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ORIGINAL ARTICLE

## Analysis of the anti-inflammatory and chemopreventive potential and description of the antimutagenic mode of action of the *Annona crassiflora* methanolic extract

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#### Abstract

*Context: Annona crassiflora* Mart. (Annonaceae) is a medicinal plant that is widely used in folk medicine, which leads to its investigation as a potential source of new pharmacological principles.

*Objective*: This study describes the anti-inflammatory, antiallodynic, and antimutagenic/ chemopreventive activities of the leaves *A. crassiflora* methanolic extract. Its antimutagenic mode of action was analyzed in a plant or animal experimental model.

*Materials and methods*: Total flavonoids were quantified by spectrophotometry at 415 nm and its composition was analyzed by <sup>1</sup>H NMR spectra. Animals received orally, 30, 100, and 300 mg/kg of extract in both tests, carrageenan-induced paw edema and myeloperoxidase activity. Animals were treated with 100 and 300 mg/kg, in all the analyzed tests, pleural cell migration and protein exudation, carrageenan-induced cell migration into the pouch, induction of joint inflammation and carrageenan-induced allodynia response in the mouse paw. To evaluate the antimutagenic/ chemopreventive activity through the *Allium cepa* test, we used 5, 10, and 15 mg/L of extract, and for the micronucleus test in the peripheral blood, we used the dose of 15 mg/kg.

Results: The fractionation of the ethyl acetate (EA) fraction, resulting from the partition of the methanol extract of the A. crassiflora, afforded through chromatographic methods resulted in the isolation of kaempferol 3-O- $\beta$ -glucoside and kaempferol 3-O- $\beta$ -diglucoside. Oral treatment with 100 and 300 mg/kg of extract significantly inhibited the carrageenan-induced edema formation, with inhibitions of  $53 \pm 7\%$  and  $47 \pm 10\%$ ; in MPO activity, the observed inhibitions were  $60 \pm 7\%$  for 100 mg/kg treatment and  $63 \pm 7\%$  for 300 mg/kg. The ACME reduced significantly the total leukocytes (an inhibition of  $78 \pm 9\%$  with 100 mg/kg and  $90 \pm 7\%$  with 300 mg/kg) and protein levels (approximately 100% inhibition with both doses) in the pleurisy model. In carrageenan-induced leukocyte migration into the pouch, the extract inhibited leukocyte migration only when administered 300 mg/kg per dose (the reduction was  $43 \pm 5\%$ ). Pretreatment with extract failed to reduce the zymosan-induced edema formation and did not inhibit the carrageenan-induced mechanical allodynia. Damage reduction in Allium cepa tested with different concentrations (5, 10, and 15 mg/L) was 66.17, 75.75, and 69.19% for the pretreatment; 72.72, 33.33, and 22.22% for the simple simultaneous treatment; 100.50, 93.93, and 102.52% for the simultaneous treatment with pre-incubation; 89.39, 79.79, and 84.34%; for the post-treatment, and 86.36, 81.31, and 93.43% for the continuous treatment. The antimutagenic evaluation in the micronucleous test showed a damage reduction of 75.00 and 64.58% for the pre-treatment and simultaneous protocols, respectively. The post-treatment protocol increased the cyclophosphamide effects in 45.83%.

*Conclusion*: These results suggest that this medicinal plant has chemopreventive and antiinflammatory therapeutic potential.

#### Keywords

Allium cepa, antimutagenesis, carrageenan, micronucleus, paw edema

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#### History

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#### Introduction

Tissue lesions usually develop into inflammation, which can cause pain as the edema (formed by increased vascular permeability and extravasation of plasma rich in proteins) compresses the nerve endings. The pain may also be caused by increased sensitivity stimulated by chemical mediators (Kantarci et al., 2005; Kumar et al., 2006; Medzhitov, 2010).

The resolution of inflammatory process requires the recruitment of specific cells, an increased rate of cell division, and the production of inflammatory mediators. The excess of these mediators leads to apoptosis inhibition. This phenomenon is particularly important for elimination of cells with genetic damage, hence the maintenance of this contribute to the development of cancer (Barreto et al., 2011).

According to the World Health Organization (WHO), it is estimated that there will be approximately 27 million new cases of cancer worldwide in 2030, and this estimate stimulates the search for natural compounds that can prevent mutagenesis, carcinogenesis, and inflammation and for tests that allow the rapid, safe, and low-cost screening of substances with these potentials.

Natural products are candidates for these tests because the insertion of food compounds or phytopharmaceuticals may be an important alternative for the treatment of inflammation and the prevention of cancer in countries that do not have sufficient resources to address these important public health issues.

The genus *Annona* L. (Annonaceae) is empirically used for the treatment of inflammatory disorders, and our research group showed that extracts, fractions, and isolated compounds are anti-inflammatory natural agents (Formagio et al., 2013a,b). Fruits from this genus are widely used in the traditional herbal medicine, such as for herbal healing; however, their toxic and pharmacological effects on inflammatory states have not been scientifically investigated (Roesler et al., 2010).

Annona crassiflora Mart. is widely used in folk medicine. Its leaves, bark, fruits, and seeds are used as stimulant, astringent, antisyphilitic, and antidiarrheal agents and also as antivenin (Corrêa, 1926; Cruz, 1979; Suleiman et al., 2008; Vilar et al., 2011). In addition, many other species of the same family are traditionally employed as ethno-medicaments against cancer (Cochrane et al., 2008; Fedel-Miyasato et al., 2014; Graham et al., 2000; Vilar et al., 2011).

Therefore, the present study evaluates the antimutagenic/ chemopreventive, anti-inflammatory, and antiallodynic activities of the *A. crassiflora* methanolic extract (ACME) and describes its antimutagenic mode of action in a plant and animal experimental model.

#### Materials and methods

## Plant material and preparation of the Annona crassiflora methanolic extracts

The leaves of *A. crassiflora* were collected from Areira farm, which is located in the Brazilian *cerrado* of Mato Grosso do Sul state (Dourados, MS, Brazil), in November 2010. The city of Dourados is located at 22°14′16″S and 54°48′02″W and at an average altitude of 452 m. The species was identified by

Prof. Dr. Zefa Valdevino Pereira of the Department of Biological and Environmental Sciences of the Federal University of Grande Dourados (Universidade Federal da Grande Dourados, UFGD). A voucher specimen was deposited at the herbarium of this university under the registration number DDMS 4596.

The dried and ground leaves (820 g) were subjected to maceration in methanol at room temperature. The evaporation of the solvent under vacuum in a rotary evaporator and its subsequent lyophilization yielded the methanolic extract (58 g).

#### Determination of total flavonoids

A volume of 1.50 mL of 95% ethanol, 0.10 mL of 10% aluminum chloride (AlCl<sub>3</sub>·6H<sub>2</sub>O), 0.10 mL of sodium acetate (NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>·3H<sub>2</sub>O) (1 mol/L), and 2.80 mL of distilled water were added to  $500 \,\mu\text{L}$  of the extract. The mixture was allowed to react at room temperature for 40 min and was read using a spectrophotometer at a wavelength of 415 nm. The same procedure was used to analyze the blank (Lin & Tang, 2007). To calculate the concentration of flavonoids, an analytical curve was prepared (2.5, 5.0, 10.0, 20.0, 25.0, 50.0, 100.0, and  $125.0 \,\mu\text{g}$ ) using quercetin as the standard, and the respective absorbances were measured. These data were analyzed through linear regression, and a straight-line equation was obtained and used for the analysis of the real samples. The results are expressed in mg of quercetin equivalents per gram of extract. The quercetin curve equation was y = 12.341x + 12.341x0.009, and its correlation coefficient was R = 0.9977. All tests were performed in triplicate.

#### Extraction and isolation of constituents

Part of the extract (29.74 g) was dissolved in a 1:1 mixture of MeOH/H<sub>2</sub>O (200 mL) and successively extracted with hexane  $(3 \times 50 \text{ mL})$ , CHCl<sub>3</sub>  $(3 \times 50 \text{ mL})$ , and EtOAc  $(3 \times 50 \text{ mL})$ . After evaporation of the solvents on a rotary evaporator, the hexane (H: 4.07 g), chloroform (C: 3.54 g), ethyl acetate (EA: 4.89 g), and hydromethanol (MA: 1.24 g) fractions were obtained. The resulting fraction of the partition into ethyl acetate (EA) was subjected to successive silica gel column chromatography (EtOAc and EtOAc/MeOH in increasing polarity) and Sephadex (H<sub>2</sub>O, 1:1 H<sub>2</sub>O/MeOH and MeOH). The <sup>1</sup>H NMR spectra of the subfractions obtained in greater quantity showed the presence of flavonoids **1** and **2** as the major components.

Kaempferol 3-*O*-β-glucoside (1): <sup>1</sup>H NMR δH (300 MHz, CD<sub>3</sub>OD): δ (H, mult., J in Hz, int.): 6.19 (H-6, d, J=2.1 Hz, 1H), 6.42 (H-8, d, J=2.1 Hz, 1H), 8.06 (H-2', H-6', d, J=8.7 Hz, 2H), 6.85 (H-3', H-5', d, J=8.7 Hz, 2H), 5.38 (H-1″, d, J=7.8 Hz, 1H); NMR <sup>13</sup>C/DEPT δc (75.5 MHz, CD<sub>3</sub>OD): 156.5 (C-2, C), 133.4 (C-3, C), 177.8 (C-4, C), 161.5 (C-5, C), 98.9 (C-6, CH), 164.4 (C-7, C), 93.9 (C-8, C), 156.6 (C-9, C), 104.2 (C-10, C), 121.3 (C-1', C), 131.3 (C-2', CH), 115.4 (C-3', CH), 160.2 (C-4', C), 115.4 (C-5', CH), 131.3 (C-6', CH), 101.9 (C-1″, CH), 71.4 (C-2″, CH), 73.4 (C-3″, CH), 68.2 (C-4″, CH), 76.1 (C-5″, CH), 60.4 (C-6″, CH<sub>2</sub>).

Kaempferol 3-*O*-β-diglucoside (**2**): <sup>1</sup>H NMR  $\delta$ H (300 MHz, CD<sub>3</sub>OD):  $\delta$  (H, mult., J in Hz, int.): 6.45

(H-6, d, J = 2.1 Hz, 1H), 6.78 (H-8, d, J = 2.1 Hz, 1H), 7.78 (H-2', H-6', d, J = 8.7 Hz, 2H), 6.90 (H-3', H-5', d, J = 8.7 Hz, 2H), 5.29 (H-1", d, J = 7.5 Hz, 1H), 5.15 (H-1"", d, J = 7.8 Hz, 1H); NMR <sup>13</sup>C/DEPT & (75.5 MHz, CD<sub>3</sub>OD): 158.0 (C-2, C), 134.7 (C-3, C), 178.1 (C-4, C), 161.1 (C-5, C), 99.7 (C-6, CH), 161.9 (C-7, C), 94.8 (C-8, C), 156.3 (C-9, C), 106.0 (C-10, C), 120.5 (C-1', C), 131.0 (C-2', CH), 115.6 (C-3', CH), 160.3 (C-4', C), 115.6 (C-5', CH), 131.0 (C-6', CH), 95.6 (C-1", CH), 73.9 (C-2", CH), 78.6 (C-3", CH), 71.2 (C-4", CH), 78.3 (C-5", CH), 62.3 (C-6", CH2), 102 (C-1", CH), 70.5 (C-2", CH), 70.4 (C-3", CH), 71.7 (C-4", CH), 74.0 (C-5", CH), 62.6 (C-6", CH2).

## Evaluation of anti-inflammatory and antiallodynic activities

Experiments were conducted using male and female Swiss mice (20-30 g), maintained on a 12 h light/dark cycle in a room kept at a constant temperature of  $22 \pm 1$  °C, controlled humidity (60–80%) with food and water provided *ad libitum*. The animals were acclimatized to the experimentation room for at least 2 h before testing and were used only once throughout the experiments. All experimental procedures were carried out in accordance with the guidelines of the U.S. National Institute of Health and were approved for research by the Ethics Committee on Laboratory Animals of UFGD sob o protocol no. 005/2010. Carrageenan was purchased from Sigma Chemical Co. (St. Louis, MO). Other drugs and reagents were of analytical grade.

#### Carrageenan-induced paw edema

Different groups of mice were orally treated with ACME (30, 100, and 300 mg/kg), or with dexamethasone by subcutaneous route (1 mg/kg) or vehicle. After 1 h, the animals received a 50 µL subcutaneous (s.c.) injection of carrageenan (300 µg/paw), into the right hindpaw, suspended in sterile 0.9% saline. The contralateral paw received only saline and was used as the control. The paw volume was measured using a digital plethysmometer at different time points (0.5, 1, 2, and 4 h) after the injection of carrageenan. Results were expressed in µm and the difference between basal and post-injection values was quantified as edema (Kassuya et al., 2009).

#### Determination of myeloperoxidase activity

To investigate whether oral treatment with ACME or vehicle could affect the cellular migration induced by carrageenan, the myeloperoxidase activity was measured into the mouse paw following experiments described in carrageenan-induced paw edema. Animals were euthanized 4 h after carrageenan injection. For myeloperoxidase (MPO) activity, the paw skins were homogenized in 5% (w/v) of 80 mM phosphate buffer, pH 5.4, containing 0.5% of hexadecyltrimethylammonium bromide. The homogenate was centrifuged at 3200 rpm and 4 °C for 20 min. Aliquots (30 µL) of each supernatant were mixed with 100 µL of phosphate buffer 80 mM, 85 µL of phosphate buffer 0.22 mol/L, and 15 µL of 0.017% H<sub>2</sub>O<sub>2</sub> on a 96-well plate. The reaction was triggered with 20 µL of 3,3,3-tetramethylbenzidine (dissolved in *N*,*N*-dimethylformamide).

The plate was kept at 37  $^{\circ}$ C for 3 min, after which the reaction was stopped by adding 30 µL of sodium acetate 1.46 mol/L, pH 3.0. The enzymatic activity was determined by measuring the optical density at 630 nm and it was expressed as mOD per mg of protein.

#### Pleural cell migration and protein exudation

Different groups of animals treated with ACME (100 and 300 mg/kg, per oral) or dexamethasone (1.0 mg/kg, subcutaneously, positive control), vehicle, and naive (0.9% negative control) were administered orally by gavage, in different groups of mice. Pleurisy was induced by the intrathoracic injection of 100 µL of 1% carrageenan as previously described (Henriques et al., 1990). The carrageenan was diluted in saline buffered. Briefly, an adapted needle was inserted into the right side of the thoracic cavity of the animals to enable intrathoracic (i.t.) administration of carrageenan. Control mice received an equal volume (100 µL) of sterile, pyrogenfree saline. After 4 h, the animals were killed and the thoracic cavity was washed with 1 mL of phosphate-buffered saline (PBS). The exudate volume was measured at room temperature, and an aliquot of  $20\,\mu L$  was diluted in the Turk solution (1:20) and used to determine the total number of leukocytes in a Neubauer chamber. For differential counting of leukocytes, the remaining fluid was centrifuged at 3200 rpm for 20 min, and the cells were resuspended. The protein exudation was evaluated directly from the lavage by Bradford's reaction, using the commercially available Bradford kit (Bioagency, São Paulo, Brazil). Total and differential cell counts were performed under light microscopy and the results are reported as the number of cells per ml of pleural fluid.

#### Carrageenan-induced cell migration into the pouch

Air pouch model of inflammation was performed as described previously (Garcia-Ramallo et al., 2002), with some modifications. Mice were briefly anesthetized with cetamine and xylasine and 4 mL of sterile air was injected with a seringle subcutaneously in the back of mice, on day 0. On the 3rd day, a second injection of 2 mL of sterile air was performed into the preformed pouch. On the 6th day, animals were treated with vehicle, dexamethasone (0.5 mg/kg s.c.) as the positive control drug or ACME (100 and 300 mg/kg orally) and 1 h later the inflammatory response was induced by a injection of a solution of carrageenan 0.1% (250 µL) directly into the pouch. Four hours after the inflammatory stimulus, mice were euthanized and the pouches were washed with 2 mL of phosphate-buffered saline (PBS) containing heparin (10 UI/mL). Exudates were collected and total cells were counted on the Neubauer chamber under light microscopy. Exsudates were then centrifuged at 1000 g for 2 min at  $4 \degree C$ and the pellet suspended in 1 mL of 3% albumin and added to previously prepared slides. Cells were stained with Rosenfeld and analyzed under light microscopy and 100 cells were counted.

#### Induction of joint inflammation

Groups of mice were orally treated with ACME (100 and 300 mg/kg) or vehicle. Another group of mice was

treated subcutaneously with the anti-inflammatory drug dexamethasone (1 mg/kg). Joint inflammation was induced by intra-articular (i.art.) injection of zymosan (500  $\mu$ g per cavity in 25  $\mu$ L sterile saline) by inserting a needle through the suprapatellar ligament into the left knee joint cavity, as previously described (Conte et al., 2008; Penido et al., 2006). Control animals received an intra-articular injection of an equal volume of sterile saline.

## Carrageenan-induced allodynia response in the mouse paw

The mechanical threshold with electronic analgesimeter (similar to von Frey hairs analysis) was measured (Chaplan et al., 1994; Kassuya et al., 2007). The mechanical threshold was expressed in milligrams (mg) and mechanical allodynia (measure after the intraplantar injection of carrageenan –  $300 \,\mu g/paw$ ) was considered as a decrease in the threshold when compared with the response of the same paws before treatment. Mice were pretreated (60 min before, per oral) with the ACME (100 and 300 mg/kg) and received a  $20 \,\mu L$  s.c. injection into the right hind paw of carrageenan ( $300 \,\mu g/paw$ ) suspended in sterile 0.9% saline. Control animals received a similar volume of vehicle ( $10 \,\text{mL/kg}$ ) by oral route or directly into the paw ( $20 \,\mu L$ ). The mechanical threshold was registered 3–4 h after injection of carrageenan.

#### Statistical analysis

Data are presented as mean  $\pm$  S.E. Differences between groups was evaluated by analyses of variance (one-way ANOVA) followed by the Newman–Keuls test. The number of animals per group is indicated in the legends. Statistical differences were considered to be significant at p < 0.05. Asterisks denote significant difference compared with the vehicle-treated group.

#### Toxicogenetic and chemopreventive evaluations

To induce DNA damage in *Allium cepa*, methyl methanesulfonate (MMS) at a concentration of 10 mg/L was used. The *Annona crassiflora* methanolic extract (ACME) diluted in 1% methanolic solution was tested at concentrations of 5, 10, and 15 mg/L.

In the micronucleus assay with mouse peripheral cells, the agent used to induce DNA damage was cyclophosphamide at a dose of 100 mg/kg of corporeal weight (c.w.). This agent was injected intraperitoneally (i.p.), and the ACME, which was diluted in 1% solution, was tested at a concentration of 15 mg/kg c.w. and administered through oral gavage.

#### Experimental design - Allium cepa test

Commercial seeds of *Allium cepa* (TOP SED – garden/blue line<sup>®</sup>; Lot: 020595) were used. These were germinated at room temperature in Petri dishes, covered with filter paper, and soaked with 3 mL of distilled water or solution. The seeds were then subjected to different treatments and protocols to evaluate the mutagenicity and antimutagenicity of the treatment (Figure 1).

The seeds were subjected to the following treatments for 120 h of cultivation:

Mutagenicity protocols:

- (I) *Negative control*: The seeds were cultivated for 120 h in 1% methanol solution.
- (II) Positive control (MMS): The seeds were cultivated in distilled water for the first 24 h and then in dishes containing aqueous MMS solution for a period of 96 h.
- (III) *ACME*: The seeds cultivated in distilled water for the first 24 h and then in ACME solutions (three different concentrations: 5, 10, and 15 mg/L) for 96 h.

Antimutagenicity protocols:

- (IV) *Pre-treatment*: The seeds were cultivated in distilled water for the first 24 h, in the ACME solution for 48 h, and then in a dish containing MMS for 48 h.
- (V) Simple simultaneous: The seeds were grown in distilled water for 72 h and then in a cultivation plate containing the MMS and ACME solutions, which were added simultaneously for a period of 48 h.
- (VI) *Simultaneous with pre-incubation*: The MMS and ACME solutions were pre-incubated at 37 °C for 1 h and subsequently transferred to the cultivation plate. The seeds were first germinated in distilled water for 72 h and then germinated in the prepared mixture for 48 h.
- (VII) *Post-treatment*: The seeds were grown in distilled water for 24 h, in a plate containing the aqueous MMS solution for a period of 48 h, and then in a plate containing the ACME for 48 h.
- (VIII) *Continuous*: The seeds were germinated in distilled water for 24 h and then in a plate containing the ACME for 48 h. The aqueous MMS solution was then added to the plate, and the seeds were cultivated in the mixture for 48 h.

Between each of the treatments, before the transfer of the seeds from one plate to the other, with the exception of the continuous phase, the seeds were washed twice in distilled water to remove the compounds from the previous treatments.

#### Preparation of slides

According to the protocol described by Mauro et al. (2014), which was slightly modified, after 120 h of cultivation, the roots were harvested at noon and subjected to fixative solution (acetic acid:ethanol 3:1) for at least 6 h. Subsequently, the roots were subjected to acid hydrolysis to expose the genetic material (1 N HCl at 60 °C for 6 min). The DNA was then stained with *Schiff*'s reagent for 2 h in the dark. The root tips were cut with a scalpel to extract the meristematic region. The cytoplasmic staining was performed by adding a drop of acetic carmine (2%). The material was then covered with a coverslip, and the meristems were gently crushed with tweezers. For the preparation of permanent slides, the whole slide was soaked in liquid nitrogen to remove the coverslip. Then, with the aid of synthetic resin (*Permout*<sup>®</sup>), a new coverslip was placed on the biological material.

#### Microscopic and statistical analysis

A total of 5000 cells per treatment (1000 cells/slide) were analyzed under a light microscope with  $400 \times$  magnification.

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Figure 1. Treatments to evaluate the mutagenicity and antimutagenicity of A. crassiflora methanolic extract (ACME). Groups: negative control = distilled water 120 h; MMS = aqueous solution of methyl methanesulfonate; pre-treatment (48 h ACME + 48 h MMS), simultaneous simple (48 h distilled water + 48 h of association of ACME and MMS), simultaneous with preincubation (48 h distilled water + 48 h MMS with ACME pre-incubated for 1 h at 37 °C in the oven), post-treatment (48 h MMS + 48 h ACME), continuous (48 h ACME + 48 h MMS) without washing the seeds.









During the analysis, the cells were classified into interphase, prophase, metaphase, anaphase, and telophase. For each of these phases, the number of chromosomal alterations was quantified, and the presence of micronuclei was evaluated.

To obtain the mitotic index (MI), the number of dividing cells (prophase, metaphase, anaphase, and telophase) was divided by the total number of analyzed cells:

$$MI = dividing cells/total analyzed cells$$

The calculation of the reduction percentage in the mitotic index (%RDMI), as suggested by Fiskesjö (1993), was calculated using the following formula:

$$\% RDMI = \frac{(MI \text{ negative control} - MI \text{ protocols}) \times 100}{MI \text{ negative control}}$$

The percentage of damage reduction (DR%), as suggested by Manoharan and Banerjee (1985) and Waters et al. (1990), was obtained by the following calculation:

$$X = \frac{\begin{pmatrix} \text{average of positive control} \\ -\text{average associated group} \end{pmatrix}}{\begin{pmatrix} \text{average of positive control} \\ -\text{average of negative control} \end{pmatrix}} \times 100$$

The statistical analysis was performed using the Chi-square test, and differences were considered to be statistically significant if p < 0.05.

#### Experimental design - Swiss mouse

Thirty Swiss female mice (n = 5/group) of reproductive age were used. The mice were obtained from the Animal Facility of the Center for Biological and Health Sciences (Centro de Ciências Biológicas e da Saúde, CCBS) located at the Federal University of Mato Grosso do Sul (Universidade Federal do Mato Grosso do Sul, UFMS), Campo Grande, MS, Brazil.

Figure 2. Experimental design in vivo.

Cyclophosphamide



The animals were maintained in propylene boxes covered with wood shavings and fed a commercial diet (Nutrivital<sup>®</sup>) and filtered water *ad libitum*. The temperature and light were controlled using 12h photoperiods (12h of light/dark) with a temperature of  $22 \pm 2$  °C and a humidity of  $55\% \pm 10$  in a ventilated AESLO<sup>®</sup> shelf (ESLO, Frankfurt, Germany). The experiment was conducted according to the standards of Brazil's National Council for the Control of Animal Experimentação Animal – CONCEA) and with the approval of the Ethics Committee on Animal Use (CEUA/UFMS) under protocol no. 311/2011.

The animals were treated and the collections of biological material were performed according to the following protocol (Figure 2):

*Control group*: On the first and second days, the animals received 0.1 mL/10 g (c.w.; oral gavage) 1% methanol solution and then 0.1 mL/10 g (c.w.; i.p.) saline solution.

*Cyclophosphamide group*: The animals were administered saline solution and 1% methanol solution on the first day and 1% methanol solution and cyclophosphamide on the second day.

Annona crassiflora methanolic extract: The animals were administered 1% methanol solution and saline solution on the first day and ACME and saline solution on the second day.

*Pre-treatment*: The animals received the ACME and saline solution on the first day and 1% methanol solution and cyclophosphamide on the second day.

*Simultaneous*: The animals were administered 1% methanol solution and saline solution on the first day and ACME and cyclophosphamide on the second day.

*Post-treatment*: The animals received 1% methanol solution and cyclophosphamide on the first day and ACME and saline solution on the second day.

The collection of peripheral blood was performed by puncture of the tail vein 24 h after the administration of the test compounds on the second day.

#### Micronucleus test in peripheral blood

The protocol described by Hayashi et al. (1990) was used with some modifications (Oliveira et al., 2009a). First,  $40 \,\mu\text{L}$  of peripheral blood were deposited on a pre-coated slide with  $20 \,\mu\text{L}$  of acridine orange (1.0 mg/mL). A coverslip was then

placed over the biological material, and the slide was placed in a freezer  $(-20 \,^{\circ}\text{C})$  for a minimum period of 7 d. The material was evaluated using a fluorescence microscope (Motic<sup>®</sup>, BA410FL, Microscopes Plus Ltd., Hertfordshire, UK) at 400× magnification with a 420–490 nm excitation filter and a 520 nm barrier filter.

Two thousand cells/animal were analyzed, and the statistical analysis was performed using ANOVA/Tukey's test (p < 0.05). The percentage of damage reduction was calculated according to the previous description.

#### Results

#### Phytochemistry

The ACME exhibited a high content of flavonoids in 427.30 mg of quercetin/g of sample. The fractionation of the ACME leaves for the identification of possible flavonoids resulted in the isolation of kaempferol 3-O- $\beta$ -glucoside and kaempferol 3-O- $\beta$ -diglucoside. The structures of the isolated compounds were elucidated based on the spectroscopic data analysis obtained through <sup>1</sup>H and <sup>13</sup>C NMR and DEPT, COSY, HMQC, and HMBC experiments.

## Evaluation of anti-inflammatory and antiallodynic activities

## Effects of oral ACME administration on carrageenan-induced paw edema

The injection of carrageenan into the paw induced edema starting 30 min after administration and peaking 2 h after administration (Figure 3). Oral treatment with ACME (100 and 300 mg/kg) significantly inhibited the carrageenan-induced edema formation. The inhibition percentages achieved with ACME doses of 100 and 300 mg/kg were  $53 \pm 7\%$  and  $47 \pm 10\%$ , respectively (Figure 3). In addition, the inhibition observed in the dexamethasone-treated group 2 h after the injection of carrageenan into the paw was  $44 \pm 9\%$  (Figure 3A).

The injection of carrageenan ( $300 \mu g/paw$ ) increased MPO activity 6 h after administration; therefore, oral treatment with ACME (100 and 300 mg/kg) altered the increase in MPO activity induced by carrageenan with inhibition percentages of  $60 \pm 7\%$  and  $63 \pm 7\%$ , respectively. The positive control (dexamethasone) was able to induce the inhibition of MPO activity compared with the control group (Figure 3B) with a reduction of  $67 \pm 5\%$ .

#### *Effects of ACME on carrageenan-induced pleural cell migration and protein exudation*

The results are expressed as leukocytes  $\times 10^6$ , and the plasma leakage results are shown as mg of proteins/mL (Figure 4). The administration of carrageenan into the pleural cavity of the animals induced an increase in the total leukocyte numbers (Figure 4A) and an increase in protein extravasation (Figure 4B) 4 h after injection. ACME significantly reduced the increase in total leukocytes (inhibition of  $78 \pm 9\%$  with 100 mg/kg and  $90 \pm 7\%$  with 300 mg/kg) and the increase in protein levels (approximately 100% inhibition with both doses) induced by the administration of carrageenan into the



Figure 3. Effect of oral administration of ACME on carrageenaninduced paw edema in mice. Animal received ACME (30, 100, or 300 mg/kg, p.o.), dexamethasone (DEX – 1 mg/kg, s.c.) or vehicle and after 1 h, an intraplantar injection of carrageenan (300 µg/paw) was performed. In (A), the inhibition induced by 30, 100, and 300 mg/kg of ACME and DEX in paw edema (µm) at 4 h after carrageenan injection is shown. In (B), bars show the effect of different doses of 30, 100, and 300 mg/kg of ACME and DEX in increasing of myeloperoxidase (MPO) activity at 6 h after carrageenan injection is shown ACME and DEX. The bars express the mean ± SE of five animals, compared with the vehicle (V) versus the treated group. \*p < 0.05, \*\*p < 0.01, one-way ANOVA followed by the Student–Newman–Keuls test.

pleural cavity (Figure 4A). The subcutaneous administration of dexamethasone (1.0 mg/kg, s.c.) 1 h before the administration of carrageenan into the pleural cavity also reduced the increases in total leukocytes (inhibition of  $93 \pm 4\%$ ) and protein levels (100%) induced by carrageenan.

#### *Effect of ACME on carrageenan-induced leukocyte migration into the pouch*

The injection of carrageenan into the pouch of sixth-day-old mice promoted plasma leakage (p < 0.001) and the migration of leukocytes, particularly polymorphonuclear leukocytes (Figure 5). The analysis of variance showed significant differences in the levels of leukocyte migration between the groups. The analysis of the inflammatory activity of ACME (100–300 mg/kg) indicated that this extract inhibited leukocyte migration only when administered at a dose of 300 mg/kg, which resulted in  $43 \pm 5\%$  inhibition. Dexamethasone, which was used as a positive control, reduced leukocyte infiltration into the pouch by 100% 4 h after carrageenan injection (Figure 5).



Figure 4. Effects of ACME on total leukocytes (A) and protein extravasation (B) induced by carrageenan in the pleural cavity of mice. Animal received the oral treatment with ACME (100 or 300 mg/kg), or vehicle, and after 1 h, they received an intrapleural injection of Cg (100 µL of a 1% solution/cavity). Control animals received only the vehicles and Naïve animal did not received carrageenan or ACME treatment. Animals were killed after Cg injection. The bars express the mean ± SEM of five animals, compared with the vehicle (V) versus the treated group. \*\*p < 0.01, \*\*\*p < 0.001, one-way ANOVA followed by the Student–Newman–Keuls test. #p < 0.001.

## *Effects of ACME on zymosan-induced articular inflammatory response*

According to previous results (Conte et al., 2008), zymosan injection induces a significant articular inflammatory response characterized by marked articular edema formation with a six-fold increase in the knee diameter of the control group compared with the naïve group (Penido et al., 2006). The results showed that pre-treatment with ACME (in both doses tested) failed to reduce the zymosan-induced edema formation, whereas the subcutaneous treatment with dexamethasone inhibited edema formation by  $59 \pm 7\%$ .

## Effects of ACME on carrageenan-induced mechanical allodynia

The intraplantar injection of  $20 \,\mu\text{L}$  of carrageenan (300  $\mu$ g/paw) induced a reduction of  $98 \pm 1\%$  in the nociceptive threshold level. The carrageenan-induced mechanical allodynia peaked within 3–4 h after injection. The oral administration of ACME (both doses: 100 and 300 mg/kg) did not inhibit the carrageenan-induced mechanical allodynia.

#### Toxicogenetic and chemopreventive evaluations

#### Allium cepa test

The *Allium cepa* test showed that the ACME is not mutagenic and exhibits significant antimutagenic activity. The damage



Figure 5. Effect of ACME on carrageenan-induced leukocyte migration and plasma leakage into the air pouch. Mice (n = 5) were pretreated 1 h before with ACME (10–300 mg/kg p.o.), dexamethasone (1 mg/kg, s.c., diluted in saline) or vehicle. Pouches were washed with PBS-containing heparin. Cells were counted and plasma leakage was analyzed. Results are expressed as cell 10<sup>6</sup>/cavity. \*\*p < 0.01, \*\*\*p < 0.001, #p < 0.001, compared with the vehicle group. Difference between groups were analyzed by analysis of variance (one-way ANOVA) followed by the Newman–Keuls test.

reduction percentages obtained with concentrations of 5, 10, and 15 mg/L were 66.17, 75.75, and 69.19% for the pre-treatment, 72.72, 33.33, and 22.22% for the simple simultaneous treatment, 100.50, 93.93, and 102.52% for the simultaneous treatment with pre-incubation, 89.39, 79.79, and 84.34% for the post-treatment, and 86.36, 81.31, and 93.43% for the continuous treatment (Table 1).

The mitotic indexes for the control and MMS groups were 3.70 and 3.57, respectively. The evaluation of the mutagenicity of ACME revealed mitotic indexes of 3.76, 4.12, and 4.79 for doses of 5, 10, and 15 mg/L, respectively. Soon after the administration, a small reduction percentage in the mitotic index (3.51%) obtained for MMS was obtained in comparison with the control. For ACME, the reduction percentages in the mitotic index were -1.61, -11.35, and -29.46% for concentrations of 5, 10, and 15 mg/L, respectively (Table 1).

The antimutagenicity evaluations revealed that the only protocol that resulted in a lower MI compared with the control was the pretreatment protocol with a concentration of 15 mg/L. However, the dose of 5 mg/L resulted in an improvement in the mitotic index for the pre-treatment, simultaneous with pre-incubation, post-treatment, and continuous protocols; the respective mitotic indexes were 4.86, 5.36, 3.79, 5.86, and 5.07, respectively, and the reduction percentages in the mitotic index ranged from -2.43 to -58.38% (Table 1).

With the concentration of 10 mg/L, all the protocols showed improvements in the mitotic index. For the pre-treatment, simple simultaneous, simultaneous with pre-incubation, post-treatment, and continuous protocols, the mitotic indexes were 6.07, 4.29, 5.02, 4.49, and 5.03, and the reduction percentages in the mitotic index were -64.05, -15.95, -35.68, -21.35, and -35.95%, respectively (Table 1).

At a concentration of 15 mg/L, only the pre-treatment protocol showed a reduced mitotic index value of 2.00, and the reduction percentage in the mitotic index was 45.95%. For the simple simultaneous, simultaneous with pre-incubation, post-treatment, and continuous treatment protocols, the mitotic indices were 4.58, 4.31, 5.06, and 5.35, and the

Table 1. Distribution of chromosomal aberrations, MI, MIDR, and DR% in Allium cepa treatments associated to A. crassiflora methanolic extract.

		Chromosomal aberrations									
Treatment	DRMI%	MI	MN	BRI	BRE	LO	SPR	DLA	MA	Total	DR%
Mutagenicity											
Control	_	3.70	19	17	0	0	0	4	0	40	-
MMS	3.51	3.57	204	16	2	5	2	9	0	$238^{\mathrm{a}}$	-
A. crassiflora 5 mg/L	-1.62	3.76	13	11	0	1	0	6	0	31 <sup>a</sup>	-
A. crassiflora 10 mg/L	-11.35	4.12	14	10	0	0	0	2	1	27 <sup>a</sup>	-
A. crassiflora 15 mg/L	-29.46	4.79	24	8	1	1	0	0	0	34 <sup>a</sup>	-
Antimutagenicity											
5 mg/L											
Pre-treatment	-31.35	4.86	322	24	6	4	1	12	0	369 <sup>b</sup> *	-66.17
Simple simultaneous	-44.86	5.36	63	20	1	4	1	5	0	94 <sup>b</sup> *	72.72
Simultaneous with pre-incubation	-2.43	3.79	21	14	0	0	0	4	0	39 <sup>b</sup> *	100.5
Post-treatment	-58.38	5.86	37	20	0	0	0	4	0	61 <sup>b</sup> *	89.39
Continuous	-37.03	5.07	36	18	0	1	0	13	0	68 <sup>b</sup> *	86.36
10 mg/L											
Pre-treatment	-64.05	6.07	52	26	0	1	1	8	0	82 <sup>b</sup> *	75.75
Simple simultaneous	-15.95	4.29	151	15	3	1	0	2	0	172 <sup>b</sup> *	33.33
Simultaneous with pre-incubation	-35.68	5.02	19	26	0	2	0	5	0	52 <sup>b</sup> *	93.93
Post-treatment	-21.35	4.49	58	14	0	1	1	6	0	$80^{b_{*}}$	79.79
Continuous	-35.95	5.03	47	22	0	1	0	7	0	77 <sup>b</sup> *	81.31
15 mg/L											
Pre-treatment	45.95	2.00	82	4	7	2	3	3	0	101 <sup>b</sup> *	69.19
Simple simultaneous	-23.78	4.58	148	33	2	3	2	6	0	194 <sup>b</sup> *	22.22
Simultaneous with pre-incubation	-16.49	4.31	10	22	0	0	1	2	0	35 <sup>b</sup> *	102.52
Post-treatment	-36.76	5.06	38	26	0	0	3	4	0	71 <sup>b</sup> *	84.34
Continuous	-44.59	5.35	33	15	1	4	0	0	0	53 <sup>b</sup> *	93.43

DRMI%, damage reduction percentages of the mitotic index; MI, mitotic index; DR%, damage reduction percentages; MN, micronucleus; BRI, bridge; BRE, break; LO, loss; SPR, sprout; DLA, delay; MAN, multipolar anaphase. MMS, methyl methanesulfonate. Experimental groups: control = distilled water 96 h; MMS = aqueous solution of MMS; pre-treatment (48 h *A. crassiflora* + 48 h MMS), simultaneous simple (48 h distilled water + 48 h of association of *A. crassiflora* and MMS), simultaneous with pre-incubation (48 h distilled water + 48 h MMS with *A. crassiflora* pre-incubated for 1 h at 37 °C in the oven), post-treatment (48 h MMS + 48 h *A. crassiflora*), continuous (48 h *A. crassiflora* + 48 h MMS without change the seeds). \*Statistically significant differences (Chi-square test, p < 0.05).

<sup>a</sup>Values compared with the control group.

<sup>b</sup>Values compared with the MMS group.

Table 2. Initial, final, and body weight gain of the animals; absolute and relative weights of the organs of animals treated with A. crassiflora methanolic extract and/or cyclophosphamide (mean  $\pm$  S.E.).

	Experimental groups						
Parameters	Control	Cycplophosphamide	ACME	Pre-treatment	Simultaneous	Post-treatment	
Initial (g)	$32.49 \pm 1.59^{a}$	$31.43 \pm 2.22^{a}$	$28.91 \pm 1.46^{a}$	$28.17 \pm 1.59^{a}$	$29.09 \pm 0.91^{a}$	$28.13 \pm 1.53^{a}$	
Final (g)	$32.44 \pm 2.56^{a}$	$31.56 \pm 1.46^{a}$	$28.79 \pm 1.29^{a}$	$27.68 \pm 1.57^{a}$	$28.97 \pm 0.58^{a}$	$27.50 \pm 1.21^{a}$	
Liquid weight gain (g)	$1.60 \pm 0.86^{a}$	$0.52 \pm 2.15^{a}$	$0.12 \pm 0.59^{a}$	$0.49 \pm 2.11^{a}$	$0.11 \pm 0.69^{a}$	$0.63 \pm 0.40^{a}$	
Absolut weight of organ	s (g)						
Lungs	$0.1939 \pm 0.0127^{a}$	$0.2175 \pm 0.0284^{a}$	$0.1762 \pm 0.0135^{a}$	$0.1864 \pm 0.0078^{a}$	$0.1862 \pm 0.0140^{a}$	$0.2057 \pm 0.0098^{a}$	
Heart	$0.1842 \pm 0.0098^{a}$	$0.1859 \pm 0.0161^{a}$	$0.1695 \pm 0.0129^{a}$	$0.1426 \pm 0.0093^{a}$	$0.1826 \pm 0.0142^{a}$	$0.1472 \pm 0.0070^{a}$	
Liver	$1.6591 \pm 0.1489^{a}$	$1.6818 \pm 0.1410^{a}$	$1.3960 \pm 0.0689^{a}$	$1.3633 \pm 0.0736^{a}$	$1.6277 \pm 0.0997^{a}$	$1.3961 \pm 0.0586^{a}$	
Spleen	$0.1644 \pm 0.0290^{a}$	$0.1148 \pm 0.0141^{a}$	$0.1325 \pm 0.0079^{a}$	$0.1051 \pm 0.0112^{a}$	$0.1697 \pm 0.0629^{a}$	$0.0889 \pm 0.0034^{\rm a}$	
Kidneys	$0.3827 \pm 0.0150^{a}$	$0.3602 \pm 0.0258^{a}$	$0.3351 \pm 0.0134^{a}$	$0.3310 \pm 0.0196^{a}$	$0.3307 \pm 0.0148^{a}$	$0.3223 \pm 0.0114^{a}$	
Relative weight of organ	s (g)						
Lungs	$0.0061 \pm 0.0006^{a}$	$0.0068 \pm 0.0006^{a}$	$0.0060 \pm 0.0003^{a}$	$0.0068 \pm 0.0003^{a}$	$0.0064 \pm 0.0004^{a}$	$0.0076 \pm 0.0004^{\rm a}$	
Heart	$0.0058 \pm 0.0003^{a}$	$0.0058 \pm 0.0003^{a}$	$0.0058 \pm 0.0002^{a}$	$0.0051 \pm 0.0002^{a}$	$0.0063 \pm 0.0004^{a}$	$0.0054 \pm 0.0002^{a}$	
Liver	$0.0513 \pm 0.0025^{a}$	$0.0528 \pm 0.0022^{a}$	$0.0479 \pm 0.0016^{a}$	$0.0493 \pm 0.0008^{a}$	$0.0561 \pm 0.0031^{a}$	$0.0508 \pm 0.0013^{a}$	
Spleen	$0.0050 \pm 0.0008^{a}$	$0.0035 \pm 0.0003^{a}$	$0.0045 \pm 0.0002^{a}$	$0.0038 \pm 0.0002^{a}$	$0.0060 \pm 0.0024^{a}$	$0.0033 \pm 0.0002^{a}$	
Kidneys	$0.0120 \pm 0.0005^{a}$	$0.0114 \pm 0.0004^{a}$	$0.0114 \pm 0.0004^{a}$	$0.0120 \pm 0.0002^{a}$	$0.0114 \pm 0.0004^{a}$	$0.0118 \pm 0.0003^{\mathrm{a}}$	

ACME Annona crassiflora methanolic extract; g, grams.

<sup>a</sup>Values compared with the control group (Chi-square test, p < 0.05).

reduction percentages in the mitotic indices were -23.78, -16.49, -36.76, and -44.59%, respectively. These data show that the ACME favors cell division (Table 1).

#### Swiss mouse test

The evaluation of the initial weight, final weight, and weight gain of the experimental animals, as well as the assessment of the absolute and relative weight of the lungs, heart, liver, spleen, and kidneys, showed no statistically significant differences (Table 2).

The ACME was not mutagenic, as determined through the mouse test. The antimutagenic evaluation showed damage reduction percentages of 75.00 and 64.58% for the pretreatment and simultaneous protocols, respectively.

Table 3. Total frequency, frequency mean  $\pm$  S.E., percentage of damage reduction related to tests for mutagenicity and antimutagenicity in micronucleus test in peripheral blood of Swiss female mice.

Experimentals groups	MN frequency	Mean $\pm$ SE	DR%	
Mutagenicity				
Control	38	$7.60 + 1.03^{a}$	_	
Cyclophosphamide	134	$26.80 \pm 1.83^{b}$	_	
ACME	55	$11.00 \pm 1.38^{a}$	_	
Antimutagenicity				
Pre-treatment	62	$12.40 \pm 1.50^{a}$	75.00	
Simultaneous	72	$14.40 \pm 0.98^{a}$	64.58	
Post-treatment	178	$35.60 \pm 2.34^{\circ}$	-45.83	

MN, micronucleus. S.E., standard error of mean. DR%, percentage of damage reduction. Different letters mean significant differences (Chi-square test, p < 0.05).

The post-treatment protocol exhibited a potentiation of the cyclophosphamide effects by 45.83% (Table 3).

#### Discussion

The results of the present study confirmed and also largely extended the previous findings by demonstrating that the *Annona crassiflora* extract exhibits important chemoprevention and antimutagenic activities also when systemically administered. Furthermore, the methanolic extract was found to effectively interfere with the inflammatory response induced by carrageenan, as demonstrated by the effects associated with paw inflammation, myeloperoxidase activity, pleural cell migration, protein exudation, cell migration into the pouch, and induction of joint inflammation. The present findings also provide the first demonstration of the antiinflammatory action of the methanolic extract and provide some insights into its inability to induce mutagenesis and exhibit antimutagenic activity.

With respect to the extract and the identified compounds of *A. crassiflora*, only a few studies have investigated their antibacterial, antileishmanial, trypanocidal, larvicidal, molluscicidal, genotoxicity, mutagenic/antimutagenic, and antispasmodic activity (De Omena et al., 2007; Dos Santos & Sant'Ana, 2001; Mesquita et al., 2005; Rodrigues et al., 2006; Takahashi et al., 2006; Weinberg et al., 1993).

The pharmacological pre-clinical studies performed with species, which belong to family Annonaceae, genus Annona, notably A. senegalensis Pers., A. reticulata L., A. muricata L., A. sylvatica A. St.-Hil., and A. dioica A. St.-Hil., have confirmed their folk medicinal properties for the treatment of inflammatory disease (Chavan et al., 2010, 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2013a,b; Yeo et al., 2011). Previous studies performed by our research group as well as others have shown that the extract, essential oil, and identified compounds obtained from the leaves and bark of Annona species exert anti-inflammatory and antinociceptive effects (Chavan et al., 2010, 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2011, 2012a,b; De Sousa et al., 2010; Formagio et al., 2013a,b; Yeo et al., 2011).

Studies conducted by our group have shown that the methanolic extract of *A. sylvatica* and the essential oil of *A. dioica* inhibit inflammatory parameters, such as edema, myeloperoxidase activity, and leukocyte migration (Formagio et al., 2013a,b). To the best of our knowledge, the present

study provides the first demonstration of the ability of the ACME, which was administered at doses in the range of 30–300 mg/kg through the oral route, to inhibit important inflammatory parameters, such as edema formation, paw inflammation, myeloperoxidase activity, pleural cell migration, protein exudation, and cell migration into the pouch. However, the nature of the active principles responsible for these actions is unclear.

Several models of experimental inflammation have been described and are used to validate natural and synthetic products or compounds. The common substance used is carrageenan, and this compound can be administered in the paw, pleura, and artificial pouches. In the carrageenan-paw, -pleural, and -pouch inflammation models, the oral administration of ACME showed similar efficacy to that observed with the subcutaneous administration of dexamethasone. In the zymosan-induced articular inflammatory model only, dexamethasone and not oral ACME induced efficacy compared with the control group. Most likely the mechanism of action is different from that of steroidal anti-inflammatory drugs and from that of nonsteroidal anti-inflammatory drugs (NSAID) because ACME did not alter the mechanical allodynia induced by carrageenan. Because the compounds contained in ACME are most likely original, and the structural diversity found in the nature could not be discussed in this step of our study, the mechanisms of anti-inflammatory action will be studied in the future.

The chronic inflammatory process obviously contributes to tissue damage and likely DNA damage, as well as a large number of inflammatory diseases. Thus, natural or synthetic agents that inhibits mutagenic and inflammatory process most likely acts through different mechanisms of action. In an attempt to evaluate the effects of the extract in both pathological alterations, there is a necessity to assess different doses for both situations. ACME was shown to be an effective anti-inflammatory agent in doses ranging from 100 to 300 mg/kg, whereas it exhibited chemopreventive effects at a dose of 15 mg/kg. Aspirin and other similar NSAIDS showed anti-inflammatory effects at a dose of 300 mg/kg (Menezes-de-Lima et al., 2006), whereas aspirin at doses of 0.5-50 mg/kg is required for micronucleus tests with peripheral blood (Niikawa et al., 2001). Thus, the doses that showed anti-inflammation effects and the induction of chemoprevention are equivalent to those found in the literature.

The present study demonstrated that the ACME has no mutagenic activity in either of the experimental models used: *Allium cepa* and mouse test. This finding was corroborated by Dragano et al. (2010) and Vilar et al. (2008), who demonstrated that neither the pulp nor the ethanol extract of *A. crassiflora* is mutagenic in mice.

With respect to antimutagenicity, the studies are contradictory for *A. crassiflora* and do not describe its antimutagenic more of action. To elucidate it, the literature suggests the use of several protocols. In particular, we used the pre-treatment, simple simultaneous, simultaneous with pre-incubation, posttreatment, and continuous protocols for the *Allium cepa* test and the pre-treatment, simultaneous, and post-treatment protocols for the test in mice. The simple simultaneous and pretreatment protocols indicate both desmutagenic and bioantimutagenic activity, the simultaneous with pre-incubation protocol indicates desmutagenic activity, and the posttreatment protocol preferentially indicates bio-antimutagenic activity (Antunes & Araújo, 2000; De Flora & Ferguson, 2005; Fedel-Miyasato et al., 2014; Felicidade et al., 2014; Ferguson, 1994; Flagg et al., 1995; Oliveira et al., 2006, 2007, 2009b, 2013a,b, 2014; Mauro et al., 2014, 2013; Pesarini et al., 2013). The desmutagenic substances chemically or enzymatically inactivate the mutagen in the extra or intracellular medium (De Flora, 1998; Ferguson, 1994; Hartman & Shankel, 1990; Kada & Shimoi, 1987; Oliveira et al., 2006, 2007). Bioantimutagenic agents are those that modulate the repair and replication of DNA and act at the cellular level by increasing the replication fidelity, stimulating error-free repair, or inhibiting repair systems subject to error (De Flora, 1998; Hartman & Shankel, 1990; Simic et al., 1998).

According to the Allium cepa test, the ACME can exert both desmutagenesis and bio-antimutagenesis activities. This assertion is supported by the high percentage of damage reduction observed with the simultaneous treatment with preincubation and post-treatment protocols, which ranged from 93.93 to 102.52% and 79.79 to 89.39%, respectively. In the test with mice, the damage reduction percentages did not confirm the mode of action because the damage reduction percentage obtained with the post-treatment protocol was -45.83%. Thus, it appears that the ACME potentiated the cyclophosphamide effects obtained with this protocol, and the rate of damage increase was approximately 45.83%. This same effect was described by Dragano et al. (2010), who reported an increased rate of micronuclei in the order of 2.32, 61.63, and 60.46% (the calculations were performed by these researchers based on data presented by the study in question) in mice supplemented with 1, 10, and 20% A. crassiflora pulp, respectively.

Another finding that stands out in the *Allium cepa* data is that the pre-treatment protocol with the smallest dose exhibited an increase in the micronuclei rate of 66.17%. However, this increase was not observed for the same protocol with the other doses. In the test with mice, this protocol showed the best damage reduction percentage (75.00%).

With respect to the potentiation of the effects of the alkylating agent, namely cyclophosphamide, Dragano et al. (2010) and Souza et al. (2012) suggest that these effects occur due to the presence of reducing agents, such as ascorbic acid, in *A. crassiflora*. Therefore, it is hypothesized that this compound increases the conversion of cyclophosphamide to its active metabolites, thereby increasing the frequency of genomic lesions (micronuclei).

For the simple simultaneous and continuous treatment protocols, through which both desmutagenesis and bioantimutagenesis can occur, an initial analysis is expected to reveal a sum of the modes of action. However, this was not verified with the *Allium cepa* test because the damage reduction percentages do not supplant those observed for the simultaneous with pre-incubation treatment protocol, which indicated that only desmutagenesis activity is observed. The evaluation of the simultaneous treatment protocol in mice revealed an intermediate damage reduction percentage, which reinforces the findings obtained with the *Allium cepa* test. According to Rank and Nielsen (1997) and Fedel-Miyasato et al. (2014), a comparison of the mutagenicity assays in *Allium cepa* with tests in rodents shows a concordance of approximately 80%, and therefore allows the confirmation of the antimutagenic mode of action. However, the data obtained in this study do not support this assertion because the post-treatment and simultaneous protocols differ by 130.17 percentage points and 42 percentage points between both modes. Additionally, if analyzed separately, the two models do not indicate the same mode of action mainly due to the inactivity of the post-treatment in the mice model.

The difference in the results between the *Allium cepa* test and the test in mice can be explained by the differences in the metabolisms of the two organisms. The cytochrome P450 enzyme is responsible for 50% of the metabolism of therapeutic agents, and the comparison of the presence of this enzyme complex leads to the conclusion that plants have a low concentration compared with mammals (Leme & Marin-Morales, 2009). Because of this increased metabolism in mammals, it can be hypothesized that the ACME was metabolized more efficiently in mice than in plant, thereby preventing the modulation of the action of enzymes by sufficient amounts of the ACME, which explains the lack of bio-antimutagenic action obtained with the post-treatment protocol.

Fiskesjö (1993) stated that the mitotic index of apical meristems of *Allium cepa* is considered normal compared with that of chemicals when there is an inhibition of cell division by up to 36%. This researcher also reports that a reduction in mitosis to values greater than 50% reveals that the substance under analysis is toxic to meristematic cells. These findings suggest that the ACME is not toxic to the meristematic culture and that it can improve the mitotic index when associated with MMS, which indicates that the ACME has the ability to modulate events to decrease the toxicity of the alkylating agent.

Based on the reference values reported by Fiskesjö (1993), it is understood that the mitotic index measured through the mutagenicity protocols demonstrates that the ACME has no genotoxic activity. The reduction percentages in the mitotic index were -1.62, -11.35, and -29.46% for concentrations of 5, 10, and 15 mg/L, respectively. Increases in the concentrations resulted in increases in the mitotic index. This finding corroborates the assertion that the substance is not mutagenic even at higher concentrations.

With respect to the antimutagenicity protocols, only the pre-treatment protocol with a concentration of 15 mg/L showed a lower mitotic index with a high rate of decrease in this index (45.95%). It is understood that this protocol (with this concentration) has not proven to be efficient at expressively reducing the toxicity caused by the MMS damage-inducing agent. The other protocols and treatments (at all concentrations) resulted in an increase in the mitotic index, which suggests that ACME is able to reverse the toxicity inducted in cultures by MMS treatment.

Based on the findings, the ACME is considered to have anti-inflammatory therapeutic potential because it is capable of inhibiting paw edema, reducing myeloperoxidase activity, reducing the total leukocyte count and protein extravasation in the pleural cavity, and reducing leukocyte migration and

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plasma extravasation in the air pouch assay, all of which are effects induced by carrageenan administration. In addition, this extract is not mutagenic in plant and animal models. Moreover, although no correspondence was found between the plant and animal models, as would be expected based on the literature, the ACME has chemopreventive potential and may act mainly through desmutagenesis in mammals. Thus, it is suggested that this medicinal plant has anti-inflammatory and chemopreventive therapeutic potential and does not induce toxicogenetic damage.

#### **Declaration of interest**

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