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Antinociceptive activity of Paederosidic Acid Methyl Ester (PAME) from the n-butanol fraction of Paederia scandens in mice

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ABSTRACT

Antinociceptive activity of Paederosidic Acid Methyl Ester (PAME), a chemical compound isolated from the n-butanol fraction of *Paederia scandens*, was evaluated in mice using chemical and thermal models of nociception. PAME given by intraperitoneal injection at doses of 20, 40 and 60 mg/kg produced significant inhibitions on chemical nociception induced by intraperitoneal acetic acid, subplantar formalin or capsaicin injections and on thermal nociception in the tail-flick test and the hot plate test. In the pentobarbital sodium-induced sleep time test and the open-field test, PAME neither significantly enhanced the pentobarbital sodium-induced sleep time nor impaired the motor performance, indicating that the observed antinociceptive activity of PAME was unlikely due to sedation or motor abnormality. Core body temperature measurement showed that PAME did not affect temperature within a 2-h period. Moreover, PAME-induced antinociception in the hot plate test was insensitive to naloxone or nimodipine but significantly antagonized by L-NAME (N (G)-nitro-L-arginine methyl ester), methylene blue and glibenclamide. These results suggested that PAME-produced antinociception was possibly related to the pathway of NO-cGMP-ATP sensitive K^+ channels, which merited further studies regarding the precise site and mechanisms of action.
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1. Introduction

Paederia scandens, a climbing plant belonging to the family Rubiaceae, is popularly known as "Ji Shi Teng" in China. It is also widely grown in India, Japan, the Philippines and the USA (Dang et al., 2002). It has been traditionally used in folk medicine and food in Southeast Asia for thousands of years (Kadota, 2000). The root, leaf, bark and fruit of *P. scandens* have long been used in India, China and Vietnam to treat toothache, chest pain, piles, inflammation of the spleen, rheumatic arthritis and bacillary dysentery (Kapadia et al., 1996; Tran, 1987). The leaves of this plant are also used as an ingredient in various foods in Vietnam (Kadota, 2000).

Recently, it has been reported that the iridoid glycosides and the dimeric iridoid glycosides paederoside, asperuloside, paederosidic acid, deacetylasperuloside, scandoside (Dang et al., 2002; Inouye et al., 1969a,b,c, 1988; Kapadia et al., 1979; Shukla et al., 1976) isolated from the MeOH extract from the stem and root of *P. scandens* (Kim et al., 2004; Zuo et al., 2006) have biological activities such as anti-viral, anti-tumor, anti-inflammatory and anti-microbial activities (Kapadia et al., 1996; Wang and Huang, 2005). Although the iridoid glycoside paederosidic acid methyl ester (PAME) was first isolated from the roots of the Vietnamese *P. scandens* in 2002 (Dang et al., 2002), there has been no report on the antinociceptive activity of this chemical compound and its mechanisms of analgesic activity thus far. Furthermore, a larger quantity of PAME has been isolated from the n-butanol fraction of *P. scandens* which has shown significant antinociceptive activity (Chen et al., 2007).

Based on the investigation above, we studied the analgesic activity of paederosidic acid methyl ester and found that the chemical compound had a powerful antinociceptive activity in preliminary experiments. During the present study, we further examined the effects of PAME on nociception model in mice that were induced by both chemical and thermal stimuli so as to elucidate the analgesic activity and mechanism of this compound, and provide a scientific basis for the clinical use of PAME.

2. Materials and methods

2.1. Plant material

P. scandens was collected in the E' mei Mountain in Sichuan Province in July, 2005. The plant was identified at the School of Pharmacy of the Second Military Medical University (Shanghai, China). A voucher specimen of *P. scandens* (#107) was deposited at the Herbarium of the Department of Pharmacognosy of the said school.

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2.2. Animals

Experimental groups consisted of 10 ICR mice (18–22 g) per group. They were housed at 21 ± 1 °C under a 12 h light/12 h dark cycle and had free access to a standard pellet diet (Purina Chow) and tap water. The animals were deprived of food for 15 h before the experiment, with free access to drinking water. Each animal was used only once in the experiment. The experimental protocols were approved by the Animal Care and Use Committee of the Second Military Medical University and complied with the recommendations of International Association for the Study of Pain (Zimmermann, 1983).

2.3. Drugs and chemicals

The following reagents and drugs were used: MeOH (AR or HPLC), $CHCL_3$ (AR), silica-gel, octadecyl silane (ODS), n-butanol (AR), formalin (AR) and acetic acid (AR) [Sinopharm chemical reagent Co., Ltd, China], morphine hydrochloride, acetyl-salicylic acid, pentobarbital sodium (Sihuan Pharmaceutical Factory, Beijing, China), diazepam, naloxone, glibenclamide, capsaicin, nimodipine, methylene blue (Chengdu Pharmaceutical Factory, Chengdu, China), L-arginine, L-NAME (Sigma, USA).

Morphine hydrochloride, nimodipine, methylene blue, L-arginine, L-NAME, acetyl salicylic acid, glibenclamide, diazepam, pentobarbital sodium and naloxone were dissolved in physiological saline (0.9% NaCl). Capsaicin was dissolved in 1% ethanol and 1% Tween 80 in saline (1:1:8). The vehicles used alone had no effect on nociceptive responses in the mice.

2.4. Sample preparation

Dried powder of the whole *P. scandens* plant (2 kg) was extracted with 85% EtOH using the Soxhlet apparatus at 80 °C. The EtOH extract was concentrated under reduced pressure to obtain a residue (588.8 g). The EtOH extract was subsequently extracted with petroleum ether, chloroform, and n-butanol, respectively. The n-butanol fraction was also concentrated under reduced pressure to obtain a residue (260 g).

The n-butanol fraction (128 g) was eluted through Gel silica H with $CHCL_3$: CH_3OH: H_2O (9:1:0.1) to obtain the sub-fraction (7.2 g). PAME (2.6 g) was obtained after the sub-fraction was eluted through the ODS with CH_3OH: H_2O (30%–70%) and then prepared using C18 reversed-phase column HPLC (Agilent liquid chromatography 1100 series with RID and DAD detectors using a preparative column-YMC ODS-A, 250 × 10 mm, S-5 μm 12 nm) with MeOH: H_2O (60: 40) as solvent (flow rate1~1.5 ml/min).

2.5. Data analysis of PAME

The chemical compound was identified separately by the methods of ^{1}H-NMR, ^{13}C-NMR as PAME (Fig. 1) (Dang et al., 2002). The spectrum data and chemical structure of the chemical compound are as follows:

White needle crystal. $[\alpha]^{20}_D$: +12.4° (c = 0.90, MeOH); ESI-MS m/z: 501.1144 [M + Na^+]; UV (MeOH): 234 (4.16); IR (KBr): 3374, 2929, 1700, 1633, 1440, 1308, 1159, 1077, 898; ^{1}H-NMR (600 MHz, CD_3OD): 5.06 (d, J = 8.5 Hz, H-1), 7.65 (d, J = 1.1 Hz, H-3), 3.03 (ddd, J = 7.4, 6.0, 1.1 Hz, H-5), 4.80 (ddd, J = 6.0, 2.5, 0.8 Hz, H-6), 6.02 (d, J = 1.7 Hz, H-7), 2.62 (dd, J = 8.5, 7.4 Hz, H-9), 4.95 (br d, J = 14.6 Hz, H_a-10), 5.10 (dd, J = 14.6, 1.3 Hz, H_b-10), 3.74 (s, -MeO-11), 2.34 (s, -SMe-12), 4.72 (d, J = 8.0 Hz, H-1′), 3.24 (dd, J = 9.1, 8.0 Hz, H-2′), 3.38 (dd, J = 9.1, 8.8 Hz, H-3′), 3.26 (dd, J = 9.3, 8.8 Hz, H-4′), 3.27 (m, H-5′), 3.63 (dd, J = 11.8, 1.9 Hz, H-6′), 3.85 (dd, J = 11.8, 6.0 Hz, H-6′); ^{13}C-NMR (150 MHz, CD_3OD): 101.3 (C-1), 155.4 (C-3), 108.1 (C-4), 42.4 (C-5), 75.3 (C-6), 132.4 (C-7), 145.5 (C-8), 46.2 (C-9), 66.2 (C-10), 172.9 (C-11), 169.3 (C-12), 51.9 (-OMe), 13.5 (-SMe), 100.7 (C-1′), 74.9 (C-2′), 77.9 (C-3′), 71.6 (C-4′), 78.6 (C-5′), 63.0 (C-6′).

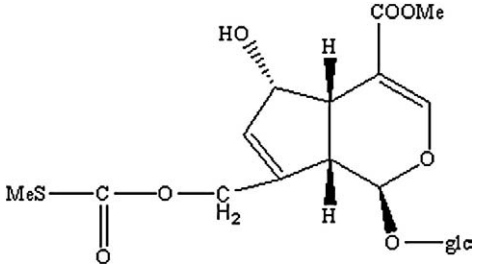

Fig. 1. Paederosidic acid methyl ester.

2.6. Protocols

Antinociceptive activity of PAME was evaluated on the chemical nociception in the test models of acetic acid-induced writhing, capsaicin and formalin-induced hind paw licking, and on the thermal nociception in the hot plate test and the tail-flick test. Conscious (un-anesthetized) mice were used in all nocifensive tests. Dosage of the positive control was determined on the basis of the principle of pharmacokinetics and clinical use. PAME was administered intraperitoneally. The dose selection of 5, 10, 20, 40 and 60 mg/kg was based on the results of preliminary experiments. Control groups were treated with an equivalent volume of the vehicle that had been used to dilute this chemical compound.

2.7. Abdominal constriction induced by acetic acid

In the acetic acid-induced writhing test (García et al., 2004), groups of mice (n = 10) fasted overnight were treated with PAME and the vehicle 30 min before administration of acetic acid (0.7%, 10 ml/kg, i.p.). Acetylsalicylic acid was administered orally at the dose of 200 mg/kg 1 h before the experiment as positive control. The number of writhings was counted for each animal, starting 3 min after acetic acid injection over a period of 12 min.

2.8. Formalin test

In the formalin test (Santos and Calixto, 1997), groups of mice were treated as above with PAME or the vehicle. 30 min later each mouse was given 20 μl of 5% formalin (in 0.9% saline, subplantar) into the right hind-paw. The duration of paw licking as an index of painful response was determined at 0–5 min (early phase, neurogenic) and 15–25 min (late phase, inflammatory) after formalin injection. Morphine administered subcutaneously 30 min before the test at the dose of 10 mg/kg was used as positive control.

2.9. Capsaicin test

In the capsaicin test (Goncales et al., 2005), mice were pretreated with PAME or the vehicle 30 min before subplantar injection of capsaicin (1.6 μg, 20 μl) into the right hind paws. The animal group receiving morphine (10 mg/kg s.c.) 30 min before the test was used as positive control. The amount of times each mouse spent licking the injected paw was recorded over the first 5 min period.

2.10. Tail-flick test

In the tail-flick test (Sánchez-Mateo et al., 2006), an apparatus consisting of a circulating immersion water heater was used. The thermostat was adjusted so that a constant temperature of 54 ± 1 °C was maintained in the water bath. Before treatment, the rear 3.5 cm of each mouse's tail was immersed into the water bath and the time taken to flick the tail was recorded in seconds.

In this thermal test, pre-treatment latencies were determined three times with 15-min intervals. Only mice showing a pretreatment reaction less than or equal to 3 s were selected for the study. Immediately after the basal latency assessment, mice were pre-

treated with PAME or the vehicle 30 min before the measurement. A morphine (10 mg/kg s.c. 30 min before the test) treated animal group was included as positive control. The cut-off time was 7 s in the tail-flick measurement in order to minimize tissue injury. The antinociceptive activity of PAME was observed within 2 h.

2.11. Hot plate test

The hot plate test (Franzotti et al., 2000) was carried out on groups of female mice using a hot plate apparatus (model YLS-6B, China) maintained at 55 ± 1 °C. Only mice that showed initial nociceptive responses between 5 and 30 s were selected for the experiment. The latency to the first sign of hind paw licking or jumping to avoid heat nociception was taken as an index of nociceptive threshold. In this test, pre-treatment latencies were determined three times with intervals of 20-min intervals. Measurement was started 30 min after pretreatment of the animals with PAME or the vehicle. A group of animals treated with morphine (10 mg/kg s.c. 30 min before the test) was used as positive control. The cut-off time was 60 s in the hot plate test in order to minimize skin damage. The antinociceptive activity of PAME was observed within 2 h.

In order to assess the possible involvement of endogenous opioids, ATP sensitive K^+ channels, endogenous NO, endogenous guanylyl cyclase and/or L-type Ca^{2+} channels in the antinociceptive activity, a high dose of PAME was examined in groups of mice pretreated with naloxone, glibenclamide, L-NAME, L-arginine, methyl blue or nimodipine. Naloxone, glibenclamide, L-NAME, methyl blue and nimodipine were administered 30 min before administration of PAME, morphine, diazoxide or L-arginine, respectively.

2.12. Pentobarbital sodium-induced sleep time

In this test (Santos et al., 2005), groups of mice ($n = 10$) were treated with PAME and the vehicle 30 min before the injection of sodium pentobarbitone. Diazepam was used as positive control. The time between losing and regaining righting reflex was considered as the duration of sleep time in seconds.

2.13. Open-field test

The effect of PAME on spontaneous locomotor activity and exploratory behavior was assessed by the open-field test (Tsuda et al., 1996). The photoelectrical spontaneous locomotor activity apparatus (model ZZ-6, China) was a round arena (34 cm in diameter) with the floor divided into 21 equal areas. Immediately after evaluation, each animal was transferred to the apparatus and observed for 5 min. The number of rearing responses, the number of areas crossed by all paws, and the total time spent immobilized (immobility) were recorded. 30 min before the test, groups of mice were pre-treated with PAME (20, 40 and 60 mg/kg, i.p.) or the vehicle. A diazepam (1.0 mg/kg, i.p.) treated animal group was included as positive control.

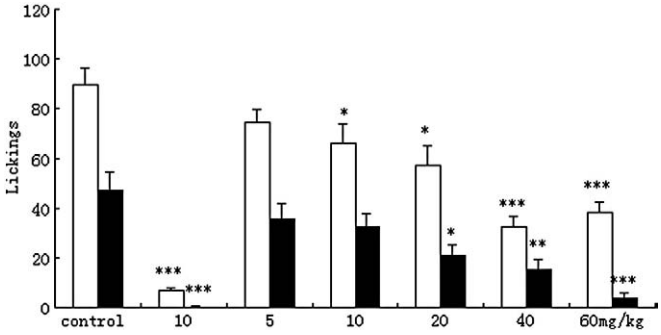

Fig. 3. The figure showed the effects of PAME and morphine (Morph) on formalin-induced nociception in mice. The total time spent in licking the injected hind-paw was measured in the early phase (0–5 min, white column) and the late phase (15–25 min, black column). The vehicle (Control, 10 ml/kg) or the compound (5, 10, 20, 40 and 60 mg/kg) was administered abdominally and morphine (10 mg/kg) subcutaneously. PAME or morphine was administered 30 min before the test. Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (ANOVA followed by Dunnett's test).

2.14. Measurement of core body temperature

Core body temperature was measured in a separate group of mice 30 min after injection of 60 mg/kg PAME or the vehicle. Mice were restrained, and a lubricated thermistor probe was inserted 3 cm into the rectum for 10–20 s to stabilize the temperature reading on the attached analog thermometer (YSI Model 432 A). Once stabilized, core body temperature to the nearest 1 °C was recorded. The effect of PAME on core body temperature was observed within 2 h.

2.15. Statistical analysis

All data were expressed as the mean ± S.E.M. Data were subjected to ANOVA followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effect of PAME on abdominal constriction induced by acetic acid

In the acetic acid-induced writhing test, treatment with PAME at the dose of 20, 40 and 60 mg/kg decreased significantly the mean

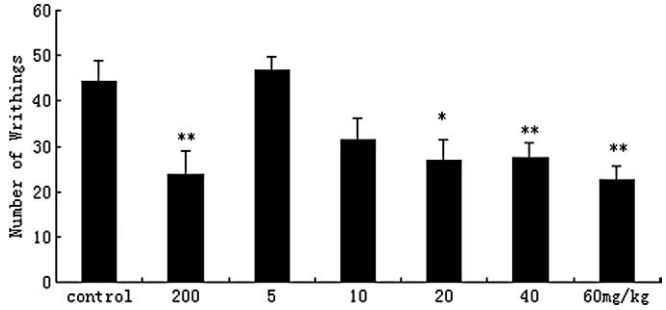

Fig. 2. Effects of the intraperitoneally injected PAME and the orally administered the acetyl salicylic acid (ASA) on acetic acid-induced writhing in mice. The vehicle (Control, 10 ml/kg, i.p.) and the chemical compound (5, 10, 20, 40, and 60 mg/kg, i.p) were administered, 30 min before the intraperitoneal administration of acetic acid (0.7%, 10 ml/kg) or ASA (200 mg/kg, o.p.) was administered orally, 1 h before the test and the number of writhes were counted over a period of 12 min. Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$ (ANOVA followed by Dunnett's test).

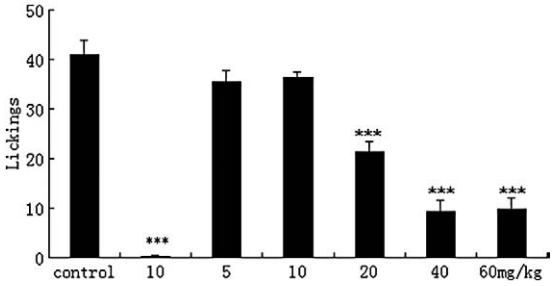

Fig. 4. The figure showed the effects of PAME and morphine on capsaicin-induced paw licking response in mice. The vehicle (Control, 10 ml/kg) or the compound (5, 10, 20, 40 and 60 mg/kg) was administered abdominally and morphine (10 mg/kg) subcutaneously. PAME or morphine was administered 30 min before the subplantar injection of capsaicin (1.6 μg, 20 μl) into the hind paw and the time in seconds (s) the animal licks the injected paw was noted over a period of 5 min. Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (ANOVA followed by Dunnett's test).

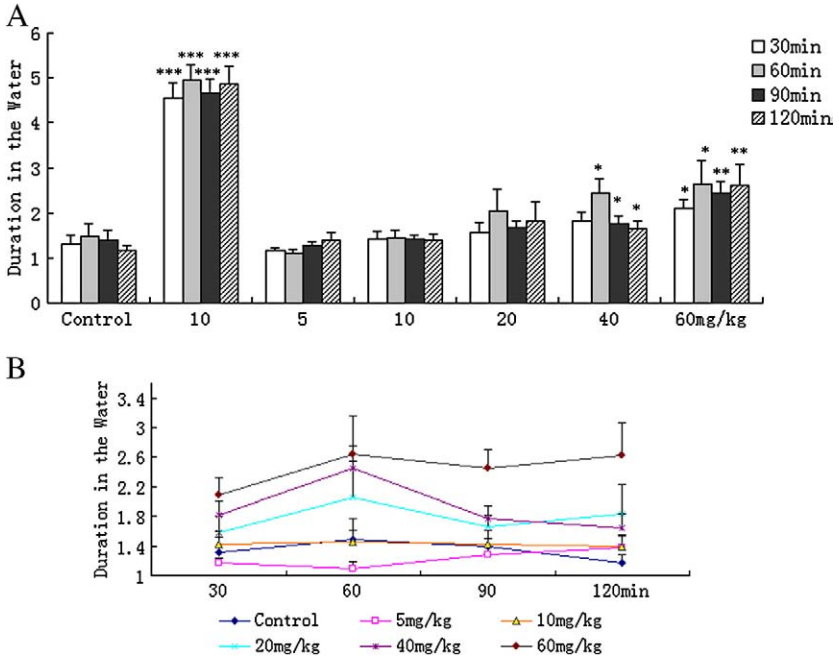

Fig. 5. Effects of PAME and morphine on thermal-induced anti-nociception within 2 h in the tail-flick test. The vehicle (Control, 10 ml/kg) or the compound (5, 10, 20, 40 and 60 mg/kg) was administered intraperitonealy and morphine (10 mg/kg) subcutaneously. PAME or morphine was administered 30 min before the test and the time in seconds taken to flick the tail was recorded. Cut-off time was 7 s. Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA followed by Dunnett's test).

number of writhes (Fig. 2). The ED_{50} was 39.07 mg/kg. The acetylsalicylic acid (200 mg/kg, o.p.) also manifested a significantly diminished the number of writhes (23.90 ± 5.07 s).

3.2. Effect of PAME on the formalin test

In the formalin test, pretreatment with PAME caused significant reduction of both the first-phase and the second-phase pain responses at the tested doses of 20, 40 and 60 mg/kg (Fig. 3). Moreover, ED_{50} was 32.39 mg/kg and 14.78 mg/kg in first phase and second phase, respectively. Morphine (10 mg/kg) also suppressed the formalin-response in both phases (first-phase, 6.67 ± 1.26 and second-phase, 0.17 ± 0.17 s).

3.3. Effect of PAME on the capsaicin test

The effects of PAME and morphine against capsaicin-induced nociception in mice were shown in Fig. 4. When compared with vehicle-treated control, a dose-dependent reduction in the duration of paw licking was observed in mice pretreated with PAME at the doses of 20, 40 and 60 mg/kg. Moreover, ED_{50} was 23.74 mg/kg. Morphine also caused significant antinociception (0.33 ± 0.21 s).

Table 1
Effects of the paederosidic acid methyl ester and morphine on thermal-induced anti-nociception in the tail-immersion test ($n = 10$).

Group	Dose (mg/kg)	Duration in the water(s) (mean ± S.E.M.)			
		30 min	60 min	90 min	120 min
Vehicle	–	1.31 ± 0.21	1.49 ± 0.29	1.4 ± 0.21	1.17 ± 0.11
Morphine	10	4.55 ± 0.33***	4.95 ± 0.35***	4.67 ± 0.32***	4.86 ± 0.41***
The paederosidic acid methyl ester	5	1.18 ± 0.06	1.1 ± 0.09	1.28 ± 0.09	1.38 ± 0.17
	10	1.42 ± 0.17	1.45 ± 0.16	1.42 ± 0.09	1.39 ± 0.15
	20	1.58 ± 0.23	2.05 ± 0.49	1.67 ± 0.15	1.83 ± 0.41
	40	1.82 ± 0.19	2.45 ± 0.3*	1.78 ± 0.17*	1.65 ± 0.19*
	60	2.09 ± 0.22*	2.64 ± 0.53*	2.46 ± 0.24**	2.63 ± 0.43**

Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control (ANOVA followed by Dunnett's test).

Table 2
Effects of the paederosidic acid methyl ester and morphine on thermal-induced anti-nociception in the hot plate test ($n = 10$).

Group	Dose (mg/kg)	Duration on the hot-plate(s) (mean ± S.E.M.)			
		30 min	60 min	90 min	120 min
Vehicle	–	14.44 ± 1.18	14.00 ± 2.46	13.67 ± 1.14	13.33 ± 1.47
Morphine	10	56.22 ± 2.07***	58.33 ± 1.67***	55.67 ± 2.39***	51.33 ± 4.17***
The paederosidic acid methyl ester	5	19.25 ± 2.21	18.14 ± 4.40	17.50 ± 1.78	15.38 ± 1.46
	10	19.67 ± 2.78	17.13 ± 1.77	18.44 ± 2.08	17.56 ± 1.68
	20	24.50 ± 3.46*	21.50 ± 2.19*	21.00 ± 2.26*	29.13 ± 6.84*
	40	23.00 ± 2.65**	22.13 ± 1.46*	22.63 ± 1.58***	30.88 ± 3.49***
	60	22.00 ± 2.40*	22.00 ± 2.23*	22.13 ± 2.11**	26.50 ± 2.11***

Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control (ANOVA followed by Dunnett's test).

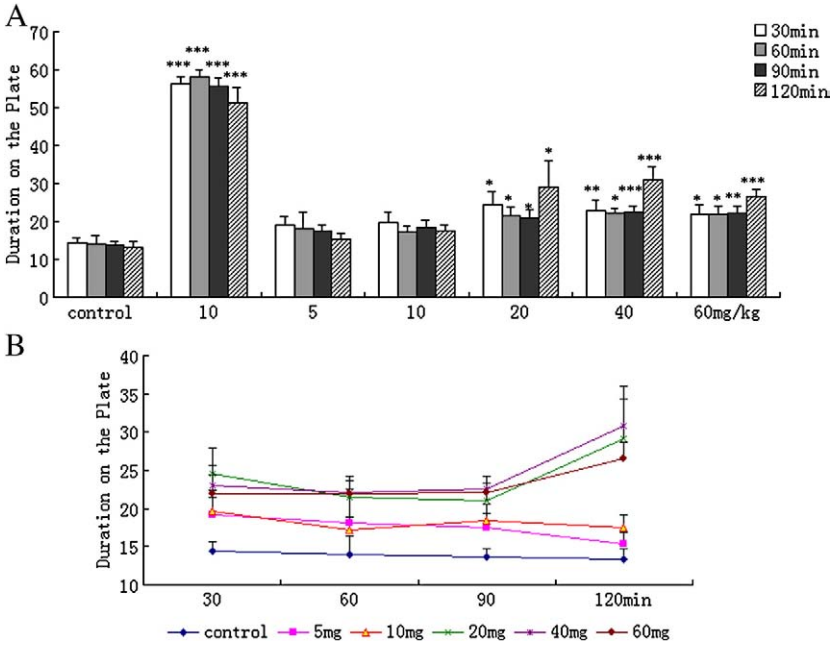

Fig. 6. Effects of PAME and morphine on thermal-induced anti-nociception within 2 h in the hot plate test. The vehicle (Control, 10 ml/kg) or the compound (5, 10, 20, 40 and 60 mg/kg) was administered intraperitonealy and morphine (10 mg/kg) subcutaneously. PAME or morphine was administered 30 min before the test and the time in seconds (s) of first sign of hind paw licking or jump response to avoid heat nociception was recorded. Cut-off time was 60 s. Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (ANOVA followed by Dunnett's test).

3.4. Effect of PAME on the tail-flick test

In the tail-flick test, PAME showed a significant effect on the duration of the mouse's tail in hot water, when compared with vehicle-treated control group (Fig. 5A, B). The mean durations in the groups of PAME dose (5, 10, 20, 40 and 60 mg/kg) and the vehicle-treated group were shown within 2 h (Table 1). Moreover, the positive control group treated with morphine (10 mg/kg) also exhibited the powerful antinociceptive activity.

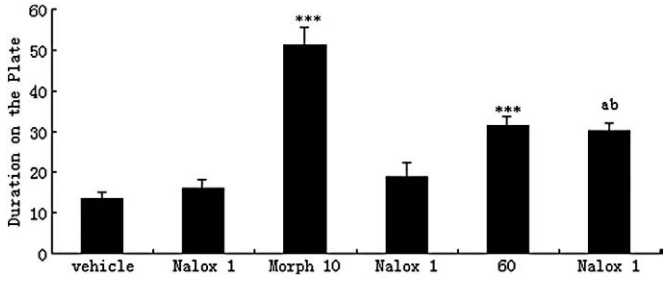

Fig. 7. The effects of naloxone on PAME and morphine anti-nociception were shown in the hot plate test. Naloxone (Nalox, 1 mg/kg, s.c.) was administered 30 min before PAME (60 mg/kg, i.p.) or morphine (10 mg/kg, s.c.). Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control, [a]$P<0.01$ vs. morphine + nalox, [b]$P<0.001$ vs. vehicle + nalox (ANOVA followed by Dunnett's test).

3.5. Effect of PAME on the hot plate test

In the hot plate test, the mean durations in the groups of the PAME dose (5, 10, 20, 40 and 60 mg/kg), the positive control group (morphine, 10 mg/kg) and control group within 2 h were shown in Table 2. The result showed that the groups treated with PAME and morphine both had powerful antinociceptive effects (Fig. 6A, B).

3.6. Mechanisms of PAME in the hot plate test

When used alone, naloxone (1 mg/kg, s.c.), a non-selective opioid receptor antagonist, L-NAME (20 mg/kg, i.p.), NO synthesis inhibitors; methylene blue (10 mg/kg, i.p.), guanylyl cyclase inhibitors; glibenclamide (2 mg/kg, i.p.), a blocker of ATP sensitive K^+ channels, and nimodipine (1 mg/kg, i.p.), a blocker of L-type Ca^{2+} channels, failed to

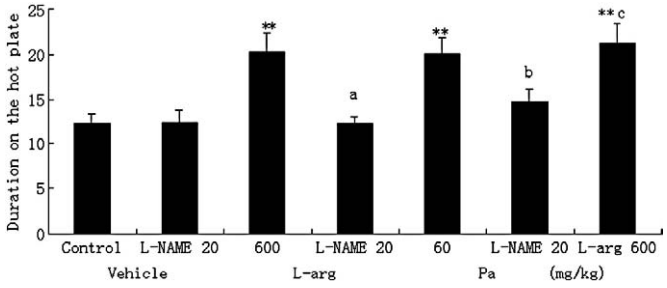

Fig. 8. The effects of L-NAME on PAME and L-arginine anti-nociception were shown in the hot plate test. L-NAME (L-NAME, 20 mg/kg, i.p.) was administered 30 min before the PAME (60 mg/kg, i.p.) or L-arginine (l-arg, 600 mg/kg, i.p.). Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control, [a]$P<0.01$ vs. L-arginine, [b]$P<0.05$ vs. the Paederosidic acid methyl ester, [c]$P<0.05$ vs. the Paederosidic acid methyl ester + L-NAME (ANOVA followed by Dunnett's test).

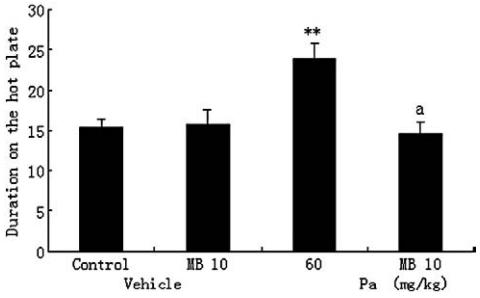

Fig. 9. The effects of methylene blue on PAME anti-nociception were shown in the hot plate test. Methylene blue (MB, 10 mg/kg, i.p.) was administered 30 min before PAME (Pa, 60 mg/kg, i.p.). Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. **$P<0.01$ vs. control, [a]$P<0.01$ vs. the Paederosidic acid methyl ester (ANOVA followed by Dunnett's test).

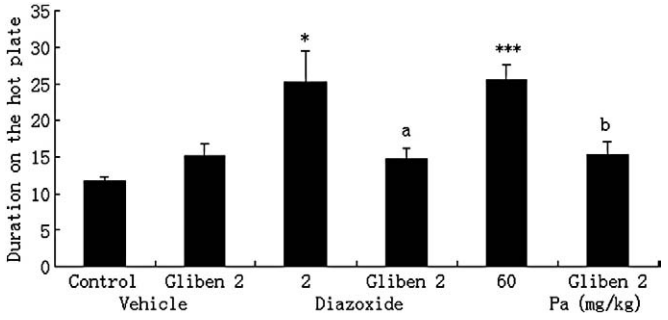

Fig. 10. The effects of glibenclamide on PAME and diazoxide anti-nociception were shown in the hot plate test. Glibenclamide (Gliben, 2 mg/kg, i.p.) was administered 30 min before PAME (Pa, 60 mg/kg, i.p.) or diazoxide (2 mg/kg, i.p.). Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control, [a]$P<0.05$ vs. diazoxide, [b]$P<0.01$ vs. the Paederosidic acid methyl ester (ANOVA followed by Dunnett's test).

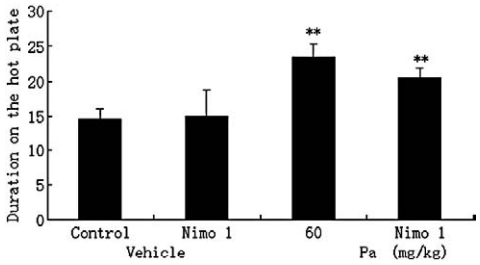

Fig. 11. The effects of nimodipine on PAME anti-nociception were shown in the hot plate test. Nimodipine (Nimo, 1 mg/kg, i.p.) was administered 30 min before PAME (Pa, 60 mg/kg, i.p.). Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. **$P<0.01$ vs. control (ANOVA followed by Dunnett's test).

modify the thermal-induced nociceptive responses in a significant manner (Figs. 7–11). During the naloxone antagonism test, naloxone was able to reverse the antinociceptive activity of morphine significantly, but not the antinociception of PAME in the combination studies. During the L-NAME antagonism test, L-NAME weakened the antinociception of both PAME and L-arginine (600 mg/kg, i.p.), NO synthesis activator. However, the antinocicepive activity was enhanced when PAME was administered in combination with L-arginine. During the methylene blue antagonism test, methylene blue exhibited antagonism to PAME in comparison with the vehicle. During the glibenclamide antagonism test, glibenclamide clearly showed antagonism to both PAME and diazoxide (2 mg/kg, i.p.), ATP sensitive K^+ channels opener, in the combination studies. During the nimodipine antagonism test, nimodipine failed to modify the antinociception of PAME in comparison with the control group.

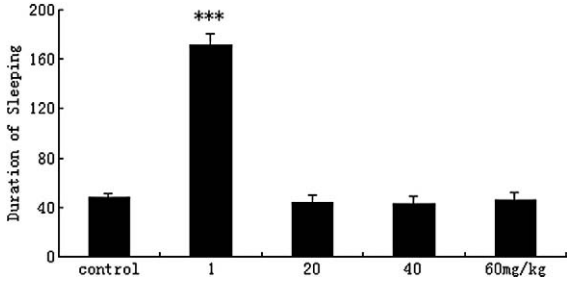

Fig. 12. The effects of PAME and diazepam on pentobarbital sodium-induced sleeping time in mice. The vehicle (Control, 10 ml/kg), the compound (20, 40, and 60 mg/kg) or diazepam (1 mg/kg) was administered intraperitonealy. PAME was administered 30 min before the injection of sodium pentobarbitone (45 mg/kg, i.p.) and the time in seconds (s) the animal slept was noted. Each column represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. ***$P<0.001$ (ANOVA followed by Dunnett's test).

Table 3
Effects of the paederosidic acid methyl ester on the core body temperature in mice ($n = 10$).

Time (min)	Vehicle	Core temperature (°C)	Paederosidic acid methyl ester	Core temperature (°C)
		(mean ± SEM)	Dose (mg/kg)	(mean ± SEM)
0	–	37.13 ± 0.14	60	37.23 ± 0.14
30	–	37.16 ± 0.13	60	37.20 ± 0.13
60	–	37.29 ± 0.15	60	36.95 ± 0.16
90	–	36.98 ± 0.20	60	37.07 ± 0.15
120	–	37.32 ± 0.11	60	37.28 ± 0.06

Asterisks indicated significant difference from control. ***$P<0.001$ vs. control (ANOVA followed by Dunnett's test).

3.7. Effect of PAME on pentobarbital sodium-induced sleep time

The effects of PAME and diazepam on pentobarbital sodium-induced sleep time were as follows: Vehicle-treated control: 48.25 ± 2.77 s; PAME dose (20, 40 and 60 mg/kg): 44.20 ± 5.30, 42.37 ± 6.55 and 46.14 ± 6.17 s, respectively; diazepam: 171.62 ± 9.40 s. Only diazepam produced a significantly prolonged sleep time ($P<0.001$) (Fig. 12).

3.8. Effect of PAME on the open-field test

PAME (20, 40 and 60 mg/kg) did not affect motor coordination in mice. The mean permanence time of animals and the length of the route in the apparatus that were obtained in the fraction-treated groups weren't statistically different from the vehicle-treated control group over a 5-min period. Only diazepam (1 mg/kg, i.p.) significantly affected the mobile performance in comparison with the control group ($P<0.01$).

3.9. Measurement of core body temperature

PAME (60 mg/kg, i.p.) did not influence the core body temperature in mice within 2 h in comparison with the vehicle (Table 3, Fig. 13).

4. Discussion

The acetic acid-induced writhing method is the most useful model in studying antinociceptive activity (Gene et al., 1998). It is able to determine antinociceptive effects of compounds and dose levels that might appear inactive in other methods like the tail-flick test (Bentley et al., 1981). However, it has previously been discovered that constriction induced by acetic acid is considered to be a non-selective antinociceptive model (Collier et al., 1968; Bighetti et al., 1999; Sánchez-Mateo et al., 2006). Our results indicated that PAME could reduce the number of writhings in animal models, implying that it had a powerful antinociceptive effect. However, the results of this writhing

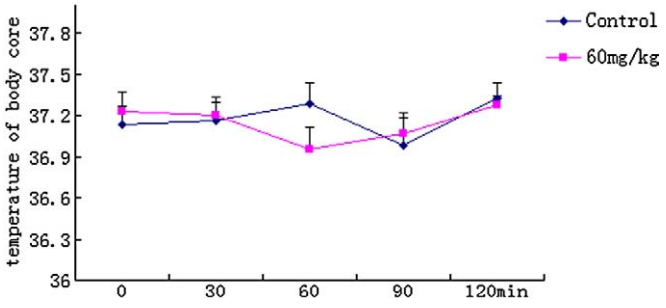

Fig. 13. The effects of PAME on core body temperature in mice within 120 min. The vehicle (Control, 10 ml/kg) and the compound (60 mg/kg) was administered intraperitonealy 30 min before test and the temperature (°C) of mice was noted. Each dot represented the mean ± S.E.M. ($n = 10$). Asterisks indicated significant difference from control. ***$P<0.001$ (ANOVA followed by Dunnett's test).

test alone were unable to ascertain whether the antinociception was central or peripheral effect.

The advantage of the formalin model of nociception is that it can discriminate pain in its central and/or peripheral components. It has been reported that formalin-induced persistent pain in mice paws produced a distinct biphasic nociception (revised by Hunskaar and Hole, 1987; Tjolsen et al., 1992). Central analgesics, such as narcotics, inhibit both phases, while peripherally acting drugs, such as steroids (hydrocortisone, dexamethasone) and NSAIDs suppress mainly the late phase (Hunskaar and Hole, 1987; Trongsakul et al., 2003). In the present test, the duration of the paw licking (s) as an index of painful response was determined at 0–5 min (early phase, neurogenic) and 15–25 min (late phase, inflammatory) after formalin injection. PAME, at more effective doses in the writhing test, reduced the duration of the paw licking (s) notably in both the first phase and the second phase of the formalin test, and exhibited a significant antinociceptive effect. It was reasonable that PAME had the same antinociception as the central analgesic drugs. Furthermore, the significant effect of PAME on tail-flick and hot plate responses in the thermal tests further confirmed that PAME had a central antinociception effect since the tail-flick test and the hot plate test are predominantly a spinal reflex and considered to be selective for centrally-acting analgesic compounds, while peripherally-acting analgesics are known to be inactive on thermal stimuli (Ramabadran et al., 1989; Srinivasan et al., 2003).

The antinociception caused by PAME seemed to be unrelated to motor impairment or sedation since the compound tested in the open-field test and pentobarbital sodium-induced sleep time test showed no significant effect on these mice's behaviors. It was interesting to mention that, at the effective doses, PAME did not significantly change the core body temperature, which might possibly confound the measurements of antinociception in the hot plate and tail-flick test, and even in the formalin test (Mogil et al., 1998).

To assess possible antinociceptive mechanisms, we examined the effect of naloxone (a non-selective opioid receptor antagonist), L-NAME (an NO synthesis inhibitor), methylene blue (a guanylyl cyclase inhibitor), glibenclamide (a blocker of ATP sensitive K^+ channels) and nimodipine (a blocker of L-type Ca^{2+} channels) on the antinociceptive activity of PAME. Interestingly, the data obtained in the hot plate test showed that the antinociception effect produced by PAME was neither naloxone-sensitive nor nimodipine-sensitive, but sensitive to L-NAME, methylene blue and glibenclamide. Since naloxone and nimodipine failed to antagonize the antinociception in hot plate test, the antinociceptive activity of PAME didn't seem to be related to opioid receptor and L-type Ca^{2+} channels, rather it might involve the NO-cGMP-ATP sensitive K^+ channels pathway.

In the present work, central opiate mechanism was first analyzed by testing the effect of s.c. naloxone on PAME-induced antinociception. The dose of naloxone (1 mg/kg, s.c.) used in the experiments was high enough to block opiate receptors, as demonstrated previously in the pain induced-functional impairment model (Yaksh, 1997). In the present study, however, naloxone was completely inactive as an antagonist, thus precluding involvement of the central opiate mechanism in the antinociception of PAME.

It's well known that NO and cyclic guanosine monophosphate (cGMP) are involved in antinociception (Asomoza-Espinosa et al., 2001; Jain et al., 2001, 2003; Patil et al., 2003). NO is an endogenous activator of guanylyl cyclase, and causes intracellular cGMP accumulation (Deguchi, 1977; Beavo, 1995; Pyne et al., 1996). Several lines of evidence have shown that the NO-cGMP signaling pathway plays an antinociceptive role. In addition, the antinociceptive effects of L-arginine and the NO donor can be blocked by the guanylyl cyclase inhibitor (Duarte et al., 1990; Ferreira et al., 1991; Jain et al., 2001, 2003; Patil et al., 2003). In our research, antinociception of PAME could be reversed by L-NAME (a NO synthesis inhibitor) and methylene blue (a guanylyl cyclase inhibitor), but was potentiated when used in combination with L-arginine. These results supported the theory that antinociceptive activity of PAME might be related to the NO-cGMP signaling pathway. We therefore hypothesized that PAME might synthesize NO or indirectly increase the intracellular NO concentration once it contacted the cell membrane. Moreover, it has been also suggested that the opening of the potassium channels is a consequence of increased cGMP via activation of the L-arginine-NO-cGMP pathway (Lazaro-Ibanez et al., 2001; Ortiz et al., 2003). However, glibenclamide, a potassium channel blocker, can reduce the antinociceptive effect of the NO donor (Soares et al., 2000).

Other studies in the literature indicate that the NO-cyclic GMP pathway can have pro-nociceptive rather than antinociceptive effects (Alley et al., 1998). This discrepancy may be due to the different experimental pain models used, diverse tissue levels and the variant NO and cGMP intracellular contents (Kawabata et al., 1994; Pehl and Schmid, 1997; Tegeder et al., 2002). Our results showed that PAME was able to activate the NO-cGMP-ATP sensitive K^+ channel pathway, in turn, produced antinociception. Therefore, the exact mechanism by which PAME activated the NO-cyclic GMP-ATP sensitive K^+ channel pathway remains to be further elucidated.

Nimodipine, an L-type Ca^{2+} channels inhibitor, had no effect on the antinociception of PAME, suggesting that there was a difference between the mechanism of antinociception of PAME and the n-butanol fraction of *Paderia scandens*, which was done previously (Chen et al., 2007). We hypothesized that the antinociception of n-butanol fraction might have a synergetic effect by the five iridoid glycosides: asperuloside, paederoside, scandoside, paederosidic acid and paederosidic acid methyl ester, which comprised a greater part of this fraction.

In conclusion, the present study demonstrated the antinociceptive activity of PAME in the test models of chemical nociception induced by acetic acid, capsaicin and formalin, as well as in the test model of nociception induced by thermal stimuli, and further suggested that antinociceptive activity of PAME might be related to the involvement of the NO-cGMP-ATP sensitive K^+ channel pathway, which merited further studies regarding the precise site and the mechanism of action.

Acknowledgement

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References

Alley KO, Mccarter G, Levine JD. Nitric oxide signaling in pain and nociceptor sensitization in the rat. J Neurosci 1998;18:7008–14.
Asomoza-Espinosa R, Alonso-Lopez R, Mixcoatl-Zecuatl T, Aguirre-Banuelos P, Torres-Lopez JE, Granados-Soto V. Sildenafil increases diclofenac antinociception in the formalin test. Eur J Pharmacol 2001;418:195–200.
Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 1995;75:725–48.
Bentley GA, Newton SH, Starr J. Evidence for an action of morphine and the enkephalins on sensory nerve endings in the mouse peritoneum. Br J Pharmacol 1981;73:325–32.
Bighetti EJB, Hiruma-Lima CA, Gracioso JS, Souza Brito ARM. Anti-inflammatory and antinociceptive effects in rodents of the essential oil of Croton cajucara Benth. J Pharm Pharmacol 1999;51:1447–53.
Chen YF, Zhang H, Zhang QY, Wu JZ, Li N, Rahman K, Zheng HC, Qin LP. Antinociceptive activity of n-butanol fraction from MeOH extracts of *Paederia scandens* in mice. Pharmazie 2007;62(12):943–8.
Collier HOJ, Dinneen LC, Johnson CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br J Pharmacol 1968;32:295–310.
Dang NQ, Toshihiro H, Masami T, Nguyen XD, Yoshinori A. Iridoid glucosides from roots of Vietnamese *Paederia scandens*. Phytochemistry 2002;60:505–14.
Deguchi T. Endogenous activating factor for guanylate cyclase in synaptosomal-soluble fraction of rat brain. J Biol Chem 1977;252:7617–9.
Duarte I, Lorenzetti B, Ferreira S. Peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. Eur J Pharmacol 1990;186:289–93.
Ferreira SH, Duarte ID, Lorenzetti BB. The molecular mechanism of action of peripheral morphine analgesia: stimulation of the cGMP system via nitric oxide release. Eur J Pharmacol 1991;201:121–2.
Franzotti EM, Santos CV, Rodrigues HM, Mourão RH, Andrade MR, Antoniolli AR. Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L. (Malva-branca). J Ethnopharmacol 2000;72:273–7.

García MD, Fernández MA, Alvarez A, Saenz MT. Antinociceptive and anti-inflammatory effect of the aqueous extract from leaves of *Pimenta racemosa var. ozua* (Mirtaceae). J Ethnopharmacol 2004;91:69–73.
Gene RM, Segura L, Adzet T, Marin E, Inglesias J. Heterotheca inuloides: anti-inflammatory and analgesic effects. J Ethnopharmacol 1998;60:157–62.
Goncales CEP, Araldi D, Panatieri RB, Rocha JBT, Zeni G, Nogueira CW. Antinociceptive properties of acetylenic thiophene and furan derivatives: evidence for the mechanism of action. Life Sci 2005;76:2221–34.
Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987;30(1):103–14.
Inouye H, Saito S, Taguchi H. Zwei neue Iridoidglucoside aus *Gardenia jasminoides*: Gardenosid und Geniposid. Tetrahedron Lett 1969a;28:2347–50.
Inouye H, Inouye S, Shimokawa N, Okigawa M. Studies on monoterpene glucosides. VII. Iridoid glucosides of *Paederia scandens*. Chem Pharm Bull 1969b;17:1942–8.
Inouye H, Okigawa M, Shimokawa N. Studies on monoterpene glucosides. VIII. Artifacts formed during extraction of asperuloside and paederoside. Chem Pharm Bull 1969c;17:1949–54.
Inouye H, Takeda Y, Nhishimura H, Kanomi A, Okuda T, Puff C. Chemotaxonomic studies of Rubiaceous plants containing iridoid glucosides. Phytochemistry 1988;27:2591–8.
Jain NK, Patil CS, Singh A, Kulkarni SK. Sildenafil-induced peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. Brain Res. 2001;909:170–8.
Jain NK, Patil CS, Singh A, Kulkarni SK. Sildenafil, a phosphodiesterase-5 inhibitor, enhances the antinociceptive effect of morphine. Pharmacology 2003;67:150–6.
Kadota S. Traditional medicine in Vietnam, Thailand and Myanmar. Investigation on Natural Drug Resources; 2000. p. 16–68.
Kapadia GJ, Shukla YN, Bose AK, Fujiwara H, Lloyd HA. Revised structure of paederoside, a novel monoterpene S-methyl thiocarbonate. Tetrahedron Lett 1979;22:1937–8.
Kapadia GJ, Sharma SC, Tokuda H, Nishino H, Ueda S. Inhibitory of iridoid glucosides on Epstein–Barr virus activation by short-term in vitro assay for anti-tumor promoters. Cancer Lett 1996;102:223–6.
Kawabata A, Manabe S, Manabe Y, Takagi H. Effect of topical administration of L-arginine on formalin-induced nociception in the mouse: a dual role of peripherally formed NO in pain modulation. Br J Pharmacol 1994;112:547–50.
Kim YL, Chin YW, Kim J, Park JH. Two new acylated iridoid glucosides from the aerial parts of *Paederia scandens*. Chem Pharm Bull (Tokyo) 2004;52(11):1356–7.
Lazaro-Ibanez GG, Torres-Lopez JE, Granados-Soto V. Participation of the nitric oxide-cyclic GMP-ATP-sensitive K(+) channel pathway in the antinociceptive action of ketorolac. Eur J Pharmacol 2001;426:39–44.
Mogil JM, Shin YH, Mccleskey EW, Kim SC, Nah SY. Ginsenoside Rf, a trace component of ginseng root, produces antinociception in mice. Brain Res 1998;792:218–28.
Ortiz MI, Granados-Soto V, Castaneda-Hernandez G. The NO-cGMP-K+ channel pathway participates in the antinociceptive effect of diclofenac, but not of indomethacin. Pharmacol Biochem Behav 2003;76:187–95.
Patil CS, Jain NK, Singh A, Kulkarni SK. Modulatory effect of cyclooxygenase inhibitors on sildenafil-induced antinociception. Pharmacology 2003;69:183–9.
Pehl U, Schmid HA. Electrophysiological responses of neurons in the rat spinal cord to nitric oxide. Neuroscience 1997;77:563–73.
Pyne NJ, Arshavsky V, Lochhead A. cGMP signal termination. Biochem Soc Trans 1996;24:1019–22.
Ramabadran K, Bansinath M, Turndorf H, Puig MM. Tail immersion test for the evaluation of a nociceptive reaction in mice. Methodological considerations. J Pharmacol Methods 1989;21:21–31.
Sánchez-Mateo CC, Bonkanka CX, Hernández-Pérez M, Rabanal RM. Evaluation of the analgesic and topical anti-inflammatory effects of Hypericum reflexum L. fil. J Ethnopharmacol 2006;107(1):1–6.
Santos ARS, Calixto JB. Further evidence for the involvement of tachykinin receptor subtypes in formalin and capsaicin models of pain in mice. Neuropeptides 1997;31(4):381–9.
Santos FA, Jeferson FA, Santos CC, Silveira ER, Rao VSN. Antinociceptive effect of leaf essential oil from Croton sonderianus in mice. Life Sci 2005;77:2953–63.
Shukla YN, Lloyd HA, Morton JF, Kapadia GJ. Iridoid glucosides and other constituents of *Paederia foetida*. Phytochemistry 1976;15:1989–90.
Soares AC, Leite R, Tatsuo MAKF, Duarte IDG. Activation of ATP-sensitive K+ channels: mechanism of peripheral antinociceptive action of the nitric oxide donor, sodium nitroprusside. Eur J Pharmacol 2000;400:67–71.
Srinivasan K, Muruganandan S, Lal J, Chandra S, Tandan SK, Raviprakash V, Kumar D. Antinoniceptive and antipyretic activities of *Pongamia pinnata* leaves. Phytother Res 2003;17:259–64.
Tegeder I, Schmidtko A, Niederberger E, Ruth P, Geisslinger G. Dual effects of spinally delivered 8-bromo-cyclic guanosine mono-phosphate (8-bromo-cGMP) in formalin induced nociception in rats. Neurosci Lett 2002;332:146–50.
Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain 1992;51:5-17.
Tran NN. Gop phan vao viec thong ke nhung loai thuc vat co ich thuoc ho ca phe (Rubiaceae Juss) o Vietnam (Contribution to the enumeration of useful species of the family Rubiaceae juss in Vietnam). J Biol 1987;9:40–4.
Trongsakul S, Panthong A, Kanjanapothi D, Taesotikul T. The analgesic, antipyretic and anti-inflammatory activity of *Diospyros variegata* Kruz. J Ethnopharmacol 2003;85:221–5.
Tsuda M, Suzuki T, Misawa M, Nagase H. Involvement of the opioid system in the anxiolytic effect of diazepam in mice. Eur J Pharmacol 1996;307:7-14.
Wang YC, Huang TL. Screening of anti-helicobacter pylori herbs deriving from Taiwanese folk medicinal plants. FEMS Immunol Med Microbiol 2005;43(2):295–300.
Yaksh TL. Pharmacology and mechanisms of opioid analgesic activity. Acta Anaesthesiol Scand 1997;41:94-111.
Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16(2):109–10.
Zuo X, Peng S, Liu X, Bai B, Ding L. Sulfur-containing iridoid glucosides from *Paederia scandens*. Fitoterapia 2006;77(5):374–7.