—Regular Article—

Anti-arthritic Active Fraction of *Capparis Spinosa* L. Fruits and Its Chemical Constituents

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The aim of this study was to ascertain the anti-arthritic active fraction of *Capparis spinosa* L. (Capparidaceae) fruits and its chemical constituents. The adjuvant arthritic rat model was developed to evaluate the anti-arthritic effects of different fractions of ethanol extraction from *C. spinosa* L. The fraction eluted by ethanol-water (50 : 50, v/v) had the most significant anti-arthritic activity. The chemical constituents of this fraction were therefore studied; seven known compounds were isolated and identified as: (1) P-hydroxy benzoic acid; (2) 5-(hydroxymethyl) furfural; (3) bis (5-formylfurfuryl) ether; (4) daucosterol; (5) α-*D*-fructofuranosides methyl; (6) uracil; and (7) stachydrine.

Key words——*Capparis spinosa* L. (Capparidaceae); arthritic; stachydrine

INTRODUCTION

Capparis spinosa L. (Capparidaceae) is a common perennate shrub and a favored plant for restoring vegetation on dry regions in west or central Asia. It is particularly widely grown in the Mediterranean basin.[1] The floral buttons of *C. spinosa* L. have long been employed as a flavoring in cooking and in traditional medicine for their diuretic, antihypertensive, poultice and tonic properties.[2,3]

In China, *C. spinosa* L. is mainly distributed in the Xinjiang Autonomy Region. Its fruits have been used to treat rheumatic arthritis and gout.[4–6] These bioactivities prompted us to continue investigating its active fractions and chemical components. In a previous study, we evaluated the anti-inflammatory and analgesic activity of distinct fractions of *C. spinosa* L. fruits *in vivo*. The fractions eluted by ethanol-water (20 : 80, v/v) and ethanol-water (50 : 50, v/v) showed significant activity.[7] As a part of this ongoing research, we investigated the anti-arthritic activity of these two fractions and their chemical constituents.

MATERIALS AND METHODS

Chemicals and Reagents Complete Freund adjuvant was supplied by the America Sigma Company. Diclofenac Sodium was made by Fujiasawa Deutschland. Petroleum ether (PE 60–90°C), chloroform, ethyl acetate, and methanol were purchased from Sinopharm Chemical Reagent Co., Ltd., China. All chemicals and reagents used were analytically pure. Macroporous resin was purchased from Haiguang Chemical Co., Ltd., China.

Animals Male Wistar rats (180–200 g) and male and female Imprinting Control Region (ICR) mice (20–22 g) were supplied by Shanghai SLAC Laboratory Animal Co., Ltd. All animals were fed a standard diet *ad libitum*. Housing conditions and all *in vivo* experiments were approved by the institutional Ethical Committee of the Faculty of Pharmacy according to the Medical Laboratory Animal Management implementation details (Ministry of Health of the People's Republic of China Order, No. 55).

Plant and Preparation of Distinct Fractions The fruits of *C. spinosa* L. were collected from Urumchi (China) and identified by Prof. Zhang Hanming, Department of Pharmacognosy, Second Military Medical University of China. The voucher specimens (NO. 070810) were deposited in the Plant Biotechnology R&D Center, Shanghai Jiaotong University, China. The fruits of *C. spinosa* L. (5000 g) were extracted with ethanol-water (70 : 30, v/v, 15 l×3).

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The extracts were concentrated under vacuum and the resulting residue dissolved in hot distilled water subjected to column chromatography over macroporous resin, using an ethanol-water gradient (20 : 80, 50 : 50, 70 : 30, 100 : 0 v/v) to afford four fractions: collected fraction 1 (402.8 g), collected fraction 2 (104.8 g), collected fraction 3 (128.9 g) and collected fraction 4 (74.3 g). Based on the result of the anti-inflammatory and analgesia activity of the four fractions in the previous study,[7] only the anti-arthritic activity of collected fraction 1 and collected fraction 2 was studied.

Induction of Adjuvant Arthritis and Treatments Adjuvant arthritis was induced by intradermal injection of 0.1 ml complete Freund adjuvant into the right hind paw of the rats. On day 7 after immunization with complete Freund adjuvant, the rats were randomized into four groups and treated, together with a normal group, as follows: group normal control, the unimmunized rats; group model control, fed with distilled water; group diclofenac sodium, fed with diclofenac sodium (7.5 mg/kg) as positive control; group collected fraction 2, fed with collected fraction 2 (240 mg/kg); group collected fraction 1 fed with collected fraction 1 (600 mg/kg). The optimal doses of collected fraction 1 and collected fraction 2 were utilized according to the clinical doses of *C. spinosa* L. (10 g/kg).[4] The total period of treatment was 27 days. The thymus and spleen coefficient, the footpad volume, and arthritis index were observed in this experiment for each group.

Hot Plate Latency Test Hot plate latency assay was carried out according to the method of Wang et al.[7] Each of the test animals was placed on a beaker maintained at 55℃, 30 min after administration of stachydrine or diclofenac sodium. The time it took for the rats to respond to the thermal stimulus (indicated by paw licking or jumping) was noted as the latency (in seconds). The mean latency for each group was thus determined. The effects of collected fraction 2, stachydrine, or DS were also determined after 15, 30, and 60 min of administration to rats.

Acetic Acid-induced Writhing in Rats The animals were pretreated with stachydrine or diclofenac sodium. After 1 h, acetic acid was administered (intraperitoneal injection). The procedures were as described by Wang.[7] The number of writhing movements (contraction of abdominal muscles and stretching of hind limbs) were counted for 30 min.

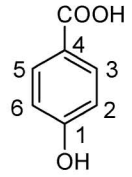
Carrageenan-induced Paw Edema in Rats The procedure used to assess anti-inflammatory activity followed the method used by Wang.[7] One hour after stachydrine or diclofenac sodium administration, edema was induced by injecting 0.02 ml of 1% carrageenan in sterile saline into the plantar side of the right hind paw. The pad thickness of each right hind paw was measured by micrometer before and at 1, 2, 3, 4, 5, and 6 h after carrageenan injection. The differences in the thickness were then calculated. The degree of foot-pad swelling was indicated by an increase in foot-pad thickness (mm).

Xylene-induced Ears Edema in Rats Xylene was smeared equably in the right ears of mice 30 min after stachydrine or diclofenac sodium was administered. The mice were killed by cervical dislocation 1 h later and the ears cut and weighed. The weight increase of the right ear *versus* the left ear was then calculated.[7]

Statistical Analysis Data are expressed as means±S.E.M. The significance of difference between drug-treated groups and the control group was evaluated by Dunnett's test.

Chemical Constituents Isolation and Analysis of Anti-arthritic Active Fraction Collected fraction 2 (104.8 g) with the strongest anti-arthritic activity was subjected to column chromatography packed with 1.1 kg of silica gel and eluted with mixed dichlormethane and MeOH (100 : 0, 100 : 1, 50 : 1, 30 : 1, 20 : 1, 10 : 1, 5 : 1, 8 l of eluent for each step) to give 15 fractions (fractions 1–15). Fraction 10 was further chromatographed on silica gel (180 g, 5×70 cm) and eluted with CH_2Cl_2-MeOH (gradient, 50 : 1, 30 : 1, 20 : 1, 10 : 1). Six fractions were collected (fractions 10.1–10.6). Fraction 10.4 was loaded on a Sephadex LH–20 column (2.0×150 cm) and eluted with CH_2Cl_2-MeOH 1 : 1 to yield compound 1 (28 mg), compound 2 (120 mg), compound 3 (22 mg). Fraction 13 was loaded on a sephadex LH-20 column (2.0×150 cm) and eluted with CH_2Cl_2-MeOH 1 : 1 to yield compound 5 (120 mg) and compound 6 (183 mg). Compound 4 (30 mg) and compound 7 (439 mg) came from fraction 16 (eluted with CH_2Cl_2-MeOH 10 : 1, 3.2 g) being chromatographed on silica gel column for the second time. Chemical structures of compounds 1–7 are shown in Fig. 1.

Compound 1: *P*-hydroxy benzoic acid, colorless needles, melting point: 215～216℃. ^{1}H-NMR (CD_3 OD) δ: 7.87 (2H, d, J=7.87, H-2, H-6), 6.82 (2H,



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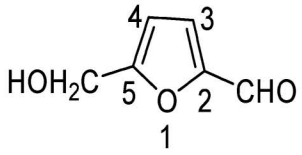

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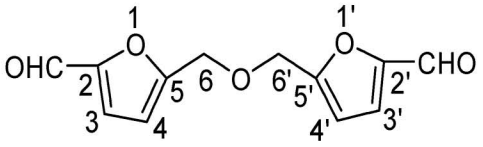

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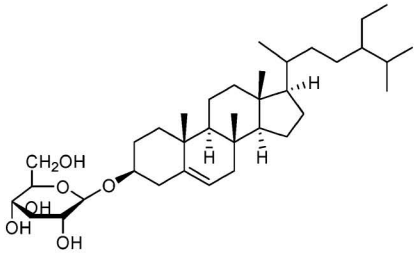

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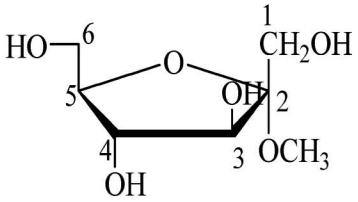

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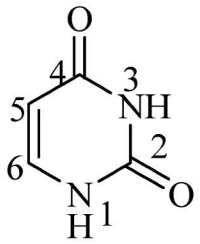

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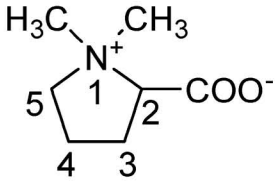

(7)

Fig. 1. Chemical Structures of Compounds (1)–(7)

d, J=7.87, H-3, H-5). ^{13}C-NMR (CD_3OD) δ: 122.8 (C, C-2), 116.0 (CH, C-3), 163.3 (C, C-4), 116.0 (CH, C-5), 133.0 (CH, C-6), 170.3 (C, COOH). ESI-MS m/z: 177 $[M+K]^+$, 315 $[2M+K]^+$ (Calcd for $C_7H_6O_3$: 138). Spectral data were in accordance with the literature,[8] so it was identified as *p*-hydroxybenzoic acid.

Compound 2: 5-(hydroxymethyl) furfural, colorless oiliness, melting point: 30～34℃. ^{1}H-NMR ($CDCl_3$) δ: 9.56 (1H, s, CHO), 7.23 (1H, d, J= 3.51, H-3), 6.51 (1H, d, J=3.51, H-4), 4.71 (2H, s, CH_2OH); ^{13}C-NMR ($CDCl_3$) δ: 152.2 (C, C-2), 123.1 (CH, C-3), 109.4 (CH, C-4), 160.9 (C, C-5), 177.7 (CHO), 57.4 (CH_2OH). ESI-MS m/z: 165 $[M+K]^+$, 275 $[2M+Na]^+$, 109 $[M\text{-}OH]^-$, (Calcd for $C_6H_6O_3$: 126). Spectral data were in accordance with the literature,[9] so it was identified as 5-(hydroxymethyl) furfural.

Compound 3: bis(5-formylfurfuryl) ether, colorless needles, melting point: 113.5～115.5℃. ^{1}H-NMR ($CDCl_3$) δ: 9.63 (2H, s, CHO), 7.21 (2H, d, J= 3.51, H-4′, H-4″), 6.57 (2H, d, J=3.51, H-3′, H-3″), 4.64 (4H, s, H-6′, H-6″). ^{13}C-NMR ($CDCl_3$) δ: 157.2 (C, C-2, C-2′), 111.8 (CH, C-3, C-3′), 121.7 (CH, C-4, C-4′), 152.8 (C, C-5, C-5′), 64.6 (CH_2, C, C-6, C-6′), 177.7 (CHO). ESI-MS m/z: 235 $[M+H]^+$, (Calcd for $C_{12}H_{10}O_5$: 224). Spectral data were in accordance with the literature,[10] so it was identi-

fied as bis (5-formylfurfuryl) ether.

Compound 4: daucosterol, blank powder. Liebermann-Burchard and Molish reaction all behaved positive. Monitored by TLC with daucosterol reference substance (3 different launch system was carried out: petroleum ether-acetic ether, petroleum ether-acetone, chloroform-methyl ethanol), the flow rate was the same.

Compound 5: *α-D*-fructofuranosides methyl, colorless oiliness. ^{1}H-NMR (CD_3OD) δ: 3.72, 3.69 (2H, H-1), 4.02 (1H, d, J=4.15, H-3), 3.88 (2H, dd, J=4.17, 6.36, H-4, H-5), 3.64, 3.60 (2H, H-6), 3.3 (3H, s, CH_3). ^{13}C-NMR (DMSO) δ: 63.1 (CH_2, C-1), 109.5 (C, C-2), 84.9 (CH, C-3), 79.2 (CH, C-4), 82.9 (CH, C-5), 60.9 (CH_2, C-6), 49.2 (OCH3). ESI-MS m/z: 217 $[M+Na]^+$, 311 $[2M+Na]^+$, 193 $[M-H]^-$ (Calcd for $C_7H_{14}O_6$: 194). Spectral data were in accordance with the literature,[11] so it was identified as *α-D*-fructofuranosides methyl.

Compound 6: Uracil, stramineous powder, melting point: 290～292℃. ^{1}H-NMR (DMSO) δ: 10.79 (1H, s, NH), 10.79 (1H, s, NH), 7.83 (1H, d, J=8.62, H-6), 5.45 (1H, d, J=8.62, H-5). ^{13}C-NMR (DMSO) δ: 151.6 (C, C-2), 164.4 (C, C-4), 100.3 (CH, C-5), 142.3 (CH, C-6), ESI-MS m/z: 111 $[M-H]^-$, (Calcd for $C_4H_4N_2O_2$: 112) Spectral data were in accordance with the literature,[12] so it was identified as uracil.

Compound 7: Stachydrine, colorless oiliness; ^{1}H-NMR (CD_3OD) δ: 4.02 (1H, t, H-2), 3.53, 3.71 (2H, m, H-3), 3.31 (3H, s, CH_3), 3.14 (3H, s, CH_3), 2.49, 2.31 (2H, m, H-3), 2.14 (2H, m, H-4); ^{13}C-NMR (CD_3OD, 100 MHz) δ: 171 (COO^-), 78 (CH, C-2), 26.9 (CH_2, C-3), 20.1 (CH_2, C-4), 68.3 (CH_2, C-5), 46.7, 53.1 ($+N(CH_3)_2$); ESI-MS m/z: 144 $[M+H]^+$, 309 $[2M+Na]^+$, (Calcd for $C_7H_{13}NO_2$: 143). Spectral data were in accordance with the literature,[13] so it was identified as stachydrine.

RESULTS

The rats were weighed on days 0, 18, 21, 24, and 27. The rate of weight gain was calculated as follows: (weight after proinflammatory − weight before proinflammatory)/the weight before proinflammatory. While a similar rate was observed in all the experimental groups, this increase was more pronounced in the group treated with *C. spinosa* L. (Table 1), which suggests that *C. spinosa* L. was not toxic *in vivo* at the doses tested.

On day 27 after injection of complete Freund adjuvant, the rat thymus and spleen weights were determined and the immune organ coefficient calculated. The immune organ coefficient = weight of organ/weight of rat × 1000. Thymus and spleen coefficient of all collected fraction 2, collected fraction 1, and diclofenac sodium groups decreased and had statistical significance compared with model group (Table 2).

In this study, rats were immunized by intradermal

Table 2. Influence on Immune Organ Coefficient after Injection of the Complete Freund Adjuvant ($\bar{x}\pm s$, n=10)

Group	Dose (mg/kg)	Thymus (g/kg)	Spleen (g/kg)
Normal		1.22±0.16*	2.63±0.28*
Model		1.66±0.44△	3.12±0.24△
DS	7.5	1.14±0.25*	2.30±0.37*
CF2	240	1.21±0.39*	2.46±0.53*
CF1	600	1.22±0.17*	2.57±0.37*

Note: DS, Diclofenac Sodium; CF1, Collected Fraction 1; CF2, Collected Fraction 2; compared with the model group * $p<0.05$, compared with the normal group △ $p<0.01$.

Table 1. Rate of Weight Gain of Rats in Each Group ($\bar{x}\pm s$, n=10)

Group	Dose (mg/kg)	Rate of weight gain (%)			
		18d	21d	24d	27d
Normal		61.78±4.23*	64.95±5.73*	66.61±7.21*	61.82±6.33*
Model		58.01±5.50	59.90±4.18	60.95±6.15	61.19±6.83
DS	7.5	58.65±4.07	60.07±8.23	61.50±7.04	64.55±9.37
CF2	240	62.98±5.23*	64.75±5.63*	67.61±9.21*	71.32±9.33*
CF1	600	63.99±6.96*	66.68±6.68*	68.13±8.62*	73.30±8.51*

Note: DS, Diclofenac Sodium; CF1, Collected Fraction 1; CF2, Collected Fraction 2; compared with the model group * $p<0.05$.

injection with Freund's complete adjuvant into the right hind footpad. The injection day was regarded as day 0. The volume of the left hind paw (non-injected) was measured on days 0, 18, 21, 24, and 27.[14] Secondary inflammation was expressed with Δml (Δml=paw volume after injection−paw volume before injection). On day 7, after complete Freund adjuvant injection, the left hind paws of the rats swelled and appeared red. The left hind paws of the rats in the model group and the other three groups were larger than those of the normal group. Δml of the group treated with diclofenac sodium and *C. spinosa* L. was significantly smaller than model group (Table 3).

The clinical symptoms of arthritis in all four limbs were evaluated by visual scoring system on a scale of 0−4: 0=no change; 1=swelling and erythema of the limb; 2=mild swelling and erythema of the limb; 3=gross swelling and erythema of the limb; 4=gross deformity and inability to use the limb. The arthritis score of each rat was the sum of the scores of the four limbs, with the maximum score being 16. Rats that showed scores of ≥ 1 were regarded to be arthritic.[15] The incidence and day of arthritis onset were also recorded. On day 15 after injection of complete Freund adjuvant, the arthritis index of collected fraction 2 group and diclofenac sodium group were significantly lower than model group (Table 4).

Analgesic and anti-inflammatory activities were studied by measuring nociception induced by acetic acid and hot-plate, and inflammation induced by carrageenan and xylene. Tables 5 and 6 and Figs. 2 and 3 disclose that stachydrine significantly delayed re-

Table 3. Influence on Footpad Swelling Induced by the Secondary Inflammation

Group	Dose (mg/kg)	Δml			
		18d	21d	24d	27d
Normal		0.13±0.08	0.14±0.06	0.15±0.09	0.15±0.07
Model		0.23±0.08	0.27±0.10	0.28±0.10	0.32±0.12
DS	7.5	0.18±0.12	0.18±0.03*	0.16±0.05**	0.15±0.05**
CF2	240	0.22±0.31	0.19±0.06*	0.17±0.07**	0.16±0.05**
CF1	600	0.23±0.08	0.26±0.09	0.23±0.05*	0.23±0.05*

Note: DS, Diclofenac Sodium; CF1, Collected Fraction 1; CF2, Collected Fraction 2; compared with the model group * $p<0.05$, ** $p<0.01$.

Table 4. Arthritis Index on the Rats after Injection of Complete Freund Adjuvant ($\bar{x}\pm s$, $n=10$)

Group	Dose (mg/kg)	Arthritis index					
		12d	15d	18d	21d	24d	27d
Model		3.50±1.18	6.40±0.74	7.90±0.88	9.30±0.82	10.50±0.92	11.60±0.85
DS	7.5	4.20±1.06	3.80±0.92*	3.60±1.17*	3.70±1.16**	3.70±0.82**	3.20±1.10**
CF2	240	3.70±1.42	4.40±0.84*	4.10±1.29*	4.30±0.57**	4.00±0.48**	3.70±0.63**
CF1	600	3.80±1.14	5.90±0.97	7.70±0.82	7.00±1.25*	7.50±0.63*	7.40±0.82*

Note: DS, Diclofenac Sodium; CF1, Collected Fraction 1; CF2, Collected Fraction 2; compared with the model group * $p<0.05$, ** $p<0.01$.

Table 5. Effects of Stachydrine on Hot Plate Test in Mice ($n=8$)

Group	Dose (mg/kg)	Reaction time (s)			
		0 min	15 min	30 min	60 min
Control		17.25±5.42	17.50±5.71	16.63±3.54	15.88±5.03
DS	5	17.50±6.95	18.50±7.52	21.25±6.20*	21.63±5.45*
Stachydrine	250	18.75±3.45	18.88±4.02	22.25±3.49*	22.13±5.77*
Stachydrine	125	14.63±5.83	13.13±4.12	16.25±6.20	16.25±5.73
Stachydrine	62.5	16.75±5.70	18.00±5.78	17.63±5.48	18.50±16.19

Note: DS, Diclofenac Sodium; compared with the control group * $p<0.05$.

Table 6. Effects of Stachydrine on Carrageenan-induced Paw Edema in Rats

Group	Dose (mg/kg)	Foot pad thickness increase (mm)					
		1 h	2 h	3 h	4 h	5 h	6 h
Control		0.25±0.42	0.21±0.03	0.18±0.05	0.17±0.03	0.16±0.03	0.14±0.03
DS	5	0.12±0.06*	0.09±0.08*	0.07±0.08*	0.05±0.05*	0.02±0.04*	0.02±0.04*
Stachydrine	250	0.16±0.07*	0.15±0.07*	0.14±0.07	0.12±0.07	0.09±0.08*	0.07±0.06*
Stachydrine	125	0.18±0.05*	0.15±0.06*	0.13±0.06	0.22±0.32	0.11±0.06	0.09±0.07
Stachydrine	62.5	0.19±0.07	0.18±0.07	0.17±0.07	0.16±0.08	0.14±0.07	0.13±0.08

Note: DS, Diclofenac Sodium; compared with the control group * $p<0.05$.

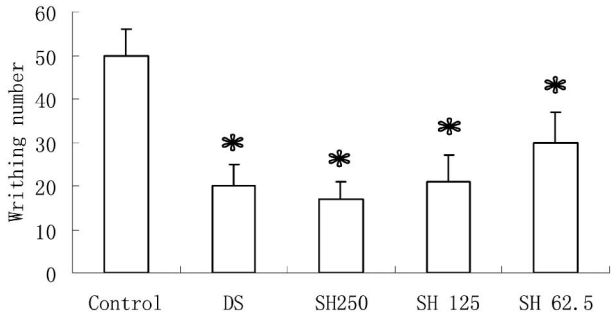

Fig. 2. Effects of Stachydrine on Acetic-acid-induced Writhing in Mice

Imprinting Control Region (ICR) mice were pretreated with stachydrine or dicolfenac sodium. After 1 h, acetic acid was administered (Intraperitoneal Injection). The number of writhing movements (contraction of abdominal muscles and stretching of hind limbs) were counted for 30 min. DS: Diclofenac Sodium (5 mg/kg); SH 125: Stachydrine (125 mg/kg); SH 250: Stachydrine (250 mg/kg); SH 62.5: Stachydrine (62.5 mg/kg). The data are expressed as mean±S.E.M. Compared with the control group *$p<0.05$.

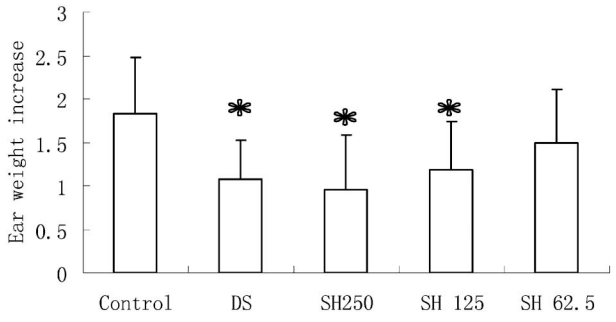

Fig. 3. Effects of Stachydrine on Xylene-induced Ear Edema in Mice

Xylene was smeared equably in the right ears of mouse. Stachydrine or diclofenac sodium were begun 30 min prior to xylene. The mouse were killed by cervical dislocation 1 h later and the ears were cut and weighed. The weight increase of the right ear *versus* the left ear was then calculated. DS: Diclofenac Sodium (5 mg/kg); SH 125: Stachydrine (125 mg/kg); SH 250: Stachydrine (250 mg/kg); SH 62.5: Stachydrine (62.5 mg/kg). The data are expressed as mean±S.E.M. Compared with the control group *$p<0.05$.

sponse to thermal stimulation and significantly inhibited abdominal constriction response caused by acetic acid. The formation of xylene-induced ear edema and carrageenan-induced paw edema was also significantly inhibited by stachydrine. The effect of stachydrine (250 mg/kg) was similar to diclofenac sodium (diclofenac sodium, 10 mg/kg), the standard analgesic and anti-inflammatory drug.

DISCUSSION

C. spinosa L. has a variety of pharmacological activities and is used in phytomedicine around the world for its anti-oxidative,[16] antifungal,[17] antihepatotoxic,[18] anti-inflammatory[19] and anti-diabetic effects.[20,21] But, the effect of *C. spinosa* L. extract on rheumatoid arthritis has not been demonstrated experimentally.

Tumor necrosis factor-α and interleukin-1β play relevant roles in the pathogenesis of adjuvant arthritis, and while tumor necrosis factor-α is involved in inflammation, differentiation, and proliferation of T and B cells as well as in bone resorption, interleukin-1β is implicated in the induction of inflammation, modification of the immune response, and activation of osteoclasts.[22] Blocking these cytokines can thus suppress inflammation and ameliorate cartilage destruction.[23] The lyophilized methanolic extract from flowering buds of *C. spinosa* L. was able to counteract the harmful effects induced by interleukin-1β.[24] Moreover, reports obtained from traditional medical practitioners in Xinjiang of China show that the fruits of *C. spinosa* L. were used for the treatment of rheumatoid arthritis.

Adjuvant arthritis in rats is an accurate assay for detecting the anti-inflammatory effect of new drugs. Adjuvant arthritis is very similar to human RA both in pathological and serological changes.[25] In this study, we found that all rats swelled and appeared red in the injected ankle on day 2, after complete Freund adjuvant injection. On day 7, the injected rats displayed secondary inflammation on the left hindpaw and forelimbs. Treatment of adjuvant arthritis rats

with *C. spinosa* L. reduced tissue swelling and decreased the edema. The anti-arthritic effects of collected fraction 2 were equivalent to those of diclofenac sodium.

One or more of these chemical compounds of collected fraction 2 is also likely to have contributed to the observed anti-adjuvant arthritis. Stachydrine was rich in collected fraction 2, which has antioxidant activity and can improve microcirculation. In this study the anti-inflammatory and analgesia activity of stachydrine was weaker in comparison with diclofenac sodium, although still present. It may therefore be one of the active principles responsible for the therapeutic effect of *C. spinosa* L. on rheumatoid arthritis, but more active compounds need to be found in future studies.

In conclusion, we demonstrated anti-arthritic effects of *C. spinosa* L. *in vivo* and analyzed the composition of collected fraction 2. These results justified the use of *C. Spinosa* L. in Xinjiang of China as an anti-inflammatory and anti-arthritic crude drug.

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