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ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF THE AQUEOUS EXTRACT OF *MIKANIA LINDLEYANA* IN RODENTS

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ABSTRACT

Mikania lindleyana is a plant widely distributed in Brazilian Amazonia, popularly known as “sucuriju” and largely used in folk medicine to treat inflammation, chronic ulcers and pain. In the present study, we identified the secondary metabolites of the aqueous extract of *M. lindleyana* (AEML) and investigate its effects on several models of inflammation and nociception in rodents. Phytochemical screening of the AEML showed the presence of saponins, proteins, amino acids, phenols, tannins, organic acids and flavonoids. Oral pretreatment of mice with AEML significantly ($P < 0.05$) reduced the abdominal constrictions evoked by acetic acid injection and the licking time in both first and second phases in the formalin test but has no significant effect on hot plate test. In rats, AEML inhibited the edema formation induced by carrageenan and croton oil while has no significant effects on the edema induced by dextran. AEML inhibited the carrageenan-induced neutrophil migration as well as the rolling and the adhesion of leukocytes. The present study shows for the first time that AEML displays antinociceptive and anti-inflammatory activities which could be attributed respectively to a possible opioid mechanism and to an inhibition of the adhesion molecules by interference on pro-inflammatory cytokines. These results support the widespread use of *M. lindleyana* in popular medicine to treat inflammation and pain.

INTRODUCTION: *Mikania lindleyana* DC (Asteraceae) is a plant native to Brazilian Amazonia where it is popularly called “sucuriju” or “sucurijuzinho” [1]. Its leaves are employed as decoction as anti-inflammatory, analgesic, wound healing as well as for the treatment of hepatitis, chronic ulcers, varicose and various skin diseases [2]. From a phytochemical view, the hydroalcoholic extract of *M. lindleyana* was found to contain alkaloids, flavonoids, tannins, steroids and terpenoids [3] while essential oil of this plant was

reported to be mainly comprised of α-phellandrene, limonene, germacrene D, α-tujeno, α-pinene, myrcene and β-caryophyllene [4].

Despite the widespread use of *M. lindleyana* in popular medicine to treat inflammation and pain, surprisingly little research has been carried out to examine its basic pharmacological properties. Therefore, the present study was undertaken to assess the effects of the aqueous extract of *M. lindleyana* (AEML) on several models of inflammation and nociception in mice and rats. The acute toxicity of the AEML as well as its preliminary qualitative phytochemical screening have been also carried out.

MATERIALS AND METHODS:

Plant material: The leaves of *M. lindleyana* were collected on April 2009, near the city of Icoaraci, State of Pará, Belém, Brazil. The identification of the plants was confirmed by Dr. Mario Augusto Gonçalves Jardim (Botanical Section of the Emílio Goeldi Museum, Belém, State of Pará, Brazil). A voucher specimen (MG 188973) was deposited in the herbarium of that Museum.

Preparation of the aqueous extract of *M. lindleyana*: Aerial parts of *M. lindleyana* (50 g) air-dried and powdered were extracted with water by decoction for 15 min according to the general method described in the Brazilian Pharmacopea [5]. The aqueous extract thus obtained was lyophilized and the yield was calculated to be 24% w/w.

Phytochemical Screening: The qualitative phyto-chemical study of the AEML was carried out according to a previously reported method [6]. Different tests were conducted to determine the presence of saponins, reducing sugars, organic acids, polysaccharides, phenols, tannins, proteins, amino acids, cardiac glycosides, catechins, derivates of quinones, flavonoids, alkaloids and purines.

Chemicals and Drugs: The following drugs and chemicals were used: acetic acid and formaldehyde used was of analytical grade (Vetec Química Fina Ltda, Rio de Janeiro, Brazil); indomethacin, carrageenan, dextran, dexamethasone, croton oil and tribromoethanol (Sigma Chemical Co., St. Louis, MO, USA); pizotifen (Novartis, Rio de Janeiro, Brazil); ketamin hydrochloride (Dopalen, Paulinia, São Paulo, Brazil), xylazine hydrochloride (Rompun, Bayer, Rio de Janeiro, Brazil), morphine sulfate and naloxone hydrochloride (Cristália, Rio de Janeiro, Brazil). The AEML was dissolved in distilled water just before the use and other drugs were dissolved in saline solution (NaCl 0.9%). AEML and drugs were administered in a volume of 0.1 mL/10g in mice and 0.1 mL/100 g in rats.

Animals: Experiments were conducted using male Swiss mice (20-30 g) and male adult Wistar rats (160-220 g) obtained from the Central Housing Facility of the Evandro Chagas Institute, Belém, State of Pará, Brazil. Animals were housed at 23±1°C under a 12 h light/dark cycle and with access to water and food *ad libitum*. On the day of experiment, animals which have been starved overnight with water ad libitum, were acclimatized to the laboratory for at least 2 h before performing any test and were used only once throughout the study. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html). All procedures described here were reviewed by and had prior approval from the local animal Ethics committee (Federal University of Pará, process number FAR 001-10).

Nociceptive tests:

1. **Acetic acid-induced abdominal writhes in mice**: Writhing test inducing somato-visceral pain was induced according to a previously described procedure [7]. Briefly, mice were randomly divided in 8 groups (n = 10 per group). An aqueous acetic acid solution (0.6% v/v) was injected by intraperitoneal (i.p., 10 ml/kg body weight) route to mice pre-treated 60 min earlier by gavage (p.o.) with vehicle (distilled water 0.1 mL/10g, control group), the standard drug indomethacin (5 mg/kg), or AEML (125, 250, 500, 750, 1000 and 1500 mg/kg). After the challenge, the mice were individually placed in a glass cylinder of 22 cm diameter. The total numbers of abdominal contractions (writhes), which consist in the contraction of the flank muscles associated with inward movements of the hindlimb or with whole body stretching, were

counted cumulatively after 10 min of stimulus over a period of 20 min.

The antinociceptive activity was determined as the difference in number of writhes between control group and each treated group, and was expressed as percent inhibition of the writhes.

2. **Hot Plate Test in mice**: The hot plate test was used to measure response latency according to a previously described method [8]. Mice (n = 10) were placed in a hot plate (Ugo Basile, model 35100, Varese, Italy) kept at a temperature of 50 ± 1°C. Animals with baselines latencies of more than 20 s were eliminated from the study and the cut-off time of 40 s was fixed to avoid damage to the paws. Mice were pre-treated orally with distilled water 0.1 mL/10g (control group) or AEML (692.6 mg/kg). Morphine (10 mg/kg), the positive reference drug, was administered subcutaneously. The latency of the reaction to nociception (licking of paw, jumping or shaking) was measured (in seconds) at 0, 30, 60, 90 and 120 min after administration.

3. **Formalin-induced Nociception in mice**: Formalin-induced nociception was induced in mice according to a previously described procedure [9]. Animals were randomly divided in 6 groups (n = 10 per group). A volume of 20 µl of a 1% formalin solution (0.92% formaldehyde) in saline was injected intraplantarly (i.pl.) in the plantar surface of the right hind paw to mice pre-treated orally 60 min earlier with distilled water 0.1 mL/10g (control group) or AEML (692.6 mg/kg, p.o.), or pretreated 30 min earlier with morphine (4 mg/kg, s.c.). After formalin injection, the mice were individually placed in a glass cylinder of 22 cm diameter and were observed from 0-5 min (neurogenic phase) and 15-30 min (inflammatory phase).

   The time spent licking the injected paw was recorded with a chronometer for both phases and considered as indicative of nociception. In order to verify the possible involvement of the opioid system in antinociceptive effect of AEML, three more groups of mice were pretreated with the nonselective opioid antagonist naloxone (0.4 mg/kg, s.c.) 15 min before receiving AEML (692.6 mg/kg, p.o.), morphine (4 mg/kg, s.c.) or vehicle.

Anti-inflammatory Activity:

1. **Ear Edema in mice**: Ear edema was induced according to a previously described procedure [10]. Three groups of 10 mice each were used. Under anesthesia with ketamine and xylazine (3:1, i.p.), cutaneous inflammation was induced by application of 20 µL of an acetone solution containing the irritant agent (2.5% of croton oil) in the inner surface of the right ear of each mouse (surface: about 1 cm^2). Left ear, used as control, received the vehicle acetone (20 µL). Sixty min before the application of the irritant agent, the animals were pretreated with vehicle (0.1 ml/10g, p.o.) (negative control), AEML (692.6 mg/kg, p.o.) or the reference steroidal anti-inflammatory drug dexamethasone (10 mg/kg, p.o.) (positive control). After 6 h, animals were sacrificed and a plug (6 mm) was removed from both ears with irritant and with acetone. The edematous response was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as a percentage of the edema reduction in pretreated mice with AEML or dexamethasone compared to the negative control group.

2. **Dextran-induced Paw Edema in rats**: Anti-inflammatory activity was evaluated by inhibition of the dextran-induced paw edema in rats [11]. Rats were randomly divided in 3 groups (n = 5 per group). A volume of 0.1 mL of a 1% dextran solution was injected i.pl. in the plantar surface of the right hind paw to rats pretreated 60 min earlier with vehicle (distilled water 0.1 mL/100g, p.o.; control group), AEML (692.6 mg/kg, p.o.), or the reference drug pizotifen (0.5 mg/kg, p.o.). The inflammation was quantified by measuring the volume (mL) displaced by the paw using a plethysmometer (Ugo Basile, model 7140, Varese, Italy) at 0 (basal time), 30, 60, 90 and 120 min after dextran injection. Results were expressed as variation in volume (mL) of both treated and control paw at each time.

3. **Carrageenan-induced Paw Edema in rats**: Carrageenan-induced paw edema was induced according to [12]. Rats were randomly divided in 3 groups (n = 5 per group). They were pretreated orally with the vehicle (0.1 mL/100g), AEML (692.6 mg/kg) or the reference anti-inflammatory indomethacin (5 mg/kg). After 60 min, edema was induced with the injection of 0.1 mL of carrageenan (100 µg/paw) in saline into the right hind paw. Left hind paw, used as control, received the vehicle saline (100 µL). The inflammation was quantified by measuring the volume (mL) displaced by the paw using a plethysmometer (Ugo Basile, model 7140, Varese, Italy) at 0 (basal time), 1, 2, 3 and 4 h after carrageenan injection. Results were expressed as variation in volume (mL) of both treated and control paw at each time.

4. **Neutrophil migration into the Peritoneal Cavity in rats**: The determination of neutrophil migration to peritoneal cavity was performed as previously described [13]. Rats (n= 4 per group) were pretreated with the AEML (692.6 mg/kg, p.o.) or vehicle (0.1 mL/100g, p.o.) 1 h before injection of carrageenan (300 µg/cavity, i.p.; 3 mL) or sterile saline (3mL) into the peritoneal cavity. Dexamethasone (1 mg/kg, i.p.), used as reference anti-inflammatory drug, was administered 30 min before carrageenan injection. The animals were then sacrificed by cervical displacement 4 h after carrageen injection. Immediately after, a volume of 10 mL of heparinized phosphate buffered saline (PBS) was injected (1 mL: 1000 mL PBS) into the peritoneal cavity and the counting of both total and differential number of the cells was achieved.

   The results obtained in the differential count were expressed as a number of neutrophils per mL in the peritoneal washing. In order to carry out the total counting, 20 µL of peritoneal washing was diluted in 380 µL Turk solution. The counting was done in a Neubauer chamber. Part of the peritoneal washing was centrifuged at 1000 rpm for 10 min. The overfloaters were re-suspended in 0.4 mL of 3% albumin solution in PBS. The cellular count was performed on a blade prepared for this purpose. The cells were colored by means of Rosenfeld's panchromatic dye and counted on an optical microscope, using an oil immersion object lens. One hundred cells were counted on each blade, and were differentiated into: neutrophils, eosinophils and lymphocytes.

   The number of differentiated cells was calculated as the percent found in relation to the total number of the cell.

5. **Real time *in situ* microscopic analysis for rolling and adhesion events of neutrophils in the Mesenteric Microcirculation**: The leukocyte rolling and adhesion were determined as previously described [14]. Briefly, mice were anesthetized with tribromoethanol (250 mg/kg, i.p.) and the mesenteric tissue was exposed for microscopic examination. The animals were maintained on a special board thermostatically controlled at 37°C. Images were recorded on a video recorder using a long-distance objective lens (×40) with a 0.65 numerical aperture.

   Vessels selected for study were third-order venules, defined according to their branch-order location within the microvascular network. These vessels corresponded to postcapillary venules, with a diameter of 10-18 µm. Rolling leukocytes were defined as the white blood cells that moved at a lower velocity than erythrocytes in the same stream and were determined at 5-min intervals 3 h after challenge with carrageenan.

   Adherent leukocytes were considered to be the white blood cells that remained stationary on the venular endothelium at the end of the observation period and were determined 3 h after challenge with carrageenan. The venular area in which the adhesion process was determined varied from 350 to 450 μm^2, and the results were expressed as the number of adherent leukocyte per 100 μm^2 of venule. The time points selected to determine rolling and adhesion (3 h) processes were based on previous studies, which observed that these processes peak at these times after inflammatory stimuli injection [15, 16].

   Three groups of mice were used, one treated orally with AEML (692.6 mg/kg, p.o.), other with saline 0.1 mL/10g, 1 h before injection of carrageenan (500 µg/cavity, i.p.; 500 µL). A third group of rats

was treated only with sterile saline (500 µL) into the peritoneal cavity.

Evaluation of Acute Toxicity of the AEML: A single oral dose of AEML (5000 mg/kg) was administered to a group of 10 male mice. The control group received only vehicle (distilled water) by the same route. Animals were maintained in a cage with free access to food and water. Besides the number of deaths, some behavioral parameters as convulsion, hyperactivity, ataxia, sedation, grooming, loss of righting reflex, increased or decreased respiration, diarrhoea, as well as food and water intake were observed over a period of 14 consecutive days. After this period animals were killed by cervical dislocation. The procedure was similar to the method previously described [17].

Statistical Analysis: The results were presented as the mean ± standard error of the mean (SEM). The ED_{50} value, defined as the dose of AEML at which 50% of the maximal response was achieved, was determined using weighted probit analysis GraphPad software (GraphPad software, San Diego, CA, USA). Groups of data were compared by unpaired Student's t-test or analysis of variance (ANOVA) followed by Bonferroni's test multiple comparison tests when appropriate. *P* values less than 0.05 ($P < 0.05$) were considered to be indicative of significance.

RESULTS:

Phytochemical screening: The phytochemical analysis of leaves AEML has detected the presence of several phytoconstituents such as saponins, organic acids, proteins, amino acids, phenols, tannins and flavonoids.

Effects of AEML on acetic acid-induced Abdominal Writhes in mice: The oral pretreatment with AEML (125-1500 mg/kg) 1 h before testing evoked a dose-dependent (**Table 1**; $P < 0.05$, one-way ANOVA) inhibition of acetic acid-induced abdominal writhes in mice when compared to control group, with an ED_{50} value of 692.6 mg/kg (95% confidence limits calculated, $r^2 = 0.99$). The standard drug reference indomethacin resulted in a significant ($P < 0.05$, **Table 1**) reduction (74.65%) of the control writhes.

TABLE 1: EFFECTS OF ORAL PRETREATMENT WITH THE AQUEOUS EXTRACT OF *M. LINDLEYANA* (AEML) OR INDOMETHACIN ON WRITHING TEST IN MICE

Pretreatments	Dose (mg/kg)	Number of writhings (20 min)	Inhibition (%)
Distilled water	-	50.50 ± 5.63	-
AEML	125	44.17 ± 3.97	12.55
AEML	250	35.00 ± 3.29*	30.69
AEML	500	26.11 ± 1.36**	48.30
AEML	750	21.78 ± 2.19**	56.87
AEML	1000	15.00 ± 2.52**	70.30
AEML	1500	9.44 ± 1.94**	81.31
Indomethacin	5	12.80 ± 2.17**	74.65

Each value represents the mean ± SEM (n = 10 per group). *$P < 0.05$, **$P < 0.001$ by ANOVA followed by Bonferroni's test with respect to mice orally pretreated with only distilled water (control group).

Effects of AEML on Hot Plate Test in mice: The pretreatment of animals with the standard drug morphine (10 mg/kg, s.c.) significantly ($P < 0.05$, one-way ANOVA) increased the response latency of the first noxious behavior at 30, 60, 90 and 120 min after administration (29.30 ± 3.93, 28.00 ± 4.36, 26.55 ± 4.53 and 17.58 ± 3.28 s, respectively) when compared to the control group (9.74 ± 0.55, 8.39 ± 0.42, 9.70 ± 1.20 and 9.22 ± 1.06 s, respectively). At the dose studied (692.6 mg/kg, p.o.), AEML did not show any antinociceptive effect at all analyzed periods.

Effects of AEML on formalin-induced Nociception in mice: Both first (neurogenic pain) and second (inflammatory pain) phases of formalin-induced nociception was significantly (**Table 2**; $P < 0.05$, unpaired Student's t-test compared to control group) inhibited in mice pretreated orally with AEML (692.6 mg/kg) with an inhibition of 25.26% and 55.1%, respectively. This effect was significantly reversed by naloxone (0.4 mg/kg, s.c.) pretreatment (**Table 2**; $P < 0.05$, unpaired Student's t-test).

Likewise, pretreatment of mice with morphine (4 mg/kg, s.c.) caused significant inhibition of both first

and second phases of formalin-induced nociception by 55.10% and 93.66%, respectively, an effect that was also abolished by naloxone (**Table 2**; $P < 0.05$, unpaired Student's t-test).

TABLE 2: **EFFECTS OF ORAL PRETREATMENT WITH THE AQUEOUS EXTRACT OF *M. LINDLEYANA* (AEML) OR MORPHINE ON THE FORMALIN-INDUCED NOCICEPTION IN MICE: INFLUENCE OF NALOXONE**

Pretreatments	Doses (mg/kg)	Licking time (s) (0-5 min)	Licking time (s) (15-30 min)
Distilled water	-	56.60 ± 5.41	183.10 ± 12.62
AEML	692.6	42.30 ± 3.88*	82.20 ± 10.69*
Morphine	4	25.67 ± 6.14*	11.67 ± 3.28*
Naloxone	0.4	65.30 ± 5.79	148.62 ± 8.78
Naloxone + Morphine	4 + 0.4	50.70 ± 3.53$^{\#}$	117.30 ± 17.37$^{\#}$
Naloxone + AEML	692.6 + 0.4	65.30 ± 6.68$^{\#}$	141.40 ± 5.69$^{\#}$

Each value represents mean ± SEM (n = 10 per group). *$P < 0.05$ by unpaired Student's t-test with respect to mice orally pretreated with only distilled water (control group). $^{\#}P < 0.05$ by unpaired Student's t-test with respect to mice pretreated with AEML or morphine alone

Effects of AEML on ear edema in mice: Pretreatment with AEML (692.6 mg/kg, p.o.) inhibited in 72.96% the ear edema formation induced by croton oil injection (**Table 3**). Likewise; pretreatment with the reference steroidal anti-inflammatory drug dexamethasone (10 mg/kg, p.o.) (positive control) inhibited the edema formation by 80.82% (**Table 3**), when compared to control group (negative control).

TABLE 3: **EFFECTS OF ORAL PRETREATMENT WITH THE AQUEOUS EXTRACT OF *M. LINDLEYANA* (AEML) OR DEXAMETHASONE ON THE EAR EDEMA INDUCED BY CROTON OIL IN MICE**

Pretreatments	Dose (mg/kg)	Edema (mg)	Inhibition (%)
Distilled water	-	6.62 ± 0.63	-
AEML	692.6	1.79 ± 0.32*	72.96*
Dexamethasone	10	1.27 ± 0.35*	80.82*

Each value represents mean ± SEM (n = 10 per group). *$P < 0.001$ by unpaired Student's t-test with respect to mice orally pretreated with only distilled water (control group)

Effects of AEML on dextran-induced paw edema in rats: Dextran 1% induced intense paw edema in rats, an effect that reached a maximum level at 1 h (0.86 ± 0.04 mL) after administration and decreased over the subsequent hours. Treatment of animals with AEML (692.6 mg/kg, p.o.) 1 h before injection of dextran did not alter the occurrence of edema, when compared to control group. However, the reference drug pizotifen (0.5 mg/kg, p.o.) produced a significant ($P < 0.001$, two-way ANOVA) inhibition of dextran-induced paw edema at 30, 60, 90 and 120 min after administration (0.12 ± 0.05, 0.15 ± 0.03, 0.10 ± 0.03 and 0.03 ± 0.02 mL, respectively), when compared to control (0.83 ± 0.09, 0.86 ± 0.04, 0.83 ± 0.06 and 0.80 ± 0.07 mL, respectively).

Effects of AEML on carrageenan-induced paw edema in rats: Carrageenan (100 µg/paw) induced significant (**Table 4**; $P < 0.001$, two-way ANOVA) and intense paw edema in rats, an effect that reached a maximum level at 3 h (0.60 ± 0.01 mL) after administration. Treatment of animals with AEML (692.6 mg/kg, p.o.) 1 h before injection of carrageenan significantly (**Table 4**; $P < 0.05$, two-way ANOVA) inhibited the edema formation at 2, 3 and 4 h when compared to control group. Indomethacin (5 mg/kg, p.o.) also produced a significant inhibition of the carrageenan-induced paw edema at 2, 3 and 4 h when compared to control (**Table 4**; $P < 0.05$, two-way ANOVA).

TABLE 4: **EFFECTS OF ORAL PRETREATMENT WITH THE AQUEOUS EXTRACT OF *M. LINDLEYANA* (AEML) OR INDOMETHACIN ON THE CARRAGEENAN-INDUCED PAW EDEMA IN RATS**

Pretreatments	Dose (mg/kg)	Time (h)				
		0	1	2	3	4
Saline (100µL/ paw)	-	0	0.13 ± 0.04	0.10 ± 0.05	0.06 ± 0.04	0.06 ± 0.04
Distilled water (p.o.)	-	0	0.30 ± 0.06$^{\#}$	0.51 ± 0.05$^{\#}$	0.60±0.01$^{\#}$	0.33 ± 0.04$^{\#}$
AEML	692.6	0	0.19 ± 0.05	0.31 ± 0.08*	0.28 ± 0.04***	0.08 ± 0.05**
Indomethacin	5	0	0.15 ± 0.07	0.12 ± 0.05***	0.13 ± 0.04***	0.13 ± 0.06*

Each value represents mean ± SEM (n = 5 per group). *P < 0.05, **P < 0.01, ***P < 0.001 by two-way ANOVA followed by Bonferroni's test with respect to rats orally pretreated with distilled water (control group). #P < 0.001 by two-way ANOVA followed by Bonferroni's test when compared to saline group (received only saline into the hind paw)

Effects of AEML on carrageenan-induced neutrophil migration into the peritoneal cavity in rats: Carrageenan (300 µg/cavity; i.p., 3 mL) induced a significant neutrophil migration (1.74 ± 0.17 neutrophils x 10^6/mL) in rats. Pre-treatment with vehicle had no effect on carrageenan-induced response. However, pretreatment with AEML (692.6 mg/kg, p.o., 1 h beforehand) significantly inhibited by 52.83% (0.82 ± 0.20 neutrophils x 10^6/mL) the neutrophil migration caused by carrageenan, when compared to control group (**Table 5**; P < 0.05, Student's t-test). The reference anti-inflammatory drug dexamethasone (1 mg/kg, i.p.) promoted significant (**Table 5**; P < 0.05, Student's t-test) reduction in neutrophil recruitment into peritoneal cavities of rats (0.60 ± 0.20 neutrophils x 10^6/mL).

TABLE 5: **EFFECTS OF THE AQUEOUS EXTRACT OF *M. LINDLEYANA* (AEML) ON CARRAGEENAN-INDUCED NEUTROPHIL MIGRATION INTO THE PERITONEAL CAVITY IN RATS**

Group	Dose (mg/kg)	Neutrophils x 10^6/mL	Inhibition (%)
Saline	-	0	-
Control	-	1.74 ± 0.17#	-
AEML	692.6	0.82 ± 0.20*	52.83*
Dexamethasone	1	0.60 ± 0.20*	65.52*

Each value represents mean ± SEM (n = 4 per group). *P < 0.05, #P < 0.05 by unpaired Student's t-test when compared to control group (treated orally with only saline) and saline group (received only saline into the peritoneal cavity), respectively

Effects of AEML on rolling and adhesion of leukocytes in mice: Carrageenan (500 µg/cavity; i.p.; 0.5 mL) caused a significant (**Table 6**; P < 0.05, Student's t-test) increase in leukocyte rolling and adhesion on endothelium 3 h after stimuli, when compared to mice pretreated only with i.p. saline. Pretreatment of mice with AEML (692.6 mg/kg, p.o.) significantly (**Table 6**; P < 0.05, unpaired Student's t-test) decreased leukocyte rolling and adhesion when compared to mice treated orally with only saline.

TABLE 6: EFFECTS OF THE AQUEOUS EXTRACT OF *M. LINDLEYANA* (AEML) ON ROLLING AND ADHESION OF LEUKOCYTES AFTER INJECTION OF CARRAGEENAN INTO PERITONEAL CAVITY IN MICE

Groups	Dose (mg/kg)	Leukocyte rolling/min	Adherent cells/100µm^2
Saline	-	11.03 ± 4.67 (n = 6)	0.24 ± 0.06 (n = 4)
Control	-	45.73 ± 2.03# (n = 9)	2.34 ± 0.36# (n = 7)
AEML	692.6	12.13 ± 2.67* (n = 6)	0.71 ± 0.12*(n = 6)

Each value represents mean ± SEM. *P < 0.05; #P < 0.05 by unpaired Student's t-test with respect to control group (treated orally with only saline) and saline group (received only saline into the peritoneal cavity), respectively.

Acute Toxicity Study: In acute toxicity study, we did not observe mortality or signs of toxicity that could be attributable to the oral administration of AEML (5000 mg/kg single dose) in mice. Therefore, no LD_{50} could be calculated.

DISCUSSION: The genus *Mikania* is known to be composed of flavonoids, coumarins and terpene compounds [18].

In the present study, AEML was shown to contain flavonoids, organic acids, saponins, phenols, tannins, proteins and amino acids, a phytochemical analysis which is similar to that previously reported [3]. In fact, these authors reported that leaves hydroalcoholic extract of *M. lindleyana* contained alkaloids, flavonoids, tannins, steroids and triterpenoids.

Some compounds such as flavonoids which are well known to display anti-inflammatory and antinociceptive activities [19, 20] are common to both studies.

The present study demonstrates for the first time that the AEML induces anti-inflammatory and antinociceptive effects in several model of nociception (acetic acid-induced abdominal writhing, hot plate and formalin) and inflammation (ear edema, paw edema,

peritonitis and intravital microscopy). Indeed, neither mortality nor signs of toxicity were detected during the behavioral observations in the acute study, indicating a lack of toxicity for the AEML. The abdominal writhes induced by acetic acid, a model largely used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents, has been described as a typical model for visceral inflammatory pain [21]. The local irritation provoked by this algogenic agent in the intraperitoneal cavity is due to liberation of several mediators such as histamine, serotonin, bradykinin and eicosanoids [22] as well as some cytokines as TNF-α, IL-1β and IL-8 [23]. Such as mediators activate chemosensitive nociceptors that contribute to the development of this type of inflammatory pain, which is known to be sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, diclofenac and indomethacin. Like indomethacin, AEML was able to reduce dose-dependently the acetic acid-induced writhing response, suggesting that in this model the antinociceptive effect can be due to a like-NSAIDs mechanism resulting a peripheral anti-nociceptive effect.

Despite AEML displayed significant antinociception on the acetic acid-induced pain, it was ineffective on hot plate test which indicates non-participation on thermal stimulation associated with central neurotransmission in what the heat activates nociceptors (Aδ and C fibers) by driving the momentum of the dorsal horn of the spinal cord and subsequently to cortical centers.

We also used the formalin test, which is one of the most used models to evaluate the neurogenic and inflammatory pain. The first phase (0-5 min) is characterized by neurogenic pain caused by a direct chemical stimulation of nociceptors. The second phase (15-30 min) is characterized by inflammatory pain triggered by a combination of stimuli, including inflammation of the peripheral tissues and mechanisms of central sensitization [24]. Substance P and bradykinin are involved in the first phase whereas histamine, serotonin, prostaglandins and bradykinin are involved in the second one [25]. Drugs that act primarily as central analgesics (morphine for instance) inhibit both phases while peripherally acting drugs (NSAIDs for instance) inhibit only the second phase [9, 21].

In the present study, oral pretreatment with AEML significantly inhibited the first (neurogenic pain) and second (inflammatory nociception) phases of formalin-induced licking in mice, an effect that was abolished by the opioid receptor antagonist naloxone. This may suggest that AEML induce its antinociceptive action by direct action on nociceptive afferent fibers where opioid receptors are involved but, the inhibition of inflammatory mediators (such as prostaglandins) can not be discarded. The present results obtained in the formalin test are not on line with the lack of efficacy of the AEML in the hot plate test. This discrepancy can be explained by the high relative polarity of the AEML, which may prevent it from crossing the blood-brain barrier.

In order to evaluate the AEML anti-inflammatory activity, this extract was tested in the edema induced by croton oil. This potent phlogistic agent induces a cutaneous inflammatory response characterized by intense vasodilatation and edema formation. The cells more important in this process are neutrophils and macrophages [10]. These irritant effects are related with phorbol esters, mainly TPA (12-o-tetradecanoil-phorbol-13-acetate) [26] which in turn are associated with increased vascular permeability, synthesis of arachidonic acid metabolites and expression of COX-2, IL-1β, TNF-α as well as adhesion molecules (ICAM-1) [27].

Oral pretreatment with AEML was able to reduce the edema formation 6 h after croton oil application, suggesting that AEML may interfere on production of mediators cyclooxygenase products, mainly PGE_2 released by cytokines such as IL-1β, TNF-α, preventing the edema formation.

The effect anti-inflammatory AEML was tested on dextran-induced paw edema. Dextran, a polymer of glucose, induces mast cell degranulation releasing histamine and serotonin which contribute to increased vascular permeability and leakage of fluid [28]. AEML was ineffective in this test suggesting that neither histamine nor serotonin is involved in the mediation of its anti-inflammatory activity.

The anti-inflammatory activity of AEML was also evaluated in carrageenan-induced rat paw edema, which is one of the most and widely used model for inflammation. Edema due to carrageenan is a

multimediated phenomenon with three distinct phases. The phase 1 (0-1.5 h) is due to liberation of histamine and serotonin, the phase 2 (1.5-2.5 h) is characterized by release of kinins (bradykinin) and the phase 3 (2.5-4 h) is sustained by liberation of prostaglandins [29].

Moreover, Rocha *et al* [30]., demonstrated that the pro-inflammatory cytokine TNF-α has an important role in this inflammatory process. This cytokine can act on inflammatory cells inducing the expression of several inflammatory proteins and promoting cytotoxicity [30, 31]. It also induce chemotaxis of neutrophils and lymphocytes T and increase the expression of adhesion molecules [32].

The oral pretreatment with AEML inhibited the edema formation in the second and especially third phase after administration of carrageenan. This allowed us to suggest that the antiedematogenic response of AEML is related with interference in the production of kinins (bradykinin) and arachidonic acid metabolites (prostaglandins).

To evaluate the anti-inflammatory activity of AEML on migration of inflammatory cells, carregeenan-induced peritonitis test was performed in rats. This test is characterized by an increase in solute transport between plasma and the porous membrane concomitantly with a cell migration that occurs in the peritoneum. These changes are due to a vasodilation of capillaries in the peritoneal membrane and to an increased microvascular pore diameter caused by the inflammatory mediators such as PGE_2, and inflammatory cells such as neutrophils [33].

The polymorphonuclear leukocytes are the main cells to migrate into the peritoneal cavity in the initial inflammatory response [34]. During inflammation, the enhanced concentration of cytokines such as TNF-α is responsible for the induction of the expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) by neutrophils [35].

In the present model of peritonitis induced by carrageenan, AEML was able to reduce the neutrophil migration into the peritoneal cavity after carrageenan injection. This result was corroborated by intravital microscopy test which evaluate the mesenteric microcirculation by means of video surveillance *in vivo*, interactions between leukocytes and the wall of blood vessels in the inflammation site [36] in which the AEML was able to reduce rolling and adhesion of leucocytes in mesenteric venules.

Our results suggest that AEML may interfere with the migration of leukocytes, especially neutrophils during the inflammatory process, which may be due to the action on the production of pro-inflammatory cytokines, as TNF-α and IL-1β. Alves *et al* [37]., showed that the hydroalcoholic extract of *M. laevigata* reduces the migration of neutrophils into the peritoneal cavity by a mechanism dependent on pro-inflammatory cytokines (TNF-α and IL-1β).

CONCLUSION: In summary, the present study shows for the first time that AEML possess antinociceptive and anti-inflammatory activities, supporting the widespread use of *M. lindleyana* in folk medicine to treat pain and inflammation. The antinociceptive action seems to be dependent on a peripheral pathway involving inhibition of liberation/synthesis of kinins and prostaglandins but opioid system seems to be also involved in this action. The anti-inflammatory activity seems to be related to the ability of AEML to reduce the rolling and adhesion of leucocytes and to inhibit the synthesis of cytokines, which are important inflammatory mediators involved in the cell migration.

Pharmacological activities shown herein with AEML could be attributed to the action of phenolic compounds such flavonoids. Further studies are necessary to identify the responsible bioactive compounds present in this extract.

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