Antioxidant and antinociceptive effect of the hydroethanolic extract and fractions of the bark of *Bowdichia virgilioides* in orofacial pain

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*Bowdichia virgilioides* is used by the population for treating inflammation in general. This study evaluated the antinociceptive activity and possible mechanisms of action of hydroethanolic extract (HEE) and hexane (HXF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fraction of HEE obtained from the plant stem bark against orofacial pain, as well as *in vitro* its antioxidant effect on the scavenging of free radical DPPH•. The antioxidant activity of the extract and fractions was evaluated against DPPH at concentrations of 5, 15, and 25 μg/mL for each sample studied, with gallic acid used as positive control. The absorbance decrease was spectrophotometrically measured at 515 nm up to 60 min to obtain the percentage of inhibition of the free radical. The antinociceptive activity was investigated in Swiss mice treated orally with HEE, EAF and HMF (100, 200, and 400 mg/kg) and morphine (5 mg/kg), using formalin, glutamate and capsaicin orofacial pain models. HEE, EAF and HMF showed the best results regarding the reduction of the DPPH radical (25 μg/mL, 60 min), with percent inhibition of 42.89, 78.52 and 54.96%, respectively. HEE, EAF and HMF significantly (p < 0.001) reduced orofacial nociception in mice in the first (56, 49 and 19%, respectively) and second phases (61, 71 and 69%, respectively) of the formalin pain model, as well as glutamate (82, 69 and 39%, respectively) and capsaicin (49, 68 and 64%, respectively) assays. Animals showed no significant changes in motor performance after treatment with HEE, EAF and HMF in the Rota rod test. In general, the potential of *B. virgilioides* to treat orofacial pain in its central and peripheral components was confirmed, with HEE, EAF and HMF (mainly at 200 and 400 mg/kg) showing antinociceptive effect in three different orofacial pain models related to opioid, glutamatergic and vanilloid receptors. In addition, it is also possible that their antioxidant activity may be related to the observed antinociceptive effect by reducing the biosynthesis of ROS and other inflammatory mediators.

**Key words:** *Bowdichia virgilioides*; black sucupira; antioxidant activity; orofacial pain; antinociception; free radical.
INTRODUCTION

Boudichia virgilioides, popularly known as "black sucupira", is a leguminous tree belonging to the family Fabaceae and occurs mainly in the North and Northeast, as well as in the Central Plateau of Brazil (Albuquerque and Guimarães, 2007). In folk medicine, B. virgilioides is used for treating diarrhea, rheumatism, headache and aneurysm (Arriga et al., 2000; Gomes et al., 2008), malaria (Mariath et al., 2009) and general inflammation (Smidler and Sousa, 2003). Previous studies have shown antimalarial activity (Deharo et al., 2001), antimicrobial (Almeida et al., 2006), anxiolytic (Vieira et al., 2013), antioxidant, anti-inflammatory and analgesic activities, which showed the plant can be used for treating diseases in which the patient is affected by pain (Thomazzi et al., 2010).

Among painful disorders is the orofacial pain, which is characterized by painful conditions in the hard and soft tissues of the head, face, neck and all intraoral structures (Macfarlane et al., 2002; Fan et al., 2012). Pain signal is carried by the trigeminal system, which is a dense nerve network that involves the fifth cranial nerve or trigeminal responsible for pain perception in the orofacial region (Viggiano et al., 2005). Anticonvulsants, antidepressants and opioids are used for treating orofacial pain. However, this therapy causes various side effects, which often prevent their use as well as prevent the patient to adhere to the treatment (Rang et al., 2003; Antonialli et al., 2012).

It is also necessary a better understanding of the physiological and pathophysiological pathways in the trigeminal system to found drugs able to reduce pain. Studies have demonstrated that reactive oxygen species (ROS) are produced during persistent facial pain. It was found that these substances are necessary for the transmission of pain signals (Meotti, 2006; Guimarães et al., 2010), thus showing that a pathway to minimize pain propagation is the reduction of ROS production. Therefore, there is a correlation with antioxidant systems (Viggiano et al., 2005; Lewis et al., 2007).

The antinociceptive and antioxidant activities of the aqueous extract of B. virgilioides were demonstrated by Silva et al. (2010) and Thomazzi et al. (2010). However, the effect of this species was not reported for orofacial pain.

This study aimed to improve the understanding of the antioxidant mechanisms based on the evaluation of the scavenging activity against free radical DPPH+ as well as to evaluate the orofacial antinociceptive effect in the models of orofacial pain induced by formalin, capsaicin and glutamate, using the hydroalcoholic extract and fractions obtained from the bark of B. virgilioides.

MATERIALS AND METHODS

Collection and identification of plant

The bark of B. virgilioides was collected in March 2011 in the village Fazenda Riachão, city of Japaratuba, state of Sergipe, Brazil (10°32′44" S and 36°53′57" W). The plant was identified by Dr. Ana Paula Silvera, botanist of the Department of Biology, Federal University of Sergipe, Brazil (DB-FUS). A voucher specimen was deposited in the DB-FUS herbarium under the registration number ASE 23107.

Preparation of the hydroethanolic extract and its fractions

A total of 1.2 kg of B. virgilioides bark were dried at room temperature, reduced to powder and subjected to maceration with 90% ethanol for 5 days. Afterwards, the material was filtered and concentrated in a rotatory evaporator under reduced pressure at 45°C to give 114.35 g of the hydroethanolic extract (HEE, yield of 9.53%). A portion of HEE (78.0 g) was dissolved in methanol: water (2:3) and subjected to liquid-liquid extraction with organic solvents to obtain hexane (HXF, 6.020 g, yield of 7.71%), chloroform (CLF, 1.590 g, yield of 2.03%), ethyl acetate (EAF, 2.010 g, yield of 2.57%) and hydromethanol (HMF, 57.920 g, yield of 74.26%) fractions.

Phytochemical screening

Extracts and fractions were qualitatively analyzed by precipitation and colorimetric methods as described by Matos (2009) to detect phenols and tannins (red and blue precipitate, respectively, after treatment with 1 mol/L alcoholic ferric chloride), flavonoids (red-orange precipitate at pH 11 with no color change at pH 3 and 8.5 after treatment with 3 mol/L sodium hydroxide or 1 mol/L hydrochloric acid), catechins (yellow precipitate after heating at pH 2 with no color change at pH 11 after treatment with 1 mol/L hydrochloric acid or 3 mol/L sodium hydroxide), steroids and triterpenoids (blue or red coloration, respectively, after a Lieberman-Buchard reaction), saponins (Lieberman-Buchard reaction and foam formation) and alkaloids (orange color in presence of the Hager, Mayer and Dragendorff reagent). Ethanol solutions (500 µg/ml) of the extract and fractions (5 ml) were treated with the different reagents.

Quantification of total phenolics

Total phenol content (TFC) was determined according to the methodology of Sousa et al. (2007) with modifications. HEE and its fractions (10 mg) were dissolved in 10 ml of methanol and an aliquot (100 µl) of the resulting solution was transferred to a Falcon conical tube with 6 ml of distilled water and 500 µl of 1N Folin-Ciocalteu reagent. The solution was stirred for 1 min. Afterwards, 2 ml of 15% sodium carbonate were added to each solution, which was again stirred for 30 s. Distilled water was then added to each Falcon tube to give a final volume of 10 ml and they were incubated for 120 min at 23°C. The absorbance of each solution was spectro-photometrically determined at 750 nm against the blank using...
Table 1. Phytochemical screening of the hydroethanolic extract (HEE) and its hexane (H XF), chloroform (CLF), ethyl acetate (EAF) and hydromethanol (HMF) fractions obtained from the bark of B. virgilioides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HEE</th>
<th>HXF</th>
<th>CLF</th>
<th>EAF</th>
<th>HMF</th>
</tr>
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<tbody>
<tr>
<td>Phenol</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Xanthones</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Catechins</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pentacyclic triterpenes and free steroid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

DPPH• free radical scavenging activity

Aliquots of HEE and its fractions solutions (0.5 mg/ml in methanol) were added to 40 μg/ml DPPH• to give final concentrations of 5, 15, and 25 μg/ml in a final reaction volume of 3 ml. The blank was a mixture of HEE/fraction with methanol, while gallic acid was used as positive control.

Absorbance values for each sample were spectrophotometrically obtained at 515 nm after 1, 5, 10, 20, 30, 40, 50, and 60 min (Sousa et al., 2007). The percentage of remaining DPPH (DPPHR_{REM}%) was calculated according to Brand-Williams et al. (1995) from the equation: DPPHR_{REM} = ([DPPH]_T/[DPPH]_0) x 100, where [DPPH]_T is the radical concentration in the reaction medium after the reaction with HEE/fractions, and [DPPH]_0 the initial concentration of DPPH. Inhibition percentage (IP) after 60 min of reaction was obtained from the DPPHR_{REM} at this same time.

Activity antinociceptive

Swiss mice (Mus musculus) males, 60 to 90 days of age, weighing 28 to 32 g each, were obtained from the Central Animal Facility of FUS. They were randomly kept in cages under controlled temperature (22 ± 3°C) with light/dark cycle of 12 h. The animals had free access to food (Purina™) and water. The Ethics Committee on Animal Research of FUS approved the experimental protocols and procedures under registration number 65/11.

Groups of mice (n=6) were systematically pretreated with vehicle (tween 80 in phosphate buffered saline), HEE, EAF and HMF (100, 200 and 400 mg/kg; p.o.) 1 h prior to administration of the pain agent, Morphine (MOR, 5 mg/kg; i.p.) was used as positive control and administered 0.5 h before the injection of the orofacial pain inducer. Nociception was quantified by measuring the time (s) that the animals remained rubbing the area of the face where the pain inducer was administered with the front or hind legs.

Nociceptive orofacial pain was induced in mice by injection of 2% (20 μl, subcutaneously, s.c) formalin on the right upper lip (perinasal area). The behavioral response characteristic of the biphasic pain-related high intensity periods was observed from 0 to 5 min (first phase) and 15 to 40 min (second phase) (Luccarini et al., 2006). The glutamate nociceptive assay was performed as described by Beirith et al. (2002) with some adaptations, wherein the glutamate (40 μl, 25 mM, s.c) was injected in the perinasal area and the animals were individually observed for 15 min after the administration. Regarding the capsaicin nociceptive pain model, capsaicin (20 μl, 2.5 μg, s.c.) was dissolved in ethanol, dimethyl sulfoxide and distilled water (1:1:8) and injected in the perinasal area, followed by observation of the behavior of experimental animals during 42 min.

To investigate whether the treatment could influence the motor activity of animals and consequently affect the assessment of the nociceptive behavior, motor activity was evaluated in a rotarod apparatus. Initially, the mice that were able to remain on the rotarod up to 180 s were selected 24 h before the test. Then, the selected animals were divided into five groups and treated with vehicle (tween 80 in phosphate buffered saline), HEE, EAF and HMF (400 mg/kg; p.o.) and diazepam (1.5 mg/kg; i.p.). After 30, 60 and 120 min of administration, each animal was tested in the apparatus and recorded over time (s) remaining in the bar up to 180 s (Dunham and Miya, 1957).

Statistical analyses

Data were evaluated by One-Way Analysis of Variance (ANOVA) followed by Dunnett’s post hoc test. Mean differences were significant for p<0.05.

RESULTS

The phytochemical screening revealed the presence of several classes of phenolic compounds (Table 1) as well as triterpenes and alkaloids, especially in HEE and EAF, which showed the greatest diversity of chemical compounds. Tannins and flavonoids appeared in HEE and all fractions, except for HXF. Triterpenes were only found in the apolar fractions HXF and CLF, while saponins were only present in EAF.

Table 2 shows the TPC of HEE and its fractions, with EAF providing the highest concentration of phenolic compounds (198.17 ± 9.06 GA mg/g fraction), while HXF was shown to have the lowest TPC (37.17 ± 8.41 GA mg/g fraction). EAF had 54.8% more TPC than HEE. Regarding the DPPH free radical scavenging activity, Table 3 shows that DPPH exposition to HEE, EAF and HMF caused the highest inhibition of the radical by 42.9, 78.5 and 54.7%, respectively.

Figure 1 shows that at the lowest dose (100 mg/kg), HEE did not reduce significantly (p > 0.05) the formalin-
induced nociceptive response in both neurogenic (Figure 1A) and inflammatory phase (Figure 1B). However, at the highest does (200 and 400 mg/kg), HEE significantly (p < 0.05) reduced the nociception in both phases by 40 to 61% compared with the control group vehicle, whereas the group treated with MOR reduced more than 80% of the nociception in both phases.

Although the highest inhibitions of the nociceptive response were observed in the inflammatory phase (Figure 1D) in a dose-dependent manner, EAF produced antinociception in both phases of the formalin test (Figure 1C and 1D). The highest inhibition of the inflammatory response (Figure 1D) at the highest dose (400 mg/kg) was similar to the inhibitory result exhibited for MOR treatment (71 and 75%, respectively, compared to the vehicle group).

HMF also showed antinociception in the two phases of the formalin test (Figure 1E and 1F). However, the reduction was significant only at highest dose for the neurogenic phase (Figure 1E), while this fraction showed an antinociceptive effect in all doses used in the inflammatory phase (Figure 1F), reducing the nociceptive behavior by 55 to 69% against 75% for MOR.

To further analyze the antinociceptive effect of the bark of *B. virgilioides*, the glutamate and capsaicin pain models were used. As shown in Figure 2, HEE, EAF and HMF showed significant (p<0.05) antinociception when glutamate was used to cause orofacial pain, with HEE inhibiting up to 82% of the nociceptive response at 400 mg/kg. This is higher than observed for MOR, which inhibited only 67% of the glutamate induced-pain.

HEE, EAF and HMF showed antinociceptive effect against pain induced by capsaicin as well (Figure 3A, 3B and 3C, respectively). However, only HEE and EAF were active at 100 mg/kg (Figure 3A and 3B), while HMF was not able to reduce significantly (p>0.05) the nociceptive effect of capsaicin (Figure 3C) compared to the vehicle group. The extract and both fractions showed similar results to MOR, with EAF and MOR reducing 71 and 68% of the nociceptive response time at 400 and 5 mg/kg, respectively, compared with the control group vehicle.

The effect of HEE, EAF and HMF on mice motor activity was investigated to evaluate its influence in the nociceptive behavior using a rotarod apparatus. Pre-treatment with HEE, EAF and HMF at the highest concentration used in the present study (400 mg/kg) or vehicle did not induce alterations in motor performance, since the animals remained on the rotating rod for 180 s at intervals of 30, 60 and 120 min after the dose administration. As expected, diazepam (1.5 mg/kg), a CNS depressant, reduced the residence time of the animal on the rotary shaft at all tested times.

**DISCUSSION**

The phytochemical prospecting (Table 1) confirms results previously seen in other studies with *B. virgilioides* (Smiderle and Sousa, 2003; Almeida et al., 2006). The presence of tannins, terpenoids, alkaloids, flavonoids and glycosides has been reported by Smith et al. (2007), with triterpenes and isoflavones being isolated in their study. Condensed tannins were also isolated in the aqueous and hydroethanolic extracts of *B. virgilioides* bark (Trugilho et al., 1997). Leite et al. (2014) isolated gallic, chlorogenic and caffeic acids and flavonoids quercetin,

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenol content (GA mg/g of extract/fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEE</td>
<td>128.00 ± 26.10</td>
</tr>
<tr>
<td>HXF</td>
<td>37.17 ± 8.41</td>
</tr>
<tr>
<td>CLF</td>
<td>169.31 ± 13.36</td>
</tr>
<tr>
<td>EAF</td>
<td>198.17 ± 9.06</td>
</tr>
<tr>
<td>HMF</td>
<td>154.21 ± 20.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>HEE IP (%)</th>
<th>HXF IP (%)</th>
<th>CLF IP (%)</th>
<th>EAF IP (%)</th>
<th>HMF IP (%)</th>
<th>GA IP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8.34±0.59</td>
<td>1.81±2.22</td>
<td>3.21±1.14</td>
<td>21.72±2.68</td>
<td>5.79±2.45</td>
<td>92.17±0.06</td>
</tr>
<tr>
<td>15</td>
<td>23.97±1.80</td>
<td>4.85±1.13</td>
<td>13.85±1.77</td>
<td>68.58±3.00</td>
<td>32.56±2.21</td>
<td>92.22±0.06</td>
</tr>
<tr>
<td>25</td>
<td>42.89±1.74</td>
<td>7.31±1.15</td>
<td>23.14±1.19</td>
<td>78.51±1.96</td>
<td>54.96±2.37</td>
<td>92.32±0.10</td>
</tr>
</tbody>
</table>

Results are expressed as inhibition percent (mean ± SD, n = 3). Gallic acid (GA) was used as positive control.
rutin and kaemferol of extracts from the stem and heartwood of the *B. virgilioides*, while β-elemene (6.9%), β-caryophyllene (44.1%), germacrene D (7.9%), bicyclogermacrene (6.4%) and caryophyllene oxide (8.9%) were found as the main components of the plant seed (Rodrigues et al., 2009). All of these compounds

Figure 1. Antinociceptive effect of the hydroethanolic extract (HEE, A and B), ethyl acetate (EAF, C and D) and hydromethanol fraction (E and F) of *B. virgilioides* in the orofacial model of pain induced by formalin. First (A, C and E) and second phase (B, D and F). Results are shown as mean ± SE (n= 6) of the time (s) that mice spent rubbing the orofacial region treated with formalin. Statistical differences were determined by ANOVA followed by Dunnett’s post hoc test. (*) Denotes significant differences compared to the vehicle control group. **p<0.01, ***p<0.001.
Figure 2. Antinociceptive effect of hydroethanolic extract (HEE, A), and ethyl acetate (EAF, B) and hydromethanol (HMF, C) fractions of *B. virgilioides* against the orofacial pain induced by glutamate. Results are shown as mean ± SE (n= 6) of the time (sec) that mice spent rubbing the orofacial region treated with glutamate. Statistical differences were detected by ANOVA followed by Dunnett’s post hoc test. (*) Denotes significant differences compared to the vehicle control group. *p<0.05, **p<0.01, ***vgw p<0.001.

were linked to the plant biological activities.

In the present study, most of these compounds found in the literature as present in *B. virgilioides* were detected in HEE and EAF, suggesting the extract and its ethyl acetate fraction are the most biologically active samples of the plant. It should be noted that saponins were detected in EAF, but not in HEE. This may be linked to the interactions of the different secondary metabolites present in the extract. Compounds may be masked by others due to coupling, polymerization, glycosides formation and simple competition for reagents (Ainsworth and Gillespie, 2007). However, these interactions can be broken due to the fractioning of the original extract in solvents with different polarities so substances that were not present in the extract are found in the fractions (Muller et al., 2006; Dias et al., 2012).

To further analyze the biological potential of the plant extract and fractions, the total phenol content was also evaluated. Phenolic compound amount in extracts or fractions is frequently linked to the biological properties besides the classes of secondary metabolites found in them. Phenolic compounds such as phenols, flavonoids and tannins are characterized by having one or more hydroxyl groups attached to an aromatic ring, which will be used to stabilize free radicals. Therefore, the higher the amount of phenolic compounds in an extract or fraction, the higher the amount of hydroxyls groups available for their antioxidant activity (Angelo and George, 2007; Simões et al., 2010), which is attributed to electron donation from hydroxyl to free radical (Sousa et al., 2007). In the present study, EAF showed a total phenol content higher than HEE, from which it was obtained (Table 2). As previously said, this can be explained by synergistic and antagonistic interactions in
Figure 3. Antinociceptive effect of hydroethanolic extract (HEE, A), and ethyl acetate (EAF, B) and hydromethanol (HMF, C) fractions of B. virgilioides against the orofacial pain induced by capsaicin. Results are shown as mean ± SE (n=6) of the time (sec) that mice spent rubbing the orofacial region treated with capsaicin. Statistical differences were detected by ANOVA followed by Dunnett's post hoc test. (*) Denotes significant differences compared to the vehicle control group. *p<0.05, **p<0.01, ***p<0.001.

HEE and their competition for the Folin-Ciocalteau reagent (Ainsworth and Gillespie, 2007). As of general, the order for TPC was EAF > CLF > HMF > HEE > HXF.

Considering that ROS are involved in pain propagation (Vigiano et al., 2005), the antioxidant potential of HEE and its fractions was evaluated using the DPPH• free radical method (Pérez-Jiménez et al., 2008; Scherer and Godoy, 2009). This method is used worldwide because the radical is stable and its reaction with the antioxidant can be easily followed using a UV/VIS spectrophotometer as the radical changes its color from purple to yellow due to its neutralization by the donation of electrons from the antioxidant to its structure (Scherer and Godoy, 2009). In the present study, HEE and its two polar fractions, EAF and HMF, were able to remove 42 to 79% of the DPPH at 40 µmol/L (Table 3). As of general, the order for the DPPH scavenging potential was EAF > HMF > HEE > CLF > HXF. Regarding HEE and its polar fractions, there is a correlation between TPC and DPPH removal: EAF > HMF > HEE. The correlation between a higher TPC and DPPH free radical removal was previously reported in other studies involving medicinal plants (Djeridane et al., 2006; Li et al., 2008; Melo et al., 2010; Vaher et al., 2010; Moura et al., 2011; Silva et al., 2011).

As previously stated, other studies have reported the antinociceptive activity of B. virgilioides. Silva et al. (2010) and Thomazzi et al. (2010) described the peripheral antinociceptive effect of the aqueous extract of the inner bark of B. virgilioides using the writhing, hot-plate and formalin tests, while the anti-inflammatory activity was evidenced by the paw oedema and peritonitis methods assays. Thus, considering those results and the findings regarding phenol content and in vitro antioxidant activity against DPPH in the present study, as well as the
use of the plant in the folk medicine to treat pain, the effect of the plant ethanol extract and fractions with the higher antioxidant activity (HEE, EAF and HMF) was also investigated against orofacial pain.

HEE, EAF and HMF showed antinociceptive responses in the inflammatory phase (second phase) of the formalin test (Figure 1B, 1D and 1F), confirming the studies of Silva et al. (2010) and Thomazzi et al. (2010) with the plant aqueous extract. However, they also caused antinociceptive responses in the neurogenic phase (first phase) of the same test (Figure 1A, 1C and 1E). These results indicate that B. virgilioides HEE, EAF and HMF have central and peripheral actions regarding the orofacial pain that may be associated with opioid receptors, which are the same used by morphine (Roseland et al., 1990; Stein et al. 2001). In their studies, Silva et al. (2010) and Thomazzi et al. (2010) did not found any central effect of the aqueous extract from B. virgilioides bark. This difference in results may be explained by the different extraction methods of the same part of plant, since more substances can be extract with ethanol, which is an organic solvent, than with water, a polar solvent that can only extract polar water soluble substances. In addition, although HMF was active in the first phase of the formalin test, this was only observed at 400 mg/kg. This may be explained by the observation of the chemical composition of HEE, EAF and HMF. The phytochemical screening showed that HEE and EAF have a similar pattern of secondary metabolites, differing only in their triterpenes, steroids and alkaloid content, while HMF lacks xanthones and saponins besides triterpenes, steroids and alkaloids, which can explain its lower antinociceptive activity (Ayres et al., 2009).

When the orofacial antinociceptive properties were further evaluated by the glutamate test, HEE, EAF and HMF showed the highest nociceptive effect at 400 mg/kg, with HEE reducing up to 82% in this concentration (Figure 2). Results were similar or even higher than morphine, suggesting there is antagonistic activity in the glutamatergic system, which is basically characterized by the action on ionotropic NMDA (N-methyl-D-aspartate) and non-NMDA receptors located in peripheral, spinal and suprastriatal structures. Glutamate is involved in the nociceptive transmission in the spinal cord and trigeminal subnucleus caudalis through primary afferent fibers that are excited by the release of nitric acid, inflammatory cytokines and other pain mediators (Bonjardim et al., 2011). Endogenous glutamate released peripherically activates NMDA receptors to stimulate the axon to release more glutamate in the periphery, creating a vicious circle that culminates in a central sensitization characterized by chronic pain in the orofacial pain. Glutamatergic antagonist prevents activation of these peripheral receptors as well as the consequent central sensitization. Chen et al. (2010) and Cardoso et al. (2006) found that ROS interfere with glutamatergic transmission, possibly by helping the release and/or uptake of glutamate as well as the activation of its receptors. The antinociceptive activity observed for HEE, EAF and HMF may also be related to the antioxidant activity of the phenolic compounds in the extract and its fractions, which may be inhibiting ROS action in the signaling pathway in the glutamatergic system besides their direct action in the nociceptive fibers and receptors, corroborating the studies of Meotti (2006), Valerio et al. (2009) and Guimarães et al. (2012).

Capsaicin activates nociceptors by direct stimulation of C fibers through their vanilloid receptor (TRPV1), which causes and influx of positive ions into neurones that depolarize with consequent release of substance P, glutamate, nitric oxide and pro-inflammatory mediators (Pelissier et al., 2002). HEE, EAF and HMF at 400 mg/kg (Figure 3) reduced the time mice remained rubbing the area where capsaicin was administered similarly to morphine, suggesting the antinociceptive activity of B. virgilioides extract and fractions is also associated with the inhibition of capsaicin action on TRPV1 or mediator production. As for glutamate, the observed effect can also be mediated by their antioxidant properties because the pro-inflammatory signaling of the TRPV1 receptor is also ROS-dependent (Akada et al. 2006).

Due to the concern for human motor impairment associated with drug administration, the Rota rod test was used to evaluate the depressive and muscle relaxant effect of HEE, EAF and HMF in mice. Results showed B. virgilioides extract and fractions did not interfere with the motor coordination of the experimental animals, which significantly differed from diazepam.

It should be noted that it is well known that flavonoids (phenolic compounds) are effective for treating pain because they inhibit enzymes related to pain mediator production such as phospholipase A2, COX, LOX and nitric oxide synthase. In this context, the production of arachidonic acid, prostaglandins, leukotrienes and nitric oxide, important mediators of inflammation mechanism, is minimized (Havsteen, 2002; Rathee et al., 2009; Valério et al., 2009; Miyashiro et al., 2010). However, the action of other metabolite secondary found in the present study cannot be discarded because the literature also report that alkaloids (Bonjardim et al., 2011) and terpenes (Guimarães et al., 2010) have antinociceptive effect.

**Conclusion**

The potential of B. virgilioides to treat orofacial pain in its central and peripheral components was confirmed. HEE, EAF and HMF (mainly at 200 and 400 mg/kg) showed antinociceptive effect in three different orofacial pain models, formalin, capsaicin and glutamate, suggesting the active metabolites extracted from the inner bark of B. virgilioides produce a peripheral and central antinociceptive response related to opioid, glutamatergic and vanilloid receptors. In addition, it is also possible that...
their antioxidant activity may be related to the observed antinociceptive effect by reducing the biosynthesis of ROS and other inflammatory mediators.

Conflicts of interest
Authors have not declared any conflict of interest.

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