesinger et al. [10] and Singh et al. [14] lost a number of research objects to follow-up; therefore, we must assume a high risk of bias [10, 14]. Singh et al. [14] and Zhang et al. [12], did not blind participants, such that the assessment of outcomes must be regarded as questionable [12, 14].

2.4. Data Synthesis. The outcomes of the selected studies were qualitatively assessed, with a focus on plasma urate

Table 2: Methodological quality assessment of the included studies.

Author	Year	Allocation generation	Allocation concealment	Double blinding	Follow-up duration	Loss to follow-up (%)	Data analysis	Other bias
Jacob et al. [9]	2003	Screened for good health by a medical history	Adequate	Unclear	1.5, 3, and 5 hours	0	ITT	
Schlesinger et al. [10]	2012	Patients with MSU crystal-proven gout were considered in this study	Adequate	Blinded participants	4 months	22	PP	
Zhang et al. [12]	2012	Internet survey	Adequate	No	3, 6, 9, and 12 months of follow-up	0	ITT	
Bell et al. [13]	2014	Volunteers	Unclear	Single blind	1, 2, 3, 5, 8, 24, 26, and 48 hours	0	ITT	
Singh et al. [14]	2015	A brief anonymous internet survey on a voluntary basis	Adequate	No	1 month	4	PP	
Martin and Coles [11]	2019	Through the use of handbills, word-of-mouth notification, and poster displays	Adequate	Blinded participant	4 weeks	0	ITT	

ITT: intention-to-treat; PP: per protocol.

Figure 2: Risk of bias summary.

level (μmol/L), the number of gout flare incidents, the risk of gout attacks (OR), serum urate levels, and serum uric acid concentrations.

3. Results

3.1. Study Characteristics. Figure 1 illustrates the results of this systematic review. Following a thorough review of all candidate papers, we identified a total of six studies that addressed the relationship between cherry intake and gout. The characteristics of the studies are listed in Table 3. Among the studies in this paper, five were conducted in the United States (US) and one was conducted in the United Kingdom (UK).

3.2. Clinical Efficacy of Cherry in Reducing Uric Acid and the Incidence of Gout Attacks. One of the studies investigated the relationship between the ingestion of cherry extract and urate concentration levels. Two of the studies focused on the correlation between the incidence of gout attacks and cherry intake. Zhang et al. [12] assessed the relationship between cherry consumption and the risk of recurrent gout attacks [12]. They reported that after ingesting cherries for a period of two days, there was a significant decrease in the risk of gout attacks. Cherry juice and cherry extract produced consistent results across groups categorized by sex, obesity status, purine intake, alcohol use, diuretic use, and use of antigout medications. They also reported that combining allopurinol with cherry could reduce the risk of gout attack.

In an investigation on gout history obtained from a website browser, Singh et al. [14] reported the a number of outcomes associated with the consumption of cherry-related supplements: (1) a significant reduction in the number of gout flares compared to the previous month; (2) a lower likelihood of being free from gout flare ups compared to the previous month; (3) a trend towards a lower proportion with urate-lowering therapy (ULT) medication possession ratio of 80%; and (4) an increase in the number of days without the need for ULT compared to the previous month [14]. Martin and Coles [11] reported significant reductions in plasma urate levels following the ingestion of tart cherry juice for 4 weeks [11]. Note however that there was a decrease in serum uric acid concentrations in the case group but an increase in the placebo group [11].

Schlesinger et al. [10] performed three pilot studies investigating the effects of cherry juice concentrate on gout

Table 3: Characteristics of included studies.

First author	Year	Country	Study design	Inclusion criteria	Intervention	Study subject	Mean age (years)	Gender (M/F)	Race	BMI	Outcome measures
Jacob	2003	USA	Follow-up study	Screened for good health by a medical history questionnaire, physical exam, and standardized blood and urine tests; including a complete blood cell count with leukocytes differentials, clinical chemistry panel, urinalysis, and tests for infectious diseases	Through consumption of two servings (280 g) of cherries after an overnight fast	10 healthy women	29.9 ± 6.1(range: 22–40)	0/10	Primarily caucasian	NA	Plasma urate decreased significantly over the 5 h period after cherry consumption, and the concentration at 5 h post-dose was significantly lower than at the baseline
Schlesinger	2012	USA	Randomized controlled trial (RCT)	Patients with monosodium urate (MSU) crystal-proven gout	The case and control group received a tablespoon of juice concentrate twice a day, with an intervention period of 4 months	Case-cherry juice ($n = 9$) Control-pomegranate juice ($n = 5$)	56.43 ± 4.10 (range 28–75)	NA	Caucasian: 11, Asian: 1, Hispanic: 1 African American: 1	30.02 ± 0.84 (range: 24.4–34.4)	Overall, serum urate levels were only slightly reduced following treatment with either cherry juice (from 8.37 ± 0.82 to 8.17 ± 1.1 mg/dL) or pomegranate juice (from 7.45 ± 1.62 to 6.14 ± 1.07 mg/dL)
Zhang	2012	USA	Case-crossover study	Gout diagnosed by a physician and that have suffered a gout attack within the past 12 months	Cherry intake, for more than 2 days	663 patients (gout patients)	54 (21–88)	494 (78%)/ 169 (22%)	Black: 19 (3%) White: 558 (88.2%) Other: 47 (7.4%) Refused to answer: 9 (1.4%)	BMI (kg/m^2, median, range) 30.6 (14.7–69.9)	(OR) = 0.65, 95% CI: 0.50–0.85) for gout risk

Table 3: Continued.

First author	Year	Country	Study design	Inclusion criteria	Intervention	Study subject	Mean age (years)	Gender (M/F)	Race	BMI	Outcome measures
Bell	2014	UK	Single blind, two-phase, randomized, crossover design	Volunteered for the study; all volunteers confirmed they were nonsmokers, had no known food allergies, and no history of gastrointestinal, renal, or cardiovascular disease or use of food supplementations	The bioavailability of anthocyanins followed by the ingestion of two different doses of montmorency tart cherry juice concentrate (MC)	12 healthy participants	26 ± 3	11/1	NA	NA	Serum urate displayed effects after a significant amount of time (F (1, 8) = 10.626, $P < 0.001$)
Singh	2015	USA	Retrospective cohort study	293 internet survey respondents 220 (75%) with gout 61 (21%)W/O gout 12 (4%) no response	Cherry intake (1 month)	220 gout patients	55.2 ± 14.1	74%/ 26%	White: 79%		Cherry intake has significantly stronger effects in producing less number of gout flare vs. none (1.54 vs. 1.91, $p = 0.047$)
Martin	2019	USA	Randomized, placebo-controlled dietary intervention	Participants were aged ≥18 y, not pregnant, not diabetic, with no unresolved infections or diseases (diabetes, cardiovascular disease, inflammatory bowel disease, cancer, or liver disease), and nonsmokers	Through consumption of 240 mL/d of either tart cherry juice (TCJ) or placebo beverage	26 participants (overweight and obese participants with body mass index (BMI) > 25.0 kg/ m^2)	41 ± 11 (range 22–61 y)	8/18	NA	Mean ± SE 31.3 ± 6.3 (range 25.1–51.3)	TCJ significantly reduced serum uric acid concentration by 19.2% ($P < 0.05$)

[10]. We selected one of these for inclusion in our review. In the selected study, Schlesinger et al. [10] compared the effects of ingesting cherry juice with those of pomegranate juice. They reported that both juices slightly decreased plasma urate levels; however, the effects of cherry juice were less pronounced [10]. Nonetheless, none of the observed changes reached the level of significance.

3.3. Time Effect in Reducing Uric Acid. The last two papers addressed in this study examined the relationship between cherry consumption and variations in uric acid and plasma urate levels over time. In research on ten women, Jacob et al. [9] observed a significant reduction in plasma urate levels for a period of 5 hours following the consumption of cherries [9]. Grapes and strawberries had similar effects; however, the observed changes did not reach the level of significance. The consumption of kiwifruit actually led to an increase in plasma urate levels. In research on 12 healthy participants, Bell et al. [13] observed a significant reduction in serum urate at 2 hours after cherry ingestion [13].

4. Discussion

4.1. Clinical Implications. Our study provided a synthesis of current findings from six studies, indicating a correlation between the ingestion of cherry extract and gout. However, there is no consensus as to the molecular association between cherry extract and gout, due to the fact that only a few studies performed statistical analysis on these variables. In addition, we deemed it impractical to conduct a meta-analysis to evaluate the possible relationship between cherry intake and gout severity.

The ingestion of cherry has proven effective in lowering urate levels, and previous studies have attributed the suppression of gout-related inflammation to the anti-inflammatory properties of cherry [7]. Key enzymes involved in inflammation (e.g., cyclooxygenase 1 and 2) are strongly inhibited by anthocyanin from cherry extract. Cherry extract has also been shown to reduce the levels of various cytokines (e.g., IL-1β, TNF-α, IL-6, and IL-17) in affected joints [17]. Evidence-based research has shown that cherry can reduce the formation of proinflammatory substances, such as NO C-reactive protein (CRP) [18]. A failure to treat hyperuricemia increases the prevalence of mortality due to cardiovascular disease as well as the incidence of complications related to diabetes mellitus [11]. These findings highlight the importance of controlling one's diet in seeking to ameliorate the accumulation of urate. A number of researchers have suggested that the effectiveness of cherry in reducing uric acid and the incidence of gout attacks can be attributed to its chemical composition. Zhang et al. [12] and Colins et al. suggested that the antioxidant and anti-inflammatory effects of anthocyanin in cherry inhibit IL-1β secretion [7, 12]. Note that anthocyanin levels in cherry are far higher than those in most other fruits.

4.2. Methodological Considerations. The strength of the study resides in the quality of the review. We separately evaluated the studies using assessment tools, and it appears that we covered most of the articles dealing with the relationship between cherry consumption and gout. The studies included in our review demonstrate the influence of time in the relationship between gout attacks or plasma uric acid levels and cherry intake. Numerous studies have investigated the relationship between cherry and gout or uric acid; however, the focus has been on the anti-inflammatory effects of IL-1 β and TNF-α, which are used as biomarkers in monitoring acute gout flare ups [7, 10, 19].

Several limitations need to be addressed. First, the small number of included studies calls into question the reliability of the findings and the strength of the conclusions. Second, it was difficult to conduct effective meta-analysis due to considerable heterogeneity in the design of the studies, the outcome variables, and the biomarkers used to assess the risk of bias (Table 3 and Figure 2). Third, the study subjects enrolled in our review may have differed in terms of health status. Fourth, there may be interference from age-dependent factors, due to the wide age range of participants (from 21 to 88). Finally, all of the included studies were conducted in Western countries (USA and UK). A lack of data from individuals of different races may have narrowed the applicability of this study.

5. Conclusions

Current evidence supports an association between cherry intake and a reduced risk of gout attacks. Note however that we were unable to conduct effective meta-analysis due to a lack of relevant studies and a high degree of variation in the methodologies and metrics used in previous studies. Furthermore, there are at present an insufficient number of studies presenting the quantitative result required to conduct effective meta-analysis. We suggest that researchers conduct randomized control trials to further assess this correlation. We also recommend research on a wider range of populations (e.g., different races) to provide more comprehensive and generalizable findings.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Phytochemistry, Antioxidant Activity, Antiproliferative Effect, and Acute Toxicity Testing of Two Moroccan *Aristolochia* Species

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Ethnopharmacological Relevance. *Aristolochia baetica* (*A. baetica*) and *Aristolochia paucinervis* (*A. paucinervis*) have been largely used in Moroccan folk medicine. The objective of the study was to investigate the phytochemical composition, the antioxidant activity, the antiproliferative effect, and the acute toxicity of the methanolic extract of *A. baetica* and *A. paucinervis* roots. *Materials and Methods*. Phytochemical composition of the methanolic extract of *A. baetica* and *A. paucinervis* roots were studied using qualitative and quantitative methods, the antioxidant activity was evaluated using DPPH assay, the antiproliferative effects against human cancer cell lines (T-24, HT-29, and Hep G-2) was assessed using WST1 assay, and the acute toxicity was carried out orally by gavage of single dose 2000 mg/kg to mice for 14 days. *Results*. The two studied plants have different classes of secondary metabolites. The concentrations of the total polyphenolic content of *A. baetica* and *A. paucinervis* root extracts were estimated at 360 ± 20 mg GAE/g and 280 ± 27 mg GAE/g, respectively. The total flavonoids content of *A. baetica* and *A. paucinervis* extracts were estimated at 35 ± 8 mg QE/g and 235 ± 7 mg QE/g, respectively. *A. baetica* and *A. paucinervis* extracts exhibited promising DPPH activity with IC_{50} values of 150 ± 8 μg/ml and 160 ± 10 μg/ml, respectively. The extracts exerted also antiproliferative effects on all tested cancer cell lines (T-24, HT-29, and Hep G-2) with IC_{50} values ranging from 6 ± 1 μg/ml to 380 ± 7 μg/ml. Regarding the results of acute toxicity study, no signs of toxicities nor mortalities were observed on the oral treated mice with 2000 mg/kg of the two investigated exacts. *Conclusion*. The methanolic extracts of *A. baetica* and *A. paucinervis* possess several phytochemicals that exhibited promising free radical scavenging activity and antiproliferative effects.

1. Introduction

Sine antiquities, plants have played an important role in our daily lives as they have been a source of food, fiber, and other necessary needs. Among these resources, medicinal plants have been widely used to protect humans against serious diseases [1, 2]. They are considered as a natural reservoir of biologically active substances with different biological

properties. These bioactive natural remedies are increasingly used to treat a wide variety of clinical diseases because of its lower side effects [3].

Currently, therapeutic chemistry has great interests in health care, with almost 80% of the population throughout the world using medicinal plants [4]. As a result, several research studies have paid more attention to medicinal plants as an important source of chemical compounds and bioactive substances with antibacterial, anti-inflammatory, antioxidant, and anticancer activity [5]. Some of the natural substances have been recognized as key players in pharmacology for the development of new drugs used in the treatment of cancer [6]. Scientific research on plants used in traditional medicine is widely required to ensure safety control and to avoid potential risks related to the ingestion of toxic herbs. Data collected from *in vitro* and *in vivo* toxicological studies of herbs are needed to consolidate the scientific validity of herbs [7].

Aristolochia species have been widely used in Moroccan traditional medicine to treat various diseases for many years ago [7]. Nowadays, some of these plants are used to treat cancer [8], digestive diseases [9], rheumatic, abortifacient, and cutaneous neoplasm. They are also used as a stimulant of blood circulatory as anti-inflammatory and antiseptic [10].

The present work was conducted to determine the scientific basis of traditional uses of *A. baetica* and *A. paucinervis*. It aims at the investigation of the phytochemical composition, the antioxidant activity, the antiproliferative effect, and the acute toxicity of the methanolic extract of the two reported plants.

2. Materials and Methods

2.1. Plant Material. *A. baetica* was collected in January 2016 from the surrounding region of Chefchaouen (Moroccan city). *A. paucinervis* was harvested in December 2016 at 30 km East of Khouribga (Moroccan city). The collected plants were identified by Dr. Mohammed Fanane (Department of Botany, Scientific Institute of Rabat, Morocco). A voucher specimen has been deposited in the herbarium under 1045 and 1046, respectively. The roots of both, *A. baetica and A. paucinervis* were initially cleaned, washed with water, dried in the shade, and chopped into small pieces using an electric mixer.

2.2. Preparation of Plant Extract. 25 g of dried powder of *A. baetica* and *A. paucinervis* roots was extracted using Soxhlet at 40°C for 2 h using methanol as an extraction solvent. The obtained extract was centrifuged, filtered, and evaporated at low pressure (40°C) to remove the excess solvent in order to obtain 3 g of crude extract.

2.3. Phytochemical Screening. The plant materials were subjected to qualitative phytochemical careening in order to qualitatively determine some type of interesting constituents that could be responsible for biological activities. Alkaloids, flavonoids, polyphenols, anthraquinones, saponins, tannins, sterols, and terpenes were the major checked groups using standard methods as reported in earlier literature with slight modifications [11].

2.4. Identification of Bioactive Constituents by GC-MS. GC-MS analysis of the methanolic extracts of the studied plants were performed using a Claus 580 Gas Chromatography according to the following acquisition parameters: oven: initial temp 50°C for 2 min, ramp 11°C/min to 200°C, hold 0 min, ramp 6°C/min to 240°C, hold 1 min, split = 10 : 1, carrier gas = He, solvent delay = 4.00 min, transfer temp = 280°C, source temp = 250°C, scan: 40 to 450 Da, and column 30.0 m × 250 μm.

2.5. Total Polyphenolic Content. The total phenolic content of *A. baetica* and *A. paucinervis* extracts was determined using the Folin–Ciocalteu method and gallic acid as standard. Aliquots of test samples (250 μL) of a diluted solution of extracts were mixed with 1.5 mL of sodium carbonate solution (7.5%). After 5 min, 1.25 mL of Folin–Ciocalteu reagent, reveal the composition of this reagent (0.2 N), was added to the mixture, allowed to stand at room temperature for 30 min in darkness. The reading was carried out versus a blank at 765 nm. The calibration curve was plotted using gallic acid as a positive control. The results were expressed as gallic acid equivalent per gram of dry extract (mg GA E/g) [12].

2.6. Total Flavonoid Content. The total flavonoid content of *A. baetica* and *A. paucinervis* extracts was identified using the aluminum chloride assay (colorimetry). Each diluted sample was mixed with 1.5 mL of $AlCl_3$ (2%) and incubated for 60 min at room temperature. The absorbance was read spectrophotometrically against a blank at 415 nm. Quercetin was used as a reference standard compound. The calibration curve was plotted using quercetin as a standard compound. The results were expressed as quercetin equivalent per gram of dry extract (mg QE/g) [13].

2.7. Antioxidant Activity. The antioxidant activity of *A. baetica* and *A. paucinervis* extracts was evaluated *in vitro* using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay according to the method described in previous literature [14]. About 1 ml of methanolic extract at different concentrations ranging from 100 μg/ml to 900 μg/ml was mixed with 500 μl of methanolic solution of DPPH (0.005%). After 30 min of incubation in darkness at room temperature, the absorbance (A) of the control and samples was read spectrophotometrically at 517 nm. Ascorbic acid was used as a positive control. The free radical scavenging activity was calculated in percentage as follows:

$$\text{Free radical scavenging activity}\,(\%) = \frac{(A)\text{control} - (\text{A})\text{sample}}{(A)\text{control}} * 100. \quad (1)$$

IC_{50} was determined graphically from the graph plotting inhibition percentage against extract [15].

2.8. In Vitro Cytotoxicity Assay

2.8.1. Cell Culture and Treatment. Three Cancerous cell lines were selected to be tested including human bladder cancer cell lines (T-24). The latter were cultured in McCoys5a; in parallel, the liver cancer cell lines (Hep G-2) and human colon cancer cell lines (HT-29) were cultured in DMEM media added with 10% heat-inactivated fetal calf serum, antibiotics (1%), and glutamine (1%). The cancerous cells were grown at 37°C in a humidified incubator set at 5% CO_2. The cultures were left until they formed a monolayer on the flask. The cells were washed with PBS and trypsinized in order to be detached and then adding a complete medium to inhibit the reaction.

Antiproliferative activity of the methanolic extract of *A. baetica* and *A. paucinervis* against T-24, Hep G-2, and HT-29 cell lines was assessed on the basis of mitochondrial metabolic activity using WST1 (disodium mono{4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-tetrazol]-3-ium-5-yl]benzene-1, 3 disulfonate}). During the exponential growth of the tested cells in 96-well microplates at a density of 8000 cells per well, the seeding medium was removed and replaced by extracts prepared in medium to concentration up (0 to 400 μg/mL). WST1 assay was performed in triplicate, and the mitomycin was used parallelly as positive control. After 72 h of incubation, briefly 100 μl of the medium was removed from each well and 10 μl of WST1 solution was added to cultured cells. The plate was incubated again for 4 h at 37°C in a dark wet atmosphere. The absorbance was read at 590 nm using a Wallac Victor X3 multiwell spectrophotometer.

The percentage of cytotoxicity was defined according to the following formula:

$$\text{cell death}\,(\%) = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} * 100. \quad (2)$$

The percentage of viability was calculated according to the following equation:

$$\text{cell viability}\ (\%) = 100 - (\%\ \text{cell death}). \quad (3)$$

IC_{50} value (the inhibition concentration required to reduce 50% of cell proliferation) was determined graphically on the basis of regression analysis performed on WST1 assay viability [16].

2.9. Animal Material. Adult Swiss albino mice weighing approximately 25 g were used for acute toxicity. The mice were purchased from the animal colony of Pasteur Institute (Casablanca, Morocco). All animals were kept in polypropylene cages. The animals were acclimatized for one week under laboratory conditions of regular light/dark cycles (12/12 h) and temperature (24 ± 2°C). The animals had free access to tap water and a normal pellet diet [17].

2.10. Study of Acute Toxicity. Nine male adult mice were randomly divided into three experimental groups of three mice each. The animals were grouped in polypropylene cages and fasted for 12 h. The organic extract of the studied plants was administered to each treatment group once as follows:

Control group—treated with vehicle (distilled water)

Group A—*A. baetica* extract (2000 mg/kg)

Group B—*A. paucinervis* extract (2000 mg/kg)

Animals were observed for signs of toxicity, mortalities, changes in general behavior, and physical appearance. This study was conducted according to the Organization for Economic Cooperation and Development (OECD) Guidelines No. 425 [18].

2.11. Statistical Analysis. Data for each test are the average of triplicate experiments ± standard deviation (SD), and statistical analysis of difference was performed using ANOVA. The means were compared using the Holm–Sidak Test. Statistically p value less than 0.05 was considered to indicate significance.

3. Results

3.1. Phytochemical Screening. The phytochemical screening of *A. baetica* and *A. paucinervis* roots revealed the presence of flavonoids, polyphenols, alkaloids, tannins, saponins, and the absence of anthraquinone, sterols, and terpenes (Table 1).

3.2. GC-MS Analysis. The GC-MS analysis of *A. baetica* methanolic extract revealed ten compounds: pseudocumene, tetracyclo [3.3.1.0.1(3,9)]decan-10-one, *p*-vinylguaiacol, 2-epi-trans-β-caryophyllene, guaia-6,9-diene, pacifigorgiol, maaliol, methylglucose, isoaromadendrene epoxide, and *trans*-sinapyl alcohol (Figure 1; Table 2).

Regarding *A. paucinervis* methanolic extract, the GC-MS identified seven compounds: dihydro-4,4,5,5-tetramethyl 2(3H)-furanone, mesitylene, dodecane, maaliol, 2-palmitoylglycerol, dioctyl terephthalate, phthalic acid, and di(2 propylpentyl) ester (Figure 2; Table 3).

3.3. Total Polyphenolic and Flavonoid Contents. Calculations of total polyphenolic content in the organic extract of the studied plants were based on the equation obtained from the standard gallic acid graph. Total polyphenolic contents of 360 ± 20 mg GAE/g and 280 ± 27 mg GAE/g were found in *A. baetica* and *A. paucinervis* methanolic extracts, respectively. Regarding the total flavonoid content calculation, the formula obtained from the standard quercetin graph was applied. Total flavonoid content of 35 ± 8 mg QE/g was obtained with *A. baetica* extract and 235 ± 7 mg QE/g with *A. paucinervis* extract.

3.4. Antioxidant Activity. The antioxidant activity of *A. baetica* and *A. paucinervis* methanolic extracts was evaluated by the DPPH free radical scavenging test. As shown in Figure 3, both extracts exhibit promising antioxidant activity in a concentration-dependent manner.

The IC_{50} value (the inhibitory concentration of extract required to inhibit 50% of the initial DPPH free radical) of the plant extracts and ascorbic acid as a positive control was

Table 1: Qualitative phytochemical screening of *A. baetica* and *A. paucinervis* roots.

Phytoconstituents	*A. baetica* roots	*A. paucinervis* roots
Polyphenols	++	+++
Alkaloids	+++	++
Flavonoids	+++	++
Anthraquinone	−	−
Sterols and terpenes	−	−
Saponins	+	+
Tannins	+++	++

+++: strong positive test; ++: positive test; +: low positive test; −: negative test.

Figure 1: GC-MS spectral chromatogram of *A. baetica* methanolic extract.

Table 2: Phytocomponents identified from methanolic extract of *A. baetica* roots using GC-MS.

S. No	Retention time (min)	Compound name	Molecular formula
1	6,249	Pseudocumene	C_9H_{12}
2	9,917	Tetracyclo [3.3.1.0.1(3,9)] decan-10-one	$C_{10}H_{12}O$
3	11,118	*p*-Vinylguaiacol	$C_9H_{10}O_2$
4	12,902	2-epi-*trans*-β-Caryophyllene	$C_{15}H_{24}$
5	13,294	Guaia-6,9-diene	$C_{15}H_{24}$
6	13,76	Pacifigorgiol	$C_{15}H_{26}O$
7	14,103	Maaliol	$C_{15}H_{26}O$
8	15,11	Methylglucose	$C_7H_{14}O_6$
9	15,163	Isoaromadendrene epoxide	$C_{15}H_{24}O$
10	18,466	*trans*-Sinapyl alcohol	$C_17H_{24}O_9$

Figure 2: GC-MS spectral chromatogram of *A. paucinervis* methanolic extract.

Table 3: Phytocomponents identified from methanolic extract of *A. paucinervis* roots using GC-MS.

S. No	Retention time (min)	Compound name	Molecular formula
10	4,893	Dihydro-4,4,5,5-tetramethyl 2(3H) furanone	$C_{11}H_{16}O_4$
17	6,246	Mesitylene	C_9H_{12}
22	9,467	Dodecane	$C_{12}H_{26}$
26	14,095	Maaliol	$C15H_{26}O$
35	18,087	2-Palmitoylglycerol	$C_{19}H_{38}O_4$
39	19,447	Dioctyl terephthalate	$C_{24}H_{38}O_4$
41	19,712	Phthalic acid, di(2 propylpentyl) ester	$C_{24}H_{38}O_4$

Figure 3: DPPH radical scavenging activity of methanolic extract of *A. baetica* and *A. paucinervis*.

Figure 4: Cell viability after 72 h of treatment with the methanolic extract of *A. baetica* roots.

Figure 5: Cell viability after 72 h of treatment with the methanolic extract of *A. paucinervis* roots.

determined graphically from the graph of DPPH inhibition percentage. The results of IC_{50} values of methanolic extract of *A. baetica*, *A. paucinervis*, and ascorbic acid were determined approximately at $150 \pm 8\,\mu g/ml$, $160 \pm 10\,\mu g/ml$, and $25 \pm 3\,\mu g/ml$, respectively. In order to perform the comparison, the IC_{50} values of DPPH radical scavenging activity of methanolic extract of *A. baetica* and *A. paucinervis* showed a significant difference compared to DPPH IC_{50} value of ascorbic acid as a standard ($p^{***} < 0.05$).

3.5. In Vitro Cytotoxicity Assay. The methanolic extract of *A. baetica* and *A. paucinervis* was investigated for potential antiproliferative effects on T-24, Hep G-2 and HT-29 human cancerous cell lines using WST1 assay. Figures 4 and 5 show important antiproliferative effects induced by the investigated extracts on the treated cells in a time- and concentration-dependent manner.

Regarding data presented in Figure 4, the methanolic extract of *A. baetica* roots induced antiproliferative effects on T-24 and HT-29 cell lines with IC_{50} $48 \pm 5\,\mu g/ml$ and $100 \pm 10\,\mu g/ml$, respectively. Both T-24 and HT-29 cell lines were more sensitive to the effect of extract of *A. baetica* than Hep G-2 cell lines with IC_{50} $380 \pm 7\,\mu g/ml$. There is a significant difference between IC_{50} value induced by the methanolic extract on all treated cancerous cells ($p^{**} < 0.05$).

In Figure 5, the methanolic extract of *A. paucinervis* roots exerts antiproliferative effects on Hep G-2 and HT-29 cell lines with IC_{50} approximately $52 \pm 5\,\mu g/ml$ and $30 \pm 6\,\mu g/ml$, respectively. The T-24 cell lines were more sensitive to organic extract of *A. paucinervis* roots than Hep G-2 and HT-29 with IC_{50} $6 \pm 1\,\mu g/ml$. To perform the

comparison, there is a significant difference between IC_{50} value induced by the methanolic extract of *A. paucinervis* roots on the treated cells (T-24, HT-29, and Hep G-2) ($p^{**} < 0.05$).

3.6. Acute Toxicity Studies. The findings of acute toxicity study showed the absence of mortalities and signs of toxicity on the oral treated mice with both 2000 mg/kg of the methanolic extract of *A. baetica* roots and 2000 mg/kg methanolic extract of *A. paucinervis* roots. Slight changes in general behavior like running after the gavage compared to the control group were observed.

4. Discussion

Since prehistoric times, the medicinal plants have played a central role in the prevention and the treatment of various diseases [19]. In the current work, we decided to scientifically highlight the traditional uses of Moroccan *A. baetica* and *A. paucinervis* roots, such as to investigate their chemical profile, antioxidant activity, antiproliferative effect, and acute toxicity.

The phytochemical profile of the two studied plants in this work revealed the presence of alkaloids polyphenols, flavonoids, tannins, saponins, and the absence of anthraquinone, sterols, and terpenes. These results were in accordance with those reported in earlier reports which showed that the phytochemical screening of *A. longa* and *A. baetica* roots revealed the presence of phenols, flavonoids, saponins, and the absence of anthraquinones, sterols, and triterpenes [17, 20]. The qualitative test of the aerial parts of *A. indica* showed positive tests for terpenes, saponins, tannins, and flavonoids [21].

Plants with high amounts of phytoconstituents such as flavonoids and polyphenols are reported to exhibit antioxidant properties [16]. In the current research work, the total polyphenolic content was quantified using the Folin–Ciocalteau assay [22]. The rate of total polyphenolic content of methanolic extract of *A. baetica* and *A. paucinervis* roots was estimated at 360 ± 20 mg GAE/g and 280 ± 27 mg GAE/g, respectively. The total flavonoids content of *A. baetica* and *A. paucinervis* extracts were estimated at 35 ± 8 mg QE/g and 235 ± 7 mg QE/g. These findings were partly comparable to those reported in previous literature [21], in which it was reported that the total polyphenolic and flavonoids content shown in aqueous extract of *A. indica* was 25.11 ± 0.18 GAE/g and 30.41 ± 0.19 QE/g, respectively.

In the present investigation, a proposing DPPH radical scavenging activity was noticed for the methanolic extract of *A. baetica* and *A. paucinervis* with IC_{50} values of $150 \pm 8\,\mu$g/ml and $160 \pm 10\,\mu$g/ml, respectively. This property could be attributed to presence of interesting total polyphenolic content [23]. The antioxidant activity of polyphenols is due to their redox properties which could play a central role in capturing and neutralizing free radicals in order to prevent their harmful effects [24]. To the best of our knowledge, no previous data were available for *A. baetica* and *A. paucinervis* antioxidant activity. Thus, other plants of genus *Aristolochia* were phytochemically and biologically evaluated to perform comparison. The obtained results were in confirmation with early reports concerning DPPH radical scavenging activity of *A. longa* organic extract which showed a DPPH IC_{50} value of IC_{50} equal $125.40 \pm 2.40\,\mu$g/mL [25].

Regarding the antiproliferative activity, the results showed that the methanolic extract of *A. baetica* roots exhibited a high antiproliferative effect on T-24, HT-29, and Hep G-2 cell lines with IC_{50} approximately of $48 \pm 5\,\mu$g/ml, $100 \pm 10\,\mu$g/ml, and $380 \pm 7\,\mu$g/ml, respectively. On the other hand, the methanolic extract of *A. paucinervis* showed IC_{50} values of $52 \pm 5\,\mu$g/ml, $30 \pm 6\,\mu$g/ml, and $6 \pm 1\,\mu$g/ml on Hep G-2, HT-29, and T-24, respectively. To perform the comparison, it was reported that the antiproliferative effect of *A. baetica* organic extracts was estimated at IC_{50}: $216.06 \pm 15\,\mu$g/mL [8]. The remarkable antiproliferative effect resulted in methanolic extracts of the studied plants was in accordance with previous data [26], in which it was reported that the IC_{50} value of *A. longa* aqueous extract against human cancer lines was determined at 15,63 μg/ml.

Dysregulation of apoptosis is one of the most important factors intervened in cancer treatment [27]. The control of cancer growth still related to the ability of cancer cells to undergo apoptosis [28]. The mechanism by which genus *Aristolochia* could induce cell death was investigated in previous literature [29], it was reported that the aqueous extract of *A. longa* induces apoptosis through the mitochondrial intrinsic pathway in BL41. This mechanism could involve in our treated cell lines with methanolic extracts of the studied plants. The phytochemical screening revealed the presence of polyphenols, alkaloids, flavonoids, and tannins. On the other hand, the pharmacological activities of plants rely on their chemical compounds, and thus, the antiproliferative activity of the studied plants is associated with the detected compounds [30]. The detected flavonoids could be the responsible compounds for the antiproliferative activity [31].

Mass spectrometry plays a crucial role in the development of the organic field because of its efficiency in the qualitative and quantitative determination of organic molecules. Gas chromatography coupled to a mass spectrometer (GC-MS) permits a mixture of small molecules rather than organic constituent of low molecular weight to be analyzed [32]. Ten compounds namely pseudocumene, tetracyclo [3.3.1.0.1(3,9)] decan-10-one, *p*-vinylguaiacol, 2-epi-*trans*-β-caryophyllene, guaia-6,9-diene, pacifigorgiol, maaliol, methylglucose, isoaromadendrene epoxide, and *trans*-sinapyl alcohol were identified in methanolic extract of *A. baetica* using GC-MS. On the other hand, seven compounds such as dihydro-4,4,5,5-tetramethyl 2(3H)-furanone, mesitylene, dodecane, maaliol, 2-palmitoylglycerol, dioctyl terephthalate, phthalic acid, and di(2 propylpentyl) ester were detected from the methanolic extract of *A. baetica* roots.

The pharmacological and biological properties of the studied plants in the current work seem to be attributed to the identified compounds. This can be a result of the action of a single molecule or by a synergy between all these molecules without excluding the potentiation effects. As an example, pacifigorgiol found in the methanolic extract of

A. baetica was reported in previous literature to exhibit antibacterial activities [33]. A cytotoxic effect against brine shrimp nauplii *Artemia salina* was attributed to dioctyl terephthalate detected in the methanolic extract of *A. paucinervis* [34].

The acute toxicity of the investigated methanolic in this work was useful to provide information concerning the safety control for potential uses in single administration. No clinical signs nor mortalities occurred in animals treated orally with 2000 mg/kg of the two plant extracts compared to control groups. Therefore, the predicted LD_{50} of the studied plant extracts would be higher than 2000 mg/kg. On the other hand, these extracts seem to be safe for animals with oral administration of single doses up to 2000 mg/kg under the conditions of acute toxicity. The present results were consolidated to other researches which showed the safety of single administered doses to mice (lower than 4 g/kg) [17, 35]. In the same way, the methanolic extract of *A. baetica* and *A. paucinervis* roots was not toxic with single doses according to the scale of Viala [32]. However, the genus *Aristolochia* exhibits high toxicity when ingested for a long time with repeated doses [36, 37].

5. Conclusion

Phytochemical profile of both *A. baetica* and *A. paucinervis* was useful to provide information on the potential of these plants as a promising source of secondary metabolites, hence, encouraging their natural potential as a source of various therapeutic agents for the treatment of cancer.

Data Availability

All data are available in the following laboratories: Laboratory of Chemistry, Biochemistry, Nutrition, and Environment, Faculty of Medicine and Pharmacy, University Hassan II, Casablanca, Morocco; Research Unit and Medical Biology, National Center for Nuclear Energy, Science and Technology, CNESTEN. Rabat 10001, Morocco; Laboratory REMTEX, Higher School of Textile and Clothing Industries, Km 8, Route d'EL JADIDA, Casablanca, Morocco; and Faculty of Sciences Ben M'Sik, University Hassan II Casablanca. B. P. 7955, Morocco Laboratory of Engineering and Materials LIMAT, Faculty of Sciences Ben M'Sik, University Hassan II Casablanca. B. P. 7955, Morocco.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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