

Free radical scavenging ability of *Ximenia americana* L. stem bark and leaf extracts

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ABSTRACT

Phytochemical investigation of the ethanol extract of stem bark and leaves of *Ximenia americana* L. revealed the presence of epicatechin and quercetin, respectively. This is the first report of the occurrence of epicatechin in this genus. The extracts, fractions and isolated compounds of *X. americana* were subjected to an *in vitro* antioxidant activity assay by the DPPH method (2,2-diphenyl-1-picrylhydrazyl). The EC₅₀ values were calculated and the results indicate that the ethyl acetate fractions of the stem bark and leaves have high antioxidant activity and a high content of total phenols. The high antioxidant activity of these fractions is justified by the presence of epicatechin and quercetin.

INTRODUCTION

Irreversible damage to proteins, carbohydrates, lipids and nucleic acids are some of the factors responsible for the development of numerous degenerative diseases, such as atherosclerosis, Parkinson's disease, Alzheimer's disease, cardiovascular diseases, cancer and diabetes (Lodyato *et al.*, 2004). Fruits and vegetables are important in human diet because they contain antioxidative components that protect against free radicals and that have been associated with reduced risk of these degenerative diseases (Zhang and Hamauzu, 2004, Sharma, *et al.*, 2015). The compounds responsible for antioxidant activity are of endogenous origin, such as enzymes and proteins, and of exogenous origin, such as low-molecular-mass substances like vitamins, carotenoids, flavonoids, anthocyanins and other phenolic compounds commonly present in plants (Argolo *et al.*, 2004; Zhang and Hamauzu, 2004; Yin *et al.*, 2015). Several methods have been developed to determine the antioxidant capacity of pure substances and extracts of plants. One of the most reliable methods involves measuring the sequestration of

free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), or other color radicals (Roginsky and Lissi, 2005, Fu *et al.*, 2010). DPPH is a stable free radical at room temperature, with a typical violet color in methanol solution, and its use is indicated as a reagent for testing the radical scavenging activity of small molecules (Roginsky and Lissi, 2005, Fu *et al.*, 2010). In this type of assay, scavenging of the DPPH• radical is verified by monitoring the decrease of absorbance at 516 nm resulting from the antioxidant activity (AH) (DPPH• + AH → DPPH-H + A•) (Argolo *et al.*, 2004; Sánchez-Moreno *et al.*, 1998). *Ximenia americana* is a thorny bush belonging to the family Olacaceae, found in tropical regions of America and Africa (Pott and Pott, 1994). In Brazil it is known by several popular names: ameixa-da-baia, ameixa-da-terra, ameixa-de-espinho and sândalo-do-brasil (Braga, 1960). All the parts of this plant are used in folk medicine (James *et al.*, 2008, Braga, 1960).

The roots have antiseptic properties and are used in the treatment of fever, jaundice and headaches. The leaves have a laxative action and are indicated in the treatment of measles (Omer and Elnima, 2003). The powdered stem is recommended for healing ulcers, as a depurative, as a menstrual regulator and for gastric upsets. The infusion of its flowers serves to reduce bloody diarrhea (Braga, 1960).

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This plant is also active against rheumatism, cancer and many infections (Mevy *et al.*, 2006). A pharmacological study of *X. americana* indicates that this species presents anticancer and antineoplastic (Voss, *et al.*, 2006), antimicrobial (Geyid *et al.*, 2005, Omer and Elimina, 2003), antibacterial (Koné *et al.*, 2004, Kawo *et al.*, 2011, Da Silva *et al.*, 2015), activity antipyretic (Soro *et al.*, 2009), radical scavenging (Maikai *et al.*, 2010, Le *et al.*, 2012) and pesticidal activity (Fatope *et al.*, 2000).

In this work, the DPPH radical scavenging activity was evaluated and the total phenolic content of ethanol extracts (EtOH) of the stem bark and leaves of *X. americana* was determined, as well as the fractions obtained from the liquid-liquid partitioning of the extracts. In addition, biomonitored fractionation of the ethyl acetate fraction of the stem bark and leaves was performed, resulting in the isolation of the flavonoids, epicatechin and quercetin as active compounds. This is the first report on the isolation and characterization of epicatechin in this specie, as well of the DPPH radical scavenging activity and total phenolic content of ethanol extract (EtOH) and fractions of the stem bark of *X. americana*.

MATERIALS AND METHODS

General experimental procedures

All the solvents and reagents used in the preparation of the extracts and fractionations were of analytical grade (PA) supplied by Vetec (gallic acid), Merck (Sephadex LH-20, SiO₂, 70 – 230 mesh; Folin-Ciocalteu), Aldrich Co (DPPH), PVP Sociedade Anônima, Parnaíba-Piauí (rutin), Synth and Ecibra. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer at 500 and 125 MHz, respectively, using CD₃OD as solvent. The absorption measurements were determined by an UV-Vis spectrophotometer PerkinElmer, Lambda 25.

Vegetable material

The stem and leaves of the species *Ximenia americana* L. were collected in April 2010 in the municipality of Campo Maior, state of Piauí, Brazil, and identified by Dr. Maria Edilene Alencar. An exsiccatum was deposited in the Grasiela Barroso herbarium at the Federal University of Piauí under number TEPB 14407.

Extraction and fractionation of the plant

The collected stems and leaves of *X. americana* were separated and dried at room temperature (26 °C±1) away from light. Dried and powdered, stem bark (3.3 kg), stem wood (2.5 kg) and leaves (1.5 kg) were extracted three consecutive times with 95% ethanol in a stainless steel extractor at a temperature of 26 °C±1.

Ethanol extracts of the stem bark (620 g, 18%), stem wood (250 g, 10%) and leaves (230 g, 15%) were obtained after concentration in a low-pressure rotary evaporator and elimination of the residual humidity by lyophilization.

The ethanol extracts of stem bark (500 g) and leaves (210 g) were suspended separately in a MeOH/H₂O mixture (2:3) and

subjected successively to the liquid-liquid partitioning process with C₆H₁₄ (3 x 700 ml), CHCl₃ (3 x 700 ml) and EtOAc (3 x 700 ml). After removal of the solvent at low pressure in the rotary evaporator and lyophilization of the hydromethanol fraction, four fractions were obtained of the ethanol extract of the leaves: in hexane (15.5 g; 7.4%), in chloroform (7.5 g; 3.2%), in ethyl acetate (80.9 g; 38.5%) and hydromethanol (101 g, 48%), and four fractions of the ethanol extract of the stem bark: in hexane (10.75 g; 1.7%), in chloroform (7.2 g; 1.2%), in ethyl acetate (220 g; 45%) and hydromethanol (240.4 g; 48%). The raw extracts in ethanol and fractions resulting from partitioning were subjected to *in vitro* antioxidant activity assays.

Phytochemical isolation

The ethyl acetate fraction (200.0 g) of stem bark was subjected to chromatography in a deactivated silica gel column with 10% water (800 g), using ethyl acetate and methanol as eluant solvents. Three subfractions were obtained from the passage of 1000 ml of each solvent: C1- EtOAc (46.54 g, 23.2%), C2- EtOAc (29.11 g, 14.5%) and C3-MeOH (76.66 g, 38%).

An aliquot of subfraction C1- EtOAc (40.0 g) was subjected to silica gel column chromatography (300 g). Seven fractions were collected with a volume of 500 mL each, using CHCl₃, MeOH and mixtures of these as eluants.

After removal of the solvent, the CHCl₃:MeOH 20% (17 g, 42.5%) fraction yielded a wine-colored amorphous solid. Part of this material (3.5 g) was subjected to crystallization in ethyl ether and ethyl acetate 1:1, three consecutive times, yielding compound **1** (1 g, 28%).

The EtOAc (70 g) fraction of the leaves was subjected to activated charcoal filtration in a Buchner funnel. Six subfractions were obtained from the passage of 1000 ml of each of the following solvents, which, after being eliminated, yielded the following fractions: H₂O (40 g, 57%), EtOH/H₂O 1:1 (10 g, 14%), EtOH (7 g, 10%), EtOAc (5 g, 7%), CHCl₃ (2 g, 3%) and C₆H₁₄ (2 g, 3%).

The EtOAc (3 g) fraction was subjected to silica gel column chromatography (30 g) and eluted with a mixture of CHCl₃ and MeOH. Thirty-five fractions of 30 mL each were collected, analyzed by silica-gel thin-layer chromatography (CCD), and grouped. The 17-24 (1.5 g, 50%) fraction was purified in a Sephadex LH-20 column using MeOH as solvent. This procedure was repeated twice and led to the isolation of compound **2** (1 g, 66.6%).

(-) *epicatechin* (**1**): brown amorphous solid, MP 239 °C (EtOH). [α]_D²⁹ -45 (c = 1.0 MeOH). IR cm⁻¹: 3502, 3412 cm⁻¹ (O-H). ¹H RMN (500 MHz, CD₃OD): δ 4.83 (d, *J* = 7.5 Hz, H-2), 4.19 (br s, H-3), 2.88 (dd, *J* = 4.5, 16.7 Hz, H-4_{syn}), 2.76 (dd, *J* = 2.7, 16.7 Hz, H-4_{anti}), 5.97 (d, *J* = 2.3 Hz H-6), 5.94 (d, *J* = 2.3 Hz H-8), 7.00 (d, *J* = 1.7 Hz H-2'), 6.78 (d, *J* = 8.2 Hz H-5'), 6.82 (dd, *J* = 1.7, 8.2 Hz H-6'). ¹³C-NMR (125 MHz, CD₃OD): δ 80 (C-2), 67.6 (C-3), 29.8 (C-4), 158.1 (C-5), 96.5 (C-6), 157.7(C-7), 96 (C-8), 157.5 (C-9), 100 (C-10), 132.4 (C-1'), 115.4 (C-2'), 145.8

(C-3'), 146 (C-4'), 116 (C-5'), 119.5 (C-6'). The data were equal to those of literature data (Lopes *et al.*, 2012, Wei *et al.*, 2013).

Quercetin (2): yellow solid, MP 312 °C (EtOH). IR cm^{-1} : 3420 cm^{-1} (O-H), 1725 cm^{-1} (C=O). ^1H RMN (500 MHz, CD_3OD): δ 6.20 (d, $J = 2.0$ Hz, H-6), 6.39 (d, $J = 2.0$ Hz, H-8), 7.74 (d, $J = 2.2$ Hz, H-2'), 6.89 (d, $J = 8.5$ Hz, H-5'), 7.65 (dd, $J = 8.5, 2.2$ Hz, H-6'). ^{13}C -NMR (125 MHz, CD_3OD): δ 147.8 (C-2), 137 (C-3), 177 (C-4), 162.1 (C-5), 99.2 (C-6), 165.2 (C-7), 94.4 (C-8), 158 (C-9), 104.4 (C-10), 124 (C-1'), 115.8 (C-2'), 146 (C-3'), 148.5 (C-4'), 116.1 (C-5'), 121.6 (C-6'). The data were equal to those of literature data (Wei *et al.*, 2013).

Determination of antioxidant activity and dosage of total phenolics

The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical consumption, and the quantification of phenolic compounds determined by Folin-Ciocalteu method and expressed in gallic acid equivalent (GAE) according to Sousa *et al.*, (2007), Costa *et al.*, (2010) and Ferreira *et al.*, (2014).

Statistical analysis

The results presented in this study correspond to the mean value of three repetitions ($n=3$) \pm standard deviation of the mean. The results of antioxidant activity that presented a probability of occurrence of the null hypothesis of less than 5% ($P < 0,05$), applying ANOVA followed by Tukey's multiple comparison test, were considered statistically different. All the analyses were performed using Microcal Origin 8.0 software.

RESULTS AND DISCUSSION

Structural identification of the isolated substances

Compound **1** and **2** (Figure 1) was identified to be (-) epicatechin e quercetin, respectively for comparison of the spectroscopic data (IR and the one- and two-dimensional NMR) with those reported in the literature Lopes *et al.*, 2012, Wei *et al.*, 2013.

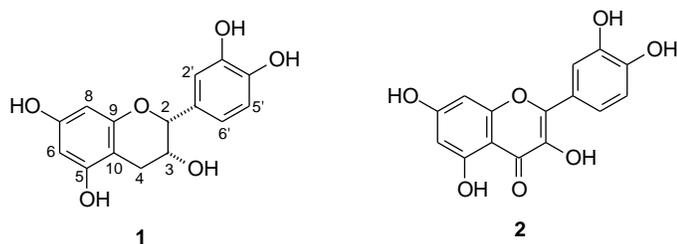


Fig. 1: Compounds isolated from the EtOH extract of *X. americana*.

In vitro radical scavenging activity

Evaluation of the antioxidant activity of extracts and molecules in assays with the DPPH radical is a widely used method because it is easy to perform and less time-consuming than other methods. The reaction can be observed visually by thin-layer chromatography (qualitative assay) and its intensity can be

evaluated by chromometric or spectrophotometric assays (Sánchez-Moreno *et al.*, 1998; Soler-Rivas *et al.*, 2000). The DPPH radical is sequestered by antioxidants through the donation of hydrogen to form the stable molecule of DPPH in the reduced form (Argolo *et al.*, 2004).

Figure 2 shows the radical scavenging activity, expressed in percent of inhibition (PI), exhibited by the ethanol extracts of the stem bark, stem wood and leaves of *X. americana*, in the concentrations of 250, 200, 150, 100, 50 and 25 $\mu\text{g}/\text{mL}$. Based on these data, it is evident that the ethanol extracts of the leaves, stem bark and wood of *X. americana* contain substances that act as hydrogen donors to the DPPH radical, although they show a differentiated action.

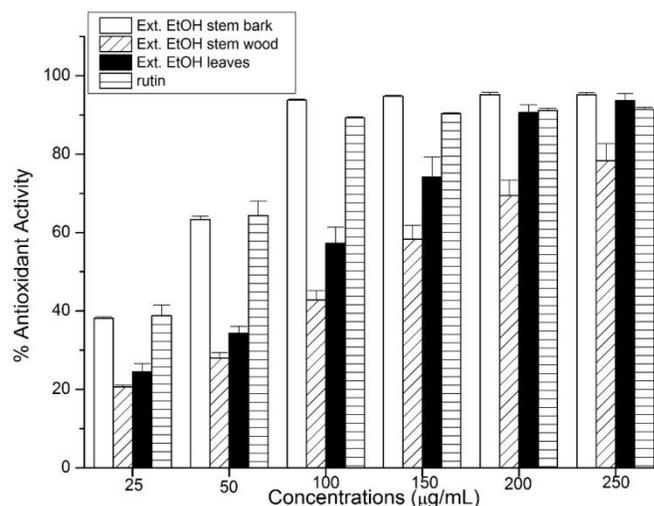


Fig. 2: Antioxidant activity of the EtOH extracts of *Ximenia americana* versus rutin (control group).

The ethanol extract of the stem bark showed the highest radical scavenging activity in all the concentrations, and was the most effective in sequestering the DPPH radical, reaching a percent of inhibition of more than 90% at the concentration of 100 $\mu\text{g}/\text{mL}$. The ethanol extract of the stem wood showed a PI = 78.29% at the concentration of 250 $\mu\text{g}/\text{mL}$, displaying a lesser capacity to sequester the DPPH radical.

Taking into consideration the statistical analysis of the data on radical scavenging activity at all the tested concentrations (25, 50, 100, 150, 200 and 250 $\mu\text{g}/\text{mL}$), and applying ANOVA and Tukey's test, it was found that the ethanol extract of stem bark showed a significant difference ($P < 0.05$) in relation to the ethanol extracts of stem wood and leaves, but did not show a significant difference ($P < 0.05$), as a source of free radical scavenging substances, in relation to the positive control rutin.

The leaf extract showed a higher radical scavenging activity than the stem wood extract and a lower activity than the stem bark extract at all the tested concentrations. A comparison of the percentages of inhibition of the extracts and that of the positive control compound rutin indicated a radical scavenging activity comparable to that of the control, as shown in Figure 2. At a concentration of 100 $\mu\text{g}/\text{mL}$, the ethanol extracts of stem bark,

leaves and stem wood showed a percentage of inhibition of the DPPH radical of $94.75\% \pm 0.27$; $57.27\% \pm 4.20$ and $42.83\% \pm 2.43$, respectively. Because the stem wood extract showed less activity, it was not fractionated.

The hexane, chloroform, ethyl acetate and hydromethanol fractions resulting from the liquid-liquid partitioning of the ethanol extracts of stem bark and leaves were subjected to quantitative antioxidant assays.

The hexane fractions of the ethanol extracts of stem bark and leaves presented a low percentage of inhibition of DPPH radical, proving to be poor in antioxidant compounds. The ethyl acetate fractions of stem bark and leaves at the concentration of $100 \mu\text{g/ml}$ reduced the DPPH by $93.49\% \pm 2.85$ and $92.33\% \pm 3.12$, respectively, indicating that these fractions were the most active, as shown in Figure 3.

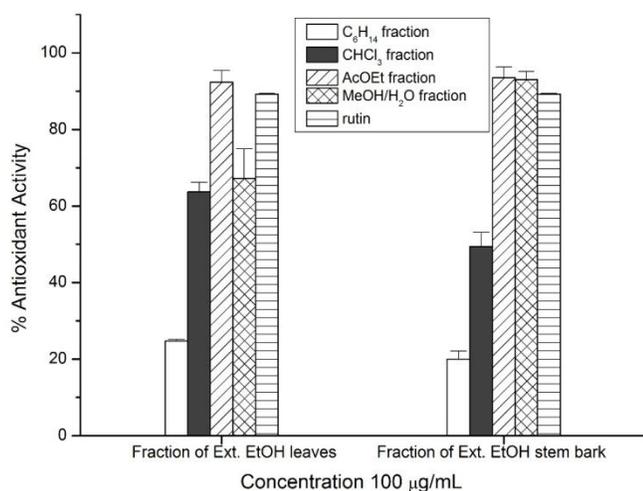


Fig. 3: Antioxidant activity of the fractions of ethanol extracts of leaves and stem bark of *X. americana*

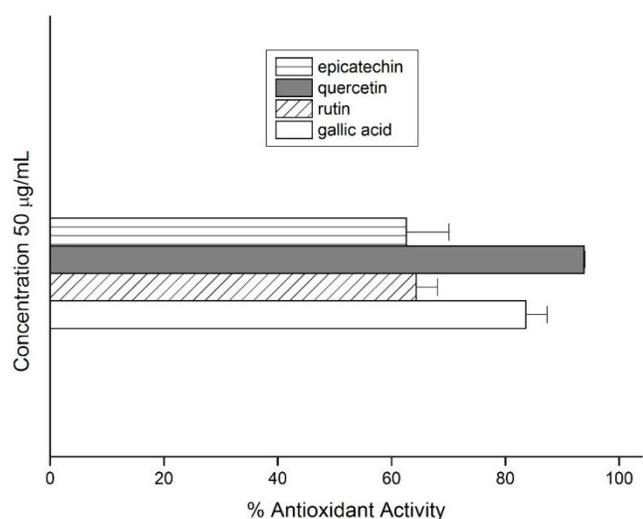


Fig. 4: Antioxidant activity of the compounds isolated from *X. americana* and controls at the concentration of $50 \mu\text{g/ml}$

The flavonoids epicatechin (**1**) from stem bark and quercetin (**2**) from leaves were isolated from the ethyl acetate

fractions. At the concentration of $50 \mu\text{g/ml}$, the isolated compounds epicatechin (**1**) and quercetin (**2**) and the positive controls gallic acid and rutin presented a percentage of DPPH inhibition of $62.62\% \pm 2.5$; $93.84\% \pm 0.13$; $83.59\% \pm 3.7$ and $64.4\% \pm 3.73$, respectively, as shown in Figure 4.

Figure 5 shows the kinetic behavior of the reaction of DPPH with each extract or control, at the concentration of $100 \mu\text{g/ml}$, based on the dose-response curve of the decrease in percentage of remaining DPPH (DPPH_{REM}) as a function of time (min). As can be seen, almost all the samples reduced the initial concentration of DPPH before of five minute of analysis, a behavior comparable to that of the positive control (rutin), and can therefore be classified as an antioxidant of rapid kinetics (0 to 5 min) (Sousa *et al.*, 2007). Only the samples of ethanol extract and hydromethanol fraction of the leaves were considered antioxidants of slow kinetics (after 30 min).

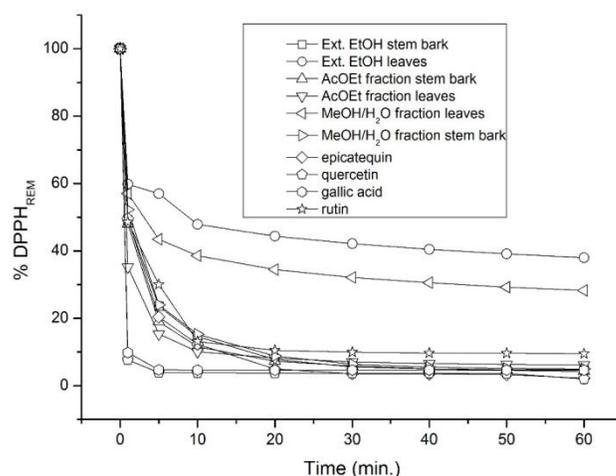


Fig. 5: % DPPH_{rem} of the samples at the concentration of $100 \mu\text{g/ml}$.

The increasing order of speed of the scavenging action was found to be: rutin control < epicatechin < MeOH/H₂O fraction (stem bark) < EtOAc fractions (leaves) < EtOH extract (stem bark) < EtOAc fractions (stem bark) < gallic acid < quercetin, as shown in Table 1. The EC_{50} is a parameter widely used to determine the antioxidant power of extracts or substances, and it can be stated that the lower the EC_{50} value the higher the free radical scavenging power (Sanchez-Moreno *et al.*, 1998).

The EC_{50} values of the extracts, fractions and compounds of *X. americana* were calculated, and the results indicate that the values of the ethyl acetate fractions of stem bark ($29.62 \mu\text{g/ml} \pm 3.00$) and of leaves ($33.03 \mu\text{g/ml} \pm 2.66$) of *X. americana* are lower than the standard compound, the rutin ($47.08 \mu\text{g/ml} \pm 4.65$) and higher than gallic acid ($24.90 \mu\text{g/ml} \pm 0.04$). The high radical scavenging activity of these fractions may be justified by the presence of epicatechin (**1**) ($34.64 \mu\text{g/ml} \pm 3.00$) and quercetin (**2**) ($16.40 \mu\text{g/ml} \pm 1.30$).

Even though the extracts present compounds such as quercetin (leaves) and epicatechin (stem bark), it is not possible to infer unequivocally that these are the only compounds responsible

for the observed action, since the raw extract of stem bark and the EtOAc fraction of leaves show a result similar to that of the epicatechin compound.

Table 1: Total phenol (TP) content and antioxidant activity (EC₅₀) of the EtOH extracts and fractions of *Ximenia americana*.

Samples	EC ₅₀ ± SD (µg/mL)	TP (mg of GAE/g of EtOH extract ± SD)
EtOH extract of stem bark	32.68 ± 0.18	678.66 ± 2.31
EtOH extract of stem wood	101.72 ± 10.33	447.47 ± 4.87
EtOH extract of leaves	68.30 ± 5.10	665.37 ± 2.50
C ₆ H ₁₄ fraction (leaves)	272.31 ± 9.67	153.29 ± 9.23
CHCl ₃ fraction (leaves)	60.31 ± 2.31	449.76 ± 7.90
AcOEt fraction (leaves)	33.03 ± 2.66	662.24 ± 3.08
MeOH/H ₂ O fraction (leaves)	53.84 ± 3.53	625.37 ± 8.01
C ₆ H ₁₄ fraction (stem bark)	NA	90.25 ± 2.38
CHCl ₃ fraction (stem bark)	84.24 ± 3.00	243.51 ± 3.00
AcOEt fraction (stem bark)	29.62 ± 2.91	878.47 ± 0.75
MeOH/H ₂ O fraction (stem bark)	33.65 ± 5.07	736.84 ± 1.50
Compound – epicatechin (stem bark)	34.64 ± 3.00	NT
Compound – quercetin (leaves)	16.40 ± 1.30	NT

NA – Not active; NT – Not tested.

The determination of total phenols is an indirect method of evaluation of antioxidant activity, providing important information that can characterize natural products as sources of free radical scavenging substances (Roginsky and Lissi, 2005). Among the various methods developed for the quantification of phenolic compounds, the Folin-Ciocalteu reagent method has been widely used (Atoui *et al.*, 2005).

The results of the analysis of total phenols represent the behavior found in the analysis of the antioxidant action. The ethanol extract of stem bark and the ethyl acetate, hydromethanol and chloroform fractions showed a total phenol content of 678.66; 878.47; 736.84 and 243.51 mg of EAG, respectively. On the other hand, the ethanol extract of leaves and the ethyl acetate, hydromethanol and chloroform fractions showed a total phenol content of 665.37; 662.24; 625.26 and 449.76 mg of EAG, respectively, as indicated in Table 1. From these results, it can be observed that the evaluated samples presented a high percentage of phenolic compounds.

The highest total phenolic content for ethanol extracts of stem bark and leaves were found in the ethyl acetate fraction. The chloroform fractions of both the stem bark and leaves presented a lower contents of total phenols, which is consistent with the lower antioxidant activity observed in the DPPH assay.

CONCLUSION

In this work, leaves and stem bark of *Ximenia americana* presented high radical scavenging activity by the DPPH photolorimetric method and high total phenol content. The stem bark showed better results than the leaves. The quercetin compound isolated from the leaves had a better free radical scavenging activity compared to the positive controls gallic acid and rutin. This is the first report of the occurrence of epicatechin in this specie, as well of the DPPH radical scavenging activity and total phenolic content of ethanol extract (EtOH) and fractions of the stem bark of *X. americana*.

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