

## ORIGINAL ARTICLE

# *Caryocar villosum* attenuates inflammation by inhibiting CXCL1 activation and peripheral hyperalgesia through opioid pathway modulation

Gutemberg Lopes SOARES<sup>1</sup>, Lívia Christine Ribeiro HILGENBERG<sup>1</sup>, Giovana Reis BATISTA<sup>1</sup>, Matheus Kleber de Jesus SOUZA<sup>1</sup>, Renata Cristina Mendes FERREIRA<sup>2</sup>, Irismara Sousa SILVA<sup>2</sup>, Tallita Marques MACHADO<sup>1</sup>, Carlos Cleomir de Souza PINHEIRO<sup>3</sup>, Fernanda GUILHON-SIMPLICIO<sup>1</sup>, Andre KLEIN<sup>2</sup>, Andrea de Castro PEREZ<sup>2</sup>, Emerson Silva LIMA<sup>1</sup>, Clarice de Carvalho Veloso MOURA<sup>1\*</sup> 

<sup>1</sup> Universidade Federal do Amazonas, Faculdade de Ciências Farmacêuticas, Manaus, Amazonas, Brazil

<sup>2</sup> Universidade Federal de Minas Gerais, Departamento de Farmacologia, Belo Horizonte, Minas Gerais, Brazil

<sup>3</sup> Instituto Nacional de Pesquisas da Amazônia - INPA, Manaus, Amazonas, Brazil

\* Corresponding author: clariceveloso@ufam.edu.br;  <https://orcid.org/0000-0002-3944-2442>

## ABSTRACT

The oil of *Caryocar villosum* is used in Amazonian folk medicine to treat pain and inflammatory conditions. So, we assessed the anti-inflammatory and antinociceptive properties of the ethanolic extract obtained from the fruit peels of this species. The acetic acid-induced writhing, carrageenan-induced mechanical hyperalgesia, formalin, carrageenan-induced paw edema and carrageenan-induced peritonitis tests were used on mice. The *C. villosum* ethanolic extract significantly inhibited the number of abdominal writhes, mechanical hyperalgesia and paw licking time in the second phase of the formalin test. At a dose of 300 mg kg<sup>-1</sup>, the extract also significantly reduced the volume of edema formed in the late phase and reduced the recruitment of leukocytes and neutrophils in the peritoneal cavity, as well as CXCL1 chemokine levels. It is suggested that the extract attenuates the leukocyte recruitment by inhibiting the CXCL1 activation. The peripheral antinociceptive activity occurred through opioid pathway modulation because pretreatment with *C. villosum* ethanolic extract reversed the naltrexone-induced antinociception.

**KEYWORDS:** pain, antinociception, chemokine, leukocyte recruitment

## *Caryocar villosum* atenua a inflamação ao inibir a ativação de CXCL1 e a hiperalgesia periférica por meio da modulação da via opioide

### RESUMO

O óleo de *Caryocar villosum* é usado na medicina popular amazônica para tratar dores e condições inflamatórias. Assim, avaliamos as propriedades antiinflamatórias e antinociceptivas do extrato etanólico obtido das cascas dos frutos desta espécie. Os testes de contorções induzidas por ácido acético, hiperalgesia mecânica induzida por carragenina, formalina, edema de pata induzido por carragenina e peritonite induzida por carragenina foram usados em camundongos. O extrato etanólico de *C. villosum* obtido das cascas dos frutos inibiu significativamente o número de contorções abdominais, a hiperalgesia mecânica e o tempo de lambida da pata na segunda fase do teste de formalina. Na dose de 300 mg kg<sup>-1</sup>, o extrato também reduziu significativamente o volume de edema formado na fase tardia e reduziu o recrutamento de leucócitos e neutrófilos na cavidade peritoneal, bem como os níveis de quimiocina CXCL1. Sugere-se que o extrato atenua o recrutamento de leucócitos por meio da inibição da ativação de CXCL1. A atividade antinociceptiva periférica ocorre por meio da modulação da via opioide pois o pré-tratamento com o extrato etanólico de *C. villosum* reverteu a antinocicepção induzida pela naltrexona.

**PALAVRAS-CHAVE:** dor, antinocicepção, quimiocina, recrutamento de leucócitos

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## INTRODUCTION

The use of medicines obtained from natural products is an alternative for the treatment of several inflammatory and painful conditions. In this context, *Caryocar villosum* (Aubl.) Pers. (Caryocaraceae), popularly known in northern Brazil as *piquiá*, stands out. This tree species reaches up to 50 m in height and is found in the Amazon rainforest, mainly in the regions close to estuaries, and also in part of the northeastern region of Brazil (Magig et al. 2006; Morais and Gutjahr 2012).

Great economic importance is attributed to *C. villosum*, since many of its constituents are used in the cosmetic, food, culinary and shipbuilding industries (Valente 2012; Chisté et al. 2014). In popular medicine, *C. villosum* oil is used in northeastern Brazil for the treatment of fungal infections, such as dermatophytosis, *Tinea capitis* and *Tinea pedis* (Magig et al. 2006; Chisté et al. 2012). In the Amazon, the bark is used to treat *Tinea pedis*, and the oil is used as an anti-inflammatory to relieve muscle pain and rheumatism, besides the treatment of bronchitis, flu, colds and vision problems (Xavier et al. 2011; Morais and Gutjahr 2012).

The demand is high for new and more effective therapeutic alternatives for pain and inflammation management that have less adverse effects. Therefore, we evaluated the antinociceptive and anti-inflammatory activities of the ethanolic extract obtained from the fruit peels of *C. villosum* in *in vivo* assays, to assess its potential medicinal use. This is the first study to determine the systemic antinociceptive and anti-inflammatory action of *C. villosum* in preclinical models.

## MATERIAL AND METHODS

### Plant material and extraction processes

The raw materials of *C. villosum* used in this study were fruit peels (280 g) separated by mother plant. The fruit samples were collected in the Adolfo Ducke Forest Reserve (2057'48.5"S 59055'18.3"W), in Manaus, Amazonas state, Brazil, in February 2012. A voucher specimen was deposited in the herbarium of Instituto Nacional de Pesquisas da Amazônia (INPA), under registry n° 73164 and its identification was performed by Dr. Raquel Medeiros.

### Fractioned extraction

After weighing, the samples of all trees were pooled into a combined sample for the extraction process. The fruit peels were transferred to a Soxhlet extraction system and extracted with 500 mL of organic solvents, in ascending polarity order (hexane, ethyl acetate and ethanol). After extraction of each fraction, the resulting extract was subjected to vacuum filtration, dried using rotary evaporation at 45 °C and stored in a refrigerator until further analysis.

### Phytochemical screening

The phytochemical screening of the *C. villosum* ethanolic extract (CvEE) obtained from the fruit peels was carried out according to the methodologies of Matos (1997) and Simões (2010) to detect the presence of phenolic compounds, triterpenes, steroids and nitrogen. The methods used for phytochemical screening included chemical reactions and use of detector reagents in test tubes and plates, which qualitatively reveal the presence of specific substances through color change, foaming and protein precipitation.

### Electrospray ionization mass spectrometry (ESI-MS)

Mass spectra were acquired using an ion trap spectrometer (LCQ Fleet™, Thermo Scientific) equipped with an electrospray source, operating in the negative ion mode and programmed to monitor the 100-1000 m/z range. The CvEE was diluted to 5 ppm in HPLC grade methanol and applied by direct insertion through the equipment's own syringe pump. To increase the sensitivity in the tandem analysis, 4 µL of glacial acetic acid were added to 10 mL of the diluted solution, with the final concentration of acetic acid in the solvent being 0.04%. To analyze the fractions, the following operating parameters were used: spray voltage: 5 kV; sheath gas: 5 arb; aux gas: 5 arb; sweep gas: 5 arb; capillary temp: 175 °C; capillary voltage: 45 V; tube lens: 115 V; syringe pump: 10 µL min<sup>-1</sup>. The ESI-MS spectra were compared with the literature for the identification of some of the compounds present in CvEE by means of the peaks.

### Test animals

Adult male *Balb/C* mice (weighing 20-30 g) and *Swiss* mice (weighing 35-40 g) were used in the *in vivo* tests according to the sensitivity of each variety for a given pharmacological test and to the availability of animals. *Balb/C* mice were subjected to acetic acid-induced writhing, and formalin- and carrageenan-induced paw edema and carrageenan-induced peritonitis tests, while *Swiss* mice were used in rotarod and carrageenan-induced mechanical hyperalgesia tests. Each experimental group or treatment consisted of 6 animals. Before the behavioral tests, the animals were kept in groups of four mice in standard cages and housed under controlled conditions (room temperature of 22 ± 2 °C, light/dark cycle of 12:12h, and access to water and food *ad libitum*). All procedures were previously approved by the commission on ethics in animal use of INPA under protocol # 0042/2016 CEUA/INPA.

### Rotarod test

To assess the influence of CvEE on animal motor coordination, *Swiss* mice were divided into three experimental treatments (groups) (n = 6) following the methodology of Dunham and Miya (1957), with modifications. Group 1 (negative control) received 3.5% ethanol solution (vehicle)

orally at a volume of 10 mL kg<sup>-1</sup> body weight; Group 2 (positive control) received xylazine at a dose of 2 mg kg<sup>-1</sup> subcutaneously and Group 3 received the CvEE orally at a dose of 300 mg kg<sup>-1</sup>. The readings on the rotarod apparatus considered the time the animal remained on the rotating bar up to a maximum of 2 minutes and occurred before the treatment (baseline reading) and 30, 60 and 120 minutes later, with the aid of a timer.

### Acetic acid-induced writhing test

Based on Koster *et al.* (1959), *Balb/C* mice were divided into four treatments (groups; n = 6) and were orally pre-treated with 3.5% ethanol solution (vehicle) at a volume of 10 mL kg<sup>-1</sup> (Group 1; negative control); indomethacin at a dose of 10 mg kg<sup>-1</sup> (Group 2; positive control); and CvEE at doses of 100 and 300 mg kg<sup>-1</sup> (Groups 3 and 4, respectively). After one hour of pre-treatment, 0.9% acetic acid was intraperitoneally administered at a volume of 10 mL kg<sup>-1</sup>, and the animals were placed in an open space during 20 minutes in order to quantify the number of abdominal contortions, which is characteristic of nociceptive behavior.

### Carrageenan-induced mechanical hyperalgesia and opioidergic pathway involvement

Following the introductory work of Randall and Selitto (1957) and the adaptation for mice by Kawabata *et al.* (1992), *Swiss* mice (n = 6 per group) were orally pre-treated with 3.5% ethanol solution (vehicle) at a volume of 10 mL kg<sup>-1</sup> (Group 1; negative control); and CvEE at a dose of 300 mg kg<sup>-1</sup> (Group 2). After one hour of pre-treatment, hyperalgesia was induced by administration of carrageenan (200 µg/20 µL) in the right hind paw. An analgesimeter was used to determine the nociceptive threshold, defined as the weight in grams needed to cause a nociceptive response (paw withdrawal). The cut-off value of 160 g was used to avoid injury to the paws.

The readings occurred in triplicate before pretreatment (baseline reading) and in the third hour after the injection of carrageenan, which is its maximum response time (Veloso *et al.* 2018), and values were expressed as the Δ value of the nociceptive threshold, i.e., the difference between the obtained means. A Δ value of the nociceptive threshold greater than zero indicates hyperalgesic action induced by carrageenan and a decrease in the value indicates antihyperalgesic action.

To determine the involvement of the opioidergic pathway, the assay was performed a second time, with new animals divided into three groups (n = 6) and the use of naltrexone (a non-selective opioid receptor antagonist administered to assess the pharmacological involvement of the opioid system). This time Group 1 received the naltrexone vehicle (saline), intraperitoneally, 30 minutes before receiving the extract vehicle (3.5% ethanol solution) orally at a volume of 10 mL kg<sup>-1</sup> for each vehicle; Group 2 received the naltrexone vehicle (saline) intraperitoneally at a volume of 10 mL kg<sup>-1</sup> 30 minutes

before the administration of CvEE orally at a dose of 300 mg kg<sup>-1</sup>; and Group 3 received naltrexone intraperitoneally at a dose of 5 mg kg<sup>-1</sup> 30 minutes before the administration of CvEE orally at a dose of 300 mg kg<sup>-1</sup>. The readings were taken in the same manner, before and after the injection of carrageenan (as previously described) to observe whether the possible antinociceptive effect is reversed by naltrexone, a non-specific opioid receptor antagonist.

### Formalin test

In accordance with Dubuisson and Dennis (1977) and Hunskaar *et al.* (1985), *Balb/C* mice (n = 6 per group) were pre-treated and then received an injection of 2.5% formalin (30 µL paw<sup>-1</sup>) in the right posterior paw. Group 1 (negative control) received 3.5% ethanol solution (vehicle) orally at a volume of 10 mL kg<sup>-1</sup>; Group 2 (positive control) received indomethacin orally at a dose of 10 mg kg<sup>-1</sup>; Group 3 (positive control) received morphine orally at a dose of 10 mg kg<sup>-1</sup>; and Group 4 received CvEE orally at a dose of 300 mg kg<sup>-1</sup>. All groups received formalin after one hour of pre-treatment.

We determined the time in seconds during which the animal continued to lick the injected paw (licking time) during two intervals after the administration of formalin: 0-5 minutes was defined as the first phase or neurogenic pain phase, and 15-30 minutes was defined as the second phase or inflammatory pain phase.

### Carrageenan-induced paw edema

Following Levy (1969), with modifications, after one hour of pre-treatment, *Balb/C* mice (n = 6 per group) received carrageenan (300 µg 30 µL<sup>-1</sup>) in the right posterior paw. The animals were orally pre-treated with 3.5% ethanol solution (vehicle) at a volume of 10 mL kg<sup>-1</sup> (Group 1, negative control); indomethacin at a dose of 10 mg kg<sup>-1</sup> (Group 2, positive control); and CvEE at a dose of 300 mg kg<sup>-1</sup> (Group 3). The readings on the hydroplethysometer (a device that measures the paw edema that was formed) were performed in triplicate before treatment (baseline reading), and one, two, three and four hours after the injection of carrageenan.

### Carrageenan-induced peritonitis

*Balb/C* mice (n = 8 per group) were intraperitoneally pretreated with CvEE (30, 100 and 300 mg kg<sup>-1</sup>) 30 min before administration of carrageenan (500 µg cavity<sup>-1</sup>). Control groups received PBS and carrageenan. Four hours later, the mice were submitted to terminal anesthesia through intraperitoneal administration of a ketamine/xylazine solution (20 µL/kg). The peritoneal cavity was washed with heparinized saline phosphate buffer, the peritoneal fluid was centrifuged at 100 g for 5 min at 4 °C and the pellet was used to determine the total cell count by using a modified Neubauer chamber and Turk's stain. Differential cell counts were performed on cytospin preparations stained with May-Grunwald and

Giemsa using standard morphological criteria to identify cell types. The results are presented as the number of cells per mL.

### Chemokine levels

*Balb/C* mice (n = 4 per group) were pretreated with CvEE (300 mg kg<sup>-1</sup>) intraperitoneally 30 min before administration of carrageenan (500 µg cavity<sup>-1</sup>). Control groups received PBS and carrageenan. The supernatants obtained from the peritoneal fluid were collected after four hours (de Souza Costa et al. 2018). The levels of chemokine CXCL1 were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA DuoSet kit (R&D Systems, Minnesota, USA), in accordance with the manufacturer's instructions. The results are expressed as picograms of cytokine per milliliter (pg mL<sup>-1</sup>). The CXCL1 levels correspond to an average obtained from triplicates for each animal representative of two independent experiments.

### Data analysis

The results were analyzed using the GraphPad 6.0 program and expressed as mean ± SEM (standard error of the mean). Normality and homogeneity of variance of variable distribution were assessed with the Shapiro-Wilk and Bartlett tests, respectively. Statistical differences among the treatments were assessed using analysis of variance (one-way ANOVA, except for the carrageenan-induced paw edema test which was used mixed ANOVA) followed by the Bonferroni test (*p* < 0.05 was considered significant).

## RESULTS

### Phytochemical screening and ESI-MS

The phytochemical prospecting tests of CvEE detected the presence of phenolic compounds, condensed tannins, saponins, flavonols, flavanones, flavanonols and xanthonones (free or their heterosides) (Table 1). Several compounds were detected through the ESI-MS spectra analysis (Table 2), however gallic acid (*m/z* 169) and *p*-coumaroylquinic acid (*m/z* 337) were the principal compounds detected (data not shown).

### Rotarod test

The motor coordination response in Group 3 (CvEE) did not differ significantly from that of Group 1 (vehicle; negative control) after 30, 60 and 120 min of pre-treatment, indicating that the extract did not cause motor deficit in the mice. The animals in (Group 2 (xylazine; positive control) spent significantly less time on the rotating bar (*p* < 0.001) after 30 and 60 min of pre-treatment compared to Group 1 (data not shown).

### Acetic acid-induced writhing test

Groups 2 (indomethacin; positive control) and 4 (CvEE 300 mg kg<sup>-1</sup>) showed significantly less abdominal writhes

compared to Group 1 (vehicle; negative control) (*p* < 0.05), while Group 3 (CvEE 100 mg/kg) did not differ significantly from Group 1 (Figure 1).

### Carrageenan-induced mechanical hyperalgesia and opioidergic pathway involvement

In the first test, the administration of CvEE produced an antinociceptive action against the hyperalgesia, as the Δ value of the nociceptive threshold was significantly lower in Group 2 (CvEE) compared to Group 1 (vehicle) (*p* < 0.01) (Figure 2).

**Table 1.** Classes of chemical substances detected by phytochemical screening in the ethanolic extract of peels of *Caryocar villosum* from Ducke Reserve (Amazonas, Brazil). + indicates the presence, and – the absence of the compounds.

Class	Occurrence
Phenolic compounds	+
Free anthraquinones	–
Coumarins	–
Condensed tannins	+
Anthocyanins, anthocyanidins, flavones, flavonols, xanthonones, chalcones, aurones, flavanonols	–
Flavonols, flavanones, flavanonols and xanthonones (free or their heterosides)	+
Saponins	+

**Table 2.** Chemical compounds detected in the ethanolic extract of peels of *Caryocar villosum* (Ducke Reserve, Amazonas, Brazil) analyzed by ESI-MS in the negative ion mode and identified by literature search.

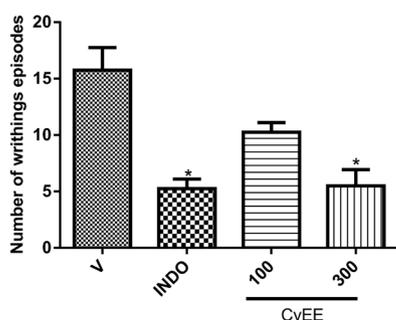
Compound	[M-H] <sup>-</sup> ( <i>m/z</i> )	References
Vanillic acid	167	Sun et al. 2007
Gallic acid	169	Roesler et al. 2008; Chisté et al. 2012
Xanthoxylin	195	Malta et al. 2013
Brevifolin	247	Zhu et al. 2015
Brevifolin carboxylate	291	Zhu et al. 2015
Ellagic acid	301	Chisté et al. 2012; Malta et al. 2013
Caffeoylshikimic acid	335	Bastos et al. 2007
<i>p</i> -Coumaroylquinic acid	337	Chisté et al. 2012; Malta et al. 2013; Zhu et al. 2015
Feruloylhexose	355	Malta et al. 2013
Quercetin hexoside	463	Malta et al. 2013; Silva et al. 2014
Apigenin deoxyhexosylhexoside	577	Silva et al. 2014
Quercetin galloylhexoside	615	Silva et al. 2014
Galloyl-HHDP hexoside	633	Chisté et al. 2012; Zhu et al. 2015
Monoacyl diglycosylated quercetin derivatives	761	Pereira et al. 2015
Tetragalloylquinic acid	799	Pereira et al. 2015

In the second test, the hyperalgesia induced by the intraplantar administration of carrageenan was also significantly reversed in Group 2 ( $p < 0.001$ ) compared to the control (Group 1), implicating a significant antinociceptive action (Figure 3). In addition, the naltrexone and extract vehicles (Group 1) did not interfere with the antinociceptive action of CvEE (Figure 3).

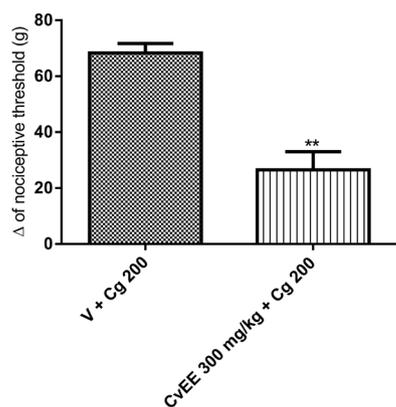
Naltrexone (Group 3) antagonized the effect of CvEE (Group 2), as shown by the increase in the  $\Delta$  value of the nociceptive threshold in Group 3 compared to Group 2 (Figure 3), which demonstrates a partial reversal of the extract's antinociceptive effect mediated by naltrexone. Therefore, it is possible to suggest that there is a participation of the opioidergic pathway in the antinociceptive mechanism of action of CvEE.

### Formalin test

In the first five minutes after formalin injection, Group 2 (indomethacin) and Group 4 (CvEE) did not differ



**Figure 1.** Effect of *Caryocar villosum* ethanolic extract (CvEE) on the number of abdominal writhes induced by 0.9% acetic acid in mice. Columns represent the mean and bars the standard error of the mean of the number of abdominal contortions for six test individuals. \* indicates statistical significance at  $p < 0.05$  relative to the control (Group 1) according to ANOVA followed by post-hoc Bonferroni tests. V = vehicle; INDO = indomethacin.

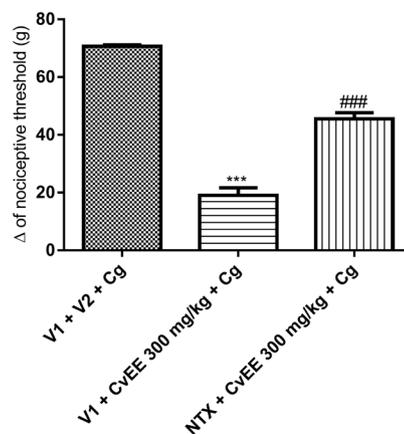


**Figure 2.** Effect of *Caryocar villosum* ethanolic extract (CvEE) on carrageenan-induced hyperalgesia in mice. Columns represent the mean and bars the standard error of the mean of the  $\Delta$  value of the nociceptive threshold (expressed in grams) for six test individuals. \*\* indicates statistical significance at  $p < 0.01$  relative to the control (Group 1) according to ANOVA followed by post-hoc Bonferroni tests. V = vehicle; Cg = carrageenan.

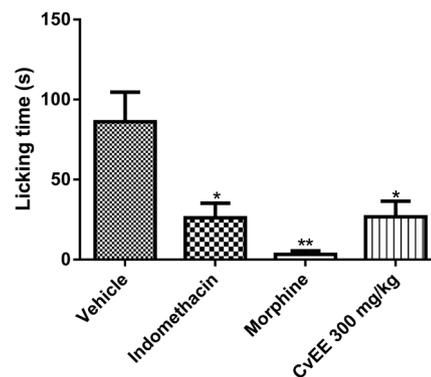
significantly from the negative control (Group 1), while the mice in Group 3 (morphine) licked their paw significantly less than those in Group 1 ( $p < 0.05$ ) (data not shown). In the second phase of the test (15-30 min after formalin injection), paw licking time was significantly lower in Groups 2 ( $p < 0.05$ ), 3 ( $p < 0.01$ ) and 4 ( $p < 0.05$ ) compared to Group 1 (Figure 4).

### Carrageenan-induced paw edema

Animals in Groups 2 (indomethacin; positive control) and 3 (CvEE) showed a peak in edema volume in the first hour, followed by a significant decrease in volume in the second ( $p < 0.001$ ), third ( $p < 0.001$ ) and fourth hour ( $p < 0.001$ ) compared to Group 1 (vehicle; negative control) (Figure 5).



**Figure 3.** Evaluation of participation of the opioidergic pathway in the mechanism of antinociceptive action of *Caryocar villosum* ethanolic extract (CvEE) in mice through the administration of naltrexone. Columns represent the mean and bars the standard error of the mean of the  $\Delta$  value of the nociceptive threshold (expressed in grams) for six test individuals. \*\*\* indicates statistical significance ( $p < 0.001$ ) relative to Group 1 (vehicles); ### indicates statistical significance ( $p < 0.001$ ) relative to Group 2 (V1 + extract) according to ANOVA followed by post-hoc Bonferroni tests. V1 = naltrexone vehicle; V2 = CvEE vehicle; Cg = carrageenan; NTX = naltrexone.



**Figure 4.** Effect of *Caryocar villosum* ethanolic extract (CvEE) on paw licking time 15-30 minutes after formalin injection. Columns represent the mean and bars the standard error of the mean of paw licking time in seconds for six test individuals. \* indicates statistical significance ( $p < 0.05$ ) relative to Group 1 (vehicle); \*\* indicates statistical significance ( $p < 0.01$ ) relative to Group 1 (vehicle) according to ANOVA followed by post-hoc Bonferroni tests.

### Carrageenan-induced peritonitis

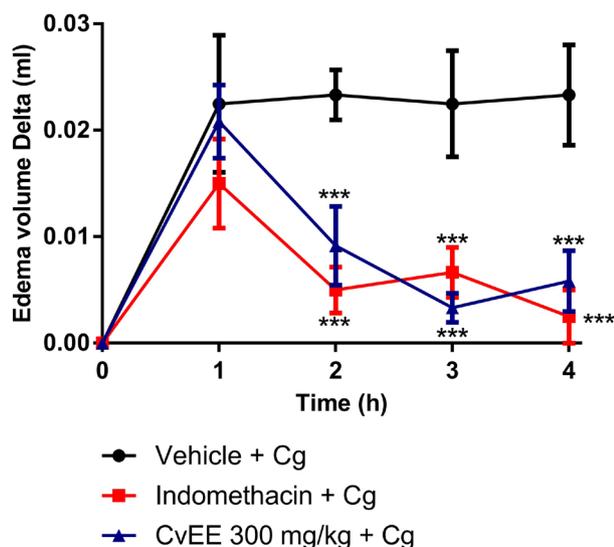
CvEE significantly inhibited leucocyte ( $p < 0.001$ ) and neutrophil ( $p < 0.001$ ) recruitment induced by carrageenan into the peritoneal cavity in mice at all doses from 30 to 300 mg kg<sup>-1</sup> (Figure 6).

### Chemokine levels

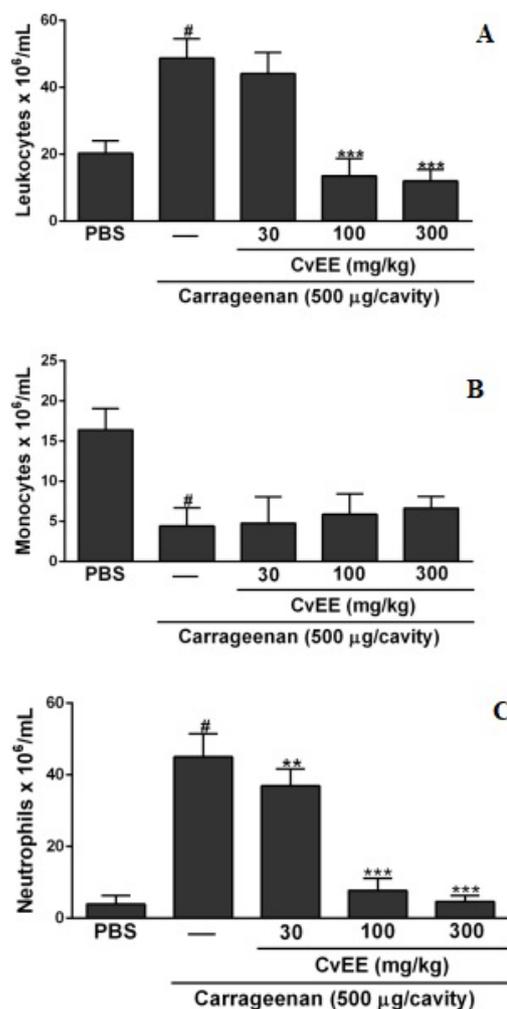
CvEE significantly reduced CXCL1 chemokine levels induced by carrageenan in the peritoneal cavity ( $p < 0.05$ ) (Figure 7).

## DISCUSSION

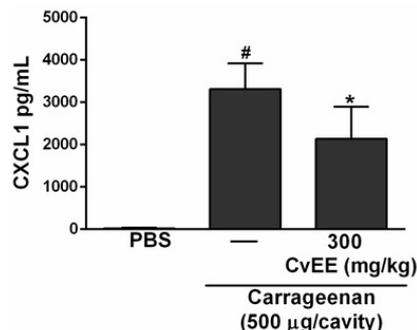
The main components of CvEE used in this study were gallic acid and *p*-coumaroylquinic acid, two phenolic compounds already found in extracts obtained from the pulp of *C. villosum* (Chisté et al. 2012). Gallic acid, or 3,4,5-trihydroxybenzoic acid, is a secondary metabolite present in most tall plants and can be found in isolated form or complexed with other compounds (Daglia et al. 2014; Fernandes and Salgado 2016). Along with its derivatives, it occurs in various parts of the plant, and is known for its antioxidant activity, but also can have anti-inflammatory, antinociceptive, anticancer and antimicrobial activity (Boeira et al. 2011; Daglia et al. 2014; Kumar and Jain 2014; Raafat and Samy 2014; Saad et al. 2014; Trevisan et al. 2014; Mard et al. 2015; Pandurangan et al. 2015; Fernandes and Salgado 2016; BenSaad et al. 2017; Cesário et al. 2018). Its



**Figure 5.** Effect of *Caryocar villosum* ethanolic extract (CvEE) on inflammation in up to four hours after carrageenan-induced paw edema in mice. Symbols are the mean and bars the standard error of the mean of the  $\Delta$  value of edema volume variation (expressed in milliliters) for six test individuals. Statistical analysis: analysis of variance followed by the Bonferroni test, \*\*\* indicates statistical significance ( $p < 0.001$ ) relative to the control (Group 1, vehicle + Cg) at each time point according to a mixed ANOVA followed by post-hoc Bonferroni tests. Cg = carrageenan. This figure is in color in the electronic version.



**Figure 6.** Effects of *Caryocar villosum* ethanolic extract (CvEE) on leukocyte recruitment in response to carrageenan injection into the peritoneal cavity of mice. Columns are the mean and bars the standard error of the mean of eight animals in each group. # indicates statistical significance ( $p < 0.01$ ) relative to the negative control (PBS); \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ) indicate statistical significance of the respective CvEE-treated group relative to the positive control (only carrageenan).



**Figure 7.** Effect of *Caryocar villosum* ethanolic extract (CvEE) on CXCL1 chemokine production in the peritoneum of carrageenan-injected mice. Columns represent the mean and bars the standard error of the mean of paw licking time in seconds for six test individuals. # indicates statistical significance ( $p < 0.01$ ) relative to the negative control (PBS); \* indicates statistical significance ( $p < 0.05$ ) relative to the positive control (only carrageenan).

pharmacological actions are related to the inhibition of the NF- $\kappa$ B signaling pathway, COX-2, histone acetyltransferase and transient receptor potential canonical 5 (Trevisan *et al.* 2014).

The *p*-coumaroylquinic acid is a phenolic acid that belongs to the class of chlorogenic acids (CGAs) (Farah and Donangelo 2006; Liang and Kitts 2016). CGAs can reduce the expression and production of pro-inflammatory mediators, such as IL-1 $\beta$ , IL-8, IL-6, among others, in cells stimulated with IFN- $\gamma$ , TNF- $\alpha$  and LPS, and can also inhibit COX-2 expression by inhibiting signaling via NF- $\kappa$ B (Liang and Kitts 2016).

CvEE did not produce any muscle relaxation or sedation in the rotarod test, since the animals that received the extract remained walking on the rotating bar before and after pre-treatment during the total time of the motor evaluation. Therefore it was possible to exclude the possibility of a false positive result due to muscle sedation or relaxation in the assays that evaluate antinociceptive activity.

CvEE reduced the number of abdominal writhes induced by acetic acid. The main prostaglandins produced and released during the nociceptive process caused by acetic acid are PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> , among other active substances such as substance P, bradykinin, serotonin, capsaicin, amines of the sympathetic system and interleukins, which facilitate the stimulation of nociceptors (Deraedt *et al.* 1980; Duarte *et al.* 1988; Ribeiro *et al.* 2000). Therefore, interference with the action of these substances through pre-treatment with opioids and cyclooxygenase inhibitors can trigger an antinociceptive effect, decreasing abdominal writhing during the test (Gawade 2012; Pavao-de-Souza *et al.* 2012). Therefore, the mechanism by which CvEE acted possibly involved the synthesis or action of inflammatory mediators.

Morphine, from the opioid class, has an antinociceptive effect in the acetic acid-induced writhing test (Smith *et al.* 1982). Therefore, another mechanism by which CvEE may have acted in this test involves the opioidergic pathway, which was tested in the carrageenan-induced mechanical hyperalgesia model. CvEE was able to reverse the hyperalgesia caused by carrageenan, demonstrating that its antinociceptive action also involves the participation of the opioidergic pathway.

CvEE showed effective antinociceptive action only in the second phase of the formalin test. The second phase is inhibited not only by NSAIDs, such as indomethacin, and corticosteroids, but also by opioids (Hunskar and Hole 1987; Malmberg and Yaksh 1992; Oluyomi *et al.* 1992). Therefore, the antinociceptive effect of CvEE only in the second phase of the formalin test seems to result from an anti-inflammatory action, such as, for example, the reduction of the action or production of inflammatory mediators, especially prostaglandins, by inhibiting cyclooxygenase. The results of the carrageenan-induced mechanical hyperalgesia test suggest that the antinociceptive action in the second phase may also

involve the opioidergic system. As CvEE only had an effect in the second phase of the formalin test, it is possible that the extract does not have a central action, as is the case with morphine, but only a peripheral action (Rosland *et al.* 1990). This is consistent with the result of the acetic acid-induced writhing test, which is a method for assessing peripheral action (Reichert *et al.* 2001; Sousa *et al.* 2012).

The carrageenan-induced paw edema test also works in a biphasic manner, with an initial phase that occurs up to two hours after carrageenan injection, and is characterized by vasodilation, increased vascular permeability and non-phagocytic edema. The second phase occurs from the second hour onwards and is characterized by an intense formation of edema. The first phase is mediated by the release of histamine, bradykinin and serotonin, while the second phase occurs due to the high production of prostaglandins (Muhammad *et al.* 2012). Oral administration of CvEE reduced edema formation, showing anti-inflammatory and antiedematogenic activity, possibly due to the inhibition of prostaglandin synthesis, an effect resulting from the inhibition of cyclooxygenase or another inflammation pathway.

The acute peritonitis model evaluates the total leukocytes, monocytes and neutrophil recruitment via the intraperitoneal injection of carrageenan, which stimulates intense chemotactic action (Guerra *et al.* 2011). CvEE reduced the polymorphonuclear leukocyte migration into the peritoneal cavity. This mechanism of action can be related to reduced levels of CXCL1, which is a chemotactic agent (Chintakuntlawar and Chodosh 2009; Guerra *et al.* 2011).

## CONCLUSIONS

The *Caryocar villosum* ethanolic extract obtained from the fruit peels showed peripheral antinociceptive action that can be due to anti-inflammatory activity and involves the participation of the opioidergic pathway. The reduced CXCL1 levels may be related to inhibition of total leukocyte and neutrophil recruitment. Anti-inflammatory and antinociceptive activities of the extract can be attributed to the presence of phenolic compounds, mainly gallic acid and *p*-coumaroylquinic acid.

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