

Full Length Research Paper

Antinociceptive and anti-inflammatory activities of ethanolic extract from atemoya (*Annona cherimola* Mill x *Annona squamosa* L.)

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Atemoya, from family Annonaceae, is an interspecific hybrid between *Annona cherimola* Mill and *Annona squamosa* L. It is a tree from a region in diversity of plants, the Caatinga, one of the main Brazilian biomes. In this research, the effects of ethanolic extract of atemoya (Acs-EtOH) were investigated in relief of pain and inflammation in experimental models in rodents. The evaluation of antinociceptive activity was carried out by the acetic acid-induced writhing, formalin and hot plate tests, while air pouch and carrageenan-induced peritonitis were used for anti-inflammatory profile. The motor coordination evaluation was performed by rota-rod test. Acs-EtOH (25, 50 and 100 mg/kg, p.o.) significantly reduced the number of writhes with significant ($p < 0.001$) and decreased ($p < 0.001$) paw licking time in both phases of the formalin test. In the hot plate test, the latency was significant, reducing painful stimulus. In the anti-inflammatory models, Acs-EtOH inhibited significantly ($p < 0.001$) the migration of leukocytes in the air pouch and peritonitis tests. Significant inhibition of migration of leukocytes in the treated groups with different doses of the extract was observed. The results indicate that Acs-EtOH may provide a source of plant compounds with antinociceptive and anti-inflammatory activities.

Key words: Annonaceae, pain, inflammation, medicinal plants, caatinga.

INTRODUCTION

The nature, in general, is responsible for producing the majority of the known organic substances. Plant kingdom has mostly contributed to the supply of useful substances

to treat diseases that affect humans (Montanari and Bolzani, 2001). The Brazilian Northeast is a region with great plant diversity. One of the main biomes the Caatinga,

has adapted vegetation to semi-arid climate, with a wide variety of endemic species in its flora. Medicinal plants are used in folk medicine in many countries to treat several painful and inflammatory conditions; however, for many plants in use, effectiveness and active compounds are unknown. Several experimental studies are demonstrating the pharmacological properties of these plants and identifying the active principles responsible for the activities (Sousa, 2002).

The Annonaceae family comprises a large number of genera and species, most of which are native to the tropical regions, with about 2500 species in about 135 genera (Chatrou et al., 2004; Anuragi et al., 2016). In Brazil, there are recorded 29 genera, comprising about 392 species (Maas and Lobão, 2015).

Atemoya (*Annona cherimola* Mill. x *Annona squamosa* L.) is a natural hybrid between cherimoya (*A. cherimola* Mill.) and pinecone or fruit-the-count (*A. squamosa* L.) (Oliveira et al., 2010) that produce edible fruits (Anuragi et al., 2016). Although pollination occurs between species naturally, the hybrid atemoya was the result of an intentional crossing, in order to obtain a fruit with a good quality and more adaptable to tropical climate. This species is a recent fruit crop in the Northeast and was first deployed in the region in 1997, specifically in the irrigation projects of the San Francisco Valley. Studies have shown that the *Annona* species have several pharmacological properties including antinociceptive (Carballo et al., 2010; Hamid et al., 2012) and anti-inflammatory activity (Foong and Hamid, 2012). From the phytochemical perspective, many secondary metabolites classes were found in the Annonaceae family. Chemotaxonomic data characterize this family for the presence of alkaloids, flavonoids and terpenoids, mainly diterpenes (Silva et al., 2009).

Considering that the most important painkillers prototypes (salicylic acid and morphine) were derived originally from the plant kingdom, the study of medicinal plants has become a useful strategy in the search for antinociceptive drugs (Elizabetsky et al., 1995). Therefore, this article aims to investigate the antinociceptive and anti-inflammatory activity of the ethanol extract of atemoya leaves in rodents.

MATERIALS AND METHODS

Plant material

The leaves of atemoya were collected in the city of Petrolina (Coordinates: 9°23'19" S and 40°29'3" W), state of Pernambuco,

Brazil, in May 2014 and was identified by a botanist from Centro de Referência para Recuperação de Áreas Degradadas (CRAD). A voucher specimen (#16310) was deposited in the Herbarium Vale do São Francisco (HVASF) from Federal University of San Francisco Valley.

Preparation of the plant extract

The leaves of atemoya were dried in an oven (Marconi®) at 45°C for 3 days. The dried and powdered leaves (575.2 g) were macerated with ethanol (EtOH) 95% at room temperature for 6 days. The extracted solution was concentrated under vacuum in a rotatory evaporator at 45°C, yielding 214 g of crude ethanol extract of atemoya (Acs-EtOH).

Animals

Swiss albino mice of both sex weighing 25 to 30 g were used, randomly housed in appropriate cages maintained at 22 ± 2°C on a 12 h light/dark cycle (lights on at 6:00 a.m.) with access to food and water *ad libitum*. Experimental protocols and procedures were approved by the Federal University of San Francisco Valley Animal Care and Use Committee, by number 0003/110414.

Pharmacological tests

Acetic acid-induced writhing in mice

The mice were divided into 6 groups, with 6 animals each. Nociception was induced by intraperitoneally (i.p.) using dose defined in study design administration of 0.1 mL acetic acid (1%). The animals were treated with Acs-EtOH (25, 50 and 100 mg/kg, p.o.) (Diniz et al., 2013) and received saline (negative control), 1 h before inoculation of the nociceptive agent. Indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg i.p.) were used as reference drugs (i.p.) for positive control 30 min before the administration of the algogen agent. The animals were observed individually and the number of writhes was counted for 20 min, starting 5 min after injection of acetic acid (Queiroz et al., 2010). The significant reduction in number of writhes of treated groups was compared to the control and the groups treated with the reference drug. The mice were observed in the chambers with a mirror and the amount of time (in sec) spent licking or/and biting the injected paw, was measured as a pain indicator. Responses were measured for 5 min (first stage, neurogenic) and 15 to 30 min after formalin injection (second phase, inflammatory) (Tjolsen et al., 1992). Treatments with saline (p.o.), Asc-EtOH (25, 50 and 100 mg/kg, p.o.), indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were injected 1 h before formalin injection (n = 6 per group).

Formalin test

Formalin-induced nociception was developed according to a previously described procedure (Hunskar et al., 1985), with some

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modifications. Formalin solution was introduced (2.5% in sterile 0.9% saline) in the subplantar paw region, injected into the right hind paw of mice (Santos et al., 2010). The mice were observed in the chambers with a mirror and the amount of time (in sec) spent licking or/and biting the injected paw, was measured as a pain indicator. Responses were measured for 5 min (first stage, neurogenic) and 15-30 min after formalin injection (second phase, inflammatory) (Tjolsen et al., 1992). Treatments with saline (p.o.), Acs-EtOH (25, 50 and 100 mg/kg, p.o.), indomethacin (20 mg/kg, i.p.) and morphine (10 mg/kg, i.p.) were injected 1 h before formalin injection (n = 6 per group).

Hot-plate test

Mice were divided into 6 groups of 6 mice each. Mice were pre-selected on the hot-plate apparatus (Insight, Brazil) one day before at $45 \pm 0.5^\circ\text{C}$. Animals showing a reaction time (defined as the latency for licking the hind feet or jumping) greater than 20 sec were discarded. Selected mice were pre-treated with saline (p.o.), Acs-EtOH (25, 50 and 100 mg/kg, p.o.), or morphine (10 mg/kg, i.p.).

Each animal was placed on the heated surface of the plate maintained at 55°C and the latency to a discomfort reaction (jumping or licking of the paws) was recorded at 30, 60, 90 and 120 min after saline administration, extract and morphine (Jacob and Ramabdran, 1978). A cut-off time of 20 sec was chosen to indicate complete analgesia and to avoid tissue injury. The latency time for each animal was recorded.

Leukocyte migration to the peritoneal cavity

The 6 groups were divided, with 6 mice each. The leukocyte migration was induced by injection of carrageenan (1%, 0.25 mL, i.p.) in the peritoneal region (Andrade et al., 2012), 1 h after administration of Acs-EtOH (25, 50 and 100 mg/kg, p.o.), and saline (p.o.) 30 min after injection of dexamethasone (2 mg/kg, i.p.) (Bastos et al., 2007).

The leukocyte migration was evaluated 4 h after exogenous stimulus. Then, euthanasia was carried out and 3 mL of saline containing 1 mM EDTA was injected at peritoneal cavity. A brief massage was done for further fluid collection, which was centrifuged (3000 rpm for 5 min) at a room temperature. The supernatant was disposed and saline was added to the precipitate. Then an aliquot of 10 μL from this suspension was dissolved in 200 μL of Turk solution and the total cells were counted in a Neubauer chamber, using an optic microscopy. All results were submitted to statistical analysis and were expressed as the number of leukocytes/mL (Melo et al., 2011).

Subcutaneous air pouch (SAP)

The SAP was performed according to a method described previously (Garcia-Ramalho et al., 2002), with some adjustments. On day 0, the animals were separated by groups and received a subcutaneous injection of 15 mL of sterile air, on the dorsal region of mice. On day 3, a new injection of 5 mL of sterile air into the preformed bag was carried out. On day 7, the animals were divided in groups: saline solution (0.9% saline p.o.), Dexamethasone (0.5 mg/kg, s.c.), Acs-EtOH (25, 50 and 100 mg/kg, p.o.). 1 h after treatment, 0.5 mL of a (1%) solution of carrageenan in saline was injected into the SAP, except the vehicle group, which received an injection of 0.25 mL of saline.

Then, 4 h after the inflammatory stimulus, the animals were euthanized and the pocket was washed with 2 mL heparinized PBS (10 IU/mL). With the aid of a pipette, exudate was collected via an incision in the dorsal pouch. The exudates were centrifuged at 3000 rpm for 5 min, and the supernatants were excluded. The total count of the number of leukocyte cells in the air pouch was carried out. A portion of fluid was diluted in a Türk solution (1:20) and subjected to counting. All counts were performed in a Neubauer chamber, with an optical microscopy.

Rota-rod test

To discard possible nonspecific effects of the Acs-EtOH in the motor coordination or the muscular relaxation, the mice had been tested in the rota-rod. The animals had been trained on of the device, one day before the experiment.

On the day of the experiment, the mice had been dealt with Acs-EtOH (25, 50 and 100 mg/kg, p.o.), diazepam (2.5 mg/kg, i.p.) and saline solution 0.9% (p.o.), after 60 min had been placed in the route-rod and registered the time of motor performance (s) of each animal using a chronometer.

Statistical analysis

All the results were presented as the mean \pm S.E.M (Standard Error Mean) and the statistical significance was determined using one-way ANOVA, followed by Tukey test which was carried out to compare the results of control and test drug groups.

The differences are considered to be significant if $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ are most significant. The analysis was performed using GraphPad Prism 5.0 program (Graph Pad Prism Software Inc., San Diego, CA, USA).

RESULTS

Acetic acid-induced writhing in mice

The results from this evaluation (Figure 1) can demonstrate that, pretreatment with ethanol extract of atemoya leaves (Acs-EtOH) (25, 50 and 100 mg/kg, p.o.) was able to inhibit acetic acid-induced writhings compared with the control group ($***p < 0.001$). The inhibition % of the doses of Acs-EtOH was 42.14, 48.88 and 63.48%, respectively.

Indomethacin and morphine reduced 91.23 and 100% of the writhes compared to the control, respectively. There was no statistical difference between the doses of Acs-EtOH when compared to each other.

Formalin test

Figure 2 shows the treatment with Acs-EtOH at doses of (25, 50 and 100 mg/kg, p.o.) produced significant antinociceptive effect compared to the control group in both phases ($***p < 0.001$). Acs-EtOH (25, 50 and 100 mg/kg, p.o.) reduced by 56.71, 64.35 and 50.09%,

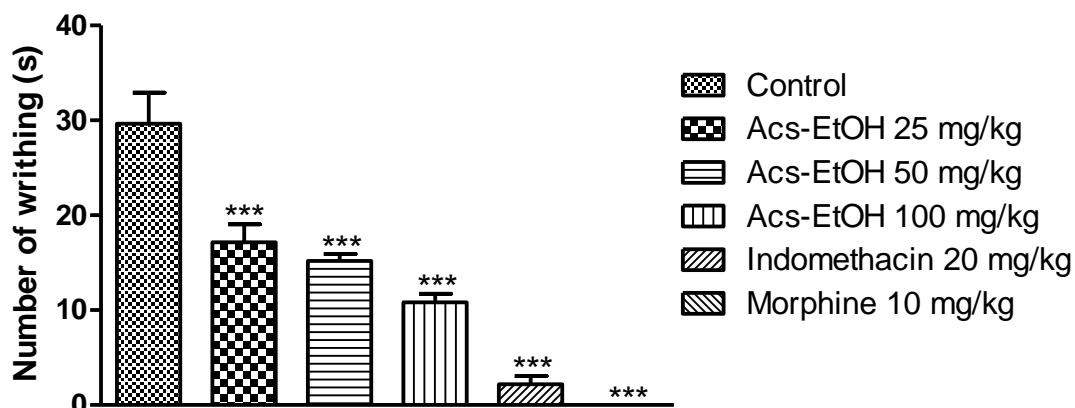


Figure 1. Abdominal writhing inhibition by crude ethanol extract of atemoya. Data are expressed as mean \pm S.E.M. of six mice. *** $p < 0.001$ indicates significant difference when compared with control group by using (one-way ANOVA) followed Tukey Test ($n = 6$, per group).

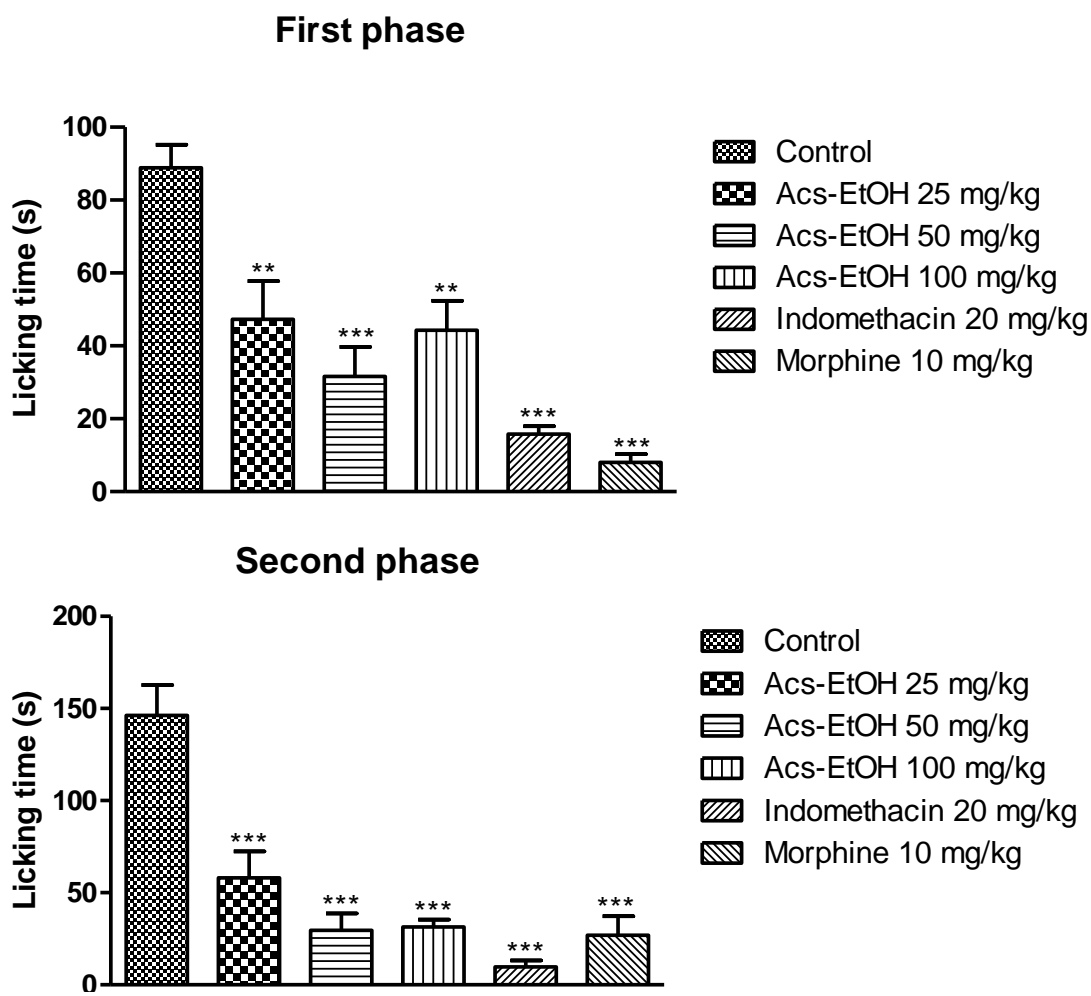


Figure 2. Effect of ethanolic extract of atemoya (Acs-EtOH) on the formalin-induced nociception assay on neurogenic phase 0-5 min (first phase) after formalin injection; and the second inflammatory pain phase 15-30 min (second phase). Data are mean \pm S.E.M. of six mice. ** $p < 0.01$, *** $p < 0.001$, significantly different from control group by using (one-way ANOVA) followed Tukey Test ($n = 6$, per group).

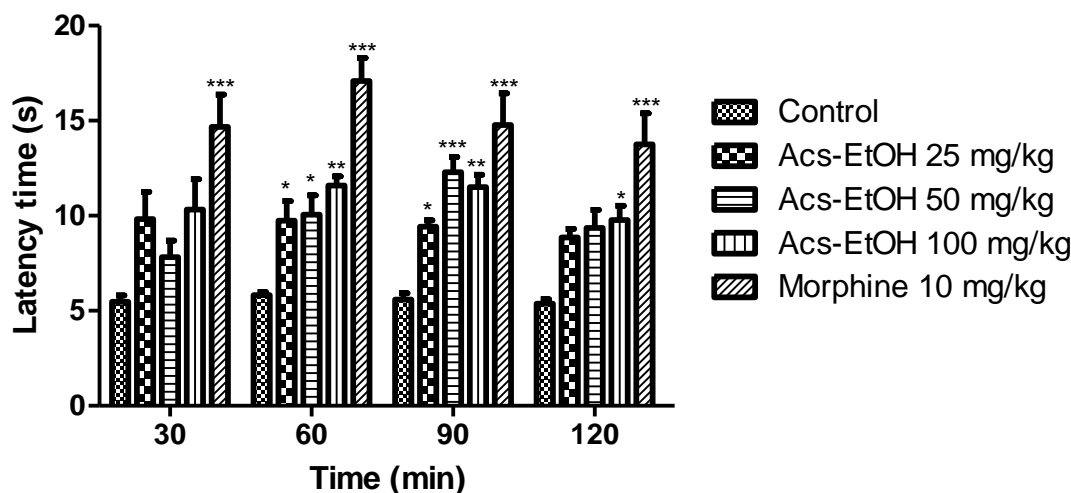


Figure 3. Effect of ethanolic extract of atemoya (Acs-EtOH) on hot plate assay in mice. Values are mean \pm S.E.M.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from control; (one-way ANOVA) followed Tukey test ($n = 6$, per group).

respectively, the time of paw licking in first phase, as well as 47.58, 73.41 and 77.79%, in the second phase of formalin test.

Indomethacin, the reference drug, demonstrated more activity in the second test phase, while morphine inhibited both phases of the pain stimulus (** $p < 0.001$). Morphine significantly inhibited pain (100%) over the control, both at early and late phases.

Hot-plate test

In this evaluation, animals treated with morphine (10 mg/kg, i.p.) had an evident increase in the latency time 30, 60, 90 and 120 min (** $p < 0.001$). For the groups treated with Acs-EtOH doses (25, 50 and 100 mg/kg, p.o.), no effect was shown in the first 30 min.

However, an increase in latency time was found in 60 and 90 min and a reduction was evident in 120 min. The morphine proved to be a potent analgesic, increasing the latency time within the time evolution (Figure 3).

Leukocyte migration to the peritoneal cavity

Acs-EtOH showed an inhibitory effect on leukocytes migration (Figure 4) (35.40, 46.20 and 63.85% at 25, 50 and 100 mg/kg, respectively, *** $p < 0.001$). The obtained results with the control group support the effect of Acs-EtOH, since the vehicle presented no activity and the control drug dexamethasone inhibited (89.55%, *** $p < 0.001$) the carrageenan-induced leukocyte migration to

the peritoneal cavity.

Subcutaneous air pouch (SAP)

Air SAP demonstrates that, the neutrophils have effective role in the defense against foreign bodies, and also in processes related to inflammatory response, due to the viability of injured cells and/or tissues. The inhibitory effect of Acs-EtOH on leukocytes migration into the air pouch was 56.17, 62.04 and 73.16% at doses of 25, 50 and 100 mg/kg, while inhibitory effect of dexamethasone was 88.15% (Figure 5).

Rota-rod test

The rota-rod assay was performed to determine if the extract Acs-EtOH (25, 50 and 100 mg/kg p.o.) compromises the motor system in mice treated sharply, when placed on the rotating rod. Mice treated showed no significant change in the performance of the device (Figure 6).

DISCUSSION

The aim of this study was to investigate the anti-nociceptive and anti-inflammatory effects of ethanol extract from atemoya leaves (Acs-EtOH), using different models with painful, chemical and thermal stimuli. These activities can possibly be associated with the chemical

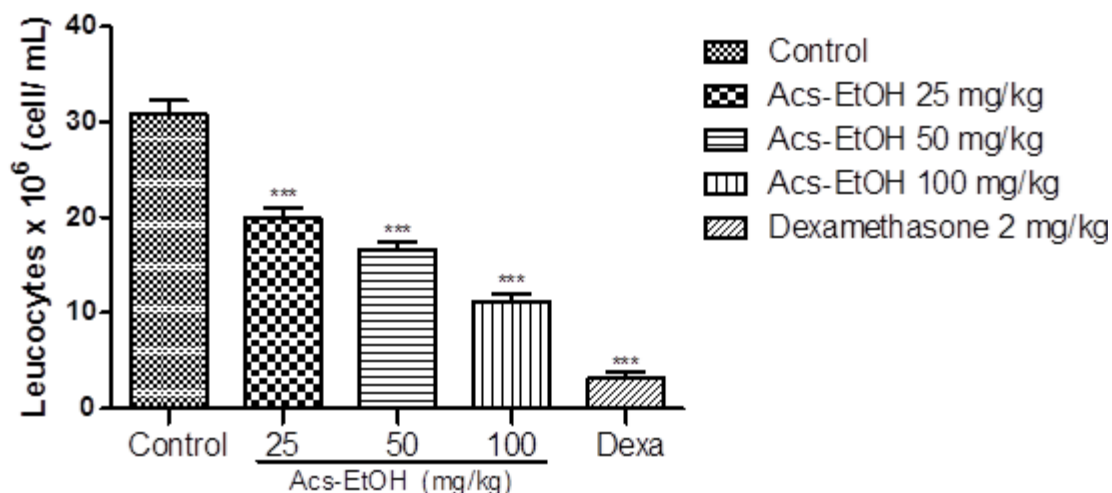


Figure 4. Effect of ethanolic extract of atemoya (Acs-EtOH) and dexamethasone on leukocytes migration into the peritoneal cavity induced by carrageenan in mice. Values are mean \pm S.E.M.; *** $p < 0.001$, significantly different from control; (one-way ANOVA) followed Tukey test ($n = 6$, per group).

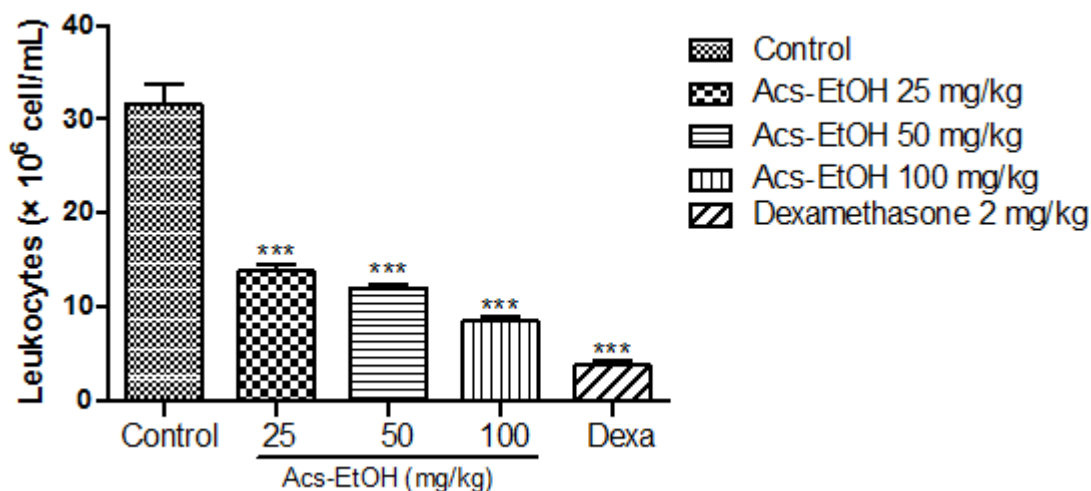


Figure 5. Effect of ethanolic extract of atemoya (Acs-EtOH) and dexamethasone on leukocytes migration into the subcutaneous air pouch induced by carrageenan in mice. Values are mean \pm S.E.M.; *** $p < 0.001$, significantly different from control; (one-way ANOVA) followed Tukey test ($n = 6$, per group).

constituents already identified in the plant, such as alkaloids, terpenes and acetogenins (Costa et al., 2012). Additionally, previous phytochemical atemoya screenings detected the presence of flavonoids, tannins, lignans, mono and diterpenes (leaves and stems). Also, positive results were noticed for the presence of other components such as alkaloids, anthracene derivatives, naphthoquinones, triterpenes and steroids (Rabêlo, 2014).

Due to the expansion of atemoya cultivation and its growing consumption in key Brazilian markets, it is necessary to investigate the chemical composition of the plant. In phytochemical study previously carried out in the São Francisco Valley, show the isolation and chemical characterization of seven alkaloids (asimilobine, pronuciferine, lanuginosine, liriodenine, lysicamine, anonaine and stepharine) by spectrometric methods (Rabêlo et al., 2015).

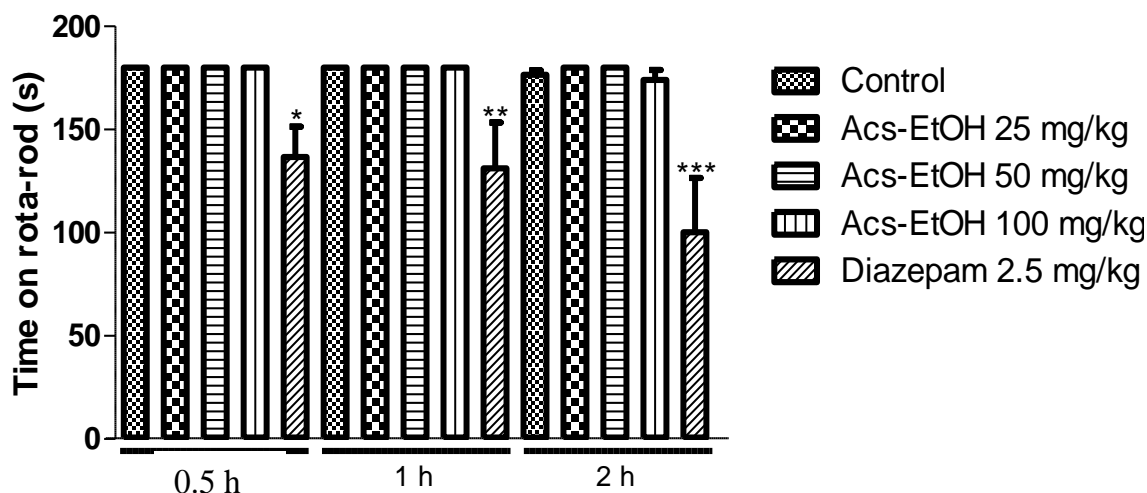


Figure 6. Effect of ethanolic extract of atemoya (Acs-EtOH) and diazepam in rota-rod assay in mice. Values are mean \pm S.E.M.; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from control; (one-way ANOVA) followed Tukey test ($n = 6$, per group).

The antinociceptive activity of Acs-EtOH in this study was investigated using the abdominal writhing, formalin and hot plate tests in mice. The writhing model induced by acetic acid in mice is commonly considered as classical peripheral inflammatory pain animal model, for evaluation of antinociceptive or anti-inflammatory drugs (Negus et al., 2006). This test of writhing induced by acetic acid has been used as a classic model for the possible antinociceptive potential of new agents (Mohamad et al., 2010).

The peripheral algescic is due to the liberation of several inflammatory mediators such as bradykinin, substance P, prostaglandins, cyclooxygenases and lipoxygenases, as well as some cytokines such as IL-1 β , TNF- α and IL-8 (Ikeda et al., 2001; Ribeiro et al., 2000).

The Acs-EtOH significantly reduced the number of writhing caused by the application of acetic acid in the intraperitoneal region. The results in Figure 1 demonstrate that the control group showed 29.67 ± 3.25 writhing 10 min after application of acetic acid. Thus, when animals were pretreated with 25, 50 and 100 mg/kg Acs-EtOH, the number of writhings was reduced in a statistically significant manner (17.17 ± 1.88 , 15.17 ± 0.74 and 10.83 ± 0.87). The Acs-EtOH reduced significantly the number of writhings at all doses compared to control groups.

This study demonstrated the significant antinociceptive activity of this plant, but the writhing model induced by acetic acid is characterized as a nonselective model to evaluate the analgesic effect of substances, like this another tests are necessary to confirm the antinociceptive potential. So, for better analysis of the analgesic effect,

the formalin test was carried out, aiming to measure the behavioral effectiveness of antinociceptive agents (Hunskar and Hole, 1987).

The administration of subcutaneous formalin injection in mice induces a biphasic nociceptive response (Ramirez et al., 2010). The first phase corresponds to neurogenic pain and is caused by the direct effect of formalin in sensory C-fibers. The second phase (or inflammatory phase) is related with the inflammatory response development, releasing the nociceptive mediators in peripheral tissue and functional changes in the dorsal medulla body (Reynoso et al., 2013). The Acs-EtOH significantly inhibited both phases of the formalin-induced pain, indicating that the extract has an antinociceptive effect in both phases.

In order to confirm the involvement of central mechanisms in the antinociceptive effect of Acs-EtOH, the hot plate assay was carried out. Analyzing the results shown in Figure 3, the animals treated with Acs-EtOH (25, 50 and 100 mg/kg, p.o.) spent more time on the heated metal plate, evidencing possible centrally acting. This pattern of response is related to a spinal reflex and it is considered, thus, a centrally acting analgesic evaluation model (Nemirovsky et al., 2001).

In relation to the evaluation of the Acs-EtOH on the inflammatory activity, the extract significantly decreased leukocyte migration. The decrease in migration was analyzed by the test in the peritoneal cavity and then confirmed by the subcutaneous air pouch test. This effect probably occurs by the inhibition mechanisms of the cyclooxygenase pathway, inhibiting the synthesis of mediators that act in the inflammatory process, whose

involvement in cell migration is well established. The inflammation induced by carrageenan involves the migration of defense cells, plasma exudation and production of mediators such as NO, PGE₂, interleukin (IL-1 β , IL-6, IL-8, and tumor necrosis factor (TNF- α)) (Loram et al., 2007; Salvemini et al., 1996)). These mediators are capable of migrating leukocytes such as neutrophils, in different experimental tests. Figure 4 shows that Acs-EtOH inhibit significantly the leukocyte migration induced by carrageenan, and the inhibition of mediators involved in cell migration may be associated with this activity.

To further confirm the effect of Acs-EtOH on the migration of leukocytes, the experiment was conducted in an animal model of carrageenan induced air pouch polysaccharide, which is a well-known model to induce migration of leukocytes, infiltrated into the bag contrived. When neutrophils stimulated release reactive oxygen species and a variety of protein granules, there is an increase in the migration process. The influx of neutrophils in inflammation active regions regulates the inflammatory process in various pathogenic stimuli (Carlson et al., 2002). The Acs-EtOH inhibition of migration of leukocytes and plasma loss observed in air pouch model is an important parameter of inflammation. These mediators are able to recruit leukocytes such as neutrophils in several experimental models in Figures 4 and 5. These results suggest that the extract contains bioactive anti-inflammatory compounds in a dose dependent manner. Several studies have shown that the use of drugs which produces Central nervous system (CNS) depression or non-specific muscle relaxing effect can reduce the coordination response that may invalidate the possible results of the behavioral tests (Quintans-Junior et al., 2010).

Thus, the results demonstrated mice treated with Acs-EtOH had no change in performance of the rota-rod device. These findings contributed to explain the use of the ethanol extract of atemoya leaves in folk medicine for the treatment of inflammatory and infectious diseases.

CONCLUSION

Considering the results of this study, the obtained ethanolic extract from the leaves of atemoya (*Annona cherimoya* Mill. x *Annona squamosa* L.) showed significant antinociceptive and anti-inflammatory effects, which may also be involved in the mechanisms that inhibit the synthesis or release of pro-inflammatory mediators.

Additional studies are being conducted by our group to clarify the mechanisms involved in these actions. Therefore, the results support the use of the species in

folk medicine, encouraging the research for new bioactive extracts, as well as the development of natural products obtained in Brazilian biomes, with the purpose of clinical application. It is noteworthy that in the present study, the atemoya ethanolic extract did not present acute toxicity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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