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Analgesic activity of the aqueous fraction from the ethanolic extract of *Chrysanthemum indicum* in mice

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The aqueous fraction (AF) of an ethanolic extract from *Chrysanthemum indicum* was evaluated for analgesic activity in mice using chemical and thermal models of nociception. Given orally, AF at doses of 300 and 600 mg/kg produced significant inhibitions on chemical nociception induced by intraperitoneal acetic acid, subplantar formalin/capsaicin injections and on thermal nociception in the tail-flick test and in the hot plate test. In the pentobarbital sodium-induced sleeping time test and the open-field test, AF neither significantly enhanced the pentobarbital sodium-induced sleeping time nor impaired the motor performance, indicating that the observed analgesic activity was unlikely due to sedation or motor abnormality. In a measurement of core body temperature, AF did not affect temperature within 80 min. Moreover, the effective dose (600 mg/kg) also showed no toxicity within 7 days. These results suggested further that AF produced analgesic activity possibly related to the flavonoid glycosides and phenolic glycosides in this fraction.

1. Introduction

Inflammation is characterized by redness, heat, swelling and pain, which has been known since ancient times. Now, it was reported that many nociception receptors, for example the capsaicin receptor TRPV I, could be activated either directly or indirectly by a multitude of pro-inflammatory agents such as prostaglandins, NO (Szallasi et al. 2007; Caterina et al. 2001), then evoke the release of pro-nociceptive and pro-inflammatory mediators in the periphery, which contributes to produce pain and deteriorate the extent of inflammation further.

Chrysanthemum indicum, a traditional anti-inflammation herb, is recorded in many ancient medical books. To date, current pharmacological research has indicated that the ethanolic extract from this plant could decrease synthesis of NO and prostaglandins in inflammatory tissue (Lee et al. 2009; Cheon et al. 2009), and this might also suppress activated nociception receptor-induced inflammatory pain activated by NO and prostaglandins. While anti-inflammation properties of *C. indicum* have widely been studied, the underlying analgesic activity may easily have been neglected. This, we studied the analgesic activity of the EtOH extract of *C. indicum* and petroleum ether, dichloromethane, *n*-butanol and aqueous fractions, respectively, and found that both the petroleum ether fraction (PEF) (Shi et al. 2010) and the aqueous fraction (AF) showed potential analgesic activity in preliminary experiments. In additional, PEF and AF in the formalin test and the capsaicin test have also shown activity against inflammatory pain. Now, we examined the effects of AF on nociception models in mice to elucidate the analgesic activity and compare the results with those of PEF.

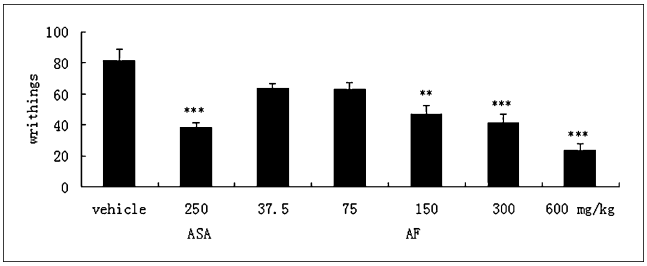

Fig. 1: Effects of AF and the acetyl salicylic acid (ASA) administered orally on acetic acid-induced writhing in mice. The vehicle (control, 10 ml/kg o.p.) and AF (37.5, 75, 150, 300 and 600 mg/kg) were administered 60 min before the intraperitoneal administration of acetic acid (0.7%, 10 ml/kg), or ASA (250 mg/kg o.p.) was administered 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 < 0.01, ***P < 0.001 (ANOVA followed by Dunnett's test).

2. Investigations and results

2.1. Abdominal constriction induced by acetic acid

In the acetic acid-induced writhing test, treatment with AF at the dose of 300 and 600 mg/kg significantly decreased the mean number of writhes (Table), and ED_{50} was 244.26 mg/kg (Fig. 1).

2.2. Formalin test

In the formalin test, treatment with AF caused significant reduction of both the first-phase and the second-phase pain responses at doses of 300 and 600 mg/kg. Moreover, ED_{50} was 214.21 and 210.28 mg/kg in first phase (0∼10 min) and second

Table: Analgesic activity of AF and reference drug in the different tests

	Acetic acid-induced writhing (Number of writhings)	The capsaicin Test (Lickings)	The tail-flick test (Duration in the water)	The hot plate test (Duration on the plate)
Vehicle (10 mg/kg o.p.)	80.90 ± 7.72	111.17 ± 15.86	3.26 ± 0.23	11.75 ± 1.09
AF (37.5 mg/kg o.p.)	63.44 ± 3.47	89.17 ± 14.12	2.93 ± 0.19	13.39 ± 2.16
AF (75 mg/kg o.p.)	62.60 ± 4.71	66.80 ± 8.50	3.28 ± 0.35	13.37 ± 2.07
AF (150 mg/kg o.p.)	$46.90 \pm 5.48^{**}$	$69.50 \pm 2.40^{*}$	$6.26 \pm 0.30^{***}$	$19.51 \pm 1.45^{***}$
AF (300 mg/kg o.p.)	$41.50 \pm 5.37^{***}$	$49.50 \pm 2.97^{**}$	$4.34 \pm 0.36^{*}$	$25.67 \pm 4.23^{**}$
AF (600 mg/kg o.p.)	$23.00 \pm 4.56^{***}$	$32.00 \pm 2.60^{**}$	$5.30 \pm 0.47^{**}$	$21.58 \pm 1.04^{***}$
ASA (250 mg/kg o.p.)	$38.20 \pm 3.51^{***}$	–	–	–
Morphine (10 mg/kg s.c.)	–	$2.67 \pm 1.31^{***}$	$6.97 \pm 0.27^{***}$	$41.02 \pm 1.69^{***}$

Each group represents 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).

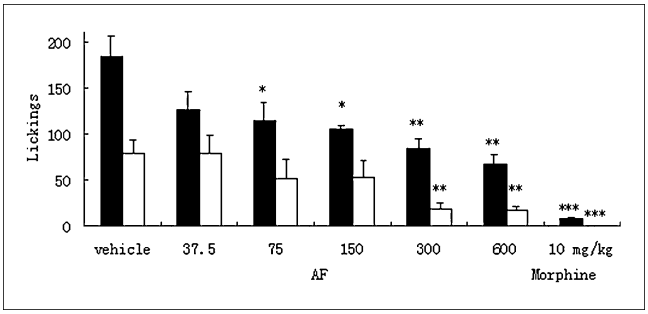

Fig. 2: Eeffects of AF and morphine on formalin-induced nociception in mice. The total time spent in licking the injected hind-paw was measured in the early phase (0∼10 min, black column) and the late phase (30∼40 min, white column). The vehicle (Control, 10 ml/kg) or the fraction (37.5, 75, 150, 300 and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. AF was administered 60 min or morphine 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).

phase (30∼40 min), respectively. Morphine also significantly suppressed the formalin-response in both phases (first-phase, 7.28 ± 1.38 s and second-phase, 0.17 ± 0.17 s) (Fig. 2).

2.3. *Capsaicin test*

The effects of AF and morphine against capsaicin-induced nociception in mice are shown in Table 1. When compared with vehicle-treated group, the reduction in the duration of paw licking was observed in mice pre-treated with AF at the appropriate doses. Moreover, ED_{50} was 205.79 mg/kg (Fig. 3).

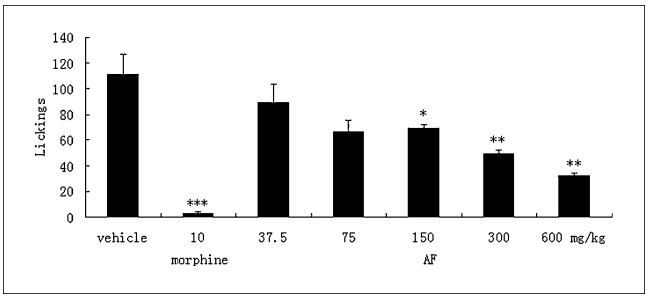

Fig. 3: Effects of AF and morphine on capsaicin-induced paw licking response in mice. The vehicle (control, 10 ml/kg) or the fraction (37.5, 75, 150, 300 and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. AF or morphine was administered 60 min or 30 min before the subplantar injection of capsaicin (1.6 μg, 25 μl) into the hind paw respectively, 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).

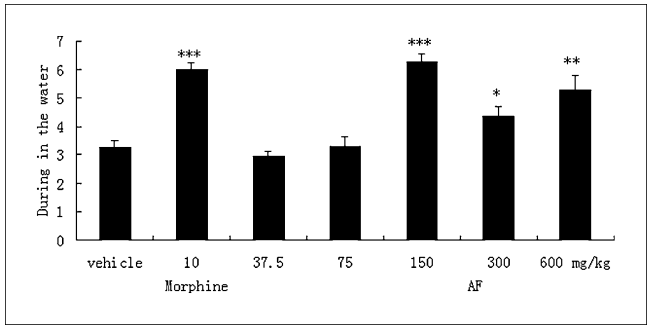

Fig. 4: Effects of AF and morphine on thermal-induced anti-nociception in the tail-flick test. The vehicle (Control, 10 ml/kg) or AF (37.5, 75, 150, 300 and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. AF or morphine was administered 60 min or 30 min before the test respectively, and the time in seconds taken to flick the tail was recorded. Cut-off time was 8 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).

2.4. *Tail-flick test*

In the tail-flick test, the groups of AF (150, 300 and 600 mg/kg) and the positive control group treated with morphine (10 mg/kg) exhibited the powerful activity when compared to vehicle-treated control group (Table) (Fig. 4).

2.5. *Hot-plate test*

The mean of the durations on the hot plate in the groups of AF, the positive control group (morphine, 10 mg/kg) and control group are shown in the Table. The results show that

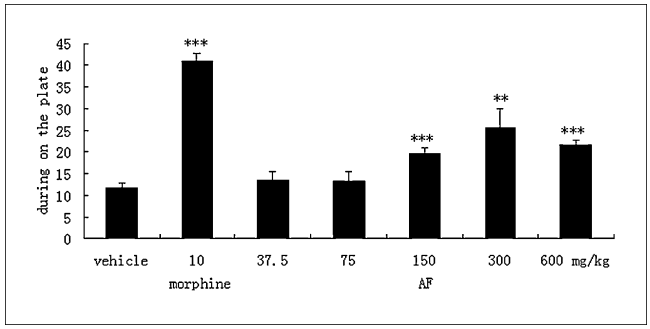

Fig. 5: Effects of AF and morphine on thermal-induced anti-nociception in the hot-plate test. The vehicle (control, 10 ml/kg) or the fraction (37.5, 75, 150, 300 and 600 mg/kg) was administered orally and morphine (10 mg/kg) subcutaneously. AF or morphine was administered 60 min or 30 min before the test respectively, 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 <0.01, ***P < 0.001 (ANOVA followed by Dunnett's test).

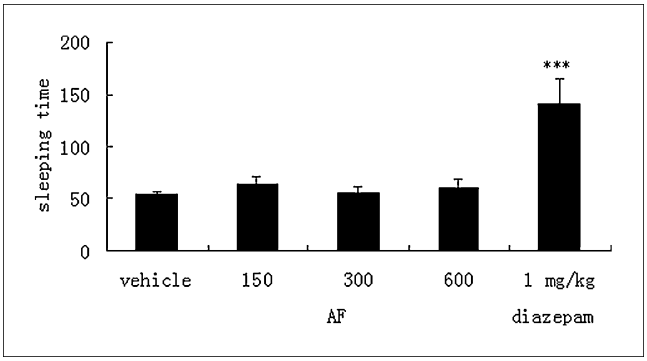

Fig. 6: Effects of AF and diazepam on pentobarbital sodium-induced sleeping time in mice. The vehicle (control, 10 ml/kg), AF (150, 300 and 600 mg/kg) was administered orally or diazepam (1 mg/kg) was administered intraperitonealy. AF was administered 60 min before the injection of sodium pentobarbitone (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).

AF (150, 300 and 600 mg/kg) and morphine had powerful anti-nociceptive effects (Fig. 5).

2.6. *Open-field test*

AF (150, 300 and 600 mg/kg) did not affect the motor coordination in mice. The mean permanence time of animals and the length of the route in the apparatus, obtained in 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 mobile performance in comparison with the control group.

2.7. *Pentobarbital sodium-induced sleeping time*

The effects of AF and diazepam on pentobarbital sodium-induced sleeping time were as follows: Vehicle-treated control: 53.43 ± 3.52 min; AF (150, 300 and 600 mg/kg): 64.29 ± 7.24, 55.13 ± 6.18 and 60.25 ± 8.05 min, respectively; diazepam: 141.43 ± 23.28 min. Only diazepam prolonged the sleeping time significantly ($P < 0.001$) (Fig. 6).

2.8. *Core body temperature*

AF (600 mg/kg.) did not influence the core body Fig. 7 temperature in mice within 80 min in comparison with vehicle.

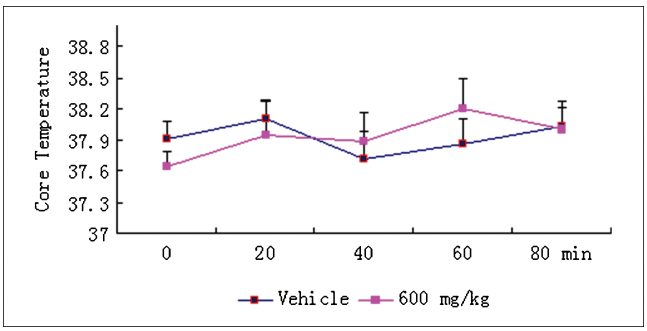

Fig. 7: Effects of AF on core body temperature in mice within 80 min. The vehicle (control, 10 ml/kg) and AF (600 mg/kg) was administered orally 60 min before test and the temperature (°C) of mice was noted. Each dot represented the mean ± S.E.M. (n = 10). No asterisks indicated any significant difference from control (ANOVA followed by Dunnett's test).

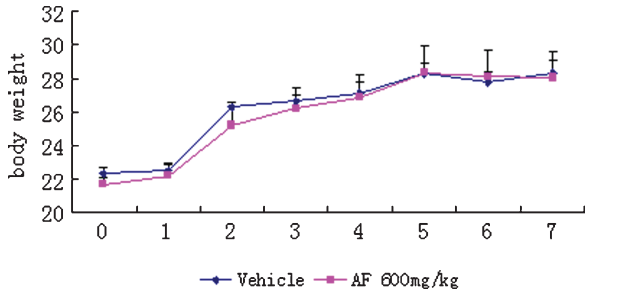

Fig. 8: Effect of AF on body weight in mice within 7 days. The vehicle (control, 10 ml/kg) and AF (600 mg/kg) was administered orally. Each dot represented the mean ± S.E.M. (n = 10). No asterisks indicated any significant difference from control (ANOVA followed by Dunnett's test).

2.9. *Acute toxicity*

In 24 h, we failed to observe the effect of convulsion, hyperactivity, sedation, grooming, vomiting, loss of righting reflex and increased or decreased respiration on mice that have given the dose of 600 mg/kg orally. Body weight in 7 days showed no differences between the treatment and plarebo groups Fig. 8. No single mouse died within 7 days.

3. Discussion

In the presented experiments, AF of *C. indicum* demonstrated significant both peripheral and central analgesic activities against chemical nociception in mice induced by intraperitoneal acetic acid, subplantar formalin/capsaicin and thermal nociception.

The acetic acid-induced writhing method has been used for the evaluation of inflammatory pain (Gene et al. 1998). Moreover, constriction induced by acetic acid was considered to be a non-selective anti-nociceptive model despite it was able to determine analgesic effect of compounds and dose levels that might appear inactive in other methods like the tail-flick test (Sánchez-Mateo et al. 2006). Our results indicated that AF at the dose of 300 and 600 mg/kg could reduce the number of writhings, showing powerful analgesic effects. However, the results of this writhing test alone did not ascertain whether the anti-nociceptive effect was central or peripheral.

The advantage of the formalin model of nociception is that it can discriminate pain in its central and/or peripheral components. The test consists of two different phases which can be separated (Tjolsen et al. 1992). Central analgesic drugs, such as morphine, could inhibit equally in both phases; while peripheral-acting drugs, such as NSAIDs, could suppress mainly in the later phase (Trongsakul et al. 2003). In this test, AF at the 300 and 600 mg/kg doses could reduce the duration of the paw licking (s) obviously in both the first phase (neurogenic) and the second phase (inflammatory) of the formalin test. So probably AF had the same analgesic mechanism as the central analgesic morphine.

In the thermal tests, the significant effect of AF at the dose of 150, 300 and 600 mg/kg on the tail-fick response and on the hot plate response provided a further confirmation of their central effect, since the tail-flick test and the hot plate test predominantly rely on a spinal reflex, and were considered to be selective for centrally acting analgesic compounds, like pethidine, while peripheral analgesics are known to be inactive on this kind of painful stimulus (Srinivasan et al. 2003).

The analgesic activity caused by AF at the effective dose seemed to be unrelated to motor impairment or sedation since the mice tested in open-field and pentobarbital sodium-induced sleeping time tests showed no significant effect on these behaviors. Moreover, AF at the effective dose did not significantly change the

core body temperature, which might possibly confound measurements of anti-nociception in the hot plate and the tail-flick test, and even the formalin test (Mogil et al. 1998).

Several reports showed that flavonoid glycosides and phenolic glycosides, which possesse significant anti-inflammatory properties, were isolated in a large quantity from the EtOH extract from *C. indicum* (Cheng et al. 2005; Yoshikawa et al. 2000). These chemical compounds are distributed easily in the AF. So it seemed to be consistent with our hypothesis that when inflammatory tissue released pro-inflammatory agents, these could activate noception receptors to release the pro-nociceptive and pro-inflammatory meditors, in turn further, induce pain and deteriorate inflammation. However, AF could suppress the release of inflammatory agents and enhance the thresholds of pain simultaneously, which might contribute to anti-inflammation and analgesic activity of this plant. Moreover, it might equally be reasonable not to obtain a clear dose–response curve for the analgesic effect in these tests, due to the synergistic effects of these compounds. However, when compared with the results from PEF, ED_{50}s obtained from AF in these chemical-induced nociception tests are significantly higher (Shi et al. 2010). Therefore, it might be hypothesized that these compounds in PEF possess enough hydrophobicity to diffuse easily through the blood-brain barrier, in turn enhanced the bio-availability; or these compounds in AF might interact with each other, narrowing the scope for anti-nociception of individual compounds.

In conclusion, the study have demonstrated the underlying analgesic activity of AF, and further suggested that analgesic activity might be related to the flavonoid glycosides and phenolic glycosides in AF.

4. Experimental

4.1. Plant material

Flowers and buds of *C. indicum* were collected in Hu bei province in October, 2008. The plant was identified at the School of Pharmacy of Shen-yang Pharmaceutical University. A voucher specimen of C. indicum (# 67) was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Shen-yang Pharmaceutical University.

4.2. Sample preparation

The dried flowers and buds of *C. indicum* (3 kg) were extracted at 80 °C with 75% EtOH in a Soxhlet apparatus. The EtOH extract was concentrated under reduced pressure to obtain a residue (459.4 g). Then, the extract was subsequently extracted with petroleum ether, dichloromethane, *n*-butanol, and water respectively. AF was concentrated under reduced pressure to obtain a residue (100.1 g) for bioactivity determination.

4.3. 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 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 Shen-yang North hospital and complied with the recommendations of International Association for the Study of Pain (Zimmermann 1983).

4.4. Drugs and chemicals

The following reagents and drugs were used: EtOH (AR), petroleum ether (AR), chloroform (AR), *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 (Chengdu pharmaceutical factory, Chengdu, China), capsaicin (Sigma, USA).

Morphine hydrochloride, acetyl-salicylic acid, diazepam and pentobarbital sodium were dissolved in physiological saline (0.9% NaCl). The vehicles used alone had no effects on the nociceptive responses in mice.

4.5. Pharmacological studies

4.5.1. Abdominal constriction induced by acetic acid

In the acetic acid-induced writhing test (García et al. 2004), groups of overnight fasted mice (n = 10) were treated orally with AF, vehicle (10 ml/kg) or acetyl-salicylic acid (250 mg/kg), 1 h before the administration of acetic acid (0.7%, 10 ml/kg, i.p.). The number of writhings was counted for each animal, starting 3 min after acetic acid injection over the period of 12 min.

4.5.2. Formalin test

In the formalin test (Santos and Calixto 1997), groups of mice were treated orally with AF or vehicle (10 ml/kg) and after 1 h, each mouse received 25 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∼10 min (early phase, neurogenic) and 30∼40 min (late phase, inflammatory) after formalin injection. Morphine was used as a positive control, which was administred at the dose of 10 mg/kg, s.c., 30 min before the test.

4.5.3. Capsaicin test

In the capsaicin test (Goncales et al. 2005), mice were pre-treated with AF or the vehicle 1 h before the subplantar injection of capsaicin (1.6 μg, 25 μl) into the right hind paws. A morphine-treated (10 mg/kg s.c. 30 min before the test) animal group was included as positive control. The time each mouse spent licking the injected paw was recorded over the first 5-min period.

4.5.4. Tail-flick test

In the tail-flick test (Sánchez-Mateo et al. 2006), the thermostat was adjusted so that a constant temperature of 54 ± 1 °C was maintained in the water bath. Pre-treatment latencies were determined three times with intervals of 25 min. Only mice showing a pre-treatment reaction for less or equal to 4 s were selected for the study. Immediately after the basal latency assessment, the mice were pre-treated with the fraction or vehicle (10 ml/kg) 1 h before the measurement. A morphine-treated (10 mg/kg, s.c. 30 min before the test) animal group was included as positive control. The cut-off time was 8 s in order to minimize tissue injuries.

4.5.5. Hot-plate test

The hot plate test (Franzotti et al. 2000) was carried out in groups of female mice using a hot plate apparatus (model YLS-6B, China) maintained at 54 ± 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 index of nociceptive threshold. In this test, pre-treatment latencies were determined three times with 25-min interval. The groups of mice were pre-treated with AF or the vehicle and 1 h later the measurement started. A morphine-treated (10 mg/kg s.c. 30 min before the test) animal group was included as positive control. The cut-off time was 60 s in order to minimize skin damage.

4.5.6. Open-field test

The effect of AF on spontaneous locomotor activity and exploratory behavior was assessed in the open-field test (Tsuda et al. 1996). The number of rearing responses, the number of areas crossed by all paws, and the total time spent on being immobilized (immobility) were recorded. 1 h before the test, the groups of mice were pre-treated with the fraction or vehicle. A diazepam-treated (1.0 mg/kg, i.p.) animal group was included as positive control.

4.5.7. Pentobarbital sodium -induced sleeping time

In this test (Santos et al. 2005), groups of mice (n = 10) were treated orally with AF and vehicle, 1 h before the injection of sodium pentobarbitone (45 mg/kg, i.p.). Diazepam (1 mg/kg, intraperitonealy) was used as the reference drug. The time between losing and regaining righting reflex was considered as the duration of sleep time in seconds.

4.5.8. Measurement of core body temperature

Core body temperature was measured in a separate group of mice 1 h after giving orally AF (600 mg/kg) or the vehicle. Mice were restrained, and a lubricated thermistor probe was inserted 3 cm into the rectum for 10 to 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 AF on the core body temperature was observed within 80 min.

4.5.9. Acute toxicity

The water and the dose of 600 mg/kg was given orally to different groups as the vehicle and the test group (n = 10). Behavior parameters including convulsion, hyperactivity, sedation, grooming, loss of righting reflex and increased or decreased respiration were also observed in 24 h. Moreover, we observed the change of body weight within 7 days.

4.6. Statistical analysis

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

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