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BIOMEDICAL SCIENCES

Samanea tubulosa Benth. (Fabaceae): Antinociceptive effect on acute pain in mice: K^+_{ATP} channel and opioid activity

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Abstract: *Samanea tubulosa* Benth. it has been widely used in traditional medicine to treat inflammatory processes. The present study aimed to investigate the antinociceptive effect and mechanism of action of the fractions obtained from the Samanea tubulosa pods in mice. The antinociceptive activity was evaluated in formalin, capsaicin and glutamate tests and the. The possible mechanisms of action involved in the antinociceptive effect of the hexane and ethyl acetate fraction in the opioid system, also the the K^+_{ATP} channels and the L-arigine pathways of nitric oxide were evaluated. The chemical characterization analysis revealed in the hexane fraction the presence of triterpenes such as lupenone and lupeol. In the glutamate test, the hexane and ethyl acetate fractions showed antinociceptive activity at the dose of 12.5 and 25 mg kg^{-1}. The antinociception produced by the hexane and ethyl acetate fractions was significantly reversed by naloxone, indicating that the fractions act through the opioid pathway. Antinociceptive response of the ethyl acetate fraction was blocked by glibenclamide, indicating that this fraction acts via the K^+_{ATP} channels activation. It is concluded that the fractions under study exert antinociceptive activity possibly related to the opioid route and through K^+_{ATP} channels activation.

Key words: Analgesic, medicinal plants, natural products, morphine.

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with tissue damage, which causes great impact on quality of life (Anand et al. 2019). Within the general context of pain, the sensory components of pain transmission are related by stimulation of the peripheral site of the lesion to the central areas of the brain through the spinal cord (ascending pain pathways) and the physiological mechanisms of modulation converge with psychological components, internal and affective, which together generates the pain process (Mccarberg & Peppin 2019, Bourne et al. 2014).

One of the major challenges in current therapy concerns painful processes that arise as a local response triggered by injury or trauma, which can be defined as a system of defense of the body to inhibit the spread of infection or injury (Burma et al. 2017, Aaltonen et al. 2012). In the market there is a range of drugs available to treat the pain such as immunosuppressants and non-steroidal anti-inflammatory drugs, but the side effects associated with them limit their

use, and although highly effective they can not be dissociated from important adverse effects such as osteoporosis, gastric lesions, arterial hypertension and allergy (Aaltonen et al. 2012, Kucukguzel & Senkardes 2015).

Studies with medicinal plants are expanding due to the ability of these species to produce molecules with medicinal activity, which can be used for therapeutic purposes (Akram et al. 2019, Naeimi et al. 2019, Souto-Maior et al. 2011, Perez et al. 2018). Therefore, natural products with anti-inflammatory and analgesic potential can be used to reduce some of the drug-related side effects that are already used in pain management (Hishe et al. 2018).

Among the medicinal plants, *Samanea tubulosa* Benth., object of this study, has been reported for the treatment of cutaneous infections, gastric inflammations, parasitic infestations and for pain (Hajdu & Hohmann 2012). The phytochemical approach of this plant pod species revealed as important constituents of the crude extract the following classes of compounds: alkaloids, saponins, flavonoids, tannins, flavones, flavanones, flavonols and catechins (Araújo et al. 2015).

Despite of existing knowledge about the plant *Samanea tubulosa* Benth. in traitional medicine, there are few scientific studies that support the analgesic potential of its pods and this research aimed to investigate the antinociceptive effect and the mechanism of action of the fractions obtained from the pods of *Samanea tubulosa* Benth. in mice.

MATERIALS AND METHODS

Collections of plant material and extraction

Samanea tubulosa (Benth.) Barneby & JW Grimes (Fabaceae) pods were collected in July 2014 at the Center for Agricultural Sciences of the Federal University of Piauí (CCA-UFPI) in the city of Teresina, Piauí, Brazil (5° 5' 13" S, 42° 48' 42" W and 72 m from sea level. After collecting the research material, an exsiccate of the species was deposited at the Graziela Barroso Herbarium of the Federal University of Piauí (TEPB 27.261).

Samanea tubulosa pods (1.6 kg) were dehydrated in a forced air circulation dryer at 40 ° C, ground in a knife mill to a fine powder, which was macerated with P.A. ethanol in triplicate at room temperature. The solvent was removed on a rotary evaporator under reduced pressure to obtain the ethanol extract which yielded 104 g (6.5%), which was suspended in MeOH-H_2O (2:1) and then partitioned liquid-liquid using hexane and ethyl acetate to give the hexane fraction (HEX, 16.76 g, 16.11%), ethyl acetate fraction (EtOAc, 5.77 g, 5.54%) and aqueous fraction (66.6 g, 64.03%).

Gas chromatography–mass spectrometry (GC-MS)

The chemical analysis of the HEX was performed by the gas chromatography technique couple to a spectrometer (GC/MS Shimadzu, QP2010S) equipped with an AOC-20i injector. STHF was dissolved in chloroform and a 1 µL alíquota of the solution was injected into the GC-MS system. The GC was equipped with an analytical column RTX-1 (30 m x 0.25 mm), 0.10 µm, dimethylpolysiloxane (Agilent, Palo Alto, CA, USA). Hellium was used as the carrier gas at a constant flow rate of 0.80 mL min^{-1}. The injector (Splitless mode, 20:1 Split ratio) was maintained at 310 ºC. The initial column temperature was set at 80 ºC and maintained for 2 min. It was then ramped by 30 ºC min^{-1} up to 290 ºC, the 25 ºC/min up to 300 ºC and, finally, 20 ºC min^{-1} up to 310 ºC, where it was maintained for 15 min and mass spectrometer operating at 70 eV. Detector MS in a scan (m/z 30 to 450 Da). The HEX constituents were identified by comparing

the mass spectra with those in the data system library (NIST version 8.0). The mass spectrum of the individual unknown compounds was compared with that of known compounds stored in the software database Library.

Electrospray Ionization Mass Spectrometry (ESI-ITMS)

The EtOAc composition was determined by electrospray ionization trap (ESI-IT-MS) mass spectrometry, a 10 mg aliquot of the acetate fraction was dissolved in MeOH (ºHPLC), filtered on nylon membrane of 0.2 µm and dissolved at 50 mg L^{-1}. An aliquot of 500 µL was injected by direct insertion into the mass spectrometer (Amazon X, Buruker Daltonics), in the following parameters: ESI negative ion source, m/z range 100-300 Da, capillary voltage 4, 5kV, mist gas (N_2) at 8.0 L min^{-1}, with sample flow 3.0 L min^{-1} and source temperature of 200 ° C.

Chemicals and reagents

Capsaicin, glibenclamide, glutamate, L-arginine, L-NOARG, naloxone and Tween 80 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Morphine was purchased from Cristália Produtos Químicos e Farmacêuticos Ltda. (SP, Brazil). Formaldehyde was obtained from Dinâmica Química Contemporânea Ltda. (SP, Brazil). Sodium chloride were from Vetec Química Fina Ltda. (RJ, Brazil). For pharmacological studies, the HEX or EtOAc fractions were dissolved in 2% Tween 80 in 0.9% NaCl (10 mL kg^{-1}). Doses were determined as milligrams of HEX or EtOAc per gram of body weight (mg kg^{-1}).

Animals

Albino mice (*Mus musculus*, Swiss line), adult males weighing 25 to 30 g, aged 50-60 days, were supplied by the Central Biotério of the Federal University of Piauí, Brazil. The animals were kept at 24 ± 1 ºC, with 12 h light/dark cycle and free access to food and water. They were fasted for 12 hours and accustomed to the test environment for one hour before each experiment. All experimental protocols were approved by the Animal Experimentation Ethics Committee of the Federal University of Piauí (nº 146/16 and 428/17). All efforts were made to minimize animal suffering and reduce the number of animals used.

The doses of these drugs were selected according to data from the literature and previous laboratory results.

Acute toxicity test

The acute toxicity test was performed in mice. For this study, methodologies recommended by the OECD Guide 423 (OECD 2001) were adopted. Male Swiss mice (25 to 30 g), fasted for 12 h solids, were used. The animals were divided into two groups (n=6), which were treated orally (p.o.) with a single dose of HEX and AcOEt fractions (300 mg kg^{-1} p.o.) or distilled water, in a single dose (10 mL kg^{-1}, p.o.). As no deaths or behavioral changes were observed, the dose used was increased up to the limit of 2000 mg kg^{-1}. The animals were clinically and behaviorally evaluated every 30 minutes during the first four hours and daily until the 14 th day. After toxicological investigation, the animals were euthanized with sodium thiopental (100 mg kg^{-1}, i.p.) and necropsied.

The activity of fractions was evaluated in the animal models of formalin, capsaicin, and glutamate test. Conscious mice were used in all the nocifensive tests. The dose range was 3,125 to 25 mg kg^{-1}, these doses were selected from tests performed previously by the research group. HEX or EtOAc were administered orally (p.o.), by gavage at different doses in order to construct a dose-response curve. Control groups were treated with a similar volume of the vehicle that had been used to dilute this compound.

Formalin test

Mice (n = 6 per group) were treated with vehicle (10 mL kg $^{-1}$ p.o.), fractions HEX or EtOAc (3.125, 6.25, 12.5, 25 mg kg $^{-1}$ p.o.), 1h before the test, or morphine (5 mg kg^{-1} s.c.) administered 30 minutes before the test as a positive control. The right hind foot pad was injected with formalin (20 µL, 2%) in the intraplantar region. The paw licking time was recorded (in seconds) from 0-5 min (1st Phase, neurogenic) and 15-30 min (2nd Phase, inflammatory) after administration of formalin (Rosland et al. 1990).

Capsaicin test

Mice (n = 6 per gruop) were treated with HEX (6.25, 12.5, 25 mg kg $^{-1}$, p.o.), EtOAc (3.125, 6.25, 12.5, 25 mg kg $^{-1}$, p.o.), vehicle (10 mL kg $^{-1}$), or morphine (5 mg kg^{-1}, s.c.). One hour after these treatments, the right hind paw was injected with capsaicin (2 µg paw^{-1}) prepared in 5% Tween solution and 2% etanol. Nociception was evaluated immediately after injection and quantied by paw licking time during a 5 min period (Sakurada et al. 2003).

Glutamate test

Mice (n = 6 per group) were treated with HEX or EtOAc (6.25, 12.5, 25 mg kg^{-1}), vehicle (10 mL kg^{-1}) or morphine (5 mg kg^{-1}, s.c.). The the other groups were treated 45 minutes before and morphine 30 minutes before intraplantar injection of glutamate in the dorsal region of the right hind paw (20 µmol paw^{-1}). The time during which the animal licks or bites its paw was observed and quantified for 15 min and compared between groups (Beirith et al. 2002).

Investigation of mechanisms of HEX and EtOAc induced antinocicetive action

In order to elucidate the mechanisms underlying HEX and EtOAc-induced (12.5 mg kg^{-1}) antinociception, three groups of mice (n = 6) were pretreated orally in the glutamate model with naloxone (2 mg kg^{-1}, i.p.), a non-selective antagonist of opioid receptor; glibenclamide (3 mg kg^{-1}, i.p.), an antagonist of K^+_{ATP} channels.

To evaluate the participation of the L-arginine-nitric oxide pathway in the antinociceptive effect induced by FHEX and FAcOEt, the animals were previously treated with L-arginine (600 mg kg^{-1}, ip, a precursor of nitric oxide) and after 20 minutes, received FHEX and FAcOEt (12.5 mg kg^{-1}, p.o.), N ω -nitro- L -arginine (L -NOARG, 75 mg kg^{-1}, ip, a nitric oxide inhibitor) or vehicle. After 1 h after FHEX, FAcOEt administration and 30 min after L-NOARG treatment, the animals were evaluated for nociception induced by the intraplantar injection of 20 µL of glutamate solution (20 µmol paw-1) (Beirith et al. 2002, Pietrovski et al. 2006).

Measurement of motor performace

The mice were divided into three groups (n = 6) and treated with vehicle (10 mL kg^{-1}, p.o.), HEX (6.25, 12.5 and 25 mg kg^{-1}, p.o.), EtOAc (6.25, 12.5 and 25 mg kg $^{-1}$, p.o.) or diazepam (4 mg kg $^{-1}$, i.p.), 30 min and 1 h before individual observation, the animals were taken individually to the open field, consisting of a square arena. (30 x 30 x 15 cm), made of acrylic and with black floor divided into nine equal parts, transparent walls and illuminated with red light. The animal was placed in the arena and left to explore the environment for a minute. After this period, the number of quadrants crossed with the four legs of the animal was evaluated over a 5 - minute period (Deacon 2013).

Rota Rod Test

The Rotarod tread mill device (Model RR, 2002, Insight equipment) consisted of a 2.5 cm diameter bar, 25 cm above the floor, subdivided into four compartments, with rotation of 14 rpm. Male mice were screened 24 h prior to the experiment by screening in 1-minute sessions

to eliminate animals that did not remain in the bar for three consecutive 60-second periods. The animals were divided into four groups (n = 6) and treated with vehicle (10 mL kg^{-1}, p.o.), HEX (6.25, 12.5, 25 mg kg^{-1}, p.o.), EtOAc (6.25, 12.5 and 25 mg kg^{-1}, p.o.) or diazepam (4 mg kg^{-1} i.p.) as a positive control. After one hour of vehicle and fractions or 30 minutes of diazepam administration, the time for performance on the bar was evaluated (Deacon 2013).

Statistical analysis

The results were expressed as the mean ± SEM and analyzed by one-way analysis of variance, followed by post hoc Tukey's or Bonferroni's. Significative differences among groups were considered when $p<0.05$ (GraphPad Prism® software version 6.00)

RESULTS

The HEX and AcOEt fractions at doses of 300 and 2000 mg kg^{-1}, p.o., did not show any signs of evident toxicity during the 14 days of observation, as well as no death and, consequently, it was not possible to determine the LD50 of FHEX, FAcOEt. Furthermore, no behavioral or clinical changes were observed after administration of HEX or AcOEt. Normal progress in body weight was observed and, at necropsy, neither macroscopic changes nor in the relative weight of organs: liver, kidney, spleen, lung, and heart were observed between groups treated with the fractions when compared to the vehicle group. Changes in biochemical parameters were also not observed. Data not shown because there were no changes compared to the control group.

Identification of constituents of STHF by Gas Chromatography and Mass Spectrometry (GC-MS)

The HEX Gas Chromatography and Mass Spectrometry (GC-MS) analysis showed 32 signals in the total ion chromatogram (Fig. 1) with retention times, relative area and more abundant peaks shown in Table I. Comparison of mass spectra obtained with data from the NIST 8.0 library allowed identification and confirmed the presence of 11 compounds.

Identification of EtOAc constituents by ESI (-) – ITMS

In the investigation of EtOAc chemical constituents by ion - trap mass spectroscopy with electrospray ionization in the negative mode (ESI (-) - ITMS), they present percussion peaks of the deprotonated molecules ($[M-H]^-$) present in the fraction (m/z range of 100-1300 Da), where it was possible to identify eleven chemical constituents, between phenolic compounds and flavonoids such as luteolin.

The ESI (-) - ITMS, MS^2 spectra and the fragmentations of the identified compounds are shown in Fig. 2 and in Table II. The detected and known substances were compared their fragmentation profile with those already existing in the literature in which they presented characteristic chemical structures of phenolic substances such as pyrogallol m/z 125, gallic acid m/z 169, ethyl gallate m/z 197, luteolin m/z 285, ellagic acid m/z 301, caffeic acid m/z 341, quercetin m/z-447, digaloyl glucose m/z 483. As well as flavonoids: luteolin-7-O-rutinoside m/z 593, Quercetin 3-O- (6-O-Ramnosyl glucoside) (rutin) m/z 609, and caffeic acid derivatives such as the dimers of caffeic acid-O-hexose m/z 683. In the spectrum (Fig. 2), some peaks with relative intensity higher than others, such as m/z 125 (93%); 169 (100%); 197 (92%), which in principle

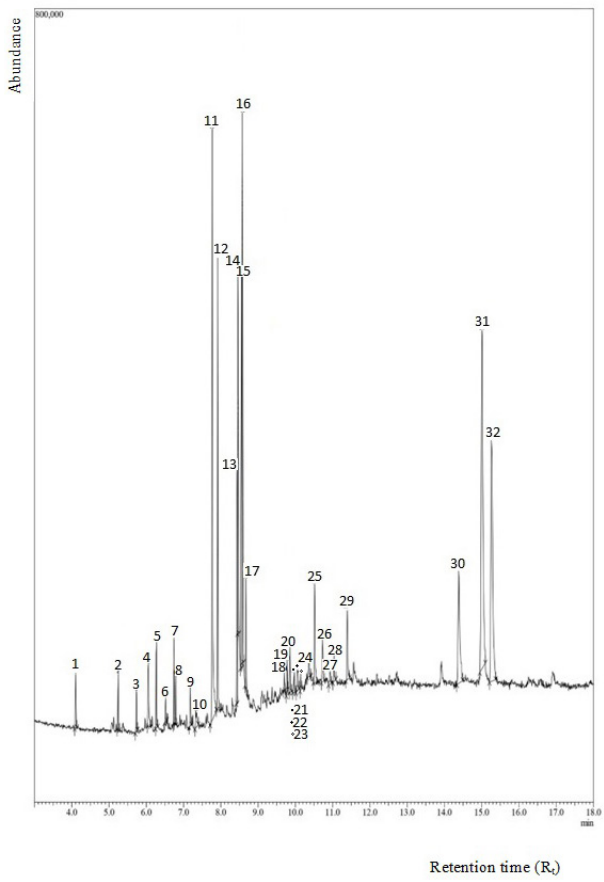

Figure 1. Gas chromatography-mass spectrometry (GC-MS) chromatogram of HEX.

characterizes fragments of the tannin class and m/z 301 (51%) of flavonoids.

Formalin test

The results shown in Table III demonstrate a significant reduction in leg licking time of HEX-treated animals. at 6.25 mg kg $^{-1}$ (43.76 ± 8.8 s), 12.5 mg kg $^{-1}$ (60.23 ± 4.27 s) and 25 mg kg $^{-1}$ (53.62 ± 4.89 s) in 63.20%, 49.36% and 54.91%, respectively, in the first phase when compared to the vehicle group that received only formalin (118.94 ± 11.22 s). HEX at doses of 6.25 and 12.5 mg kg^{-1}, promoted an inhibition of 53.72% (55.62 ± 6.66 s) and 41.95% (69.77 ± 14.64 s) respectively in the second phase. As shown in Table I, EtOAc was able to significantly inhibit (The constituents of HEX and theirbe p <0.0001) licking time in 52.02% (57.06 ± 2.25 s), 51.08% (58.18 ± 5.68 s) and 57.84% (50.14 ± 2.96 s), at the doses of 6.25, 12.5 and 25 mg kg $^{-1}$, respectively in the first phase of the formalin test. These same doses were also able to reduce the response time, in the second phase corresponding to an inhibition of 69.83%, 45.44% and 58.43%, when compared to the vehicle group (120.19 ± 6.05 s). The group treated with the standard drug morphine (5 mg kg $^{-1}$) decreased the analyzed parameter by 79.95% (23.84 ± 8.66 s) and 92.22% (9.35 ± 0.97 s) in the first and second phases of the test, respectively.

Capsaicin test

Figure 3a and 3b show the effect of HEX and EtOAc on capsaicin-induced nociception in mice. A significant reduction in paw lick time (**** p <0.0001) was observed in mice given HEX (Figure 3a) at doses (12.5 and 25 mg kg^{-1}, p.o.), promoting an inhibition of 72.86% (14.33 ± 2.15 s) and 81.41% (9.44 ± 1.98 s), when compared to the vehicle group (46.50 ± 3.07 s). As shown in Figure 3b, EtOAc was able to significantly inhibit (**** p<0.0001) licking time in 48.10% (24.13 ± 3.21 s), 33.16% (31.08 ± 2.79 s) and 47.03% (24.63 ± 3.05 s), at the doses of 6.25, 12.5 and 25 mg kg^{-1}, respectively. Morphine (5 mg kg^{-1}, s.c.) was used as a positive control and showed a reduction in response by 81.63% when compared to vehicle (**** p <0.0001).

Glutamate test

The results obtained during the glutamate test show that HEX (Figure 4a) administered (p.o.) (6.25, 12.5 and 25 mg kg^{-1}) caused significant inhibition (*** p <0.001 and * p <0.05) at doses of 12.5 and 25 mg kg $^{-1}$, exhibiting an inhibition of 68.39% (40.61 ± 9.64 s) and 36.40% (81.72 ± 13.03 s), respectively, when compared to the vehicle group (128.5 ± 18.78 s). On the other hand, EtOAc (Figure 4b) administered (p.o.) (6.25, 12.5 and 25 mg kg $^{-1}$) caused significant inhibition (* p <0.05)

Table I. Chemical compounds (%) identified by gas chromatography-mass spectrometry (GC-MS) analysis of hexane fraction (HEX).

Compound	*R_t (min)	Composition %
p-Toluamide, N, N-diethyl	6.055	1.53
Hexadecane	6.272	1.36
Palmitic Acid	7.773	12.26
Palmitic acid, ethyl ester	7.917	6.62
Linoleic acid	8.450	5.34
Oleic acid	8.461	6.15
Linoleic acid ethyl ester	8.557	7.67
Oleic acid, ethyl ester	8.582	8.84
Stearic Acid, Ethyl Ester	8.676	1.56
Phytic acid, dioctyl ester	9.859	0.77
Lupenone	15.012	16.53
Lupeol	15.268	12.05

*Rt = Retention time.

at doses of 6.25, 12.5 and 25 mg kg $^{-1}$, exhibiting an inhibition of 45.26 (76.02 ± 14.81 s) and 43.21% (78.87 ± 9.22 s), respectively, when compared to the vehicle group (138.9 ± 18.95 s). The group treated with morphine (5 mg kg^{-1}), standard drug, also significantly reduced this parameter presenting 95.24% (6.61 ± 3.16 s) inhibition.

Analysis of possible antinociceptive mechanisms of action of the HEX and EtOAc in mice

As can be seen in Figure 5 (a-b), naloxone significantly inhibited the antinociceptive effect of HEX and EtOAc, as well as glibenclamide (Figure 5 (c-d)).

The results of the evaluation of the participation of the L-arginine-nitric oxide pathway show that the group treated only with L-arginine (127.0 ± 32.91) presented values close to those of the control (166.3 ± 18.50), ruling out the existence of an antinociceptive effect of the nitric oxide precursor. L-arginine associated with L-NOARG (106.4 ± 26.25 s) reversed the effect of L-NOARG alone (53.05 ± 10.38 s). The association of L-arginine with FHEX (12.5 mg kg^{-1}) (75.78 ± 18.85 s) did not significantly change the licking time of the animals when compared to FHEX (48.57 ± 8 .35 s) alone, the same was observed for the FAcOEt (45.00 ± 6.95 s). It was thus evidenced that the antinociceptive effect of FHEX and FAcOEt is not related to the nitric oxide pathway.

Measurement of motor performance and locomotor activity

In order to evaluate the effect of motor coordination and spontaneous locomotion of HEX and EtOAc, the mice were submitted to the open field test and rotated rod. For the open-set test, HEX and EtOAc (6.25, 12.5 and 25 mg kg^{-1}, p.o.) do not interfere with the number of invasions of the animals, neither do they alter the movement frequency of the animals nor the duration time the animals stayed in the bar on the rota-rod test for 1 min when compared to vehicle. Not shown, because there was no difference.

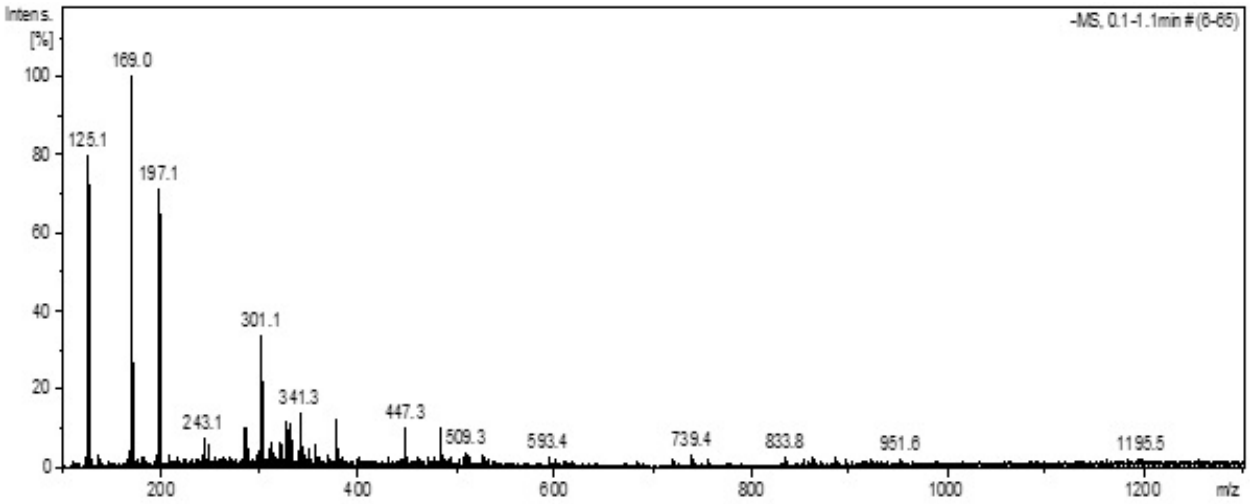

Figure 2. Spectrum ESI-ITMS of EtOAc evaluated in negative mode in the range of ions with m/z of 100-1300 Da.

Table II. Proposed compounds detected in EtOAc by ESI(-)-ITMS in negative ion modes.

Tentative Identification	$[M-H]^-$ (*m/z*)	MS^2 Ions (*m/z*)
Pyrogallol	125	---
Gallic acid	169	139, 125
Ethyl gallate	197	169, 159, 151, 125
n.i.	243	225, 197, 181, 169, 151, 139, 125, 109
Luteolin	285	265, 241, 221, 199, 175, 155, 141, 125
Ellagic acid	301	284, 257, 229, 221, 201, 185, 169, 153, 139, 125,109
Caffeic acid-O-hexose	341	323, 297, 279, 251, 227, 197, 183, 179, 169, 161, 143, 131, 119
n.i.	377	357, 341, 215, 179
Quercetin	447	401, 370, 341, 301, 255, 179, 151
Digaloyl-glucose	483	447, 331, 313, 301, 271, 255, 211, 193, 169
Luteolin-7-O-Rutinoside	593	588, 555, 499, 447, 341, 285, 255, 169
Quercetin-3-O- (6-O-Ramnosyl-glucoside) (Routine)	609	531, 513, 445, 314, 301, 263
Caffeic acid dimer-O-hexose	683	678, 635, 509, 447, 341

*n.i. (unknown).

Table III. Antinociceptive effect partitioning fractions in the fornalin induced nociceptive response in mice.

Treatment	Dosage (mg kg^{-1})	Total time spent licking (s)			
		0 – 5 min	Inhibition (%)	15 – 30 min	Inhibition (%)
Vehicle	-	118.94 ± 11.22	-	120.19 ± 6.18	-
HEX	3.125	94.35 ± 4.58	-	101.55 ± 6.66	-
	6.25	43.76 ± 8.88****	63.20	55.62 ± 9.51****	53.72
	12.5	56.23 ± 6.29****	52.72	59.24 ± 12.71****	50.71
	25	53.62 ± 4.89****	54.91	94.42 ± 6.05	-
EtOAc	3.125	91.90 ± 8.66	-	99.67 ± 8.29	-
	6.25	47.26 ± 5.12****	60.59	29.44 ± 9.16****	75.50
	12.5	58.18 ± 5.68****	51.08	65.57 ± 11.15****	45.44
	25	50.14 ± 2.96****	57.84	49.96 ± 7.88****	58.43
Morphine	5	13.30 ± 3.02****	88.81	13.26 ± 2.58****	88.96

Mice were treated with fractions 60 min (p.o.) before formalin test. Data represent the mean ± S.E.M of 6 animals. ** Siginificance level of $p<0.0001$. * (HEX - hexane fraction; EtOAc - ethyl acetate fraction).**

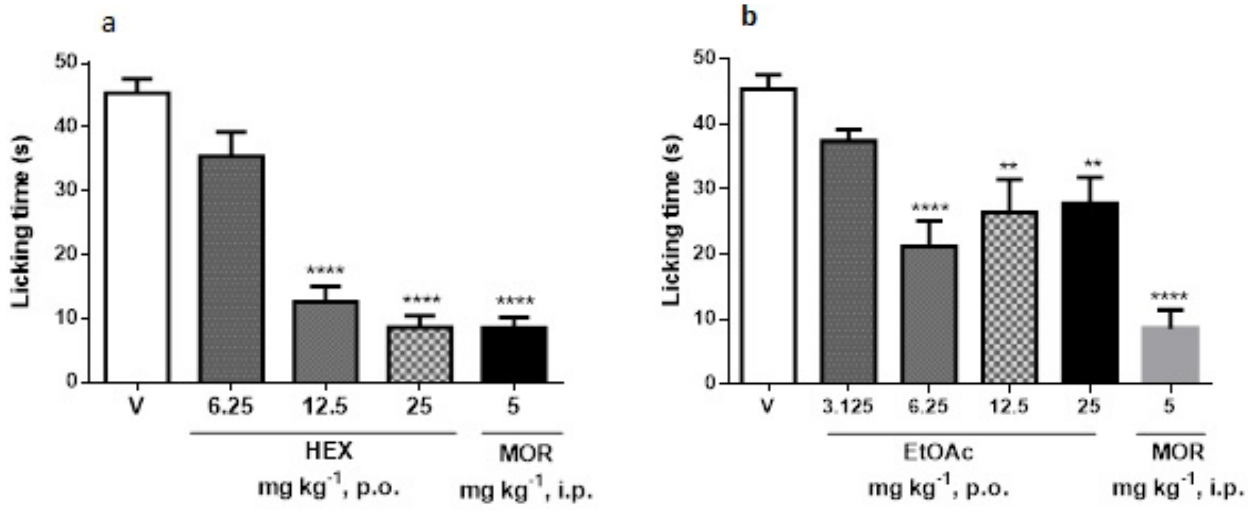

Figure 3. Effect of hexanic fraction – HEX (a), ethyl acetate fraction – EtOAc (b) administred orally and morphine (MOR) against capsaicin-induced nociception in mice. Each column representes the mean ±S.E.M. of 6 animals. Vehicle value (V) indicates the animals treated with vehicle and the asterisks denote the significance levels, when compared with control groups (one-way ANOVA followed by Bonferroni test) ($p<0.01$; **** $p<0.0001$).**

DISCUSSION

In the acute toxicity test, with observations up to 14 days, no changes were observed in: behavioral, body weight, relative weight or macroscopic aspects of the organs, nor biochemical changes. According to OECD 423, since all animals survived up to the dose of 2000 mg kg^{-1}, until the end of the observation period, the fractions can be considered as having low toxicity and can be included in category 5, with a LD50 estimated between 2000-5000 mg kg^{-1} (OECD 2001).

Most secondary metabolites are divided into three main classes according to the metabolic pathway, namely terpenoids,

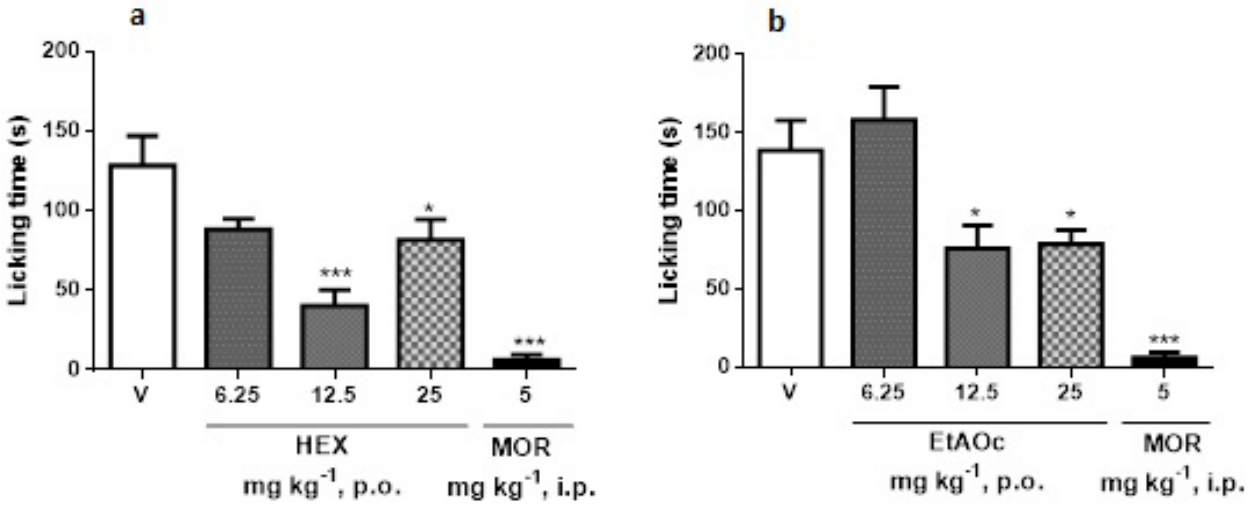

Figure 4. Effect of hexanic fraction – HEX (a), ethyl acetate fraction – EtOAc (b) administred orally and morphine (MOR) against glutamate-induced nociception in mice. Each column representes the mean ±S.E.M. of 6 animals. Vehicle value (V) indicates the animals treated with vehicle and the asterisks denote the significance levels, when compared with control groups (one-way ANOVA followed by Bonferroni test) (* *p*<0.05; * *p*<0.001).**

phenolic compounds and alkaloids and exhibit relevant pharmacological activity such as anti-inflammatory, anticancer among others. Which due to their complementary mechanism of action serve as sources of many potent drugs (Islam et al. 2017, Lakshmi & Nair 2017).

The constituents of HEX and their calculated peak areas include mostly fatty acids and their esters (52.1%), such as palmitic acid, and lupane-derived triterpenes (28.58%) such as lupenone (16, 53%) and lupeol (12.05% (Table I).

The known substances detected in EtOAc were compared in their fragmentation profile with those already existing in the literature, in which they presented chemical structures characteristic of phenolic and flavonoid substances, such as pyrogallol, gallic acid, ethyl gallate (Figure 2), luteolin and ellagic acid (Table II).

Lupeol has several pharmacological activities under in vitro and in vivo conditions. These include its beneficial activity against inflammation, cancer, arthritis, diabetes, heart disease, kidney toxicity and liver toxicity (Fernández et al. 2001, Kallubai et al. 2015). The anti-inflammatory action of Lupeol probably involves the opioid system, as indicated by the complete blockade of the opioid antagonist naloxone (Lucetti et al. 2010), as well as acting through the inhibition of IL-1β and TNF-α, during inflammatory pain, (Lima et al. 2013).

Among the biological activities of gallic acid, there is the anti-inflammatory activity with inhibition of the production of NO, PGE-2 and IL-6 (Bensaad et al. 2017) and protection against liver damage induced by carbon tetrachloride in rats, in addition to of antioxidant activity with inhibition of lipid peroxidation (Tung et al. 2009).

Luteolin, in preclinical studies have shown that this flavone possesses a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer activities (López-Lazaro 2009)

Gallic acid has an anti-inflammatory effect through the mechanisms mainly involved MAPK and NF-κB signaling pathways. It thus weakens the inflammatory response by reducing the release of inflammatory cytokines, chemokines, adhesion, and cell infiltration (Bai et al. 2021).

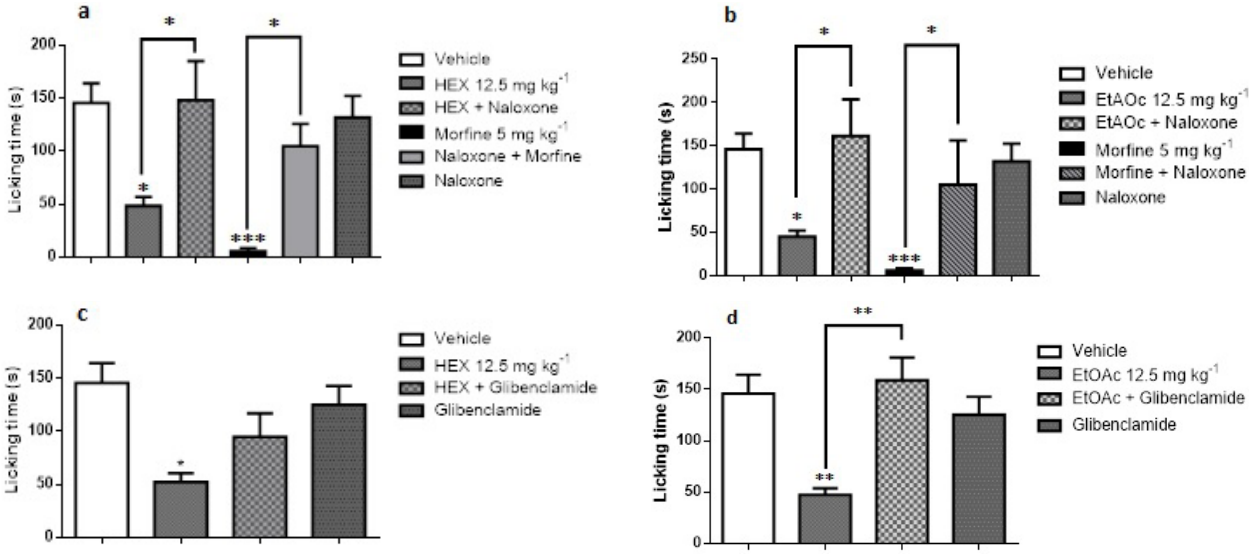

Figure 5. Effect of the HEX (hexane fraction) (12.5 mg kg^{-1}, p.o.) e EtOAc (ethyl acetate fraction) (12.5 mg kg^{-1}, p.o.) against the action of naloxone (2 mg kg^{-1}, i.p.) (a-b) and glibenclamide (3 mg kg^{-1}, i.p.) (c-d) on glutamate-induced nociception (20 µL 20 µmol^{-1} paw) in mice. Data represent mean ± SEM of 6 mice. Figure 5 (a), the symbols indicative the level of significance: $^{*}p< 0.05$ compared with vehicle, $^{***}p< 0.001$ compared with morphine (one-way analysis of variance, Bonferroni's test).

Study carried out with other plants of the Ingeae tribe, to which *Samanea tubulosa* Benth. belongs, demonstrated that the leaves of *Albizia zygia* (DC.) J.F. Macbr. have anti-inflammatory, antipyretic and analgesic properties (Abotsi et al. 2017). In addition, the ethanolic extract of *Archidendon clypearia* Jack., as well as the extract of *Abarema cochliacarpos* Gomes., have active substances that exert a marked protective effect on NF-ß. acute colitis (Da Silva et al. 2010)

In the present study, the antinociceptive activity of HEX and EtOAc was evaluated in experimental models of nociception, thus contributing to the pharmacological knowledge of this plant. The results of this study show for the first time that HEX and EtOAc obtained from the ethanolic extract of the pods of *Samanea tubulosa* Benth., have antinociceptive activity when administered orally in different models of chemical nociception in mice.

The formalin test is sensitive to several classes of analgesics. Our results show that HEX and EtOAc produced antinociception in both phases of the formalin test. Intraplantar injection of formalin demonstrates two distinct phases of pain sensitivity. The first phase, when neurogenic pain is caused by the direct effect of formalin on sensory C fibers, releasing neuropeptides as substance P, among others. The second phase is characterized as inflammatory pain, related to the release of nociceptive mediators such as histamine, serotonin, bradykinin, prostaglandins and excitatory amino acids, which can be inhibited by analgesics and anti-inflammatories (Hunskaar & Hole 1987, Baliki et al. 2014).

In addition, HEX and EtOAc were able to inhibit neurogenic nociception caused by capsaicin, an alkaloid extracted from spicy pepper, which stimulates nociceptive and thermal nerve endings causing severe pain. Capsaicin acts by activating the transient potential receptors (TPR) (Gees et al. 2013, Hwang et al. 2000). An injection of capsaicin into the hind paw of mice produces characteristic nociceptive behavior patterns, such as licking and biting the affected paw, producing significant changes in the thresholds

of thermal and mechanical nociception. Such events occur through direct stimulation of unmyelinated C-type fibers and Aδ thin and poorly myelinated fibers through the transient potential vanilloid receptor1 (TRPV-1) located in nociceptive neurons, opening a non-selective cation channel, which allows the influx of cations, mainly Ca^{2+} and Na^{+}. This causes depolarization and initiation of action potentials and the release of various neuropeptides involved in painful neurotransmission, including substance P (Bourinet et al. 2014, Palazzo et al. 2008).

In order to evaluate the involvement of the excitatory amino acids in the antinociceptive effect of HEX and EtOAc, the nociception test was performed induced by glutamate. Glutamate is an important excitatory neurotransmitter that transmits nociceptive signals promoting the direct activation of receptors in nociceptive fibers (Baliki et al. 2014). It is linked to the mediation of nociception in the dorsal spine and periphery, since it is released in response to nociceptive stimulation and tissue or nerve damage (Diaz & Dickenson 1997). For this reason, glutamate can be used as an agent in the induction of nociception (Kuner 2010, Kryzhanovskii 1999). The nociceptive response generated by glutamate has its activity mediated by the activation of glutamatergic receptors, which are divided into two large groups: ionotropic and metabotropic. The ionotropes can be of three types: N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-isoxazol-4-propionic acid (AMPA) and kainate (KA) (Kuner 2010). The stimulation of these receptors, especially NMDA, may lead to the activation of intracellular enzymes and the production of second messengers, such as nitric oxide (NO), which will produce hyperalgesia (Beirith et al. 2002). In this sense, substances that decrease glutamate-induced nociception may be acting on their receptors as antagonists or inhibiting NO synthesis by NOS blockade (Tiwari et al. 2014).

Medications that may block sensory hyperexcitability include anticonvulsants, antidepressants and analgesics. They exert their effects on calcium channels, sodium channels, monoamine uptake mechanisms, and G-protein coupled membrane receptors typically (Khalid & Tubbs 2017). In order to clarify whether the mechanism of action involved in the antinociceptive response of HEX and EtOAc would be related to the inhibitory pathways of pain, we used pharmacological antagonists capable of preventing the connection between the agonist and a specific receptor. In the present study, we showed that pretreatment with glibenclamide before glutamate injection in the paw was effective in altering the antinociceptive effect of EtOAc. This shows that the antinociceptive effect of EtOAc on the test was mediated by the activation of K^{+}_{ATP} channels pathway (Ghorbanzadeh et al. 2014).

The results of the evaluation of the participation of the L-arginine-nitric oxide pathway show that the group treated only with L-arginine presented values close to those of the control, ruling out the existence of an antinociceptive effect of the nitric oxide precursor. L-arginine associated with L-NOARG reversed the effect of L-NOARG alone. The association of L-arginine with FHEX did not significantly change the licking time of the animals when compared to FHEX alone, the same was observed for the FAcOEt. It was thus evidenced that the antinociceptive effect of FHEX and FAcOEt is not related to the nitric oxide pathway.

The antinociception produced by the hexane and ethyl acetate fractions was significantly reversed by naloxone, indicating that the fractions act through the opioid system. The antinociceptive response of the ethyl

acetate fraction was blocked by glibenclamide, indicating that this fraction acts via the K^+_{ATP} channels.

Among the components of the studied fractions, FEX and EtOAc, which presents the greatest similarity of actions with the results of the fractions, was Lupeol, which also acts via the opioid pathway.

It is known that drugs with sedative activity may also inhibit the behavior of licking and scratching the animal's paw and also influence the motor response (Burma et al. 2017). However, the present study showed that the HEX and EtOAc did not alter the locomotor activity of the animals. Locomotor activity is a result of cerebral activation, which manifests as an excitation of central neurons and an increase in brain metabolism (Calabresi et al. 2000). The open field and Rota-Rod tests were used to exclude the possibility that the antinociceptive action of the fractions is related to nonspecific disturbances in the locomotor activity of the animals (Goulding et al. 2008).

The present study showed that treatment with HEX and EtOAc had low toxicity by the acute toxicity test and had no effect on motor and locomotor coordination, as reflected by the open field test and rotated rod. Therefore, suppression of the licking response induced by capsaicin, formalin, and glutamate, caused by the treatment with HEX and EtOAc, are complementary indications of the antinociceptive action of this plant.

In conclusion, the results contribute to the pharmacological knowledge of *Samanea tubulosa*, because it was shown that the partition fractions (HEX, EtOAc) have antinociceptive properties effect in models of chemical nociception and this action possibly occurs with the involvement of the opioid system and via K^+_{ATP} channels.

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