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Sida cordifolia Leaf Extract Reduces the Orofacial Nociceptive Response in Mice

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In this study, we describe the antinociceptive activity of the ethanol extract (EE), chloroform (CF) and methanol (MF) fractions obtained from Sida cordifolia, popularly known in Brazil as "malva branca" or "malva branca sedosa". Leaves of S. cordifolia were used to produce the crude ethanol extract and after CF and MF. Experiments were conducted on Swiss mice using the glutamate and formalin-induced orofacial nociception. In the formalin test, all doses of EE, CF and MF significantly reduced the orofacial nociception in the first ($p < 0.001$) and second phase ($p < 0.001$), which was also naloxone-sensitive. In the glutamate-induced nociception test, only CF and MF significantly reduced the orofacial nociceptive behavior with inhibition percentage values of 48.1% (100 mg/kg, CF), 56.1% (200 mg/kg, CF), 66.4% (400 mg/kg, CF), 48.2 (200 mg/kg, MF) and 60.1 (400 mg/kg, MF). Furthermore, treatment of the animals with EE, CF and MF was not able to promote motor activity changes. These data demonstrate that S. cordifolia has a pronounced antinociceptive activity on orofacial nociception. However, pharmacological and chemical studies are necessary in order to characterize the responsible mechanisms for this antinociceptive action and also to identify other bioactive compounds present in S. cordifolia.

Keywords: medicinal plant; Sida cordifolia; orofacial pain; mice.

INTRODUCTION

The orofacial region is one of the most densely innervated areas of the body, which focuses some of the most common acute pains, i.e. those accompanying pathological states of the teeth and related structures (Raboisson and Dallel, 2004). Many of the difficulties in the management of acute and chronic orofacial pain conditions stem from a lack of recognition and understanding of pain mechanisms (Miranda *et al.*, 2009). Thus, the management of orofacial disorders is one of the most challenging in the pharmacology field (Botelho *et al.*, 2008).

A significant portion of drugs are obtained from plants and several synthetic drugs are obtained using natural products as models (De Souza *et al.*, 2009). The potential of biodiversity as a source for new drugs remains unexplored, since of all the plant species estimated on the planet (250000–300000), fewer than 10% have been investigated in relation to their chemical and pharmacological activities. Although in recent years, notable progress has been made concerning the development of natural therapies (Botelho *et al.*, 2009, 2010), there is an urgent need to discover effective and potent analgesic agents (Calixto *et al.*, 2000). Additionally, due to their low cost and easy access in the natural flora of several countries, medicinal plants could be used as synthesis models of more selective and powerful drugs (Almeida *et al.*, 2001; Quintans-Júnior *et al.*, 2008; Melo *et al.*, 2010).

Sida cordifolia L. (Malvaceae) is a native species of the Brazilian Northeast, popularly known as 'Malva branca'. It is used in folk medicine for its antirheumatic, antipyretic, antiasthmatic, nasal anticongestant activities (Silveira *et al.*, 2003; Santos *et al.*, 2005). In addition, it is reported to possess anticancer, diuretic, laxative, hypoglycemic, hepatoprotective (Kubavat and Asdaq, 2009), antiinflammatory and analgesic (Sutradhar *et al.*, 2006 Franzotti *et al.*, 2000), hypotension and bradycardia activities (Santos *et al.*, 2005). It still has a depressant effect on the central nervous system without interfering with motor coordination with a low toxicity (Franco *et al.*, 2005).

The leaves of *S. cordifolia* L. contain mainly alkaloids, oils, resin acid, mucin, potassium nitrate, sympathomimetic amines, ephedrine, pseudoephedrine (vasoconstrictor), vasicinone, vasicinol, vasicine (bronchodilator), steroids, flavonoids and saponins (Kubavat and Asdaq, 2009; Franco *et al.*, 2005; Franzotti *et al.*, 2000). The aim of this study was to evaluate the pharmacological activity of the extracts and fractions from *S. cordifolia* in two animal models of orofacial nociception.

MATERIAL AND METHODS

Plant collection. Leaves of *Sida cordifolia* (voucher specimen no. 30171, deposited in the herbarium of the

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Department of Biology, Universidade Federal de Sergipe, Brazil) were collected in the city of Lagarto (10° 55' 02'' S, 37° 39' 00''W), state of Sergipe, Brazil.

C: Preparation of the ethanol extract. Leaves of *S. cordifolia* (1250 g) were dried in an oven at 40 °C for 48 h, then powdered and macerated with 99.5% ethanol for 72 h. The solution was filtered and concentrated under reduced pressure in a rotary evaporator at 54 °C, producing the crude ethanol extract (EE).

Fractionation of the ethanol extract. The EE was submitted to liquid–liquid partitions successively with hexane, chloroform and methanol:water. Thereafter, the solvents were evaporated under reduced pressure in a rotary evaporator at 54 °C, producing chloroform (CF) and methanol fractions (MF). A chemical study of the samples was carried out as described previously by Matos (1997).

Pharmacological assays. Experiments were conducted using adult male Swiss mice weighing 26–31 g each, at about 8 weeks of age. The animals were maintained with free access to food and water and kept in a 25–27 °C room under a controlled 12 h light/dark cycle. The numbers of animals (8 per group) and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments. All nociception tests were carried out by the same visual observer. Experimental protocols were approved by the Animal Care and Use Committee (CEPA/UFS # 14/08) at the Federal University of Sergipe.

Drugs and reagents. The chemicals and the drugs used in this study were morphine sulphate, naloxone and diazepam which were purchased from Cristalia (São Paulo, Brazil) and glutamate from Sigma (USA). A solution of formalin (2.0%) was prepared with formaldehyde (Merck) in distilled water. The phases were used as suspensions in 0.2% Tween 80 concentration.

Formalin-induced orofacial nociception. The procedure used was described previously (Clavelou *et al*., 1995; Luccarini *et al*., 2006; Quintans-Júnior et al., 2010). Animals received (s.c. into the right upper lip, perinasal area) a dose of 20 μL of a 2.0% formalin solution (formaldehyde, in saline). The animals were observed for 5 min (neurogenic phase) and from 15 to 40 min (inflammatory phase) and the time (s) that they spent rubbing the injected area with the ipsilateral fore- or hindpaw was recorded and considered as indicative of nociception. The animals ($n = 8$, per group) were pretreated systemically with EE, CF and MF (100, 200 and 400 mg/kg, p.o.) 60 min before the formalin administration. The reference drug utilized (morphine, MOR, 5 mg/kg, i.p.) was administered 30 min before the formalin administration. Control animals received only the vehicle (saline + Tween 80 0.2%). The possible involvement of the opioid system in the antinociception produced by EE, CF and MF was tested by administration of naloxone, NAL, (1.5 mg/kg), an opioid antagonist, 30 min before administration of drugs mentioned above.

Glutamate-induced orofacial nociception. The procedure used was similar to that described previously by Beirith *et al*. (2002) and Quintans-Júnior et al. (2010) with some alterations. A volume of 40 μL of glutamate (25 μM) was injected in the right upper lip (perinasal area). The animals were observed individually for 15 min following glutamate injection. Nociception quantification was performed at this period measuring the time (s) that the animals spent face-rubbing the injected area with fore- or hindpaws. Animals ($n = 8$, per group) were pretreated with EE, CF and MF (100, 200 and 400 mg/kg, p.o.) 60 min before the glutamate administration. The reference drug utilized (morphine, 5 mg/kg, i.p.) was administered 30 min before the glutamate administration. Control animals received only the vehicle (saline + 0.2% Tween 80).

Evaluation of motor activity. To investigate whether the treatments influenced the motor activity of the animals and consequently impaired the assessment of the nociceptive behavior in the experimental models, the motor performance of the animals was evaluated in a rota-rod apparatus, according to Dunham and Miya (1957) with some modifications. Initially, mice that were able to remain on the rota-rod apparatus (AVS®, Brazil) for longer than 180 s (7 rpm) were selected 24 h before the test. Then, the selected animals were divided into five groups ($n = 8$) and treated with vehicle (control, v.o.), EE, CF, MF (400 mg/kg, v.o.) and diazepam (DZP, 1.5 mg/kg, i.p.). Each animal was tested on the rota-rod and the time (s) they remained on the bar for up to 180 s was recorded 60 min after administration.

Statistical analysis. Data obtained from animal experiments were expressed as mean and standard error of the mean (mean ± SEM). Statistical differences between the treated and the control groups were evaluated by ANOVA and Tukey's tests. In all cases, $p < 0.05$ was considered to be significant. The percent inhibition by an antinociceptive agent was determined by the following formula:

$$Inhibition\,\% = 100 \times \frac{(control - experiment)}{control}$$

RESULTS

Administration of EE, CF and MF from *S. cordifolia* produced a reduction in face-rubbing behavior induced by formalin (Table 1). All doses tested significantly increased ($p < 0.001$) antinociception both in the first and second phase compared with the control (vehicle). MOR was able to reduce nociceptive behavior in both phases. The effects of EE, CF, MF and MOR were antagonized by naloxone.

On average, the percent inhibition of nociceptive behavior induced by EE, CF and MF in the first phase was 79.6%, 78.4% and 65.6%, respectively, and in second phase was 77.9%, 69.7% and 81.1%, respectively (Table 1).

Table 2 shows that the treatment with CF and MF significantly reduced the nociceptive behavior induced by glutamate in relation to the control group. Conversely, these outcomes were not observed for EE that was not able to inhibit the glutamate-induced nociception.

Table 1. Effects of EE, CF and MF of *S. cordifolia* in the formalin-induced rubbing response

Treatment	Dose (mg/kg)	Time of face-rubbing (s)					
		0–5 min			15–40 min		
		Time[a]	% inhibition	% IM	Time[a]	% inhibition	% IM
Vehicle	-	84.3 ± 6.6	-	-	170.2 ± 21.2	-	-
EE	100	17.5 ± 3.2[b]	79.2	79.60	49.8 ± 15.6[b]	70.7	77.9
EE	200	23.9 ± 5.2[b]	71.6		38.2 ± 7.3[b]	77.6	
EE	400	10.3 ± 3.0[b]	87.8		24.9 ± 6.5[b]	85.4	
CF	100	24.5 ± 3.7[b]	70.9	78.40	55.6 ± 6.4[b]	67.3	69.7
CF	200	17.5 ± 4.6[b]	79.2		54.4 ± 14.1[b]	68.0	
CF	400	12.6 ± 2.5[b]	85.1		44.6 ± 8.3[b]	73.8	
MF	100	26.8 ± 4.4[b]	68.2	65.60	28.4 ± 5.8[b]	83.3	81.1
MF	200	36.1 ± 3.8[b]	57.2		57.6 ± 11.9[b]	66.2	
MF	400	24.1 ± 3.5[b]	71.4		10.5 ± 3.0[b]	93.8	
MOR	5	2.6 ± 0.9[b]	96.9		1.0 ± 0.5[b]	99.4	
NAL + MOR	1.5 + 5	59.0 ± 5.6[c]	30.0		70.1 ± 0.9[d]	58.8	
NAL + EE	1.5 + 400	56.8 ± 7.4[e]	32.6		80.5 ± 13.3[f]	52.7	
NAL + CF	1.5 + 400	63.5 ± 7.7[g]	24.7		101.3 ± 6.9[h]	40.5	
NAL + MF	1.5 + 400	50.7 ± 2.7[i]	39.9		87.5 ± 14.5[j]	48.6	

$n = 8$; % IM, inhibition mean.
[a]Values represent mean ± SEM.
[b]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from control.
[c]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from MOR (5 mg/kg).
[d]$p < 0.01$ (one-way ANOVA and Tukey's test), significantly different from MOR (5 mg/kg).
[e]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from EE (400 mg/kg).
[f]$p < 0.05$ (one-way ANOVA and Tukey's test), significantly different from EE (400 mg/kg).
[g]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from CF (400 mg/kg).
[h]$p < 0.05$ (one-way ANOVA and Tukey's test), significantly different from CF (400 mg/kg).
[i]$p < 0.01$ (one-way ANOVA and Tukey's test), significantly different from MF (400 mg/kg).
[j]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from MF (400 mg/kg).

Table 2. Effects of EE, CF and MF of *S. cordifolia* on the glutamate-induced nociception

Treatment	Dose (mg/kg)	Time of face-rubbing (s)[a]	% inhibition	% inhibition (mean)
Vehicle	-	68.4 ± 5.4	-	-
EE	100	49.0 ± 4.8	28.4	20.0
EE	200	57.1 ± 11.3	16.5	
EE	400	58.1 ± 9.6	15.1	
CF	100	35.5 ± 6.5[b]	48.1	56.87
CF	200	30.0 ± 4.9[c]	56.1	
CF	400	23.0 ± 5.4[d]	66.4	
MF	100	49.4 ± 5.3	27.8	45.36
MF	200	35.4 ± 2.4[b]	48.2	
MF	400	27.3 ± 5.0[d]	60.1	
MOR	5	2.6 ± 1.2[d]	96.2	-

$n = 8$.
[a]Values represent mean ± SEM.
[b]$p < 0.05$ (one-way ANOVA and Tukey's test), significantly different from control.
[c]$p < 0.01$ (one-way ANOVA and Tukey's test), significantly different from control.
[d]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from control.

On average, the percent inhibition of nociceptive behavior induced by CF and MF in the glutamate-induced nociception test was 56.9% and 54.2%, respectively (Table 2).

In the rota-rod test, the mice treated with EE, CF and MF (400 mg/kg) did not show any significant alterations in motor performance (Fig. 1). As might be expected, the CNS depressant diazepam (1.5 mg/kg, i.p., standard drug) reduced the time that treated animals could remain on the rota-rod after 30 min (7.9 ± 3.0 s) compared with the control group.

DISCUSSION

The orofacial region is one area of the body most densely innervated by the trigeminal nerve and focuses

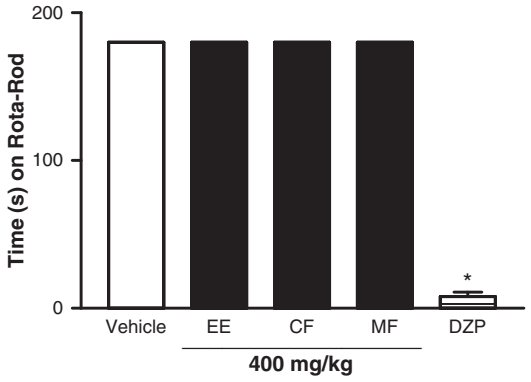

Figure 1. Time (s) on the rota-rod observed in mice after v.o. treatment with vehicle, EE, CF and MF (400 mg/kg) or diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s after drug treatment. Statistical differences versus control group were calculated using ANOVA, followed by Tukey's test ($n = 8$). *$p < 0.001$.

some of the most common acute pain. It is also the site of frequent chronic post-herpetic neuralgia, migraine and referred pain (Baron, 2009). Consequently, orofacial pain is a major concern for patients who are suffering and it is very difficult to treat by health professionals (Merril, 2010).

Recent studies have shown that natural products, such as medicinal plants, have been an important source for the development of new therapeutic leads for orofacial painful conditions (Botelho *et al*., 2007; Holanda Pinto *et al*., 2008; Quintans-Júnior *et al*., 2010; Siqueira *et al*., 2010). Our study assessed the pharmacological activity of extracts and fractions of *S. cordifolia* in two of the most important experimental models of cutaneous orofacial nociception (Raboisson and Dallel, 2004; Luccarini *et al*., 2006). Therefore, this research sought to confirm its popular use (for orofacial pain disorders), and also to contribute to the pharmacological knowledge about this plant using EE from its leaves and partition fractions obtained with solvents of different polarities (CF and MF).

The results of this study showed for the first time that an ethanol extract of *S. cordifolia* leaves, and its fractions, had antinociceptive activity when administered orally in different models of orofacial nociception in mice.

The orofacial formalin test represents a useful animal model of acute inflammatory nociception in the trigeminal region (Raboisson and Dallel, 2004). It generates behavioral as well as electrophysiological responses that last from several minutes up to more than 1 h and which are sensitive to a wide range of analgesics. The typical time course of the response to formalin is biphasic, with an early and short-lasting first phase followed, after a quiescent period, by a second prolonged (tonic) phase (Clavelou *et al*., 1995). In the first phase, the neurogenic pain is caused by direct activation of type fibers C nociceptive nerve endings, releasing neuropeptides such as substance P, among others (Luccarini *et al*., 2006). The second is characterized as inflammatory pain, related to the release of chemical mediators such as histamine, serotonin, bradykinin, prostaglandins and excitatory amino acids, which can be inhibited by non steroidal antiinflammatory drugs (NSAIDs) and central analgesics (Hunskaar and Hole, 1987; Pereira *et al*., 2010). In mice, formalin injection into the upper lip induced sustained face-rubbing episodes with vigorous face-wash strokes directed to the perinasal area with the ipsilateral forepaw, sometimes with the hindpaw. Thus, the behavioral response followed the typical biphasic time course seen in all formalin models.

In this study, an ethanol extract of *S. cordifolia* leaves and its fractions induced a reduction in face-rubbing behavior in the first and second phases of the formalin test. These results suggest that EE, CF and MF have a central analgesic effect. To confirm such an effect, naloxone, a non-selective opioid antagonist, was used in the formalin test (Belvisi *et al*., 1998). Because naloxone reversed the effect of EE, CF and MF (400 mg/kg), it may suggest the participation of the opioid system in the modulation of nociception induced by EE of *S. cordifolia* leaves, as well as CF and MF.

It is well known that excitatory amino acids, primarily glutamate, are involved in nociceptive transmission as excitatory neurotransmitters in the spinal cord and trigeminal subnucleus caudalis. A recent study demonstrated that peripheral glutamate participates in pain modulation (Ahn *et al*., 2004). Glutamate is involved in nociceptive transmission through primary afferent fibers, as well as in the development and maintenance of the pain response. It is well documented that glutamate acts on ionotropic receptors such as methyl-D-aspartate (NMDA), located in the peripheral, spinal and supra spinal structures, which can be directly linked to the release of nitric oxide at the spinal cord level (Pereira *et al*., 2010).

The MF and CF exhibited significant activity when the glutamate model was used. Thus, it is suggested that the constituents of this fraction could interfere in the glutamatergic system, through activation of NMDA receptors, which would limit the production of nitric oxide and other mediators (Ribas *et al*., 2008).

Previous studies suggested that the CNS depression and the non-specific muscle relaxation effect can reduce the response of motor coordination and might invalidate the behavior tests results (De Sousa *et al*., 2006). Our results revealed that none of mice treated with EE, CF and MF (400 mg/kg) had any performance alterations in the rota-rod test.

The hydroalcohol extract and total alkaloid fraction of *S. cordifolia* induced hypotension and bradycardia, mediated by activation of muscarinic receptors, with participation of nitric oxide (Medeiros *et al*., 2006; Santos *et al*., 2005). These results, taken together with the study conducted by Silveira *et al.* (2003), suggest that these effects appear to be mainly due to the presence of vasicine, an alkaloid present in the leaves of *S. cordifolia*. Moreover chemical studies of the leaves of this plant revealed the presence of other alkaloids such as vasicinone and vasicinol. Furthermore, a large number of different types of naturally occurring alkaloids with antinociceptive and antiinflammatory activity have been reported, such as pronuciferine, glaucine, nuciferine and pukateine (Calixto *et al*., 2000).

A recent study reported the same activity of compounds isolated from *S. cordifolia*, as 5,7-dihydroxy-3-isoprenyl flavone and 5-hydroxy-3-isoprenyl flavones isolated from the chloroform extract and 3′-(3′′7′′ dimethyl-2′′6′′octadiene)-8-C-β-D-glucosyl-kaempferol 3-O-β-D-glucoside from the ethyl acetate extract (Sutradhar *et al*., 2006, 2008).

Thus, it is suggested that the central and peripheral antinociceptive effect presented by the ethanol extract from *Sida cordifolia* and its chloroform and methanol fractions in the tests of orofacial nociception induced by formalin and glutamate could be because of the presence of alkaloids and flavones described for this species. Additionally, it seems, at least in part, that this antinociceptive action of EE, CF and MF involves the opioid and the glutamatergic systems. However, other mechanisms of action may be responsible for this effect, such as an action on muscarinic receptors and the nitrergic system, since previous studies have demonstrated the action of *S. cordifolia* on these systems.

CONCLUSION

Within the limitation of this trial, it was demonstrated that the extracts from *S. cordifolia* produced a consistent antinociceptive activity in experimental models of orofacial nociception that may be associated with central and peripheral mechanisms. These activities support the continued investigation of this plant as a potential therapeutic agent in order to characterize the mechanism responsible for the antinociceptive action and also to identify other active compounds present in *S. cordifolia* for orofacial pain management. The results may have an important impact for creating an effective and inexpensive agent for use in health programs.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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