

Antiinflammatory and Analgesic Activities of *Thesium chinense* Turcz Extracts and its Major Flavonoids, Kaempferol and Kaempferol-3-*O*-glucoside

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The ethyl acetate, chloroform extracts, and the two flavonoids kaempferol **1** and kaempferol-3-*O*-glucoside **2** isolated from whole plants of *Thesium chinense* Turcz were investigated for their antiinflammatory and analgesic activities. For the antiinflammatory activity, carrageenan-induced hind paw edema and xylene-induced mouse ear edema models, and for the analgesic activity, the acetic acid-induced abdominal constriction test was used. The ethyl acetate extract and two flavonoids showed significant ($p < 0.05$ and $p < 0.01$) and dose-dependent antiinflammatory and analgesic activity. The chloroform extract was inactive in the assay.

Key words—*Thesium chinense* Turcz; antiinflammatory activity; carrageenan-induced paw edema; ear edema; kaempferol; kaempferol-3-*O*-glucoside

INTRODUCTION

Since ancient times, people have relied on plants as either a prophylactic or therapeutic arsenal to restore and maintain health, and plants are well known as important source of many biologically active compounds. There has been a growing interest in plants as a significant source of new pharmaceuticals.¹⁾ *Thesium chinense* Turcz is a perennial herb widely distributed in China belonging to the family Santalaceae. The whole plant is claimed to possess antiinflammatory, analgesic, antipyretic, and antibacterial activities.²⁾ In traditional Chinese medicine it is used for the treatment of mastitis, tonsillitis, pharyngitis, pneumonia, and upper respiratory tract infections.³⁾

A phytochemical investigation revealed the presence of flavonoids, glycosides, essential oils, alkaloids, steroids, and organic acids in *T. chinense* Turcz.⁴⁾ This plant is rich in flavonoids and from the whole plant several flavonoids like rutinoid, kaempferol-3-*O*-glucoside, naringenin-3-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, and kaempferol were isolated,^{5,6)} but relatively little is known about their pharmacologic activities.

Flavonoids are reported to possess antiinflammatory and analgesic activities. The analgesic activity of kaempferol-3-*O*-sophoroside from the leaf extract of *Cassia alata* was assessed using the tail clip, tail flick,

tail immersion, and acetic acid-induced writhing methods in mice and rats.⁷⁾ It was reported that kaempferol-3-*O*-galactoside, besides the five flavonoids studied, possesses *in vivo* (carrageenan-induced mouse paw edema and 12-*O*-tetradecanolyphorbol-3-acetate-induced ear edema) and *in vitro* (phospholipase A₂ enzyme) antiinflammatory effect.⁸⁾ Inducible nitric oxide synthase (iNOS) is believed to be responsible for the inflammatory response and eventually modulation of this enzyme may be important for the prevention of inflammation. It was reported that kaempferol and related flavonoids are potent inhibitors of iNOS.⁹⁾ It was also reported that kaempferol exhibits potent *in vitro* inhibitory effects on nitric oxide (NO) release and iNOS expression, whereas kaempferol-3-*O*-rutinoside (rutin) showed no significant effects even at the highest concentrations.¹⁰⁾

The present study investigated the possible antiinflammatory and analgesic effects of chloroform, ethyl acetate extracts (EAE), and isolated flavonoids from *T. chinense* Turcz using *in vivo* experimental models *i.e.*, carrageenan-induced hindpaw edema and xylene-induced ear edema for antiinflammatory activity and the acetic acid-induced abdominal constriction test for analgesic activity.

MATERIALS AND METHODS

General ¹H-NMR and ¹³C-NMR experiments were performed on a Bruker AR400 MHz using tetramethylsilane (TMS) as an internal standard.

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ESI-MS are recorded on Agilent 1100 Series LC/MS Trap. For column chromatography (CC), Silica gel (200–300 mesh, Ocean Chemical industry, China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used. Thin-layer chromatographic (TLC) analysis was performed on precoated silica gel plates F₂₅₄ (Atlantic, USA). HPLC was done on Elite P 230 HPLC.

Indomethacin, lambda carrageenan, and Aspirin were purchased from Sigma Chemical Co. (St. Louis, MO., USA). Tween-80, acetic acid, and xylene were purchased from Beijing Chemical Co. (Beijing, China).

Plant Material The whole plants of *T. chinense* Turcz were collected from Dongzhi county, Anhui province, China, and identified by Dr. H.-B. Chen, Department of Pharmaceutical Botany, Peking University, Beijing, China. A voucher specimen was deposited in the Beijing Institute of Technology, Beijing, China (voucher no. BIT122004).

Extraction and Isolation of Flavonoids 1 and 2 Air-dried whole plants (1 kg) of *T. chinense* Turcz (comprising leaves, flowers, stalks, roots, and seeds) were ground and sieved to obtain a particle size of less than 2 mm. The ground material was extracted with aqueous ethanol (80%, v/v) under reflux for 3 h. After extraction, the extracted slurry was filtered to collect the extract alone. The above procedures were repeated three times, and the extracts obtained from the first, second, and third extractions were combined and concentrated using a vacuum evaporator to give a solid mass (200 g). The dry residue was suspended into water, extracted with chloroform and ethyl acetate to give 5.03 (CE) and 10.5 g (EAE) of each respective residue. Part of the EAE (5 g) was subjected to silica gel CC using a CHCl₃ : CH₃OH gradient system (9 : 1 → 1 : 1). Thirty fractions were collected. TLC of these fractions was done using the CHCl₃ : CH₃OH solvent system. Fractions showing similar TLC patterns were combined. Flavonoids **1** and **2** isolated from these fractions and purified by Sephadex LH-20 and their purity was monitored on HPLC using acetonitrile: water (40% in 40 min).

Structures of the flavonoids were elucidated using several spectral techniques (¹H-NMR, ¹³C-NMR, LC-MS) and by comparing the data with those reported in the literature.

Pharmacologic Procedures

Animals Swiss albino mice (20–30 g) of both

sexes were used for the oral analgesic and antiinflammatory study. The animals were kept and maintained under laboratory conditions of temperature, humidity, and light and were allowed free access to food and water. The animals were divided into drug-treated “test” and 3% (v/v) Tween-80 in normal saline-treated “control” groups of 6 animals per group. All animals were fasted for 12 h although still allowed free access to water before the commencement of the experiments. The experimental protocol was approved by the Animal Ethics Committee of Beijing Institute of Technology, in accordance with the Principles of Laboratory Animal Care and Use in Research (Ministry of Health, Beijing, China).

Preparation of Test Samples for Bioassay The extracts and flavonoids were administered orally at doses of 50, 100 and 200 mg/kg dose after suspending in 3% (v/v) Tween-80 in normal saline. The control group animals received the same experimental handling as those in the test groups except that the drug treatment was replaced with an appropriate volume of vehicle.

Antiinflammatory Activity

Carrageenan-Induced Hindpaw Edema The previously described method of carrageenan-induced paw edema model in mice was used.¹¹⁾ The difference in footpad thickness between the right and left foot was measured with a gauge calliper. Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. One hour after the oral administration of test sample or dosing vehicle, each mouse was injected with freshly prepared (0.5 mg/25 μ l) suspension of carrageenan in physiological saline into the subplantar tissues of the right hindpaw. As the control, 25 μ l of saline solution was injected into that of the left hindpaw. The paw edema was measured at 60, 120, 180, 240 min with the gauge calliper. The percentage inhibition of paw volume in the drug-treated group was compared with the control group. Indomethacin (10 mg/kg *p.o.*) was used as the reference drug.

Xylene-Induced Ear Edema Mice were allotted to groups of 6 animals each. Thirty minutes after oral treatment of mice with 3% (v/v) Tween-80 (10 ml/kg), Aspirin (100 mg/kg), extracts (100 and 200 mg/kg), and flavonoids **1** and **2** (50 and 100 mg/kg), edema was induced in each mouse by applying a drop of xylene to the inner surface of the right ear. Fifteen minutes later, the animals were killed by cervical dis-

location and a 7-mm diameter section of the right and left ears were cut and weighed.¹²⁾ The level of inhibition (%) was calculated according to the following equation:

$$\text{Inhibition (\%)} = [1 - \text{Et}/\text{Ec}] \times 100$$

Where Et=average edema in the treated group and Ec=average edema of the control group.

Acute Toxicity Animals employed in the carrageenan-induced paw edema experiment were kept alive and observed for 24 h and morbidity or mortality was recorded for each group at the end of the observation period.

Analgesic Activity

Acetic Acid-Induced Writhing Test The previously described acetic acid-induced writhing model in mice was used.¹³⁾ Acetic acid (0.7% v/v) was administered intraperitoneally in a volume of 10 ml/kg body weight. Vehicle (normal saline), Aspirin (100 mg/kg), and test samples (50, 100, and 200 mg/kg) were administered *p.o.* 30 min before the acetic acid injection. The number of writhings and stretchings produced in each group for the succeeding 15 min was counted and compared with the response in the control group. Immediately after the injection of the analgesic compounds, each animal was isolated in an individual box to be observed for 15 min. The number of writhings and stretchings was recorded and expressed as the percentage of protection.

Statistical Analysis Data obtained from animal experiments were expressed as mean \pm standard error (\pm S.E.). Statistical differences between treatments and the control were evaluated using Student's *t*-test and $p < 0.05$ was considered to represent a statistically

significant difference.

RESULTS

Chemical Study Two compounds were isolated and identified from the EAE, flavonoid **1** and **2**. These flavonoids were identified by LC-MS, ¹H-NMR, and ¹³C-NMR data.^{14,15)}

Antiinflammatory Activity

Carrageenan-Induced Paw Edema Subplantar injection of carrageenan in mice resulted in a time-dependent increase in paw thickness (Table 1); this increase was observed at 1 h and was maximal at 4 h after administration of carrageenan in the vehicle-treated group. Carrageenan-induced inflammation was significantly ($p < 0.01$) reduced in all phases of the experiment after treatment with EAE, flavonoids **1** and **2**, and indomethacin while CE was ineffective (Table 1).

Xylene-Induced Ear Edema Results obtained from xylene-induced ear edema are shown in Table 2. EAE, flavonoids **1** and **2** significantly reduced ear edema induced by xylene. This effect was dose-dependent. CE was ineffective in this test.

Acute Toxicity EAE and flavonoids **1** and **2** used in pharmacologic study did not show any acute toxicity. Common side effects such as mild diarrhea and depression were not recorded.

Analgesic Activity

Acetic Acid-Induced Writhing The effects of EAE, CE, and flavonoids **1** and **2** on writhing response in mice are shown in Table 3. EAE (100 and 200 mg/kg), flavonoids **1** and **2** (50 and 100 mg/kg) caused dose-dependent inhibition of the writhing

Table 1. Antiinflammatory Effects of Extracts and Flavonoids **1** and **2** on Carrageenan-Induced Paw Edema in Mice

Treatment	Dose (mg/kg)	Swelling thickness \pm S.E. (mm) (inhibitory %)			
		60 min	120 min	180 min	240 min
Control		1.47 \pm 0.04	1.82 \pm 0.05	1.93 \pm 0.04	2.06 \pm 0.04
EAE	100	1.30 \pm 0.02 (11.6)	1.41 \pm 0.01 (22.6)	1.25 \pm 0.07 (35.3)	1.20 \pm 0.03 (41.8) ^{b)}
	200	1.15 \pm 0.03 (21.8)	1.01 \pm 0.07 (45.0)	0.89 \pm 0.03 (53.9) ^{a)}	0.75 \pm 0.04 (63.6) ^{a)}
CE	100	1.45 \pm 0.06 (NI)	1.85 \pm 0.03 (NI)	1.83 \pm 0.01 (5.2)	2.10 \pm 0.03 (NI)
	200	1.48 \pm 0.07 (NI)	1.70 \pm 0.06 (6.6)	1.75 \pm 0.02 (9.4)	1.78 \pm 0.06 (13.6)
Flavonoid 1	50	1.29 \pm 0.04 (12.3)	1.45 \pm 0.03 (20.4)	1.30 \pm 0.07 (32.7)	1.25 \pm 0.01 (39.6) ^{b)}
	100	1.20 \pm 0.07 (18.4)	1.15 \pm 0.03 (36.9)	1.12 \pm 0.07 (42.0) ^{a)}	0.89 \pm 0.05 (56.8) ^{a)}
Flavonoid 2	50	1.31 \pm 0.02 (10.9)	1.49 \pm 0.03 (18.2)	1.43 \pm 0.07 (26.0)	1.37 \pm 0.02 (33.5) ^{b)}
	100	1.25 \pm 0.05 (15.0)	1.35 \pm 0.07 (25.9)	1.25 \pm 0.02 (35.5) ^{b)}	1.10 \pm 0.05 (46.7) ^{a)}
Indomethacin	10	0.98 \pm 0.05 (34.4)	0.90 \pm 0.03 (50.5)	0.78 \pm 0.01 (59.6) ^{a)}	0.70 \pm 0.04 (66.5) ^{a)}

Values are mean \pm S.E. $n=6$. ^{a)} $p < 0.01$ compared with control values. ^{b)} $p < 0.05$ compared with control values.

Table 2. Anti-inflammatory Effects of Extracts and Flavonoids **1** and **2** on Xylene-Induced Ear Edema in Mice

Treatment	Dose (mg/kg)	Edema weight (mg)	% Inhibition
Contro		13.16±0.7	—
EAE	100	4.51±0.4 ^{b)}	65.8
	200	2.10±0.2 ^{a)}	84.0
CE	100	12.01±1.3	8.8
	200	11.85±0.7	10.0
Flavonoid 1	50	5.12±0.3 ^{b)}	61.1
	100	3.13±0.6 ^{a)}	76.30
Flavonoid 2	50	7.85±0.5 ^{b)}	40.4
	100	6.05±0.3 ^{a)}	54.1
Aspirin	100	1.593±0.4 ^{a)}	87.9

Values are mean ± S.E. *n* = 6. *a)* *p* < 0.01 compared with control values. *b)* *p* < 0.05 compared with control values.

Table 3. Analgesic Effects of Extracts and Flavonoids **1** and **2** on Acetic Acid-Induced Nociception in Mice

Treatment	Dose (mg/kg)	No. of writhings (Mean ± S.E.)	% Inhibition
Control		42.33 ± 5.5	—
EAE	100	21.10 ± 4.4 ^{b)}	52.2
	200	8.89 ± 2.9 ^{a)}	79.0
CE	100	40.00 ± 4.0	5.6
	200	36.90 ± 2.3	12.90
Flavonoid 1	50	25.05 ± 2.3 ^{b)}	40.9
	100	15.15 ± 0.6 ^{a)}	64.3
Flavonoid 2	50	30.50 ± 3.5 ^{b)}	28.0
	100	20.50 ± 0.3 ^{a)}	51.6
Aspirin	100	1.593 ± 0.4 ^{a)}	87.9

Values are mean ± S.E. *n* = 6. *a)* *p* < 0.01 compared with control values. *b)* *p* < 0.05 compared with control values.

response induced by acetic acid. CE was ineffective.

DISCUSSION

Carrageenan-induced paw edema is a widely used primary test for the screening of new anti-inflammatory agents¹⁶⁾ and is believed to be biphasic. The initial phase (1–2 h) of this assay is mainly mediated by histamine and serotonin. The second phase of edema is due to the release of prostaglandin (PG).¹⁷⁾ PGs are metabolites of arachidonic acid, which are synthesized and released by most cell types, and cyclooxygenase (COX) enzymes catalyze the first steps in the biosynthesis of PGs.¹⁸⁾

The results of this study indicate that *T. chinense* Turcz crude extract and pure compounds significantly inhibit the development of carrageenan-induced

mouse paw edema. The mechanism of action may involve inhibition of histamine, serotonin, or PGs synthesis.

The xylene-induced mouse ear edema method has certain advantages for natural product testing and has good predictive values for the screening of anti-inflammatory agents.^{19,20)} Xylene causes instant irritation of the mouse ear, which leads to fluid accumulation and edema characteristic of an acute inflammatory response. Suppression of this response is a likely indication of antiphlogistic effect.²¹⁾ In the present study, EAE and two flavonoids showed significant antiphlogistic effects against xylene-induced ear edema.

EAE and both flavonoids were shown to possess analgesic effects in the acetic acid-induced writhing test and the effects are dose dependent. The acetic acid-induced writhing test is widely used for the evaluation of peripheral antinociceptive activity.²²⁾ It is reported that local peritoneal receptors are partly involved in the abdominal constriction response.²³⁾ The method has been associated with prostanoids in general, *e.g.*, increased levels of PGE₂ and PGE_{2α} in peritoneal fluids as well as lipooxygenase products.^{24–26)} Therefore the results of the acetic acid-induced writhing test strongly suggest that the mechanism of action of extract and flavonoids may be linked partly to lipooxygenase and/or cyclooxygenase.

The results of present study reveal that EAE and both flavonoids showed a potent activity profile against all the models employed and flavonoids are the major anti-inflammatory and analgesic principles of the *T. chinense* Turcz.

CONCLUSIONS

In conclusion, this is the first study evaluating the anti-inflammatory and analgesic activity of *T. chinense* Turcz EAE and two flavonoids. Flavonoids **1** and **2** are major contributor of the analgesic and anti-inflammatory activity of EAE. The results also provide further supportive data that kaempferol and kaempferol-3-*O*-glucoside possess the relevant *in vivo* activities, although further detailed studies are required to determine the mechanism of action of flavonoids **1** and **2** in the inflammatory and analgesic processes.

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