Original Article

Evaluation of the orofacial antinociceptive profile of the ethyl acetate fraction and its major constituent, rosmarinic acid, from the leaves of *Hyptis pectinata* on rodents


**A R T I C L E   I N F O**

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**A B S T R A C T**

*Hyptis pectinata* (L.) Poit., Lamiaceae, popularly known as “sambacaitá,” is an aromatic shrub largely grown in the Brazilian northeastern. We investigated the antinociceptive effects of the ethyl acetate fraction obtained from the leaves of *H. pectinata* and of its main constituent rosmarinic acid, on formalin (2%), glutamate (25 µM)- and capsaicin (2.5 µg)-induced orofacial nociception in rodents. Male mice were pretreated with ethyl acetate fraction (100, 200 or 400 mg/kg, p.o.), rosmarinic acid (10 or 20 mg/kg, p.o.), morphine (5 mg/kg, i.p.), or vehicle (distilled water + 0.2% Tween 80). Ethyl acetate fraction reduced the nociceptive face-rubbing behavior during the two phases of the formalin test, whereas pretreatment with rosmarinic acid decreased the pain behavior in the second phase. Ethyl acetate fraction produced significant antinociceptive effects in the capsaicin and glutamate tests. This study showed that oral administration of ethyl acetate fraction produced potent antinociceptive effects compared to treatment with rosmarinic acid.

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**Introduction**

Pain in the oral and craniofacial system represents a major medical and social problem (Hargreaves, 2011). Indeed, a report from the U.S. Surgeon General on orofacial health concludes that, “…oral health means much more than healthy teeth. It means being free of chronic oral-facial pain conditions…” (National Institutes of Health, 2000). Moreover, orofacial pain is derived from many unique target tissues, such as the meninges, cornea, tooth pulp, oral/nasal mucosa, and temporomandibular joint, and thus has several unique physiologic characteristics compared with the spinal nociceptive system (Bereiter et al., 2008; Hargreaves, 2011). Thus, the management or treatment of orofacial pain conditions represents a significant health care problem and a challenge for the pharmaceutical industry.

In the last couple of decades, important progress has been made regarding the development of natural therapies. However, there is an urgent need to discover effective and safe analgesic agents (Calixto et al., 2000) and natural products have been shown to be strong candidates for development of new drugs for pain control (Quintans et al., 2014; Siqueira-Lima et al., 2014). Besides, a current approach is to develop new biological compounds from natural products that manage orofacial pain with enhanced efficacy and minimal side effects; these compounds are derived from medicinal plants or their secondary metabolites (Bonjardim et al., 2012; Guimarães et al., 2013; Quintans-Júnior et al., 2010; Venâncio et al., 2011).

*Hyptis pectinata* (L.) Poit., Lamiaceae, is a medicinal plant known as “sambacaitá” or “canudinho” in northeastern Brazil that is widely used to treat gastrointestinal disorders, skin infections, nasal congestion, fever, cramps, inflammation and pain (Bispo et al., 2001; Raymundo et al., 2011). Some studies have demonstrated that *H. pectinata* possesses antinociceptive and anti-inflammatory activities (Bispo et al., 2001; Lisboa et al., 2006; Raymundo et al.,...
Nascimento et al. (2008) described how *H. pectinata* leaves are used as treatment for several oral diseases, such as dental caries and orofacial pain. This pharmacological profile was corroborated by Paixão et al. (2013, 2015), who demonstrated an important neurogenic and inflammatory orofacial antinociceptive profile of the crude aqueous extract obtained from *H. pectinata* leaves and its possible application against other orofacial diseases such as periodontitis.

Besides, Arrigoni-Blank et al. (2005) demonstrated anti-edematogenic effect of the aqueous extract of *H. pectinata*. Hasanen and Mohammad Zaheri (2014) showed that rosmarinic acid (1), an ester of caffeic acid found in *Hyptis* species, reduces noiception in painful diabetic neuropathy model. Nevertheless, little is known how *H. pectinata* extract modulates orofacial pain transmission.

The present study proposed to verify the antinociceptive properties of the ethyl acetate fraction (EtOAc) of *H. pectinata* leaves and, in particular, rosmarinic acid (1) (RA), which was the major compound isolated from this fraction. The antinociceptive properties of EtOAc fraction were tested on mice following orofacial noiception induced by formalin, capsaicin and glutamate. three algogens agents that promote pain through different mechanisms and also by activation of different neuronal populations.

**Materials and methods**

**Plant material**

Plant material was obtained and extracted according to protocols described in Falcão et al. (2013). Voucher specimen (88157) is deposited at the Instituto Agronômico de Pernambuco, Recife, PE. Briefly, the leaves were dried, crushed and successively extracted with EtOH to obtain 7 g dry extract. This extract was dissolved in MeOH:H_{2}O (1:1) and successively fractionated with hexane and EtOAc. A portion of the EtOAc fraction (3.5 g) was subjected to chromatography on a Sephadex LH-20 column, and the compounds were purified using a semi-preparative HPLC column. This fractionation resulted in the isolation of sambacaitaric acid (2), 3-O-methyl-sambacaitaric acid (3), rosmarinic acid (1), 3-O-methyl-rosmarinic acid (4), ethyl caffeate (5), nepetoidin A (6), nepetoidin B (7), cirsiilol (8), cirsimaritin (9), 7-O-methyluteolin (10) and genkwanin (11) (Falcão et al., 2013). Rosmarinic acid was the major compound isolated from the EtOAc fraction, as shown in the chromatogram (Fig. 1) obtained by HPLC-DAD analysis.

**Animals**

Male Swiss mice (28–34 g), 2–3 months of age, were used throughout this study. The animals were randomly housed in appropriate cages at 22 ± 2 °C on a 12 h light/dark cycle (lights on between 6 am and 6 pm) with free access to food and water. All experiments were carried out between 9 am and 2 pm in a quiet room. All noiception tests were carried out by the same visual observer, and behavioral tests were performed under blind conditions. Experimental protocols were approved by the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS # 10/11).

**Drug and treatments**

Morphine hydrochloride (União Química, Brazil), 37% formaldehyde (Vetec, Brazil), diazepam (Roche, Brazil), Tween 80 (polyoxyethylene-sorbitan monooleate), glutamate, capsaicin and rosmarinic acid (RA) were purchased from Sigma (USA). During the noiception tests, the extract or agent was administered by oral gavage (p.o., per os) or intraperitoneally (i.p.) at a dose volume of 0.1 ml/10 g, with the exception of the noiception inducing agents, such as formalin, glutamate and capsaicin, which were injected subcutaneously (s.c.) into the right upper lip.

**Fig. 1.** Chromatogram from HPLC-DAD analysis and the compounds isolated from the EtOAc fraction of the leaves of *Hyptis pectinata*.

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Fig. 2. Effects of EtOAc fraction (*Hyptis pectinata*), rosmarinic acid (RA) or morphine (MOR) on formalin-induced orofacial noiception in mice. Vehicle (control), EtOAc (100, 200 or 400 mg/kg, p.o.), RA (10 or 20 mg/kg, p.o.) or MOR (5 mg/kg, i.p.) were administrated 1 h before formalin injection. (A) First phase (0–5 min) and (B) second phase (15–40 min). Each column represents the mean ± S.E.M. (n = 8 per group). *p < 0.05 or **p < 0.001 vs. the control (ANOVA followed by Tukey’s test).
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Nociception studies

Formalin test

Orofacial nociception was induced in mice by s.c. injection of 20 μl 2% formalin into the right upper lip (perinasal area), using a 27-gauge needle (Lucarini et al., 2006; Pelissier et al., 2002) according to previously reported methods with changes (Quintans-Júnior et al., 2010). This volume and concentration of formalin was selected during our pilot studies because it showed an intense nociception-related biphasic behavioral response (face-rubbing) at periods of 0–5 min (first phase) and 15–40 min (second phase) (Quintans-Júnior et al., 2010). Nociceptive behavior was quantified during these periods by measuring the time (s) that the mice spent rubbing their face at the injection area with its fore or hind paws (Lucarini et al., 2006). To assess the effects of the test drugs, groups of mice (n = 8 per group) were pretreated systemically with a vehicle control (distilled water + 0.2% Tween 80), EtOAc (100, 200 or 400 mg/kg, p.o.), or RA (10 or 20 mg/kg, p.o.) 1 h before the injection of formalin. Morphine (MOR, 5 mg/kg, i.p.) was administered 1 h before of nociceptive agent, as a positive control.

Capsaicin and glutamate tests

The orofacial nociception induced by capsaicin or glutamate in rodents was performed as described previously by Quintans-Júnior et al. (2010). Mice (n = 8 per group) were injected with capsaicin (20 μl, 2.5 mg) or glutamate (40 μl, 25 μM prepared in phosphate buffered saline) subcutaneously into the right upper lip (perinasal area), using a 27-gauge needle. Capsaicin was dissolved in ethanol, dimethyl sulfoxide and distilled water (1:1:8). An additional group received a similar volume of the capsaicin vehicle. Nociception quantification was performed by measuring the time(s) that the animals spent rubbing the orofacial region with greater intensity for a period of 42 and 30 min after the injection of capsaicin and glutamate, respectively. The EtOAc fraction (100, 200 or 400 mg/kg) or vehicle (distilled water + 0.2% Tween 80) was given by oral gavage to animals 1 h before the injection of capsaicin or glutamate, similar to the formalin test. MOR was used as a positive control and was administered (5 mg/kg, i.p.) 1 h before the nociception-inducing agent.

Evaluation of motor activity

In order to evaluate a possible non-specific muscle-relaxant or sedative effect of the extract, mice were submitted to the Rota-rod apparatus, as described by Quintans-Júnior et al. (2010). Initially, 24 h before the test, the mice that were able to remain on the Rota-rod apparatus (AVS® Brazil) longer than 180 s, at a speed of 9 rpm, were selected. Mice were treated with the EtOAc fraction (100, 200 or 400 mg/kg, p.o.), vehicle or diazepam (3 mg/kg, i.p.) 1, 2 and 4 h before being placed on the Rota-rod; each animal was tested on the Rota-rod apparatus, and the time (s) that the animal remained on the bar for up to 180 s was recorded.

Statistical analysis

Data obtained from the animal experiments were expressed as the mean ± the standard error of the mean (mean ± S.E.M.). Significant differences between the treated and the control groups were evaluated using the ANOVA and Tukey tests. Differences were considered to be statistically significant when p < 0.05. All statistical analyses were performed using GraphPad Prism 5 (Graph Pad Prism Software Inc., San Diego, CA, USA).

Results

The chemical analysis of the EtOAc fraction from H. pectinata leaves showed the presence of eleven compounds and the chromatogram (HPLC-DAD) is shown in Fig. 1. As seen in the chromatogram, rosmarinic acid (1) was the main component of this fraction. Four of the eleven isolated compounds are derived from rosmarinic acid, and compounds 2 (sambacaitaric acid) and 3 (3-O-methyl sambacaitaric acid) were identified as new natural compounds.

Acute treatment of mice with the EtOAc fraction (100, 200 or 400 mg/kg, p.o.) produced a significant reduction (p < 0.05 or p < 0.001) in the face-rubbing behavior (Fig. 2A and B) in both phases of formalin-induced nociception. Additionally, treatment with rosmarinic acid (10 or 20 mg/kg, p.o.) alone was effective in significantly reducing the nociceptive behaviors during the second phase, p < 0.05 or p < 0.001 respectively.

As shown in Fig. 3A, pretreatment with the EtOAc fraction (100, 200 or 400 mg/kg, p.o.) significantly reduced (p < 0.001) the face-rubbing behavior induced by capsaicin treatment. As expected, the group that received the vehicle treatment (ethanol, dimethyl sulfoxide and distilled water 1:1:8) did not present any nociceptive behavior (data not shown). Besides, acute treatment with the EtOAc fraction produced a significant reduction (p < 0.05 or p < 0.001) of glutamate-induced nociception when compared with the control group (Fig. 3B).

In the rota-rod test, ETOAc-treated mice did not show any significant motor performance alterations with the doses of 100, 200 or 400 mg/kg (Fig. 4). As expected, the CNS depressant diazepam (3 mg/kg, standard drug), reduced the time of animals on the rota-rod after 60, 120 and 240 min.
Discussions

In folk medicine used in northeastern Brazil, the H. pectinata plant ("sambacaita") is used in the treatment of several orofacial pathological disorders, including orofacial pain. However, there are no pharmacological studies that report this effect. For the first time, we demonstrate that oral administration of the EtOAc fraction from H. pectinata leaves exerts protective effects against formalin-, capsaicin-, and glutamate-induced orofacial pain in mice; this effect may be related to the presence of rosmarinic acid in this fraction.

In this study, the eleven compounds of the EtOAc fraction from H. pectinata leaves were isolated and identified according to the protocols described by Falcão et al. (2013). Rosmarinic acid (RA), a polyphenol commonly found in other species of this genus as Hyptis atrorubens, was identified as the main component of this fraction.

During the formalin test, the presence of two distinct phases is at least partially due to the mechanisms of nociception. The first phase is associated with the direct stimulation of the C-nociceptors by mechanisms dependent on TRPA1, whereas the second phase reflects the integration between the peripheral (nociceptors) and central (spinal/brainstem) signaling pathways (Capuano et al., 2009; Dallel et al., 1995; McNamara et al., 2007). All tested doses of EtOAc produced antinociception in both the first and second phases of the formalin test compared to the vehicle control. Synergistic effect of compounds in the extract, as polyphenols and flavonoids, certainly contributed to the reduction of formalin-induced nociception during the first phase, since flavonoids and phenols are among the main natural products studied pain, as demonstrated by Quintans et al. (2014).

Interestingly, Bispo et al. (2001) demonstrated that pretreating mice with the aqueous extract of H. pectinata at the doses of 200 and 400 mg/kg had no significant effect during the first phase of the formalin test, when the painful agent was applied in the paw. However, Paixão et al. (2013) showed a marked antinociceptive effect of the aqueous extract of H. pectinata in both phases of the orofacial formalin test and attributed this profile to a possible antioxidant effect of the extract. The discrepancies in the results of Bispo et al. (2001) and Paixão et al. (2013) may be due to the greater sensitivity of the orofacial region, the involvement of the trigeminal pathway (Sessle, 2011), or due to the chemical variation of plant secondary metabolites generated by environmental and genetic (Moore et al., 2014).

RA reduced only late phase of formalin test. A previous study showed that the administration of RA at higher doses reduces both phases of formalin-induced nociception, with the involvement of opioid mechanisms (Boonyarikpunchai et al., 2014; Hasanein and Mohammad Zaheri, 2014). Furthermore, this compound and some analogs could be useful in the prevention and treatment of inflammatory diseases, and inhibit inflammatory responses, such as neutrophilic migration and edema in the lungs of mice (Gamaro et al., 2011).

Capsaicin, an active ingredient in hot chili peppers, directly stimulates transient receptor potential cation channel subfamily
V member 1 (TRPV1), which are involved in the transmission and modulation of nociceptive activity, through selective actions on unmyelinated C-fibers and thinly myelinated A primary sensory neurons (Dray, 1992). Antagonists of TRPV1 receptors have been reported to exhibit a pain-relieving activity (Trevisani, 2013) and were effective in reducing nociception from inflammatory as well as neuropathic pain models in rats (Khairatkar-Joshi and Szallasi, 2009). Based on our finding, the oral administration of EtOAc produced a neurogenic inhibition against capsaicin-induced nociception indicating its ability to inhibit nociceptive transmission initiated by TRPV1 activation.

When glutamate is injected into the right upper lip (perinasal area) elicits a nocuous stimulus characterized by a behavioral response of rubbing the orofacial region (Quintans-Jünior et al., 2010; Siqueira et al., 2010). Noxious stimulation of primary afferent fibers results in the release of glutamate from the peripheral and central terminals of the trigeminal and spinal afferent fibers (Keast and Stephens, 2000; Lam et al., 2005). Our results demonstrated that EtOAc fraction reduced orofacial pain induced by glutamate. Thus, suggest that the suppression of glutamate-induced nociception by EtOAc treatment can be associated to its interaction with the glutamatergic system. These results are similar to those found by Guginski et al. (2009), who demonstrated that rosmarinic acid blocks the pain induced by glutamate on the mouse paw.

In summary, we demonstrated the EtOAc effect on orofacial pain induced by three algogens agents, which promote nociception by different mechanisms as TRPA1, TRPV1 and glutamate receptors (Caterina et al., 1997; McMamara et al., 2007; Willcockson and Valtoschano, 2008) with the possible participation of peptidergic and non-peptidergic primary afferent neurons (Kobayashi et al., 2005; Willcockson and Valtoschano, 2008). Additionally, previous study has shown the CNS action of the aqueous extract from H. pectinata leaves on rodent central nervous system (Bueno et al., 2006), with possible involvement of opioid system (Bispo et al., 2001). Thus, it is suggested that EtOAc has central antinociceptive actions and can be acting on perception of antinociceptive stimuli, signal transduction or its conduction to the central nervous system (CNS).

In this sense drugs that act on the CNS, such as diazepam or morphine, can impair motor coordination and generate confusion in analysis of results of nociceptive behavioral tests, such as those used here (Quintans-Jünior et al., 2010). Therefore, we evaluated whether pretreatment with EtOAc can impair the motor coordination of mice using a Rota–rod apparatus. However, all tested doses of EtOAc were ineffective in producing changes in motor coordination.

Taken altogether, our data led to the hypothesis that EtOAc had a protective role in orofacial nociception in mice. These effects may be related to the presence of rosmarinic acid and other compounds. These findings also support the hypothesis that H. pectinata has potential therapeutic use for painful facial and, perhaps, dental disorders. Nevertheless, further studies are needed to determine the precise mechanism of action.

Taken altogether, our data led to the hypothesis that EtOAc had a protective role in orofacial nociception in mice. These effects may be related to the presence of rosmarinic acid and other compounds. These findings also support the hypothesis that H. pectinata has potential therapeutic use for painful facial and, perhaps, dental disorders. Nevertheless, further studies are needed to determine the precise mechanism of action.

Authors’ contributions

REAF, SAS and CAC contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper, JSSQ, PLS, and AANL contributed to biological studies. REAF, SAS and CAC contributed in plant identification, herbarium confection and contributed to chromatographic analysis. JSSQ, LJQ, MTSC, TMSS contributed to critical reading of the manuscript. AGG and LJQ designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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