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Antinociceptive activity of *n*-butanol fraction from MeOH extracts of *Paederia scandens* in mice

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The *n*-butanol fraction of the MeOH extract from *Paederia scandens* was evaluated for antinociceptive activity in mice using chemical and thermal models of nociception. The n-butanol fraction, given orally at doses of 150, 300 and 600 mg/kg produced significant inhibition of chemical nociception induced by intraperitoneal acetic acid and subplantar formalin or capsaicin injections, and of thermal nociception in the tail-flick test and in the hot plate test. More significant inhibition of nociception was observed at a dose of 600 mg/kg. In the pentobarbital sodium-induced sleeping time test and the open-field test, the *n*-butanol fraction neither significantly enhanced pentobarbital sodium-induced sleeping time nor impaired motor performance, indicating that the observed anti-nociception was unlikely to be due to sedation or motor abnormality. Moreover, the *n*-butanol fraction-induced anti-nociception in both the capsaicin and formalin tests was insensitive to naloxone or glibenclamide but was significantly antagonized by nimodipine. These results suggest that the *n*-butanol fraction produced anti-nociception possibly related to nimodipine-sensitive L-type Ca^{2+} channels, which merits further studies regarding the precise site and mechanism of action. Furthermore, four iridoid glycosides isolated from the *n*-butanol fraction might be related to its antinociceptive action fraction. Therefore, the effect on analgesic activity of each chemical compound in large quantity might well be considered in a further study.

1. Introduction

Paederia scandens (Lour.) Merri., a climbing plant belonging to the family Rubiaceae, popularly known as "Ji Shi Teng" in Chinese, is widely grown in India, China, Japan, Philippines and the USA (Dang et al. 2002). It has been traditionally used as a folk medicine and food in south-east Asia for thousands of years (Kadota 2000). The leaves of the plant are used as an ingredient in various foods in Vietnam (Kadota 2000).

Recently, it was reported that the iridoid glycosides and the dimeric iridoid glycosides paederoside, asperuloside, paederosidic acid, deacetylasperuloside and scandoside (Inouye et al. 1969a–c; Kapadia et al. 1979; Dang et al. 2002), had been isolated from the MeOH extract from the stems and roots of *P. scandens* (Zuo et al. 2006; Kim et al. 2004). These chemical constituents of *P. scandens* have biological activities such as anti-virus, anti-tumor, anti-inflammation and anti-microbial activities (Kapadia et al. 1996; Wang et al. 2005). In folk medicine, the roots, leaves, bark and fruits of *P. scandens* have been used to treat toothache, chest pain, piles, inflammation of the spleen and rheumatic arthritis, as a diuretic and emetic, and to cure bacillary dysentery in China, Japan, Vietnam and other countries in south-east Asia for thousands of years (Tran 1987; Kapadia et al. 1996). Although *P. scandens* is particularly useful for pain-relief in folk medicine, there has been no report on the anti-nociceptive activity of this plant and its mechanisms of analgesic activity so far.

Based on the investigation above, we studied the analgesic activity of MeOH extracts of *P. scandens* and the anti-nociceptive activity of the petroleum ether, chloroform, *n*-butanol and water fractions from the MeOH extracts, and found that the *n*-butanol fraction had a powerful anti-nociceptive activity in preliminary experiments. In the present study, we further examined the effects of the *n*-butanol fraction on nociception models induced by chemical and thermal stimuli in mice to elucidate the analgesic activity and possible mechanism of action of the *n*-butanol fraction, and to provide a scientific basis for the clinical use of *P. scandens.*

2. Investigations and results

2.1. Effect on abdominal constriction induced by acetic acid

In the acetic acid-induced writhing test, treatment with the *n*-butanol fraction significantly decreased the mean number of writhes (Fig. 1). These were 44.22 ± 4.74, 25.1 ± 5.84, 24.91 ± 3.88, and 27.18 ± 2.54 s, respectively, for the controls and the *n*-butanol fraction at the tested doses

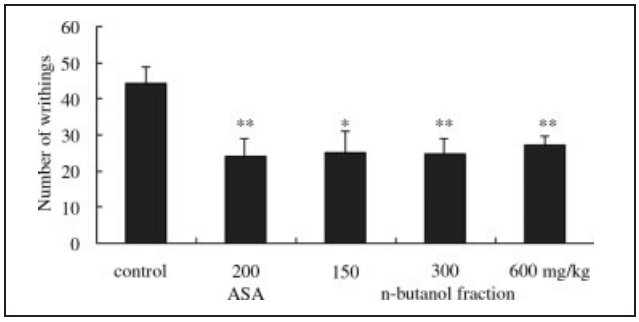


Fig. 1: Effects of the orally administered *n*-butanol fraction of *P. scandens* and acetyl salicylic acid (ASA) on acetic acid-induced writhing in mice. The vehicle (control, 10 ml/kg), the fraction (150, 300, and 600 mg/kg) or ACS (200 mg/kg) were administered orally, 1 h before the intraperitoneal administration of acetic acid (0.7%, 10 ml/kg) and the number of writhes were counted over a period of 12 min. Each column represents 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)

of 150, 300 and 600 mg/kg. The positive drug control, acetylsalicylic acid (200 mg/kg), also significantly diminished the number of writhes (23.9 ± 5.07 s). Therefore, the data showed that the fraction dose-dependently inhibited writhing on nociception.

2.2. Effect on formalin test

In the formalin test, the vehicle-treated animals showed mean licking times (s) of 83.83 ± 13.56 in the first-phase and 45.17 ± 12.86 in the second-phase (Fig. 2A). Pretreatment with the fraction caused significant diminution of both the first-phase (46.5 ± 9.88, 34.83 ± 7.38, and 25.67 ± 6.38 s) and the second-phase (11.33 ± 6.09, 10.5 ± 5.05, and 1.17 ± 1.17 s) pain responses, at the tested doses of 150, 300 and 600 mg/kg, respectively. Morphine (10 mg/kg), the reference drug also significantly suppressed the formalin-response in both phases (first-phase, 6.67 ± 1.26 and second-phase, 0.17 ± 0.17 s). When used alone, naloxone (1 mg/kg, s.c.), glibenclamide (2 mg/kg, i.p.) and nimodipine (1 mg/kg, i.p.), respectively opioid-receptor, K^{+}-ATP channel and L-type Ca^{2+} channel antagonists, failed to modify the formalin-induced nociceptive responses in a significant manner (Fig. 2B) (naloxone: first-phase, 85.83 ± 9.17 and second-phase, 47.5 ± 6.57; glibenclamide: first-phase, 89.33 ± 18.81 and second-phase, 46.5 ± 8.06; nimodipine: first-phase 80.83 ± 8.47, and second-phase 40.83 ± 6.19 s). In combination studies, naloxone markedly antagonized anti-nociception only by morphine but not by the fraction [naloxone + morphine: first-phase, 74.33 ± 11.01 and second-phase, 47 ± 8.25 s; naloxone + dose (600 mg/kg): first-phase, 34.5 ± 4.77 and second-phase, 2 ± 1.44 s], and glibenclamide manifested no antagonism to either the fraction or morphine [glibenclamide + dose (600 mg/kg): first-phase, 39.5 ± 5.58 and second-phase, 3.83 ± 1.14 s; glibenclamide + morphine: first-phase, 10.83 ± 1.94 and second-phase, 0.83 ± 0.48 s]. In contrast, nimodipine exhibited significant antagonism to the fraction but not to morphine (nimodipine + high dose: first-phase, 56.17 ± 6.48 and second-phase, 38 ± 9.54 s; nimodipine + morphine: first-phase, 6.33 ± 3.84 and second-phase, 0 ± 0 s) (Fig. 2B).

2.3. Effect of the capsaicin test

The effects of the *n*-butanol fraction and morphine against capsaicin-induced nociception in mice are shown in Fig. 3A. When compared to vehicle-treated controls (43.5 ± 5.3 s), a dose-dependent reduction in the duration of paw licking was observed in mice pretreated with the fraction (29.17 ± 3.66, 21.17 ± 6.46, and 16 ± 3.39 s, for doses of 150, 300 and 600 mg/kg, respectively). Morphine, the positive control used in the study, also caused significant anti-nociception (0.33 ± 0.21 s). The effects of naloxone (1 mg/kg, s.c.), glibenclamide (2 mg/kg, i.p.) and nimodipine (1mg/kg, i.p.) on the anti-nociceptive activities of morphine (10 mg/kg, s.c.) and the fraction (600 mg/kg) are shown in Fig. 3B. Employed alone, naloxone, gliben-

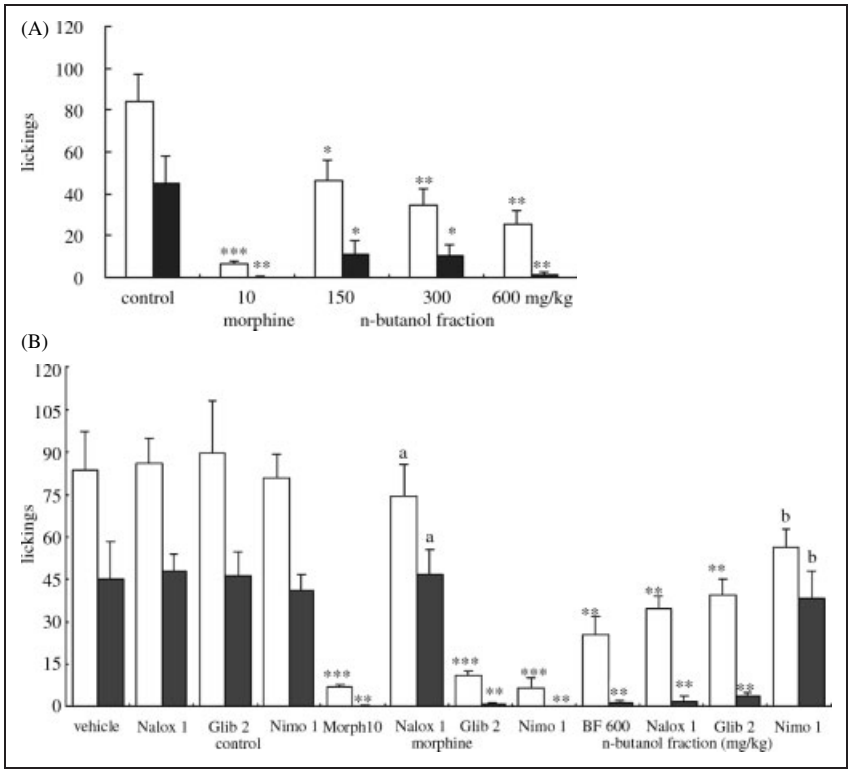


Fig. 2:
The upper panel (A) showed the effects of the *n*-butanol fraction of *P. scandens* and morphine (Morph) on formalin-induced nociception in mice. The total time spent in licking the injected hind-paw was measured in the early phase (0–5 min, white column) and the late phase (20–25 min, black column). The vehicle (control, 10 ml/kg) or the fraction (150, 300, and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. The fraction was administered 1 h before and morphine 30 min before the test. The effects of naloxone, glibenclamide and nimodipine on the fraction and morphine antinociception were shown in lower panel (B). Naloxone (Nalox, 1 mg/kg s.c.), glibenclamide (Glib, 2 mg/kg, i.p.) or nimodipine (Nimo, 1mg/kg, i.p.) were administered 15 min before the fraction or morphine. Each column represents the mean ± S.E.M. (n = 10). Asterisks indicated significant difference from control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control; $^{a}P < 0.001$ vs. Morph; $^{b}P < 0.01$ vs. the fraction (ANOVA followed by Dunnett's test)

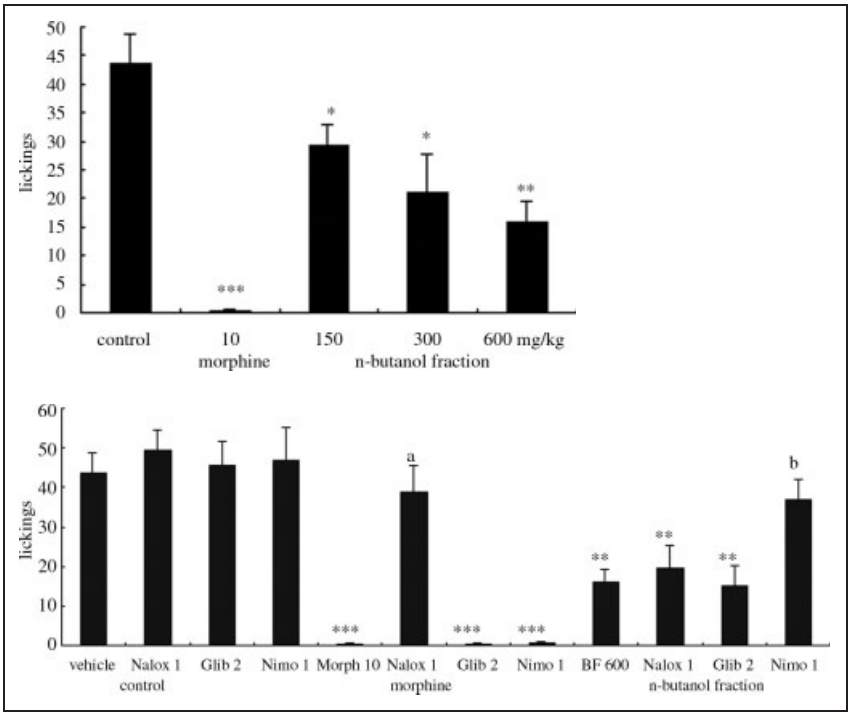


Fig. 3:
The upper panel (A) showed the effects of the *n*-butanol fraction of *P. scandens* and morphine on capsaicin-induced paw licking response in mice. The vehicle (Control, 10 ml/kg) or the fraction (150, 300, and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. The fraction was administered 1 h before and morphine 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. The lower panel (B) shows the pretreatment effects of naloxone (Nalox, 1 mg/kg, s.c.), glibenclamide (Glib, 2 mg/kg, i.p.) and nimodipine (Nimo, 1 mg/kg, i.p.) on the fraction and morphine antinociception. Nimodipine, glibenclamide and naloxone were administered 15 min before the fraction or morphine administrations. Each column represents the mean ± S.E.M. (n = 10). Asterisks indicated significant difference from control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control; $^{a}P < 0.001$ vs. Morph; $^{b}P < 0.01$ vs. the fraction (ANOVA followed by Dunnett's test)

clamide and nimodipine produced no effect on capsaicin-induced paw licking response (naloxone: 49.5 ± 5.17; glibenclamide: 45.5 ± 6.13; nimodipine: 47 ± 8.32 s). In combination studies, while naloxone pretreatment selectively antagonized the anti-nociceptive effect of morphine [morphine + naloxone: 38.83 ± 6.85; dose (600 mg/kg) + naloxone: 19.67 ± 5.68 s], glibenclamide pretreatment showed no effect on anti-nociception by morphine and the fraction [morphine + glibenclamide: 0.33 ± 0.21; dose (600 mg/kg) + glibenclamide: 15 ± 5.06 s]. Interestingly, nimodipine exhibited significant antagonism to the fraction but not to morphine [nimodipine + morphine: 0.5 ± 0.5; nimodipine + dose (600 mg/kg): 36.83 ± 5.22 s].

2.4. Effect on tail-flick test

In the tail-flick test, the fraction showed a significant effect on duration in hot water, when compared to the vehicle-treated control group (1.52 ± 0.15 s) (Fig. 4). The mean durations of the fraction-treated group (150, 300 and 600 mg/kg dose) were 2.36 ± 0.26, 2.23 ± 0.12, 2.72 ± 0.27 s, respectively. However, the positive control group treated with morphine (10 mg/kg) exhibited powerful activity (4.95 ± 0.35 s).

2.5. Effect on hot-plate test

In the hot-plate test, the mean durations of the fraction group (150, 300 and 600 mg/kg dose), the positive control group (morphine, 10 mg/kg) and control group were as follows: fraction group (150, 300 and 600 mg/kg) 19.96 ± 1.53, 24.06 ± 1.15, 36.19 ± 3.54 s; positive control group 44.4 ± 3.5 s; control group 12.92 ± 1.47 s, respectively. The results showed a powerful anti-nociceptive effect in the group treated with the fraction and with morphine (Fig. 5).

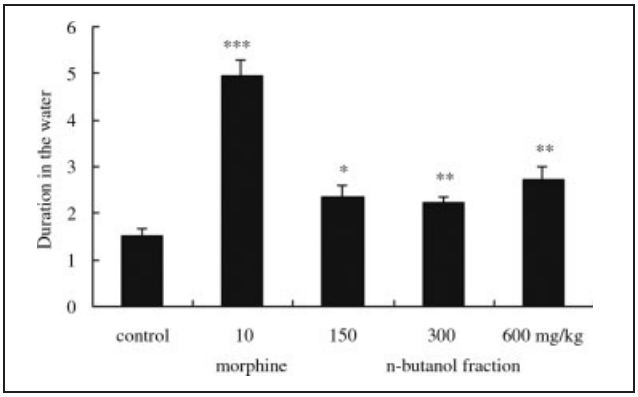


Fig. 4: Effects of the *n*-butanol fraction of *P. scandens* and morphine on thermal-induced antinociception in the tail-flick test. The vehicle (control, 10 ml/kg) or the fraction (150, 300, and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. The fraction was administered 1 h before and morphine 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 represents the mean ± S.E.M. (n = 10). Asterisks indicats significant difference from control. $^{*}P < 0.05$, $^{**}P < 0.01$ (ANOVA followed by Dunnett's test)

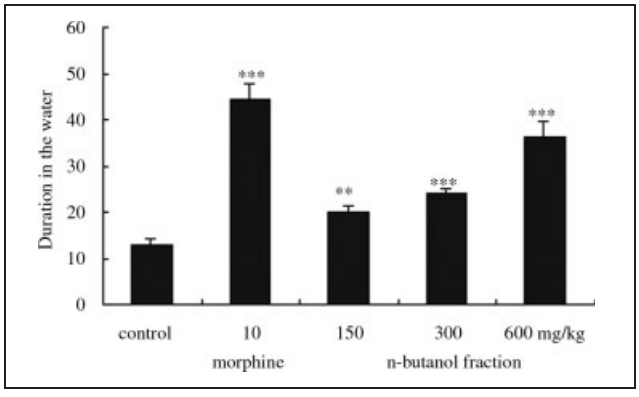


Fig. 5: Effects of the *n*-butanol fraction of *P. scandens* and morphine on thermal-induced antinociception in the hot plate test. The vehicle (control, 10 ml/kg) or the fraction (150, 300, and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. The fraction was administered 1 h before and morphine 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 represents the mean ± S.E.M. (n = 10). Asterisks indicate significant difference from control. $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA followed by Dunnett's test)

2.6. Effect on open-field test

The fraction (150, 300 and 600 mg/kg) did not affect motor coordination in mice. The mean residence time of animals and the route length in the apparatus, obtained with the fraction-treated groups, were not statistically different from those of the vehicle-treated control group over a 5 min period. Only diazepam (1 mg/kg, i.p.) significantly ($P < 0.01$) affected the mobilily performance in comparison with the control group.

2.7. Effect on pentobarbital sodium-induced sleeping time

The effects of the *n*-butanol fraction and diazepam on pentobarbital sodium-induced sleeping time were as follows: vehicle-treated controls: 42.25 ± 1.85 s; fraction (150, 300 and 600 mg/kg): 39.5 ± 4.94, 39.87 ± 6.32 and 40.14 ± 4.88 s, respectively; diazepam: 166.12 ± 7.67 s. Only diazepam, and not the fractions prolonged the sleeping time significantly ($P < 0.001$) (Fig. 6).

2.8. Chemical compounds in n-Butanol fraction of P. scandens

Four known iridoid glycosides, paederoside, asperuloside, paederosidic acid and scandoside, were isolated from the whole plant of *P. scandens*. Their structures were elucidated on the basis of spectroscopic analysis (UV, MS, IR, ^{1}H and ^{13}C NMR) (Inouye et al. 1969a–c, Kapadia et al. 1979). The four chemical compounds represented 2.17% of the n-butanol fraction of *P. scandens* – paederoside (1.23%), asperuloside (0.068%), paederosidic acid (0.7%), scandoside (0.18%).

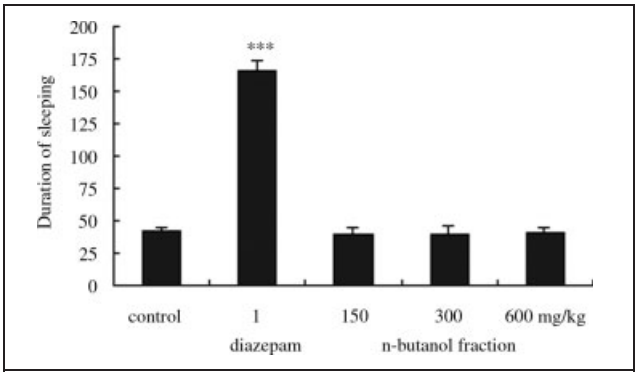
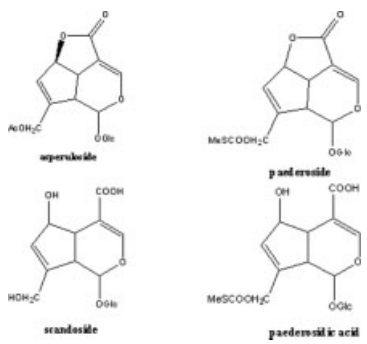


Fig. 6: Effects of the *n*-butanol fraction of *P. scandens* and diazepam on pentobarbital sodium-induced sleeping time in mice. The vehicle (control, 10 ml/kg) or the fraction (150, 300, and 600 mg/kg) was administered orally and diazepam (1 mg/kg) intraperitonealy. The fraction was administered 1 h before the injection of sodium pentobarbitone (45 mg/kg, i.p.) and the time in seconds (s) the animal slept was noted. Each column represents the mean ± S.E.M. (n = 10). Asterisks indicate significant difference from control. *** $P < 0.001$ (ANOVA followed by Dunnett's test)



3. Discussion

In the present experiments, the *n*-butanol fraction of *Paederia scandens* demonstrated significant analgesic activities, both peripheral and central, against chemical nociception in mice induced by intraperitoneal acetic acid, subplantar capsaicin or formalin and thermal nociception.

The acetic acid-induced writhing method has been widely used for the evaluation of peripheral anti-nociceptive activity, and is able to determine the anti-nociceptive effect of compounds at dose levels that might appear inactive in other methods like the tail-flick test (Bentley et al. 1983; Gene et al. 1998). However, it is known that acetic acid induced constriction may be considered a non-selective anti-nociceptive model, since acetic acid indirectly induces the release of endogenous mediators stimulating nociceptive neurons sensitive to non-steroidal antiinflammatory drugs (NSAIDs) (S'anchez-Mateo et al. 2006). Our results indicated that the *n*-butanol fraction of *P. scandens* could reduce the number of writhings in an animal model, showing powerful anti-nociceptive effects. However, the results of this writhing test alone did not ascertain whether the anti-nociceptive effect was central or peripheral.

To confirm this, the formalin test was carried out. The advantage of the formalin model of nociception is that it could discriminate between the central and/or peripheral components of pain. The test consists of two different phases which can be separated in time: the first is generated peripherally through the activation of nociceptive neurons by the direct action of formalin and the second phase occurs through the activation of the ventral horn neurons at the spinal chord level (Tjolsen et al. 1992). Central analgesic drugs, such as narcotics, inhibit both phases equally, while peripherally acting drugs, such as steroids (hydrocortisone, dexamethasone) and NSAIDs (aspirin) suppress mainly later phase (Trongsakul et al. 2003). In this test, the *n*-butanol fraction of *P. scandens* at doses which had been more effective in the writhing test obviously reduced the duration of paw licking (s) in both the first phase (neurogenic) and the second phase (inflammatory) of the formalin test. It was considered reasonable that the *n*-butanol fraction of *P. scandens* had the same anti-nociceptive activity as the central analgesic morphine. Furthermore, the significant effect of the *n*-butanol fraction of *P. scandens* on tail-flick response and hot plate response, in the thermal tests, provided a further confirmation of their central effect, since the tail-flick test and the hot plate test are predominantly a spinal reflex, and are considered to be selective for centrally acting analgesic compounds, like pethidine, while peripheral analgesics are known to be inactive to this kind of painful stimulus (Srinivasan et al. 2003).

The capsaicin-induced paw-licking responses are mediated by release of the excitatory amino acid glutamate and by sensory neuropeptides like substance P released from sensory neurons at the spinal cord, corresponding to the mechanism of the late phase of the formalin test (Santos and Calixto 1997; Otuki et al. 2001). Thus, the data obtained in the capsaicin test and the late phase of the formalin test indicated that the fraction had anti-nociceptive activity. The anti-nociception caused by the *n*-butanol fraction of *P. scandens* seemed to be unrelated to motor impairment or sedation since the mice tested in the open-field and pentobarbital sodium-induced sleeping time tests showed no significant effect on these behaviors.

To verify possible anti-nociceptive mechanisms, we examined the effect of naloxone, a non-selective opioid receptor

antagonist, glibenclamide, a K^+-ATP channel blocker, and nimodipine, an L-type Ca^{2+} channel blocker, on the anti-nociceptive activity of the *n*-butanol fraction of *P. scandens*. Interestingly, the data obtained in both the formalin and capsaicin tests showed that the anti-nociception produced by the *n*-butanol fraction of *P. scandens* was neither naloxone-sensitive nor glibenclamide-sensitive, but was sensitive to nimodipine. Since naloxone and glibenclamide failed to antagonize anti-nociception in the formalin and capsaicin tests, the anti-nociceptive activity of the fraction might involve of L-type Ca^{2+} channels. It is well known that nociception in the formalin test and hot plate test, as well as the capsaicin test, results from the accumulation of intracellular Ca^{2+} concentration that, in turn, initiates a number of second/third messenger-mediated intracellular cascades (Mayer and Miller 1990; Collingridge and Singer 1990; Mayer and Miller 1990; Sommer and Seeburg 1992; Schoepp and Conn 1993). In addition, several reports have indicated that nimodipine is a selective L-type Ca^{2+} channel blocker. When nimodipine is bound with the L-type Ca^{2+} channel, it could inhibit Ca^{2+} influx through L-type Ca^{2+} channels. So nimodipine could enhance anti-nociception in the acute and chronic nociception model (Gurdal et al. 1992). In our formalin test, the dose of nimodipine alone failed to affect the duration of licking in comparison with the vehicle group, that is, nimodipine could not completely block the influx of ex-cellular Ca^{2+}. Interestingly, the nimodipine dose did affect the anti-nociceptive activity of the *n*-butanol fraction. Therefore, we hypothesized that the chemical compounds of the *n*-butanol fraction might bind with specific receptors near the L-type Ca^{2+} channels on the cell membrane and pass through the cell membrane by the way of receptor-mediated endocytosis, then interact with free Ca^{2+} ion in the cell to produce significant anti-nociceptive activity in the formalin test. However, after nimodipine had bound with the L-type Ca^{2+} channels, the channels might affect the binding of chemical compounds in the fraction with the specific receptors near it, so that the chemical compounds failed to pass through the cell membrane and interact with free Ca^{2+}; thus nociception in the formalin test could not be inhibited. A further study could be carried out by the of patch clamp, voltage clamp method to ascertain the precise mechanisms.

It has been further reported that the *n*-butanol fraction of *Paederia scandens* has a high content of iridoid glycosides and dimeric iridoid glycosides (Zuo et al. 2006; Kim et al. 2004). Among the iridoid glycosides and dimeric iridoid glycosides, four chemical compounds – asperuloside, paederoside, scandoside and paederosidic acid – comprise a great part of the *n*-butanol fraction (Kapadia et al. 1996; Wang et al. 2005). Therefore, the anti-nociceptive activity of *P. scandens* might be related to these four chemical compounds.

In conclusion, the study demonstrated the anti-nociceptive activity of the *n*-butanol fraction of *P. scandens* in the test models of nociception induced by chemical and thermal stimuli, and further suggested that the anti-nociceptive activity might be related to the involvement of L-type Ca^{2+} channels which merits further studies regarding the precise site and the mechanism of action.

4. Experimental

4.1. Plant material

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

4.2. 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 our institute and complied with the recommendations of the International Association for the Study of Pain (Zimmermann 1983).

4.3. Drugs and chemicals

The following reagents and drugs were used: MeOH (AR or HPLC), $CHCL_3$ (AR), silica-gel, *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 (Chengdu Pharmaceutical Factory, Chengdu, China).

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

4.4. Sample preparation

The dried powders of whole plant of *P. scandens* (500 g) were extracted with MeOH using a Soxhlet apparatus. The MeOH extract was concentrated under reduced pressure to obtain a residue (137.2 g). The MeOH extract was subsequently extracted with petroleum ether, chloroform and *n*-butanol, respectively. The *n*-butanol fraction was concentrated under reduced pressure to obtain a residue (47.7 g) for bioactivity determination.

4.5. Protocol

The anti-nociceptive activity of the *n*-butanol fraction of *P. scandens* was evaluated in chemical nociception in the test models of acetic acid-induced writhing, and capsaicin- and formalin-induced hind paw licking, and in thermal nociception in the hot-plate test and the tail-flick test. In all of the nocifensive tests, conscious (un-anesthetized) mice were used. The doses of the positive drugs were determined on the basis of their pharmacokinetics and clinical use. The *n*-butanol fraction of *P. scandens* was administered orally. The doses selected – 150 mg/kg, 300 mg/kg and 600 mg/kg – for the fraction were based on the results of preliminary experiments. Control groups were treated with a similar volume of the vehicle used to dilute the *n*-butanol fraction.

4.6. Abdominal constriction induced by acetic acid

In the acetic acid-induced writhing test (Garcia et al. 2004), groups of overnight fasted mice ($n = 10$) were treated with the *n*-butanol fraction of *P. scandens*, vehicle or acetylsalicylic acid, 1 h before the administration of acetic acid (0.7%, 10 ml/kg, i.p.). The number of writhings was counted for each animal over a period of 12 min, starting 3 min after acetic acid injection.

4.7. Formalin test

In the formalin test (Santos and Calixto 1997), groups of mice were treated as above with the *n*-butanol fraction of *P. scandens* or vehicle and after 60 min, each mouse was given 20 µl of 5% formalin (in 0.9% saline, subplantar) into the right hind-paw. The duration of paw licking (s) as an index of painful response was determined at 0–5 min (early phase, neurogenic) and 20–25 min (late phase, inflammatory) after formalin injection. Morphine was used as a positive control drug, and was administered at a dose of 10 mg/kg, s.c., 30 min before the test. In order to verify the possible mechanism of action of the *n*-butanol fraction of *P. scandens*, anti-nociception (600 mg/kg) animal groups pretreated with naloxone, glibenclamide or nimodipine were used. Naloxone, glibenclamide and nimodipine were administered 15 min before the *n*-butanol fraction of *P. scandens* or morphine.

4.8. Capsaicin test

In the capsaicin test (Goncales et al. 2005), mice were pretreated with the *n*-butanol fraction of *P. scandens* or vehicle 60 min before the subplantar injection of capsaicin (1.6 µg, 20 µl) into the right hind paw. A morphine (10 mg/kg, s.c. 30 min before the test) – treated animal group was included as a positive control. The amount of time each mouse spent licking

the injected paw was recorded over the first 5-min period. In order to verify the possible involvement of endogenous opioids, K^+-ATP channels, and/or L-type Ca^{2+} channels in anti-nociceptive activity, the high dose of the fraction was studied in groups of mice pretreated with naloxone, glibenclamide or nimodipine. Naloxone, glibenclamide and nimodipine were administered 15 min before the fraction or morphine.

4.9. Tail-flick test

In the tail-flick test (Sanchez-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 in 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 intervals of 15 min. Only mice showing a pretreatment reaction of 3 s or less were selected for the study. Immediately after the basal latency assessment, the mice were pre-treated with the fraction or vehicle 1 h before the measurement. A morphine (10 mg/kg, s.c. 30 min before the test) – treated animal group was included as a positive control. The cut-off time for tail-flick measurements was 7 s in order to minimize tissue injuries.

4.10. 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 s and 30 s were selected for the experiment. The latency to 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. The groups of mice were pre-treated with the fraction or vehicle and 1 h after the measurement had started. A morphine (10 mg/kg, s.c. 30 min before the test) – treated animal group was included as a positive control. The cut-off time in the hot-plate test was 60 s in order to minimize skin damage.

4.11. Open-field test

The effect of the chemical compounds 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. One hour before the test, the groups of mice were pre-treated with the fraction or vehicle. A diazepam (1.0 mg/kg, i.p.) – treated animal group was included as a positive control.

4.12. Pentobarbital sodium-induced sleeping time

In this test (Santos et al. 2005), groups of mice ($n = 10$) were treated orally with the fraction or vehicle 60 min before the injection of sodium pentobarbitone. Diazepam was used as the reference drug. The time between losing and regaining righting reflex was considered as the duration of sleep time in seconds.

4.13. Statistical analysis

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

4.14. Chemical isolation from n-butanol extract

Silica-gel column chromatography of *n*-butanol extract (47.7 g), using $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5, lower phase) as eluent, partly resulted in the isolation of eight fractions (fraction 1–8). Fractions 1 (1.5g) and 2 (0.39 g) were subjected to C18 reversed-phase column HPLC (Agilent liquid chromatography 1100 series with RID and DAD detectors using a preparative column-YMC ODS-A, 250 × 10mm, S-5 μm 12 nm) with MeOH–H_2O (40 : 60) as solvent (flow rate 1–1.5 ml/min). Paederoside (586.3 mg) was obtained from fraction 1, while asperuloside (32.5 mg) was obtained from fraction 2. Fractions 3 (1.326 g) and 4 (1.366 g) were purified by HPLC with a reversed phase column [flow rate 1.5 ml/min, solvent MeOH–H_2O (60 : 40)] to give paederosidic acid (333.7 mg) from fraction 3 and scandoside (83.6 mg) from fraction 4.

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