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Antinociceptive effects of ethanolic extract from the flowers of *Acmella oleracea* (L.) R.K. Jansen in mice

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ABSTRACT

Ethnopharmacological relevance: In Brazil, *Acmella oleracea* (L.) R.K. Jansen, popularly known as "jambu", has been used by some communities from Amazon region to treat toothache. In this study we examined the antinociceptive effect of the ethanolic extract obtained from the flowers of *Acmella oleracea* (EEAO) in animal models of nociceptive (chemical and thermal) and neuropathic (partial sciatic nerve ligation) pain.

Materials and methods: Adult male mice were treated by intraperitoneal route (i.p.) with EEAO before the induction of nociceptive response by formalin, capsaicin and cinnamaldehyde, thermal heat hyperalgesia (hot plate test) and mechanical allodynia (traumatic sciatic nerve injury). Acute toxicity and non-specific sedative effects were evaluated.

Results: EEAO (10, 30 and 100 mg/kg) reduced both neurogenic and inflammatory phases of the formalin- and also capsaicin- and cinnamaldehyde-induced orofacial nociception. Interestingly, EEAO at 100 mg/kg (i.p.) also reversed capsaicin-induced heat hyperalgesia assessed as the latency to paw withdrawal in the hot plate test. Also in the hot plate test, paw withdrawal latency was increased by EEAO (100 mg/kg) and this response was only partially reversed by naloxone. Furthermore, EEAO (100 mg/kg) also reduced mechanical allodynia caused by partial sciatic nerve ligation for 3 h. The estimated LD50 value was 889.14 mg/kg and EEAO did not alter the locomotion of animals in the open-field test.

Conclusion: Taken together, our data show that EEAO produces prevalent antinociceptive effects and does not cause adverse effects. The presence of N-alkylamides, including spilanthol, suggests that the therapeutic effect of EEAO is related to its highest anesthetic activity.

1. Introduction

Pain sensation serves as warning system that triggers adequate reactions to avoid and to minimize contact with damaging stimuli (Basbaum et al., 2009; Woolf, 2010). On the other hand, under pathological conditions, such as inflammatory and neuropathic pain, this system can become sensitized and pain turn into chronic and debilitating (Basbaum et al., 2009). The need for better treatment of pain is evident because many patients, mainly who suffer with neuropathic pain, do not experience sufficient pain relief. Also importantly is the pain in the orofacial region, which is associated with pathological states of the teeth and the trigeminal related structures, that is very difficult to treat by health professionals (Miranda et al., 2009). For this reason, the search for new therapeutic alternatives is arising and some of this relies on medicinal plants and their bioactive compounds.

In northern Brazil, *Acmella oleracea* (L.) R.K. Jansen (bas. *Spilanthes oleracea*; syn. *Spilanthes acmella* var. *oleracea*; Asteraceae) is widely used as a condiment in local dishes because of its pungent flavor. Popularly known as "jambu", "agrião bravo" or "agrião do Pará", its leaves and yellow flowers cause a mild tingling and numbness of the tongue (Nascimento et al., 2013). According to folk medicine, *Acmella oleracea* is an important medicinal plant whose leaves and flowers are used by the population as local anesthetic to treat toothache (Villachica et al., 1996; Tiwari et al., 2011).

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In addition, *Acmella oleracea* is also taken for the treatment of stomatitis and cold (Prachayasittikul et al., 2013). Several studies demonstrated that *Spilanthes acmella* presents a variety of biological effects such as local anesthetic, analgesic, antipyretic, anti-inflammatory, antioxidant, diuretic and vasorelaxant (Chakraborty et al., 2004; Ratnasooriya et al., 2004; Wongsawatkul et al., 2008; Chakraborty et al., 2010). Despite the great number of bioactive compounds found in *Spilanthes acmella* (Prachayasittikul et al., 2009), lipophilic alkylamides or alkamides such as spilanthol are the major metabolites isolated and studied in this plant, which seems to be responsible for its anesthetic and anti-inflammatory activities (Ley et al., 2006; Wu et al., 2008; Tiwari et al., 2011). Furthermore, phenolic compounds, coumarin and triterpenoids were also found in *S. acmella* (Prachayasittikul et al., 2009). Recently, our group isolated a polysaccharide (rhamnogalacturonan) from leaves of *Acmella oleracea* with gastroprotective activity against acute gastric lesions induced by ethanol in rats (Nascimento et al., 2013).

However, despite of *Acmella oleracea* is consumed as food or tea, to the best of our knowledge, there has not been a chemical composition investigation confirming the putative analgesic effect of *Acmella oleracea* in painful conditions. Thus, considering the traditional use of this plant for treatment of toothaches, the goal of this study was to perform a chemical characterization and to investigate the acute toxicity and antinociceptive properties of systemic administration of ethanolic extract of flowers from *Acmella oleracea* in experimental models of nociception in mice. Reported herein are the pronounced analgesic effect on formalin-, capsaicin- and cinnamaldehyde-induced orofacial nociception, thermal antihyperalgesic and mechanical antiallodynic of an extract that contains N-alkylamides, without promote acute toxicity or interfere with the motor performance, confirming and reinforcing the therapeutic potential of *Acmella oleracea*.

2. Materials and methods

2.1. Plant collection and preparation of ethanolic extract

The plant *Acmella oleracea* (L.) R.K. Jansen (Asteraceae) was collected in the State of Acre (Northern Brazil), region of Cruzeiro do Sul, in August 2010. The plant was identified by Rosângela de A. P. H. e Souza M.Sc. (Zoobotanical Park, Federal University of Acre, Rio Branco, Brazil) and a voucher specimen was deposited in the Herbarium of UFAC, under no. 15099.

Briefly, fresh flowers of *Acmella oleracea* (60 g) were subjected to extraction with absolute ethanol under reflux for 3 h (adapted from Dias et al., 2012). The ethanolic extract of *Acmella oleracea* flowers (EEAO) was evaporated under reduced pressure, resuspended in water, and freeze-dried, yielding 2.7 g.

2.2. Sample analysis (liquid chromatography–mass spectrometry)

The sample was examined by ultra performance liquid chromatography (UPLC, Waters-Acquity) composed by a binary solvent delivery and a photodiode-array (PDA) detector. The sample at 1 mg/ml was prepared in H_2O–MeOH (1:1 v/v) and the analysis was carried out on a reversed phase BEH-C18 column 1.7 μm (2.1 × 50 mm). The binary solvent was composed by (A) 0.1% aqueous formic acid (v/v) and (B) methanol. The linear solvent gradient was: initial (B) at 5–80% at 5 min, hold to 7 min, returning to 5% at 8 min, and additional 3 min at 5% (B) was held to system re-equilibration. The column was heated at 60 °C and the samples held at room temperature (22 °C). The injection volume was 2 μl and the compounds were detected at λ=200–400. The mass spectrometry was electrospray ionization (ESI), triple quadrupole (Quattro-LC, Waters), operating at atmospheric pressure ionization (API). The positive ionization mode was used for detecting compounds, at m/z 100–600, with energies at 2.6 kV on capillary and 70 V on cone. Nitrogen was used as nebulizer and desolvation gas at 850 l/h.

2.3. Animals

Experiments were conducted using male Swiss mice (25–35 g), housed at 22 ± 2 °C under a 12 light/12 h dark cycle and with free access to food and water. The experimental procedures were performed after approval of the respective protocols by the local Ethics Committee (CEUA/BIO-UFPR; Approval no. 544) and were carried out in strict compliance with the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The animals were divided into groups of 5–8 mice and each animal was used only once in the experiment.

2.4. Drugs and reagents

The following reagents and drugs were used: capsaicin and cinnamaldehyde were obtained from Sigma-Aldrich (St Louis, MO, USA), morphine hydrochloride and naloxone from Cristália (São Paulo, Brazil), fluoxetine from Eurofarma (São Paulo, Brazil), methanol and formic acid were HPLC grade (Tedia Company Inc., USA), water was Milli-Q (Millipore Corporation, USA) and all other chemicals (absolute ethanol, formalin and tween 80) were of analytical grade and obtained from Vetec (Rio de Janeiro, Brazil). Drugs were immediately dissolved in 0.9% NaCl solution before administration.

2.5. Mice orofacial nociception tests

2.5.1. Nociception induced by orofacial formalin

Measurement of formalin-induced nociception was carried out as described by Luccarini et al. (2006), with some modifications. Briefly, after the environmental habituation period, the mice were intraperitoneally pretreated with vehicle (saline plus 30 μl tween 80, 10 ml/kg) or EEAO (10, 30 and 100 mg/kg) 30 min before injection of 20 μl of a 2.5% formalin solution (0.92% formaldehyde in saline) into the right upper lip, just lateral to the nose. Following the injection of formalin, mice were immediately replaced into a glass cylinder of 20 cm diameter, and the time that each animal spent rubbing the injected area with the forepaws was recorded with chronometer for both the early neurogenic phase (0–5 min) and late inflammatory phase (15–40 min) of this model and was considered as index of nociception.

2.5.2. Nociception induced by orofacial capsaicin or cinnamaldehyde

The procedure used was similar to that described by Rodrigues et al. (2012). Mice were intraperitoneally pretreated with vehicle (10 ml/kg) or EEAO (10, 30 and 100 mg/kg), 30 min before a single injection of 20 μl of capsaicin (1.6 μg/lip) or cinnamaldehyde (13.2 μg/lip). Then, mice were returned immediately to the observation glass cylinder and the time that each animal spent rubbing the injected area with the forepaws was recorded in 5 min and it was considered as an index of nociception.

2.6. Thermal heat hyperalgesia induced by capsaicin

Capsaicin-induced thermal hyperalgesia was measured using the hot-plate apparatus according to the method described previously by Garcia-Martinez et al. (2002). First, untreated animals were placed on an heated surface (Socrel DS-35, Ugo Basile, Comerio, VA, Italy) kept at a constant temperature of 55 ± 1 °C and the time

between placement and shaking or licking the hindpaws or jumping off plate was recorded as paw withdrawal latency (s). Animals that remained on the apparatus for an average of 10 s were previously selected on the basis of their reactivity in the model. Next, mice were intraperitoneally treated with vehicle (10 ml/kg) or EEAO (10, 30 e 100 mg/kg) and, after 30 min received an injection of 20 μl of capsaicin (1.6 μg/paw) into the right hindpaw. The paw withdrawal latency was recorded for each animal at 0, 5, 15, 30, 60 and 120 min after capsaicin injection and a 30 s cut-off time was used to prevent tissue damage.

2.7. Thermal heat hyperalgesia and involvement of opioid system

First of all, animals were selected using the hot plate test as previously described. Then, in order to test the hypothesis of whether the opioid system could be involved in the antinociceptive activity of ethanolic extract, animals were pretreated with vehicle (saline, 10 ml/kg, i.p.) or naloxone (1 mg/kg, i.p., a non-selective opioid receptor antagonist), 20 min before the administration of vehicle (10 ml/kg, i.p.), morphine (1 mg/kg, s.c., a non-selective opioid receptor agonist) or EEAO (100 mg/kg, i.p.). After 30 min, mice were replaced on the hot plate and the paw withdrawal latency was recorded for each animal at 0, 5, 15, 30, 60 and 120 min. A 30 s cut-off time was used to prevent tissue damage.

2.8. Mechanical allodynia induced by partial sciatic nerve ligation

The measurement of the mechanical hindpaw withdrawal threshold was carried out using von Frey monofilaments (Stoelting, Chicago, USA). Briefly, mice were first acclimatized (1 h) in individual clear Plexiglass boxes (9 cm × 7 cm × 11 cm) on an elevated wire mesh platform to allow access to the plantar surface of the hindpaws. Mechanical allodynia was evaluated as the withdrawal response frequency to 10 applications of 0.6 g von Frey hairs to the hindpaw plantar surface with a pressure high enough to bend the filament (Rodrigues et al., 2012). For the induction of peripheral traumatic neuropathy (Malmberg and Basbaum, 1998), mice were anaesthetized with an i.p. injection of a mixture of ketamine and xylazine (100 and 5 mg/kg, respectively). Then, a partial ligation of the right sciatic nerve was made by tying one-third to one-half of the dorsal portion of the sciatic nerve, using a silk 8-0 suture, followed by suture of the wound. Seven days after the surgical procedure, mice were intraperitoneally treated with vehicle (10 ml/kg), fluoxetine (10 mg/kg, a selective serotonin reuptake inhibitor) or EEAO (10, 30 and 100 mg/kg) and replaced in the Plexiglas boxes. Thirty minutes after, mechanical allodynia was evaluated as the withdrawal response frequency to 10 applications of 0.6 g of von Frey hairs, repeatedly at 1, 2, 3, 4, 5 and 6 h.

2.9. Assessment of side effects

2.9.1. Hippocratic screening test and acute toxicity

In order to study the effect of EEAO on the general behavior of conscious animals (Malone and Robichaud, 1962), mice were fasted overnight (12 h) with free access to water prior to intraperitoneal treatment with single doses of EEAO (5, 50, 500 and 5000 mg/kg). The signs and symptoms produced by EEAO administration were observed in freely moving animals at 0, 30, 60, 120, 180, 240 and 300 min afterwards and once daily thereafter for 7 days. Finally, the number of survivors was noted at the end of experiment. Thus, the toxicological effect was assessed on the basis of mortality, which was expressed by the required dose in mg/kg body weight to cause death in 50% of animals tested (LD50) (Litchfield and Wilcoxon, 1949).

2.9.2. Measurement of locomotor activity

Exploratory behavior and locomotor activity were tested using the open field test as previously described (Rodrigues et al., 2012). Mice were intraperitoneally treated with vehicle (10 ml/kg) or EEAO (10, 30 and 100 mg/kg) and, 30 min after, the animals were placed in the middle of a circular arena (42 cm in diameter and 24 cm in height) divided in 19 squares into center, middle and outer ring to explore freely. The number of sectors crossed with all paws (crossings) was counted for 5 min.

2.10. Statistical analysis

Data are presented as means ± standard error of the mean (S.E.M.). Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by Newman Keul's test when appropriate. Statistical comparisons of mechanical allodynia and hot plate data were performed by two-way ANOVA followed by Bonferroni's multicomparison post-hoc test. ID50 values (dose capable of reducing nociceptive response by 50% relative to the control value) were determined by nonlinear regression analysis and reported as geometric mean with 95% confidence limits. P values less than 0.05 were considered as indicative of significance.

3. Results

3.1. Ultra performance liquid chromatography (UPLC) analysis of ethanolic extract of Acmella oleracea flowers

The presence of three N-alkylamides in EEAO were detected by ultra performance liquid chromatography using photodiode-array and electrospray mass spectrometry. Thus, N-alkylamides structures were identified on the basis of their UV-absorption and m/z profiles as follows: peak 1 at Rt 4.64 min, m/z 230 $[M+H]^+$, and λ_{max} 259 nm [(2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide]; peak 2 at Rt 5.15 min, m/z 222 $[M+H]^+$, and λ_{max} 233 nm [(2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide, spilanthol]; and peak 3 at Rt 5.41 min, m/z 236 $[M+H]^+$, and λ_{max} 261 nm [(2E,6Z,8E)-N-(2-methylbutyl)-2,6,8-decatrienamide] (Fig. 1).

3.2. Effect of ethanolic extract of Acmella oleracea flowers on nociception induced by orofacial formalin

Intraperitoneal administration of mice with EEAO (10, 30 and 100 mg/kg), given 30 min beforehand significantly inhibited the neurogenic phase of formalin in 34 ± 6%, 35 ± 3% and 41 ± 8%, respectively (Fig. 2A). Moreover, all tested doses of EEAO also reduced the inflammatory phase of nociception with an ID_{50} value (and their 95% confidence limits) of 28.6 (20.6–39.7) mg/kg and inhibitions of 19 ± 9%, 47 ± 7% and 92 ± 4% for 10, 30 and 100 mg/kg, respectively (Fig. 2B).

3.3. Effect of ethanolic extract of Acmella oleracea flowers on nociception induced by orofacial capsaicin and cinnamaldehyde

Animals that received an orofacial injection of 20 μl saline following vehicle (10 ml/kg) as control were observed during 5 min and rubbing the injected area with the forepaws for 9.4 ± 1.7 s (data not shown). On the other hand, orofacial administration of capsaicin or cinnamaldehyde caused marked nociceptive behavior in mice (42.3 ± 3.3 and 62.7 ± 7.2 s, respectively) (Fig. 3A and B). The intraperitoneal treatment with EEAO (30 and 100 mg/kg) reduced the time of nociception induced by orofacial capsaicin in 65 ± 9% and 69 ± 6%, respectively (Fig. 3A). Similarly, EEAO (30 and 100 mg/kg, i.p.) dose-dependently inhibited the

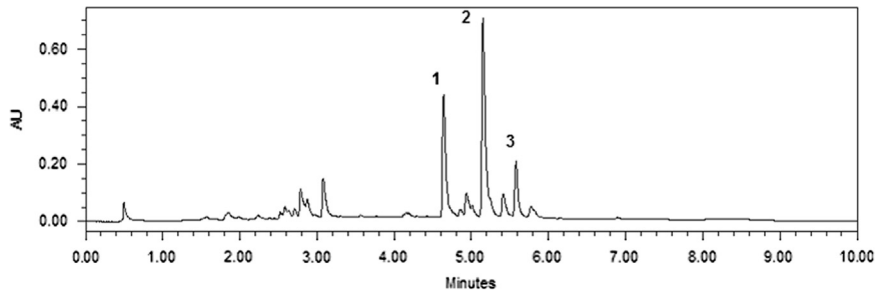

Fig. 1. Representative ultra performance liquid chromatography of ethanolic extract of *Acmella oleracea* flowers (EEAO) at λ 250 nm. [(2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide] (peak 1), [(2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide, spilanthol] (peak 2) and [(2E,6Z,8E)-N-(2-methylbutyl)-2,6,8-decatrienamide] (peak 3). Chromatographic conditions are described in the Methods section.

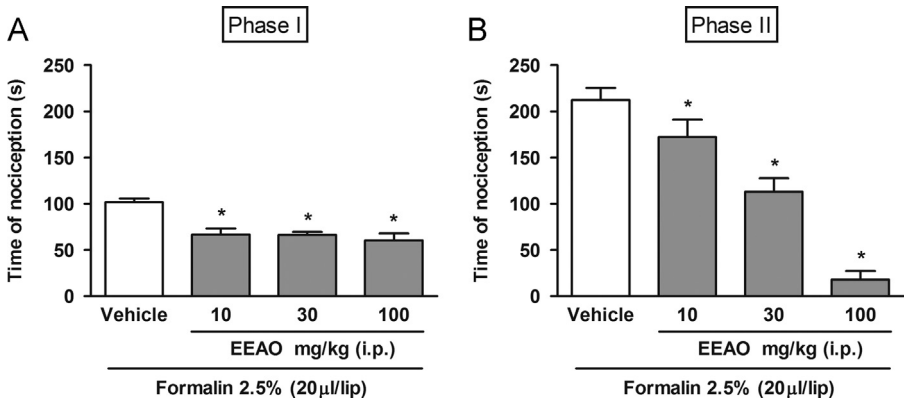

Fig. 2. Effect of ethanolic extract of *Acmella oleracea* flowers (EEAO) on neurogenic (A) and inflammatory (B) phase of nociception induced by orofacial 2.5% formalin in mice. The animals were intraperitoneally treated with vehicle (10 ml/kg) or EEAO (10, 30 and 100 mg/kg). Data are expressed as the means ± SEM (n=5–8). *$P < 0.05$ compared to the control (vehicle) group. (ANOVA followed by post-hoc Newman Keul's test).

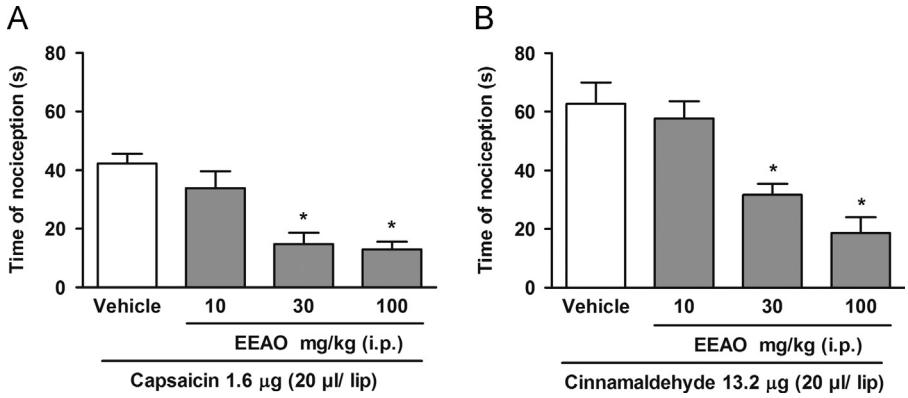

Fig. 3. Effect of ethanolic extract of *Acmella oleracea* flowers (EEAO) on nociception induced by orofacial capsaicin (1.6 µg/lip, A) and cinnamaldehyde (13.2 µg/lip, B) in mice. The animals were intraperitoneally treated with vehicle (10 ml/kg) or EEAO (10, 30 and 100 mg/kg). Data are expressed as the means ± SEM (n=5–8). *$P < 0.05$ compared to the control (vehicle) group. (ANOVA followed by post-hoc Newman Keul's test).

time of nociception induced by orofacial cinnamaldehyde with an ID_{50} value of 39.7 (25.5–62.0) mg/kg and inhibitions of 50 ± 6% and 70 ± 9%, respectively (Fig. 3B).

3.4. Effect of ethanolic extract of Acmella oleracea flowers on thermal hypersensitivity induced by capsaicin

Animals that received an intraplantar injection of capsaicin developed thermal hyperalgesia characterized by a significant reduction in the latency to noxious heat at 15 and 30 min (11.1 ± 2.4 s at baseline to 4.8 ± 0.7 s at 15 min). Interestingly, treatment of animals with EEAO (100 mg/kg, i.p.) was able to increase the paw withdrawal latency in 140%, 221% and 257% at 5, 15 and 30 min, respectively (Fig. 4A). Furthermore, EEAO 10 and 30 mg/kg also reduced capsaicin-induced thermal hyperalgesia, increasing the paw withdrawal latency in 113% at 30 min and in 147% at 15 min, respectively (Fig. 4A).

3.5. Involvement of opioid system on antinociceptive effect of ethanolic extract of Acmella oleracea flowers

Control animals that received vehicle treatments do not manifest any significant alterations in responsiveness to heat stimuli in the hot plate test. On the other hand, treatment of mice with EEAO (100 mg/kg, i.p.) increased the paw withdrawal latency in 31 and

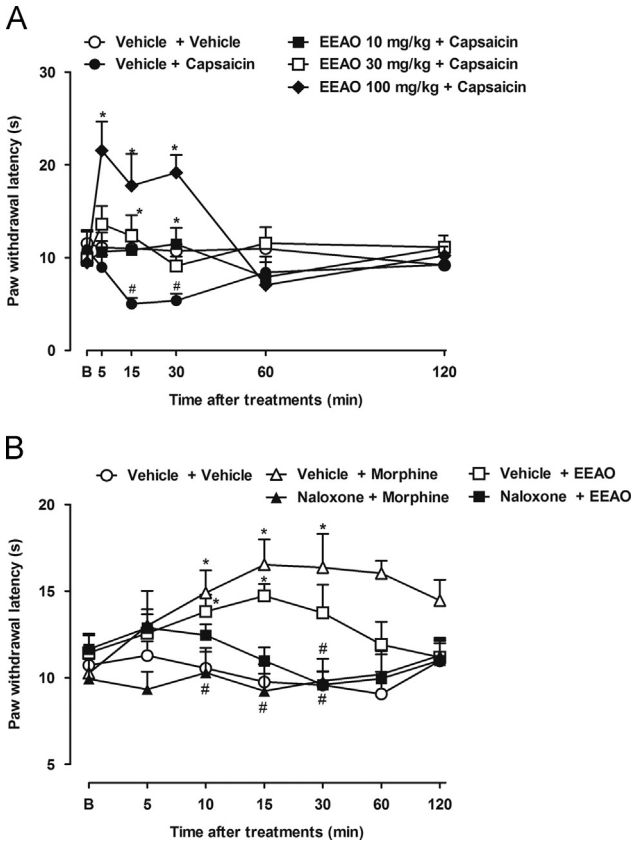

Fig. 4. Effect of ethanolic extract of *Acmella oleracea* flowers (EEAO) on heat hyperalgesia in mice. The animals were intraperitoneally treated with vehicle (10 ml/kg) or EEAO (10, 30 and 100 mg/kg) and the thermal hypersensitivity was induced by intraplantar injection of capsaicin (1.6 μg/paw, A). Effect of treatment with naloxone (1 mg/kg, i.p.) on the antihyperalgesic effect of morphine (1 mg/kg, s.c.) and EEAO (100 mg/kg, i.p., B). Each point represents the means ± SEM (n=5–8) of paw withdrawal latency (s) evaluated on hot plate at 55 ± 1 °C at the times indicated thereafter. $^{*}P<0.05$ compared to the control (vehicle) group and $^{\#}P<0.05$ compared to morphine or EEAO groups. (Two-way ANOVA followed by post-hoc Bonferroni's test).

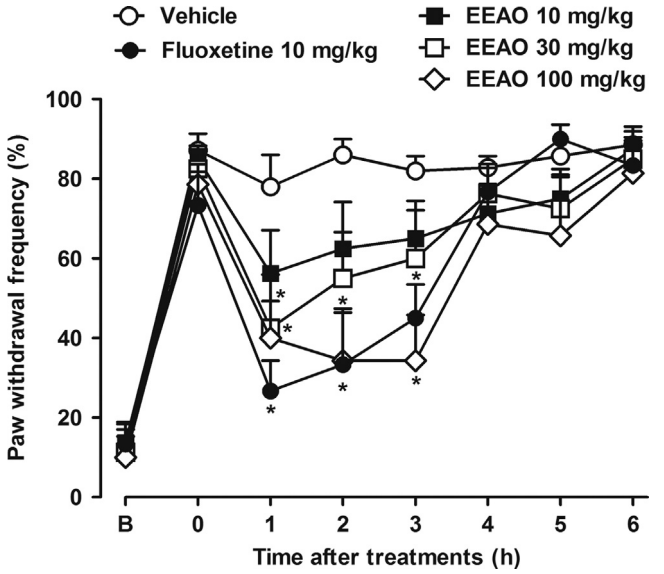

Fig. 5. Effect of ethanolic extract of *Acmella oleracea* flowers (EEAO) on mechanical allodynia induced by partial sciatic nerve ligation in mice. Seven days after nerve injury, the animals were intraperitoneally treated with vehicle (10 ml/kg, i.p.), EEAO (10, 30 and 100 mg/kg, i.p.) or fluoxetine (10 mg/kg, i.p.). Each point represents the mean ± SEM (n=5–8) of paw withdrawal frequency to von Frey hair (0.6 g) stimulation to the ipsilateral hind paw at the times indicated thereafter. $^{*}P<0.05$ compared to the control (vehicle) group. (Two-way ANOVA followed by post-hoc Bonferroni's test).

51% at 10 and 15 min, respectively. Morphine (1 mg/kg, s.c., used as positive control), also increased the paw withdrawal latency in 41%, 69% and 71% at 10, 15 and 30 min, respectively. Moreover, pre-treatment of mice with naloxone (1 mg/kg, i.p.) significantly reversed the antinociception caused by the reference opioid analgesic morphine (1 mg/kg, s.c.) at 10, 15 and 30 min, whereas was only effective in reversed the antinociception caused by EEAO at 30 min (Fig. 4B).

3.6. Effect of ethanolic extract of Acmella oleracea flowers on mechanical allodynia induced by partial sciatic nerve ligation

Animals that received a partial sciatic nerve ligation injury developed mechanical allodynia, which was characterized by a significant increase in the frequency of paw withdrawal threshold in response to 0.6 g von Frey filaments (12.9% at baseline vs. to 87.1% at 1 h for vehicle group). The treatment of neuropathic mice with EEAO (100 mg/kg) significantly reduced the mechanical allodynia induced by nerve injury, an effect that started at 1 h and was maintained up to 3 h after, reaching inhibitions of 49 ± 12%, 60 ± 15% and 58 ± 14%, respectively. Moreover, EEAO at the dose of 30 mg/kg was able to decrease the mechanical allodynia only at 1 h after treatment in 46 ± 17%. Finally, treatment of neuropathic mice with fluoxetine (10 mg/kg, used as positive control of the test) also reduced the mechanical allodynia in 66 ± 10%, 61 ± 15% and 45 ± 10% at 1, 2 and 3 h after treatment, respectively (Fig. 5).

3.7. Analysis of possible side-effects

The general behavioral changes of the mice were observed following a single intraperitoneal injection of EEAO at doses of 5, 50, 500 and 5000 mg/kg. Doses of 5, 50 and 500 mg/kg did not cause any detectable changes, signs or symptoms of toxicity. In addition, no death was recorded for these doses until the 7th day of observation. In contrast, treatment with the high dose of ethanolic extract (5000 mg/kg) caused respiratory alterations and convulsion, culminating with the death of all animals in up to 3 min after treatment. The calculated LD50 value was 889.14 mg/kg (95% confidence intervals: 740.95–1066.97 mg/kg). The internal organs of all treated mice did not show any unusual signs and were found to be normal in both size and color.

Lastly, the treatment of animals with EEAO (10, 30 and 100 mg/kg, i.p) did not affect the locomotor activity in the open-field test when compared to control group (Fig. 6).

4. Discussion

This study assessed the pharmacological activity of *Acmella oleracea* in experimental models of chemical, thermal and mechanical nociception, as well as acute toxicity. Thus, this study sought to confirm the analgesic properties of this antitoothache plant, and also to contribute to the pharmacological knowledge about it using ethanol extract from its flowers, which contains spilanthol, a relatively abundant component among other N-alkylamides, that induces tingling paresthesia and numbing analgesia in humans.

Surprisingly, the results of this study provides for the first time a phytochemical profile associated with pharmacological data about extracts of *Acmella oleracea* flowers. Recently, a phytochemical analysis of *Spilanthes acmella* reported that this plant contains amino

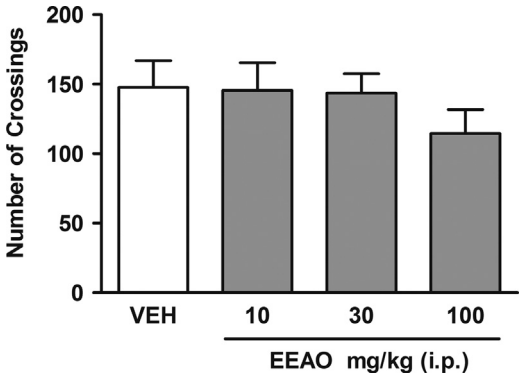

Fig. 6. Effect of ethanolic extract of *Acmella oleracea* flowers (EEAO) on locomotor activity in mice on the open field test. The animals were treated with vehicle (10 ml/kg, i.p.) or EEAO (10, 30 and 100 mg/kg, i.p.) and the number of total crossings were recorded for 5 min. Data are expressed as the means ± SEM (n=5–7).

acids, triterpenoids, α- and β-amyrin esters, stigmasterol, myricyl alcohol and alkaloids being particularly rich in alkylamides (for review see Dias et al., 2012). In addition, previous phytochemical investigations for the same genus attribute the presence of alkylamides for the biological effects of *Acmella oleracea* (for review see Tiwari et al., 2011; Sharma et al., 2011; Dias et al., 2012; Prachayasittikul et al., 2013). In EEAO, a preliminary UPLC analysis identified, with their different chromatographic peaks, three N-alkylamides, namely (2E,4Z)-N-isobutyl-2,4-undecadiene-8,10-diynamide, (2E,6Z,8E)-N-isobutyl-2,6,8-decatrienamide or spilanthol and (2E,6Z,8E)-N-(2-methylbutyl)-2,6,8-decatrienamide. Unfortunately, the phytochemical quantification of N-alkylamides in our ethanolic extract of *Acmella oleracea* flowers is currently unknown; however, considering the detected compounds, when comparing our data with others that investigated the chemical composition of ethanolic extracts of *Spilanthes acmella* (Sharma et al., 2011; Dias et al., 2012), it was clear that the pungent taste promoted when *Spilanthes* flowers are chewed is associated to the presence of alkylamides, with spilanthol recognized as the most abundant and responsible for the local anesthesia (Nakatani and Nagashima, 1992; Sharma et al., 2011; Tiwari et al., 2011; Dias et al., 2012; Prachayasittikul et al., 2013).

It is known that the trigeminal ganglion neurons have comparable neuronal populations to dorsal root ganglion, but their innervation is responsible for the pain sensation in orofacial area (Sessle, 2005). It is well established that formalin causes a biphasic nociception, initially characterized by the neurogenic phase, due to releasing of substance *P* and glutamate and more also by direct activation of TRPA1 and TRPV1 channels expressed on nociceptors (Luccarini et al., 2006; McNamara et al., 2007). Thus, after a short quiescent period, the following pain-related behaviors are associated to the release of inflammatory mediators like bradykinin and prostaglandins (Shibata et al., 1989). Regarding the largest group of noxious stimulus detectors, the transient receptor potential (TRP) channel family such TRPV1 is activated by heat (>42 °C) and capsaicin, whereas TRPA1 is activated by noxious cold (<18 °C) and cinammaldehyde, among various others stimuli (Caterina et al., 1997; Jordt et al., 2004; Patapoutian et al., 2009; Premkumar and Abooj, 2013). Perhaps the tingling effect promoted by N-alkylamides (i.e. spilanthol) may be mediated by interaction with TRPV1 or TRPA1 receptors. Thus, it appears reasonable to suggest that the analgesic effect of EEAO on the orofacial nociception mediated by capsaicin and cinammaldehyde could be related to TRPV1 and TRPA1 receptors modulation and/or blockade.

Previous studies indicated that a methanolic extract from flowers of *Acmella uliginosa* reduced the antinociceptive activity on formalin- and capsaicin-induced paw nociception in mice (Ong et al., 2011). Another study showed that an aqueous extract of aerial parts from *Spilanthes acmella* (a synonym of *Acmella oleracea*) possess antinociceptive effects in acute models such acetic acid-induced abdominal constrictions and tail flick tests and anti-inflammatory action in the carrageenan-induced paw edema (Chakraborty et al., 2004). Anti-inflammatory in vitro effects are also observed with a chloroform extract and isolated spilanthol obtained from flowers of *Spilanthes acmella*, through inhibitions of production of NO, iNOS protein expression, production of inflammatory cytokines (IL-1β, IL-6 and TNF-α), COX-2 expression and inhibitory effect on NF-κB activation in mouse macrophage RAW 264.7 cells (Wu et al., 2008). Altogether, these results suggest that one of the mechanisms whereby this genus of plants promotes antinociception could be associated to its anti-inflammatory properties. Of note is the fact that compounds that typically inhibit the first phase of formalin test include local anesthetics, such as lidocaine whereas the inflammatory phase is affected by intrathecal nonsteroidal anti-inflammatory drugs and morphine (for review see McNamara et al., 2007). In the case of genus *Spilanthes*, its antinociceptive effects have been related to diverse processes including inhibition of prostaglandins synthesis (Ratnasooriya et al., 2004), activation of opioidergic (Ong et al., 2011), serotoninergic and GABAergic systems (Acosta et al., 2009) and anesthetic activity through blockage of voltage-gated Na^+ channels (Chakraborty et al., 2010) (for review see Prachayasittikul et al., 2013). Taking into account, the present study extends and confirms previous reports that *Acmella oleracea* displays potent antinociceptive properties against nociception caused by formalin and TRPs channels agonists in the orofacial region of mice, reinforcing its popular use to relieve toothache.

We also investigated the possible antinociceptive effects of EEAO against thermal hyperalgesia in the hot plate test. Mice that received an intraplantar injection of capsaicin developed heat hyperalgesia that was completely blockade by high dose of ethanolic extract (100 mg/kg). Similarly, heat hyperalgesia induced by carrageenan in rats was attenuated by a cold-water extract of *Spilanthus acmella* flowers (Ratnasooriya and Pieris, 2005). It is noteworthy that both thermal hyperalgesia caused by intraplantar injection of capsaicin and that seen in the second phase of the hindpaw formalin test, reflects the development of central nociceptive sensitization in the dorsal horns of the spinal cord, which in turn is mediated by several mechanisms that ultimately lead to an enhancement of excitatory transmission at the synapses in the dorsal horns of the spinal cord (Coderre and Melzack, 1992; Tominaga et al., 2001; McNamara et al., 2007). However, it is not possible to conclude from the heat pain data whether the effect of ethanolic extract of *Acmella oleracea* flowers reported here is centrally or peripherally mediated, since hyperalgesic response on hot plate is attributed to the combination of both central and peripheral mechanisms (Kanaan et al., 1996).

Another finding of the preset study was the fact that the higher dose of EEAO shows an antihyperalgesic effect per se, that was only partially reduced by naloxone, a non-selective opioid receptor antagonist. Conversely, Ong et al. (2011) showed that the antihyperalgesic effect of methanolic extract from flowers of *Acmella uliginosa* on the hot plate test was significantly reverted by naloxone, suggesting the involvement of opioid system in this antinociceptive activity. Among the various possibilities which may have contributed to this apparent discrepancy are the differential plants species investigated (*Acmella oleracea* versus *Acmella uliginosa*), the contrast in the preparation of extracts (extraction solvents, i.e. ethanol versus methanol) as well as differences between doses of naloxone. Nonetheless, it is highly unlikely that the antihyperalgesic effect observed with EEAO involves an opioidergic mechanism.

Another important novelty of the present study results is that EEAO was also effective in reduce the nerve injury-induced mechanical allodynia, presenting similar antinociceptive effects to fluoxetine. Besides numerous analgesic agents are available, chronic neuropathic

pain is still difficult to treat and our data is quite relevant, suggesting for the first time the effectiveness of the *Acmella oleracea* for neuropathic pain treatment. Although the precise mechanisms underlying the pronounced antinociceptive effects of *Acmella oleracea* remain to be adequately elucidated, a recent study revealed that a structurally similar alkylamide named hydroxy-α-sanshool, produced by plants of the *Zanthoxylum* genus cause tingling sensations by blocking two-pore potassium channels (Bautista et al., 2008) and induces analgesia by blocking voltage-gated sodium channels in myelinated mechanosensitive nociceptors (Tsunozaki et al., 2013). Nonetheless, is pertinent to note that systemic administration of EEAO was active against orofacial and hind paw pain, at doses that promotes antinociceptive, antihyperalgesic and anti-allodynic effects without produced any impairment of locomotor activity in the open-field test, excluding the nonspecific disturbances on motor performance or sedation. Moreover, another interesting point we have considered is that EEAO did not show signs or symptoms of toxicity, indicating that this extract have a reasonably low toxicity profile, since according to Loomis and Hayes (1996) classification, chemical substance with a LD50 within the range of 5–15 g/kg is considered as practically non-toxic.

In summary, the results of the present study demonstrate for the first time that EEAO, rich in alkylamides, displays significant antinociceptive effects that resemble the pattern of an anesthetic activity in models of pain in mice, supporting at least in part, the ethnomedical uses of this plant. Although, to date, the precise mechanism underlying the antinociceptive action of *Acmella oleracea* remains unclear, this plant, rich in bioactive N-alkylamides, might be of potential interest in the future development of new clinically relevant drugs for the management of pain.

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References

Acosta II, M., Castaneda-Hernandez, G., Cilia-Lopez, V.G., Carino-Cortes, R., Perez-Hernandez, N., Fernandez-Martinez, E., Ortiz, M.I., 2009. Interaction between *Heliopsis longipes* extract and diclofenac on the thermal hyperalgesia test. Phytomedicine 16, 336–341.

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D., 2009. Cellular and molecular mechanisms of pain. Cell 139, 267–284.

Bautista, D.M., Sigal, Y.M., Milstein, A.D., Garrison, J.L., Zorn, J.A., Tsuruda, P.R., Nicoll, R.A., Julius, D., 2008. Pungent agents from *Szechuan* peppers excite sensory neurons by inhibiting two-pore potassium channels. Nature Neuroscience 11, 772–779.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D., 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389, 816–824.

Chakraborty, A., Devi, B.R., Sanjebam, R., Khumbong, S., Thokchom, I.S., 2010. Preliminary studies on local anesthetic and antipyretic activities of *Spilanthes acmella* Murr. in experimental animal models. Indian Journal of Pharmacology 42, 277–279.

Chakraborty, A., Devi, R.K., Rita, S., Sharatchandra, K., Singh, T.I., 2004. Preliminary studies on antiinflammatory and analgesic activities of *Spilanthes acmella* in experimental animal models. Indian Journal of Pharmacology 36, 148–150.

Coderre, T.J., Melzack, R., 1992. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. Journal of Neuroscience 12, 3665–3670.

Dias, A.M.A., Santos, P., Seabra, I.J., Junior, R.N.C., Braga, M.E.M, Sousa, H.C., 2012. Spilanthol from *Spilanthes acmella* flowers, leaves and stem obtained by selective supercritical carbon dioxide extraction. The Journal of Supercritical Fluids 61, 62–70.

Garcia-Martinez, C., Humet, M., Planells-Cases, R., Gomis, A., Caprini, M., Viana, F., De La Pena, E., Sanchez-Baeza, F., Carbonell, T., De Felipe, C., Perez-Paya, E., Belmonte, C., Messeguer, A., Ferrer-Montiel, A., 2002. Attenuation of thermal nociception and hyperalgesia by VR1 blockers. Proceedings of the National Academy of Sciences 99, 2374–2379.

Jordt, S.E., Bautista, D.M., Chuang, H.H., McKemy, D.D., Zygmunt, P.M., Hogestatt, E.D., Meng, I.D., Julius, D., 2004. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature 427, 260–265.

Kanaan, S.A., Saade, N.E., Haddad, J.J., Abdelnoor, A.M., Atweh, S.F., Jabbur, S.J., Safieh-Garabedian, B., 1996. Endotoxin-induced local inflammation and hyperalgesia in rats and mice: a new model for inflammatory pain. Pain 66, 373–379.

Ley, J.P., Krammer, G., Looft, J., Reinders, G., Bertram, H., 2006. Structure-activity relationships of trigeminal affects for artificial an naturally occurring alkamides related to spilanthol. Developments in Food Science 43, 21–24.

Litchfield Jr., J.T., Wilcoxon, F., 1949. A simplified method of evaluating dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics 96, 99–113.

Loomis, T.A., Hayes, A.W., 1996. Loomis's Essentials of Toxicology, 4th ed. Academic Press, San Diego.

Luccarini, P., Childeric, A., Gaydier, A.M., Voisin, D., Dallel, R., 2006. The orofacial formalin test in the mouse: a behavioral model for studying physiology and modulation of trigeminal nociception. Journal of Pain 7, 908–914.

Malmberg, A.B., Basbaum, A.I., 1998. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain 76, 215–222.

Malone, M.H., Robichaud, R.C., 1962. A Hippocratic screen for pure and crude drug materials. Lloydia 25, 320–332.

McNamara, C.R., Mandel-Brehm, J., Bautista, D.M., Siemens, J., Deranian, K.L., Zhao, M., Hayward, N.J., Chong, J.A., Julius, D., Moran, M.M., Fanger, C.M., 2007. TRPA1 mediates formalin-induced pain. Proceedings of the National Academy of Sciences 104, 13525–13530.

Miranda, H.F., Sierralta, F., Prieto, J.C., 2009. Synergism between NSAIDs in the orofacial formalin test in mice. Pharmacology Biochemistry and Behavior 92, 314–318.

Nakatani, N., Nagashima, M., 1992. Pungent Alkamides from *Spilanthes acmella* L. var. oleracea Clarke. Bioscience, Biotechnology, and Biochemistry 56, 759–762.

Nascimento, A.M., de Souza, L.M., Baggio, C.H., Werner, M.F., Maria-Ferreira, D., da Silva, L.M., Sassaki, G.L., Gorin, P.A., Iacomini, M., Cipriani, T.R., 2013. Gastroprotective effect and structure of a rhamnogalacturonan from *Acmella oleracea*. Phytochemistry 85, 137–142.

Ong, H.M., Mohamad, A.S., Makhtar, N., Khalid, M.H., Khalid, S., Perimal, E.K., Mastuki, S.N., Zakaria, Z.A., Lajis, N., Israf, D.A., Sulaiman, M.R., 2011. Antinociceptive activity of methanolic extract of *Acmella uliginosa* (Sw.) Cass. Journal of Ethnopharmacology 133, 227–233.

Patapoutian, A., Tate, S., Woolf, C.J., 2009. Transient receptor potential channels: targeting pain at the source. Nature Reviews Drug Discovery 8, 55–68.

Prachayasittikul, V., Prachayasittikul, S., Ruchirawat, S., Prachayasittikul, V., 2013. High therapeutic potential of *Spilanthes acmella*: a review. EXCLI Journal 12, 291–312.

Prachayasittikul, S., Suphapong, S., Worachartcheewan, A., Lawung, R., Ruchirawat, S., Prachayasittikul, V., 2009. Bioactive metabolites from *Spilanthes acmella* Murr. Molecules 14, 850–867.

Premkumar, L.S., Abooj, M., 2013. TRP channels and analgesia. Life Sciences 92, 415–424.

Ratnasooriya, W.D., Pieris, K.P., Samaratunga, U., Jayakody, J.R., 2004. Diuretic activity of *Spilanthes acmella* flowers in rats. Journal of Ethnopharmacology 91, 317–320.

Ratnasooriya, W.D., Pieris, K.P.P., 2005. Attenuation of persistent pain and hyperalgesia by *Spilanthus acmella* flowers in rats. Pharmaceutical Biology 43, 617–619.

Rodrigues, M.R., Kanazawa, L.K., das Neves, T.L., da Silva, C.F., Horst, H., Pizzolatti, M.G., Santos, A.R., Baggio, C.H., Werner, M.F., 2012. Antinociceptive and anti-inflammatory potential of extract and isolated compounds from the leaves of *Salvia officinalis* in mice. Journal of Ethnopharmacology 139, 519–526.

Sessle, B.J., 2005. Peripheral and central mechanisms of orofacial pain and their clinical correlates. Minerva Anestesiologica 71, 117–136.

Sharma, V., Boonen, J., Chauhan, N.S., Thakur, M., De Spiegeleer, B., Dixit, V.K., 2011. *Spilanthes acmella* ethanolic flower extract: LC-MS alkylamide profiling and its effects on sexual behavior in male rats. Phytomedicine 18, 1161–1169.

Shibata, M., Ohkubo, T., Takahashi, H., Inoki, R., 1989. Modified formalin test: characteristic biphasic pain response. Pain 38, 347–352.

Tiwari, K.L., Jadhav, S.K., Joshi, V., 2011. An updated review on medicinal herb genus *Spilanthes*. Zhong Xi Yi Jie He Xue Bao 9, 1170–1178.

Tominaga, M., Wada, M., Masu, M., 2001. Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proceedings of the National Academy of Sciences 98, 6951–6956.

Tsunozaki, M., Lennertz, R.C., Vilceanu, D., Katta, S., Stucky, C.L., Bautista, D.M., 2013. A 'toothache tree' alkylamide inhibits Adelta mechanonociceptors to alleviate mechanical pain. Journal of Physiology 591, 3325–3340.

Villachica, H., Carvalho, J.E.U., Muller, C.H., Diaz, S.C., Almanza, M., 1996. Frutales y Hortalizas Promissórios de la Amazônia. Tratado de Cooperacion Amazônica, Secretaria Protempore, Lima.

Wongsawatkul, O., Prachayasittikul, S., Isarankura-Na-Ayudhya, C., Satayavivad, J., Ruchirawat, S., Prachayasittikul, V., 2008. Vasorelaxant and antioxidant activities of *Spilanthes acmella* Murr. International Journal of Molecular Sciences 9, 2724–2744.

Woolf, C.J., 2010. What is this thing called pain? Journal of Clinical Investigation 120, 3742–3744.

Wu, L.C., Fan, N.C., Lin, M.H., Chu, I.R., Huang, S.J., Hu, C.Y., Han, S.Y., 2008. Anti-inflammatory effect of spilanthol from *Spilanthes acmella* on murine macrophage by down-regulating LPS-induced inflammatory mediators. Journal of Agricultural and Food Chemistry 56, 2341–2349.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.