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Original Article

Phytochemical study and anti-inflammatory and antioxidant potential of *Spondias mombin* leaves

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ABSTRACT

Spondias mombin L., Anacardiaceae, is a plant native of Brazil, where it is known as "cajá". In order to find a potential application for this native species, the anti-inflammatory and antioxidant effects were investigated. The anti-inflammatory activity was evaluated using the *in vivo* model carrageenan-induced peritonitis in mice. The *in vitro* antioxidant potential as well the cytotoxicity against 3T3 fibroblast cells also were evaluated. Through High Performance Liquid Chromatography-diode array detector analysis, an analytic method was developed and validated. It allowed the identification and quantification of ellagic acid and chlorogenic acid in hydroethanolic extract of *S. mombin* leaves. This extract showed anti-inflammatory effect at 100, 200, 300 and 500 mg/kg, however, the ethyl acetate fraction, at 200 mg/kg, showed the highlighted results. Ellagic acid and chlorogenic acid (2.5, 5 and 10 mg/kg) also inhibited the leukocyte migration to the site of inflammation. The extract, fractions and compounds showed significant antioxidant potential when evaluated in different assays. The results shown in this work suggest the anti-inflammatory potential of the leaf extract of *S. mombim* on peritonitis model induced by carrageenan, it was also observed antioxidant properties associated with an absence of cytotoxicity in cell culture. Further *in vivo* studies are required to confirm the anti-inflammatories action of *S. mombin* and its possible anti-inflammatory mechanisms of action.

Introduction

The ethnopharmacology associated with the chemical study has become an important tool in bioprospecting. Studies have associated information on the traditional use of medicinal plants with phytochemical and pharmacological studies, searching for new drugs and herbal medicines (Medeiros et al., 2013). Brazil has one of the world's highest levels of biodiversity, including several plants of economic interest (Albuquerque et al., 2007). The North and Northeast regions are those where most of the existing biodiversity is concentrated, which allows access to numerous kinds of plants and fruit specie (Mattietto et al., 2010).

Spondias mombin L. is a plant belonging to the Anacardiaceae family. This species is native of Brazil and their fruits are commonly known as "cajá". It occurs widespread through tropical regions in America, Africa and Asia. In Brazil, it is mainly found in the North and Northeast (Soares, 2005). This native fruit has potential use for processing to make jelly, juice, jams and ice cream mainly in northeastern Brazil and their leaves are used in folk medicine for the treatment of several topic and systemic diseases like inflammation of the mouth and throat and in cases of prostatitis and herpes labialis (Lorenzi and Matos, 2008). Despite this therapeutic approach, the chemical studies are scarce and some phenolic acids, flavonoids, tannins and triterpenes have been isolated from *S. mombin* leaves so far (Corthout et al., 1991; Fred-Jaiyesimia et al., 2009). A survey of the relevant literature revealed that the leaves of *S. mombin* exhibit antimicrobial, leishmanicide, antiviral, antiedematogenic, hypoglycemic and antioxidant properties (Corthout et al., 1994; Fred-Jaiyesimia et al., 2009; Nworu et al.,

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2011; Silva et al., 2011, 2012). Regarding the anti-inflammatory effect only one report support its popular use (Nworu et al., 2011). The chemical characterization of the leaf extract from this species as well as the identification of chemical markers that could be useful in extract standardization for applying it in herbal drugs was not developed. In addition, it is important to highlight that the extract standardization enable us to guarantee reliable results regarding biological activities.

Taken together, the aims of this study were: (i) to develop an analytic methodology by HPLC-DAD in order to quantify chemical markers in extract from *S. mombin* leaves and (ii) to evaluate their anti-inflammatory potential *in vivo* model, (iii) to investigate the antioxidant potential since the oxidative stress is an important aspect associated to inflammation and (iv) and to evaluate the cytotoxicity against 3T3 cells.

Materials and methods

Phytochemical procedures

Plant material and extraction

Leaves of *Spondias mombin* L., Anacardiaceae, were collected in Dom Marcolino Dantas, Rio Grande do Norte, Brazil (SS-RN°28′14″ W 35°27′37″), on November 2011. The plant material was identified by Alan de Araújo Roque and a voucher specimen was deposited at Herbarium of Federal University of Rio Grande do Norte, Brazil, under the reference number 12252. *S. mombin* leaves were dried at room temperature and triturated mechanically. The extract from *S. mombin* leaves was prepared by maceration in ethanol: water (70: 30, v/v), for seven days, filtered and later lyophilized. It was obtained the hidroethanolic extract from *S. mombin* leaves (HE).

In order to characterize the active compounds from HE, a portion of the extract was resuspended in water and subjected to liquid-liquid partition with solvents of increasing polarity: hexane (Hex) (3× 300 ml), dichloromethane (DCM) (3× 300 ml), ethyl acetate (EtOAc) (3× 300 ml) and butanol (ButOH) (3× 300 ml). Five fractions were obtained (Hex, DCM, EtOAc, ButOH) and an aqueous residual fraction (ARF). All fractions were dried under reduced pressure at 45 °C.

Chromatographic procedures

The analytical standards were purchased from: chlorogenic acid hemihydrate (Sigma–Aldrich®, ≥98%), ellagic acid (Roth®, ≥98%) and isoquercitrin (Hwi Analytik GmbH®, 94.16%). The solvents used in extract preparation were Analytical Grade and in HPLC analysis were used HPLC grade solvents. The water was purified with a Milli-Q system (Millipore®, Bedford, USA). All solutions prepared for HPLC analysis were filtered through a 0.45 mm membrane before use.

Quantitative high-performance liquid chromatography analysis

The quantification of the phenolic compounds was performed in a high performance liquid chromatography (HPLC) using a Perkin Elmer 200 series equipped with a diode array detector (DAD), a quaternary pump, a degasser online and an autosampler. All HPLC data were processed using the TotalChrom® Workstation software. Chromatographic analyzes were performed using a reversed phase column (Phenomenex®) C-18 (4.6 mm × 250 mm, 5 μm). An elution system with acetonitrile 100% (solvent A) and acetic acid 1%, adjusted to pH 3 (solvent B) at the follow gradient flow was used: 0–30 min, a linear change from A:B (13:87, v/v) to A:B (15:85, v/v); 30–40 min, an isocratic elution with A:B (15:85, v/v); 40–45 min, a linear variation up to A:B (20:80, v/v). The flow rate was kept constant at 1 ml/min, and the chromatograms were recorded at 340 nm, whereas the UV spectra were monitored at wavelength of 450–200 nm. The peaks were characterized by comparison of their retention times and UV spectra with the reference standards and by a co-injection (extract + reference standard). The reference standard solutions were prepared in H_2O:methanol (3:2, v/v) at different concentrations: chlorogenic acid – 10, 20, 30, 40, and 50 μg/ml; ellagic acid – 20, 30, 40, 50, and 60 μg/ml. The HE from *S. mombin* leaves was analyzed at 2.5 mg/ml. The reference standards were analyzed in triplicate and the average peak areas measured.

Validation of analytical procedure

The validation of analytical procedures was performed in accordance with ICH guidelines (ICH, 2005). The validated parameters were specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ) and limit of detection (LOD).

Pharmacological assays

Anti-inflammatory activity

Animals. Experiments were carried out using male BALB/c mice (6–8 weeks old). All mice were housed, 5–6 per cage at a room temperature of 22 ± 2 °C, and a 12 h:12 h light/dark cycle, with *ad libitum* access to water and food. Groups of six animals were used in each test group and control animals received saline only. All *in vivo* experiments were approved by the ethical committee of the Federal University of Rio Grande do Norte, Brazil (protocol number 013/2013) and were carried out according to the current guidelines for laboratory animal care. Each animal was used only once.

Carrageenan-induced peritonitis. The inflammation was induced in mices by administration of 1% carrageenan, intraperitoneally (*i.p.*). The animals ($N = 5$) were *i.p.* pretreated with (i) vehicle (saline, 0.1 ml/10 g) or (ii) dexamethasone (0.5 mg/kg) or (iii) HE from *S. mombin* leaves (100, 200, 300 or 500 mg/kg) or (iv) fractions DCM, EtOAc, ButOH and ARF (200 mg/kg) or (v) chlorogenic acid and ellagic acid (2.5, 5 or 10 mg/kg). Thirty minutes after treatment with the samples, the animals received an injection into the peritoneal cavity of 50 μl of 1% carrageenan. After 4 h, the animals were sacrificed with an overdose of thiopental at 80 mg/kg, *i.p.*, followed by cervical dislocation. Peritoneal exudates were collected with 2 ml of ice-cold PBS for abdominal laparoscopy. The exudates were centrifuged at 250 × *g* for 5 min at 4 °C. The cell pellet was diluted 1:10 in Türk solution and the total leukocyte number was determined in a Neubauer chamber. These doses of HE are based on previous work published in literature (Nworu et al., 2011) and pilot studies in our laboratory (*data not shown*). Chlorogenic acid (Sigma–Aldrich®, ≥98%) and ellagic acid (Roth®, ≥98%) used in experiment *in vivo* and *in vitro* model were purchased commercially.

Antioxidant activity. The antioxidant activity was performed with HE from *S. mombin* leaves, DCM, AcOEt, ButOH and ARF fractions and the compounds ellagic acid and chlorogenic acid. The DPPH radical scavenging, Scavenger of superoxide ion (O_2^-), Reducing power and Sequestration of the hydroxyl radical (OH^-) activity was evaluated. The extracts and fractions were tested at 60, 125, 250 and 500 μg/ml and the compounds at 5, 15, 30 and 60 μg/ml.

Viability cell. The 3T3 cells were grown in 75 cm^2 flasks with 9 ml of medium Dulbecco modified Eagle culture medium (DMEM). Then, for the assay cells, they were transferred to 96 well plates and it was grown at 5×10^3 cell/well. These cells were grown overnight in order to attach in 200 μl medium at 37 °C and 5% CO_2. After that, HE from *S. mombin* leaves, fractions or compounds were added in three different concentrations according results obtained in antioxidant assays (60, 125 μg/ml and 500 μg/ml in order to verify if the HE, fractions or compounds showed cytotoxicity in higher concentration during the periods of 24 h). After incubation, the samples

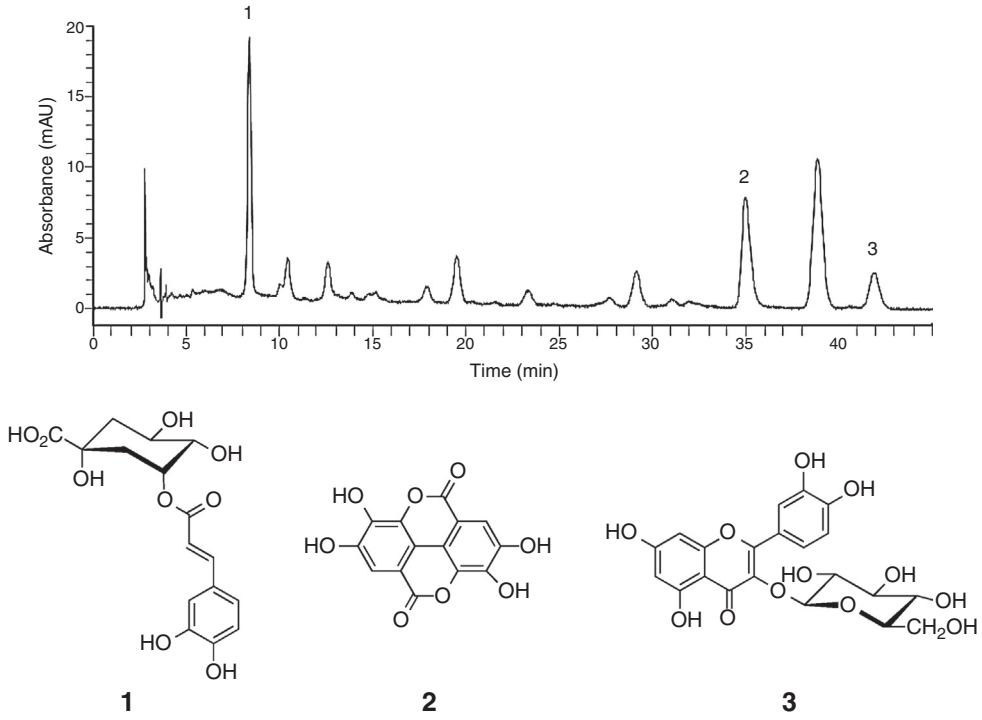

Fig. 1. Chromatogram at 340 nm obtained by HPLC-DAD of hydroethanolic extract from *Spondias mombin* leaves. Chlorogenic acid (**1**), ellagic acid (**2**) and isoquercetrin (**3**).

were removed and cells were washed with 200 μl PBS (twice), after that it was added 100 μl of fresh medium. Then, it was added 100 μl of MTT reagent (5 mg/ml) (Sigma, M5655) dissolved in fresh DMEM. The cells were then incubated for 4 h at 37 °C and 5% CO_2. To solubilize the formazan crystals it was used 100 μl of ethanol which was added to each well and after 20 min the absorbance was read at 570 nm using the ELISA reader (Biotec μQuant). As a control, untreated normal cells were cultured only in the presence of DMEM. The cell proliferation (%) was expressed as: [(Sample Abs 570 nm × 100)/Control Abs 570 nm].

Statistical analysis

Results were expressed as means ± standard device. Statistic analysis was conducted by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. Statistical significance was considered of 95% ($p < 0.05$). The statistical program INSTAT, Graph-Pad, San Diego, CA, was used.

Results and discussion

Phytochemical analysis of S. mombin

Analysis by HPLC-DAD though UV analysis of each peak showed that the HE of *S. mombin* leaves presents a large amount of phenolic and flavonoid derivatives. The qualitative analysis of HE enabled us to indicate the occurrence of three phenolic major compounds: chlorogenic acid, ellagic acid and isoquercetin and one no identified peak at UV 340 nm, by the retention time, the UV spectra and by co-injection of the HE and reference standard (Fig. 1).

In the literature there is no report about the identification of chlorogenic acid and isoquercetin in *S. mombin* species, however, ellagic acid has already been identified in methanol extract from the leaves (Silva et al., 2012). Due to this, an analytical methodology was developed and validated for the quantification of chlorogenic acid and ellagic acid by HPLC for HE from *S. mombin* leaves. Since the presence of flavonoids and phenolic acids in species of *Spondias* genus is described in literature (Corthout et al., 1992; Satpathy et al., 2011; Silva et al., 2012; Engels et al., 2012), these compounds could be highlighted as chemical markers for this genus. The flavonoid isoquercetin cannot be quantified in HE due to unavailability of suitable amount of their reference standard. The method developed showed a chromatogram of the HE from *S. mombin* leaves with symmetrical peaks (tailing factor: 0.9 and 0.75, chlorogenic acid and ellagic acid, respectively) and appropriated resolution (2.0 to both compounds).

Table 1
Phenolic acids content in hydroethanolic extract from *Spondias mombin* leaves.[a]

Species	Compound	Content/g extract
Spondias mombin leaves (2.5 mg/ml)	Chlorogenic acid (*Rt* = 8.28 min)	12.0 mg/g extract
	Ellagic acid (*Rt* = 34.21 min)	19.4 mg/g extract

[a] Expressed as mg/g of extract ± SD ($n = 3$)/*Rt* = retention time.

Standard solutions of chlorogenic acid and ellagic acid were prepared, at a concentration range from 10 to 60 μg/ml, and the chromatographic method showed a suitable linear relationship ($r^2 > 0.995$) for both standard solutions. This enabled us to measure the content of the chlorogenic acid and ellagic acid in HE (Table 1). The limit of quantification (LOQ) and the limit of detection (LOD) were defined by relative standard deviation (RSD > 5%) and by a signal: noise ratio of 3:1 respectively (Table 2).

The precision was determined by repeatability (intra-day assay) and intermediate precision (inter-day assay) (Table 3). The intra-day assay was performed by triplicate analysis of three different concentrations of reference standard solutions, and expressed as relative standard deviation. Good repeatability was obtained from lower, medium and higher concentrations of the curve, with an RSD <5% for all standard analyses. The inter-day assay was determined by the analysis of a medium concentration in the curve, three times a day, on three different days. As well as other parameters of precision, the RSD value did not exceeded the limits recommended in

Table 2
Calibration, LOD and LOQ data of phenolic acid standards.

Compound	Linearity range (μg/ml)	Calibration equation[a]	Correlation factor (r^2)	LOD[b] (μg/ml)	LOQ[b] (μg/ml)
Chlorogenic acid	10–50	$y = 9727.8x - 6129$	0.9936	0.625	5.0
Ellagic acid	20–60	$y = 13976x - 28{,}598$	0.9901	1.25	5.0

[a] Six data point ($n = 3$).
[b] LOD = limit of detection; LOQ = limit of quantification.

Table 3
Repeatability, intermediate precision and accuracy data of phenolic acids standards.

Compound	Repeatability		Intermediate precision		Recovery[a]	
	Concentration (μg/ml)	R.S.D. (%)	Concentration (μg/ml)	R.S.D. (%)	Mean %	R.S.D. (%)
Chlorogenic acid	10	1.0	10	1.0	110	1.0
	30	2.4	30	0.3		
	50	0.4	50	1.0		
Ellagic acid	20	0.8	20	2.5	102	0.8
	30	1.0	30	1.2		
	50	4.5	50	0.8		

[a] Recovery was determined by injection of spiked samples, in triplicate, with reference standard.

the literature (Cass and Degani, 2001; ICH, 2005). Concerning accuracy, an important recovery was observed for the standards in both extracts, which was determined by spiking samples (2.5 mg/ml) with the standard solutions of ellagic acid (50 μg/ml) and chlorogenic acid (10 μg/ml) (1:1, v/v) (Table 3).

The quantification of individual compounds was performed using an analytical regression curve ($r^2 > 0.991$). The standards were analyzed in triplicate and the average of the measured peak areas. After obtaining the equation of the straight the compounds were quantified according to the area under the curve. It was found 19.4 mg/g of ellagic acid and 12 mg/g of chlorogenic acid in the HE of *S. mombin* leaves (Table 1). A previous report about quantification of ellagic acid in *S. mombin* showed a content of 41.56 ± 0.01 mg/g in the methanol: water (80:20, v/v) leaves extract (Silva et al., 2012) but the environmental conditions were not described. This difference can be attributed to the solvent used in the extraction process as well as to the edafoclimatic features in Brazilian northeast, where phenolic derivatives are particularly susceptible to environmental changes in planting the behavior (Araújo et al., 2012). Another possibility is the biosynthesis of secondary metabolites, since the biogenesis of phenolic acids, flavonoids and tannins are very similar and have the same precursors, which can generate a compensation mechanism for the production of a particular secondary metabolite.

In this work the harvest of plant material was carried out in summer and in rainforest, the solvent used for extraction was composed of ethanol: water (70:30, v/v). There are no reports in the literature about the quantification of chlorogenic acid in the species *S. mombin* thus it highlights our results since it was quantified as 12 mg metabolite/g extract.

Assessment of anti-inflammatory activity

Previous studies with species of *Spondias* genus showed anti-inflammatory activity. According to literature studies with *Spondias mangifera* using butanol and ethyl acetate fractions obtained of ethanolic extract administered orally at doses 75, 150, 300 mg/kg b.w. showed a significant reduction in paw volume when compared with the respective control group challenged by carrageenan (Sachan et al., 2011). It was also noted the study of ethyl acetate extract of *Spondias pinnata* had anti-inflammatory effects at doses of 200, 400 mg/kg, b.w. ($p < 0.001$) in reducing rat paw edema induced by carrageenan assay (Rao et al., 2009).

Additionally was reported the anti-inflammatory activity of *S. mombin* leaves in paw edema model (Nworu et al., 2011). The study showed that treatment with the methanol extract from *S. mombin* leaves at 100, 200 and 400 mg/kg inhibited by a dose dependent way the paw edema induced by carrageenan besides causing reduction of the LPS-induced by TNF-α and in production of nitric oxide (Nworu et al., 2011). Based on these studies and in popular use of *S. mombin* in inflammatory processes, our study evaluated the *in vivo* anti-inflammatory extract potential by acute peritonitis model using carrageenan-induced dexamethasone (5 mg/kg, *i.p.*) as reference compound.

This study aimed to test the extract to another inflammation model in order to assess other inflammatory parameters such as leukocyte migration. The carrageenan-induced peritonitis is a well characterized experimental model of acute inflammation widely used to test new anti-inflammatory therapies, it enable the quantification and the correlation of cellular migration on inflammatory exudate (Sedgwick and Lees, 1986; Paulino et al., 2008; Bitencourt et al., 2014; Lima et al., 2014).

Carrageenan is an indirect phlogistic agent that in rats' peritoneum induces neutrophil migration which is dependent of resident macrophage activation (Souza et al., 1988). Furthermore, studies showed that carrageenan injection into the peritoneum of rats induces the expression of nitric oxide synthase and COX-2 resulting so in liberation of a large amount of nitric oxide and prostaglandins (Tomlinson et al., 1994; Hatanaka et al., 1999). We have found that intraperitoneal injection of carrageenan was able to induce an inflammatory reaction which is revealed by high cell migration to the peritoneal cavity.

Our results demonstrated that the anti-inflammatory potential of HE of the *S. mombin* leaves at 100, 200, 300 and 500 mg/kg reduced the number of leukocytes influx to peritoneal cavity of treated animals. In comparison with the control group treated only with carrageenan, the HE at 300 and 500 mg/kg showed the most significant effect with an inhibition of leukocyte migration of 51.75% and 55.54%, respectively (Fig. 2A). The signs of acute inflammation are vasodilatation, edema and leukocyte infiltration (Sherwood and Toliver-Kinsky, 2004). This study showed that the extract of *S. mombin* could act in one of the inflammation mechanism inhibiting significantly the leukocyte migration to site of inflammation.

In order to obtain an enriched extract of active components was carried out liquid-liquid partition, which resulted in fractions DCM, EtOAc, ButOH, and ARF. These fractions were tested and showed significant anti-inflammatory activity at 200 mg/kg (Fig. 2B). However, the EtOAc fraction showed better inhibition of leukocyte migration (83.67%), when compared with the control group treated only with carrageenan. The strategy of use a liquid-liquid partition

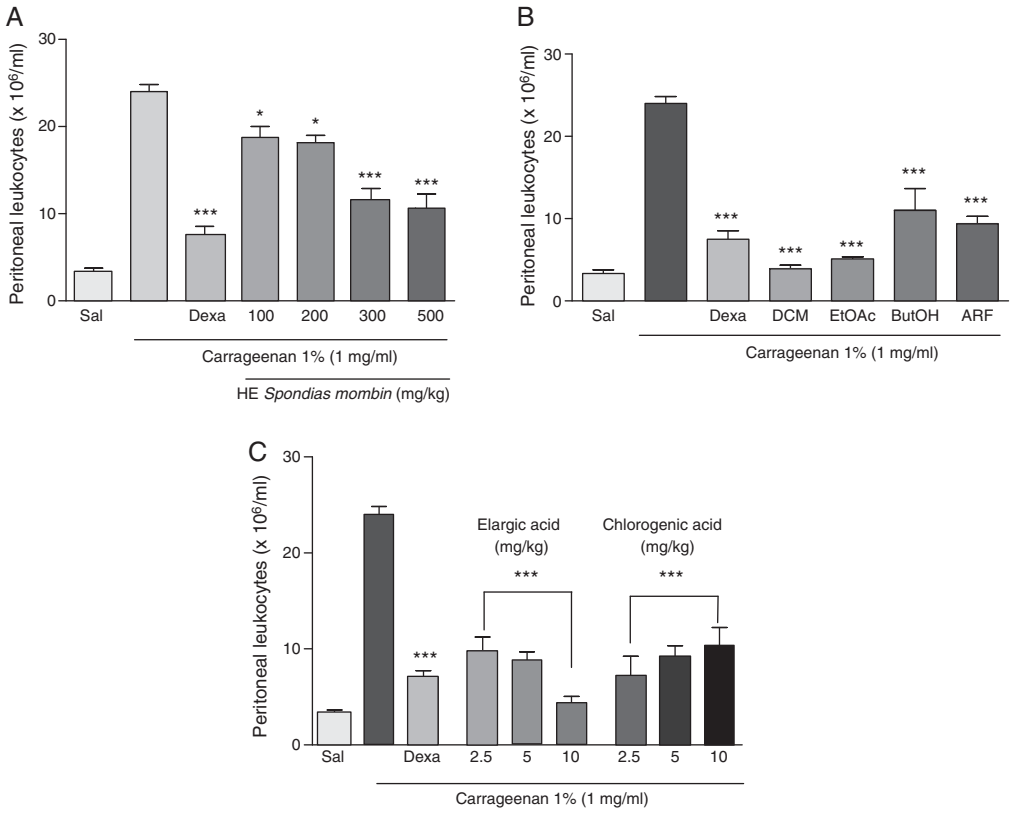

Fig. 2. Effect of hydroethanolic extract of the *Spondias mombin* leaves on leukocyte migration induced by carrageenan in an acute peritonitis model. [a]HE tested at concentrations of 100, 200, 300 and 500 mg/kg, *i.p.* [b]Fractions: DCM: dichloromethane, EtOAc: ethyl acetate, ButOH: *n*-butanol and ARF: aqueous residual fraction at 200 mg/kg, *i.p.* [c]ellagic acid, chlorogenic acid tested at concentrations of 2.5, 5 and 10 mg/kg. Sal: saline. Dexa: dexamethasone (0.5 mg/kg, i.p.). Data are expressed as mean ± standard deviation. $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* the positive control group (treated with carrageenan only). $N = 6$.

with several polarity solvents could be considered useful since one fraction (EtOAc) showed a better potential in comparison to HE.

HE and fractions were analyzed by HPLC-DAD (340 nm) and show the presence of phenolic compounds. This is an important finding, since many phenolic are related to the anti-inflammatory activity of various plants (Rotelli et al., 2003; Santangelo et al., 2007). In EtOAc fraction were identified and quantified two phenolic compounds ellagic acid and chlorogenic acid, and these were evaluated your potential anti-inflammatory, in order to verify the contribution of these compounds for the bioactivity of the extract.

These compounds were assayed at 2.5, 5 and 10 mg/kg, *i.p.* No statistical differences between the treatment groups (Fig. 2C) were observed. It is important to emphasize that the dose of 10 mg/kg showed the best inhibition profile to the ellagic acid and chlorogenic acid that was demonstrating by inhibition of leukocyte migration of 82 and 70%, respectively, to the site of inflammation.

Besides the inhibition of leukocyte migration the ellagic acid and chlorogenic acid it has activity in other ways, as reported in other studies.

In the literature chlorogenic acid is able to inhibit nitric oxide production, inhibit the expression of both COX-2 and pro-inflammatory cytokines (including IL-1β and TNF-α) (Esposito et al., 2014), as well as it can also act by inhibiting the nuclear translocation and activation of NF-κB, a master regulator of inflammation (Francisco et al., 2013; Hwang et al., 2014). Also, *in vivo* studies showed that chlorogenic acid at 50 and 100 mg/kg inhibited the rat paw edema induced by carrageenan (Santos et al., 2006). In addition, important anti-inflammatory and antinociceptive activities by chlorogenic acid were demonstrated since this compound has taking greater action than salicylic acid (Yonathan et al., 2006).

Regarding ellagic acid, this compound was able to inhibit COX-2 and NF-κB (Marin et al., 2013) and presented anti-inflammatory activity at 10 and 50 mg/kg when tested in induced arthritis animal model and did not show any effect on disease development in a lower dose, but inhibited the paw volume ($p < 0.05$) with the higher dose. In our analysis, ellagic acid and chlorogenic acid inhibited a leukocyte migration in acute peritonitis model, thus providing a new and additional evidence of its anti-inflammatory potential. Taken together, these results suggest that ellagic acid and chlorogenic acid could be involved in the anti-inflammatory action observed for HE extract.

The inhibition of leukocyte migration into the peritoneal cavity may be by two mechanisms: by inhibiting the production of chemotactic substances and/or expression of adhesion molecules, this because the leukocytes require chemotactic substances that facilitate your migration to the place of injury (Sherwood and Toliver-Kinsky, 2004).

In summary, can said that the HE of *S mombin* has the ability to inhibit leukocyte migration to the peritoneal cavity by acute peritonitis model and that the compounds ellagic acid and chlorogenic acid may be involved with this activity. However it takes more studies to justify the use of the leaf extract from *S. mombin* to treat inflammatory problems and to elucidate the mechanisms involved in this action.

Assessment of antioxidant activity

Inflammation is a pathological condition that could be pointed as a cause of cancers and express aging processes, since the inflammatory process induces oxidative stress and reduces the antioxidant capacity (Khansari et al., 2009). Thus, the antioxidant

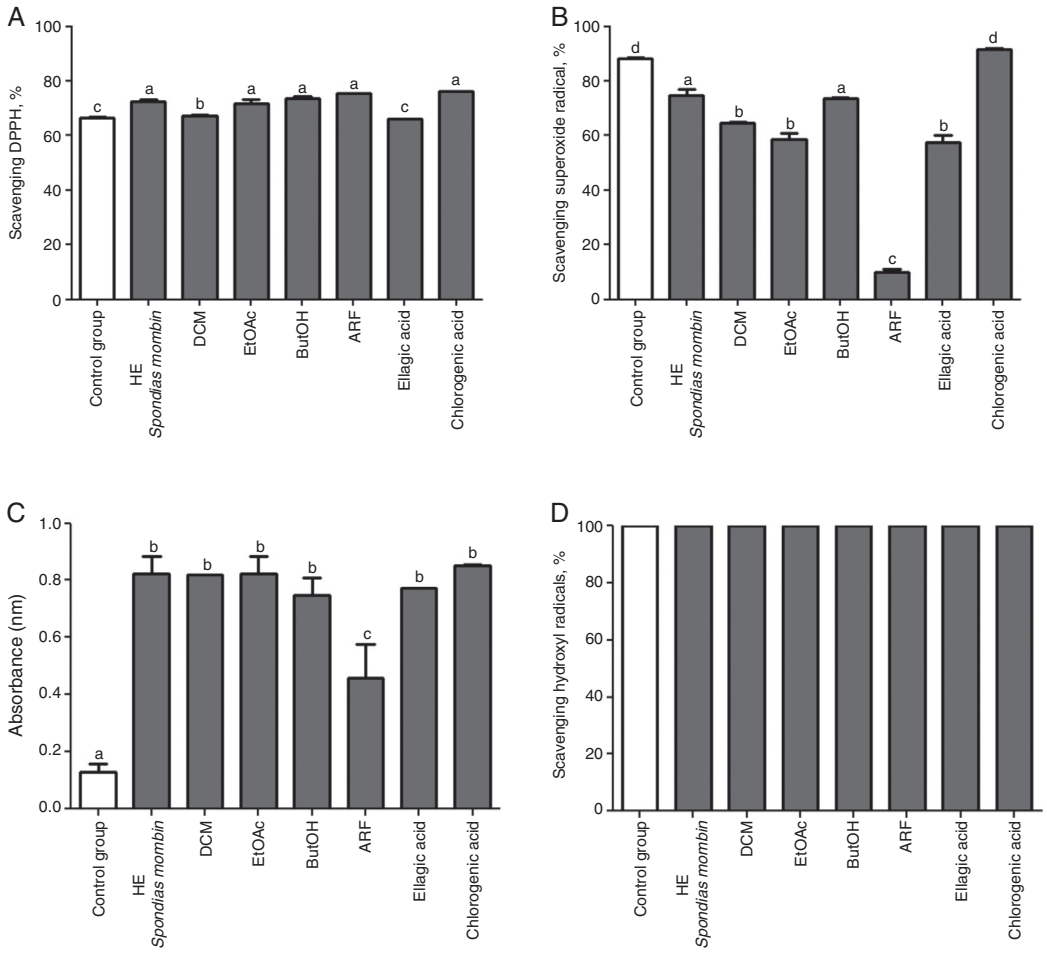

Fig. 3. Antioxidant activity of hydroethanolic extract of the *Spondias mombin* leaves. *X* axis corresponds to hydroethanolic extract from *S. mombin* leaves (HE); fractions: DCM: dichloromethane, EtOAc: ethyl acetate, ButOH: *n*-butanol, ARF: aqueous residual fraction and ellagic acid and chlorogenic acid. The graphs represent the data obtained for scavenging DPPH radical (control group: gallic acid 30 mg/ml) a; superoxide radicals (control group: gallic acid 30 mg/ml) b; reducing power (control group: ascorbic acid 0.2 mg/ml) c; scavenging hydroxyl radicals (control group: gallic acid 15 mg/ml) d; expressed as mean ± standard deviation of percentage of sequestration DPPH at a concentration of 60 μg/ml of the HE and fractions and 30 μg/ml of chlorogenic acid and ellagic acid. a, b, c. Different letters mean significant difference between the crude extract fractions and compounds identified ($p \leq 0.05$).

capacity associated with an anti-inflammatory potential are desirable features for a bioactive compound. Considering the anti-inflammatory potential demonstrated by HE, DCM, EtOAc, ButOH, ARF fractions, and ellagic acid and chlorogenic acid compounds, it is important to evaluate their antioxidant potentials. This investigation was performed in several approaches: (i) scavenging free radical DPPH assay, (ii) superoxide radical scavenging activity, (iii) reducing power, and (iv) hydroxyl radical scavenging activity.

In the DPPH assay (Fig. 3) it was observed a potential donation of electrons or H^+ ions with values ranging from scavenging 66% to 76% for all samples tested. The HE, ButOH and chlorogenic acid were able to sequester 74.53, 73.71 and 91.47%, of the free radical, respectively. In the assay of reducing power to evaluate the reductive capacity it was observed a positive result for all samples analyzed (Fig. 3). Furthermore, it was observed that all samples tested for the hydroxyl radical scavenging activity showed sequestration percentage higher than 100%. The antioxidant capacity of the leaves can be associated to their bioactive compounds, mainly antioxidant polyphenols, because of their ability to scavenge free radicals (Mandic et al., 2008). On this way, we suggest that the phenolic acids present in the extract and fractions of *S. mombin* could contribute for the extract antioxidant activity.

Finally, a preliminary evaluation of the potential cytotoxicity of HE, fractions (DCM, AcOEt, ButOH, ARF), and the ellagic acid and chlorogenic acid compounds was carried out. It was observed that there was an increase in mitochondrial metabolism of 3T3 fibroblasts, suggesting that there is also an increase in the proliferation of these cells. The lineage of 3T3 fibroblasts showed a proliferation rate from 25 to 100% more than the control for the HE, DCM,

Table 4
Viability cell effects of the hidroethanolic extract, fractions and chlorogenic acid and ellagic acid from *Spondias mombin* leaves. HE and fractions were assayed at 60, 125 and 250 μg/ml and compounds at 5, 15 and 60 μg/ml on 3T3 fibroblasts. Results are expressed as percentage of proliferation media ± standard deviation from triplicate assays.

	Fibroblasts 3T3 (24 h)		
Extract and fractions	60 μg/ml	125 μg/ml	500 μg/ml
HE	125 ± 0.0205	129 ± 0.0095	181 ± 0.185
DCM	253 ± 0	128 ± 0.0005	142 ± 0.0025
EtOAc	168 ± 0.0205	204 ± 0.034	128 ± 0.0445
ButOH	141 ± 0.0195	93 ± 0.0005	158 ± 0.048
ARF	100 ± 0.01	108 ± 0.0025	67 ± 0.022
Compounds	5 μg/ml	15 μg/ml	60 μg/ml
Chlorogenic acid	122 ± 0.0355	106 ± 0.002	105 ± 0.0025
Ellagic acid	154 ± 0.0535	127 ± 0.0015	181 ± 0.1205

HE = hidroethanolic extract of *Spondias mombin* leaves, DCM = dichloromethane fraction EtOAc = ethyl acetate fraction. ButOH = *n*-butanol fraction. ARF = aqueous residual fraction.

AcOEt and ButOH (Table 4). It was observed a cytotoxic effect only at the maximal concentration 500 μg/ml for the residual fraction (ARF).

In summary, to the best of our knowledge, this is the first time that is reported antioxidant/anti-inflammatory property (*in vitro*/*in vivo*) to the leaf extract from *S. mombin* along with characterization of their chemical composition. The evidence founded in this work, makes it a suitable candidate for further consideration as an alternative treatment to reduce oxidative stress and inflammation.

Conclusions

The results shown in this work suggest the anti-inflammatory potential of the leaf extract of *S. mombim* on peritonitis model induced by carrageenan, It was also observed antioxidant properties associated with an absence of cytotoxicity in cell culture. Furthermore, it was found that chlorogenic acid and ellagic acid contribute to action pharmacologically of *S. mombin*.

Therefore, these results indicate the potential of the leaf of *S. mombin* as a source of new anti-inflammatory herbal drug and/or molecules, and seem to justify part of its main popular uses in traditional medicine. Further studies are required to confirm the anti-inflammatories action of *S. mombin* and its possible anti-inflammatory mechanisms of action.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

BECVF participated of phytochemical analysis, interpretation of data and drafted the manuscript. MMM participated in the evaluation of anti-inflammatory activity, acquisition and interpretation of data. AHK participated in antioxidant analysis and revised it critically the manuscript. RS participated in its design and coordination, and helped to draft the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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