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Chemical characterization and pharmacological assessment of polysaccharide free, standardized cashew gum extract (*Anacardium occidentale L.*)

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ABSTRACT

Ethnopharmacological relevance: The cashew gum (*Anacardium occidentale L.*) is used in traditional Brazilian medicine in the treatment of inflammatory conditions, asthma, diabetes, and gastrointestinal disturbances.
Aim of the study: In the present study, we aimed at forming a chemical characterization and investigation of the antinociceptive and anti-inflammatory activities of the aqueous extract of cashew gum without the presence of polysaccharides in its composition (CGE).
Materials and methods: The CGE was obtained after the precipitation and removal of polysaccharides through the use of acetone. After, the acetone was removed by rotaevaporation, and the concentrated extract was lyophilized. The chemical characterization of CGE was performed by liquid chromatography mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS) analyses. Mice were used for the evaluation of the antinociceptive and anti-inflammatory activities. CGE was analyzed via the Irwin test, acetic acid-induced writhing test, formalin-induced pain test, and carrageenan-induced paw edema test. The motor activity or probable sedation was verified through the chimney, open-field, and sodium pentobarbital-induced sleep tests. We investigated if the analgesic and anti-inflammatory effects of CGE depend of reduction in PGE_2 levels, were performed the carrageenan or PGE_2-induced hyperalgesia tests.
Results: The chemical characterization of CGE showed the presence of anacardic acids as the predominant phytoconstituents. The treatment with CGE (75, 150, and 300 mg/kg, p.o.) inhibited the number of writhing in a dose-dependent manner. With an intermediate dose, CGE did not cause motor impairment with the chimney test or alterations in either the open-field or sodium pentobarbital-induced sleep. In the formalin-induced pain test, CGE (150 mg/kg, p.o.) produced an antinociceptive effect only in the first phase of the test, suggesting anti-inflammatory activity. With the same dosage, CGE also reduced the carrageenan-induced paw edema at all hours of the test, confirming its anti-inflammatory effect. Furthermore, CGE (150 mg/kg, p.o.) presented an anti-hyperalgic effect at all hours of the carrageenan-induced hyperalgesia test. However, this dose of CGE was not able to reduce the hyperalgesia induced by PGE_2, suggesting that the anti-inflammatory effect of this extract depends on the reduction in the PGE_2 levels.
Conclusion: The anacardic acids are the predominant phytoconstituents identified in the CGE. The action mechanisms of CGE suggest the reduction in the PGE_2 levels. These findings support the use of cashew gum in popular medicine and demonstrate that part of its antinociceptive and anti-inflammatory effects should also be attributed to the presence of anacardic acids in its composition, independent of the presence of polysaccharides.

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1. Introduction

Anacardium occidentale Linn, the member of the family Anacardiaceae, is a plant with considerable economic and medicinal value. It is commonly known as the cashew tree and is native in Africa, India, and northeastern Brazil. Extracts obtained from different parts of the cashew tree have found wide application in traditional medicine for the treatment of various diseases, such as asthma (Giron et al., 1991; Mazzetto and Lomonaco, 2009), gastrointestinal disorders (Agra et al., 2007; Coe and Anderson, 1996; Giron et al., 1991), skin diseases (Coe and Anderson, 1996; Mazzetto and Lomonaco, 2009), and fever (Patro and Behera, 1979; Sudarshan et al., 1985). In addition, its stem-bark and leaves have been used in topical preparations or decoctions for the treatment of pain (Coe and Anderson, 1996; Mazzetto and Lomonaco, 2009) and various inflammatory conditions (Agra et al., 2007; Coe and Anderson, 1996), such as arthritis (Iwu, 1993; Oliver-Bever, 1986).

The epithelial cells from the bark of the cashew tree naturally produce and store an exudate known as cashew gum, which is released in response to mechanical injury or attacks by pathogens, as a form of protection (Miranda, 2009). Cashew gum, in water-soluble preparations, has been commonly used as an anti-inflammatory, analgesic, anti-asthmatic, and antidiabetic agent (Lima et al., 2006). Furthermore, it has been used to treat gastrointestinal diseases, including diarrhea (Anderson et al., 1974), warts, coughs, and wounds (Agra et al., 2007).

The ethnopharmacological properties of cashew gum have been evaluated in preclinical studies and have demonstrated significant antibacterial (Campos et al., 2012; Torquato et al., 2004), antitumor (Florêncio et al., 2007; Mothe et al., 2008), antidiarrheal (Araújo et al., 2015), and hypoglycemic effects (Ojewole, 2003). A study conducted by Yamassaki et al. (2015), using immuno-stimulant and anti-inflammatory in vitro assays of murine peritoneal macrophage activities, shows the anti-inflammatory activity of cashew gum, which was attributed to the presence of polysaccharides in its composition. In this study, our aims were to evaluate the antinociceptive and anti-inflammatory activities of cashew gum aqueous extract without the presence of polysaccharides in its composition (CGE) and characterize the chemical profile of this extract, in order to better elucidate the therapeutic effects of this plant and point out its value in traditional medicine.

2. Materials and methods

2.1. Origin and preparation of cashew gum extract

Samples of cashew gum were collected from the trees of *Anacardium occidentale* at the CIALNE farm in Pacajus, Ceará, Brazil. The tree was identified through a comparison with a voucher specimen of HDELTA herbarium from the Federal University of Piauí, Parnaíba, Piauí, Brazil (voucher specimen number 52). Nodules were milled, immersed in distilled water in a proportion of 20% (w/v), and kept at room temperature (25 °C) for 24 h. The solution was sifted in nylon mesh to remove bark fragments and then precipitated with acetone in a ratio of 1:3 (v/v) for 24 h. The precipitated cashew gum polysaccharide (CGP) was separated by centrifugation, washed with acetone, dried, and stored at room temperature (25 °C) in hermetically closed vials. After removal of the polysaccharides, the acetone solvent was removed from the filtration by rotaevaporation; subsequently, this concentrated extract was lyophilized. The process of obtaining the CGE yielded 2.27% on the starting material. For the pharmacological tests, CGE was emulsified in distilled water containing 2% Tween 80.

2.2. Liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS) analyses

The LC-MS analyses were executed using a liquid chromatograph Shimadzu LC-20 CE (Kyoto, Japan) with a binary pump, which was connected to a micrOTOF-Q III instrument (Bruker Daltonics, Bremen, Alemanha) and equipped with an ESI commercial ionization source (electrospray ionization). For the chromatography separation, a Zorbax Eclipse Plus – C18 (4,6 mm × 150 mm, 5 μm) reversed-phase HPLC column (Agilent Technologies, Palo Alto, CA – USA) was used. Milli-Q water (2.0% v.v-1, ammonium hydroxide) (Eluent A) and ACN (0.5% v.v-1, ammonium hydroxide) (Eluent B) were used as mobile phase solvents. For the LC-MS experiments, samples were dissolved in methanol (1 μL: 999 μL v.v-1). A flow rate of 1 mL min-1 was maintained isocratically in a proportion of 50:50 for 20 min. The column temperature was 40 °C, and 20 μL of each sample was injected into the LC-MS/MS system. The MS/MS experiments were performed by direct infusion of the CGE sample on ESI (-) Q-TOF at a concentration of 1 μL mL-1 (0.1% v.v-1, ammonium hydroxide) and a flow of 3 μL/min. The electrospray source was operated in the negative mode in all analyses. High purity nitrogen (> 98%) was used as a desolvation (220 °C; 12 L min-1) nebulizer and collision gas. The nebulizer pressure was kept at 5 bars, and the capillary voltage was set at 3500 V. The Q-TOF conditions were as follows: End plate offset: 500 V, Funnel 1: 2200 Vpp, Funnel 2: 300 Vpp, Hexapole RF: 400 Vpp, Collision RF: 400 Vpp, Transfer Time: 120 μs, Pre Pulse Storage: 10 μs, Ion Energy Quadrupole: 5 eV, Rolling Average: 3 × 1 Hz. Data were collected in a full scan mode from 300 to 400 m/z and 100–400 m/z in the MS/MS mode, for selected precursors, using collision-induced fragmentation. When the MS/MS mode was applied, the collision energy increased from 10 to 35 eV. The mass spectra were acquired and processed using the Bruker Compass DataAnalysis Software (Bruker Daltonik, GmbH).

2.3. Animals

Experiments were performed using female Swiss albino mice (25–35 g) from the Central Animal House of the Universidade Federal de Goiás (UFG). The animals were kept in plastic cages at 22 ± 2 °C, with free access to pellet food and water and under 12 h light/dark cycle, in compliance with the International Guiding Principles for Biomedical Research Involving Animals. The animals were acclimatized for 7 days before the experiments began. All of the experimental protocols were developed and approved according to the principles of ethics and animal welfare designated by the Ethics Committee on Animal Use of UFG (number: 057/15).

2.4. Drugs and chemicals

The chemicals used in this study were acetic acid (Merck, USA), acetone (Synth, Diadema, SP, Brazil), carrageenan (Sigma Chemical, USA), dexamethasone (Prodome, Campinas, SP, Brazil), formaldehyde (Synth, Diadema, SP, Brazil), indomethacin (Merck Sharp & Dohme Farmacêutica Ltda, SP, Brazil), morphine hydrochloride (Dimorf®, Cristalia, SP, Brazil), PGE_2 (Cayman Chemical, Michigan, USA), and polyoxyethylenesorbitan monooleate (Tween 80®, Sigma-Aldrich, Brazil).

2.5. Effects on gross behavior or the Irwin test

This test was used to assess preliminary drug effects on mice behavior and toxicity and to determine an effective dose range. Experimental groups of mice (n = 1) were treated orally (p.o.), intraperitoneally (i.p.), or subcutaneously (s.c.) with CGE at doses of 30 mg/kg, 100 mg/kg, 300 mg/kg, and 1000 mg/kg, whereas the control groups received a vehicle (2% Tween 80, 10 mL/kg) by the same routes. Animals were observed in free ambulation on a flat surface for 3 min, at 0, 5, 10, 20, 30, and 60 min; 4, 8, 24, and 48 h; 4 and 7 days after the treatment. The presence or absence of mortality, seizure, erection of the tail (Straub sign), sedation, excitation, motor incoordination, abdominal torsion, or spontaneous ambulation was recorded. The observed effects were compiled using a standard

pharmacological screening approach, adapted from that described by Irwin (1968).

2.6. Acetic-acid-induced abdominal writhing test

The acetic-acid-induced writhing test was performed as described by Koster et al. (1959). Groups of mice (n = 8) were treated by gavage (p.o.) with a vehicle (2% Tween 80, 10 mL/kg), CGE (75, 150, and 300 mg/kg), or indomethacin (10 mg/kg – a positive control for antinociceptive activity) 60 min before the application of the acetic acid solution (1.2% v/v; 10 mL/kg, i.p.). The number of abdominal constrictions (writhing) was counted for each animal over a period of 30 min after the acetic acid injection, and the results are expressed as the mean ± S.E.M. of the number of writhing incidents.

2.7. Central activity assessment

2.7.1. Chimney test

The chimney test permits the detection of muscle relaxing agents and/or drugs that produce motor incoordination. Briefly, after 60 min of treatment with a vehicle (2% Tween 80, 10 mL/kg p.o.), CGE (150 mg/kg, p.o.), or diazepam (5 mg/kg p.o.), the animals (n = 8) were placed in a tube (25 cm length, 3 cm diameter) with a rough inner surface. Motor impairments were indicated by the animals' inability to climb backward up a tube at time of 30 s (Coleta et al., 2006). The results are expressed as the mean ± SEM of time, in seconds, that the animals spent climbing up the tube backward.

2.7.2. Open field test

The open field test assessed the ambulatory behavior of mice and detected anxiolytic-like or anxiogenic-like agents. The open field area was made of white acrylic (40 cm diameter and 30 cm wall height). The floor had eight squares of equal area. After 60 min of treatment with a vehicle (2% Tween 80, 10 mL/kg p.o.), CGE (150 mg/kg, p.o.), or diazepam (5 mg/kg, p.o.), the animals (n = 8) were placed individually in the center of the open field and observed for 5 min. In the exploratory activities of animals were registered fecal boluses, rearings, grooming, immobility, total crossings, and crossings in the central area (%) (Archer, 1973). The results are expressed as the mean ± SEM of the different parameters evaluated.

2.7.3. Pentobarbital-induced sleeping test

This sleeping test is used to detect agents with sedative/depressant or stimulant effects; stimulant drugs reduce sleeping time, and depressant drugs increase this time. After 60 min of treatment with a vehicle (2% Tween 80, 10 mL/kg p.o.), CGE (150 mg/kg, p.o.), or diazepam (5 mg/kg, p.o.), all of the groups of mice (n = 8) were treated with sodium pentobarbital (50 mg/kg i.p.). In this test, we evaluated the latency (s), that is, the time between the injection of the pentobarbital and the loss of the righting reflex. We also evaluated the sleeping duration (min), that is, the time between the loss of the righting reflex and the regaining of the righting reflex (Carlini and Burgos, 1979). The results are expressed as the mean ± SEM of the latency (s) and sleeping duration (min).

2.8. Formalin-induced pain

The formalin-induced nociception was performed as described by Hunskaar and Hole (1987). Mice (n = 9) were treated by gavage (p.o.) with a vehicle (2% Tween 80, 10 mL/kg), CGE (150 mg/kg), indomethacin (10 mg/kg – a positive control for antinociceptive activity in the second phase), or morphine (5 mg/kg, s.c. – a positive control for antinociceptive activity in the first and second phases). Sixty minutes after the treatment, 20 µL of formalin (3%) was injected into the plantar surface of the right hind paw. After, the mice were placed in an acrylic box, and a mirror was placed under this box to enable an unhindered observation of the formalin-injected paw for 30 min. The pain reaction time (licking time) was assessed during two periods: from 0 to 5 min during the first phase, when neurogenic pain is caused by a direct stimulation of the nociceptors, and from 15 to 30 min during the second phase, when inflammatory pain is caused by a release of inflammatory mediators. These results were expressed as the mean ± SEM of the licking time, in seconds (s).

2.9. Carrageenan-induced paw edema

Carrageenan-induced paw edema in mice was used as a model of acute inflammation, according to the method of Passos et al. (2007). Initially, the animals (n = 9) were treated with a vehicle (2% Tween 80, 10 mL/kg p.o.), CGE (150 mg/kg, p.o.), or indomethacin (10 mg/kg, p.o). One hour later, 50 µL of carrageenan (1%) was injected intraplantar in the right hind paw, and 50 µL of saline (0.9% NaCl) was injected intraplantar in the left hind paw (used as the control). The paw volume (µL) was measured using a plethysmometer (Model 7141, Ugo Basile, Italy) at 1, 2, 3, and 4 h after the injection of carrageenan.

2.10. Mechanical hyperalgesia induced by carrageenan or PGE_2

The paw pressure test based on the method described by Randall and Selitto (1957) was used to measure the mechanical nociceptive thresholds. Initially, the groups of animals (n = 8) were treated with a vehicle (2% Tween 80, 10 mL/kg, p.o.), CGE (150 mg/kg, p.o.), or indomethacin (10 mg/kg, p.o. – a positive control). One hour later, 50 µL of carrageenan (1%) was injected intraplantar in the right hind paw, and 50 µL of saline (0.9% NaCl) was injected intraplantar in the left hind paw (used as the control). The pain threshold was measured at 1, 2, 3, and 4 h after the intraplantar carrageenan injection by using a mechanical nociceptive stimulation (Insight Apparatus EFF-440 - Brazil). The nociceptive threshold in response to a mechanical stimulus was measured according to the exposure of the inflamed and noninflamed hind paw to an increasing force, until the appearance of a nociceptive reaction (vocalization or paw withdrawal) was observed. The stimulation was stopped when the mice struggled to withdraw the corresponding paw, and the threshold value was recorded. Measurements were alternately performed for each paw. The cut-off for each paw was 450 g. A baseline was performed before the treatments (time 0) for each animal occurred (Kawabata et al., 1992). To evaluate the effect of the CGE treatment on hyperalgesia induced by prostaglandin E_2 (PGE_2), the mechanical nociceptive thresholds were measured following the same protocol previously described. However, instead of carrageenan, the animals received PGE_2 (100 ng/paw) in a volume of 50 µL in the right hind paw and the same volume of saline (0.9% NaCl) in the left hind paw (used as the control); then the response to the mechanical stimulus was evaluated (Barbosa et al., 2013). The results of both tests were expressed as the mean ± SEM of the difference between the nociceptive threshold of the paws in grams.

2.11. Statistical analysis

The data were analyzed statistically by a one-way ANOVA, followed by the Newman-Keuls' test as a post-hoc or two-way ANOVA, followed in turn by Bonferroni' test as post-hoc (Sokal and Rohlf, 1981). All statistical analyses were carried out using GraphPad Prism version 5.0. The values of $P \leq 0.05$ were considered significant.

3. Results

3.1. Chemical analysis

Fig. 1 shows the extracted ion chromatogram (EIC), referring to the cashew gum extract (CGE) sample. As shown in Fig. 1, seven different anacardic acids were found, separated, and identified satisfactorily by

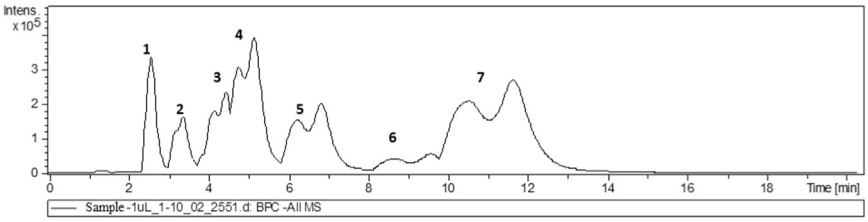

Fig. 1. Extracted ions chromatogram (EIC) referring to cashew gum extract (CGE) sample.

Table 1
ESI (-) MS/MS data for anacardic acids, from cashew gum extract (CGE).

Anacardic Acid	Peak	$[M-H]^-$	*m/z* (rel. intensity, %)	
			MS	*Major fragmente ions*[a]
An 15:3	1	341.2071	33.3	**297** (100) and 119 (3.5)
An 15:2	2	343.2225	20.8	**299** (100) and 119 (3.9)
An 15:1	4	345.2389	99.5	**301** (100) and 199 (2.1)
An 15:0	6	347.2531	17.4	**303** (100)
An 17:3	3	369.2383	44.0	**325** (100), 231 (3.6), and 119 (9,7)
An 17:2	5	371.2544	41.9	**327** (100) and 119 (3.5)
An 17:1	7	373.2696	100	**329** (100) and 119 (0.9)

[a] Base peak in bold face.

Table 2
Concentrations of anacardic acids in cashew gum extract (CGE).

Anacardic Acid	Peak	$[M-H]^-$	EIC (area)	Equation: y = 1373,4x + 394296 R^2 = 0,9911 Concentration ($\mu g\ mL^{-1}$)
An 15:3	1	341.2071	5981599	4.07
An 15:2	2	343.2225	4413431	2.93
An 15:1	4	345.2389	16910624	12.03
An 15:0	6	347.2531	4421529	2.93
An 17:3	3	369.2383	7994983	5.53
An 17:2	5	371.2544	11637954	8.19
An 17:1	7	373.2696	27935610	20.05

LC-MS. In some cases, the assignment of two peaks to ions of the same *m/z* was also observed, probably due the presence of isomers. The highest intensity in the extracted ion chromatogram was obtained for molecular ions of anacardic acid, with 15 carbon atoms and 1 unsaturation in the alkyl chain, detected as deprotonated molecules $[M-H]^-$. To confirm the chemical structure and connectivity of anacardic acid, collision induced dissociation (CID) experiments were performed, and the ESI(-)MS/MS data are shown in Table 1. The tandem mass spectra of deprotonated anacardic acids depend significantly on the degree of unsaturation of alkyl chains. With an increasing number of double bonds in the alkyl chain, more fragments are formed from the precursor ion. With regard to the quantitative experiments, the anacardic acid An 15:0 was used as the standard. A calibration curve (supplementary material) was constructed of chromatogram areas of extracted ions in the concentration range of 500 – 4000 ng mL-1, and it showed a coefficient of linear correlation (r2) greater than 0.99. The concentration of anacardic acid 15:0 in the real sample was calculated by a line equation arranged in Table 2. As shown in Table 2, the concentration of other acids present in the real sample was calculated by proportionality, using the same line equation found for anacardic acid 15:0.

3.2. Effects on gross behavior or Irwin test

The CGE at dose 1000 mg/kg (p.o., i.p., s.c.) reduced spontaneous ambulation and induced analgesia 30 min after treatments. The administration of this dose by the intraperitoneal pathway produced the death of the animal on the seventh day. The treatment with CGE at a dose of 300 mg/kg (i.p., s.c.) also reduced spontaneous ambulation and induced analgesia 4 h after treatment. However, the treatment with CGE at a dose of 30 or 100 mg/kg (p.o., i.p. s.c.) or 300 mg/kg (p.o.) did not produce behavior alterations. Based on these results, we opted for CGE at the doses of 75, 150, and 300 mg/kg, and an oral pathway to assess the subsequent pharmacological activities.

3.3. Acetic-acid-induced abdominal writhing test

In the acetic-acid-induced abdominal writhing test, in a dose-dependent manner, the CGE decreased the number of writhing incidents. The treatment with CGE at doses of 75, 150, or 300 mg/kg decreased the number of abdominal writhing incidents by 14%, 40%, and 55%, respectively, when compared to the group vehicle. Indomethacin (10 mg/kg) reduced the abdominal writhing by 46%, as shown in Table 3.

3.4. Central activity assessment

3.4.1. Chimney test

CGE, at the dose used in the nociception tests (150 mg/kg, p.o.), did not induce impairment of motor coordination in the chimney test; however, with diazepam (5 mg/kg, p.o.), an impairment of motor coordination was observed (Table 4).

3.4.2. Open field test

The oral treatment with CGE (150 mg/kg) did not significantly alter the parameters observed in the open field test. On the other hand, diazepam (5 mg/kg, p.o.) increased the immobility time of the animals and reduced the parameters of the fecal boluses, rearing, grooming, total crossings, and percentage of the crossings in the central area (Table 4).

3.4.3. Pentobarbital-induced sleep test

The oral treatment with CGE (150 mg/kg) did not significantly alter the sleep latency or the sleeping time when compared to the control group, whereas diazepam (5 mg/kg, p.o.) reduced the latency time and increased the sleeping duration (Table 4).

Table 3
Antinociceptive activity of cashew gum extract (CGE) in acetic acid-induced abdominal writhing test.

	Number of writhes	
	(Mean ± SEM)	% Inhibition
Vehicle 10 mL/kg, p.o.	94.1 ± 3	–
CGE 75 mg/kg, p.o.	81 ± 2.8**	14
CGE 150 mg/kg, p.o.	56.8 ± 3.7***	40
CGE 300 mg/kg, p.o.	42.2 ± 2.6***	55
Indomethacin 10 mg/kg, p.o.	50.7 ± 3***	46

Results are expressed as mean ± S.E.M. and percentage of inhibition of number of writhings in 30 min for each experimental group (Number of mice per group = 8) and percentage of inhibition. ** $P \leq 0.01$ and *** $P \leq 0.001$ compared to the group treated with vehicle, according to ANOVA followed by post-hoc Newman-Keuls' test.

Table 4
Effects of cashew gum extract (CGE) on chimney and pentobarbital-induced sleep tests.

	Central activity assessment		
	Vehicle 10 mL/kg, p.o.	CGE 150 mg/kg, p.o.	Diazepam 5 mg/kg, p.o.
Fecal boluses	1.9 ± 0.7	1.7 ± 0.5	0.8 ± 0.4**
Rearing	63.9 ± 4.0	65.3 ± 4.9	26.2 ± 6.6***
Grooming	4.5 ± 0.8	4.7 ± 0.6	1.6 ± 0.2*
Immobility (s)	11.2 ± 1.0	11.5 ± 1.4	229.2 ± 13.4***
Total crossings	83.5 ± 7.2	81.1 ± 5.3	52.3 ± 13**
Crossings in the central area (%)	55.4 ± 3.8	62.9 ± 1.4	23.5 ± 3.2***
Chimney (s)	6.9 ± 0.9	7.2 ± 1.0	27.4 ± 3.9***
Latency of sleep (s)	236 ± 8.1	247 ± 7.2	147.8 ± 7.0***
Duration of sleep (min)	46.6 ± 6.7	49.8 ± 5.1	132 ± 13.7***

Results are expressed as mean ± SEM of number of fecal boluses, rearings and grooming, immobility time (s), total number of crossings, crossings in the central area (%), time in the chimney (s), latency (s) and duration (min) of sleep in the pentobarbital-induced sleeping test for each experimental group (Number of mice per group = 8).

* $P \leq 0.05$.

** $P \leq 0.01$.

*** $P \leq 0.001$ compared to the group treated with vehicle, according ANOVA followed by Student-Newman-Keuls' test.

3.5. Formalin-induced pain

The treatment with CGE at a dose of 150 mg/kg or indomethacin (10 mg/kg) decreased the licking time only in the second phase of the test, by 33% and 40%, respectively, when compared to the group treated with a vehicle (licking time in the second phase = 188.1 ± 8.5 s). However, morphine (5 mg/kg, s.c., - a positive antinociceptive control) decreased both phases of this test significantly, by 90% and 96%, respectively, compared to the group treated with a vehicle (licking time in the first phase = 73.6 ± 3.9 s), as shown in Table 5.

3.6. Carrageenan-induced paw edema

The oral treatment with CGE at a dose of 150 mg/kg reduced the paw edema in all hours of the test. In the first hour, CGE reduced the difference between the paws by 13%; in the second hour, by 19%; in the third hour, by 25%; and finally, in the four hour, by 24%, compared to the group treated with a vehicle (difference between the paws was 135 ± 1.7, 136 ± 3.4, 125 ± 3.7, and 114 ± 3.1 μL, respectively). The treatment with indomethacin (10 mg/kg, p.o.) reduced the paw edema in all hours of the test by 35%, 45%, 53%, and 52% in the first, second, third, and fourth hour of the test, respectively, compared to the group treated with a vehicle (Table 6).

Table 5
Antinociceptive activity of cashew gum extract (CGE) in formalin-induced pain test.

	Licking time (s) (Mean ± SEM and % Inhibition)			
	Neurogenic pain		Inflammatory pain	
Vehicle 10 mL/kg, p.o.	73.6 ± 3.9	–	188.1 ± 8.5	–
CGE 150 mg/kg, p.o.	65.4 ± 3.9	–	126.4 ± 6.6***	33%
Indomethacin 10 mg/kg, p.o.	72.1 ± 4.0	–	112.3 ± 7.3***	40%
Morphine 5 mg/kg, s.c.	7.4 ± 2.8***	90%	8.2 ± 0.8***	96%

Results are expressed as mean ± SEM. of licking time during the first (0–5 min) and second phase (15–30 min) of the test, in seconds, for each experimental group (Number of mice per group = 9) and percentage of inhibition.

*** $P \leq 0.001$ compared to the group treated with vehicle, according to ANOVA followed by post-hoc Newman–Keuls' test.

3.7. Mechanical hyperalgesia induced by carrageenan or PGE_2

In the experimental protocol in which the animals received carrageenan in the paw, the treatment with CGE (150 mg/kg, p.o.) or indomethacin (10 mg/kg, p.o.) reduced the difference of the nociceptive threshold between the noninflamed and inflamed paw of the animals in response to a mechanical stimulus in all hours of the test. This treatment significantly decreased the difference between the nociceptive threshold, respectively, by 41% and 72% in the first hour, 33% and 68% in the second hour, 36% and 71% in the third hour, and 39% and 53% in the fourth hour of the test, compared to the control group (Table 7).

However, in the experimental protocol in which the animals received PGE_2 in the paw, the treatment with CGE (150 mg/kg, p.o.) or indomethacin (10 mg/kg, p.o.) did not significantly alter the difference of the nociceptive threshold between the noninflamed and inflamed paw of the animals (Table 7).

4. Discussion

The current study investigated the antinociceptive and anti-inflammatory activity, as well as the possible action mechanism of cashew gum extract, polysaccharides free, in several experimental models of pain and inflammation.

Cashew gum is a water-soluble branched acidic heteropolysaccharide that forms a solution with low viscosity, and it is precipitated by organic polar solvents, such as acetone (Marques et al., 1992). According to Silva et al. (2010), the cashew gum possesses in its chemical composition 70% galactose, 11% glucose, 6% glucuronic acid, 5% arabinose, 4% rhamnose, and 1% mannose. However, in this study, the CGE was obtained after the precipitation and removal of polysaccharides, and this process yielded 2.27%.

The chemical characterization by LC-MS and tandem (MS/MS) analyses indicated the presence of seven anacardic acids in CGE, with a difference in the number of carbons and unsaturations. Anacardic acid is a blanket term applied to a family of closely related compounds consisting of salicylic acid with a 15-carbon alkyl chain, which exists either in a fully saturated form or as a monoene, diene, or triene (Hemshekhar et al., 2012). In our chemical analysis, the highest intensity in the extracted ion chromatogram was obtained for molecular ions of anacardic acid with 15 carbon atoms and 1 unsaturation in the alkyl chain. However, the highest concentration was found for anacardic acid 17:1.

Previous studies have shown a considerable number of biological activities for anacardic acids, among them antitumor (Harsha Raj et al., 2016; Sung et al., 2008), anti-acne (Sharma et al., 2013), antibacterial (Kubo et al., 1999; Mamidyala et al., 2013), antioxidant (Trevisan et al., 2006), molluscicidal (Sullivan et al., 1982), antifungal (Begum et al., 2002), and anti-inflammatory activities (Ha and Kubo, 2005; Paramashivappa et al., 2003). Grazzini et al. (1991) showed the ability of anacardic acids to inhibit the enzymes COX and lipoxygenase, which are responsible for the synthesis of lipid mediators that participate in the inflammatory process. Furthermore, Sung et al. (2008) showed that anacardic acids are a potent inhibitor of NF-κB activation, which may explain its antiangiogenic, antiproliferative, proapoptotic, antimetastatic, anti-inflammatory, and immunomodulatory effects.

For preliminary pharmacological screening with CGE, gross behavior or the Irwin test was carried out. This test is often used to evaluate the effects of the extracts of medicinal plants on physiological functions and the behavior of experimental animals, and it permits the determination of potential toxicity, appropriate dose, and routes of administration (Irwin, 1962, 1968; Roux et al., 2005). Our results showed that CGE in its highest dose, intraperitoneally administered, promoted some behavior alterations and the death of an animal on the seventh day. However, the oral treatment with CGE at doses of 30, 100, or 300 mg/kg did not promote behavior alterations. Therefore, for the

Table 6
Anti-inflammatory activity of cashew gum extract (CGE) in the carrageenan-induced paw edema test.

	Difference between the paws (μL) (Mean ± SEM and % Inhibition)							
	1st Hour		2nd Hour		3rd Hour		4th Hour	
Vehicle 10 mL/kg, p.o.	135 ± 1.7	–	136 ± 3.4	–	125 ± 3.7	–	114 ± 3.1	–
CGE 150 mg/kg, p.o.	118 ± 4.7***	13%	110 ± 2.9***	19%	94 ± 3.4***	25%	86.6 ± 3.1***	24%
Indomethacin 10 mg/kg, p.o.	87.6 ± 5.2***	35%	75 ± 4.7***	45%	59 ± 4.9***	53%	54.7 ± 5.5***	52%

Results are expressed as mean ± S.E.M. of the difference between the paws, in μL, for each experimental group (Number of mice per group = 9) and percentage of inhibition.
*** $P \leq 0.001$, compared to the vehicle, according to two-way ANOVA followed by Bonferroni's post-hoc test.

Table 7
Anti-hyperalgic activity of cashew gum extract (CGE) in mechanical hyperalgesia induced by carrageenan or PGE_2.

	Difference between the nociceptive thresholds of paws (g) (Mean ± SEM and % Inhibition)			
	Carrageenan		PGE_2	
	1st Hour			
Vehicle 10 mL/kg, p.o.	164 ± 8.2	–	190 ± 7.1	–
CGE 150 mg/kg, p.o.	96.7 ± 6.5***	41%	174 ± 5.1	–
Indomethacin 10 mg/kg, p.o.	45.9 ± 9.2***	72%	178 ± 6.3	–
	2nd Hour			
Vehicle 10 mL/kg, p.o.	152 ± 3.6	–	184 ± 9.3	–
CGE 150 mg/kg, p.o.	102 ± 4.2***	33%	188 ± 10.2	–
Indomethacin 10 mg/kg, p.o.	48.9 ± 1.9***	68%	183 ± 13.2	–
	3rd Hour			
Vehicle 10 mL/kg, p.o.	136 ± 4.3	–	188 ± 5.8	–
CGE 150 mg/kg, p.o.	87 ± 4.7***	36%	178 ± 9.7	–
Indomethacin 10 mg/kg, p.o.	44 ± 6.7***	71%	188 ± 7.5	–
	4th Hour			
Vehicle 10 mL/kg, p.o.	138 ± 3.9	–	168 ± 5.8	–
CGE 150 mg/kg, p.o.	84 ± 5.8***	39%	166 ± 4.0	–
Indomethacin 10 mg/kg, p.o.	65.2 ± 4.6***	53%	170 ± 3.5	–

Results are expressed as mean ± S.E.M. of the difference between the nociceptive thresholds of paws, in grams, for experimental group (Number of mice per group = 8) and percentage of inhibition. ** $P \leq 0.01$, *** $P \leq 0.001$, compared to the vehicle, according to two-way ANOVA followed by Bonferroni's post-hoc test.

pharmacological tests, we opted for an oral administration of CGE at doses of 75, 150, and 300 mg/kg.

The model of abdominal writhing induced by acetic acid was the first test performed in this study to evaluate the possible antinociceptive activity of CGE. The administration of the acetic acid in the peritoneal cavity induces pain and acute inflammation from the liberation of chemical mediators, such as bradykinin, substance P, prostaglandins, and cytokines (Malvar et al., 2014; Ribeiro et al., 2000; Zakaria et al., 2006). As a behavioral response, the mice produced abdominal contractions with extension followed by rotating one or both hind legs. In this test, CGE at doses of 75, 150, or 300 mg/kg decreased, in a dose-dependent manner, the number of writhing incidents induced by acetic acid, suggesting antinociceptive activity for the extract. To reduce the number of animals experimented upon, as recommended by the CEUA, only the intermediate dose of CGE was used in the following tests.

To verify if the analgesic effect was not a result of motor deficits or sedation, the mice were subjected to chimney, open-field, and pentobarbital-induced sleep tests after the oral administration of CGE. Our results showed that the treatment with CGE, in the analgesic dose, did not cause motor impairment in the chimney test, nor did it cause alterations in the parameters of open-field or changes in the latency and sleep time, confirming that the antinociceptive effect of CGE was not a false-positive result due to the possible peripheral neuromuscular blockade or sedation.

The formalin-induced pain test was conducted to differentiate the antinociceptive effect of CGE. This test is well described to evaluate the neurogenic pain (first phase of test) caused by direct stimulation of the nociceptors by formalin and preformed nociceptive mediators (Dubuisson and Dennis, 1977; Hunskaar and Hole, 1987; Shibata et al., 1989; Corrêa and Calixto, 1993; Munron, 2007) and to evaluate the inflammatory pain (second stage of the test) caused by the stimulation of nociceptors and the release of many pro-inflammatory mediators, such as the prostaglandins and cytokines (Fujimaki et al., 1992; Shibata et al., 1989; Omote et al., 1998). The pain, in this test, can be inhibited by central analgesics (both phases) and anti-inflammatory drugs (second phase) (Hunskaar and Hole, 1987; Le Bars et al., 2001). As can be observed, CGE in an intermediate dose (150 mg/kg, p.o.) reduced the licking time only in the second phase of the test, suggesting an antinociceptive effect dependent on an anti-inflammatory activity.

Some studies have shown that others species in the family Anacardiaceae, such as *Mangifera indica* L. (Dhananjaya and Shivalingaiah, 2016; Oliveira et al., 2017), *Spondias tuberosa* (Siqueira et al., 2016), *Antrocaryon klaineanum Pierre* (Fongang et al., 2017), *Pistacia integerrima galls* (Rauf et al., 2014, 2016), *Pistacia vera L* (Orhan et al., 2006; Grace et al., 2016), and *Lannea coromandelica* (Imam and Moniruzzaman, 2014) have antinociceptive and anti-inflammatory effects, which were demonstrated in different experimental models.

The anti-inflammatory effect of CGE was confirmed by the carrageenan-induced edema test. The intraplantar administration of carrageenan promotes an inflammatory process with the extravasation of cells and proteins, leading to the formation of edema by action of pro-inflammatory mediators (Liew and McInnes, 2002). The results show that the treatment of animals with CGE (150 mg/kg, p.o.) reduced the edema formation at all hours of the test, showing that this extract has an anti-edema activity and confirming the action to be anti-inflammatory, as suggested in the second phase of the formalin test.

Similar to cashew gum, other parts of *Anacardium occidentale* also have been described in the literature to have anti-inflammatory and antinociceptive activities. The use of leaf extracts of this plant in preclinical studies has shown a significant reduction in the paw edema induced by carrageenan, in the paw licking time from the formalin injection, and in the number of abdominal writhing incidents induced by acetic acid (Onasanwo et al., 2012; Pawar et al., 2000). Furthermore, studies involving the stem bark of *Anacardium occidentale* showed an excellent anti-inflammatory effect in different in vivo models (Mota et al., 1985; Olajide et al., 2004; Swarnalakshmi et al., 1981; Vilar et al., 2016) and in the inhibition of the NF-κB pathway as a mechanism of action (Olajide et al., 2013). In addition, cashew apple juice has also been presented to have a potential anti-inflammatory effect and wound healing effect in animal models (Vasconcelos et al., 2015).

In the present study, the treatment with CGE also reduced the hyperalgesia induced by carrageenan in the paw pressure test. It has been reported that the peripheral mechanism of carrageenan-induced hyperalgesia is due in part to the formation of PGE_2, which is responsible for the sensitization of nociceptive fibers (Kawabata et al., 1992; Treede et al., 1992; Ferreira, 1972, 1990). PGE_2 is synthesized by the action of

cyclooxygenases enzymes on arachidonic acid, which is formed by the action of the phospholipase A_2 enzyme on membrane phospholipids. Logically, inhibitor anti-inflammatory agents of cyclooxygenases or phospholipase A_2 suppress the formation of prostaglandins and present an antihyperalgesic effect in the carrageenan-induced hyperalgesia test (Juárez-Rojop et al., 2015; Chopade and Mulla, 2010; Tsuchida et al., 2015). CGE reduced the hyperalgesia induced by carrageenan and, therefore, may suggest a modulation of this extract on arachidonic acid metabolism.

To determine whether the anti-inflammatory effect of CGE depends on the inhibition of PGE_2 synthesis by a possible inhibition or modulation in the expression of enzymes involved in arachidonic acid metabolism, we performed the paw pressure test, injecting PGE_2 directly into the paw of animals to induce hyperalgesia. In this test, the same dose of CGE used in the previous test did not reduce the hyperalgesia caused by PGE_2, similar to the positive control, indomethacin, a non-selective COX inhibitor. Considering the ability of anacardic acids to inhibit COX (Grazzini et al., 1991) and the NF-κB pathway (Sung et al., 2008), this work indicates that CGE may be directly inhibiting COX or PLA_2 activity or inhibiting the expression of these enzymes, due to the presence of anacardic acid in its composition. However, more pharmacological and chemical studies are necessary to evaluate the real interaction of CGE with these enzymes and to quantify the cytokines involved in the inflammatory process in order to achieve a better understanding of the mechanisms involved in the anti-inflammatory effect of this extract.

5. Conclusion

In this study, the results obtained with CGE demonstrated the potential antinociceptive and anti-inflammatory effects of cashew gum extract in different experimental models and justify its use in traditional medicine to treat painful conditions and inflammatory diseases. Also, our results suggest that the action mechanism of CGE depends on a reduction in the PGE_2 levels. The chemical characterization of CGE showed that the anacardic acids are the predominant phytoconstituents in this extract, and therefore, part of the analgesic and anti-inflammatory effects of cashew gum should also be attributed to them, independent of polysaccharides. Finally, this study supports the use of cashew gum in popular medicine and shows its therapeutic potential for the development of analgesic and anti-inflammatory phytomedicines. However, more pharmacological studies will be needed to better clarify the mechanism(s) responsible for the antinociceptive and anti-inflammatory action of cashew gum.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

Author Contributions

Daiany P. B. Silva and E. A. Costa (Head) conceived the study, designed the pharmacological assays, and drafted the manuscript. Iziara F. Florentino, Lorrane K. S. Moreira, and Adriane F. Brito carried out the pharmacological assays. Verônica V. Carvalho, Marcella F. Rodrigues, and Géssica A. Vasconcelos carried out the study, and Boniek G. Vaz (Head) carried out the mass assays. Marcus A. Pereira-Junior and Kátia F. Fernandes (Head) carried out the identification and collection of the vegetable material and preparation of the extract. All authors critically revised the manuscript for important intellectual content and approved its final version.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.jep.2017.11.021.

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