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Antioxidant Activity and Phytochemical Screening of Extracts of *Erythroxylum suberosum* A.St.-Hil (Erythroxylaceae)

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ABSTRACT

Erythroxylum suberosum (Erythroxylaceae) is used in traditional Brazilian medicine as antidiarrheal, astringent, antirheumatic, anesthetic and against indigestion. The objectives of this study were to perform classical phytochemical screening, determine the total phenolic content and the antioxidant activity of ethanol and ethyl acetate extracts of *E. suberosum*. The extracts of the leaves were obtained by maceration. Phytochemical screening was performed using Thin Layer Chromatography (TLC) and specific reactions. Total phenolic content was determined using the Folin-Ciocalteu method. The antioxidant activity of the extracts was performed by reduction of DPPH, reduction of Fe⁺³ and Cyclic Voltammetry (CV). Phytochemical screening suggested the presence of alkaloids, coumarins, flavonoids, anthocyanins and condensed tannins in ethanol extract and flavonoids and triterpenes/steroids in the ethyl acetate extract. The amount of total phenolics detected was expressive, especially in the ethanol extract (575.50±2.50 mg g⁻¹ of ethanol extract and 242.20±9.53 mg g⁻¹ of extract in ethyl acetate). The ethanol extract showed higher antioxidant activity in the three methodologies employed (DPPH: IC₅₀ of 0.18 mg mL⁻¹, reduction of metal ion at 0.3 mg mL⁻¹: 0.903±0.006-absorbance at 700 nm, CV: Increase of anodic current at 1.0 V at 2 mg extract mL⁻¹) in comparison with the extract in ethyl acetate (DPPH: IC₅₀ of 0.74 mg mL⁻¹, reduction of metal ion at 0.3 mg mL⁻¹: 0.871±0.007-absorbance at 700 nm, CV: Lower increase of anodic current at 1.0 V at 2 mg extract mL⁻¹). The highest antioxidant activity of the ethanol extract may be associated with a higher content of phenolic compounds.

Key words: *Erythroxylum suberosum*, phytochemical, antioxidant activity

INTRODUCTION

Erythroxylum suberosum A.St.-Hil., popularly known as “galinha choca” and “mercúrio do campo”, is commonly found in the Brazilian Cerrado. The stem bark, leaves and roots of this plant are traditionally used as antidiarrheal agents, astringent, anesthetic, antirheumatic and against indigestion (Barbosa and Pinto, 2003; Brandao, 1993).

This species was described by European naturalists, who traveled to Brazil in the nineteenth century. The French botanist Auguste de Saint-Hilaire, in his "Plantes Usuelles des Brésiliens" reports the use of their barks to obtain reddish-brown pigment in Minas Gerais. The German naturalist Karl FP Martius in "Systema Materiae Medicae Vegetabilis, Brasiliensis" describes that the bark of this species is abundant in "stryphno" (astringency) that precipitates in the presence of iron, yielding a blue color and can be used as a tonic and to dye cloth.

The Erythroxylaceae family comprises four genres and about 240 species with pantropical distribution. The genus *Erythroxylum* P. Browne is the most representative, with approximately 230 species that are found in South America, Africa and Madagascar (De Oliveira *et al.*, 2011; Loiola *et al.*, 2007; Brachet *et al.*, 1997). Brazil is considered a center of endemism and diversity of this genus (Plowman and Hensold, 2004).

Chemically, *Erythroxylum* genus is characterized by the presence of tropane alkaloids (Oliveira *et al.*, 2012; Zuanazzi *et al.*, 2001; Brachet *et al.*, 1997), among which stands out the biosynthesized cocaine almost exclusively by *E. coca* and *E. novogranatense* (Loiola *et al.*, 2007; Bieri *et al.*, 2006). Researches on the chemical composition of the genus *Erythroxylum* also indicate the occurrence of diterpenes (Santos *et al.*, 2006; Ansell *et al.*, 1993), flavonoids and triterpenes (Chavez *et al.*, 1996). Phenolic compounds and alkaloids have been detected and isolated from *E. suberosum* (Lucas-Filho, 2009; Bohm *et al.*, 1988). Some biological activities have been reported for the genus, such as anti-inflammatory, cytotoxic, antioxidant, ACE inhibition (Lucas-Filho *et al.*, 2010; Cordova *et al.*, 2012; De Oliveira *et al.*, 2011; Chaves *et al.*, 1988). For the *E. suberosum*, antimicrobial and cytotoxic activities were observed in the extracts evaluated (Violante *et al.*, 2012). The phenolic compounds of plant origin generally act in the capture and neutralization of reactive oxygen species or radical species, in addition to bind metals, preventing them from acting as catalysts in the production of radicals (Evans, 2002; Sousa *et al.*, 2007). Thus, they can be used as antioxidants, including pathological processes.

Oxidative stress, a consequence of the imbalance between pro-oxidant and antioxidant factors in the body, plays a very important role in the pathogenesis of several diseases, such as diabetes, cardiovascular diseases, cancer, inflammation and atherosclerosis (Ramalho and Jorge, 2006; Droge, 2002). Thus, the medicinal activity of natural products can, in certain cases, such as anti-inflammatory activity, be associated with the antioxidant power of their secondary metabolites.

Erythroxylum suberosum is a medicinal plant with recorded usage in Brazil, since the nineteenth century and be used in pathologies in which free radicals may be involved (as in different types of rheumatism). However, the chemical composition and biological activity of this species has been little studied. So, the objectives of this study were to conduct screening phytochemical, determine the total phenolic content and the antioxidant capacity of ethanol and ethyl acetate extracts from the leaves of this species.

MATERIALS AND METHODS

Chemical and solvents: The solvents ethyl acetate and ethanol and Folin-Ciocalteu reagent were purchased from Dinamica, the gallic acid from Impex and DPPH from Aldrich.

Plant material: The leaves of *E. suberosum* were collected on April 20th, 2012, near the Campus JK of the Federal University of the valleys of Jequitinhonha and Mucuri (UFVJM) in Diamantina, Minas Gerais, Brazil (18°14'2.20"S and 43°35'12.95"W, 1284 m altitude). The species was identified by means of specific literature and consultation herbal and its voucher specimen deposited in the Herbarium Dendrologic Jeanine Felfili (HDJF/UFVJM) under the registration number 1189.

Preparation of crude extracts: The leaves were dried in an air circulating oven at 35°C to constant weight and then were ground in a knives mill. After grinding, two hundred and forty grams of the plant material was macerated at room temperature with 250 mL of ethyl acetate for 72 h. The extraction process was repeated three times to obtain extractable components of the plant. Then, the same maceration process was performed with the solvent ethanol. The extracts were filtered and concentrated on a rotary evaporator Fisatom® 801 at 40°C, under reduced pressure. The yield was 1.56% for the extract in ethyl acetate and 17.3% for the ethanol extract.

Phytochemical screening: The presence of the major groups of secondary metabolites in the extracts in ethyl acetate and ethanol was evaluated by conventional tests involving chromogenic and precipitation reactions and analysis by Thin Layer Chromatography (TLC). The tests were based on the methods described by Raaman (2006) and Farnsworth (1966) as the polarity of each extract, such as: Mayer, Bertrand, Dragendorff and Bouchardat reactions (alkaloids), reactions in alkaline medium (anthocyanins and coumarins), Borntraeger reaction (anthraquinone), Shinoda reaction (flavonoids), reactions with ferric chloride and gelatin (tannins), Liebermann-Burchard reaction (steroids and triterpenes). The TLC analyzes were performed according Wagner and Bladt (2009), with specific reagents for visualization.

Determination of total phenolic content: The dosage of phenolic compounds present in the extracts of *E. suberosum* was carried out using the Folin-Ciocalteu reagent, according to the method of Singleton *et al.* (1999) with modifications. Folin-Ciocalteu reagent consists of a mixture of phosphomolybdic and phosphotungstic acids in the presence of certain reducing agents, such as phenolic compounds, are reduced to molybdenum blue and blue tungsten whose color allows the determination of the concentration of reducing substances (Sousa *et al.*, 2007).

The extracts were analyzed at a concentration of 0.1 mg mL⁻¹. So, 100 µL of the samples were mixed with 100 µL of Folin-Ciocalteu reagent and 0.8 mL of distilled water. The mixture was stirred vigorously and allowed to rest for 5 min. Then was added 1.0 mL of sodium carbonate solution of 1 mol L⁻¹. The solution was stirred and allowed to stand for 90 min. The absorbances were measured at 750 nm in UV-Vis spectrophotometer (Femto® Espectro 600).

Gallic acid was used as a standard at concentrations of 0, 0.02, 0.04, 0.06, 0.08 and 0.1 mg mL⁻¹ to prepare the analytical curve.

The assay was performed in triplicate and the amount of phenolic compounds in mg, was expressed as gallic acid equivalents (Mean±standard deviation).

Activity of withdrawal using the DPPH radical method: The activity of removing or inhibiting free radical chemical species was determined by the ability of the substances present in the extracts sequester radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) according to the methodology described by Singh *et al.* (2002) and Blois (1958). The DPPH is a stable radical, soluble in methanol

or ethanol having a violet color and absorption at 517 nm. The antioxidant action causes the reduction of DPPH forming diphenyl-picryl hydrazine, yellow in color which decreases the absorbance at 517 nm (Deng *et al.*, 2011; Li *et al.*, 2009; Sousa *et al.*, 2007).

Different and increasing concentrations of the standard (gallic acid) and the extracts for determination of IC₅₀ were used. So, 100 µL of the extracts and of the standard were dissolved in methanol, followed by addition of 5.0 mL of 0.1 mM DPPH methanolic solution with stirring and after standing at 27°C for 20 min. Samples containing methanol and DPPH were used as a blank. The absorbance was measured at 517 nm in UV-Vis spectrophotometer. All tests were performed in triplicate and the activity of withdrawing of radical (AW) was expressed as percentage inhibition related to the Mean±standard deviations (Mean±SD) of the absorbance (Abs.), which is calculated by the formula:

$$AW (\%) = \left(\frac{\text{Mean} \pm \text{SD Abs. blank} - \text{Mean} \pm \text{DP Abs. sample}}{\text{Mean} \pm \text{DP Abs. blank}} \right) \times 100$$

The IC₅₀ of the standard and the extracts were calculated by linear regression.

The reducing power of metal ion: The power of the extracts to reduce metal ion was evaluated according to the methodology described by Yildirim *et al.* (2001). In determining the reducing power of metallic ions, phenolic substances present in the extracts are reacted with ferricyanide ion [Fe (CN)₆]³⁻ and are oxidized, while the [Fe (CN)₆]³⁻ is reduced to ferrocyanide ion [Fe (CN)₆]⁴⁻. This then reacts with ferric ion (Fe³⁺) form ferric ferrocyanide or hexacyanoferrate III (Fe₄[Fe (CN)₆]₃), also known as Prussian blue. Hence, blue color formation measured at 700 nm is used to monitor the concentration of Fe²⁺ (Graham, 1992).

So, 1 mL of extracts was used at concentrations of 0.05, 0.1, 0.2 and 0.3 mg mL⁻¹ in ethanol. Solution of gallic acid at the same concentrations was used as standards. It was added to each sample extract and gallic acid, 1.0 mL of phosphate buffer 0.2 mol L⁻¹ (pH 6.6) and 1.5 mL of 1% potassium ferricyanide. Then the samples were incubated at 50°C for 30 min. After this time was added 1.5 mL of 10% trichloroacetic acid and the mixtures were centrifuged at 2500 rpm for 8 min. Then, 2.0 mL were removed from the top layer of each sample and added 2.0 mL of distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm in UV-Vis spectrophotometer. Tests were performed in triplicate and the reducing power was considered directly proportional to the absorbance observed.

Statistical analysis: The results presented for the evaluated samples are Mean±standard deviation. Groups (extracts at different concentrations and control) were compared by Analysis of Variance (ANOVA) followed by Tukey test to identify significant differences between means. Graph Pad Prism 3 program was used, where the average level of 5% (p<0.05) were considered significant.

Cyclic voltammetry for the evaluation of antioxidant activity: The technique is based on scanning voltammetry potential on a surface of a working electrode, where the electrochemical behavior of one or more species is evaluated in a certain potential range. A linear voltammetry, the potential is varied with time, starting from an initial value up to a final potential. The scan in cyclic voltammetry, after reaching the final potential is reversed to get back to the starting potential (full cycle). The result is a record of current as a function of applied potential, commonly called

voltammogram or cyclic voltammogram. Thus, if any chemical species present in the sample, which oxidation or reduction processes of this range the applied potential on the electro work, current peaks of oxidation and/or reduction will be observed in the voltammogram.

Voltammetric experiments were performed according to the methodology described by Kilmartin (2001) with some modifications. One Autolab Potentiostat (model 128N) connected to the GPES software for data acquisition was used. A 10 mL electrochemical cell containing three electrodes (working, reference and auxiliary) was used to evaluate the antioxidant activity. The working electrode used was the boron-doped diamond electrode (8.000 ppm doping and area of 3.14 mm²) acquired by the company Adamant Technologies SA, La-Chaux-de Fonds, Switzerland. The reference electrode was Ag/AgCl (saturated KCl) constructed in the laboratory and a Pt wire was used as auxiliary electrode. Different amounts of the extracts were dissolved in 10 mL of ethanol: H₂SO₄ 0.1 mol L⁻¹ (1:1), standing at rest for 12 h. For the experiments 500 µL of each extract solution and 4.5 mL of Britton-Robinson buffer were used, to provide extracts concentrations of 1 and 2 mg mL⁻¹. It was also carried out on blank reading, consisting of 500 µL ethanol: H₂SO₄ 0.1 mol L⁻¹ (1:1) and 4.5 mL of Britton-Robinson buffer for comparison with the results of the samples.

RESULTS

Phytochemical screening: The result of the phytochemical screening of extracts of *E. suberosum* is shown in Table 1. The tests suggested the presence of triterpenes/steroids and flavonoids in the extract in ethyl acetate. In the analysis of the ethanol extract obtained positive for alkaloids, coumarins, flavonoids (including anthocyanins) and condensed tannins result.

Determination of total phenolic content: The dosage of total phenolic compounds in both extracts was determined by obtaining a calibration curve using standard Gallic Acid (GA). The equation of the line was found: $A = 0,0047C$, with $R^2 = 0.9999$, where $A =$ Absorbance and $C =$ Concentration; and substituting the Absorbance (A) of each extract in the line equation obtained C and then calculated the amount of phenolic (mg) in equivalents of GA per gram of extract. The values were 242.20 ± 9.53 mg of phenolic compounds per gram of extract in ethyl acetate and 575.50 ± 2.50 mg of phenolic compounds per gram of ethanol extract.

Activity of withdrawal using the DPPH radical method: The presence of phenolic compounds suggests an antioxidant potential of extracts of *E. suberosum*, being therefore conducted tests to verify this activity. Table 2 shows the inhibitory activity of the extracts of *E. suberosum* and gallic acid standard against DPPH.

Table 1: Results of phytochemical screening of extracts of *Erythroxylum suberosum*

Secondary metabolites	Ethyl acetate extract	Ethanol extract
Alkaloids	Negative	Positive
Anthocyanins	*	Positive
Anthraquinones	Negative	Negative
Coumarins	Negative	Positive
Flavonoids	Positive	Positive
Condensed tannins	*	Positive
Gallotannins	*	Negative
Triterpenes/steroids	Positive	Negative

*: Test not performed due to the polarity of the sample

From the data of Table 2, the IC₅₀ was calculated, which is defined as the final concentration in mg mL⁻¹ of the extract was required to decrease the initial concentration of DPPH by 50%. The IC₅₀ value of gallic acid was 0.07 mg mL⁻¹. Among the extracts, the ethanol had the lowest IC₅₀ (0.18 mg mL⁻¹), whereas the ethyl acetate extract exhibited an IC₅₀ of 0.74 mg mL⁻¹.

The reducing power of metal ion: Table 3 shows the results of the reducing power of Fe³⁺ by extracts of *E. suberosum*. The components of the extracts were able to reduce the Fe³⁺. The ethanol extract showed greater activity than the extract in ethyl acetate.

Cyclic voltammetry: Evaluation of reducing power (electron donating) a compound or group of compounds by cyclic voltammetry reflects its antioxidant activity (Chevion *et al.*, 2000). In this context, cyclic voltammetry has been reported as a simple and rapid analytical tool for assessing total antioxidant capacity of different samples for example in plant extracts (Cosio *et al.*, 2006). The choice of the electrode used for voltammetric analysis of plant extracts is an important parameter, which should highlight mainly the range of anodic electrode potential and sensitivity for detection of compounds. The purpose of this study was based on the use of boron-doped diamond electrode, which has been used in various analytical applications due to its high stability, high sensitivity and wide range of anodic potential, allowing the analysis of several compounds (Lima *et al.*, 2014, 2013). Consequently, the characteristics above of this electrode increase the power to evaluate the antioxidant activity in plant extracts.

The components of the extracts in ethyl acetate and ethanol of *E. suberosum* were evaluated by cyclic voltammetry under the working electrode with boron-doped diamond to confirm the antioxidant activity of the extracts observed in trials to reduce DPPH and Fe³⁺. As can be observed in voltammogram obtained in Fig. 1, although the extracts evaluated did not show a sharp oxidation process, it is possible to check an increase of anodic current at 1.0 V, indicating an antioxidant activity in the range of applied potential and the concentration of 2 mg extract mL⁻¹ (the concentration of 1 mg extract mL⁻¹ was not detected change in current).

Table 2: Activity of withdrawing of radical (AW), in percentage, of extracts of *Erythroxylum suberosum* and gallic acid in different concentrations (mg mL⁻¹)

Concentration (mg mL ⁻¹)	Abs±SD	AW (%)
GA-0.01	0.964±0.006	7.8
GA-0.03	0.829±0.002	20.7
GA-0.05	0.633±0.002	39.4
GA-0.07	0.517±0.003	50.5
GA-0.1	0.270±0.003	74.2
EE-0.1	0.646±0.016	36.7
EE-0.2	0.524±0.009	48.7
EE-0.3	0.299±0.007	70.7
EE-0.4	0.096±0.003	90.6
EAc-0.5	0.722± 0.002	31.8
EAc-0.7	0.547±0.012	48.4
EAc-0.9	0.394±0.006	62.7
EAc-1.1	0.260±0.006	75.4
EAc-1.3	0.157±0.005	85.2

GA: Gallic acid, EE: Ethanol extract, EAc: Ethyl acetate extract, Abs±SD: (n = 3), Abs: Mean absorbance

Table 3: Reducing power of the extracts in ethyl acetate and ethanol of *Erythroxylum suberosum* and gallic acid on the metal ion Fe³⁺

Parameters (mg mL ⁻¹)	EAc	EE	AG
0.05	0.249±0.008*	0.384±0.001*	0.821±0.007
0.1	0.367±0.004*	0.853±0.022	0.865±0.029
0.2	0.633±0.006*	0.883±0.003	0.882±0.001
0.3	0.871±0.007*	0.903±0.006	0.919±0.004

Values of absorbance at 700 nm are expressed as the Mean±SD (n = 3), *: p<0.05

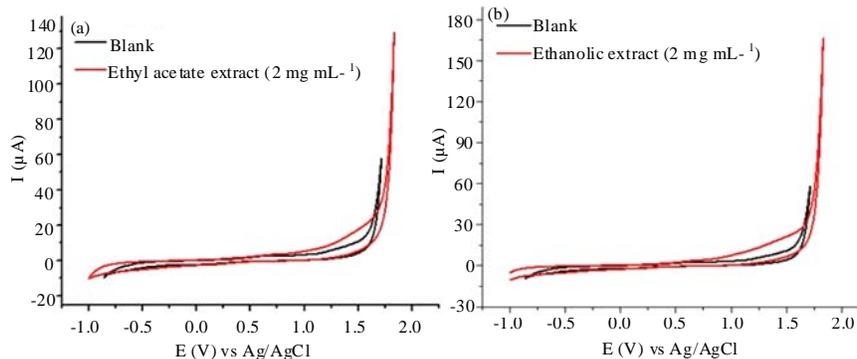


Fig. 1(a-b): Cyclic voltammograms of the extracts in (a) Ethyl acetate and (b) Ethanol *Erythroxylum suberosum* (2 mg mL⁻¹) on the boron-doped diamond electrode in a solution of ethanol solution of H₂SO₄ 0.1 mol L⁻¹ (1: 1). Scan speed: 50 mV sec⁻¹

DISCUSSION

Phytochemical screening and total phenolic compounds: Detection of alkaloids in ethanol extract and detection of phenolic compounds (especially flavonoids and condensed tannins) (Table 1) in two extracts is in agreement with previous studies with *E. suberosum* (Lucas-Filho, 2009; Bohm *et al.*, 1988). The presence of triterpenes/steroids detected in the extract in ethyl acetate is common in the genus *Erythroxylum* (Lucas-Filho *et al.*, 2010; Chavez *et al.*, 1996).

In the dosage of total phenolic compounds were detected an increased amount of total phenolics in the polar extract. It is in accordance with the result of phytochemical screening, which suggested the presence of only one class of phenolic extract in ethyl acetate (flavonoids) and the ethanol extract there was positive for flavonoids, anthocyanidins, coumarins and condensed tannins.

Antioxidant activity: The results presented by extracts are significantly different from the IC₅₀ of gallic acid standard in DPPH radical method. However, gallic acid is a pure phenolic compound, while in extracts phenolic compounds are mixed with other substances, which can interfere with its antioxidant capacity; hence, probably higher IC₅₀ values for the extracts compared to the IC₅₀ of the standard.

The reducing power of extracts was found to be concentration dependent. The ethyl acetate extract showed a gradual increase in activity, while for the ethanol extract, there was a greater increase in reducing power between the concentrations of 0.05 and 0.1 mg mL⁻¹, but from this concentration, there was stabilization in the activity. All results of the extract in ethyl acetate were statistically different (p<0.05) from values obtained with the control (gallic acid). However, the results of the ethanol extract at concentrations 0.1, 0.2 and 0.3 mg mL⁻¹ were not significantly different from those presented by gallic acid, these same concentrations, indicating that the ethanol extract has a pronounced ability to reduce the metal ion, comparable to that of gallic acid.

As previously mentioned, antioxidants organic compounds are reducing agents, whose antioxidant potential can be assessed in terms of two parameters obtained from cyclic voltammetry (Gandra *et al.*, 2004): (1) the first parameter is the anodic peak potential that is related to processes of oxidation of the species present in the sample and the lower the value presented for the anodic peak potential, the greater the antioxidant capacity of the species present. However, the species present in the extract of this plant can have various oxidation processes, which can be

superimposed only allowing a complete analysis of antioxidant capacity in this sample (2). The second parameter is the value of the anodic peak current, which is dependent on concentration of antioxidant present in the sample which was analyzed.

The sharpness of the peak oxidation in cyclic voltammetry analyses may have been compromised by the presence of other electroactive compounds in the sample extract. Anyway, it can be highlighted the wide swath anodic electrode diamond doped with boron, which was up to 1.8 V (vs Ag/AgCl), because other electrodes would not be able to evaluate the antioxidant activity observed in this region of potential. It is noteworthy that in both voltammogram presented in Fig. 1, there is a discharge current generated around 1.75 V, which is produced from the oxidation of water present in the electrolyte solution (H_2SO_4 0.1 mol L^{-1}) used in analyzes. The voltammogram in Fig. 1, also indicate that the ethanol extract showed higher antioxidant capacity as the current increase started earlier and at a higher intensity compared to the extract in ethyl acetate.

Some studies have used cyclic voltammetry to correlate oxidation potentials and current intensity with the antioxidant capacity of plant extract and is also useful, as an additional criterion for identifying samples that have high complexity (Alves *et al.*, 2010; Chevion *et al.*, 2000; Kilmartin, 2001; Lucio and Gil, 2007).

Although the assay of cyclic voltammetry confirms the antioxidant activity observed for the ethanol extract in the assays for inhibition of DPPH and reduction of ferric ion, this methodology was less sensitive than the other methods, since the activity was detected in the ethanol extract at a concentration of 2 mg mL^{-1} .

The presence of phenolic compounds in extracts is not the only factor responsible for antioxidant activity by stabilization of radicals by donating electrons or by metal ion complexation. Other aspects must be considered; in particular the structure of phenolic compounds, since some structural requirements, such as the presence of vicinal hydroxyl groups are essential in a pronounced antioxidant activity (Rice-Evans *et al.*, 1996). The improved performance of the ethanol extract compared to the ethyl acetate extract in all assays are performed according to the results of the phytochemical screening, which suggested a greater variety of phenolic compounds in the first extract (flavonoids, coumarins, tannins) and greater concentration of total phenols.

CONCLUSIONS

In pathological processes, such inflammation can be formed radicals that damage cells and tissues, increasing the pathological processes. Thus, the presence of phenolic compounds and antioxidant activity observed for the extracts may be related to reported medicinal activities for *E. suberosum*, like antirheumatic activity.

The species *E. suberosum* is traditionally have been used for over 200 years by the Brazilian population, according to the description of European naturalists. Nevertheless, still, few studies exist about the plant; thus, the results of this work are a contribution to the knowledge of this plant.

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