Journal of Ethnopharmacology 146 (2013) 192–197

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Orofacial antinociceptive effect and antioxidant properties of the hydroethanol extract of *Hyptis fruticosa* salmz ex Benth

Amanda C.B. de Lima, Monica S. Paixão, Mônica Melo, Marilia T. de Santana, Nicole P. Damascena, Antonio S. Dias, Yasmin C.B.S. Porto, Ximene A. Fernandes, Clisiane C.S. Santos, Clésio A. Lima, Lucindo J. Quintans Júnior, Charles dos S. Estevam, Brancilene S. Araújo *

Departamento de Fisiologia, Universidade Federal de Sergipe (UFS), Campus Universitário, 49100-000, São Cristóvão-SE, Brazil

ARTICLE INFO

Article history:
Received 2 August 2012
Received in revised form
27 November 2012
Accepted 2 December 2012
Available online 28 December 2012

Keywords:
Hyptis fruticosa
Orofacial pain
Antioxidant activity
Lipoperoxidation
Oxygen reactive species
Antinociceptive activity

ABSTRACT

Ethnopharmacological relevance: Hyptis fruticosa is a plant native to Brazil with antinociceptive and antiinflamatory properties. This study evaluated the antinociceptive activity of the hydroethanol extract of the plant leaves (CHEE) against orofacial pain as well as its in vitro effect against lipid peroxidation.

Materials and methods: The antinociceptive activity was investigated in mice orally treated with different doses of the CHEE (50, 100, and 200 mg/kg) and morphine (5 mg/kg) using formalin, glutamate, and capsaicin orofacial pain models using. Lipoperoxidation was induced in egg yolk by AAPH and $FeSO_4$ in the absence and presence of the CHEE (5, 50, 100, and 150 μg/mL).

Results: CHEE (200 mg/kg) significantly reduced ($\rho < 0.001$) the pain response in the first (69.6%) and second (81.8%) phases of the formalin test, while the nociception caused by capsaicin was significantly ($\rho < 0.001$) reduced by up to 62% at 200 mg/kg of extract. When glutamate was used as algogen, a significant ($\rho < 0.001$) nociception reduction of up to 85% at 200 mg/kg extract was observed. CHEE showed a higher protection against lipoperoxidation caused by $FeSO_4$ (82.3% TBARS inhibition) than AAPH (35.7% TBARS inhibition) at 150 μg/mL.

Conclusion: Hyptis fruticosa leaf CHEE is of pharmacological interest because it was able to inhibit the peripheral and central transmission of orofacial pain, while reducing the spreading of the inflammatory processes by neutralizing reactive oxygen species, which are by-products in the biosynthesis of pain mediators.

1. Introduction

Pain is a complex multidimensional phenomenon linked to nociception recognition, which can be defined as the perception of a lesion in tissues. It is controlled by a receptor system that sends information to the brain through specialized nervous fibers (Heredia and Rodrigues, 2008; Szumita et al., 2010). In this context, the orofacial region is a common part of the body for pain to occur, which is acute and frequently associated with respiratory, head, and neck syndromes (Macfarlane et al., 2001; Luccarini et al., 2006; Bonjardim et al., 2011). Despite its importance, the mechanisms by which the orofacial pain occurs are still poorly understood, probably due to few animal models to study nociception in this area (Bonjardim et al., 2011). In addition, there are difficulties in treating both acute and chronic orofacial pain when they are caused by neuropeptides or leukotrienes because nonsteroidal anti-inflammatory drugs (NSAIDs) are not effective in inhibiting these inflammatory mediators (Lindenmeyer et al., 2010). Therefore, the development of alternative therapies is necessary.

Hyptis fructicosa (Lamiaceae) is known as "alecrim de tabuleiro" and can be found in the coastal tablelands and lowlands of northeast Brazil, where it is an important medicinal plant whose leaves are directly chewed or the leaf tea is used by the population to treat pain (Menezes et al., 2007; Agra et al., 2008; Franco et al., 2011). *Hyptis fruticosa* has been the subject of some studies, which reported the toxicities of the essential oil and leaf aqueous extract, antibacterial and antineoplasic activities of compounds isolated from the plant roots, and cardiovascular effects of the essential oil (Silva et al., 2006; Menezes et al., 2007; Santos et al., 2007). Regarding pain and inflammation, the analgesic and antinociceptive activities of the *Hyptis fruticosa* essential oil and leaf aqueous extract were also demonstrated (Menezes et al., 2007; Franco et al., 2011). Nevertheless, despite of

* Corresponding author. Tel./fax: +55 79 2105 6647.
E-mail address: bsa@ufs.br (B.S. Araújo).

0378-8741
http://dx.doi.org/10.1016/j.jep.2012.12.031

the plant being used to treat oral conditions, no study was done to demonstrate its effect on the orofacial pain. Therefore, the present study aims to improve the knowledge of the antinociceptive activity of this plant by studying its potential to treat orofacial pain using formalin, glutamate, and capsaicin pain models, as well as its possible antioxidant and anti-lipoperoxidative activity.

2. Materials and methods

2.1. Plant material

Hyptis fruticosa Salzm. Ex Benth. leaves were collected in July 2009, in the village Beans, São Cristóvão, Sergipe (10° 56'S, 37° 05'W). The plant was identified by Profa. Dra. Ana Paula Silver, botanist of the Department of Biology, Federal University of Sergipe, Brazil (DB–UFS). A voucher specimen was deposited in the Herbarium of the DB–UFS under the registration number SEA 13649.

2.2. Extract preparation

Leaves were dried at environment temperature and triturated in a knife mill to give a fine powder (900.5 g), which was subjected to extraction by maceration with 90% ethanol for 7 days. The extract was concentrated by solvent evaporation in a rotary evaporator under reduced pressure to give the crude hydroethanol extract (CHEE, 381.3 g, yield 42.3%).

2.3. Phytochemical analysis

The qualitative identification of the main classes of the secondary metabolites presents in the CHEE was done by using the colorimetric methods (Table 1) proposed by Sousa et al. (2007) and Matos (2009).

2.4. Total phenol quantification

The total phenol content was spectrophotometrically determined using the Folin–Ciocalteu method (Sousa et al., 2007). The reaction mixture was composed of extract (0.1 mL), distilled water (7.9 mL), Folin–Ciocalteu reagent (0.5 mL), and 20% sodium carbonate (1.5 mL). After 2 h, the absorbance of the resulting solutions was measured at 750 nm and applied in the calibration curve built with known concentrations of gallic acid (GA) standards (0.01 to 0.35 mg/mL). Results were expressed as milligram equivalents of gallic acid per g of extract (EAG mg/g). All analyses were carried out in triplicate.

Table 1
Phytochemical analysis of the crude methanol extract of *Hyptis fructicosa* leaves and its fractions.

Metabolites	Assays	CHEE
Alkaloids	Mayer's reagent/Dragendorff's reagent	–
Phenols in general	Folin Ciocaultey reagent	+
Flavonoids in general	Hydrochloric acid/magnesium	+
Flavones	pH change	+
Flavononols	pH change	+
Tannins	Cerric sulphate	+
Saponins	Clorofórmio	+
Steroids	Lieberman–Bouchard's reagent	+
Triterpenes	Anysaldehyde/Sulfuric acid	+
Xanthones	pH change	+

2.5. In vitro lipid peroxidation inhibitory activity

The degree of lipid peroxidation prevented by the hydroethanol extract was monitored by measuring the production of thiobarbituric acid-reactive substances (TBARS) (Budni et al., 2007). Briefly, egg yolk homogenate (1%w/v, 1 mL) in phosphate buffer (pH 7.4) was sonicated (10 s) and mixed with freshly prepared solutions of the hydroethanol extract and controls at 5, 50, 100 and 150 μg/mL. Lipid peroxidation was induced by adding either 2′-azobis(2-amidinopropane) dihydrochloride (AAPH, 0.17 mol/L, 0.1 mL) or ferrous sulphate ($FeSO_4$, 0.17 mol/L, 0.1 mL). Trolox was used a positive control, while the negative control was the vehicle (water or ethanol). The mixture was incubated at 37 °C for 30 min. Upon cooling, samples (0.5 mL) were centrifuged with 15% trichloroacetic acid (TCA, 0.5 mL) at 1200 rpm for 10 min. Supernatant was taken (0.5 mL), mixed with 0.67% thiobarbituric acid (TBA, 0.5 mL), incubated at 95 °C for 60 min and, after cooling, the formation of TBARS was measured by reading the supernatant absorbance at 532 nm. Results were expressed as inhibition percentage of TBARS formation.

2.6. Antinociceptive activity

2.6.1. Animals

Male Swiss mice weighing 20–35 g each, obtained from the Central Animal House of the Federal University of Sergipe, were randomly maintained in cages under controlled temperature (22 ± 3 °C) with light/dark cycles of 12 h (lights on from 06h00 to 18h00). The animals had free access to food (Purina™) and tap water. Animal Care and Use Committee of Federal University of Sergipe approved the experimental protocols and procedures under the registration CEPA/UFS number 65/09.

2.6.2. Formalin test

Orofacial nociception was induced in mice by injection of 2% formalin (20 μL, s.c) in the right upper lip (perinasal area) using a 27-gauge needle (Luccarini et al., 2006). The volume and formalin concentration were selected from pilot studies that showed a nociceptive-related biphasic behavioral response of great intensity at periods from 0 to 5 min (first phase) and 15 to 40 min (second phase). Nociception was quantified by measuring the time (sec) animals spent rubbing their faces in the injected area with their fore- or hind paws. To assess the effects of the test drugs, groups of mice ($n=8$) were systematically pretreated with vehicle (one drop of 0.2% Tween 80 in distilled water) and CHEE (50, 100, and 200 mg/kg, v.o.) 1 h before the formalin injection. Morphine (MOR, 5 mg/kg, i.p.), administered 1 h before the algogen, was included as positive control.

2.6.3. Capsaicin test

Orofacial nociception was induced by capsaicin (Pelissier et al., 2002) as described for formalin. Capsaicin (20 μL, 2.5 μg, s.c.) was injected in the perinasal area and the drug effects were evaluated using the same groups as described previously, which were administered 1 h before the algogen. Capsaicin was dissolved in ethanol, dimethyl sulfoxide and distilled water (1:1:8), and the face-rubbing behavior was observed during 42 min. MOR (5 mg/kg, i.p.) was used as positive control.

2.6.4. Glutamate test

In an attempt to provide evidence of the CHEE interaction with the glutamatergic system, its antagonistic effect on glutamate-induced orofacial nociception in mice was studied using the method previously described by Beirith et al. (2002) with modifications. Glutamate (20 μL, 25 μmol/L in phosphate buffered

saline) was injected in the perinasal area of mice and the animals were observed individually during 15 min. Nociception was quantified by measuring the time (sec) the animals spent rubbing the injected area with their fore- or hind paws. Animals in the groups were injected with the CHEE, MOR or vehicle, as previously described for formalin, 1 h before the glutamate injection.

2.7. Statistical analysis

Data obtained were evaluated by One-Way Analysis of Variance (ANOVA) followed by Dunnett's test for mean differences to be considered statistically significant ($\rho < 0.05$).

3. Results

The phytochemical screening of the *Hyptis fruticosa* CHEE, whose results are shown in Table 1, revealed the presence of two main classes of secondary metabolites: phenolic compounds and terpenes. The total phenol concentration determined for the CHEE was 360.51 ± 16.29 EGA mg/g extract.

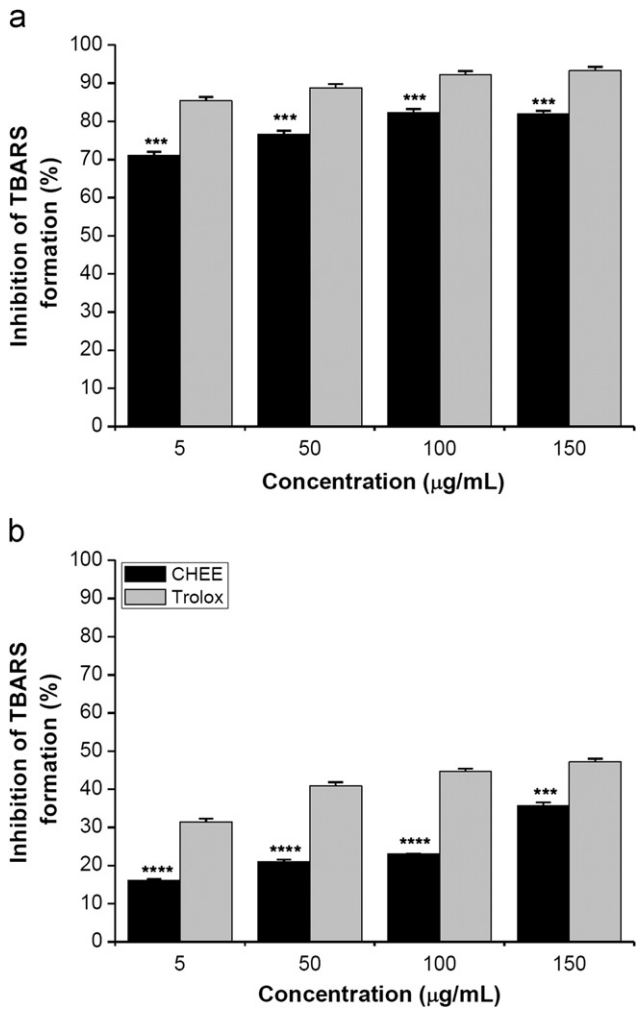

Fig. 1. Effect of *Hyptis pectinata* CHEE on the amount of TBARS (Thiobarbituric Acid-Reactive Substances) produced in the presence of the free radical inducers $FeSO_4$ (A) and AAPH (B). The effect of different concentrations of the CHEE and the standard antioxidant Trolox in preventing egg yolk lipoperoxidation were spectrophotometrically measured at 532 nm. Bars represent mean ± SEM. $^{***}P < 0.001$, when compared to control. One-way ANOVA followed by Dunnett's post hoc test was applied to all data.

In the present study, in vitro lipoperoxidation of egg yolk was induced by two chemical substances, AAPH and $FeSO_4$, to study the antioxidant effect of the CHEE against lipid oxidation. In presence of $FeSO_4$, the extract showed a high protection against lipoperoxidation with inhibition of the TBARS ranging from 71 to 83%. The highest inhibition (82.3%) was observed at 150 μg/mL (Fig. 1A). When the lipoperoxidation was induced by the AAPH, its inhibition varied from 16 to 36% and the highest value was observed at 150 μg/mL (Fig. 1B). However, lipoperoxidation inhibition was significantly ($\rho < 0.01$, $\rho < 0.001$, respectively) lower than the controls for both inducers (Fig. 1A and B).

The effect of CHEE on the nociception induced by formalin, which was characterized by the reduction of the face rubbing time after the chemical stimulus, is shown in Fig. 2. The extract in all doses used significantly reduced ($\rho < 0.001$) pain response in both phases of the test in comparison with the control as follow: 37.7%, 38.1%, and 69.6% (first phase) and 61.9%, 74.3%, and 81.8% (second phase) for 50, 100, and 200 mg/kg, respectively.

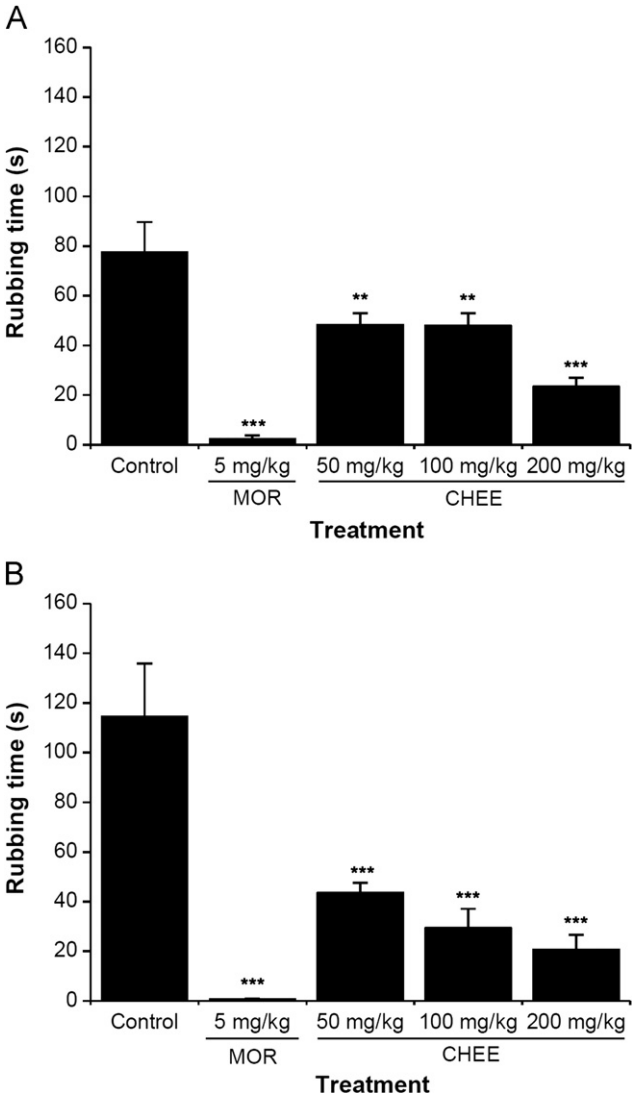

Fig. 2. Antinociceptive effect of *Hyptis fruticosa* CHEE at different concentrations and morphine (MOR) in the pain response induced during the first (A) and second (B) phases of the formalin test. Orofacial nociception was induced in mice by injecting 2% formalin in the right upper lip with a needle and quantified by measuring the time (sec) animals spent rubbing their faces in the injected area with their fore- or hind paws. Bars represent mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$, when compared to control. One-way ANOVA followed by Dunnett's post hoc test was applied to all data.

According to these findings, both phases of pain are affected in a dose-dependent manner by CHEE at 100 mg/kg and higher doses.

A dose-dependent antinociceptive effect was observed when capsaicin was used as a nociceptive inducer (Fig. 3A), with inhibition of mice face rubbing varying from 14 to 62%, which was significant at doses of 100 ($\rho < 0.01$) and 200 ($\rho < 0.001$) mg/kg of extract. As shown in Fig. 3B, when the *Hyptis fruticosa* CHEE was administered orally to mice, it caused a dose-dependent inhibition of the glutamate-induced nociception by reducing mice face rubbing from 43 to 85%. In addition, all doses of the extract showed a significant reduction of the nociception ($\rho < 0.05$, $\rho < 0.01$, $\rho < 0.001$, respectively) caused by glutamate when compared with the control. ED_{50} values for the extract were 76.07 and 150.63 mg/kg, respectively, for glutamatate and capsaicin antinociceptive tests.

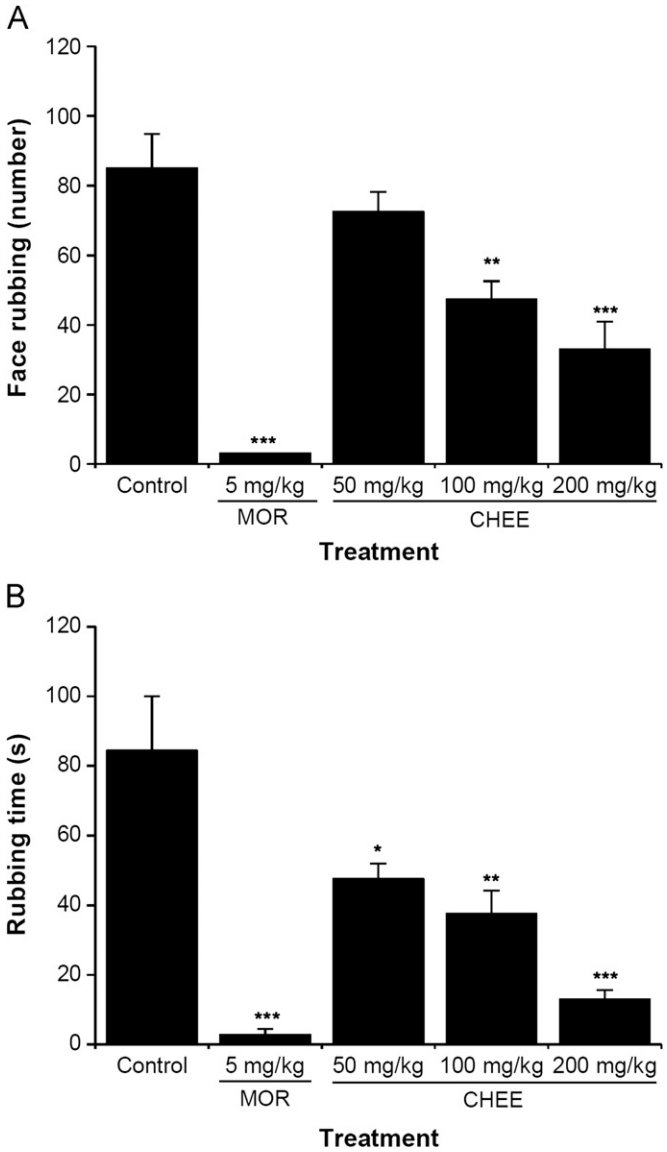

Fig. 3. Antinociceptive effect of *Hyptis fruticosa* CHEE at different concentrations and morphine (MOR) in the pain response induced by 2.5 μg/kg capsaicin (A) and 25 μM glutamate (B) injection in right upper lip of mice with a needle. Nociception was quantified by measuring the time (sec) animals spent rubbing their faces in the injected area with their fore- or hind paws. Bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, when compared to control. One-way ANOVA followed by Dunnett's post hoc test was applied to all data.

4. Discussion

Face sensory information is processed by the trigeminal nerve, which is also related to motor activities such as biting, chewing, and swallowing. This nerve is divided into three main branches called the ophthalmic nerve (sensory), maxillary nerve (sensory), and mandibular nerve (sensory and motor). The maxillary nerve carries sensory information caused by chemical, thermal, and mechanical stimulus from the lower eyelid and cheek, the nares and upper lip, the upper teeth and gums, the nasal mucosa, the palate and roof of the pharynx, the maxillary, ethmoid, and sphenoid sinuses, and parts of the meninge, towards the central nervous system, where the noxious stimuli are frequently translated as orofacial pain. CHEE effect in preventing orofacial pain caused by chemical stimulation of the maxillary nerve in the upper lip of mice was investigated in the present since the extract and essential oil of the plant previously showed antinociceptive activity (Silva et al., 2006, Menezes et al., 2007; Franco et al., 2011).

In the formalin orofacial test, an established pre-clinical model to investigate the analgesic potential of substances on facial pain, formaldehyde induces damages in tissues to imitate acute post-lesion pain in humans with a biphasic response (Bonjardim et al., 2011). In the acute phase, called neurogenic, nociception is caused by direct activation of the nociceptive fibers, which release substance P, glutamate, and bradykinin, among other pain mediators (Luccarini et al., 2006; Bonjardim et al., 2011). The late phase, which is inflammatory, is mediated by the release of inflammatory mediators such as nitric oxide, excitatory amino acids (aspartate and glutamate), prostacyclins and prostaglandins (PGE2), and leukotrienes, which will later stimulate the spinal cord and cause pain (Tjølsen et al., 1992; Bonjardim et al., 2011; Venâncio et al., 2011). Results in the present study showed the CHEE inhibited both nociceptive phases induced by formalin, suggesting the extract has central and peripheral antinociceptive activity, similarly to morphine, the standard drug used for nociception.

The effect of CHEE on orofacial pain was further investigated by the glutamate test. Glutamate, an excitatory amino acid, is found primarily in Type C sensory fibers and transmits nociceptive information involving peripheral, spinal, and supraspinal sites of action. (Beirith et al., 2002; Bonjardim et al., 2011). Glutamate is released from afferent fibers sensitized by nerve or tissue injury or by response to noxious stimuli (Keast and Stephensen, 2000; Lam et al., 2005). The main mechanisms used by agonists of glutamate receptors to cause pain include the intracellular release of Ca^{2+}, activation of cellular mediators, and the opening of ion channels (Lipton and Rosenberg, 1994; Millan, 1999), which are associated with an increase in the production of NO, protein kinase C activity, and calcium inflow (Liu et al., 1997; Ji and Stricharstz, 2004). In addition, glutamate combined with other mediators and pro-inflammatory cytokines (TNF-α and IL-1β) act synergistically in neuronal excitation (Liu et al., 1997; Millan, 1999; Bernardino et al., 2005). The results of the present study showed that CHEE inhibited the nociception caused by glutamate with significant results for the highest concentration used (200 mg/kg), suggesting that the extract can be active against sensory information transport associated with this amino acid. This may be related to the inhibition of glutamatergic receptors such as *N*-methyl-D-aspartate (NMDA), kainate (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (Beirith et al., 2002).

Vanilloid receptors (TRPV1) are involved in orofacial nociception by contributing to the integration and detection of chemical and thermal stimulus in peripheral sensory neurons such as Type C and Aδ. These receptors can be activated by capsaicin, the

pungent principle of the red pepper, which mimics a heat sensation acting selectively on sensory neurons to promote pain (Caterina and Julius, 2001). TRPV1 activation by capsaicin will induce a cation inflow to produce neuron depolarization and excitation (Pelissier et al., 2002) so that neuropeptides such as tachykinins (neurokinin A), substance P, calcitonin peptide, excitatory amino acids (aspartate and glutamate), nitric oxide, and other pro-inflammatory substances are released from peripheric terminals to transfer the painful stimulus through the trigeminal nerve (fifth cranial nerve) (Holzer, 1991; Waning et al., 2007; Honda et al., 2008). CHEE at 100 and 200 mg/kg was able to reduce the capsaicin nociceptive effect, suggesting it is an antagonist of vanilloid receptors and modulates the transfer of pain sensory information through the trigeminal nerve.

During inflammatory processes, free radicals such as nitric oxide and reactive oxygen species derived from the prostaglandin biosynthesis are released in the organism, where they will propagate pain and inflammation by activating nociceptive receptors such as TRPV1 and NMDA (Ma et al., 2009; Chen et al., 2010) and cause the oxidation of membrane components such as lipids. This will damage the cell membrane, its metabolism and create the conditions needed for the establishment of free radical related diseases such as cancer and diabetes (Wilhelm, 1990). Therefore, CHEE was investigated for its action against lipid oxidation using the lipoperoxidative inducers AAPH to generate peroxyl radicals able to remove hydrogen from peroxidable lipids, and $FeSO_4$ to generate hydrogen peroxide or hydroxyl radicals, which cause lipid peroxidation and give lipid alkoxyl radicals (Hanlo and Seybert, 1997; Budni et al., 2007; Mendez-Sanchez et al., 2008). The results found in the present study showed that the phenolics present in CHEE, as it was shown by the colorimetric methods in Table 1 as well as the total phenol quantification, are able to reduce the peroxidation of lipids caused by hydrogen peroxide and hydroxyl radicals, while they are less effective against the peroxidation caused by peroxyl radicals. The selectivity for certain types of free radicals is related to the hydroxyl groups in the aromatic rings of phenolics. Hydroxyl groups can donate their hydrogen to the free radical and the aromatic ring turned into a radical itself is then stabilized by electron resonance. It was pointed out the hydrogen donation and stabilization by resonance depend of the position and number of hydroxyl as well as the number of conjugated aromatic rings and/or double bonds so the free electron can generate several resonance forms of the molecule. Therefore, the higher the number of resonance forms the higher the probability of a certain phenolic to scavenger free radicals (Van Acker et al., 1996; Ainsworth and Gillespie, 2007; Budni et al., 2007).

In conclusion, the *Hyptis fruticosa* leaf CHEE at 100 and 200 mg/kg can be of pharmacological interest because it is not only able to inhibit the peripheral and central transmission of orofacial pain, but also shows potential in reducing the spreading of the inflammatory processes by neutralizing reactive oxygen species, which are by-products in the biosynthesis of pain mediators.

Aknowledgments

The authors wish to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) for financial support of this research.

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