Anti-inflammatory and anti-arthritic effects of Guge Fengtong Formula: in vitro and in vivo studies

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[ABSTRACT] Rheumatoid arthritis (RA) is the most common inflammatory arthritis and a major cause of disability. Presently, the clinical therapeutic medicines for inflammatory and arthritic diseases are unsatisfactory due to severe adverse effects or ineffectiveness. The Guge Fengtong formula (GGFT), containing the standardized extracts of Dioscoreae Nipponicae Rhizoma, Spatholobi Caulis, and Zingiberis Rhizoma, has long been used for RA treatment in China. However, the detailed anti-inflammatory and anti-arthritic activity of GGFT has not been reported so far. In the present work, we aimed to evaluate the anti-inflammatory and anti-arthritic effects of GGFT using three in vivo animal models and tried to uncover the underlying mechanism of action in RAW 264.7 macrophages. The obtained results indicated that GGFT significantly attenuated ear edema, decreased carrageenan-induced paw edema, reduced the arthritis score, and reversed the weight loss of the complete Freund’s adjuvant (CFA)-injected rats. Additionally, decrease in synovial inflammatory infiltration and synovial lining hyperplasia in the joints and decline of inflammatory factors (TNF-α and IL-1β) in the serum were observed in the GGFT-treated rats. In lipopolysaccharide-activated RAW264.7 macrophages, GGFT reduced the production of NO, PGE2, and IL-6 and inhibited the expression of iNOS, COX-2, and NF-κB. Our results demonstrated that GGFT possessed considerable anti-inflammatory activity and had potential therapeutic effects on adjuvant induced arthritis in rats, providing experimental evidences for its application in the treatment of RA and other inflammatory diseases.

[KEY WORDS] Guge Fengtong formula; Rheumatoid arthritis; Anti-arthritis; Anti-inflammatory; RAW264.7 macrophages


Introduction

Rheumatoid arthritis (RA), also called “immortal cancer”, is the most common type of inflammatory disease and characterized by inflammatory cell infiltration, proliferation of synovial tissue, and bone destruction [1-3]. It affects 1% of the population and progresses into multisystem inflammation with irreversible joint damage, causing mortality and disability, and compromising the quality of life in most patients [4]. Currently, the etiology and pathogenesis of RA is poorly understood. Nevertheless, various cytokines, particularly pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta (IL-1β), and interleukin-6 (IL-6), are known to play an important role in RA [5]. It is well established that the pro-inflammatory cytokines induce RA progression through mediators such as cyclooxygenase-2 (COX-2), which increases prostaglandin E2 (PGE2) production, synovial inflammation, and matrix metalloproteinases (MMPs) levels [6]. A recent study has also shown that an imbalance between pro- and anti-inflammatory cytokine activities favors the induction of autoimmunity, chronic inflammation, and thereby joint damage [7]. The severity of RA often depends on the “balance” between the effects of pro- and anti-inflammatory cytokines. Therefore, downregulation of pro-inflammatory cytokines may be an appropriate therapeutic strategy for RA.

Nowadays, the therapeutic interventions conventionally employed for RA include the use of disease-modifying...
anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), steroid hormone, and biologics. Newly diagnosed RA patients are usually recommended to be treated with NSAIDs for relieving nociceptive pain and controlling inflammation [8]. However, these clinical therapeutic medicines only transiently suppress inflammation and ameliorate symptoms, without significantly improving the long-term outcome [9]. Furthermore, long-term treatment with NSAIDs may result in serious side effects, including gastrointestinal lesions, cardiovascular complications, and reproductive toxicity [10]. To date, there is no therapeutically active agent which could effectively treat RA, and a more effective and safe therapeutic strategy is desired [11]. In traditional oriental medicine, many herbal preparations have been reported to afford protection against the deleterious effects of rheumatic and arthritic diseases [12-14]. Novel therapeutic agents originated from herbal medicines may not only prevent structural damage of arthritic joints caused by tissue and bone breakdown, but also be safe and better tolerated in RA patients [15]. A recent study has shown that about 60%–90% of people suffering from RA are most likely to seek the option of botanicals [16]. This growing interest in alternative medical practices clearly indicates a need for more safe and effective anti-RA botanicals from traditional medicine.

Guge Fengtong formula (GGFT), an herbal formula composed of Dioscoreae Nipponicae Rhizoma, Spatholobi Caulis, and Zingiberies Rhizoma, has long been used for arthritis treatment in China (Table 1). All these herbs exert potent anti-inflammatory activity and have well-established histories of use for treatment of rheumatic and arthritic diseases in China and Korea [17-18]. Although GGFT possesses significant curative effects, its detailed anti-inflammatory and anti-arthritic activity has not been reported so far. Therefore, the present study was conducted to investigate the anti-inflammatory and anti-arthritic effects of GGFT using experimental animal models such as xylene-induced mouse ear edema, carrageenan-induced rat paw edema, and complete Freund’s adjuvant (CFA)-induced rat arthritis models. Furthermore, to understand the underlying mechanisms of action, the production of inflammatory mediators (NO, PGE2, IL-6) and the protein expression of inducible nitric oxide synthase (iNOS), COX-2, and nuclear factor-kappa B (NF-kB) were determined in lipopolysaccharide (LPS)-activated RAW264.7 macrophages in vitro.

Table 1  Different components in the formula of GGFT

<table>
<thead>
<tr>
<th>Pharmaceutical name</th>
<th>Botanical plant name</th>
<th>Family</th>
<th>Part used</th>
<th>Chinese name</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioscoreae Nipponicae Rhizoma</td>
<td>Dioscorea nipponica Makino</td>
<td>Dioscoreaceae</td>
<td>Rhizome</td>
<td>Chuanshanlong</td>
<td>4.5</td>
</tr>
<tr>
<td>Spatholobi Caulis</td>
<td>Spatholobus ssuberectus Dunn</td>
<td>Leguminosae</td>
<td>Caulis</td>
<td>Jixueteng</td>
<td>4.5</td>
</tr>
<tr>
<td>Zingiberies Rhizoma</td>
<td>Zingiber officinale Rose</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>Ganjiang</td>
<td>1</td>
</tr>
</tbody>
</table>

Materials and Methods

Drugs and chemicals

GGFT, a GMP (Good Manufacture Practice)-approved product, was kindly provided in the form of ethanol-free lyophilisate by Xiuzheng Pharmaceutical Co., Ltd. (Jilin, China). The lyophilized extract was reconstituted with distill water to a specific concentration and kept at 4 °C before use. Complete Freund’s adjuvant, xylene, carrageenan, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), LPS (Escherichia coli serotype 055 : B5), and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-6, TNF-α, and IL-1β ELISA (enzyme-linked immunosorbent assay) kits were purchased from eBioscience (San Diego, CA, USA), and PGE2 ELISA kits were obtained from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Ultrapure water obtained from a Milli-Q system (Millipore Corporation, Bedford, USA) was used for the experiments. All the other reagents were of analytical grade and obtained from commercial sources.

Antibodies against iNOS and COX-2 were bought from Cell Signaling Technology (Beverly, MA, USA); Antibodies against NF-κB p65, p-NF-κB p65, and β-actin were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase labeled anti-goat IgG and anti-mouse IgG were purchased from KPL Company (Gaithersburg, MD, USA).

Liquid Chromatography /Mass Spectrometry (LC/MS) analysis

A LC/MS system (Agilent Technologies, Santa Clara, CA, USA) containing a Agilent 1100 HPLC system, a 6520 quadrupole time-of-flight tandem mass spectrometry with an electrospray ionization (ESI) source, and a Masshunter Qualitative Analysis Software B 02.00 was used in the present study. Chromatographic separation was optimized at 25 °C on an Agilent Extend -C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was consisted of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.5 mL·min⁻¹. A gradient program was used as follows: 0 min, 15% B; 20 min, 30% B; 40 min, 50% B; 55 min, 55% B; 80 min, 70% B; and 85 min, 100% B. The mass spectrometer was operated in the positive ESI mode using the selected ion recording data acquisition. Mass spectrometric conditions were optimized as follows: −3.0 kV capillary voltage, 120 °C source temperature, 32 °C desolvation temperature, 10 L·min⁻¹ desolvation gas flow, and −30 cone voltage.
Experimental animals and ethics statement

In vivo experiments were performed with male ICR mice or male Sprague-Dawley rats housed under SPF (specific-pathogen free) conditions. The animals were kept in plastic cages at a constant temperature of 22 ± 2 °C and relative humidity at 55% ± 5%, with a 12 : 12 h light/dark cycle and free access to pellet food and tap water. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Pharmaceutical University, Nanjing, China. All experimental procedures were performed according to IACUC policy and the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People’s Republic of China (2006)398.

Xylene-induced ear edema in mice

The experiment with xylene-induced ear edema model was performed as described previously [19]. The mice (weighing 18–22 g) were divided randomly into the following five groups (10 mice per group) for oral gavage treatment once a day for 6 days: control group (0.2 mL of distill water); positive treatment group (1 mg·kg⁻¹ of dexamethasone dissolved with distill water, volume of injection 0.1 mL/10 g); and GGFT groups (340, 680 and 1360 mg·kg⁻¹ of GGFT, volume of gavage 0.1 mL/10 g). At 30 min after the last drug administration, ear edema was induced by applying 0.2 mL of xylene on the outer and inner surfaces of the right ear of each mouse. The untreated left ear served as a control. At 1 h after the application, an ear disk with diameter of 9.0 mm was punched out and weighed. The weight difference between the left and the right ear disks of the same animal was considered as the extent of edema. The inhibition percentage was calculated by equation as follows:

Inhibition (%) = (Econtrol – Etreated) / Econtrol × 100

Where Econtrol and Etreated are the extent of edema from the control and treated groups, respectively.

Carrageenan-induced hind paw edema in rats

The acute anti-inflammatory effects of GGFT were evaluated by the carrageenan-induced rat paw edema model, according to method described in literature [20]. Edema was induced by injecting carrageenan (1% W/V, 0.1 mL/paw) into the plantar surface of the right hind paw of each rat. The GGFT (242, 484, and 968 mg·kg⁻¹, 0.1 mL/10 g) was administered orally for 6 days before injection of carrageenan. The control group received an equal volume of distill water (vehicle) and the positive control group received dexamethasone (i.p. 1 mg·kg⁻¹·d⁻¹, p.o.). The paw volumes before and after carrageenan injection (at various time points up to 6 days) were measured using a plethysmometer. Percentage change in paw volume at each time point was calculated by the following formula:

Change in paw edema (%) = (A – B) / B × 100

Where A is the paw volume at different time points after injection and B is paw volume before injection.

CFA-induced arthritis in rats

The induction of arthritis in animals was performed by the subplantar injection of 0.1 mL of Complete Freund’s Adjuvant on day 0. Following the onset of arthritis, the rats were randomly divided into five groups: (i) normal control (without CFA injection); (ii) CFA plus vehicle; (iii) CFA plus dexamethasone (1 mg·kg⁻¹·d⁻¹, p.o.); (iv) CFA plus GGFT (484 mg·kg⁻¹·d⁻¹, p.o.); (v) CFA plus GGFT (984 mg·kg⁻¹·d⁻¹, p.o.). Each treatment group contained ten rats. The treatment was performed since CFA injection and the experiment lasted for 26 days.

Clinical evaluation of arthritis

Weight: In the course of the experiment, the body weights of rats were measured every 4 days.

Hind paw swelling: The volume of the hind paw swelling was measured using a plethysmometer on Days 0, 1, 5, 9, 13, 22, and 26.

Arthritis score: The rats were examined every 4 days for clinical parameters, and incidence of arthritis was judged macroscopically. Each joint was examined for swelling and redness. The severity of arthritis was evaluated using a well-established, widely-used scoring system [21]. Paws were examined and graded on a scale of 0–4 for each paw for degree of redness and swelling: 0 = no signs of disease, 1 = signs involving the ankle/wrist, 2 = signs involving the ankle plus tarsal of the hind paw and/or wrist plus carpals of the forepaw, 3 = signs extending to the metatarsals or metacarpals, and 4 = severe disease involving the entire hind or fore paw. A severity score was calculated based on the total scores of the four limbs (maximum 16 points for each individual rat).

Histological examination

On Day 26, all the rats were sacrificed via anesthesia after blood sample collection. Hind paws were removed above the knee joint for histological examination. The paws were fixed in 10% phosphate buffered formalin, decalcified in 10% EDTA for 30 days at 4 °C, and then embedded in paraffin. Serial paraffin sections (5 µm) were made and stained with hematoxylin and eosin dye and then examined under a light microscope for any bony destruction, presence of mononuclear infiltration, and pannus formation.

Measurement of serum TNF-α and IL-1β concentrations

The blood sample was allowed to clot for 30 min, and the serum sample was obtained by centrifugation at 3 000 r·min⁻¹ for 15 min and stored at –20 °C until analysis. Serum interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) levels were quantified by ELISA, according to the manufacturer’s protocol. All the samples were measured in triplicate using the test kits.

RAW 264.7 cell culture

The Raw 264.7 cell line derived from murine macrophages was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS and antibiotics (100 U·mL⁻¹ of penicillin and 100
were determined using BCA Protein Assay Kit (Beyotime). The cells containing a density of $4 \times 10^4$ cells/mL were activated by incubation in medium containing LPS (1 µg·mL$^{-1}$). LPS was added to various concentrations of GGFT extracts dissolved in dimethyl sulfoxide (DMSO). The cells treated with 0.05% (V/V) DMSO were used as negative control.

**Determination of NO, PGE2, and IL-6**

The RAW 264.7 cells were seeded in 96-well plates at a density of $4 \times 10^4$ cells/well in a total volume of 100 µL, and incubated in serum-free medium for 24 h. Plated cells were treated with LPS (1 µg·mL$^{-1}$) and/or indicated concentrations of GGFT. After a 24-h incubation, MTT at 0.5 mg·mL$^{-1}$ (10 µL) was added to each well and incubated for 4 h. After discarding all medium from the plates, 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and the plates were shaken for 5 min at room temperature. Thereafter, the absorbance at 490 nm was measured using a spectrophotometric plate reader (Benchmark; Bio-Rad Laboratories, CA, USA). Cell viability was defined as the ratio (expressed as a percentage) of absorbance of the treated cells to the untreated cells. All experiments were performed in triplicate.

**Cytotoxicity assay**

Cytotoxicity of GGFT was examined using MTT assay. The cells were seeded in 96-well plates at a density of $4 \times 10^4$ cells/well with 100 µL of culture medium, and incubated for 24 h. The cells were then treated with various concentrations of GGFT and/or LPS at a final concentration of 1 µg·mL$^{-1}$ before incubation for 24 h. The nitrre concentration in the medium was assessed using the Griess reaction. Briefly, the conditioned medium (50 µL) was mixed with the same volume of Griess reagent and incubated for 15 min. The absorbance of the mixture at 540 nm was measured with a plate reader as described above. By comparing with the standard concentrations of sodium nitrite dissolved in DMEM, the nitrre concentrations in the conditioned media of the treated cells were calculated.

In this experiment, the generation of PGE2 and IL-6 by the RAW 264.7 cells were assessed with ELISA kits. Briefly, the RAW 264.7 cells (4 \times 10^5 cells/well, 2 mL each well) were cultured in 24-well plates with serum-free medium and treated with different concentrations of GGFT and/or LPS for 24 h. The PGE2 and IL-6 levels were determined in conditioned medium with a PGE2 and IL-6 ELISA kit (BD Biosciences, USA), respectively, according to the manufacturer’s instructions. Absorbance at 450 nm was measured with a microplate reader. PGE2 and IL-6 standard curves were used to quantify the PGE2 and IL-6 released by the cells.

**Western blot analysis**

The RAW 264.7 cells treated with GGFT and/or LPS were lysed in lysis buffer containing protease inhibitors. The lysates were centrifuged at 15 000 r·min$^{-1}$ for 10 min at 4 °C, and the protein concentrations in the supernatants were determined using BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Suzhou, China). The proteins (10 µg/lane) were size-fractionated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. The separated proteins were transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated (overnight at 4 °C) with antibodies against iNOS, COX-2, NF-κB p65, and p-NF-κB p65 (all diluted 1 : 1 000) after blocking with 5% skim milk. Equal protein loading was verified using β-actin antibody. The membranes were then incubated with horseradish peroxidase labeled anti-goat IgG or anti-mouse IgG (all diluted 1 : 2 000) and immuno-reactive bands were detected with ECL.

**Statistical analysis**

The statistical differences between the values of various experimental groups were analyzed using one-way analysis of variance (ANOVA). The data are expressed as means ± SD, and differences were considered statistically significant at $P < 0.05$.

**Results and Discussion**

**LC/MS analysis of GGFT**

To elucidate the chemical profile of GGFT, a LC/MS method was developed for detection of the main compounds in GGFT. Our previous phytochemical studies on Dioscoreae Nipponicae Rhizoma, Spatholobi Caulis, and Zingiberis Rhizoma revealed the abundance of saponins, flavonoids, and gingerols [22-23]. Through comparing the retention times ($t_R$) and MS spectra of GGFT with those of corresponding standards, six major components in the HPLC-MS profiles were unequivocally identified. As shown in Fig. 1, the main compounds in GGFT are protodioscin ($t_R$: 22.65 min), protogracillin ($t_R$: 23.60 min), pseudoprotodioscin ($t_R$: 29.41 min), 6-gingerol ($t_R$: 42.33 min), dioxcin ($t_R$: 48.68), and 6-shogaol ($t_R$: 60.24 min).

**Effects of GGFT on xylene-induced ear edema in mice**

The xylene-induced ear edema model is a widely used for identifying potential anti-inflammatory agents [19]. Application of xylene markedly elicits an inflammatory response in mice, as judged by edema formation determined by the increase in the weight of the ear. As presented in Table 2 and Fig. 2a, GGFT inhibited xylene-induced ear edema in a dose-dependent manner, with 8.3%, 32.3%, and 59.1% inhibition occurring at doses of 340, 680, and 1360 mg·kg$^{-1}$, respectively. At 1360 mg·kg$^{-1}$, it reduced the ear edema to a greater extent than that of dexamethasone ($P < 0.01$, Fig. 2a). These results indicated that GGFT possesses a remarkable anti-inflammatory activity against acute inflammation.

**Effects of GGFT on carrageenan-induced paw edema in rats**

The carrageenan-induced rat paw edema model has been commonly employed as an assay to reflect edema that occurs during the early stages of acute inflammation [24]. In the present study, the rats were given different doses of GGFT or dexamethasone to evaluate their effect on edema induced by...
Fig. 1  Total ion chromatograms of GGFT in positive ion mode, and mass spectra of dioscin, protodioscin, protogracillin, pseudoprotodioscin, 6-gingerol, and 6-shogaol

Table 2  Effects of GGFT on xylene-induced ear edema in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg·kg⁻¹)</th>
<th>Ear Edema (mg)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Distilled water</td>
<td>25.2 ± 2.5</td>
<td>–</td>
</tr>
<tr>
<td>DSMS</td>
<td>1.4</td>
<td>13.1 ± 3.1‡</td>
<td>47.9 ± 12.3</td>
</tr>
<tr>
<td>GGFT</td>
<td>340</td>
<td>23.1 ± 1.3‡</td>
<td>8.3 ± 5.0</td>
</tr>
<tr>
<td>GGFT</td>
<td>680</td>
<td>17.4 ± 2.7‡</td>
<td>32.3 ± 10.8</td>
</tr>
<tr>
<td>GGFT</td>
<td>1360</td>
<td>10.3 ± 2.2‡</td>
<td>59.1 ± 8.7</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of 10 animals for each group.
DSMS, dexamethasone.
‡P > 0.05, ‡P < 0.01 vs the vehicle control group.

carrageenan. As expected, the edema volume of the rats in model control group was significantly increased and showed its maximum value at 1 h after the carrageenan injection. Oral treatment with GGFT inhibited the swelling of hind paw induced by carrageenan (Fig. 2b). Compared to the model control group, GGFT (968 mg·kg⁻¹) significantly decreased the edema volume in all time points (P < 0.05), with inhibitory rate being 43.6%, 42.2%, 40.5%, 35.7%, 33.8%,...
and 58.5% at 0.5, 1, 3, 4, and 5 h, respectively. In the positive control group, dexamethasone at 1 mg·kg⁻¹ also significantly inhibited the paw edema, compared with the model control group (P < 0.01 at 0.5–5 h), validating the usefulness of the model in the present study.

Effects of GGFT on CFA-induced arthritis in rats

Effects of GGFT on paw swelling, arthritis score and weight growth

At 24 h after CFA injection, the primary arthritis of the hind paw was induced with a significant increase in paw volume in all CFA-injected animals, compared with normal control group. The initial development in the manifestations of inflammation occurred from Day 1 to Day 5 of administration, and the inflammatory signs decreased from Day 9 to Day 10. This phase was followed by a quick reappearance of inflammation on Days 13 to 14. The secondary arthritis of the left hind paw occurred by the immune response on 13 days after the CFA injection. Administration of GGFT at 484 mg·kg⁻¹ led to a significant decrease in the edema volume of right hind paw swelling and polyarthritis symptoms on Days 13, 17, 22 and 26 after immunization, with the inhibitory rates being 23.3%, 27.6%, and 29.6%, respectively. GGFT at 968 mg·kg⁻¹ also exhibited notable efficacy in inhibiting the paw edema of the arthritic rats. The inhibition rate on Days 9, 13, 17, 22 and 26 were 40.9%, 32.8%, 35.0%, 42.2%, and 46.1 %, respectively (Table 3). In addition to the suppression of paw swelling, an evident reduction in the clinical arthritis score was observed in rats treated with GGFT at both 484 and 968 mg·kg⁻¹ throughout the treatment period from the 17th

Table 3 Inhibition of right and left hind paw edema by GGFT on CFA-induced arthritis in rats

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Adjuvant-treated</th>
<th>GGFT (484 mg·kg⁻¹)</th>
<th>GGFT (968 mg·kg⁻¹)</th>
<th>DSMS (1 mg·kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Edema volume of right hind paw (mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.09 ± 0.03</td>
<td>1.12 ± 0.03**</td>
<td>0.92 ± 0.04*</td>
<td>0.87 ± 0.01**(22.3)</td>
<td>0.55 ± 0.02**(50.9)</td>
</tr>
<tr>
<td>9</td>
<td>0.09 ± 0.01</td>
<td>0.93 ± 0.02**</td>
<td>0.66 ± 0.03</td>
<td>0.55 ± 0.01**(40.9)</td>
<td>0.30 ± 0.01**(67.7)</td>
</tr>
<tr>
<td>13</td>
<td>0.15 ± 0.01</td>
<td>1.28 ± 0.02**</td>
<td>1.05 ± 0.04</td>
<td>0.86 ± 0.08**(32.8)</td>
<td>0.47 ± 0.01**(63.3)</td>
</tr>
<tr>
<td>17</td>
<td>0.19 ± 0.03</td>
<td>1.20 ± 0.04**</td>
<td>0.92 ± 0.02**(23.3)</td>
<td>0.78 ± 0.01**(35.0)</td>
<td>0.38 ± 0.01**(68.3)</td>
</tr>
<tr>
<td>22</td>
<td>0.19 ± 0.02</td>
<td>1.16 ± 0.01**</td>
<td>0.84 ± 0.03**(27.6)</td>
<td>0.67 ± 0.08**(42.2)</td>
<td>0.32 ± 0.01**(72.4)</td>
</tr>
<tr>
<td>26</td>
<td>0.24 ± 0.02</td>
<td>1.15 ± 0.01**</td>
<td>0.81 ± 0.01**(29.6)</td>
<td>0.62 ± 0.02**(46.1)</td>
<td>0.25 ± 0.02**(78.3)</td>
</tr>
<tr>
<td></td>
<td>Edema volume of left hind paw (mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.12 ± 0.00</td>
<td>0.10 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.00 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>9</td>
<td>0.12 ± 0.01</td>
<td>0.42 ± 0.03*</td>
<td>0.25 ± 0.02</td>
<td>0.11 ± 0.01**</td>
<td>0.10 ± 0.01**(76.2)</td>
</tr>
<tr>
<td>13</td>
<td>0.16 ± 0.01</td>
<td>0.65 ± 0.01**</td>
<td>0.34 ± 0.01**(47.7)</td>
<td>0.17 ± 0.00**(73.8)</td>
<td>0.08 ± 0.02**(87.7)</td>
</tr>
<tr>
<td>17</td>
<td>0.18 ± 0.00</td>
<td>0.64 ± 0.01**</td>
<td>0.26 ± 0.02**(59.4)</td>
<td>0.19 ± 0.01**(70.3)</td>
<td>0.07 ± 0.02**(89.1)</td>
</tr>
<tr>
<td>22</td>
<td>0.22 ± 0.03</td>
<td>0.80 ± 0.01**</td>
<td>0.32 ± 0.02**(66.3)</td>
<td>0.27 ± 0.01**(66.3)</td>
<td>0.08 ± 0.02**(90)</td>
</tr>
<tr>
<td>26</td>
<td>0.23 ± 0.01</td>
<td>0.87 ± 0.01**</td>
<td>0.45 ± 0.01**(48.3)</td>
<td>0.30 ± 0.03**(65.5)</td>
<td>0.13 ± 0.02**(85.1)</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of 10 animals for each group; Each value in parenthesis indicates the percentage inhibition rate (%); DSMS, dexamethasone; *P < 0.05, **P < 0.01 vs the adjuvant-treated group; *P < 0.05, **P < 0.01 vs the vehicle control group.
day (Fig. 3a). In GGFT-treated groups, the clinical scores decreased to $8.8 \pm 0.45$ (484 mg·kg$^{-1}$) and $7.33 \pm 0.75$ (968 mg·kg$^{-1}$), respectively, compared with $14.7 \pm 0.52$ for adjuvant-treated group. The potent efficacy of dexamethasone (1 mg·kg$^{-1}$) was also confirmed from Day 17 to Day 26. Body weight of each animal was determined daily throughout the course of the experiment. The data showed animals treated with CFA gained weight more slowly than the normal control group, and the arthritic rats showed a marked weight loss ($P < 0.05$), compared with the vehicle control from the third week (Fig. 3b). However, the weight loss of the CFA-injected rats could be reversed by administration of GGFT at 968 mg·kg$^{-1}$. In the dexamethasone-treated group, the rats had greater weight loss compared with adjuvant-treated, possibly indicating the potential toxicity of dexamethasone at the dose used in the present study (Fig. 3b).

![Fig. 3](image-url) Effects of GGFT on CFA-induced arthritis. (a) Mean clinical scores of arthritis rats. (b) Body weight changes of arthritis rats. (c) Serum levels of IL-1$\beta$ and TNF-\(\alpha\) (d) in rat adjuvant arthritis. $^{\#\#}P < 0.01$ vs the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs adjuvant-treated group. DSMS, dexamethasone

**Histopathological assessment of the arthritic knee joints**

To further evaluate the inhibitory effects of GGFT on inflammation in arthritis rats, histological assessment of the ankle joints was performed. On Day 26, histological evaluation of the joints in vehicle-treated CFA rats demonstrated classic features of severe arthritis, including synovial hyperplasia, massive mixed (neutrophil, macrophage, and lymphocyte) infiltration (Fig. 4b), articular cartilage, and bone erosion. The dexamethasone-treated group revealed a marked decrease in synovial inflammatory cell infiltration and synovial hyperplasia with moderate obliteration of the joint cavity (Fig. 4c). Similarly, in the CFA rats treated with GGFT, the cartilage damage, massive cell influx along with minimal evidence of inflammation was significantly reduced compared with the model group (Figs. 4d & 4e). Histopathological studies showed the evidence of GGFT in suppressing chronic inflammation and bone destruction of in the arthritis rats.

**Effects of GGFT on CFA-induced expression of IL-1$\beta$ and TNF-\(\alpha\) in serum**

TNF-\(\alpha\) and IL-1$\beta$, the key inflammatory mediators mainly secreted by macrophages, are believed to play crucial roles in the procession of RA. To investigate the effects of GGFT on cytokine modulation, the serum levels of TNF-\(\alpha\) and IL-1$\beta$ were measured...
Histology of ankle joint of the CFA-treated paw. GGFT (484 and 986 mg·kg$^{-1}·d^{-1}$) and dexamethasone (DSMS, 1 mg·kg$^{-1}·d^{-1}$) were used. Massive inflammation with severe erosion and many infiltrated inflammatory cells were seen in the CFA-treated paw. Marked reductions of all parameters were observed in GGFT- and DSMS-treated groups with H&E staining.

Cytotoxicity of GGFT in RAW264.7 cells

The potential cytotoxicity of GGFT extract was examined after incubating RAW 264.7 cells with 37.5, 75, 150, or 300 µg·mL$^{-1}$ GGFT for 24 h, by MTT assay. The results demonstrated that GGFT had little cytotoxic effects on the viability of RAW 264.7 cells at a dose range of 37.5–300 µg·mL$^{-1}$ (Fig. 5a).

Effects of GGFT on the production of NO, PGE2, and IL-6 in LPS-stimulated RAW264.7 cells

The RAW 264.7 macrophage cells stimulated with LPS would generate massive inflammatory mediators and cytokines (e.g. NO, PGE2, IL-6, TNF-α, IL-1β), which would cause kinds of inflammatory diseases [25]. In the present study, the LPS-activated RAW 264.7 cell model was chosen to reveal the anti-inflammatory mechanism for GGFT.

We firstly evaluated the effects of GGFT on modulating the production of inflammatory mediators NO and PGE2 in the LPS-induced RAW264.7 macrophages. The cells were treated by LPS with or without GGFT at different concentrations. As a positive control, 10 µmol·L$^{-1}$ of luteolin was used, which has been reported to possess potent anti-inflammatory activity [26]. The levels of NO and PGE2 in cell supernatants were determined by the Griess assay and ELISA, respectively. As shown in Fig. 5b, GGFT demonstrated significantly inhibitory effects on NO production. GGFT extract at a concentration of 37.5 µg·mL$^{-1}$ inhibited more than 22% of NO production and its inhibitory effect was increased dose-dependently. At a concentration of 300 µg·mL$^{-1}$, GGFT extract reduced NO production up to 52%. Stimulating RAW 264.7 cells with LPS also resulted in significant increase of PGE2 production ($P < 0.01$; Fig. 5c). However, this effect could be strongly reversed by GGFT administration. As seen in Fig. 5c, GGFT at 150 and 300 µg·mL$^{-1}$ could inhibit the PGE2 level in RAW 264.7 cells by 66% and 72%, respectively. Since GGFT exhibited elevated inhibitory effects on NO and PGE2 production, we further investigated the effects of GGFT on LPS-induced cytokine secretion. The concentration of IL-6 in RAW264.7 cell supernatants was determined by ELISA. After co-treatment with LPS and GGFT for 24 h, the LPS-induced IL-6 expression in the medium was decreased remarkably in a concentration-dependent manner. The IC$_{50}$ value of GGFT in inhibiting LPS-induced IL-6 production was 155.2 µg·mL$^{-1}$ (Fig. 5d).
Effects of GGFT on the expression of COX-2, iNOS and NF-κB activation

To investigate whether the inhibition of GGFT on NO and PGE2 production was related to the down-regulation of iNOS and COX-2, we tested the effects of GGFT on expression of COX-2 and iNOS protein in RAW 264.7 cells by Western blot analysis. Treatment of RAW 264.7 cells with LPS alone resulted in a significant increase in iNOS and COX-2 protein levels, compared to the control group. However, GGFT extract inhibited the expression of these proteins in a dose-dependent manner (Figs. 5e & 5f).

As the activation of NF-κB is critically required for the LPS-induced activation of iNOS, COX-2, PEG2, and IL-6[27], we further determined the effects of GGFT on LPS-induced NF-κB activation. Since p65 is the major component of NF-κB activated by LPS in the macrophage, the protein levels of NF-κB p65 in RAW 264.7 cells were measured by Western blot experiment [28]. As revealed in Fig. 5g, the difference of total NF-κB p65 protein expression was not statistically significant between groups, while the expression of p-NF-κB p65 was markedly increased 1.6-fold upon exposure to LPS alone. GGFT suppressed LPS-induced production of p-NF-κB p65 protein ($P < 0.05$, $P < 0.01$, respectively), suggesting that GGFT potentially suppressed iNOS, COX-2, PEG2 and IL-6 expression, at least in part, via an NF-κB- dependent mechanism.

Discussion

RA is a chronic debilitating autoimmune disease characterized by cytokine-mediated inflammation of the joints, which can lead to deformities and disability [29]. Pain, stiffness, swelling, deformity and, eventually, loss of function of the joints are common manifestations in patients with RA [30]. Many potent anti-arthritis drugs, including pain killers (analgesics), NSAIDs, DMARDs, and corticosteroids (steroids), are available to alleviate symptoms of arthritis. However, a prolonged use of these drugs is associated with severe adverse effects and most drugs are ineffective in a substantial proportion of patients. Accordingly, an increasing number of patients with RA in the Western countries are...
turning to herbal products and other complementary and alternative medicine (CAM) approaches for their healthcare needs [31-33]. Herbal medicines with high efficacy and fewer side effects have become more and more popular and well explored in recent decades [34]. GGFT is an herbal formula composed of the standardized extracts of Dioscoreae Nipponicae Rhizoma, Spatholobi Caulis, and Zingiberis Rhizoma (the total amount of water-soluble total saponins ≥ 4.8%). Among them, Dioscoreae Nipponicae Rhizoma is an important medicinal crude drug commonly used in TCM for the treatment of various inflammation related disorders, in particular rheumatism and inflammation of joints [35]. Spatholobi Caulis, derived from vine plant Spatholobus suberecots Dunn. (Leguminosae), also has long history of use to alleviate pain in joints and muscles, to relieve fever and stiffness in the limbs, and to expel “wind-dampness”, which can be interpreted as signs of rheumatoid arthritis in modern medicine [36]. In northeast of China, GGFT are widely used for the treatment of rheumatism and inflammation of joints. However, its anti-inflammatory and anti-arthritic activities have been poorly explored. Therefore, the present study aimed to investigate the anti-inflammatory and anti-arthritic effects of GGFT through in vivo and in vitro studies.

Pro-inflammatory mediators, such as NO and PGE2, play a key role in the pathogenesis of inflammation and RA. NO is synthesized by nitric oxide synthase that converts arginine into citrulline, producing NO in the process. At adequate concentration, NO can inhibit adhesion molecule expression, cytokine and chemokine synthesis, leukocyte adhesion, and transmigration, inducing an anti-inflammatory effect [37]. However, a large amount of NO is considered as a pro-inflammatory mediator that can cause tissue destruction and cell death [38]. In inflammation, PGE2, a major cyclooxygenase (COX) product, is of particular interest, because it is involved in all processes leading to the classic signs of inflammation: redness, swelling, and pain [39]. Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 are also considered to be important initiators of the inflammatory response and mediators of the development of inflammation, contributing to tissue damage and multiple organ failure [40]. A drug capable of preventing the release of pro-inflammatory mediators and cytokines in inflammatory cells could potentially possess anti-inflammatory activities. In the present study, both in vitro (RAW 264.7 cells) and in vivo experiments (xylene-induced mouse ear edema and carrageenan-induced rat paw edema) were conducted to determine the anti-inflammatory activity of GGFT. For in vitro study, the RAW 264.7 cells produced 72.85 ± 4.56 pg·mL$^{-1}$ of PGE2 and 12.59 ± 0.55 μmol·L$^{-1}$ of NO after a 24-h treatment with LPS (Basal levels were 7.14 ± 0.23 pg·mL$^{-1}$ and 4.31 ± 0.46 μmol·L$^{-1}$, respectively). GGFT concentration-dependently inhibited COX-2-catalyzed PGE2 and iNOS-catalyzed NO production at 37.5–300 μg·mL$^{-1}$. Furthermore, GGFT strongly inhibited IL-6 production from the same LPS-treated RAW 264.7 cells (Fig. 5). To elucidate the PGE2 and NO inhibitory mechanisms, the expression levels of COX-2 and iNOS were compared by Western blot analysis. The results suggested that GGFT inhibited PGE2 and NO production mainly by COX-2 inhibition and iNOS down-regulation, respectively. The anti-inflammatory activity of GGFT was further confirmed in vivo. In xylene-induced ear edema model, GGFT at 340–1 360 mg·kg$^{-1}$ showed potent inhibitory action towards mouse ear edema. The inhibition of GGFT at 1 360 mg·kg$^{-1}$ was stronger than that of dexamethasone. On carrageenan-induced paw edema in rats, the inhibition effect was also observed after GGFT treatment.

To evaluate the in vivo anti-arthritic activity, the adjuvant-induced arthritides (AA) in rats was used. AA animal model is a widely used experimental method for screening potential anti-RA drugs. It is characterized by infiltration of the synovial membrane and associated with joints destruction, which resembles closely to those clinical and pathological features of human RA [41]. In the present study, AA in the rats was induced by subcutaneous injections of CFA into the footpad of the right hind paw. After adjuvant injection, indices such as paw swelling and arthritis scores were recorded to measure the anti-arthritic activity of various tested drugs and employed here to evaluate the activity of GGFT at doses of 484 and 986 mg·kg$^{-1}$. The increase in the paw volumes in both CFA-injected and non-CFA-injected paws indicates the primary and secondary arthritic lesions respectively. Our results revealed that oral administration of GGFT clearly inhibited the arthritic inflammation in rats, in a dose-dependent manner (Fig. 3). Against secondary inflammation (arthritic inflammation) in the adjuvant-treated paws, GGFT (968 mg·kg$^{-1}·d^{-1}$) inhibited the paw volume by 32.8% and 35.9% on Days 13 and 17, respectively, while dexamethasone (1 mg·kg$^{-1}·d^{-1}$) inhibited the paw volume by 63.3% and 68.3%, respectively at the same time points (Table 3). Against arthritic inflammation in non-treated paws, GGFT inhibited the paw volume by 73.8% and 70.3% on Days 13 and 17, while dexamethasone (1 mg·kg$^{-1}$) showed 87.7% and 89.1% inhibition, respectively (Table 3). Histological observation also showed similar results (Fig. 4). In addition, treatment with an adjuvant led to a reduction in the body weight increase (Fig. 3a). Dexamethasone further reduced the body weight, which was well known as an adverse effect of steroidal anti-inflammatory drugs. However, treatment of GGFT reversed this decrease in body weight loss. Since inflammatory cytokines levels have been reported to be associated with weight loss in various human diseases, the anti-inflammatory activity of GGFT can be responsible for its attenuation of weight loss in CFA-induced rats [42]. In recent years, TNF-α inhibitors have been widely used for the treatment of RA and other inflammatory diseases, such as atherosclerosis, psoriasis, diabetes, and Crohn’s disease [43]. TNF-α not only acts as a critical mediator of joint inflammation but also contributes significantly to articular destruction in human rheumatoid
arthritis \[44]. Overproduction of TNF-α can stimulate synoviocytes to mediate synovial hyperplasia and produce extracellular matrix-degradative enzymes and chemokines, facilitating the progress of the arthritic erosion \[7]. The blockade and inhibition of TNF-α suppresses the pathological processes of RA \[45]. IL-1β is a pro-inflammatory cytokine driving joint inflammation as well as systemic signs of inflammation, playing a vital role in the pathogenesis of RA. In the present study, the levels of TNF-α and IL-1β raised significantly in AA model group. Administration of GGFT markedly attenuated TNF-α and IL-1β expression in serum, suggesting the mechanism for the beneficial effects of GGFT on RA may involve down-regulating the levels of TNF-α and IL-1β.

Based on the LC/MS analyses, we found that the main components of GGFT were dioscin, protodioscin, protagelcillin, pseudoprotodioscin, 6-gingerol, and 6-shogaol. Since these compounds have been proven to be effective as anti-arthritic and anti-inflammatory agents, we suggested that the saponins and gingerols in GGFT can be responsible for the observed anti-inflammatory and anti-arthritic activity observed in the present study, which merits further studies regarding the precise site and the underlying mechanism of action \[46-47].

**Conclusion**

In the present study, GGFT was evaluated for its anti-inflammatory and anti-arthritic activities *in vitro* and *in vivo* for the first time. Our study showed that GGFT, with abundant saponins and gingerols, presented potent anti-inflammatory and anti-arthritic activities, as evidenced by both signs and pathology scores. The observed activities of GGFT can be attributed to the down-regulation of TNF-α and IL-1β as well as the inhibition of inflammatory enzymes COX-2 and iNOS, thus suggesting GGFT as a potent strategy for the treatment or prevention of RA. In our future investigations, we will focus on a full elucidation of the anti-RA constituents and their mechanisms of action.

**References**


