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ORIGINAL ARTICLE

# Anti-arthritic Effects of Total Flavonoids from *Juniperus sabina* on Complete Freund's Adjuvant Induced Arthritis in Rats

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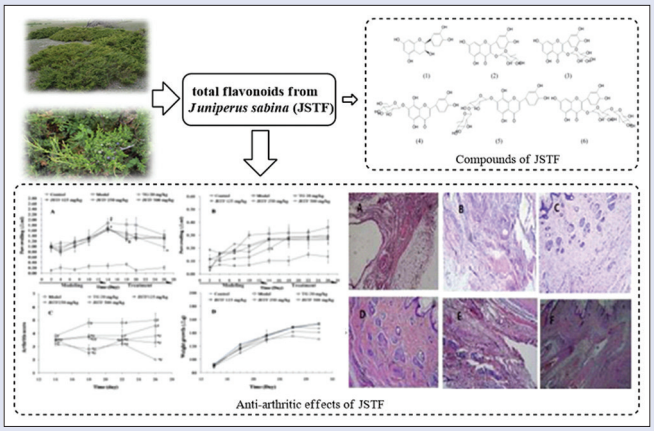
## ABSTRACT

**Context:** Twigs and leaves of *Juniperus sabina* L. have been traditionally used as the medicinal herb in China for the treatment of many ailments including rheumatoid arthritis (RA). **Aims:** To confirm the therapeutic effect of total flavonoids from *J. sabina* (JSTF) on RA-induced by Complete Freund's Adjuvant (CFA) in rats. **Settings and Design:** Wistar rats (200 ± 20 g) were immunized by intradermal injection of 0.1 mL of CFA into the right hind metatarsal footpad. JSTF was administered orally at the dose of 125,250 and 500 mg/kg on 14 days after the induction of adjuvant arthritis. Tripterygium glycoside (20 mg/kg) was used as a positive control. Paw swelling, arthritic score, body weight loss, serum cytokines, inflammatory mediators, and histological change were measured. **Results:** We found that JSTF could ameliorate paw swelling of CFA rats, and significantly inhibit arthritic score ($P < 0.05$). The overproduction of tumor necrosis factor alpha and interleukin 1beta were remarkably suppressed in the serum of JSTF (125,500 mg/kg) treated rats ($P < 0.05$). Histopathological studies also showed a marked decrease of synovial inflammatory infiltration and synovial lining hyperplasia in the joints of JSTF-treated animals. Six flavonoids were isolated and from JSTF by various chromatographic methods and identified as follows: Catechin, quercitrin, isoquercitrin, isoscutellarein 7-*O*-β-D-xylopyranoside, isoscutellarein 7-*O*-β-D-xylopyranose-(1 → 3)-α-L-rhamnoside, and rutin. **Conclusions:** These results suggest the potential therapeutically effect of JSTF as an anti-arthritis agent toward CFA-induced arthritis in rats, and verified therapeutic applications of *J. sabina* on RA in folk medicine.

**Key words:** Anti-arthritic effect, flavonoids, *Juniperus sabina L*

## SUMMARY

- Twigs and leaves of *Juniperus sabina* L. have been traditionally used as the medicinal herb in China for the treatment of rheumatoid arthritis
- JSTF could ameliorate paw swelling of CFA rats, and significantly inhibit arthritic score
- Histopathological studies showed a marked decrease of synovial inflammatory infiltration and synovial lining hyperplasia in the joints of JSTF-treated animals
- Six flavonoids were isolated and from JSTF including: Catechin, quercitrin, isoquercitrin, isoscutellarein 7-*O*-β-D-xylopyranoside, isoscutellarein 7-*O*-β-D-xylopyranose-(1 → 3)-α-L- rhamnoside, and rutin.




**Abbreviations used:** JSTF: Total flavonoids from *Juniperus sabina*; CFA: Complete Freund's Adjuvant; TG: Tripterygium glycoside; TNF-α: Tumor necrosis factor alpha; IL-1β: Interleukin 1beta; IL-6: Interleukin 6; H and E: Hematoxylin and eosin.

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## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease, with characteristic pathological changes of joint swelling, synovium hyperplasia, inflammatory cell infiltrates, and cartilage or bone damage.[1,2] The repeated bout of inflammation often leads to irreversible damage of bone joint and cartilage tissue, eventually led to the patient's disability, and compromised the quality of life in the industrialized and developing the world, so RA is called "immortal cancer" by people also.[3] At present, the nonsteroidal anti-inflammatory drugs and biologics remain a prominent group of drugs used in the treatment of RA.[4] However, administration of these drugs is associated with serve adverse effects including gastrointestinal lesions, cardiovascular complication, reproductive, etc.[5-7] Therefore, more and more attention has been focused on traditional folk medicine and natural medicine with high efficacy and few side effects.[8]

*Juniperus* L (Cupressaceae) species have been used to treatment of various inflammatory and infectious diseases in European countries and the United States, such as bronchitis, colds, cough, fungal infections, hemorrhoids, gynecological diseases, and wounds.[9,10] The extracts of fruits and leaves



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from *Juniperus oxycedrus* to *Juniperus communis* (100 mg/kg) exhibited significantly anti-inflammatory effect by carrageenan-induced edema and prostaglandin E2 ($PGE_2$) induced edema model.[11] Different extracts of *J. phoenicea* have obvious inhibitory effect on lipoxygenase and (5-HPETE) biosynthesis.[12] The traditional use of *Juniperus* on an anti-inflammatory agent may have potential anti-arthritic effects in the treatment of RA. Twigs and leaves of *Juniperus sabina* are used in the treatment of RA in Uyghur folk medicine in China.[13] This plant contains an abundance of bioactive compounds, such as terpene, lignans, flavonoids, and essential oil, of which flavonoids were mainly water-soluble constituents and its contents is 3.12%.[14-16] This study was designed to investigate the anti-arthritic effect of total flavonoids from *J. sabina* (JSTF) on adjuvant-induced arthritis (AA) in rats and its mechanism.

## SUBJECTS AND METHODS

### Plant material

The twigs and leaves of *J. sabina* were collected at September 17 in 2012 from the southern mountain at Urumqi in China, and authenticated by associate researcher Jiang He (Xinjiang Institute of Materia Medica, Republic of China). A voucher specimen has been deposited in Xinjiang Institute of Materia Medica in China.

### Preparation of total flavonoids from *Juniperus sabina* and isolation of compounds

The powdered twigs and leaves of *J. sabina* (10.0 kg) were defatted at reflux condition with petroleum ether and extracted under reflux at 80°C with 30% ethanol for 1 h in three batches to yield a dark brown residue (2.16 kg). After being dissolved in water, the extract was purified by D101 adsorption macroporous resin and polyamide resin to obtain total flavonoids (JSTF, 420 g). Total flavonoids content in JSTF was determined according to described methods in Chinese pharmacopeia.[17] Flavonoids content was calculated with rutin as the standard and total flavonoids content of JSTF was 69.21 mg/100 mg.

JSTF were applied to ODS RP-18 column and eluted with mixtures of MeOH: $H_2O$ (0:1 → 1:0) successively. Elutes were combined into fourteen fractions according to TLC behavior using solvent systems EtOAc: Actone: $H_2O$ (6:4: 1) (spots were visualized under 254 nm or after spraying 10% $H_2SO_4$). Various fractions were repeatedly purified by Sephadex LH-20 column with methanol, and six flavonoids were isolated from JSTF, and their structures were identified as catechin (1), quercitrin (2), isoquercitrin (3), isoscutellarein 7-O-β-D-xylopyranoside (4), isoscutellarein 7-O-β-D-xylopyranose-(1 → 3)-α-L-rhamnoside (5), and rutin (6) respectively.by their spectroscopic data (MS, 1H NMR, and $^{13}C$ NMR) comparison with spectral data obtained from the literature,[16] or co-TLC with authentic samples [Figure 1].

### Animals

Wistar rats, aged 6–8 weeks (200 sr20 g), were purchased from the Experimental Animal Center of Xinjiang Medical University (Urumqi, China). All rats were allowed to acclimatize for 1 week before the experiments were started. The rats were housed under standard laboratory conditions (room temperature 25°C, relative humidity 40–70% and free access to water) maintained on a 12 h light/dark cycle. This experiment was approved by the Bioethics Committee of Xinjiang Institute of Materia Medica (Urumqi, China), and the procedures of the experiment strictly adhered to generally accepted international rules and regulations.

### Induction of adjuvant arthritis and treatment

Freund's complete adjuvant (FCA, sigma) was prepared by suspending heat-killed BCG in liquid paraffin at 10 mg/mL.[18] Wistar rats were immunized by intradermal injection 0.1 mL FCA into the right hind paw of rat. The rats were divided into 5 groups randomly, in which the rats with AA received JSTF at 125, 250, and 500 mg/kg once daily by intragastric (i.g.) from 14 to 30 days. Tripterygium glycoside (TG) (20 mg/kg) was used as a positive control. The model and control group rats were given an equal volume of vehicle (1% CMC-Na) at the same time.

### Assessment of arthritis

The volume of the hind paw swelling was measured with water plethysmographicall before first immunization (basic value, day 0) and repeat on days 14, 18, 22 and 26. Meanwhile, the body weight of rats was measured every 4 days, and the changes in body weight are shown as weight growth (g). Arthritis score was used to evaluate the clinical severity of AA rats.[19] Paws were examined and graded for severity of erythema, swelling and induration using a 5-point scale: 0 = no signs of disease, 1 = mild swelling and erythema of the ankle/wrist, 2 = swelling and erythema of the ankle/wrist, 3 = severe swelling and erythema of the ankle/wrist, and 4 = severe disease involving the entire hind or fore paw. The maximum arthritic score per rat was set at 8 (4 points 2 hind paws).

### Measurement of serum cytokines concentrations

When the blood was standing for 30 min, the serum was collected by centrifugation at 3000 rpm for 10 min and stored at 20°C before analysis. The concentrations of cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL 1β), IL-6 and $PEE_2$ were quantified by ELASA assay according to the manufacturer's protocol.

### Histological changes

When the rats were sacrificed via anesthesia after serum collected on day 30. Knee joints were removed from the rats for histological analysis. The joints 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 mm) were stained with hematoxylin and eosin (H and E).

### Statistical analysis

The data represent the mean ± standard error of the mean ($n$ = 6). Differences between experimental groups were tested using one-way ANOVA; $P < 0.05$ were considered significant.

## RESULTS

### Effects of total flavonoids from *Juniperus sabina* on paw swelling, arthritis score and weight change

JSTF was administered intragastrically from 14 to 30 days after AA immunization. Paw swelling and arthritis score were measured every 4 days from 14 to 26 days. In the model group, injection resulted in progressive swelling of the left hind paw following the onset of the secondary phase arthritis that increased over time up to day 26, the swelling of right hind paw increased remarkably also and peaked at 14 days. TG (20 mg/kg) and JSTF (125, 500 mg/kg) significantly lowered the light paw volumes of the rats from 14 to 18 days [$P < 0.05$, Figure 2a]. On day 14, administration of TG (20 mg/kg) and JSTF at 500 mg/kg showed a significant inhibitory effect on the left hind paw swelling of AA rats [Figure 2b]. The arthritis score of the model group increased gradually and then peaked around 18 days after the Complete Freund's Adjuvant (CFA) injection. After treatment with JSTF (125, 250, 500 mg/kg) or TG (20 mg/kg), the arthritic scores in rats were

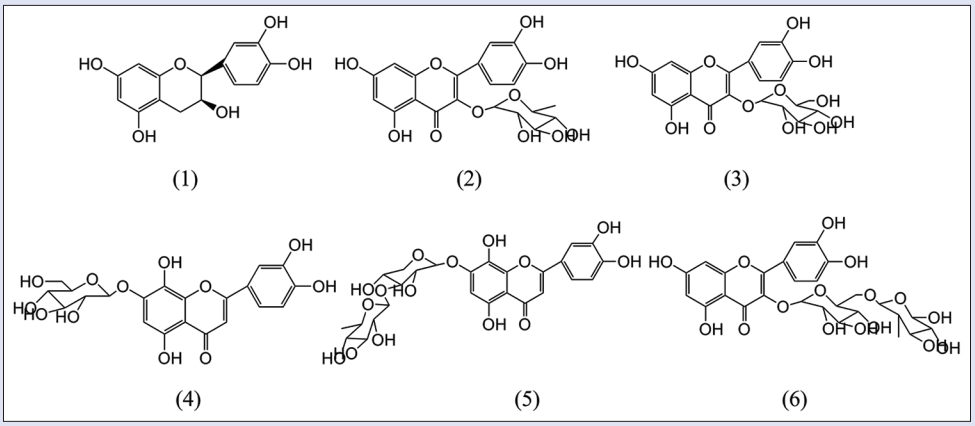


**Figure 1:** Compounds of JSTF

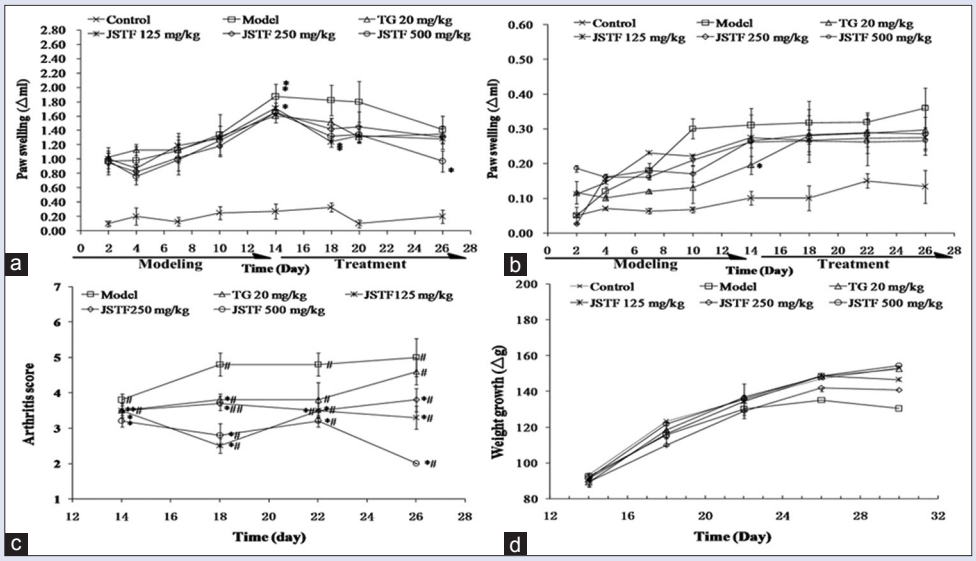


**Figure 2:** Pharmacological effects of total flavonoids from *Juniperus sabina* on adjuvant-induced arthritis rats. (a) Right paw volume in rats, (b) left paw volume in rats, (c) comparison of arthritic scores in rats, and (d) body weight changes. The increment pf paw swelling calculates as follows: Δ (mL) = volume day 14 (18, 22 or 26) volume day 0. The weight increment calculated as follows: Δ (g) = weight (day 1, 14, 18, 22, 26, 30) initial weight (day 0). Data represent the mean ± standard error of the mean ($n = 6$). Values are statistically significant at $^*P < 0.05$, compared with the model group. #$P < 0.05$ compared with the model group

successfully reduced compared with the control rats significantly from 14 to 26 days [Figure 2c]. It is observed that the body weight gain restored slightly in JSTF and TG treated groups, while compared with the AA models the difference was not obvious [Figure 2d].

## Effects of total flavonoids from *Juniperus sabina* on relative cytokine production in the serum of adjuvant induced arthritis rats

Cytokines (such as IL 1β, IL-6 and TNF-α) play an important role the pathogenesis of RA. Therefore, levels of these cytokines in the serum of AA rats were analyzed by ELISA kits [Figure 3]. As shown in the results, the levels of inflammatory cytokine TNF-α, IL-1β and IL-6 in model group rats were significantly elevated respectively ($P < 0.05$, vs. the control group). TG (20 mg/kg) and JSTF (125,500 mg/kg) significantly reduced the levels of TNF-α, especially in the 500 mg/kg dose, was almost equivalent to that by the reference drug TG ($P < 0.05$); decreasing in levels of IL-1β in serum of rats were observed in JSTF treatment group (125,500 mg/kg) ($P < 0.05$), the same effect was observed in TG (20 mg/kg) treatment group. However, both concentrations of JSTF treatment groups did not show any significant reduction in the IL-6 and $PGE_2$ levels when compared with the model group.

## Effects of total flavonoids from *Juniperus sabina* on histopathological changes

In the histopathological evaluation by H and E staining, AA rats exhibited extensive inflammation, pannus formation, cartilage destruction, synovial hyperplasia and vascular proliferation [Figure 4b], while no inflammation or joint destruction was seen in normal rats [Figure 4a]. The TG group revealed a marked decrease of the synovial inflammatory cell infiltrate and synovial lining hyperplasia with moderate obliteration of the joint cavity [Figure 4c]. The rats treated with 125,250 and 500 mg/kg of JSTF showed a remarkable reduction in synovial hyperplasia and inflammatory cell infiltration compared with control

rats [Figure 4d and f]. Histological analysis suggested that JSTF inhibited synovial hyperplasia, bone or cartilage destruction, and inflammatory cells infiltration in a dose-dependent manner.

## DISCUSSION

Traditional Chinese herbal medicine (TCHM) has a long history for the treatment of RA, and many components with better antirheumatic activity were isolated from TCHM in recent years, such as TG, total glucosides from peony and sinomenine.[20-23] Among them, TG has been widely applied to treatment of RA in clinic.[24] Ethnopharmacology survey showed that twigs and leaves from *J. sabina* are used to treat RA in folk medicine in China.[25] As reported, more than 100 compounds have been isolated from this plant, and flavonoids are the main characteristic components in this plant. Therefore, to find the chemical responsible of flavonoids from *J. sabina* for its significant anti-RA, we has studied as follows: JSTF were enriched and purified by macroporousresin and polyamide resin; on the above basis, we investigated the inhibitory effects of JSTF on adjuvant arthritis rats and its possible immunomodulatory mechanisms.

CFA induced arthritis in rats is a chronic inflammatory disease characterized by infiltration of the synovial membrane and associated with the destruction of the joints resembling closely to the human RA.[26,27] Paw swelling and arthritic scores are an index of measuring the anti-arthritic activities of various drugs in this model. As shown in the results, the levels of inflammatory cytokine TNF-α, IL-1 β and IL-6 in model group rats were significantly elevated respectively ($P < 0.05$, vs. the control group). TG (20 mg/kg) and JSTF (125, 500 mg/kg) significantly reduced the levels of TNF-α, especially in the 500 mg/kg dose, was almost equivalent to that by the reference drug TG ($P < 0.05$); decreasing in levels of IL-1β in serum of rats were observed in JSTF treatment group (125,500 mg/kg) ($P < 0.05$), the same effect was observed in TG (20 mg/kg) treatment group. However, both concentrations of JSTF treatment groups did not show any significant reduction in the IL-6 and $PGE_2$ levels when compared with the model group. JSTF administered groups showed marked reduction in arthritic scores when compared with the control group. The rats were sacrificed via anesthesia after serum collected on day 30. Knee joints and hind paws were removed from the rats and fixed in 10% phosphate buffered formalin, decalcified in 10% EDTA for 30 days, then embedded in paraffin. Serial paraffin sections (5 mm) were stained with H and E. TG (20 mg/kg) and JSTF (125, 250, 500 mg/kg) treatment group showed less inflammatory cell infiltration, well-preserved joint spaces and minimal synovia hyperplasia.

Chronic inflammation involves the release of a number of mediators which are responsible for the pain, destruction of bone and cartilage that can lead to severe disability.[28] Cytokines, such as TNF-α, IL-1 and IL-6, plays an important role on the pathogenesis process of RA. In the early stage of RA, IL-1 associates with the expression of leukocyte migration and stimulated endothelial cell, and the leukocyte and adhesion molecules were pooled into the joint cavity

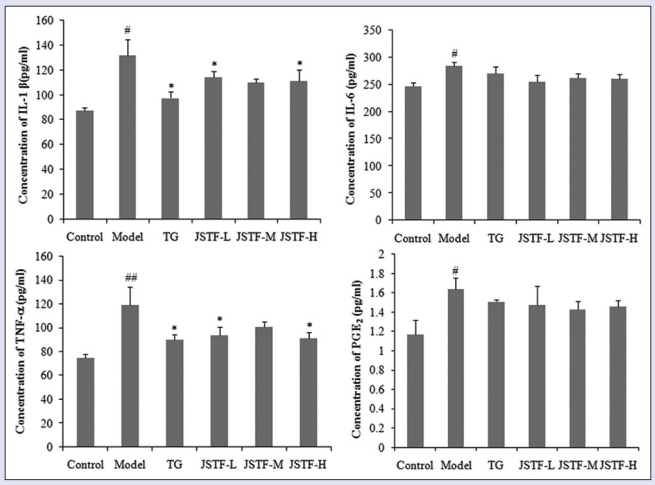


**Figure 3:** Effects of total flavonoids from *Juniperus sabina* on cytokine production in serum (tripterygium glycoside 20 mg/kg; total flavonoids from Juniperus Sabina L., 125 mg/kg; total flavonoids from *Juniperus sabina* M., 250 mg/kg; total flavonoids from *Juniperus sabina* H., 500 mg/kg). On day 30, the rats were sacrificed and the blood was collected when the observation finished. When the blood was standing for 30 min, the serum was collected by centrifugation at 3000 rpm for 10 min. The levels of tumor necrosis factor alpha, interleukin-1beta, interleukin-6 and prostaglandin E2 in serum were determined using an ELISA immunoassay kits according to the manufacturer's instructions. The data represent the mean ± standard error of the mean ($n = 6$). Values are statistically significant at $^*P < 0.05$ compared with the model group, $\#P < 0.05$ compared with the control group

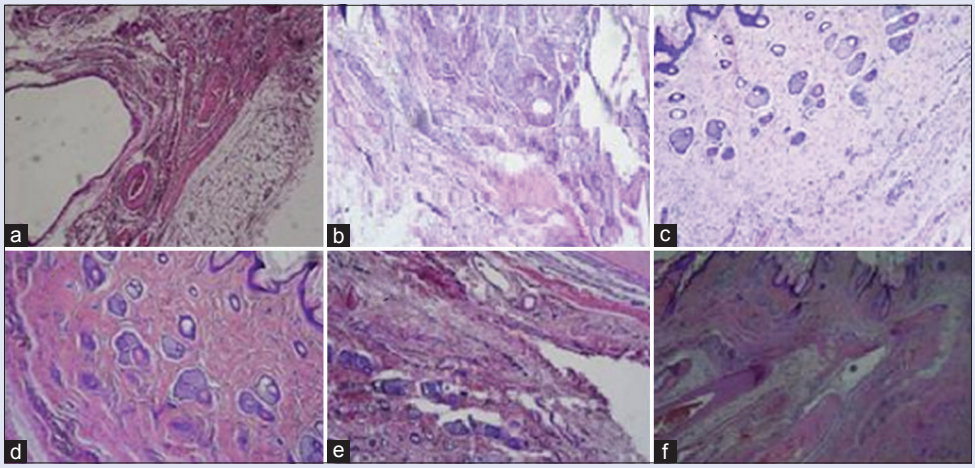


**Figure 4:** Effect of total flavonoids from *Juniperus sabina* on histopathological changes. (a) Normal group rats showed the normal articular cartilage, absence of damage in the synovium; (b) model group rats showed marked infiltration of inflammatory cells and synovial hyperplasia; (c) tripterygium glycoside (20 mg/kg) treatment group; (d-f) total flavonoids from *Juniperus sabina* (125, 250, 500 mg/kg) treatment group, and (d-f) showed less inflammatory cell infiltration, well-preserved joint spaces and minimal synivia hyperplasia

to induce arthritis by their interaction.[29] TNF-α plays a central role in the cytokine network of RA, and can stimulate mononuclear macrophage to produce IL-1, IL-6; stimulate fibroblasts to produce Granulocyte-macrophage colony-stimulating factor and collagenase; also osteoclast activating factor.[30] The pathogenic role of IL-6 in RA is the effect of enhancement TNF-α, and can promote the liver synthesis of acute phase proteins and promote the synthesis of rheumatoid factors, IL-6 may be an important pathological factor for the development of RA disease mainly.[31] In our experiments, both doses of JSTF (125, 500 mg/kg) significantly reduced the serum TNF-α and IL-1 β levels. This result indicates that anti-inflammatory effect of JSTF could be associated with its inhibition TNF-α, IL-1 β level. To find the chemical responsible of JSTF for its significant anti-inflammatory effect, we investigated the chemical profile of JSTF, and six flavonoids were identified as follows: Catechin, quercitrin, isoquercitrin, isoscutellarein 7-*O*-β-D-xylopyranoside, isoscutellarein 7-*O*-β-D-xylopyranose-(1 → 3)-α-L-rhamnoside, and rutin. Rutin has been showed a significant effect on the subchronic and chronic process of adjuvant arthritis and play a role by inhibiting the release of nitric oxide (NO), TNF-α, IL-1, IL-6 as well as T-cells proliferation.[32-34] Catechin (60, 120 mg/kg, i.g.) significantly suppressed secondary inflammatory paw swelling, pain reponse, and polyarthritis index in rats with AA by inhibiting production of IL-1, TNF-α, and $PGE_2$ in synoviocytes.[35] Moreover, quercitrin (50,100 and 200 mg/kg, p.o.) inhibited the rat hind paw edema induced by various phlogistics (carrageenin, dextran, histamine, serotonin and bradykinin) in a dose-dependent manner.[36]

## CONCLUSIONS

In summary, the results of the present study suggest that JSTF effective on CFA-induced arthritis in rats. The anti-arthritic activity of an extract of JSTF probably related to downregulate in the levels of pro-inflammatory cytokines TNF-α and IL-1 β in the serum of rats with CFA. JSTF be regarded as a potential candidate for use in general therapeutics and as an immunemodulatory medicine in RA.


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Nil.


### Conflicts of interest

There are no conflicts of interest.

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