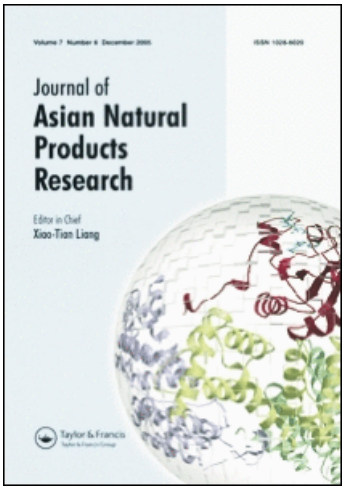

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Inhibitory effects of 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-d-glucoside on experimental inflammation and cyclooxygenase 2 activity

Y.-Z. Zhang[a]; J.-F. Shen[a]; J.-Y. Xu[a]; J.-H. Xiao[a]; J.-L. Wang[a]
[a] Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China

To cite this Article Zhang, Y.-Z. , Shen, J.-F. , Xu, J.-Y. , Xiao, J.-H. and Wang, J.-L.(2007) 'Inhibitory effects of 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-d-glucoside on experimental inflammation and cyclooxygenase 2 activity', Journal of Asian Natural Products Research, 9: 4, 355 — 363
To link to this Article: DOI: 10.1080/10286020600727772
URL: http://dx.doi.org/10.1080/10286020600727772

Journal of Asian Natural Products Research, Vol. 9, No. 4, June 2007, 355–363

Inhibitory effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside on experimental inflammation and cyclooxygenase 2 activity

Y.-Z. ZHANG, J.-F. SHEN, J.-Y. XU, J.-H. XIAO and J.-L. WANG*

Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, China

(Received 16 November 2005; revised 8 March 2006; in final form 14 March 2006)

The inhibitory effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside (THSG), extracted from the roots of *Polygonum multiflorum* Thunb, on inflammatory activity in animal models and cyclooxygenase-2 (COX-2) activity in lipopolysaccharide (LPS)-induced mouse RAW264.7 macrophage cells were investigated. The carrageenin (CGN)-induced rat paw oedema model and dimethylbenzene-induced mouse ear oedema model were prepared; MTT assay, semi-quantitative RT-PCR, Western blot and ELISA were adopted. THSG 2.3, 4.6 and 9.2 mg kg^{-1} by oral administration inhibited mouse ear oedema and the percentage of inhibition of THSG 9.2 mg kg^{-1} is 87%. THSG 3.2, 6.4 and 12.8 mg kg^{-1} by oral administration dose-dependently inhibited rat paw oedema and the percentage of inhibition of THSG 12.8 mg kg^{-1} is 56% at 6 h. Indomethacin 13 and 9 mg kg^{-1} showed 90% and 57% inhibition in the same animal models, respectively. LPS 1 μg ml^{-1} significantly up-regulated prostaglandin E_2 (PGE_2) production (inducing COX-2 activity) by 35% (exogenous arachidonic acid, AA), which was dose-dependently decreased by THSG 1, 10, and 100 μmol L^{-1} and the percentage of inhibition of THSG 10 μmol L^{-1} was 40%. NS-398 10 μmol L^{-1} decreased PGE_2 production by 42%. THSG 1, 10, 100 μmol L^{-1} was shown to markedly inhibit the LPS-induced COX-2 protein and mRNA expression in RAW264.7 cells ($P < 0.05$) but had no effect on COX-1 protein and mRNA ($P > 0.05$). In summary, the data showed that THSG possessed an anti-inflammatory effect, which was perhaps related to the inhibition of COX-2 enzyme activity and expression in RAW264.7 macrophage cells.

Keywords: 2,3,5,4′-Tetrahydroxystilbene-2-*O*-β-D-glucoside; *Polygonum multiflorum* Thunb; Lipopolysaccharide; Cyclooxygenase-2; Prostaglandin E_2; Inflammation

1. Introduction

Prostaglandins (PGs) biosynthesised by cyclooxygenases (COX) in many cell types are important inflammatory mediators and are deeply associated with inflammatory disorders, acute as well as chronic inflammation. An inhibition of PG production is one of the important therapeutic strategies for various inflammatory diseases. Among the eicosanoid generating enzymes, an inducible isoform of cyclooxygenase, COX-2, was found to be pivotal to produce prostanoids in the inflammatory site, but another isoform of cyclooxygenase,

*Corresponding author. Email: wangjialingtjmu@yahoo.com.cn

Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2007 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020600727772

COX-1, is constitutively expressed with near constant levels and activity in many tissues [1]. In accordance with this aspect, development of COX-2 inhibitors or modulators of COX-2 expression may be a significant way to find new anti-inflammatory agents. In the course of our search for anti-inflammatory effects of natural products-derived compounds including resveratrol, curcumin and other polyphenols, these compounds were also shown to potentially inhibit COX-2 activity [2,3].

Polygonum multiflorum Thunb, a traditional Chinese medicine, possesses extensive pharmacological effects, including an obvious anti-inflammatory effect [4] and anti-atherosclerotic effect, strongly suppressing oxidation and cleaning the active oxygen free radicals [5]. 2,3,5,4′-Tetrahydroxystilbene-2-*O*-β-D-glucoside (THSG) is an active compound isolated from the roots of *Polygonum multiflorum* Thunb [6] that has been reported to possess the effects mentioned above [6] except the anti-inflammatory effect. The chemical structure of THSG and resveratrol is very similar and belongs to hydroxystilbene compounds. Resveratrol suppresses the activation of COX-2 gene expression and directly inhibits COX-2 activity [2]. So we considered whether THSG might have an anti-inflammatory effect and suppress COX-2 activity and expression in LPS-stimulated mouse RAW264.7 macrophage cells.

2. Results and discussion

2.1 Effects of THSG on the drug-induced inflammatory models

In experimental inflammatory models, dimetheylbenzene was able to induce mouse ear oedema and carrageenin (CGN) evoked rat paw oedema. The ear oedema value (weight) was raised from 7.1 ± 0.7 mg (as control) to 10.7 ± 2.9 mg ($P = 0.05$) and the paw oedema value (circumference) was increased from 21.59 ± 0.49 mm to 25.66 ± 1.03 mm ($P = 0.01$), as shown in tables 1 and 2. THSG 2.3, 4.6 and 9.2 mg kg^{-1} by oral administration inhibited mouse ear oedema and the percentage of inhibition of THSG 9.2 mg kg^{-1} is 87% (table 1). THSG 3.2, 6.4 and 12.8 mg kg^{-1} by oral administration also antagonised the inflammatory activity in a dose-dependent manner and the percentage of inhibition of THSG 12.8 mg kg^{-1} is 56% at 6 h (table 2). Indomethacin 13 mg kg^{-1}, 9 mg kg^{-1}, a reference compound, showed 90% and 57% inhibition in ear oedema and paw oedema models, respectively (tables 1 and 2).

2.2 Influence of THSG on cell viability of LPS-stimulated RAW264.7 cells

As shown in figure 1, RAW264.7 cells were treated with LPS 1 μg ml^{-1} for 6 h. The viability of cells was significantly increased from 100% to 110.47% ($P > 0.05$). After treatment with

Table 1. Effects of THSG on mouse ear oedema induced by dimetheylbenzene.

Group	*Dose (mg kg^{-1})*	*Increased weight (mg)*
Saline		4.6 ± 2.5
THSG	2.3	4.4 ± 2.8
THSG	4.6	1.0 ± 0.4**
THSG	9.2	0.6 ± 0.2**
	0.013	0.5 ± 0.2**

*$P = 0.05$, **$P = 0.01$, compared with Saline group. Mean ± SD, $n = 7$–9.

Table 2. Effects of THSG on rat paw oedema induced by CGN.

	Increased circumference (mm)			
Group/dose (mg kg^{-1})	*1 h*	*2 h*	*4 h*	*6 h*
Saline	1.00 ± 0.51	1.21 ± 0.76	2.60 ± 1.07	4.07 ± 0.92
THSG 3.2	0.75 ± 0.67	0.91 ± 0.63	1.47 ± 0.54*	2.28 ± 1.03**
THSG 6.4	0.67 ± 0.51	0.83 ± 0.28	1.56 ± 0.38*	2.02 ± 0.25**
THSG 12.8	0.55 ± 0.45	0.78 ± 0.41	1.40 ± 0.53**	1.81 ± 0.46**
Indo 0.009	0.37 ± 0.1*	0.75 ± 0.31	1.34 ± 0.75*	1.73 ± 0.45**

*$P = 0.05$, **$P = 0.01$, compared with Saline group. Mean ± SD, $n = 6–8$.

THSG 1, 10, 100 μmol L^{-1} for 2 h, no obvious potential effect could be found (LPS + THSG group *vs*. LPS group, $P > 0.05$). THSG 1, 10, and 100 μmol L^{-1}did not affect cell viability as determined by MTT assay (figure 1).

2.3 Effects of THSG on PGE$_2$ production in LPS-activated RAW264.7 cells

As shown in figure 2; LPS 1 μg ml^{-1} for RAW264.7 cells was able to change endogenous AA and then significantly increase the production of PGE_2 from 44.87 ± 2.48 pg ml^{-1} (control level) to 69.07 ± 5.73 pg ml^{-1}. THSG 1, 10 and 100 μmol L^{-1} dose-dependently decreased LPS-induced PGE_2 production (figure 2). THSG 100 μmol L^{-1} decreased PGE_2 production by 50%. NS-398 100 μmol L^{-1}, a selective COX-2 inhibitor, was used as a positive control in this assay and showed 71% inhibition. In addition, resveratrol, a naturally occurring COX-2 inhibitor, also showed inhibition of PGE_2 production in this assay (data not shown).

2.4 Effects of THSG on LPS-induced COX-2 and COX-1 expression in RAW 264.7 cells

LPS was shown previously to induce COX-2 expression in RAW 264.7 cells. THSG 1, 10 and 100 μmol L^{-1} concentration-dependently suppressed LPS-stimulated COX-2 protein and

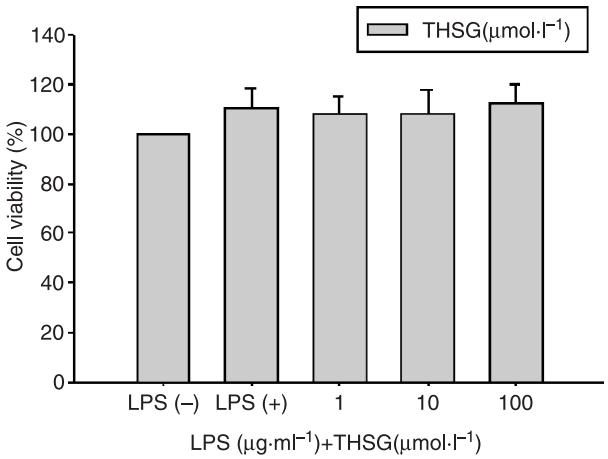

Figure 1. No obvious effects of THSG on cell viability. RAW264.7 cells were stimulated with LPS 1 μg ml^{-1} for 6 h and treated with THSG 1, 10, 100 μmol L^{-1} simultaneously for 2 h. Cell viability was measured by MTT assay. Mean ± SD, $n = 8$. $P > 0.05$ compared with LPS(+) group.

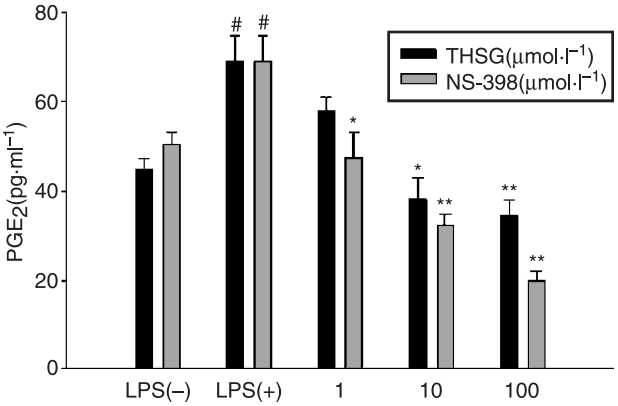

Figure 2. Inhibitory effects of THSG or NS-398 on LPS-induced PGE_2 production in RAW 264.7 cells. RAW264.7 cells was stimulated with LPS 1 μg·ml^{-1} for 6 h and then treated with THSG or NS-398 1, 10, 100 μmol L^{-1} simultaneously for 2 h. Then, the supernatant was removed and the amount of PGE_2 production was analysed. Mean ± SD, $n = 3$. $^{\#}P = 0.05$ compared with LPS(−) group; $*P = 0.05$, $**P = 0.01$ compared with LPS(+) group.

mRNA expression (figure 3). LPS 1 μg ml^{-1} after treatment for 6 h clearly induced COX-2 protein from 65.14 ± 3.6 (as control) to 103.01 ± 9.2 and had no effect on COX-1 protein from 100.77 ± 14.56 to 102.43 ± 13.32. THSG 10 μmol L^{-1} inhibited COX-2 protein from 103.01 ± 9.2 to 90.49 ± 8.12 ($P = 0.05$) and had no obvious effect on COX-1 protein from 102.43 ± 13.32 to 100.34 ± 12.95 ($P > 0.05$) (figure 3A). LPS 1 μg ml^{-1} for RAW 264.7 cells for 6 h clearly induced COX-2 mRNA from 0.152 ± 0.016 (as control) to 0.734 ± 0.015 and had no effect on COX-1 mRNA from 0.343 ± 0.015 to 0.334 ± 0.010. THSG 10 μmol L^{-1} inhibited LPS-induced COX-2 mRNA from 0.734 ± 0.015 to 0.498 ± 0.016 ($P = 0.05$) and had no obvious effect on COX-1 mRNA from 0.334 ± 0.010 to 0.325 ± 0.022 ($P > 0.05$) (figure 3B).

2.5 Effects of THSG on COX-2 enzyme activity (exogenous AA) in LPS-activated RAW264.7 cells

As depicted in figure 4, LPS 1 μg ml^{-1} for RAW264.7 cells for 6 h increased dramatically the PGE_2 production from the basal level of 59.25 ± 8.17 pg ml^{-1} to 79.95 ± 5.53 pg ml^{-1} (1×10^5 cells in a 96-well plate). THSG 1, 10, 100 μmol L^{-1} concentration-dependently inhibited GE_2 production from exogenous AA and the percentage of inhibition of THSG or NS-398100 μmol L^{-1} is 56% and 68%, respectively.

2.6 Discussion

Previous study had demonstrated that roots of *Polygonum multiflorum* Thunb could increase non-specificity immune organ weight (thymus gland, adrenal gland and spleen), enhance normal leucocyte sum, raise in mice the percentage of C3b receptor rosette and decrease immune complex rosette content [7]. But the anti-inflammatory effect of THSG on animal models had not so far been reported.

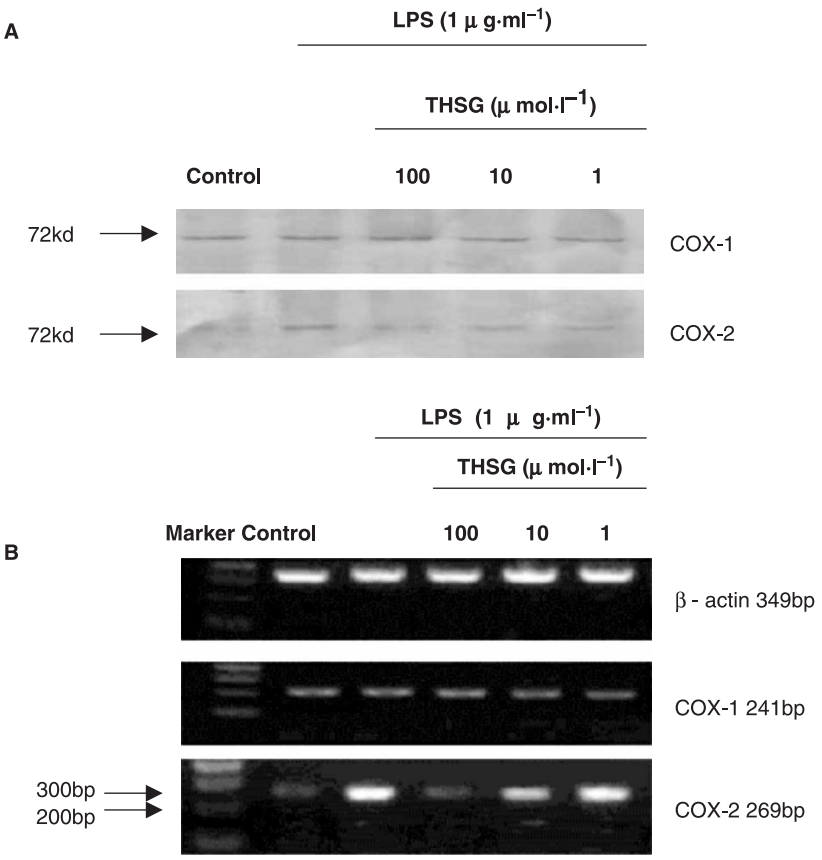

Figure 3. Inhibitory effects of THSG on COX-2 and COX-1 protein and mRNA expression in LPS-activated RAW264.7 cells. (A) RAW264.7 cells were stimulated with LPS $1\,\mu g\,m^{-1}$ for 6 h and treated with THSG simultaneously for 2 h. Total protein was analysed for the expression of COX-2 and COX-1 gene by Western blot analysis (B) RAW264.7 cells were stimulated with LPS $1\,\mu g\,ml^{-1}$ for 6 h and then treated with THSG 1, 10, $100\,\mu mol\,L^{-1}$ simultaneously for 2 h. Total RNA was analysed for the expression of COX-2 and COX-1 gene by RT–PCR, as described in *Materials and methods*. Expression of β-actin gene was used as internal standard as described in *Materials and methods*.

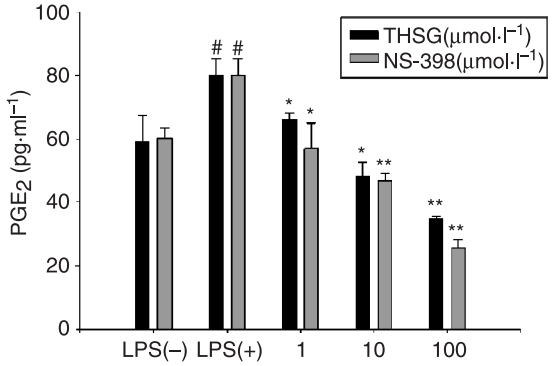

Figure 4. Inhibitory effects of THSG on COX-2 enzyme activity in LPS-activated RAW264.7 cells. RAW264.7 cells were stimulated with LPS $1\,\mu g\,ml^{-1}$ for 6 h and treated with THSG or NS-398 1, 10, $100\,\mu mol\,L^{-1}$ simultaneously for 2 h before exogenous AA as substrate was added. After 30 min, the supernatant was removed and the amount of PGE_2 production was analysed. Mean ± SD, $n = 3$. $^{\#}P = 0.05$ compared with LPS(−) group; $*P = 0.05$, $**P = 0.01$ compared with LPS(+) group.

In the present paper, our results first demonstrated that THSG possessed acute anti-inflammatory activity *in vivo* against dimethylbenzene and CGN in mouse and rat inflammatory models, although the potency of anti-inflammatory activity by THSG was weaker than that of Indomethacin. These results are consistent with those demonstrating that the root extract of *P. multiflorum* Thunb exerts anti-inflammatory effects in whole animal models [4] and that *P. multiflorum* Thunb has a history of use in the treatment of rheumatic arthralgia [8].

CGN-induced paw oedema as an *in vivo* model of inflammation has been frequently used to assess the anti-oedematous effect of natural products [9]. The process of CGN-induced rat paw oedema consists of two phases and the inflammation mediators mainly include histamine (phase 1) and PGs (phase 2). The latter is sensitive to most clinically effective anti-inflammatory drugs [10], such as brucine and brucine *N*-oxide [9]. In the present study, we prove that THSG could obviously inhibit CGN-induced rat paw oedema, suggesting it might be related to the inhibition of prostaglandin synthesis.

THSG degraded dimethylbenzene-induced ear oedema, which indicates that other mechanisms such as capillary vessel oedema, except for the inhibition of PGs, could be involved in the inflammation modulation of THSG.

Many studies have shown that lipopolysaccharide (LPS) increases the production of PGE_2 and induces COX-2 expression in RAW264.7 cells [11]. Our results also demonstrated that LPS could increase PGE_2 production and induce COX-2 expression and activity. In addition, THSG dose-dependently reduced the PGE_2 production from endogenous and exogenous AA in LPS-activated RAW 264.7 cells. These results provide further support that the anti-inflammatory activities of THSG are probably due to the inhibition of the release or synthesis of PGE_2.

To obtain more clearly inhibitory mechanisms of PGE_2 production, the effects of THSG on COX-2 protein and mRNA expression and enzyme activity were investigated. Results showed that THSG significantly inhibited LPS-induced COX-2 protein and mRNA expression but without an obvious effect on COX-1. These results suggest that the inhibition of PGE_2 production by THSG is closely related to the suppression of COX-2 gene expression. In addition, THSG also directly inhibited COX-2 enzyme activity in intact cells, indicating that the inhibition of LPS-induced PGE_2 production by THSG might be due to either suppression of COX-2 expression or direct inhibition of COX-2 enzyme activity. This result was consistent with that showing inhibition of COX-2 activity by roots of *Polygonum multiflorum* Thunb to 65.3% and 44.3% [12.13]. THSG at the tested concentration did not show any cytotoxicity judged by MTT assay, indicating that the inhibition of PGE_2 production by THSG was not associated with its cytotoxicity.

Recent reports point out that clinical use of selective COX-2 inhibitors (i.e. Celebrex, Celecoxib, Refecoxib) increased the number of cardiovascular events, which may be related to the "misbalance" of PGI_2 (in vascular endothelial cells, COX-2) and TXA_2 (in platelet, COX-1). But THSG possesses an anti-atherosclerotic effect [6] compared with traditional selective COX-2 inhibitor, so extracted compounds from natural products may be more safe; however, many details are still unclear.

In conclusion, our results demonstrated for the first time that THSG possesses an anti-inflammatory effect, suppresses COX-2 gene expression or directly inhibits COX-2 enzyme activity. The findings of the present study can shed light on the pharmacological basis for the clinical application of THSG in inflammatory diseases.

3. Materials and methods

3.1 Drug and reagents

Polygonum multiflorum Thunb was purchased from DeQing county, GuangDong province and identified by Wang XiaoMin, Chief Pharmacist. THSG (chemical purity = 99% by HPLC) [14], a main water-soluble compound, was extracted from *Polygonum multiflorum* Thunb, isolated by conventional methods and provided by the phytochemistry laboratory of the Pharmacological Department, Tongji Medical College. Dulbecco's modified Eagle's medium (DMEM), trypsin and Trizol total RNA kit were purchased from Gibco. Foetal bovine serum was purchased from SanLi Co. (ZheJiang, China). Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) was purchased from Sigma Chemical Co. NS-398 was purchased from Cayman Co. ELISA test kit and AA were purchased from R&D Co. DAB test kit and Western blot kit were purchased from Wuhan Boster Co. All the other reagents were of analytical grade. The sense primer for COX-2 was 5′-AAA TGC TGG TGT GGA AGG TG-3′ and the antisense primer was 5′-GAA GTT CAG CCT GGC AAG TCT-3′ (product size, 269 bp); the sense primer for Cox-1 was 5′-GTG GGC TTC AAC CTT GTC AAC-3′ and the antisense primer was 5′-ATC AGC TCT CAC AAT GCC AAG-3′ (product size, 241 bp); the sense primer for β-actin was 5′-TGG AAT CCT GTG GCA TCC ATG AAAC-3′ and the antisense primer was 5′-TAA AAC GCA GCT CAG TAA CAG TCCG-3′ (product size, 349 bp). All primers were synthesised by Beijing Aoke Co.

3.2 Animals

Kunming mice (18–21 g) and Sprague–Dawley rats (180–210 g) were obtained from the animal laboratory of Tongji Medical College of Huazhong University of Science and Technology. All procedures involving animals were approved by the institutional animal care investigation committee. Animals were kept under the conditions of 20–22°C, 40–60% relative humidity, and 12 h/12 h light/dark cycle, and the mice had access to water and food *ad libitum*.

3.3 In vivo *anti-inflammatory activity*

3.3.1 Dimethylbenzene-induced ear oedema in mice. Kunming mice of both sexes were divided into 5 groups consisting of 10 animals: 3 groups for THSG 2.3, 4.6 and 9.2 mg kg^{-1}, 1 group for Indo 13 mg kg^{-1} and 1 group for saline. Vehicle, THSG, and Indo were administered once a day for 3 consecutive days by oral administration. Dimethylbenzene-induced ear oedema was measured according to previously published procedures [16]. Briefly, dimethylbenzene was topically applied to ears of mice (20 μl per ear). One hour later, the per ear weight using a single hole punch was measured in electronic scale (Panyu Scientific Instrument, China). Percent inhibition was estimated as = [1 − (Drug-treated group)/(Saline-treated group)] × 100%.

3.3.2 CGN-induced rat paw oedema in rats. Sprague–Dawley rats of both sexes were divided into 5 groups and each consisting of 8 animals: 3 groups for THSG 3.2, 6.4 and 12.8 mg kg^{-1}, 1 group for Indo 9 mg kg^{-1} and 1 group for saline. After the last time the test

substance had been administered by oral administration, the rats were injected subcutaneously with 0.1 ml of 1% CGN solution in saline (0.9% NaCl, w/v) into the sub-plantar region of the left hind paw as reference [15]. The swelling of the CGN and its contralateral were measured before and 1, 2, 4 and 6 h after injection of CGN. Saline solution was used for the control group, while Indo served as reference. The difference in footpad thickness between the right and left foot was measured with tape (precision to 0.2 mm). Percent inhibitory effects were estimated as mentioned above.

3.4 Measurement of PGE_2 accumulation by COX-2 in cultured LPS induced RAW264.7 cells

RAW264.7 macrophage cells were maintained in DMEM supplemented with 200 U ml^{-1} penicillin, streptomycin and 10% FBS at 37°C, 5% CO_2 in humidified air. The cells were allowed to adhere for 2 h in the presence of Indo 10 μg ml^{-1} to inactivate endogenous COX in 96-well culture plate, washed three times with media, and then incubated in fresh medium with LPS 1 μg ml^{-1} for 6 h. Test materials were simultaneously added to each well for 2 h. After 8 h incubation, the media were removed and analysed by PGE_2 enzyme immunometric assay (EIA). PGE_2-EIA was performed according to the literature [17] with the minor modifications. The percentage of inhibition was expressed as [1 − (PGE_2 level of sample)/(PGE_2 level of vehicle treated-control)] × 100%.

3.5 COX-2 enzyme activity and cell viability

RAW264.7 cells (1×10^5 cells in a 96-well plate) were pre-treated with Indomethacin 10 μg ml^{-1} for 2 h to inactivate endogenous COX, and then washed with PBS and fed with fresh medium. Induction of COX-2 was achieved with the addition of LPS 1 μg ml^{-1} for 6 h. Cells were washed with PBS three times, then the sample was pre-incubated for 2 h before exogenous substrate AA 10 μmol L^{-1} was added. After 30 min, supernatants were removed and analysed by PGE_2 enzyme immunometric assay (EIA) as mentioned above.

Cell viability was determined by MTT. After the supernatant was removed for PGE_2 determination, cells were incubated at 37°C with MTT (0.5 mg ml^{-1}) for 4 h. The cytotoxicity effects of THSG were evaluated by MTT assay [18]. The percentage of cell viability was derived from the following formula: the percentage of inhibition = $A_{\mathrm{LPS/LPS+THSG}}/A_{\mathrm{control}} \times 100\%$.

3.6 Western blot analysis of COX-2 and COX-1 protein in RAW264.7 cells

The RAW 264.7 cells were seeded in 100-ml culture flasks at the same density and incubated in DMEM with 10% FBS at 37°C. The cells were treated with or without THSG 1, 10, 100 μmol L^{-1} and LPS 1 μg ml^{-1} for 8 h. The total protein solutions were collected and quantified as 20 μg. Then all solutions were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described previously [19]. COX-2 or COX-1 on the membranes was assayed by antigen–antibody reaction according to the instructions of the Western blot test kit. The immunoreactive bands were visualised by a DAB detection kit and quantified by grey degree that was measured by the GeneGenius system (Sygene, USA).

3.7 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of COX-2 and COX-1 mRNA in RAW264.7 cells

RAW264.7 cells (5×10^6 cells in a 10-cm dish) were incubated for 8 h with or without THSG 1, 10, 100 μmol L^{-1} and LPS 1 μg ml^{-1}. After washing with PBS twice, total RNA was isolated from the cell pellet using an RNA isolation kit. The total amount of RNA was determined by absorbance at 260 nm. One microgram (μg) of RNA was reverse transcribed into cDNA and amplified to PCR products as described previously [20]. The PCR amplification was performed under the following conditions: a first cycle at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C, 45 s for extension at 72°C for 45 s, and last extension at 72°C for 10 min, using a thermal cycle (Hybaid Thermal Cycle, USA). The amplified PCR products were run on a 1.6% agarose gel and visualised by the GeneGenius bioimaging system. The results were expressed as $A_{\text{COX-2/COX-1}}/A_{\beta\text{-actin}}$.

3.8 Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analysed using the Student *t*-test. In all cases, differences were considered significant at $P < 0.05$.

References

[1] K. Seibert, Y. Zhang, K. Leahy, S. Hauser, J. Masferrer, W. Perkins, L. Lee, P. Isakson. *Proc. Natl. Acad. Sci. USA*, **91**, 12013 (1994).
[2] Y.L. Lin, J.K. Lin. *Mol. Pharmacol.*, **52**, 465 (1997).
[3] M.M. Chan, H.I. Huang, M.R. Fenton, D. Fong. *Biochem. Pharmacol.*, **55**, 1955 (1998).
[4] J.S. Lu, D.S. Meng, M.F. Xiang. *Chin. Pharm.*, **12**, 712 (2001).
[5] G. Ryu, J.H. Ju, Y.J. Park, S.Y. Ryu, B.W. Choi, B.H. lee. *Arch. Pharm. Res.*, **25**, 636 (2002).
[6] Q.L. Liu, J.H. Xiao, R. Ma, Y. Ban, J.L. Wang. *J. Asian Nat. Prod. Res.*, (2006) in press.
[7] H.C. Liu, W.S. Chen. *J. Pharm. Pract.*, **18**, 232 (2000).
[8] G.S. Tu, Q.Z. Fang, S.C. Yuan. *Pharmacopoeia of the People's Republic of China*, (English edition), pp. 18–21, Gaungdong Science and Technology Press, Guangzhou, China (1992).
[9] C.H. Hong, S.K. Hur, O.J. Oh, S.S. Kim, K.A. Nam, S.K. Lee. *J. Ethnopharmacol.*, **83**, 153 (2002).
[10] J.S. Mossa, S. Rafatullah, A.M. Galal, M.A. Al-Yahya. *Int. J. Pharm.*, **33**, 242 (1995).
[11] E. Uhlmann, A. Ryte, A. Peyman. *Antisense Nucleic Acid Drug Dev.*, **7**, 3545 (1997).
[12] W. Yin, T.S. Wang, F.Z. Yin, B.C. Cai. *J. Ethnopharmacol.*, **88**, 205 (2003).
[13] W. Rachel, G. Li, L. David, S.P. Myers, D.N. Leach. *J. Ethnopharmacol.*, **85**, 61 (2003).
[14] Y. Ban, Q.L. Liu, Y. Jin, J.L. Wang. *Chin. Tradit. Herb. Drugs*, **35**, 1340 (2004).
[15] C.A. Winter, E.A. Risley, G.W. Nuss. *Proc. Soc. Exp. Biol. Med.*, **111**, 544 (1962).
[16] X.Y. Zhu. In: J.T. Zhang ed. *Modern Experimental methods in pharmacology. Beijing: The Union Press of Beijing Medical University and Chinese Academy of Medical Sciences, Peking Union College*, pp. 1382–1383 (1998).
[17] P. Pradelles, J. Grassi, J. Maclouf. *Anal. Chem.*, **57**, 1170 (1985).
[18] T. Mosmann. *J. Immunol. Methods*, **65**, 55 (1983).
[19] D.E. Smith, P.A. Fisher. *J. Cell Biol.*, **99**, 20 (1984).
[20] J. Sambrook, E.F. Fritsch, T. Maniatis. *Molecular Cloning: A Laboratory Manual*, 2nd ed., pp. 679–684, Cold Spring Harbor Laboratory Press (1989).