

The Tea and Polysaccharide Fractions of *Ximenia  
americana* Stem Barks Inhibits Inflammatory  
Parameters Induced by Zymosan in Mice

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## Abstract

*Ximenia americana* or “ameixa-do-sertão” is a plant used in the folk medicine of Northeast-Brazil in inflammatory and painful processes. The literature has shown anti-inflammatory and antinociceptive activities for the aqueous or hydroalcoholic extracts and polysaccharide fractions of *X. americana* stem barks. This study investigated the anti-inflammatory effect of polysaccharide fractions and tea prepared from *X. americana* stem barks in mice models of paw edema and peritonitis induced by zymosan. Tea (50,100, 150 mg/kg; p.o.) and polysaccharide fractions (FI, FII: 1 mg/kg; i.v.), containing carbohydrate (44.8, 25.0, 44.4%) and polyphenols (28.3, 0.78, 1.50%), respectively, were administered 1 h before zymosan (500 µg; s.c. or i.p). FT-IR analysis of the tea showed bands indicating C=O and C-O groups vibration, typical of phenolic compounds. Zymosan-induced paw edema (AUC: 355.0 ± 38.1) was inhibited by the tea by 38% (100 mg/kg) and 36% (150 mg/kg). FI and FII reduced the edema by 47% (933.8 ± 158.1 AUC) and 30% (1263 ± 124.8 AUC), respectively. Zymosan i.p. induced hypernociception (4.7 ± 0.3 g) and increased the peritoneal leukocyte migration (10979 ± 1475 cells/µl). FII increased by 66% the nociceptive threshold (13.87 ± 1.59 g) and reduced by 63% leukocyte migration (3993 ± 439.1 cells/µl). FII decreased plasma protein by 64% (92.6 ± 4.5 vs. zymosan: 119.6 ± 5.4 µg/ml) and MDA by 63% (57.5 ± 3.5 vs. zymosan: 88.9 ± 6.3 µg/ml). The tea, containing polysaccharides and polyphenols, and polysaccharide fractions FI/FII of *X. americana* barks possess antiinflammatory effect in mice models of acute inflammation induced by zymosan.

**Keywords:** medicinal plant, *Ximenia americana*, polysaccharides, decoction

## 1. Introduction

Natural compounds isolated from medicinal plants have been widely investigated due to its anti-inflammatory and analgesic properties, allowing the validation of the popular use and directing several clinical researches. These compounds can be obtained from various plant

parts, such as stem barks, leaves, fruits, seeds, roots, and are popularly used as infusion, maceration, decoction, etc (Balunas & Kinghorn, 2005).

Among plant compounds, polysaccharides are highly bioavailable and have low or no toxicity (Ovodov, 1998). Regarding its biological activities in the inflammatory process, the polysaccharide fractions obtained from pods of *Caesalpinia ferrea* (jucá) and seeds of *Azadirachta indica* (neem) inhibited leukocyte migration and acute phase inflammatory mediators in rats (Pereira et al., 2012a; 2012b). In nociceptive processes, *Thladiantha dubia* fruit polysaccharides reduced the abdominal contortions elicited by acetic acid (Wang et al., 2011).

*Ximenia americana* L. (Olacaceae), popularly known as ameixa-do-sertão, is widely distributed in Northeast Brazil (Le et al., 2012), being its barks prepared as decoction (“tea”) to treat inflammatory and painful conditions. Experimental studies demonstrated the antiinflammatory effect for the hydroethanolic bark extracts in acute and chronic inflammation models (da Silva et al., 2018). The aqueous extract of *X. americana* roots reduced paw edema, neutrophil migration and vascular permeability induced by carrageenan in mice (Olabissi et al., 2011). The antinociceptive activity of the aqueous extract obtained from stem barks was also demonstrated in the inflammatory phase of the formalin test (Soro et al., 2009). In addition, total polysaccharide and fractions isolated from *X. americana* presented inhibitory effect in inflammatory models (Silva-Leite et al., 2017, 2018). Moreover, the inhibitory effect on neutrophil migration was demonstrated in the gastritis model for the tea of *X. americana*, prepared following the same protocol used in the present study (da Silva-Pantoja et al., 2018).

This study aimed to evaluate the anti-inflammatory activity of the tea and polysaccharides obtained from *X. americana* stem barks in the models of paw edema and peritonitis induced by zymosan in mice.

## 2. Material and Methods

### 2.1 Experimental Design

The anti-inflammatory and anti-hypernociceptive activities of the tea preparation and polysaccharide fractions of *X. americana* stem barks were evaluated after stimulation of mice paws or peritonium with the flogistic agent zymosan for analysis of the following parameters: edema, protein leakage, leukocyte infiltrate, oxidative stress markers (malondialdehyde) and hyperalgesia. Control animals received sterile saline by subcutaneous (s.c.) route in the paws or intraperitoneal (i.p.).

### 2.2 Material

#### 2.2.1 Plant

Barks of *X. americana* were collected in Custódio county (Quixadá-CE) and the plant exsiccate was deposited at FECLESC/Quixadá and Herbarium Prisco Bezerra - UFC (n° 46794). In accordance to the Brazilian Federal Law No. 13123/2015, the assessment activity was registered in the Brazilian System for the Management of the Genetic Heritage and the Associated Traditional Knowledge (SISGEN - A8B2C79).

### 2.2.2 Tea Preparation, Extraction and Isolation of Polysaccharides of *X. americana*

The tea was obtained by decoction of *X. americana* stem barks in distilled water (100°C/5 min) and lyophilization before used in the experimental protocols (da Silva-Pantoja et al., 2018).

For the polysaccharides extraction and isolation, barks were washed, dried (40°C) and macerated into powder (5 g), suspended in methanol (1:50, w/v), homogenized (76 °C, 2 h) and filtered (2x). The insoluble portion was added to 0.1 M NaOH (97 °C, 2 h), filtered and centrifuged (2496 g, 15 min, 25 °C). The alkaline supernatants were neutralized (1 M HCl), precipitated in ethanol and centrifuged under the previous conditions. The final precipitate was dialyzed and the supernatant centrifuged (Silva-Leite et al., 2017), which was precipitated with trichloroacetic acid (TCA) until reaching pH 2.0 or 3.0, over 4 h in ice bath (4 °C) (Zhao et al., 2014). The protein precipitate was separated by centrifugation (2496 g, 30 min, 25 °C) and the supernatant dialyzed and lyophilized to obtain the total polysaccharides deproteinized, named TPL-Xa.

### 2.2.3 Fractioning, Biochemical and Structural Characterization of Tea and Polysaccharides of *X. americana*

TPL-Xa was dissolved in distilled water (1:2, w/v) and applied to chromatographic column (resin: DEAE-cellulose), equilibrated and washed with distilled water, and eluted with NaCl (0.1 and 0.25M). Polysaccharide fractions were monitored for the carbohydrate content by the phenol–sulfuric acid method (Silva-Leite et al., 2017; Dubois et al., 1956).

Tea and polysaccharide fractions were quantified for total carbohydrate (Dubois et al., 1956), hexoses and hexuronic acid (Dische, 1947), total protein (Bradford, 1976), and polyphenolic compounds (Siddiqui et al., 2017), in comparison with standard curves of galactose, galacturonic acid, bovine serum albumin and gallic acid, respectively.

The tea was analyzed by infrared spectroscopy (FT-IR). For this, samples (2 mg) were mixed with powdered potassium bromide and pressed into a disc in the medium infrared region at 500-4000 cm<sup>-1</sup>, with resolution of 3 cm<sup>-1</sup> (Perkin Elmer Spectrum 100, USA).

## 2.3 Experimental Protocols

### 2.3.1 Animals

Swiss mice (25-35 g) were maintained with free Access to food and water and controlled environmental conditions (12/12 h light/dark cycle, temperature of 25 °C). The experimental protocols were approved by the Animal Care and Use Committee of the State University of Ceara (UECE n°: 4153018/2018).

### 2.3.2 Animals Treatment

Decoction of *X. americana* barks (50,100, 150 mg/kg) was administered *per oral* (p.o.) and the polysaccharide fractions FI (0.1 M) and FII (0.25 M) injected intravenously (1 mg/kg; i.v.) 1 h before edema or peritonitis induction by zymosan.

### 2.3.3 Paw Edema Model

Paw edema was induced by zymosan (500 µg; s.c.) and measured by hydroplethysmometry at

zero time and selected time intervals thereafter (0.5, 1 – 5 h). Control animals received saline (0.1 mL/10 g body mass). Results were expressed as the variation in paw volume ( $\mu\text{l}$ ) or area under curve (AUC).

#### 2.3.4 Peritonitis Model

Zymosan (500  $\mu\text{g}/\text{cavity}$ ) or saline was administered via intraperitoneal (i.p.) 4 h before hypernociception measurement. Immediately after euthanasia peritoneal fluid was harvested with 3 ml saline (5 IU heparin) for total and differential leukocyte count (neutrophils and mononuclear cells) and quantification of total protein (Bradford, 1976) and malondialdehyde-MDA (TBARS) (Huong et al., 1998).

For evaluation of mechanical visceral hypernociception, mice were placed in clear acrylic box 15 min before the test for adaptation to the environment. The nociceptive threshold of the animals was tested by application of a gradual pressure (g) using polypropylene tip, coupled to a hand-held force transducer (Electronic Von Frey Anesthesiometer; Insight). Hypernociception was also evaluated at baseline (time zero) (Le Bars et al., 2001).

#### 2.4 Statistical Analysis

Results were presented as mean  $\pm$  SEM. Analysis of variance (ANOVA) followed by Bonferroni's test were applied. P values < 0.05 were considered significant.

### 3. Results

#### 3.1 Tea Preparation, Purification and Partial Chemical Characterization of Polysaccharides of *X. americana* Barks

The tea prepared by decoction of *X. americana* barks revealed 1.8% yield, presenting carbohydrate (44.8%, including 10.4% uronic acid) and polyphenols (28.3%) as major constituents, but low content of proteins (1.34%). TPL-Xa (2.0 yield) was extracted from *X. americana* barks by the combination of light alkaline solution followed by precipitation with ethanol. The deproteinated TPL-Xa and major polysaccharide fractions, obtained by DEAE-cellulose, presented similar content of carbohydrate, specially TPL-Xa and FII (TPL-Xa: 43%; FI: 25%; FII: 44%), but different content of uronic acid (TPL-Xa: 5.2%; FI: 9.8%; FII: 25%), proteins (TPL-Xa: 0.6%; FI: 0.9%; FII: 0.8%), showing relevant decrease in the polyphenols content (TPL-Xa: 4.64; FI: 0.78; FII: 1.50 GAE) compared to the tea. In addition, the uronic acid/hexoses ratio (0.56) was higher for FII (Table1).

Table 1. Yield and chemical analysis of *X. americana* tea and deproteinated polysaccharide fractions

Samples	Yield (%)	<sup>a</sup> Carbohydrates (%)	<sup>b</sup> Uronicacid (%)	<sup>c</sup> Uronicacid/Hexoses	<sup>d</sup> Polyphenols (GAE)	<sup>e</sup> Proteins (%)
Tea	1.8	44.8	10.4	0.23	28.3	1.34
TPL-Xa	2.0	43.0	5.2	0.12	4.64	0.6
FI	9.0	25.0	9.8	0.39	0.78	0.9
FII	34.0	44.4	25.0	0.56	1.50	0.8

<sup>a</sup>Dubois et al., 1956; <sup>b</sup>Dische 1947; <sup>c</sup>Molar ratio; <sup>d</sup>Siddiqui et al., 2017; <sup>e</sup>Bradford, 1976. GAE: gallic acid equivalent (mg/g). Yield: from 5 g dry powder of *X. americana* barks.

FTIR spectra of *X. americana* tea revealed absorption peaks around 3375 cm<sup>-1</sup> and 2925 cm<sup>-1</sup> corresponding to stretching of O-H and C-H, respectively (Sun et al., 2009). Peaks at 1442 cm<sup>-1</sup>, 1525 cm<sup>-1</sup> and 1612 cm<sup>-1</sup> were also detected, indicating presence of uronic acid (group COOH) (LI et al., 2014). Absorption regions between 1500 and 700 cm<sup>-1</sup> correspond to carbohydrate range (Karnik et al., 2016), in which peak at 1113 cm<sup>-1</sup>, characterizes glycosidic bonds (Xu et al., 2016). The peak observed at 1442 cm<sup>-1</sup> was attributed to the elongation of the C=O group of aromatic rings, typical of phenolic compounds and the peak at 1296 cm<sup>-1</sup> to C-O in the flavonoids found in the tea (Bagheri et al., 2013) (Figure 1).

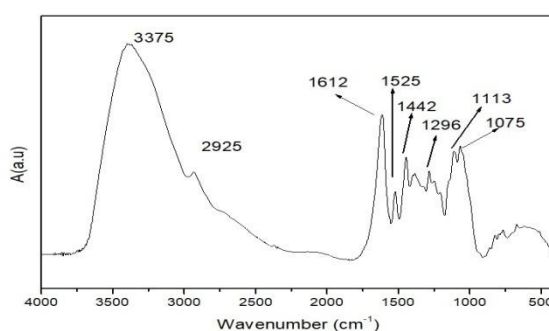


Figure 1. *X. americana* tea analysis by infrared spectroscopy (FTIR)

The spectral region examined was from 500 to 4000 cm<sup>-1</sup>, with resolution of 3 cm<sup>-1</sup>.

### 3.2 Edematogenic Effect of Tea and Polysaccharides From *X. Americana* Barks

Zymosan subcutaneous injection (500 (µg) induced paw edema, that reached maximal effect 3 h after induction (211.4 ± 24.1 (µl vs. saline: 2.5 ± 2.5 (µl), and was sustained until 5 h. The animals treatment with tea preparation of *X. americana* showed better inhibition of edema at 3 h by 43% (100 mg/kg: 120.0 ± 10.2 (µl) and 40% (150 mg/kg: 131.4 ± 15.3 (µl) (Figure 2A). The area under curve (2-4 h) revealed that the tea at 150 mg/kg and 100 mg/kg reduced the zymosan-elicited edema (355.0 ± 38.1 AUC vs. saline: 4.2 ± 4.2 AUC) by 38% (222.5 ± 18.7 AUC) and 36% (229.2 ± 15.6 AUC), respectively (Figure 2B). FI and FII (1 mg/kg) reduced the edema at 2 h by 55% (38.7 ± 6.9 (µl vs. zymosan: 86.6 ± 9.5 (µl) and 65% (30.0 ± 6.6 (µl vs. zymosan: 86.6 ± 9.5 (µl) (Figure 2C). The area under curve showed that FI and FII reduced the edema by 47% (933.8 ± 158.1 AUC) and 30% (1263 ± 124.8 AUC), respectively (Figure 2D).

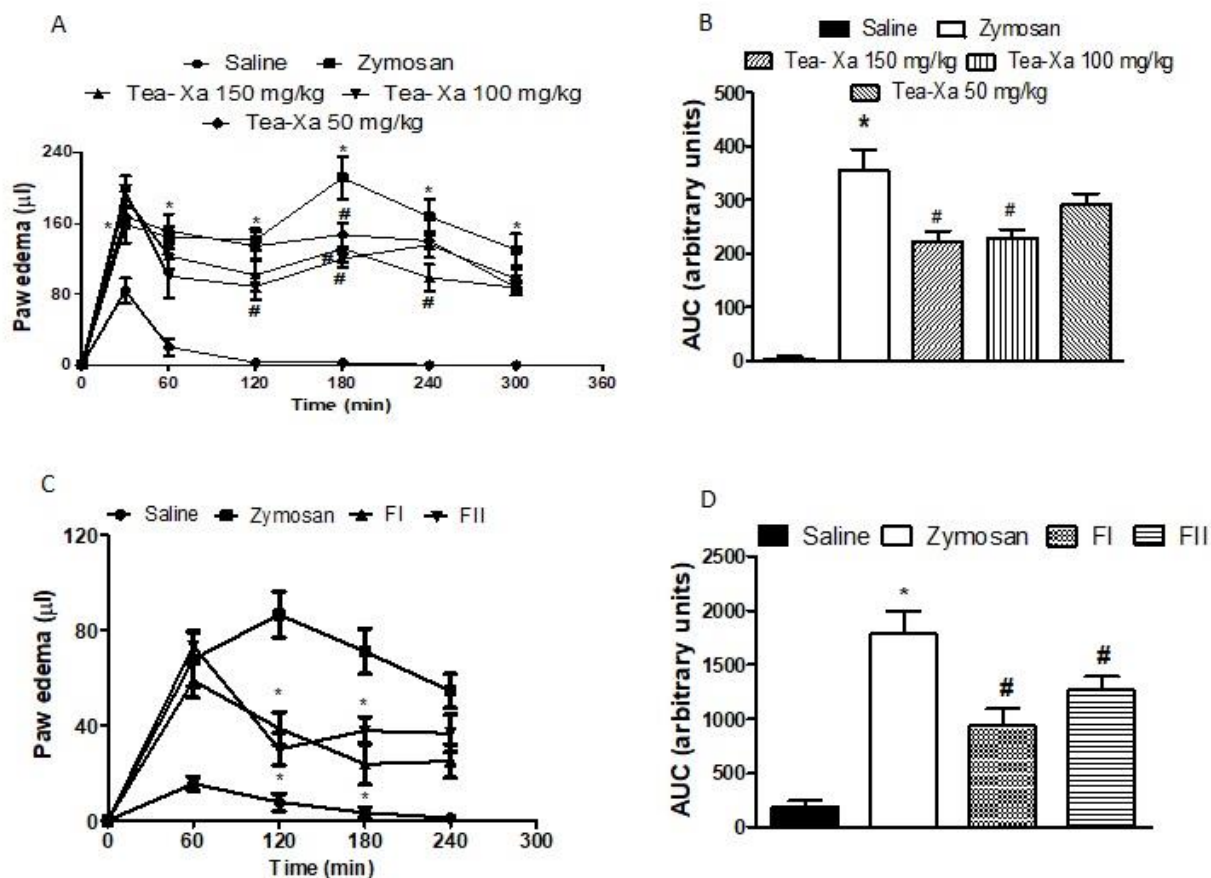


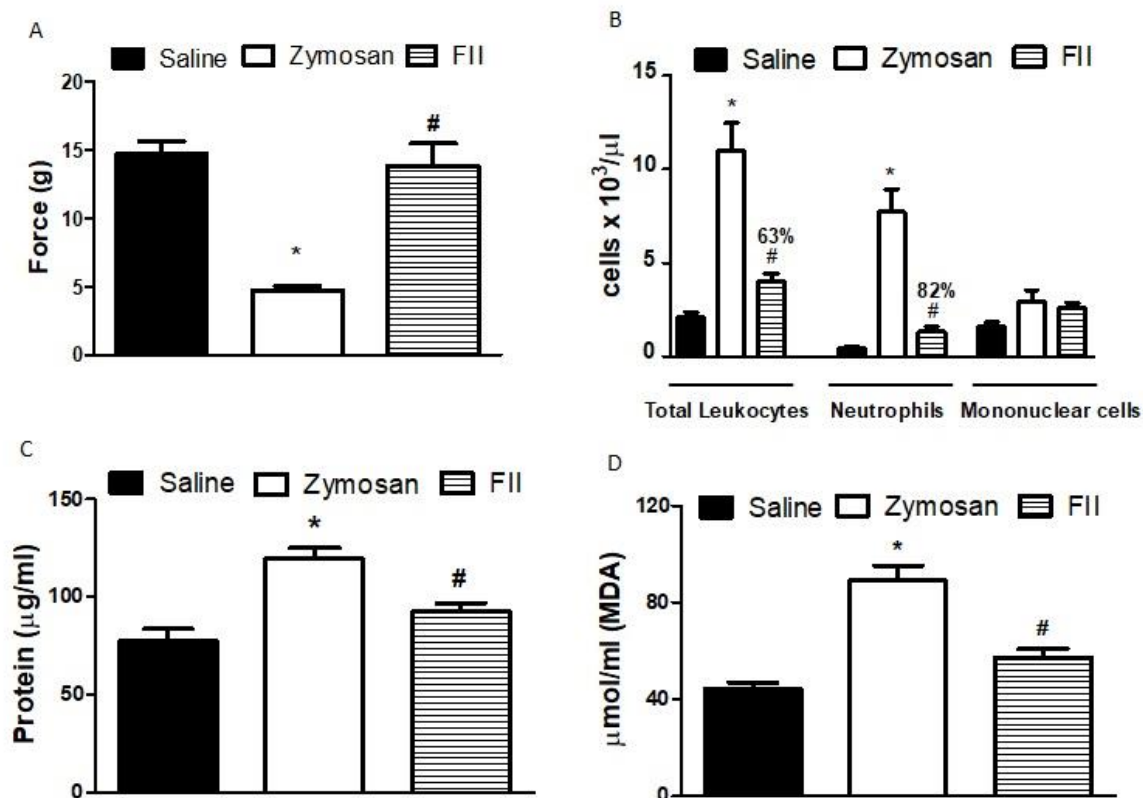
Figure 2. Inhibitory effect of the tea preparation and polysaccharide fractions (FI e FII) on zymosan-induced edema

(A) Mice were treated with tea (50, 100, 150 mg/kg; p.o.) or (C) FI and FII (1 mg/kg) 1 h after zymosan (500 (µg/paw, s.c); control animals received saline. Edema was evaluated by hydropleysmometry after (1-5 h) and before treatment (B, D) Area under curve. Mean ± SEM (n = 8). ANOVA and Bonferroni \*p<0.05 vs. saline #p<0.05 vs. zymosan.

### 3.3 FII Inhibits Inflammatory Parameters and Oxidative Stress Makers in the Peritonitis Induced

Zymosan i.p. (500 (µg) elicited hypernociception in mice, observed by the behavioral response of abdomen withdrawal in response to the pressure applied, reducing the nociceptive threshold compared to the control (zymosan:  $4.7 \pm 0.3$  g vs. saline:  $14.7 \pm 0.9$  g). FII increased by 66% the animal's nociceptive threshold, restoring the values close to the controls (FII:  $13.87 \pm 1.59$ ) (Figure 3A).

Zymosan i.p. (500  $\mu\text{g}$ ) also increased in 5 fold the number of total leukocytes/ $\mu\text{l}$  in the animals peritoneum ( $10979 \pm 1475$  cells/ $\mu\text{l}$  vs. saline:  $2106 \pm 266.7$  cells/ $\mu\text{l}$ ). FII treatment reduced by 63% total leukocytes ( $3993 \pm 439.1$  cells/ $\mu\text{l}$ ), mainly neutrophils, by 82% ( $1350 \pm 238.9$  vs. zymosan:  $7773.2 \pm 1191$  cells/ $\mu\text{l}$ ) (Figure 3B). Besides, FII decreased plasma protein by 64% ( $92.6 \pm 4.5$  vs. zymosan:  $119.6 \pm 5.4$   $\mu\text{g/ml}$ ) (Figure 3C) and MDA by 63%



( $57.5 \pm 3.5$  vs. zymosan:  $88.9 \pm 6.3$   $\mu\text{g/ml}$ )(Figure 3D).

Figure 3. Inhibitory effect of the polysaccharide fraction FII on inflammatory parameters induced by zymosan

Mice were treated with FII (1 mg/kg) 1 h after zymosan (500  $\mu\text{g/cavity}$ , i.p.); control animals received saline. Inflammatory parameters were assessed in the peritoneal fluid 4 h after stimuli (A) Abdominal hypernociception; (B) total and differential leukocyte; (C) Protein; (D) MDA. Mean  $\pm$  SEM (n = 8). ANOVA and Bonferroni \* $p < 0.05$  vs. saline. # $p < 0.05$  vs. zymosan.

#### 4. Discussion

The present study demonstrates the obtention and partial chemical characterization of deproteinized polysaccharide fractions and tea from *X. americana* stem barks, along with its anti-inflammatory activity in the mice models of paw edema and peritonitis induced by zymosan.



The tea presented high content of total carbohydrates and polyphenols with low protein content. Previous studies had already demonstrated this biochemical pattern in teas and polysaccharide fractions obtained from plants (Zhu et al., 2019, Yang et al., 2018, Chen et al., 2018).

TPL-Xa and FII fraction presented carbohydrate content similar to the tea, but different uronic acid content, being FII superior in the ratio uronic acid/hexoses, and substantial lower levels of polyphenols compared to the tea. It is well reported in the literature that polysaccharide fractions of several plants present similar biochemical composition compared to the decoction extracts (teas) from which they were extracted (Chen et al., 2016).

The tea FTIR spectra demonstrates peaks related to carbohydrate range, uronic acid (group COOH), phenolic compounds and flavonoids (Sun et al., 2009; Karnik et al., 2016; Li et al., 2014; Bagheri et al., 2013). Our data reinforce the suggestion that despite of different extraction methods the total carbohydrates content does not significantly change. These results corroborate the absorption ranges found for the non-deproteinated FII fraction extracted from *X. americana* barks, already published by our research group (Silva-Leite et al., 2017).

The animal's treatment *p.o.* with the tea and *i.v.* with the polysaccharide fractions of *X. americana* inhibited the zymosan-induced paw edema. Zymosan, a polysaccharide of yeast cell wall, induces an inflammatory response involving dose and time-dependent edema, accompanied by severe leukocyte infiltrate (Suo et al., 2014). Studies had shown the antiedematogenic effect of plant polysaccharide extracts, such as those from *Caesalpinia ferrea*, *Azadirachta indica* (Pereira et al., 2012a; 2012b) and *X. americana* (roots aqueous ethanol extract) (Olabissi et al., 2011) in carrageenan-induced edema, which is also accompanied by protein exudate and intense leukocyte infiltrate (DiRosa et al., 1971).

Zymosan, administered *i.p.* induces acute inflammation characterized by severe leukocyte infiltrate, increase in vascular permeability, hypernociception and inflammatory mediators (Leite et al., 2015). The animal's treatment with FII reduced neutrophil migration and vascular permeability, seen by the decrease in protein leakage, and also increased the abdominal nociceptive threshold, showing significant action on inflammatory pain. In previous studies, similar results had been reported for other plant polysaccharides (Pereira et al., 2012a; 2012b; Wang et al., 2011; Olabissi et al., 2011; Silva-Leite et al., 2017).

Moreover, our results showed that the polysaccharide fraction FII was able to reduce MDA levels in the peritoneal fluid of animals stimulated with zymosan. Malondialdehyde is one of the main products of lipid peroxidation, therefore an important oxidative stress determinant in clinical situations, such as inflammation (Giera et al., 2012). Accordingly, antioxidant and free-radical scavenging activities had been described for plant polysaccharides (Chen et al., 2004; Liu et al., 2011; Mengome et al., 2014; Lin et al., 2017).

The chemical analysis showed high carbohydrate content in the tea and polysaccharide fractions composition, especially FII, which reinforces the supposition that both the anti-inflammatory effect of tea and fractions, as well as the antioxidant effect of FII may be accounted for the polysaccharides. In addition, the tea also presents considerable content of

polyphenols, substance well described for its anti-inflammatory and antioxidant properties (Xu et al., 2017).

In conclusion, the polysaccharide fractions and the tea, containing polysaccharides and polyphenols, obtained from *X. americana* stem barks possess anti-inflammatory effect in the mice models of paw edema and peritonitis induced by zymosan via reduction of edema, protein exudate, neutrophil migration and inflammatory pain. These effects may validate the popular use of *X. americana* as anti-inflammatory.

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