

Anti-inflammatory and anti-allergic activity of the methanolic extract from *Annona sylvatica* (Annonaceae)

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ABSTRACT. *Annona sylvatica* (Annonaceae) is a common medicinal plant used in folk medicine for fever and cough; it is found in several Brazilian states, including Mato Grosso do Sul. The local population uses *A. sylvatica* leaves for treating fever and cough; however, how this medicinal plant affects the patients is little understood. We evaluated the anti-inflammatory and anti-allergic properties of a methanolic extract of *A. sylvatica* (MEAS) leaves in mouse models of inflammation and allergy. The study employed male C57bL/6 mice for allergy models and male Swiss mice for the inflammation study. Oral treatments with MEAS (30 to 150 mg/kg) significantly inhibited the carrageenan-induced leukocyte migration and protein extravasation to the cavity in an air pouch model. In the allergic animal models, oral treatment with MEAS (150 mg/kg) significantly reduced histamine- and ovalbumin (OVA)-

induced paw edema. Moreover, in the OVA-induced allergic lung inflammation model, oral treatment with MEAS (150 mg/kg) significantly inhibited neutrophil, eosinophil, and mononuclear cells migration to the lung. Pretreatment of neutrophils with MEAS (3, 150, 300 µg/mL) significantly reduced neutrophil chemotaxis induced by N-Formyl-Met-Leu-Phe (fMLP) and complement 5a (C5a, in a concentration-dependent manner. We conclude that *A. sylvatica* has anti-inflammatory and anti-allergic properties. Flavonoids and acetogenins, compounds found in the MEAS, could be responsible for these anti-inflammatory and anti-allergic effects.

Key words: Araticum; Allergy; Inflammation; Ovalbumin; Air pouch; Histamine

INTRODUCTION

The inflammatory response is involved in several kinds of diseases and also in host defense response to infection. The endogenous or exogenous aggressive stimuli perturbates cell homeostasis and can induce the activation responses. A recent example is the SARS-CoV-2 virus that potentially binds to anticoagulant protein S (PROS1) to initiate the inflammatory transduction cascade, resulting in the production of interferons, proinflammatory cytokines (such as IL-6 and TNF) and other mediators (Lemke and Silverman, 2020).

Proinflammatory cytokines, histamine, serotonin, eicosanoids and complement products could be produced or released, leading to the classical signs of inflammation (Medzhitov, 2008), allergy, and pain. Inflammation could be controlled using nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (Lin et al., 2020), or cytokine blockers (Nathan et al., 2002; Schett et al., 2018).

In allergic inflammation, the allergens are processed by antigen-presenting cells to T helper cells. And T-helper cells, in turn mediate the B cells activation to produce immunoglobulin E (IgE), which plays a role in mast cell sensitization. When there is re-exposure to the allergen, the allergen binds to IgE on mast cells, triggering an inflammatory response (Galli et al., 2008).

Annona sylvatica (former name *Rollinia sylvatica*) is a native Brazilian species known as “araticum”, “araticum-do-mato”, “embira”, “cortiça” and “cortiça-amarela” (Lorenzi et al., 2005). The leaves have been used in folk medicine against fever, cough, ulcers caused by syphilis, as an antispasmodic, and for angina and diarrhea (Vendruscolo et al., 2005). Low toxicity of a methanolic extract of *A. sylvatica* leaves (MEAS) in acute toxicity test and the finding of luteolin, quercetin, laherrandurin, almunequin, otivarin and 2.5 dihydroalmunequin in MEAS has been reported (Araujo et al., 2014). These facts together suggest an anti-allergic and anti-inflammatory potential for MEAS. Consequently, we investigated the anti-inflammatory and anti-allergic activities of MEAS in animal models.

MATERIAL AND METHODS

Chemicals, kits and reagents

λ -Carrageenan, Ovalbumin (OVA), histamine, phosphate-buffered saline (PBS), Tween 20%, dexamethasone, ethylenediaminetetraacetic sodium salt (EDTA), N-Formyl-methionyl-leucyl-phenylalanine (fMLP) and zymosan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ketamin and xylazine was obtained from Syntec (Santana de Paraiba, SP, Brazil). Aluminium hydroxide was purchased from EMS Sigma Pharma (São Paulo, Brazil).

Plant material, preparation of the methanolic extract and phytochemical studies

Leaves of a mature *A. sylvatica* plant were collected in a natural Atlantic forest area in Dourados, Mato Grosso do Sul state, Brazil, at coordinates 22°11'48.9" S 54°56'11.4" W, in August 2009). The plant was identified by Dr. Zefa Valdevina Pereira (Faculdade de Ciências Ambientais e Biológicas - UFGD, MS, Brazil) and a voucher specimen was deposited in the DDMS/UFGD herbarium (no. 4600). Authorization for accessing and studying samples from the Brazilian genetic heritage site was obtained from the "Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado" (SisGen -A163977).

Air-dried and powdered leaves (230 g) were macerated for seven days using methanol as solvent at room temperature (25°C). After filtration, the sample underwent two more extractions using the same procedure. Subsequently, the solvent was eliminated in a rotaevaporator at 45°C and lyophilized to obtain the methanolic extract (25 g). The lyophilized MEAS, was stored at 4°C and protected from light. Phytochemical studies of MEAS had been made by Araujo et al. (2014).

Animals

The experiments were conducted using male C57Bl/6 (n = 8; 28-32 g) provided by Oswaldo Cruz Foundation breeding unit (CECAL-FIOCRUZ) and male Swiss (n = 7; 28-32 g) from the Universidade Federal da Grande Dourados (UFGD). The animals were maintained under a 12 h light-dark cycle, with controlled temperature (22 ± 1°C) and relative humidity (60-80%). The ethics committee for research on laboratory animals of the UFGD (Nbr. 005/2010) approved the research. The MEAS obtained was dissolved in a solution of Tween 80 (20%) and water for administration at doses of 3, 30, 150 mg/kg. The vehicle was a solution of Tween 80 (20%) and water.

Air pouch model

The Air Pouch model was used as described by Garcia-Ramallo et al. (2002) and Piornedo et al. (2011), with some modifications. Swiss mice were anesthetized with intraperitoneal injections of 100 mg/kg of ketamin and 10 mg/kg of xylazine (Syntec, Santana de Paraiba, SP, Brazil). After this, a subcutaneous injection was made with 4 ml of sterile air in the back of the mice, on day 0. On day 3, a second injection of 2 mL of sterile air was performed into the pouch. On day 6, animals were treated with vehicle, DEX (1 mg/kg s.c.) as a positive control drug or with MEAS (3, 30, 150 mg/kg, orally). After one hour a solution of carrageenan 0.1% (350 μ L) was injected directly into the pouch. 4 h later

the mice were submitted to euthanasia and the pouches were washed with 1 mL of PBS/EDTA 10 mM. Exudates were collected and total cells were counted (Neubauer chamber). Exudates were then centrifuged (1000 g for 2 min at 4°C) and the pellet suspended to prepare slides. Leukocytes were stained by May-Gruenwald-Giemsa method and analyzed under light microscopy and 100 leukocytes (polymorphonuclear (PMN) and mononuclear (MN)) were counted, while protein were measured in the supernatants by Bradford's reaction (Bradford, 1976).

Histamine- or ovalbumin (OVA)-induced paw edema

Male C57Bl/6 mice were actively sensitized by subcutaneous injection of 0.2 mL of a mixture of ovalbumin (OVA, 50 µg) and aluminum hydroxide (Al OH₃, 5 mg). Fourteen days later, 12 h fasted animals received MEAS by oral route (30 and 150 mg/kg, n = 8, orally), the positive control promethazine (H1-receptor antagonist, 10 mg/kg, orally) or vehicle were administered by oral route. One hour later mice received into right paw an injection (50 µL) of OVA, while into the left paw they received an injection of 50 µL of sterile saline (used as a control). The edema was measured using a plethysmometer (Ugo Basile, Italy), 15 min after stimulation.

Another group of C57Bl/6 mice was treated with MEAS (30 and 150 mg/kg, p.o., n = 8 for each treatment), promethazine (10 mg/kg, orally n = 8) and vehicle (a solution of Tween 80 (20%) and water, n = 8); after 1 h they received an injection of histamine (100 µmol/paw) and the induction of paw edema was evaluated 30 min after stimulation. Paw edema was expressed in µL/paw as the difference between stimulated and non-stimulated paws (Cavalher-Machado et al., 2008).

Allergen sensitization and airway challenge

Male C57Bl/6 mice were sensitized by a subcutaneous injection of 0.2 mL of a mixture of OVA (100 µg) and Al(OH)₃ (5 mg). A second sensitization was made with the same mixture after 14 days (Park et al. 2009). From day 21 to day 23 after the last immunization, mice were orally treated (MEAS, 30 or 150 mg/kg, n = 8) once per day. 1 h after the last treatment in day 23, animals received anesthesia (intramuscular, ketamin 35 mg/kg and xylazine 5 mg/kg) and then received an intranasal challenge (OVA 4 mg/mL) in a final volume of 25 µL. The control group received the vehicle DEX (i.p., 1 mg/kg), used as a reference inhibitor.

The bronchoalveolar lavage fluid was examined 24 h after the last intranasal OVA challenge. The evaluation of leukocyte influx into the bronchoalveolar lavage fluid was made 24 h after final challenge, when the mice were killed by pentobarbital overdose (i.p., 150 mg/kg). Subsequently, a polyethylene cannula was introduced into the trachea, and PBS containing EDTA (10 mM) was instilled in two aliquots (0.8 mL each). The bronchoalveolar lavage fluid (BALF) was recovered placed on ice and centrifuged (150 g, 10 min). The cell pellets were resuspended in 0.25 mL of PBS/EDTA and the total leukocyte performed in an automatic particle counter Z2: Counter (Coulter, Beckman Coulter, Fullerton, California, USA) Differential cell counts were made using stained cytospin smears (Shadon, Pittsburgh, Pennsylvania, USA) by the May-Gruenwald-Giemsa

method under light microscopy (100x). Counts are reported as number of leukocytes/mL ($\times 10^5$ /mL).

Neutrophil isolation and chemotaxis in vitro assay

Peripheral blood with EDTA was obtained from healthy volunteers and neutrophils were isolated by density gradient centrifugation using Ficoll-Hypaque as described by Boyum (1968). Mononuclear cells were removed and red cells were lysed twice in the PMN-red cell pellet with a cold isotonic NH_4Cl solution. The cells were washed twice with Hanks balanced salt solution (HBSS) and resuspended with the same buffer solution supplemented with 10% heat-inactivated fetal calf serum. Subsequently, the cells were counted (Neubauer Chamber after dilutions in Türk fluid) and cell concentration was adjusted to cells/mL. Neutrophil viability was subsequently estimated via the trypan blue exclusion method for each experiment.

Different groups of neutrophils were treated with extract (30, 150 and 300 $\mu\text{g/mL}$) diluted with Tween 80 solution at 1% HBSS. Another group received only Tween 80 (1%) and all groups were incubated for one hour at 37°C. The chemotaxis assay was performed employing a Boyden chamber (Boyden, 1962; Neves et al., 2009). Aliquots of cell suspension containing 1.5×10^6 neutrophils treated (with extract/Tween) or not were placed in upper chamber that was separated from the chemotactic agent in the lower chamber by a 8 μm average pore size cellulose nitrate filter. The lower wells of the chamber were filled with chemotactic stimulus, fMLP at 10^{-9} M or C5a at 5%, or only with HBSS to measure random migration. The cells were allowed to migrate in humidified air for 60 min at 37°C, followed by removal of the filters for fixation and staining of the cells. Neutrophil migration within the filter was determined under light microscopy by the “leading front” method (Zigmond and Hirsch, 1973). The distance was measured from the top of the filter to the farthest plane still containing two cells with a 40 X objective. For each treatment, duplicate wells were always run. Five fields were counted for each filter. C5a was obtained from opsonized zymosan in human plasma of healthy volunteers. The Institutional Committee for Human Ethics approved this study protocol to obtain human plasma and neutrophils of healthy volunteers; all protocols were performed as described by Neves et al. (2009).

Statistical analysis

All data are presented as mean \pm S.E.M. Differences between groups were evaluated by analysis of variance (one-way ANOVA), followed by a Student Newman-Keuls test. The number of animals per group is indicated in the legends of the Figures. Statistical differences were considered to be significant at $P < 0.05$. Statistical analysis and graphing were performed using the GraphPad Prism 7 Software (San Diego, CA, USA).

RESULTS

Effects of MEAS on carrageenan-induced leukocytes migration and protein extravasation to air pouches

On the 6th day, after induction of air pouch in the dorsal region of mice, carrageenan injection (350 μ L at 0.1%) was applied, which in 6 h promoted protein extravasation and leukocyte migration, mainly polymorphonuclear leukocytes (Figure 1A-B). The oral treatment of the animals with MEAS significantly inhibited leukocyte migration to air pouches in $90 \pm 1\%$ and $52 \pm 13\%$ for doses of 30 and 150 mg/kg, respectively. In protein extravasation, the percent of inhibition was $86 \pm 6\%$ for 30 mg/kg of MEAS and $71 \pm 15\%$ for 150 mg/kg of MEAS (Figure 1C). DEX was used as a positive control, and as expected reduced leukocytes migration and plasma extravasation.

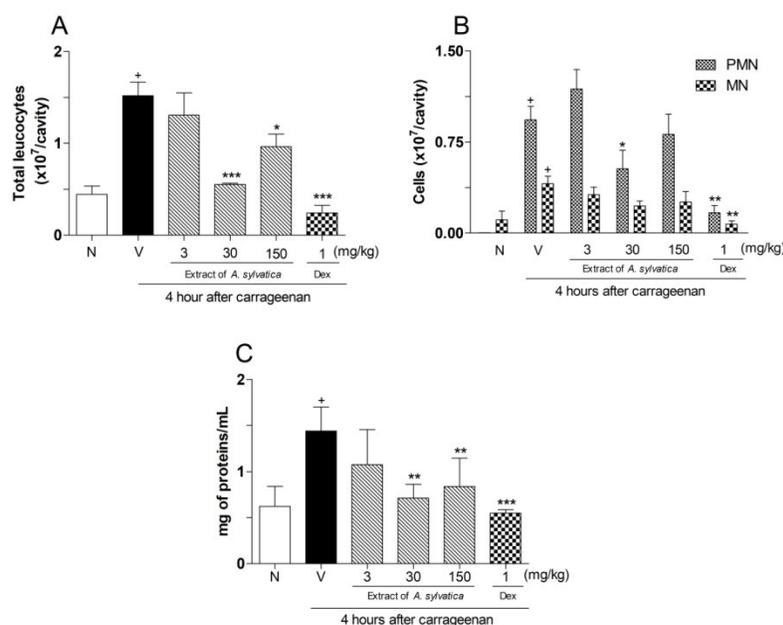


Figure 1
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Figure 1. Effect of methanolic extract of *Annona sylvatica* (MEAS) on carrageenan-induced leukocyte migration and plasma leakage into the air pouch. Mice were pretreated one hour before with MEAS (3, 30 and 150 mg/kg p.o.), dexamethasone (1 mg/kg, s.c. in sterile saline) or filtered water (vehicle). Pouches were washed with PBS/EDTA 10mM. Cells were counted and plasma leakage was analyzed. In A, number of cells that migrated to the pouches 4 h after carrageenan. Results are expressed as 10^7 cells /cavity. In B, Differential cell counting was performed. In B, plasma leakage was measured by Bradford's reaction; results are expressed as protein/mg-l of protein compared with vehicle (V) vs. treated group. The bars express the mean \pm SEM of six animals, compared with vehicle (V) vs treated group. *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Student-Newman-Keuls. + denotes significant difference (P < 0.05) between naive (N) and vehicle group.

Effects of MEAS on Histamine - or OVA-induced paw edema

As observed in Figure 2, the histamine and OVA injection induced edema formation 30 min later. The oral treatment with MEAS (150 mg/kg) significantly inhibited edema formation. There was no difference between the group treated with MEAS 30 mg/kg and the vehicle group. A single dose of 150 mg gave a significant response, with inhibitions

of $64 \pm 6\%$ ($P < 0.01$) and $74 \pm 7\%$ ($P < 0.001$) for OVA and histamine edema, respectively (Figure. 2A-B). As expected, the positive control, promethazine (10 mg/kg), also inhibited edema formation, with inhibitions of $52 \pm 6\%$ (OVA) and $69 \pm 10\%$ (histamine). Based on these results, *A. sylvatica* leaf extract has anti-allergic properties. The effect of MEAS in an OVA-induced allergic inflammation model was shown (Figure 2).

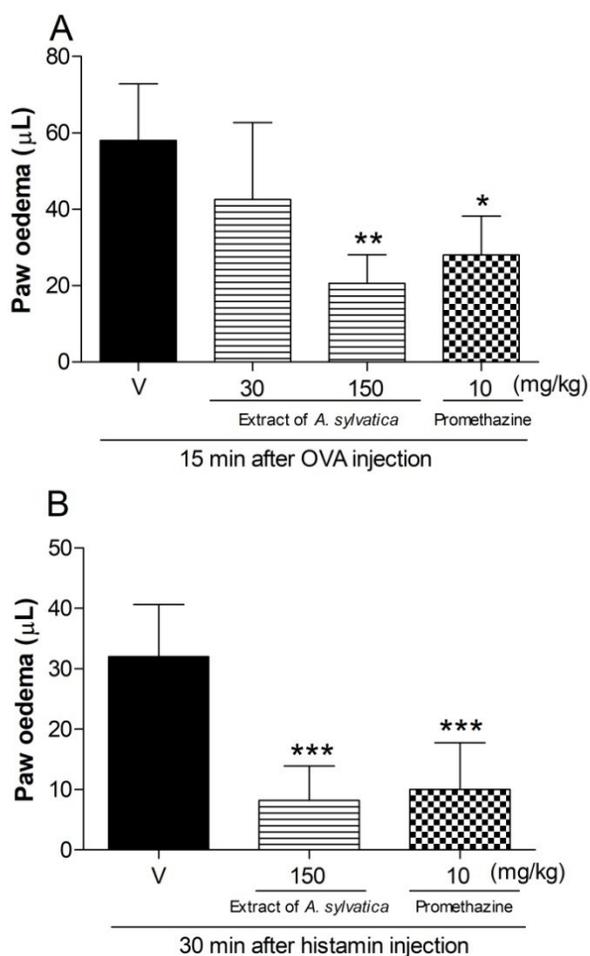


Figure 2
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Figure 2. Anti-allergic evaluation of methanolic extract of *Annona sylvatica* (MEAS) on histamine and OVA-induced paw edema in mice. In the upper panels, animals received extract (30 and 150 mg/kg, p.o), promethazine (10 mg/kg, p.o) was used as a reference drug or filtered water (control) and 1 h after was given an intraplantar injection of histamine (100 µg/paw) or OVA (3 µL/paw). In A, the inhibition induced by extract and promethazine 30 min after OVA injection. In B, the extract (150 mg/kg) in paw edema 30 min after histamine injection. The bars express the mean \pm SEM of eight animals, compared with vehicle (V) vs treated group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA followed by Student-Newman-Keuls. + denotes significant difference ($P < 0.05$) between saline and OVA group.

Evaluation of leukocyte influx into the bronchoalveolar lavage fluid (BALF) in an OVA-induced allergic inflammation model

BALF was collected twenty-four hours after antigen final challenge; subsequently the mice were submitted to euthanasia with a pentobarbital overdose. As shown in Figure 3, the OVA caused a marked leukocyte influx into the lung, mainly eosinophils. In the naive animal group, eosinophils constituted 0.6% of total leukocytes after allergen challenge. In contrast, the control group (challenged with OVA + vehicle) showed an increase of eosinophils, constituting 51.9% of total leukocytes (Figure 3D). The oral treatment with MEAS significantly inhibited cells influx into the lung. In total leukocytes, the extract at dose 150mg/kg inhibited $88 \pm 6\%$ and DEX (1 mg/kg) $86 \pm 5\%$ cells migration. In differential cell analysis, the MEAS (150 mg/kg) significantly decreased the influx of the mononuclear cells, neutrophils and eosinophils, 75 ± 15 , 91 ± 7 , and $84 \pm 10\%$, respectively. The dose of 30 mg/kg of MEAS inhibited ($81 \pm 11\%$) only the mononuclear cell influx.

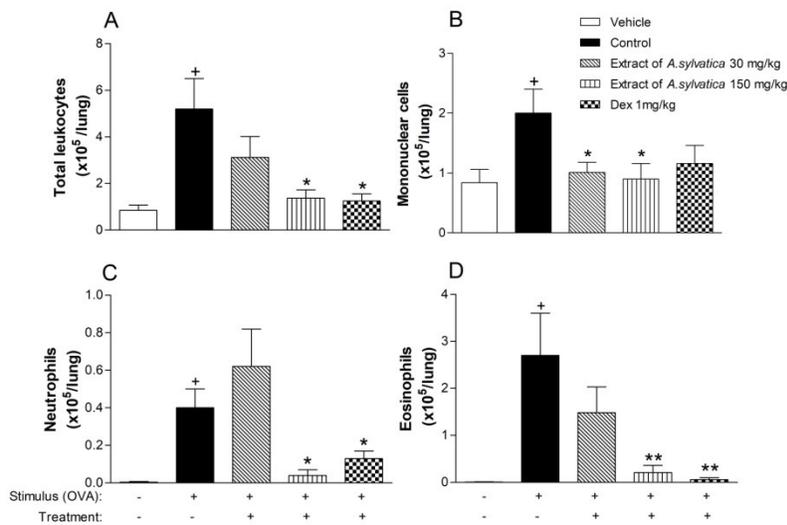


Figure 3
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Figure 3. Effects of methanolic extract of *Annona sylvatica* (MEAS) on proinflammatory cells in a model of allergic inflammation of OVA-induced in mice. After period of sensitization the animals were challenged and treated with extract (30 and 150 mg/kg, p.o), dexamethasone (1 mg/kg, s.c) or filtered water (control) for three days. After 24 h the mice were euthanized and collected of BALF. In the upper panels, the effects of oral treatment of extract on total leukocytes (A), mononuclear cells (B), neutrophils (C) and eosinophils (D). The bars express the mean \pm SEM of eight animals, compared with vehicle (V) vs treated group. *P < 0.05, **P < 0.01, one-way ANOVA followed by Student-Newman-Keuls. + denotes significant difference (P < 0.05) between saline and OVA group.

Effect of the MEAS on neutrophils migration *in vitro*

The direct effect of MEAS on the neutrophil chemotaxis *in vitro* was determined. The neutrophil viability ($\geq 95\%$) was confirmed in trypan blue assays after neutrophils were

incubated for 1 h with MEAS (results not shown). As shown in Figure 4, the chemotactic agents fMLP and C5a increased the neutrophils migration through the cellulose membrane. The neutrophils pretreatment with MEAS (3, 150, 300 $\mu\text{g/mL}$) significantly reduced neutrophil chemotaxis toward both chemotactic agents, fMLP (Figure 4A) and C5a (Figure 4B) in a concentration-dependent manner.

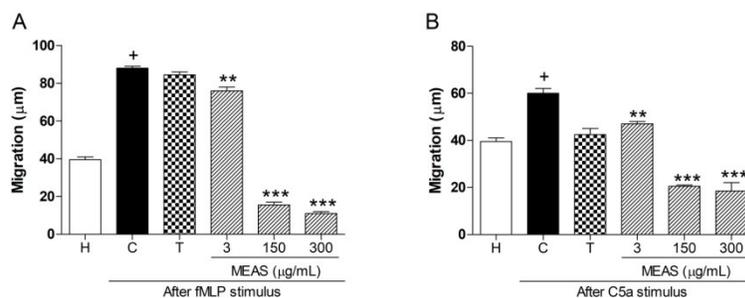


Figure 4
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Figure 4. Effects of methanolic extract of *Annona sylvatica* (MEAS) on neutrophil chemotaxis. In A and B, different cell groups were incubated with extract (3, 30 and 150 $\mu\text{g/mL}$) or with Tween (T) for 1 h. After this period, these cells were transferred to a Boyden chamber and the chemotaxis started. HBSS (H) was used as a diluent of C5a and fMLP. Results are expressed as μm . Each bar represents the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with control (C) vs. treated group.

DISCUSSION

We found that *A. sylvatica*, a native species of Brazil, has anti-allergic and anti-inflammatory activity, which still is poorly characterized pharmacologically. The essential oil obtained from *A. sylvatica* was shown to have anti-edematogenic effects against carrageenan- and complete Freund's adjuvant (CFA)-induced mouse paw edema (Formagio et al., 2013, Quílez et al., 2018). Using phytochemical analysis of the MEAS by LC-MS, we detected flavonoids flavones and flavonol and Annonaceous acetogenins (Araujo et al., 2014). The leaves of *A. sylvatica* are popularly used against cough, which is a common symptom associated with airway inflammation, such as asthma and eosinophilic bronchitis (Brightling et al., 1999; Birring et al., 2004). In relation to development of cough, some studies suggest involvement of inflammatory mediators, i.e. histamine (Choudry et al., 1989) and increased expression of TRPV-1 receptor (Groneberg et al., 2004).

Therefore, we evaluated the activity of MEAS on OVA- and histamine-induced paw edema. The results showed that MEAS can inhibit edema formation. Formagio et al., (2013) showed that the oral administration of essential oil obtained from *A. sylvatica* (20 and 200 mg/kg) leaves was effective to inhibit carrageenan- and complete Freund's adjuvant-induced mouse paw edema. Other species of the *Annona* genus also showed anti-inflammatory properties (Rocha et al., 2016, Quílez et al., 2018). Based on these results, it is possible to suggest that the MEAS has an anti-edematogenic effects; so it was decided to also test MEAS on OVA-induced allergic lung inflammation.

In airways allergic disorders, such as asthma, allergen exposure produces a response dominated by Th2 cells that lead to increase of cytokines, such as, IL-4, IL-5 and IL-13 and recruiting inflammatory cells, mainly eosinophils. IL-5 is an important cytokine in

eosinophil recruitment (Kay, 2001; Bisset and Schmid-Grendelmeier, 2005). We found that compared with vehicle-treated mice, MEAS-treated animals showed significant reduction of eosinophilia (92.3%) caused by allergen (OVA) after challenge. This effect of MEAS extract treatment may result from a decrease in the levels of Th2 cytokines responsible for eosinophil recruitment into the lung. The results demonstrated that MEAS was effective in reducing experimental allergic inflammation in mice, suggesting an anti-allergic potential of *A. sylvatica*.

In a non-allergic air-pouch model, we evaluated the effects of MEAS on carrageenan-induced inflammation. MEAS inhibited leukocyte migration and plasma leakage, revealing its anti-inflammatory activity. Thus, this is the first study showing that oral administration of MEAS reduces, in a dose-dependent manner, leukocyte migration and plasma leakage.

Previous studies demonstrated that flavonol derivatives and other flavonoids inhibit various parameters of the inflammatory process. In another study, the flavonol quercitrin prevented acute and fatal anaphylactic shock in 75% of the challenged animals. Anaphylactic shock is an extreme and potentially fatal allergic reaction (Cruz et al., 2008). These findings allow us to suggest that part of the effects observed in our study may be mediated by flavonoids characterized in MEAS as described above.

We also investigated the effect of MEAS on neutrophil migration. For this, we performed a chemotaxis assay using different types of stimuli, such as, synthetic fMLP (derived from bacterial endotoxin) and C5a (from opsonized zymosan), which react with specific sites on PMN cell membrane, leading to the activation of these cells (Chenoweth and Hugli, 1978; Andersson et al., 1987). The results suggest that the anti-inflammatory effects of MEAS may be, at least in part, related to the capacity of this extract to interfere in mechanisms involved in cell recruitment during the inflammatory process.

In summary, this is the first study showing the anti-inflammatory and anti-allergic activity of *A. sylvatica*, indicating that the methanolic extract is able to reduce inflammatory parameters in mice. Studies with extracts of medicinal plants are useful for developing medicines, such as phytomedicines. In the composition of vegetal extracts, there are many chemical constituents; consequently, the pharmacological effects of extracts can be attributed to the interaction of these molecules (Calixto, 2000; Calixto, 2003)

Our results show that the effects observed by methanolic extract of *A. sylvatica*, may be associated with the high levels of the flavonoid and acetogenins found in this plant. Studies are now in progress to isolate and characterize other constituents present in the plant that could account for the reported pharmacological effects and also to further characterize their sites of action.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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