

## Artigo

## Antinociceptive and Anti-inflammatory Activities of the Ethanolic Extract, of Fractions and of Epicatechin Isolated from the Stem Bark of *Ximenia americana* L. (Oleaceae)

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### Atividades Antinociceptivas e Anti-inflamatórias do Extrato Etanólico, das Frações e da Epicatequina Isolada da Casca do Caule da *Ximenia americana* L. (Oleaceae)

**Resumo:** As ações antinociceptivas e anti-inflamatórias do extrato etanólico, das frações e da epicatequina (XM-Catequina) isolada a partir dos extratos etanólicos das cascas de *Ximenia americana* L. foram determinadas utilizando modelos *in vivo* como contorções induzidas pelo ácido acético, teste de formalina, teste de placa quente, peritonite induzida por zimosan e ensaio *in vitro* de inibição da ciclo-oxigenase. O teste de contorção revelou efeitos inibitórios maiores que 90% do extrato etanólico, das frações e da XM-Catequina (este isolado apresentou  $ID_{50} = 32,03 \mu\text{mol} / \text{kg}$  e efeito máximo = 99,60%). O teste de formalina demonstrou um efeito antinociceptivo nas fases inicial (64.23%) e tardia (86.80%) com a XM-catequina, enquanto a fração clorofórmica mostrou menor efeito antinociceptivo na fase inicial (31.51%). A fração aquosa e o extrato etanólico causaram uma inibição significativa na fase tardia (73.68% e 82.40%, respectivamente). O tratamento de ratos com XM-catequina ou com a fração de acetato de etila não teve efeito antinociceptivo central no teste de placa quente. Os efeitos anti-inflamatórios foram determinados para a inflamação peritoneal induzida por zimosan e os dados indicaram que a XM-catequina, a fração hidrometanólica, o extrato etanólico e a fração em acetato de etila reduziram o número de células recrutadas em 46.02%, 35.06%, 41.45% e 38.61%, respectivamente. Estes resultados mostram que o extrato, as frações e a XM-catequina produzem respostas antinociceptivas e anti-inflamatórias. De acordo com os resultados obtidos nos ensaios de inibição da ciclooxigenase, os efeitos observados estão relacionados com a inibição de COX-1 e COX-2. As ações biológicas demonstradas no presente estudo apoiam o uso etnomedicinal desta planta.


**Palavras-chave:** Plantas medicinais; anti-inflamatórios; corticosteroides; ciclooxigenase.

### Abstract

The antinociceptive and anti-inflammatory actions of the ethanolic extract, fractions and epicatechin (XM-Catechin) isolated from the ethanolic extracts of bark of *Ximenia americana* L. were determined utilizing *in vivo* models such as acetic acid-induced writhing, the formalin test, hot plate test, zymosan-induced peritonitis and *in vitro* cyclooxygenase inhibition assay. The writhing test revealed inhibitory effects higher than 90% of the ethanolic extract, fractions and XM-Catechin (this isolate presented  $ID_{50} = 32.03 \mu\text{mol}/\text{kg}$  and maximum effect = 99.60%). The formalin test demonstrated an antinociceptive effect in both early (64.23%) and late (86.80%) phases by XM-catechin, while the chloroform fraction showed a lower antinociceptive effect at the early phase (31.51%). The aqueous fraction and ethanolic extract caused significant inhibition at the late phase (73.68% and 82.40%, respectively). Treatment of mice with XM-catechin or with the ethyl acetate fraction had no central antinociceptive effect in the hot plate test. Anti-inflammatory effects were determined for zymosan-induced peritoneal inflammation and the data indicated that XM-catechin, the hydromethanol fraction, the ethanol extract and the ethyl acetate fraction reduced the number of recruited cells by 46.02%, 35.06%, 41.45%, and 38.61%, respectively. These results demonstrate that the extract, fractions and XM-Catechin produce antinociceptive and anti-inflammatory responses. According to the results obtained in the cyclooxygenase inhibition assays, the observed effects are related to inhibition of COX-1 and COX-2. The biological actions demonstrated in the present study support the ethnomedicinal use of this plant.

**Keywords:** Medicinal plants; anti-inflammatory drugs; corticosteroids; cyclooxygenase.

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## Antinociceptive and Anti-inflammatory Activities of the Ethanolic Extract, of Fractions and of Epicatechin Isolated from the Stem Bark of *Ximenia americana* L. (Oleaceae)

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## 1. Introduction

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Drugs currently used to manage pain and inflammatory conditions are non-steroidal anti-inflammatory drugs, corticosteroids and opiate analgesics. All these drugs exhibit well known toxic side effects. In contrast, many medicines of plant origin have been used for long periods with no adverse effects. It is therefore essential that efforts continue to be made to introduce new medicinal plants for the development of more efficacious and less expensive drugs.<sup>1</sup> Plants represent a still largely untapped source of structurally novel compounds that may serve in the development of novel drugs.<sup>2</sup>

*Ximenia americana* L., which belongs to the Oleaceae family, is a thorny bush very common in the state of Piauí (Brazil) and found in tropical regions of America and Africa.<sup>3</sup> In Brazil, it is commonly known as plum. In north-eastern Brazil, it is used in folk medicine to treat several disorders including pain, obesity, inflammation, wound healing, diabetes, cough, hoarseness, constipation, venereal disease and osteoporosis.<sup>4</sup> This plant is also active against rheumatism and many infections.<sup>4</sup> Several studies have demonstrated its pharmacological activity, e.g., antiviral,<sup>5</sup> antimicrobial,<sup>6</sup> antitumor,<sup>7</sup> antioxidant<sup>8</sup> and antipyretic activities,<sup>9</sup> with hepatoprotective and hypoglycemic properties.<sup>10</sup>

With the purpose of identifying plants and natural products with pharmacological activities, our research group performed previous ethnopharmacological studies with many plants with antinociceptive and anti-inflammatory activities.<sup>11-13</sup> Thus, the major

aim of the current study was to evaluate the ethanol extract, fractions and one pure compound, an epicatechin derivative obtained from the stem bark of *X. americana*, for their antinociceptive and anti-inflammatory activities. *In vitro* models were used to help in the assessment of their medicinal properties, supporting the ethnopharmacological use of this plant.

## 2. Materials and Methods

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### 2.1. General experimental procedures

All the solvents and reagents used in the preparation of the extracts (EtOH) and fractionations (C<sub>6</sub>H<sub>14</sub>, CHCl<sub>3</sub>, EtOAc, MeOH) were of analytical grade (PA) supplied by Vetec, Synth and Ecibra, Sephadex LH-20, SiO<sub>2</sub>, 70 – 230 mesh (Merck). Analytical TLC was performed on precoated Kieselgel 60 F<sub>254</sub> plates (Merck). <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer at 500 and 125 MHz, respectively, using CD<sub>3</sub>OD as solvent.

### 2.2. Plant material

*Ximenia americana* L. stem was collected in April 2012 in the city of Campo Maior, state of Piauí and identified by Dr. Maria Edilene Alencar. A voucher specimen under the reference number 14407 was deposited at the Graziela Barroso Herbarium at the Federal University of Piauí (Teresina, PI, Brazil).

### 2.3. Extraction and fractionation of the plant

The collected stems of *X. americana* were separated and dried at room temperature (26 °C) away from light. Ethanol extract and liquid-liquid partitioning process of the stem bark were performed according to Uchôa et al., 2016.<sup>8</sup> The raw extract in ethanol and fractions resulting from partitioning were subjected to *in vivo* antinociceptive and anti-inflammatory activity assays.

### 2.4. Phytochemical isolation

The ethyl acetate fraction of stem bark was subjected to chromatography in a deactivated silica gel column with 10% water, using ethyl acetate and methanol as eluant were performed according to Uchôa et al., 2016,<sup>8</sup> yielding compound **1** (1 g, 28%), named as XM-Catechin, known as (-) epicatechin (Figure 1).

### 2.5. Animals

Swiss mice of both sexes, 6–8 weeks of age with an average weight of 20–30 g, were obtained from the Central Animal House at the Federal University of Alagoas (Maceió, Brazil) and were used throughout the experiments. They were housed in single-sex cages under a 12 h light/dark cycle at constant temperature (22 °C) conditions with free access to water and pelletized food. All animals were manipulated according to the norms established by the Ethics Commission—UFAL for handling animals (Protocol number: 14/2013). Animal care and research protocols were in accordance with the principles and guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals.<sup>14,15</sup>

### 2.6. Drugs and Reagents

The following substances were used: acetic acid and indomethacin (Merck), dipyron (Sigma Chemical), morphine sulfate (Dimorf-Cristalia, Brazil) and Tween 20 (Sigma). A solution of 2.5% formalin was prepared with formaldehyde (Merck) in saline (NaCl 0.9%). The ethanol extract, hydromethanol fraction, hexane fraction, chloroform fraction, acetate fraction and XM-Catechin (**1**) were suspended in Tween 20 (0.05%) and distilled water (vehicle) for all the experiments.

### 2.7. Acetic acid-induced writhing in mice

At the beginning of the experiment, the mice were administered the ethanolic extract, hydromethanol fraction, hexane fraction, chloroform fraction and ethyl acetate fraction (all in doses of 100 mg/kg i.p.). The XM-Catechin (**1**) isolated from *X. americana* L. and dipyron were administered in doses of 300, 100, 30, 10 and 1 µmol/kg, i.p. Least-squares linear regression analysis of the log dose-response curves allowed the calculation of the dose that produced 50% antinociception (ID<sub>50</sub>) by XM-Catechin (**1**) and the reference drug, which were injected 40 min before the intraperitoneal injection (i.p.) of acetic acid 0.6% (10 mL/kg body wt.). The control animals received only the vehicle and acetic acid. Dipyron (10 mg/kg body wt.) was used as the standard drug. Five minutes after administration of acetic acid, the number of writhes was recorded over a period of 20 min.<sup>15,16</sup>

### 2.8. Formalin test in mice

The mice were pre-treated with XM-Catechin (**1**) (100 µmol/kg, i.p.), the ethanol extract and the fractions from *X. americana* L. (all in doses of 100 mg/kg, i.p.) with

indomethacin (100  $\mu\text{mol}/\text{kg}$ , i.p.) as the standard drug. Control animals received the vehicle. Then, 40 minutes after pre-treatment the animals were given 20  $\mu\text{L}$  of a 2.5% formalin solution (0.92% formaldehyde, in saline) in the ventral surface of the right hind paw (i.pl.). They were then observed from 0 to 5 min (neurogenic phase) and from 15 to 30 min (inflammatory phase), and the time they spent licking the injected paw was recorded and considered as indicative of nociception.<sup>16,17</sup>

### 2.9. Hot plate test in mice

The animals were placed on a hot plate (54  $\pm$ 1 $^{\circ}\text{C}$ ) (model EFF 361, Insight, Brazil). The reaction time (paw licking, jumping, shaking) was recorded 30, 60, 90 and 120 min after intraperitoneal administration of the ethyl acetate fraction (100 mg/kg,i.p.) and XM-Catechin (**1**, 100 $\mu\text{mol}/\text{kg}$ ,i.p.) isolated from *X. americana* L. The vehicle and morphine (15  $\mu\text{mol}/\text{kg}$ ,i.p.) were used as the control and the standard drug.<sup>16,18</sup>

### 2.10. Zymosan-induced peritonitis in mice

For this series of experiments, the method described by Doherty *et al.* (1985) and Kolaczowska *et al.* 2001,<sup>19,20</sup> was used. Zymosan A (Sigma Aldrich) was freshly prepared (2 mg/mL) in sterile 0.9% w/v saline, and 0.5 mL was injected i.p. The animals were then killed by cervical dislocation. The peritoneal cavity was washed with 1.5 mL cold PBS, and after 6 hs of gentle manual massage, the exudate was retrieved and its volume measured. The exudate was collected and used freshly for cell counts and cytospin preparations. The ethanol extract and the fractions (100 mg/kg, i.p.), XM-Catechin (**1**) (100  $\mu\text{mol}/\text{kg}$ , i.p.), the vehicle (control, i.p.) and indomethacin (100  $\mu\text{mol}/\text{kg}$ , i.p.) were administered 30 min before the zymosan A injection.

### 2.11. *In vitro* cyclooxygenase inhibition assay

The flavonoid XM-Catechin (**1**) was also evaluated for its ability to inhibit COX in accordance with the method described by Uddin *et al*

.2004,<sup>22</sup> using both the kit for determining the inhibition of sheep COX-1/COX-2 (Cayman Chemicals, MI, USA), and according to the manufacturer's instructions. Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid (AA) to PGH<sub>2</sub>. PGF<sub>2 $\alpha$</sub> , produced from PGH<sub>2</sub> by reduction with stannous chloride, is measured by enzyme immunoassay (ACETM competitive EIA, Cayman Chemicals, MI, USA). The test compound solutions were prepared using DMSO as a solvent and reaction buffer (supplied in the kit) to a concentration of 2  $\mu\text{M}$ . Thereafter, a series of supplied reaction buffer solutions (960  $\mu\text{L}$ , 0.1 M Tris-HCl, pH 8.0, containing 5 mM EDTA and 2 mM phenol) were aliquoted into plastic tubes, which then had the enzyme COX-1 or COX-2 (10  $\mu\text{L}$ ) added in the presence of heme (10  $\mu\text{L}$ ). Later, 10  $\mu\text{L}$  of the XM-Catechin (**1**) solution and indomethacin (0.2  $\mu\text{M}$  in a final volume of 100  $\mu\text{L}$ ) were added. These solutions were incubated for a period of 5 min at 37  $^{\circ}\text{C}$ . Thereafter, 10  $\mu\text{L}$  of a solution of arachidonic acid (100  $\mu\text{M}$ ) were added and the COX reaction stopped by the addition of 50  $\mu\text{L}$  of HCl (1 M) after 2 min from the start of the reaction. PGF<sub>2 $\alpha$</sub> , produced from PGH<sub>2</sub> by reduction with stannous chloride was measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG able to connect to the PG antiserum is inversely proportional to the inhibition of COX, since the concentration of the PG-acetylcholinesterase conjugate is kept constant, while the concentration of PGs varies. This antibody-PG complex binds to an anti-monoclonal antibody previously placed in the wells of the plate. The plate is washed to remove any unbound reagents and the Ellman's reagent

(Sigma Aldrich), which contains the substrate for acetylcholine esterase, is then added to the well. The yellow product of this enzymatic reaction is determined spectrophotometrically in a Microplate Reader (Thermo Scientific Multiskan® Spectrum, Thermo Fisher Scientific Corporation, Finland) at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG-acetylcholinesterase conjugate that is connected to the PG antiserum, which is inversely proportional to the amount of PGs present in the well, during the incubation period.<sup>21,22</sup>

### 2.12. Statistical analysis

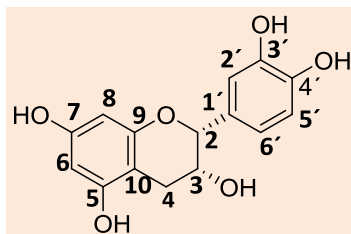
The percentages of inhibition were reported as the mean  $\pm$  S.E.M inhibition obtained in each individual experiment at the peak of the nociceptive or inflammatory response, depending on the experimental protocol. The data were compared statistically by analysis of variance (ANOVA)

followed by the Dunnett's test. *P* values [ $p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ ] were considered significant, using GraphPad Prism 5.0. When appropriate, the mean ID<sub>50</sub> values accompanied by their respective 95% confidence limits were determined by linear regression from individual experiments, using linear regression software.

## 3. Results and Discussion

### 3.1. Structure elucidation

Structural identification of the isolated substance Compound **1** (Figure 1), extracted from the ethyl acetate fraction of the stem bark of *X. americana*, was identified to be (-) epicatechin for comparison of the spectroscopic data (IR and the one- and two-dimensional NMR), were compared according to Uchôa et al. 2016.<sup>8</sup>



**Figure 1.** Chemical structure of compound 1 (XM-Catechin)

### 3.2. Acetic acid-induced writhing

In the acetic acid-induced writhing assay (Table 1), the ethanol extract (98.20%), hexane fraction (99.60%), chloroform fraction (95.00%), ethylacetate fraction (95.30%) hydromethanol fraction (98.50%) (all at the dose of 100 mg/kg, i.p), and XM-Catechin (**1**) (98.50%) (at the dose of 100  $\mu$ mol/kg) showed an inhibitory effect of nociception after intraperitoneal administration.

Dipyron (85.60%) also exerted a significant antinociceptive effect (Table 1).

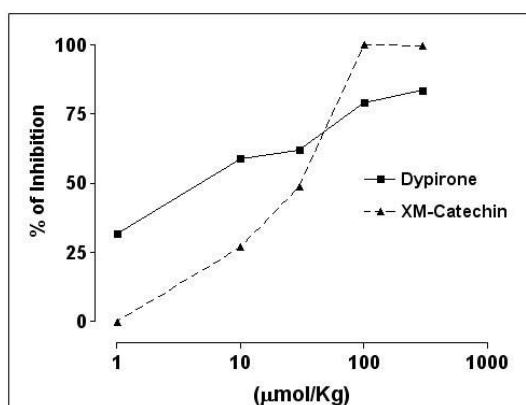
Utilizing the writhing test, a dose-response curve of XM-Catechin (**1**) was made. The results, depicted in Figure 2 and Table 2, show that XM-Catechin (**1**) (ID<sub>50</sub>= 32.03  $\mu$ mol/kg, maximum effect=99.60%) produced the same order of potency as that observed for the antinociceptive activity of dipyron (ID<sub>50</sub>= 29.30  $\mu$ mol/kg, maximum effect= 83.50%).



**Table 1.** Antinociceptive effect of the extract, fractions and XM-Catechin (**1**) isolated from *X. americana* on acetic acid-induced writhing in mice

Group	Dose (mg/kg) or $\mu\text{mol/kg}^{\#}$	Number of abdominal constrictions ( $M \pm \text{S.E.M.}$ )	Inhibition (%)
Control (vehicle)	---	44.40 $\pm$ 1.86	---
Dipyron <sup>#</sup>	100	6.50 $\pm$ 0.50**	85.60
Ethanol Extract	100	0.80 $\pm$ 0.58**	98.20
Hydromethanol Fraction	100	2.00 $\pm$ 2.00**	98.50
Hexane Fraction	100	3.40 $\pm$ 0.98**	99.60
Chloroform Fraction	100	0.20 $\pm$ 0.20**	95.00
Acetate Fraction	100	2.20 $\pm$ 1.36**	95.30
XM-Catechin	100	0.67 $\pm$ 0.67**	98.50

<sup>a</sup>Number of animals; \*\* $p < 0.01$  (One-way ANOVA followed by the Dunnett's test);  $n = 8$ .



**Figure 2.** Dose-response curves of XM-Catechin ( $\blacktriangle$ ) and dipyron ( $\blacksquare$ ) (all in doses of 1, 10, 30, 100 and 300  $\mu\text{mol/kg}$ , i.p.) in the acetic acid-induced writhing assay. Data expressed as the inhibition percentage of total writhings calculated from eight animals. \*\* $p < 0.01$  (One-way ANOVA followed by the Dunnett's test)

**Table 2.** Power and efficacy of dipyron and XM-Catechin (**1**) in acetic acid-induced writhing in mice

Substances	Inhibitor Dose ( $\text{ID}_{50}$ )	Maximum Effect
Dipyron	29.27 $\mu\text{mol/kg}$	83.50%*
XM-Catechin	32.03 $\mu\text{mol/kg}$	99.60%*

Number of animals (7-8); \* $p < 0.05$  (One-way ANOVA followed by the Dunnett's test).

It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons which are sensitive to nonsteroidal anti-inflammatory

drugs (NSAIDs) and to opioids,<sup>23</sup> and that it is possible to quantify prostaglandins by radioimmunoassay in the peritoneal exudates of rats collected after intraperitoneal injection of acetic acid.<sup>24</sup> The most important transmission pathways for inflammatory pain are those comprising peripheral polymodal nociceptors sensitive to protons, such as acid sensitive ion channels and via endogenous mediators, such as bradykinin, serotonin, histamine, substance P, glutamate, ROS, cytokines and prostaglandins.<sup>23,25,26</sup>

Moreover, it was demonstrated that the intraperitoneal administration of acetic acid induces the release not only of prostaglandins but also of mediators of the sympathetic nervous system.<sup>27</sup> Despite the poor specificity (e.g., tricyclic antidepressants, anticholinergic, antihistaminic and other agents showed activity in this test) of the writhing test, it is a very sensitive method for screening the antinociceptive effects of compounds.<sup>23,28</sup>

Our results show that the ethanol extract, all the fractions and XM-Catechin (1) mice, induce antinociceptive effects. These findings

are indicative for the potential use of *X. americana* an antinociceptive agent. In addition, these data are consistent with previously studies in animals showing that epicatechin exerts antinociceptive effects in several models of chemical nociception.<sup>29,30</sup>

### 3.3. Formalin test

The formalin test in mice revealed an antinociceptive effect (Table 3). The XM-Catechin (1) (100 µmol/kg, i.p.) caused a significant reduction in nociceptive responses during both the neurogenic (64.23%) and the inflammatory (86.80%) phase. The chloroform fraction (100 mg/kg, i.p.) induced reduction (31.51%) in the neurogenic phase, but not in the inflammatory phase. Indomethacin (100 µmol/kg, i.p.), the aqueous fraction and the ethanolic extract (all at doses of 100 mg/kg, i.p.) caused significant inhibition (49.86%, 73.68% and 82.40%, respectively) in the inflammatory phase, but this effect was not observed in the neurogenic phase.

**Table 3.** Anti-inflammatory profile of XM-Catechin (1), ethanol extract and fractions of *X. americana* in the formalin test

Group	Dose (mg/kg) or µmol/kg#	n <sup>a</sup>	1 <sup>st</sup> phase neurogenic <sup>b</sup> Time (s)	1 <sup>st</sup> phase inhibition (%)	2 <sup>nd</sup> phase inflammatory <sup>b</sup> Time (s)	2 <sup>nd</sup> phase inhibition (%)
Control (vehicle)	---	10	65.70 ± 2.48	---	207.40 ± 24.67	---
Indomethacin <sup>#</sup>	100	8	51.20 ± 4.78	21.32	104.00 ± 1.38**	49.86
XM-Catechin (1)	100	8	23.50 ± 7.05**	64.23	27.38 ± 12.77**	86.80
Ethanol Extract	100	10	51.60 ± 5.20	21.46	36.50 ± 14.68**	82.40
Acetate Fraction	100	8	60.43 ± 5.52	8.02	181.70 ± 24.88	12.40
Hexane Fraction	100	8	61.33 ± 2.65	6.65	169.30 ± 12.68	18.37
Chloroform Fraction	100	8	45.00 ± 2.86*	31.51	203.20 ± 23.43	2.03
Hydromethanol Fraction	100	8	62.00 ± 8.49	5.63	54.60 ± 27.10**	73.68

<sup>a</sup>Number of animals; <sup>b</sup>Values are expressed as mean ± S.E.M, \*\**p* < 0.01, \**p* < 0.05 (One-way ANOVA followed by the Dunnett's test).



The formalin test is believed to represent a more valid model for clinical pain.<sup>17</sup> The formalin test is a very useful method for not only assessing antinociceptive drugs but also helping in the elucidation of the mechanism of action. The neurogenic phase (first phase) is probably a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance P, while the late phase (second phase) is due to the release of histamine, serotonin, bradykinin and prostaglandins. Drugs that act primarily on the central nervous system, such as narcotics, inhibit both phases equally, while peripherally acting drugs such as anti-inflammatory non-steroidal and anti-inflammatory steroidal drugs only inhibit the late phase.<sup>31</sup>

Previous results have shown that the formalin test involves the release of various inflammatory mediators.<sup>32</sup> It is well known that NSAIDs (e.g., aspirin and diclofenac), which are known to inhibit cyclooxygenase (COX) activity, are largely ineffective or cause very weak inhibition against the early phase in the formalin test.<sup>33</sup> Our results, however, show that XM-Catechin (**1**), i.p. administered, induced a significant reduction in nociceptive

response during both the neurogenic phase (64.23%) and the inflammatory phase (86.80%), while indomethacin, the aqueous fraction, and the ethanol extracts caused significant inhibition of the inflammatory phase, and the chloroform fraction induced reduction only in the neurogenic phase of the formalin test in mice.

Moreover, it has been recently suggested that epicatechin exerts antinociceptive effects in formalin test by activation of the NO-cyclic GMP-K channels pathway, 5-HT<sub>1A/1B/1D/5A</sub> serotonergic receptors, and  $\mu/\kappa/\delta$  opioid receptors.<sup>34</sup> In addition, acute pre-treatment with epicatechin (0.03–30 mg/kg, i.p.) also prevented formalin-induced nociception in diabetic rats.<sup>35</sup>

### 3.4. Hot plate test

The hot plate test was used to determine whether XM-Catechin (**1**) or the ethyl acetate fraction had any central antinociceptive effect, but they showed no significant activity (Table 4). Morphine was used as a positive control in the hot plate test.

**Table 4.** Effects of the ethyl acetate fraction and XM-Catechin of *X. americana* in the hot plate test

Group	Pre-treatment (min)	Mean latency in the hot plate test (s)			
		Post-treatment (min)			
		30 min	60 min	90 min	120 min
Control	2.75 ± 0.46	2.50 ± 0.44	2.03 ± 0.37	4.24 ± 0.71	3.28 ± 0.78
Morphine	1.89 ± 0.55	9.03 ± 1.64*	7.40 ± 0.89*	5.39 ± 0.85*	2.58 ± 0.23
Acetate Fraction	5.87 ± 0.89	4.35 ± 0.39	4.89 ± 1.28	6.63 ± 1.84	5.56 ± 0.92
XM-Catechin ( <b>1</b> )	4.32 ± 0.94	3.25 ± 0.48	4.59 ± 0.66	5.17 ± 0.92	5.64 ± 1.3

Data represented as mean ± S.E.M, (%) – Percent inhibition of total hot plate response, number of animals = 8, \* $p < 0.05$  (One-way ANOVA).

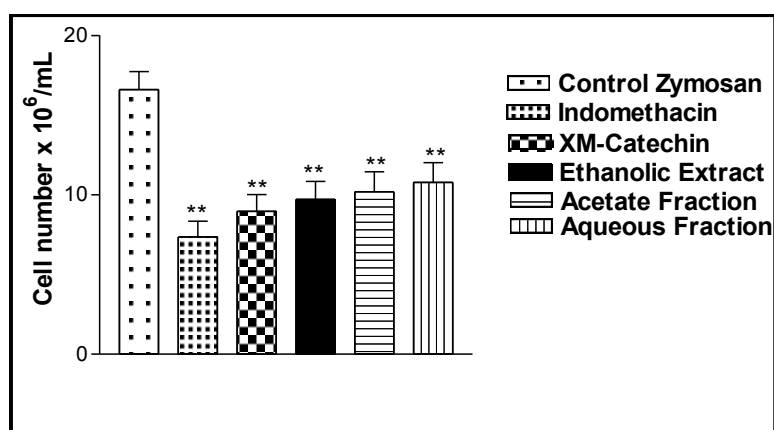
The thermal stimulus of the hot plate test is employed to evaluate antinociceptive

activity via the central mechanism.<sup>18</sup> In this model, the XM-Catechin (**1**) and ethyl acetate

fraction did not significantly increase the latency time. These results allow us to infer that the XM-Catechin (**1**) and acetate fraction do not possess a central antinociceptive effect. Morphine produced a marked and significant antinociception effect in the hot plate assay. Although the hot plate test is commonly used to assess narcotic analgesics, other centrally acting drugs, including sedatives and muscle relaxants or psychotomimetics have shown activity in this test.<sup>18</sup> However, unlike morphine, indomethacin and other NSAIDs have no effect in the hot plate test.<sup>28</sup>

### 3.5. Zymosan-induced peritonitis

The number of leukocytes in peritoneal exudates from mice treated with XM-Catechin (**1**), the hydromethanol fraction, the ethanolic extract and ethyl acetate fraction decreased by 46.02%, 35.06%, 41.45%, and 38.61%, respectively, when compared to exudates from the vehicle-treated control. The inhibition in response to indomethacin was 55.65% (Figure 3).



**Figure 3.** Effect of indomethacin and XM-Catechin (**1**) (both 100  $\mu$ mol/kg, i.p.), the ethanolic extract, ethyl acetate fraction and aqueous fraction (both 100 mg/kg, i.p.) on cell migration. Data represent mean  $\pm$  S.E.M. from at least six animals. \*\* $p$ <0.01

Another interesting result of the current study was the fact that XM-Catechin (**1**), the ethanol extract and the acetate fraction inhibited cell migration in zymosan-induced peritonitis, confirming their anti-inflammatory activity. Zymosan A is an insoluble polysaccharide component of the cell wall of *Sacharomyces cerevisiae* yeast. Among other actions, zymosan induces mast cell degranulation and activates macrophages. When administered in the peritoneal cavity of mice, zymosan induces increased vascular extravasation, one of the main signs of inflammation. This is a key step in the formation of inflammatory exudate and is followed by a time-dependent recruitment of migratory cells, especially

neutrophils.<sup>19</sup> There are many mediators that coordinate the initial events of acute inflammation, such as vasoactive amines, lipid-derived eicosanoids, chemokines, reactive oxygen species (ROS), nitric oxide, and cytokines.

In this test, the results showed that the ethanol extract, ethylacetate, hydromethanol fraction and XM-Catechin (**1**) of *X. americana* reduced the number of recruited cells, indicating that they contain active anti-inflammatory agents. When administered into the peritoneal cavity of mice, zymosan A induces an increase in vascular leakage, one of the primary signs of inflammation.<sup>36</sup> In this model, pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , activate the signaling

pathway in endothelial cells, which regulates the expression of adhesion molecules to initiate the recruitment of both circulating leukocyte migrating cells and partially activated leukocyte cells.<sup>37</sup> Moreover, Quiñonez-Bastidas et al. 2017 previously reported that oral (-)-epicatechin also reduced carrageenan-induced inflammation by about 59%.<sup>34</sup>

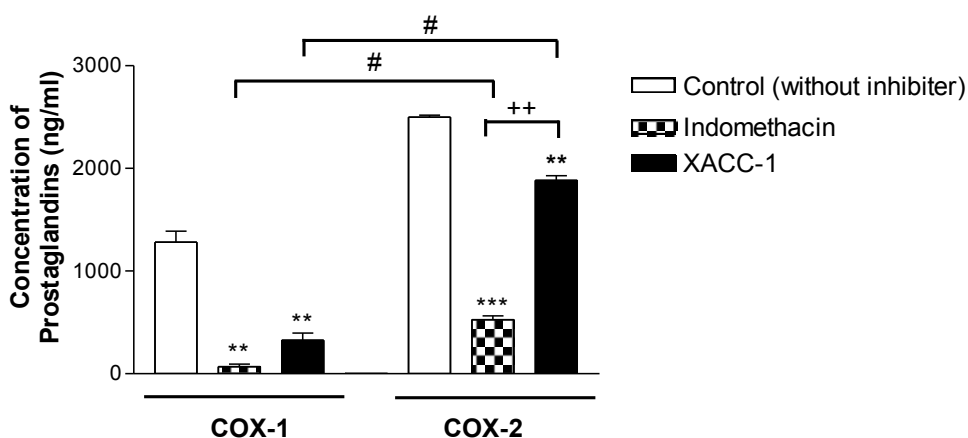
Therefore, the ethanol extract of stem bark, ethyl acetate, hydromethanol fractions and isolated XM-Catechin (**1**) of *X. americana* decreased leukocyte recruitment likely due to their ability to decrease production of pro-inflammatory cytokines. This probably occurs because flavonoids have an anti-inflammatory activity, which was verified by significant inhibition of inflammatory nociception in the formalin test. These data are consistent with results verified by Soro & Sakande 2009 and Olabissi et al. 2011,<sup>28,38</sup> when they respectively studied the

antinociceptive activity of aqueous extract of stem bark and anti-inflammatory property of the aqueous ethanol extract of root bark of *X. americana*.

### 3.6. Inhibition of COX

The ability of XM-Catechin to inhibit COX-1 and COX-2 was determined using an ovine enzyme immunoassay kit, according to the methodology described by Uddin et al., 2004.<sup>16</sup> These results are shown in figure 4.

It was observed that XM-Catechin and indomethacin were able to significantly inhibit more COX-1 than COX-2 at a concentration of 0.2  $\mu$ M. Moreover, indomethacin decreases more COX-2 activity than XM-Catechin, but both treatments, at this concentration, inhibit COX-1 similarly.



**Figure 4.** *In vitro* inhibition of COX-1/COX-2 sheep enzymes by XACC-1=XM-Catechin (**1**) and indomethacin, both at concentrations of 0.2  $\mu$ M. Values are expressed as mean  $\pm$  S.E.M; \*\*\* $p$  < 0.001, \*\* $p$  < 0.01 when compared to the control group using one-way ANOVA followed by the Dunnett's test; # $p$  < 0.05 when compared to the treatment against COX-1 versus COX-2 using the *t* student test; ++ $p$  < 0.01 when compared to the inhibition of the COX isoform by treatment with indomethacin versus XM-Catechin (**1**) using the *t* student test

The inhibition of COX by flavonoids can occur indirectly or directly. Indirectly, flavonoids can inhibit COX by a combination of scavenging free radicals and interaction with enzymes, modifying their functions.<sup>39</sup> Multiple hydroxyl groups in the molecular structure of flavonoids confer substantial

antioxidant and chelating properties, however, pro-oxidant activity cannot be excluded. Hydroxyl groups on the B-ring donate a hydrogen and alternatively, electrons and proton, to hydroxyl and peroxy radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. 5,7-

Dihydroxy arrangement in the A ring, such as present in XM-Catechin (**1**), increase antioxidant effects and 5-OH enhances peroxynitrite scavenging ability.<sup>17,28,40</sup>

Free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH in the C-ring. The torsion angle of the B-ring with respect to the rest of the molecule strongly influences free radical scavenging ability. Flavonoids with a 3-OH are planar and this planarity permits conjugation, electron delocalization, and a corresponding increase in flavonoid phenoxyl radical stability.<sup>17,31,41</sup>

Directly, flavonoids can bind to COX and thereby inhibit the enzyme. As described in the literature, regards the structural requirements of flavonoids for direct inhibition of PLA<sub>2</sub>, COX and LOX, it is the phenyl ring that is responsible for the inhibition of PLA<sub>2</sub>. The benzopyran ring is the portion which binds to COX, and both the ring and benzopyran hydroxyls at C5 and C7 of the A ring are the pharmacophore groups for inhibition of LOX.<sup>32</sup>

As such, it was verified that the mechanisms of actions of antinociceptive and anti-inflammatory activities by XM-Catechin (**1**) involve the inhibition of COX using *in vitro* cyclooxygenase (COX) inhibition assays at a concentration of 0.2 μM. It was observed that XM-Catechin (**1**) inhibited COX-1 and COX-2. COX-2 is the inducible COX isoform expressed in inflammatory cells and tissues in response to cellular activation by endotoxins, cytokines, mitogens and other stimuli.<sup>32,42</sup>

These data suggest that XM-Catechin (**1**), hydromethanol fraction and the ethanol extracts have antinociceptive and anti-inflammatory actions. This effect of the XM-Catechin (**1**) flavonoid occurs at least in part due to COX inhibition, and consequently, decreased prostaglandin synthesis. Also, it is reasonable to assume that the XM-Catechin (**1**) and the chloroform fraction may act in other ways, considering the action evidenced in the first phase of the formalin test.

Unpublished findings of our group also demonstrate that the treatment of mice with

*X. americana* extracts, fractions or XM-Catechin (**1**) produces no changes in behavior, such as the appearance of involuntary movements, piloerection, stimulatory or sedative effects, respiratory depression or gastric toxic effects.

There is a compelling evidence that many plants or their active principles used in traditional medicine may be useful for the treatment of pain or inflammatory conditions.<sup>43</sup> Then, the biological actions demonstrated in this study support the ethnomedicinal use of this plant, which is used for treatment for pain and inflammatory ailments (such as joint pain, headache, tooth ache and arthritis) and prepared although decoction, maceration, powder of the leaves, bast, seeds, roots, and fruits.<sup>44-46</sup>

Further, this work shows, for the first time, that the ethanol extract, fractions and/or XM-Catechin (**1**) from *X. americana*, administered intraperitoneally in mice, produce significant antinociceptive and anti-inflammatory action against chemical models of nociception (acetic acid-induced writhing or formalin test) or zymosan-induced peritonitis.

#### 4. Conclusion

This study has shown that the extract, fractions and XM-Catechin (**1**) isolated from *X. americana*, according to Uchôa et al. 2016,<sup>8</sup> possess significant antinociceptive and anti-inflammatory effects in mice at the doses investigated. Our results support the traditional use of this plant for some painful and inflammatory conditions, and its activity is attributed to the flavonoid (XM-Catechin (**1**)) reported here which is present in the ethyl acetate fraction of the stem bark extract of the plant. Furthermore, the antinociceptive and anti-inflammatory actions, demonstrated in the present study, support, at least partially, the ethnomedicinal uses of this plant.

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