Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas
ISSN: 0717-7917
editor.blacpma@usach.cl
Universidad de Santiago de Chile
Chile

HOLANDA LEITE MAIA, Ana Karina; BEZERRA DE QUEIROZ, Ronaldo; VILAR DA FONSÊCA, Diogo; SILVEIRA PINHO, André; GOLZIO DOS SANTOS, Sócrates; LIMA DE ALENCAR, Jacicarlos; FECHINE TAVARES, Josean; NOBRÉGA DE ALMEIDA, Reinaldo; CLÉBIA SOARES LIMA DE MORAIS, Liana

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Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, vol. 13, núm. 2, 2014, pp. 152-162
Universidad de Santiago de Chile
Santiago, Chile

Available in: http://www.redalyc.org/articulo.oa?id=85631009004

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Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 13 (2): 152 - 162
ISSN 0717 7917
www.blacpma.usach.cl

Artículo Original | Original Article

Antinociceptive activity of the extract of *Erythroxylum caatingae*

[Actividad antinociceptiva del extracto de *Erythroxylum caatingae*]

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Abstract*:* The genus *Erythoxylum* plants are popularly used as anti-inflammatory, anti-bacterial, and diuretic agentes, and for treating of respiratory problems. This study investigated the antinociceptive activity of methanolic extract of *Erythroxylum caatingae* (EcME) in chemical (formalin test and acetic acid test) and thermal models (hot plate test) of nociception. Intraperitoneal pre-treatment with EcME reduced the number of abdominal contortions and the licking time in the second phase of the formalin test. EcME did not show a significant effect in the hot plate test, rota-rod test, and the elevated plus maze test. These findings indicate that the antinociceptive activity of EcME is not because of a depressor effect on the central nervous system, and EcME is not a muscle relaxant. Nevertheless, *Erythroxylum caatingae* demonstrated peripheral antinociceptive activity, which confirms its popular use and contributes to the scientific knowledge of the species.

Keywords: *Erythroxylum caatingae*, medicinal plant, antinociceptive activity, pain.

Resumen: El género *Erythoxylum* es popularmente utilizado como agente anti-inflamatorio, antibacteriano y para el tratamiento de problemas respiratorios. Este estudio tiene como objetivo investigar la actividad antinociceptiva del extracto metanólico *Erythroxylum caatingae* (EcME), utilizando modelos químicos (prueba de la formalina y prueba de ácido acético) y térmico (prueba de la placa caliente) de nocicepción. El pretratamiento EcME por la vía intraperitoneal (i.p.), fue capaz de reducir el número de contorsiones abdominales y el tiempo de lamida en la segunda fase del test de formalina. EcME no tuvo efecto significativo en el test de la placa caliente, Rota-Rod y Laberinto en Cruz Elevado, mostrando que el efecto antinociceptivo no está relacionado con un efecto depresor del sistema nervioso central o miorelajante. Los datos experimentales muestran que *Erythroxylum caatingae* posee una actividad antinociceptiva periférica que confirma su uso popular, contribuyendo para el conocimiento científico de la especie.

Palabras Clave: *Erythroxylum caatingae*, planta medicinal, actividad antinociceptiva, dolor.

Recibido | Received: March 17, 2013
Aceptado en versión corregida | Accepted in revised form: September 19, 2013
Publicado en línea | Published online: March 30, 2014
Este artículo puede ser citado como / This article must be cited as: AK Holanda Leite Maia, R Bezerra de Queiroz, D Vilar da Fonseca, A Silveira Pinho, S Golzio dos Santos, J Lima de Alencar, J Fechine Tavares, R Nobrega de Almeida, L Clebia Soares Lima de Morais. 2014. Antinociceptive activity of the extract of Erythroxylum caatingae. **Bol Latinoam Caribe Plant Med Aromat 13(2): 152 – 162.**

INTRODUCTION

The genus *Erythroxylum* is one of the four genera which belong to the *Erythroxylaceae* family and comprises approximately 200 species indigenous to Venezuela, Brazil and Madagascar (Daly, 2004). *Erythroxylum* species are characterized by production of tropane alkaloids, which include cocaine, a natural alkaloid produced by *Erythroxylum coca* that has been used as a local anesthetic in small surgeries (Bohm *et al.*, 1982; Griffin and Lin, 2000). However, cocaine has gained notoriety for its psychoactive activity in the Central Nervous System (CNS), becoming one of the major public health problem nowadays (Alagille *et al.*, 2005).

The species *Erythroxylum caatingae* has a restricted distribution in Northeastern Brazil; it is found only in the states of Bahia, Ceará, Paraíba, Pernambuco, and Rio Grande do Norte (Plowman and Hensold, 2004). It presents as a shrub or small tree that can grow, up to 3.0 m in height, and has colorless, oblong, and obovate leaves, and whitish pedicellate, longistylus flowers with triangular thrums (Loiola *et al.*, 2007). This species has been investigated in some studies that have demonstrated antimicrobial and cytotoxic activity that occurs via apoptosis (Aguiar *et al.*, 2012).

Due to the absence of studies that reinforce the popular use of *Erythroxylum caatingae*, this work aims to evaluate the antinociceptive activity of the methanolic extract of the stem of *Erythroxylum caatingae* (EcME) on nociception induced by chemical and physical stimuli. In addition, we performed the elevated plus maze test and the rota-rod test to rule out a possible sedative or muscle relaxant effect.

MATERIAL AND METHODS

Animal

Male Swiss mice (*Mus musculus*; 30 - 40 g) were used in this study. Mice were kept under controlled temperature conditions 21 ± 2º C, were subjected to a 12-h light/dark cycle, and had free access to food and water. The mice were assigned to one of 5 groups (n = 8), and were used only once. The experimental procedures were reviewed and approved previously by the CEPA: The Ethics Committee for the Use of Animals LTF/CCS/UFPB, under certificate N° 0305/08.

Drugs

The chemical substances used in this study were: glacial acetic acid (Synth, USA), Morphine hydrochloride (Merck, USA), diazepam (Sigma, USA), formaldehyde 37% (Vetec, Brazil), and Tween 80 (Merck, USA). The EcME was dissolved in distilled water and Tween 80, and then administrated via intraperitoneal (i.p.) injection (0.1 mL/10 g).

Plant material

The botanical material (leaves) of *Erythroxylum caatinga*, were collected in Picuí - PB, Serra Branca - PB, Brazil and identified by Dra. Maria de Fátima Agra from the Pharmaceutical Technology Lab at the Federal University of Paraíba. Samples of this species are kept at the Professor Lauro Pires Xavier Herbarium (JPB/UFPB). Collection of M.F. Agra *et al.*, 6700.

Extraction

The botanical material was passed through a drying process, followed by a spray in a mechanical mill. This yielded 4.0 kg of stem powder that was thoroughly macerated with methanol (MeOH) for 72 h. The extraction solution was concentrated in rot vapor under reduced pressure at 35° C, which yielded the methanolic crude extract (500 g).

Standards used in the extract standardization

The compounds 3α-3.4.5 trimethoxybenzoyl-6β-benzoyl tropane (Catuabine B) (AL-1) and 3α-(3.5 dimethoxybenzoyl)-6-β benzoyl tropane (AL-2) were used with standards in the extract standardization, and were isolated, identified, and assigned according to Oliveira *et al*., (2011).

Extraction of total alkaloid fraction (FAT)

The extraction of the total alkaloids, which weighed 200 mg from the extract, was diluted into 5 mL of solution in a ratio of 3:7 of H_2O and MeOH. This solution was homogenized in an ultrasound device for 1.5 min. Next, it was added to 1 mL of the extract solution in a test tube and then added to 1 mL of HCl (8.10×10^3 M, pH = 2.0), and then manually homogenized. The solution was then supplemented with 1 mL of $CHCl_3$, and homogenized in ultrasound for 1.5 min. It was discarded the organic phase ($CHCl_3$) of the test tube, and then 1 mL of NH_4OH (3.25×10^1 M, pH = 11.0) was added, it was again homogenized manually, and 1.5 mL of $CHCl_3$ was

added to the solution, and then homogenized in ultrasound for 1.5 min. It was discarded in the aqueous phase (NH_4OH) and transferred in the organic phase ($CHCl_3$) to a micro-tube, which was previously weighed when empty, and then subjected to 40° C for evaporation in Dry Block (Brand: Tecnal, model TE-021) for 6 h. After drying, the micro-tube with the precipitate was weighed for measuring the mass yield (FAT). Soon after, the precipitate was resuspended with 1.5 mL of a solution of 1:1 (H_2O: MeOH).

Characterization and quantification of the Alkaloids

Analysis was performed with high performance liquid chromatography (SHIMADZU 10AV) using a ShimPack C18, 4.6 mm × 150 mm, 5-µm column particles at 40º C, with a flow rate of 1.0 mL/min, and an injection volume of 20 µL. The chromatographic system was coupled to a UV/Vis detector (SPD-10A vp) that operated in Δλ (225 nm). Isocratic elution was performed using (30:70 | 1.60 × 10-3M:MeCN) (Figures 1 and 2). The quantification of the main constituents AL-1 and AL-2 was made from EcME, in quadruplicate, with the analysis and extraction methodology previously validated.

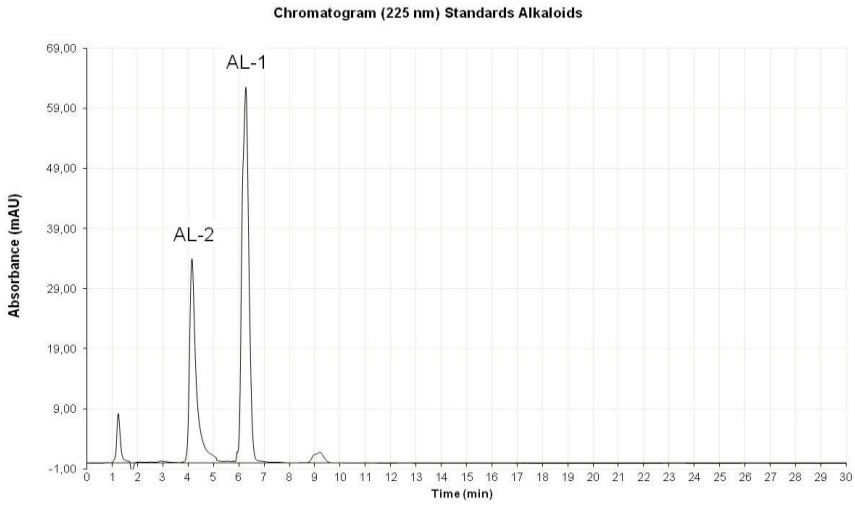

Figure 1
Chromatogram standards of alkaloids observed at 225 nm. Isocratic elution was performed using (30:70 | 1.60 × 10^{-3}M:MeCN)

Acute toxicity

EcME was given to groups of mice (n = 8) via i.p. injection in doses randomly selected from 500, 650, 750, 850, or 1000 mg/kg. The control group received only the vehicle. After the treatment, the mice were observed for 4 h for pharmacological and/or toxic effects, and for deaths over a 72-h period.

Acetic acid-induced writhing test

This test is based on the fact that i.p. administration of acetic acid solution at 0.85% in mice causes peritoneal irritation that is characterized by abdominal contortions followed by extensions of the hind limbs (Koster *et al.*, 1959). This nociceptive behavior was quantified for 15 min following acetic acid administration. The mice were divided into 5 groups (n = 8) that were pre-treated with the drug standard (morphine 6 mg/kg, i.p), vehicle, and EcME (50, 100, and 200 mg/kg, i.p.). After 30 min, the mice were treated with acetic acid at 0.85% (0.1 mL/10 g, i.p.) and subsequently were placed into a polyethylene box to observe the number of abdominal contortions.

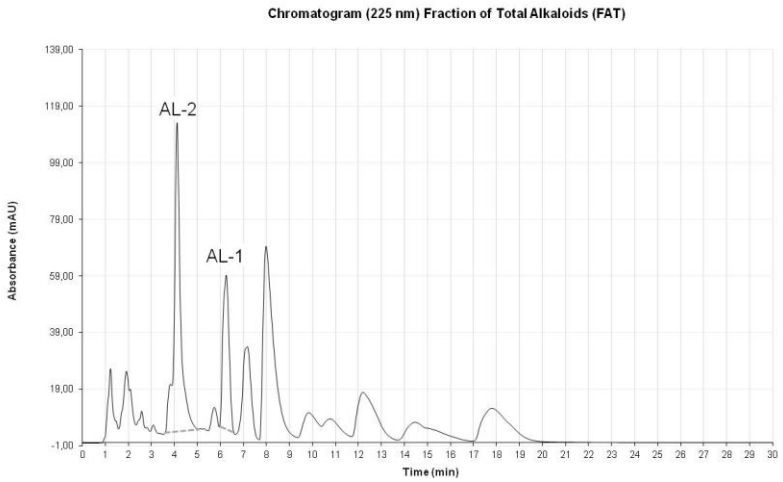

Figure 2
Chromatogram fraction of total alkaloids (FAT) observed at 225 nm. Isocratic elution was performed using (30:70 | 1.60×10^{-3}M:MeCN).

Formalin test

In the formalin test, mice (n = 8) were pre-treated with the vehicle, EcME (50, 100, and 200 mg/kg, i.p.), and morphine (10 mg/Kg, i.p.). After 30 minutes, 20 μL (2.5% formalin solution) was injected in the sub-plantar region of the right hind paw. After the formalin administration, the animals were placed into observation boxes, which consisted of a triangular apparatus with two walls made of transparent glass and mirror. The indication for nociception was the total duration of paw licking that was counted in two phases. The first phase usually occurs in the first 5 min after the formalin administration (neurogenic response), which is followed by a ~10-min period characterized by pain-inhibiting mechanisms. The second phase (15–30 min) is known primarily for an inflammatory response (Hunskaar and Hole, 1987).

Hot Plate test

This test quantifies the reaction time to a thermal stimulus provided by a hot plate (46.5 ± 1º C). The nociceptive effect is characterized by the stand-up behavior (jump attempt) or licking one of the hind paws. The response was quantified by the latency, which was the duration between placing the mouse on the plate and the reaction time. Groups of 8 mice were treated after a pre-selection with the vehicle, EcME (50, 100, and 200 mg/kg, i.p.), or morphine (10 mg/kg, i.p.) and were evaluated for 30, 60, 90, and 120 min after the treatments. The mice were placed on the hot plate for no more than 30 sec to avoid tissue damage.

Elevated plus maze test

This test examines the animal's exploratory behavior and is based on the rodent's dislike of open spaces and height. When anxiolytic substances are administered in animals, the number of entries is greater and/or the time they remain in the open arms are longer than those for controls. The mice were sorted into 5 groups (n = 8) that received EcME (50, 100, or 200 mg/kg, i.p.), vehicle, or diazepam (0.5 mg/kg, i.p.). Each animal was placed individually in the center of the apparatus and the number of entries and the time spent in the open and closed arms were recorded during a 5-min period.

Rota-rod test

The rota-rod apparatus consists of a rotating bar, subdivided into 5 compartments, delimiting a total of 4 segments. Animals were pre-selected without providing treatment based on the time the animal remained on the rotating bar of the rota-rod apparatus for at least a minute at a constant speed of 7 rotations per minute (7 r.p.m.) (Mendes *et al.*, 2002). Twenty-four hours after the pre-selection, the mice (n =8) were pre-treated with EcME (50, 100, or 200 mg/kg, i.p.), vehicle, or diazepam (1 mg/kg, i.p.). The mice were observed at different time intervals (30, 60, and 90 min) to determine the duration for which the animals remained on the apparatus.

Statistical analysis

The data were analyzed with an analysis of variance (ANOVA) to identify significant differences among groups, followed by Dunnett's *post hoc* test. The descriptive data were expressed as the mean ± S.E.M; statistical significance was determined when $p < 0.05$. The LD_{50} was determined using non-linear regression.

RESULTS

Characterization and quantification of the Alkaloids

Based on the characterization method used, we quantified the alkaloids AL-1 and AL-2 (AL-1: 3α-3.4.5 trimetoxibenzoiloxi-6β-benzoil tropane (Catuabine B); AL-2: 3α-(3.5 dimetoxibenzoiloxi)-6β-benzoil tropane). We identified 28.60 mg of AL-1 and 18.97 mg AL-2 in 1 g of EcME (Table 1).

Table 1
Values of the mass of alkaloids AL-1 and AL-2 in 1.0g of EcME

Mass of EcME (mg)	Mass of FAT in the EcME (mg)	Recovery of the FAT in EcME (%)	Recovery of the AL-1 in the FAT (%)	Mass of the AL-1 in EcME (mg)	Recovery of the AL-2 in the FAT (%)	Mass of the AL-2 in EcME (mg)
200	21.49	10.74	26.62	5.72	17.66	3.79
1000	107.44	10.74		28.60		18.97

(AL-1): 3α-3.4.5 trimetoxibenzoiloxi-6β-benzoil tropane (Catuabine B); (AL-2) 3α-(3.5 dimetoxibenzoiloxi)- 6β-benzoil tropane; EcME: Methanol Extract of *Erythroxylum caatingae

Acute toxicity of the EcME

The EcME showed a LD_{50} of 732.8 mg/Kg and a confidence limit of 678.1-791.8 mg/Kg.

Effects of EcME in the acetic acid-induced writhing test

In the acetic acid test, we observed a significant reduction ($p < 0.001$) in the number of contortions with the experimental doses of 50, 100, and 200 mg/kg i.p. when compared with the control group. These findings were similar for the morphine positive control (Figure 3).

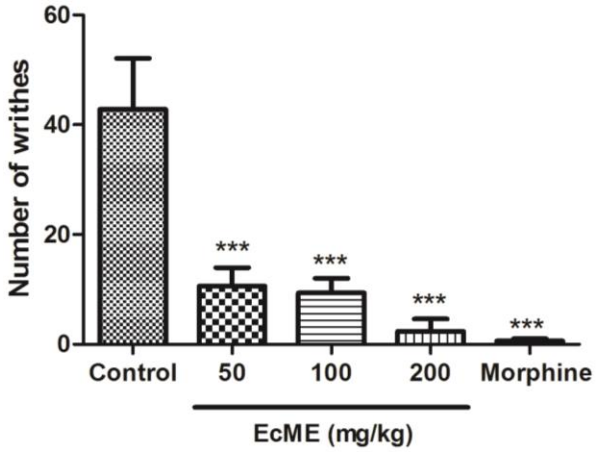

Figure 3
Effect of intraperitoneal administration of methanolic extract of *Erythroxylum caatingae* (EcME) in the number of abdominal contortions induced by acetic acid in mice (n = 8 per group). *** p< 0.001 versus the control group. The values are expressed as mean ± S.E.M. (ANOVA - Dunett test).

Effect of the EcME in the formalin test

The mice treated with EcME (50, 100, and 200 mg/kg, i.p.) showed no significant reduction in the duration of paw licking compared to the control. As shown in Figure 4, the animals treated with doses of 50, 100, and 200 mg/kg i.p. of EcME showed significantly decreased ($p < 0.001$) paw licking time in the second phase of the test, as compared to the control group (Figure 5). The morphine positive control (10 mg/kg, i.p.) showed a reduced licking time in both phases.

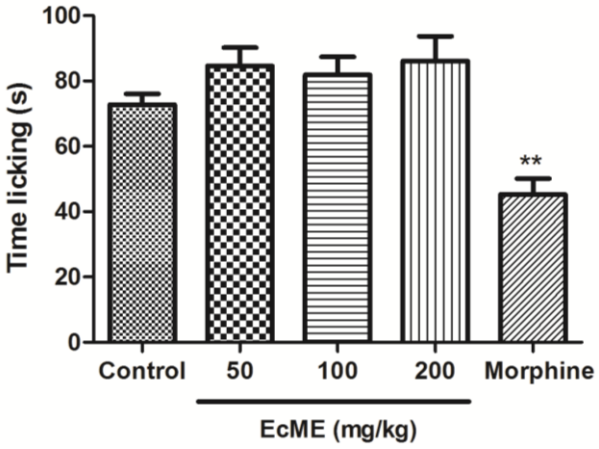

Figure 4
Effect of intraperitoneal administration of methanolic extract of *Erythroxylum caatingae* (EcME) in the licking time in the first phase of the Formalin test in mice (n = 8 per group). ** p <0.01 versus the control group. The values are expressed as mean ± S.E.M. (ANOVA - Dunett test).

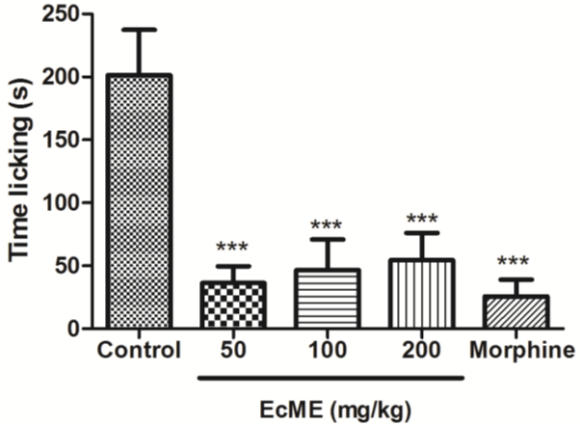

Figure 5
Effect of intraperitoneal adminstration of methanolic extract of *Erythroxylum caatingae* (EcME) in the licking time in the second phase of the Formalin test in mice (n = 8 per group). *** p < 0.001 versus the control group. The values are expressed as mean ± S.E.M. (ANOVA - Dunett test).

Effect of the EcME in the hot plate test

Compared to the control group, EcME (50, 100, and 200 mg/kg, i.p.) showed no significant change in latency during the hot plate test at any of the analyzed time (Figure 6).

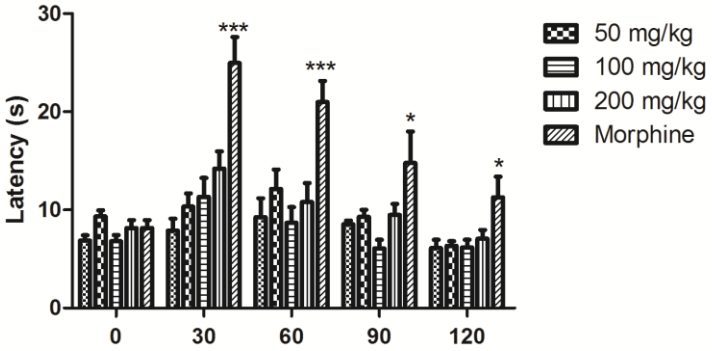

Figure 6
Effect of intraperitoneal administration of methanolic extract of *Erythroxylum caatingae* (EcME) on the stay time on the hot plate in mice (n = 8 per group). *p < 0.05, ***p < 0.001 versus the control group. The values are expressed as mean ± S.E.M. (ANOVA - Dunett test).

Effect of the EcME in the elevated plus maze test

Compared to the control group, the animals treated with EcME (50, 100, and 200 mg/kg) failed to show significant changes in both the number of entries and in the time spent in the open and closed arms, thus indicating an absence of a sedative effect. Diazepam was able to reduce the time and the number of entries in the open arms, as compared to the control group (Table 2).

Table 2
Effects of intraperitoneal administration of methanolic extract of *Erythroxylum caatingae* (EcME) on mice in the elevated plus maze test

Treatment (mg/kg, i.p.)	Permanence time (s)		Entries number	
	Open arms	Closed arms	Open arms	Closed arms
Control	46.6 ± 13.1	210.9 ± 17.4	4.4 ± 0.9	8.0 ± 0.8
EcME (50)	41.4 ± 7.7	187.4 ± 10.3	4.8 ± 0.7	9.0 ± 0.7
EcME (100)	53.2 ± 8.5	183.6 ± 12.2	5.6 ± 1.6	8.8 ± 1.0
EcME (200)	50.3 ± 17.0	207.9 ± 22.4	4.6 ± 0.8	8.1 ± 0.8
Diazepam (0,5)	126.0 ± 25.0**	108.4 ± 23.4***	10.7 ± 2.9*	4.7 ± 0.8*

The values are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 versus the control group (ANOVA - Dunett test).

Effect of the EcME in the Rota-Rod Test

EcME treatment at any dose had no significant effect on the time that animals remained on the rotating bar, indicating that EcME does not impair motor activity. The positive control diazepam, however, reduced the amount of time on the rota bar (Figure 7).

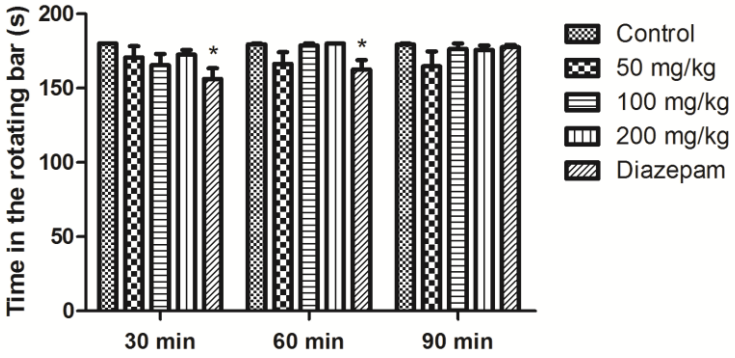

Figure 7
Effect of intraperitoneal administration of methanolic extract of *Erythroxylum caatingae* (EcME) on the stay time in the rotating bar in the Rota-Rod test in mice (n = 8 per group). *p < 0.05 versus the control group. The values are expressed as mean ± S.E.M. (ANOVA - Dunett test).

DISCUSSION

By determining the acute toxicity and the lethal dose 50 (LD_{50}), we can investigate the possible toxic effects of substances and extracts, and determine the dose responsible for the death in 50% of the animals studied (Litchfield and Wilcoxon, 1949). These findings provide data that allow pharmacological tests at safe doses. The estimation of the LD_{50} for EcME delivered i.p., which was approximately 732.8 mg/kg (678.1 - 791.8mg/kg), allowed us to determine doses that could be used in the present antinociceptive tests. During the observation period, EcME administration at high doses induced some toxic signs such as piloerection, ataxia, and increased defecation.

From the data obtained through acute toxicity evaluation, we determined the EcME doses used in the present study (50, 100, and 200 mg/kg, i.p.). As the results in Table 1 indicate, we found the following masses of alkaloids (AL-1): 3a-3.4.5 trimetoxibenzoiloxi-6b-benzoyl tropane (Catuabine B), 1.43 mg to 50 mg/mL, 2.86 mg to 100 mg/mL and 5.72 mg to 200 mg/mL of EcME. As for (AL-2) 3a-(3.5 dimetoxibenzoiloxi)-6b-benzoyl tropane there is 0.95 mg to 50 mg/mL, 1.90 mg to 100 mg/mL, and 3.79 mg to 200 mg/mL of EcME.

The writhing test is characterized by its high sensitivity, although it is not as selective as it is sensitive to sedative drugs, muscle relaxants, analgesics, non-steroidal anti-inflammatory drugs, and narcotics (Collier *et al.*, 1968). The nociceptive response to acetic acid may involve direct stimulation of the nociceptive afferent fibers. This may occur through a reduction in pH or synthesis of inflammatory mediators such as the arachidonic acid, metabolism via COX, or with consequent biosynthesis of prostaglandins (Duarte *et al.*, 1988; Franzotti *et al.*, 2000) that may be indicated by increased prostaglandin levels E_{α} and $F_{2\alpha}$ in the peritoneal fluid (Deraedt *et al.*, 1976; Oliveira *et al*, 2001; Almeida, 2006). Studies carried out by Ribeiro *et al.* (2000) have shown that the nociceptive activity of acetic acid can be due to cytokine release by macrophages and peritoneal mast cells, including tumor necrosis factor-alpha, interleukin-β1, and interleukin-8 (Bastos *et al.*, 2006).

There was a significant decrease in the number of contortions in groups treated with EcME (50, 100, and 200 mg/kg i.p.). From these results, it can be determined that increasing the dose causes a reduction in the number of contortions, demonstrating the effectiveness of the EcME in the writhing test. We therefore propose that this extract presents antinociceptive activity and/or inhibits release of inflammatory mediators and cytokines.

In order to evaluate the antinociceptive activity of EcME observed in the acetic acid-induced writhing test, we was used a hot plate test. The hot

plate test uses a methodology that has specificity in evaluating substances that act in the CNS and the formalin test.

The hot plate test measures the animal reaction time to the thermal stimulus. The reaction time is considered the time at which the animal shows the stand-up behavior (jump attempt) or licks one of its paws, which indicates nociception. These behaviors are understood as integrated responses in the brain, while the unrest of the hind paws (tap dance) occurs as a response to medullary integration. The latency of the response is quantified in a maximum time of 30 sec in order to avoid tissue damage, and may be modified by analgesics of central action (Almeida, 2006). At doses of 50, 100, and 200 mg/kg, EcME showed no significant responses at any of the analyzed times. This finding differed from that for the standard group (morphine 10 mg/kg, i.p.).

The formalin test constitutes a valid and safe nociception model and is sensitive to several classes of analgesic drugs. Formalin produces a distinct biphasic response where analgesic drugs may act differently in the first and second phases of the test (Morteza-Semnani *et al.*, 2002).

The first phase of the formalin model occurs due the release of substance P and direct nociceptive chemical stimulation of afferent fibers, especially direct C fiber stimulation (Heapy *et al.*, 1987). This phase is sensitive to drugs that act at the central level, such as morphine. The second phase results from local action of inflammatory mediators, such as prostaglandins, histamine, serotonin, and bradykinin (Rujjanawate *et al.*, 2003), or by facilitating spinal synaptic transmission (Tjolsen *et al.*, 1992; França *et al.*, 2001).

Between the first and second phase of the formalin test is the "inter-phase" that occurs when mechanisms that inhibit pain release substances such as GABA, serotonin, and endorphins, which are hyperpolarizing substances (Henry *et al.*, 1999).

Drugs that act at the central level, such as the analgesic opioids, inhibit both phases of the formalin test; however, drugs with peripheral actions, such as anti-inflammatories, are only effective in the second phase (Adeyemi *et al.*, 2004).

We observed that EcME did not reduce nociceptive responses in the first phase of the formalin test. In the second phase, however, the doses of 50, 100, and 200 mg/kg of the EcME produced a significant reduction in the RT for paw licking, indicating a possible peripheral analgesic effect by inhibiting the release of chemical mediators (Bastos *et al.*, 2006; Ferreira *et al.*, 2006; Fisher *et al.*, 2007).

We performed the elevated plus maze test and the rota-rod test to attempt to eliminate false positive results that might be due to neuromuscular block or central sedative effects. The elevated plus maze test is based upon the fact that mice and other rodents avoid exposing themselves in the open and high areas of the labyrinth, and prefer to be enclosed within protected areas and surrounded by walls. In general, anxiolytic drugs increase the number of entries into open spaces and increase the time they remain in the open arms (Grundmann *et al.*, 2007). We observed no behavioral modification with EcME in this test, indicating a possible anxiolytic effect for EcME.

The rota-rod test is used for drug screening to identify psychomotor inhibition. This test evaluates the animal's body movement using the total time the animal remains on the rotating bar (Capasso, 1996). Poor coordination in the rotating bar test is a feature of pharmacological agents, such as skeletal muscle relaxants or drugs that decrease CNS activity, such as neuroleptics, sedatives, anxiolytics, and hypnotics (Sen and Chaudhuri, 1992; Pultrini *et al.*, 2006). However, the rotating bar test is a non-specific method, as it measures indistinctly with either stimulants or depressant effects on body movement, which is also attributed the term neurotoxicity (De Sousa *et al.*, 2007). Therefore, there was no significant reduction in the time animals remained on the rotating bar at 30, 60, or 90 minute intervals following EcME treatment. This may indicate that the treatment does not interfere with body movement, thus ruling out a muscle relaxant effect or even neurotoxicity.

Based on our findings, we can infer that *Erythroxylum caatingae* presents antinociceptive activity as evidenced by decreasing the abdominal contortions induced by acetic acid and the decrease licking time observed in the second phase of the formalin test. The extract did not provide central antinociceptive activity, as it was unable to increase latency in the hot plate test. The EcME did not show sedative or muscle relaxant effects, which again confirms its peripheral actions.

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