Antinociceptive effect of *Encholirium spectabile*: A Bromeliaceae from the Brazilian caatinga biome

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ABSTRACT

Background: *Encholirium spectabile* is a species found in outcrops rocky throughout the Brazilian Caatinga. **Objective:** This study was carried out to evaluate the antinociceptive effects of ethanolic extract of the leaves from *E. spectabile* (Es-EtOH) in mice using chemical and thermal models of nociception. **Material and Methods:** HPLC was used to determine the fingerprint chromatogram. The Es-EtOH was examined for its antinociceptive activity at the doses of 100, 200 and 400 mg/kg intraperitoneal (i.p.). The evaluation of antinociceptive activity was carried out by the acetic acid-induced writhing, formalin and hot plate tests in mice. Rota-rod test was used for the evaluation of motor coordination. **Results:** In the acetic acid-induced writhing test, the Es-EtOH (100, 200 and 400 mg/kg, i.p.) reduced the number of writhings by 68.59, 79.33 and 65.28%, respectively. Additionally, Es-EtOH (100, 200 and 400 mg/kg, i.p.) decreased by 34.14, 52.61 and 60.97% the paw licking time in the first phase, as well as 89.56, 79.90 and 96.71% in the second phase of the formalin test, respectively. Es-EtOH also showed effect in the hot plate test, since increased the latency time at dose of 100 mg/kg after 60 minutes. In addition, Es-EtOH did not impair motor coordination. The presence of phenolic compounds in the extract was confirmed using HPLC. These results indicate that Es-EtOH has antinociceptive activity, probably of peripheral origin. The mechanism involved is not completely understood but, at least in part there is the participation of opioid receptors.

Key Words: Antinociceptive effect, Bromeliaceae, *Encholirium spectabile*, Pain

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INTRODUCTION

Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care.[1] Natural products of plant origin are still a major part of traditional medicinal systems in developing countries. There is also a resurgence of interest in herbal medicines in western countries as an alternative source of drugs often for diseases as inflammation and pain.[2] The need for safer and effective analgesic drugs justifies the interest in the study of medicinal plants because many species of plants are used worldwide for the relief of pain.

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The Bromeliaceae family is predominantly Neotropical and comprises 58 genera and approximately 3172 species.[3] The phytochemistry of this family is characterized by the presence of flavonoids, triterpenoids, steroids, diterpenes, cinnamic acid derivatives, lignans, nitrogen compounds among others.[4] Some studies have demonstrated that species of the Bromeliaceae family have pharmacological properties such as antioedematogenic and free radical scavenging,[5] antinociceptive and anti-inflammatory,[6] anti-allergic[7] and cytotoxic activity.[8]

Encholirium is a Brazilian genus of Bromeliaceae which occurs exclusively in rocky landscapes in areas of Cerrado, Caatinga and Atlantic Forest and is constituted by 23 species. This genus is exclusively Brazilian and has restricted distribution of most endemic species.[9,10]

Encholirium spectabile is the species best known of the genus because the frequency with which it is found in outcrops rocky throughout the Brazilian Caatinga.[11] Is popularly

known as "macambira-de-flecha" and "macambira-de-pedra".[12] It is one of the plants that have been included in a conservation program of the "Reference Centre for Recovery of Flora in Priority Areas of the San Francisco River Basin – The Caatinga Biome".

Little is known about the chemistry and pharmacology of *Encholirium* species. Previous study realized by our research group demonstrated that the ethanolic extract of *Encholirium spectabile* has gastroprotective activity against gastric mucosal damage induced by ethanol, HCl/ethanol, ibuprofen, ischemia and reperfusion, which suggests that the extract may activate cytoprotective mechanisms that increase the release of prostaglandins. The presence of phenolic compounds, mainly flavonoids, contributes to the gastroprotective activity of the extract.[13] Except this study, so far no other biological studies were carried out on *Encholirium spectabile*.

One of most important side effects of conventional analgesic and anti-inflammatory drugs (NSAIDs, for example) is their ulcerogenic activity. Flavonoids are good analgesic and anti-inflammatory compounds and are also able to protect the gastric mucose against a variety of ulcerogenic agents. Many studies were performed examining the antiulcerogenic activity of flavonoids using both naturally derived and synthetic compounds. Flavonoids are reported to act in the gastrointestinal tract as antisecretory and antiulcer agents.[14]

Considering the effect shown by the ethanolic extract of *E. spectabile* using different standard experimental models of induced acute gastric ulceration, the objective of this work was to evaluate the antinociceptive effect of the extract of this plant in mice using experimental models of pain. There is no previous report on the analysis of the antinociceptive activity of *Encholirium spectabile*.

MATERIALS AND METHODS

Plant material

The leaves of *Encholirium spectabile* Mart. ex Schult. f. were collected in the city of Petrolina (Coordinates: S 09º 07' 30"; W 40º 26' 00"), State of Pernambuco, Brazil, in January of 2010. The samples were identified by André Paviotti Fontana, a botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (6443) was deposited at the Herbarium Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley.

Extraction

The leaves dried and pulverized (1196 g) were macerated with ethanol 95% at room temperature for 72 hours. The solution was filtered and concentrated under reduced pressure in a rotatory evaporator oven at 50ºC, producing 64 g of crude ethanol extract (Es-EtOH).

Analysis of Es-EtOH by High Performance Liquid Chromatography (HPLC)

The solvents used in high performance liquid chromatography are of analytic grade from Merck®. A Milli-Q System® (Bedford, MA, USA) was used to purify the water. Analyses of high performance liquid chromatography was performed on a Merck-Hitachi liquid chromatograph LaChrom Elite® equipped with a VRW HITACHI L- 2130 pump, a VRW HITACHI L-2300 Diode-Array Detector (DAD), and an auto sampler with a 100 µl loop. The data was acquired and processed using Ezchrom Elite software. The extract was analyzed using a reverse-phase HPLC column: Purospher® STAR RP-18e (250 mm × 4.6 mm i.d., 5 µm) column (Merck). The mobile phase was composed of solvent (A) H_2O/H_3PO_4 0.1% and solvent (B) MeOH. The solvent gradient was composed of A (75-0%) and B (25-100%) for 20 minutes, then 100% B for 4 minutes, then again at the initial conditions (75% A and 25% B) for 10 minutes. A flow rate of 1.0 ml/minutes was used in a 30ºC oven, and 20 µl of each sample was injected. The procedure was repeated three times for each sample. Samples and mobile phases were filtered through a 0.22 µm Millipore filter prior to HPLC injection. Spectra data were recorded from to 200 to 400 nm during the entire run.

Animals

Male and female adult albino Swiss mice (25-35 g), were used throughout this study. The animals were randomly housed in appropriate cages at 22 ± 2°C on a 12 hours light/dark cycle (lights on at 6:00 a.m.) with free access to food and water. When necessary, animals were deprived of food 12 hours prior to the experiments. They were used in groups of five or six animals each. All nociception tests were carried out by the same visual observer. Experimental protocols and procedures were approved by the Federal University of San Francisco Valley Animal Care and Use Committee by number 21051023.

Acute toxicity

In the inquiry of the acute toxicity, groups of five male and five female Swiss mice (n = 10) were administered intraperitoneally 2.0 g/kg and 5.0 g/kg orally of the crude ethanol extract of *E. spectabile* (Es-EtOH). Control group received vehicle. Mortality in each group within 72 hours was recorded and the animals were observed during a period of 14 days for the presence of toxicity signals.[15]

Acetic acid-induced writhing in mice

This test was performed as described by Koster *et al.*[16] with modifications. Male mice (n = 6) were intraperitoneally

pre-treated 30 minutes before the nociceptive agent, acetic acid 0.9% (v/v, 10 ml/kg). Vehicle (saline), Es-EtOH (100, 200 and 400 mg/kg, body weight), acetylsalicylic acid (ASA, 200 mg/kg) and morphine (10 mg/kg) were administered before acetic acid injection. Following the injection of acetic acid, the nociceptive behavior was quantified by counting the total number of writhes (a response consisting of contraction of the abdominal wall, pelvic rotation followed by hind limb extension) occurring between 5 and 15 minutes after stimulus injection.[17]

Formalin test

The method used was similar to that described previously.[18-20] Twenty microliters of 2.5% formalin was injected subcutaneously into the right hind paw of male mice. The time (in seconds) spent in licking and biting responses of the injected paw was taken as an indicator of nociceptive behavior. Responses were measured for 5 minutes after formalin injection (first phase, neurogenic phase) and 15-30 minutes after formalin injection (second phase, inflammatory phase). Es-EtOH (100, 200 and 400 mg/kg), ASA (200 mg/kg) and morphine (10 mg/kg) were administered intraperitoneally 60 minutes before formalin injection. Control animals received the same volume of saline. Mice were observed in the chambers with a mirror mounted on three sides to allow view of the paws. For evaluation of the involvement of opioid receptors in the effect of the extract, naloxone (1.5 mg/kg, i.p.) was administered 30 minutes before administration of the extract (400 mg/kg) and morphine.

Hot plate test

Male mice were pre-selected on the hot plate at 55 ± 0.5°C. Licks on the rear paws were the parameters of observation. Animals showing a reaction time (latency for licking the hind feet or jumping) greater than 20 seconds were discarded. The animals were then treated with vehicle (saline, 0.1 ml/10 g), morphine (10 mg/kg) and Es-EtOH (100, 200 and 400 mg/kg) via *i.p.* Latency time (in seconds) for each mice was determined on the hot plate during the maximum period of 20 seconds, at intervals of 30, 60, 90 and 120 minutes after the administration of the extract.[21]

Motor coordination test (rota-rod test)

A Rota-rod tread mill device (Insight, Brazil) was used for the evaluation of motor coordination.[22] Initially, the capable mice to remain on the Rota-rod apparatus longer than 180 seconds (7 rpm) were selected 24 hours before the test. Thirty minutes after the administration of either Es-EtOH (100, 200 and 400 mg/kg, i.p.), vehicle (saline/Tween 80 0.2%; control group) diazepam (DZP; 2.5 mg/kg, i.p.), each animal was tested on the Rota-rod apparatus and the time (s) remained on the bar for up to 180 seconds was recorded after 0.5, 1 and 2 hours.

Statistical analysis

The data obtained from animal experiments were analyzed using the GraphPad Prism program version 4.0 and expressed as mean ± S.E.M. Significant differences between groups were calculated by the application of analysis of variance (ANOVA) followed by Dunnett's test. Values were considered significantly different at $P < 0.05$.

RESULTS

Analysis of Es-EtOH by high performance liquid chromatography (HPLC)

Phytochemical screening showed that the ethanolic extract of leaves from *E. spectabile* (Es-EtOH) contains phenols, flavonoids, steroids and terpenoids. Phenolic profiles at 320 nm for the Es-EtOH evaluated are presented in Figure 1. The chromatogram shows the presence of ten peaks with different retention times: 10.89 (**1**), 11.12 (**2**), 11.78 (**3**), 12.86 (**4**), 13.37 (**5**), 14.44 (**6**), 15.16 (**7**) 15.58 (**8**), 16.68 (**9**) and 17.63 (**10**). Based on their UV-Vis spectral data and their retention time, the compounds have UV band characteristic for cinnamic acid, coumarin and flavonoid derivatives. These compounds are under investigation.

Acute toxicity

Acute toxicity results showed no signs of toxicity and mortality of the animals. Therefore, an LD_{50} >2.0 g/kg intraperitoneally and 5.0 g/kg orally may be assumed.

Acetic acid-induced writhing in mice

The intraperitoneal administration of Es-EtOH (100, 200 and 400 mg/kg) decreased significantly the action of acetic acid used to induce writhes in mice, when compared to the control group. The percentages of inhibition were of 68.59, 79.33 and 65.28%, respectively. ASA (200 mg/kg) also significant inhibited the writhes, while the morphine

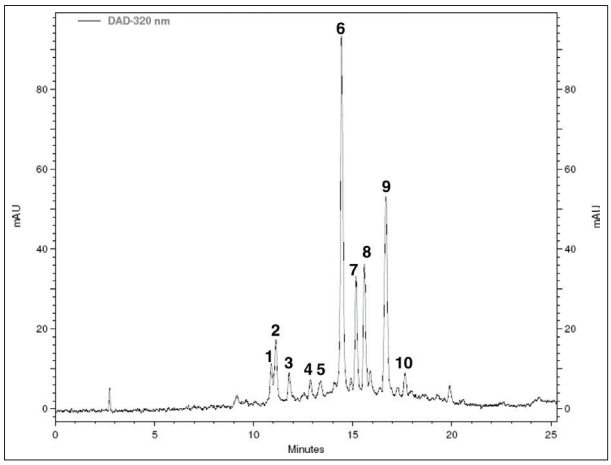

Figure 1: High performance liquid chromatography with diode array detector (HPLC-DAD) profiles of *Encholirium spectabile* ethanolic extract recorded at 320 nm

abolished the nociception induced by acetic acid. The results can be seen in Figure 2.

Formalin test

The extract (100, 200 and 400 mg/kg) injected 60 minutes before formalin showed a significant effect to reduce the licking time in both phases of this test. In the first phase (neurogenic pain) the effects were dose-dependent with percentages of inhibition of 34.14, 52.61 and 60.97%, respectively. The effect was more significant in the second phase (inflammatory pain), 89.56, 79.90 and 96.71%, respectively. Similarly, morphine (10 mg/kg) caused significant inhibition of 61.67 and 81.06% of formalin-induced nociceptive behavior in the first and second phases, respectively. ASA (200 mg/kg) caused inhibition of 88.79% in the second phase of this test. The pre-treatment with naloxone (1.5 mg/kg, i.p.) partially reversed the effect of the extract at dose of 400 mg/kg in the first phase of this test. The effect of morphine (10 mg/kg) was also reversed by naloxone in both phases. The results of formalin test are showed in Figure 3.

Hot plate test

Using the hot plate test, the administration of the extract induced alteration in the latency time when compared to the control, and increased the latency time at dose of 100 mg/kg after 60 minutes. The results are shown in Figure 4.

Motor coordination test (rota-rod test)

In this test, Es-EtOH did not impair motor coordination 0.5, 1 and 2 hours after administration. Diazepam (2.5 mg/kg) caused significant decrease of remaining time of animals on the Rota-rod apparatus, when compared to control group [Figure 5].

DISCUSSION

The acetic acid-induced writhing reaction in mice has been largely used as screening tool for assessment of analgesic or anti-inflammatory properties of new agents as well as a typical model for visceral inflammatory pain.[23,24] The local irritation provoked by a test agent in the intraperitoneal cavity triggers a variety of mediators, such as bradykinin, substance P and prostaglandins, especially PGI_2, as well

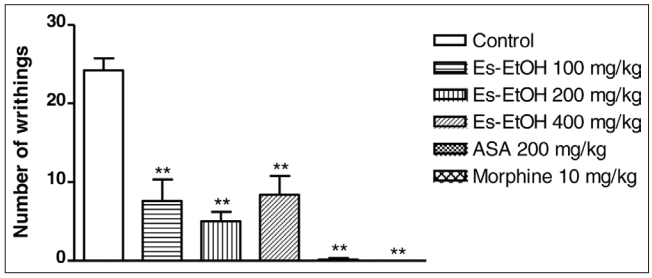

Figure 2: Effect of ethanolic extract of the *Encholirium spectabile* (Es-EtOH), acetylsalicylic acid (ASA) and morphine on acetic acid induced writhing test. Values are mean ± S.E.M. $^{**}P < 0.01$, significantly different from control; ANOVA followed Dunnett's test (n = 6, per group)

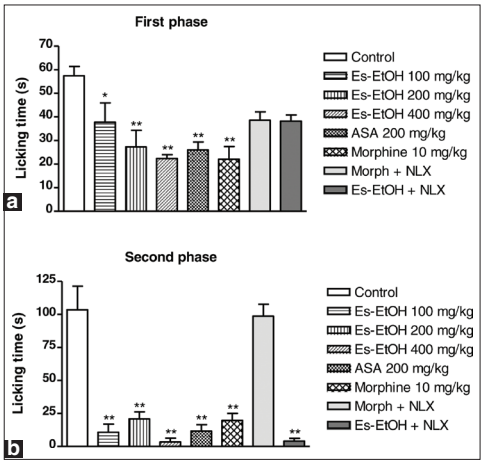

Figure 3: Effect of ethanolic extract of *Encholirium spectabile* (Es-EtOH), acetylsalicylic acid (ASA), morphine, morphine + naloxone (Morph + NLX; 10 mg + 1.5 mg/kg) and Es-EtOH + NLX (400 mg + 1.5 mg/kg) on formalin test. Values are mean ± S.E.M.; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control; ANOVA followed Dunnett's test ($n = 6$, per group)

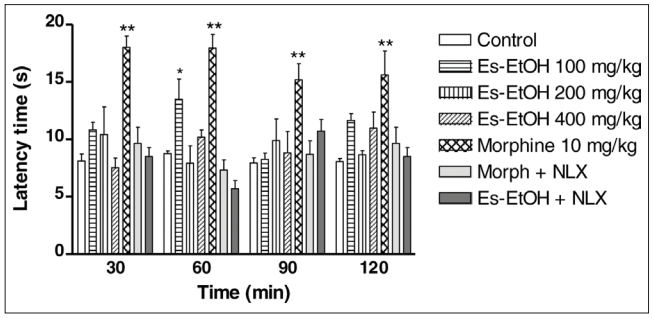

Figure 4: Effect of ethanolic extract of *Encholirium spectabile* (Es-EtOH), morphine, morphine + naloxone (Morph + NLX; 10 mg + 1.5 mg/kg) and Es-EtOH + NLX (400 mg + 1.5 mg/kg) on hot plate test. Values are mean ± S.E.M.; $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control; ANOVA followed Dunnett's test ($n = 6$, per group)

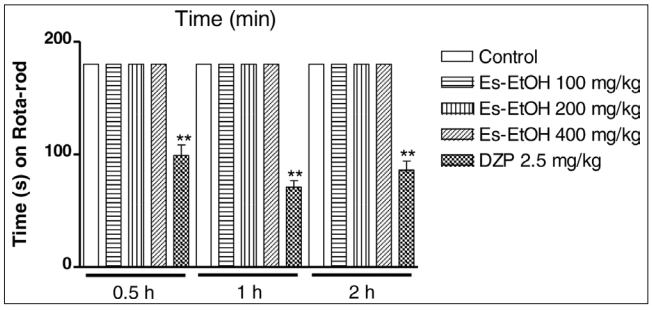

Figure 5: Effect of ethanolic extract of *Encholirium spectabile* (Es-EtOH) and diazepam (DZP) on Rota-rod test. Values are mean ± S.E.M.; $^{**}P < 0.01$, significantly different from control; ANOVA followed Dunnett's test ($n = 6$, per group)

as some cytokines such as IL-1β, TNF-α and IL-8.[25] Es-EtOH was able to reduce the writhing in all doses tested, suggesting that its antinociceptive effect could be related to inhibition of mediators released in response to acetic acid.

The formalin test is believed to represent a significant model of clinical pain. This test allows the evaluation of two distinct phases. It is known that the first phase is result of the direct chemical activation of myelenated and unmyelenated nociceptive afferent fibers while the second phase response is considered as a consequence of noxious stimulus-evoked long term changes in the properties of spinal dorsal horn neurons.[26] The first phase occurs during the first 5 minutes after the formalin injection and is characterized by the direct stimulation of nociceptors presents on afferent C and in part by Aδ fibers (glutamate and substance P release). The second phase occurs between the 15th and 30th minute after formalin injection and is putatively caused by the release of pro-inflammatory mediators such as adenosine, bradykinin, histamine, prostaglandins and serotonin. The results presented in this test (inhibition of both phases, although higher inhibition was seen in the second phase) suggest that the Es-EtOH might possess anti-inflammatory activity. The extract exerts its antinociceptive effects connected with peripheral mechanisms. However, the inhibition presented in the first phase suggests a disruption of either the production or release of some central neurotransmitters.[27] In another set of experiments, naloxone (1.5 mg/kg, *i.p.*) was injected 30 minutes before the morphine (10 mg/kg) and Es-EtOH (400 mg/kg). The pre-treatment of animals with naloxone had significant effect on the antinociception of the first phase. The naloxone reversed the effect caused by morphine in both phases of formalin-induced licking. At least in part, the antinociceptive effect presented by the extract may involve the participation of opioid receptors.

The hot plate is a method used to investigate central antinociceptive activity. The effect of the extract was observed only at time of 60 minutes with a dose of 100 mg/kg. The present study lead us to the conclusion that the opioid system, at least in part is involved in the antinociceptive effect of the extract. However, in this method the pre-treatment with naloxone, a non-selective opioid receptor antagonist not reversed significantly the antinociceptive effect presented by the extract. This could indicate that the effect is really by peripheral mechanisms.

In addition, to assess whether Es-EtOH produces loss of motor coordination of animals was performed to Rota-rod apparatus. The result showed that the extract did not produce changes in motor coordination of animals treated.

CONCLUSION

In summary, we demonstrated that the ethanolic extract of *Encholirium spectabile* exhibit antinociception when assessed in chemical models of nociception in mice. Novel antinociceptive agents could be discovered from medicinal plants containing a wide variety of phytoconstituents. The presence of phenolic compounds in the extract was confirmed using HPLC. The results indicate that Es-EtOH has antinociceptive activity, probably of peripheral origin. The mechanism involved is not completely understood but, at least in part there is the participation of opioid receptors. The peripheral mechanisms appear to be more important than central. Further research would be of interest to explain the exact mechanism of this antinociceptive effect.

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