

Full Length Research Paper

## Evaluation of the antioxidant activity, antimicrobial effect and acute toxicity of leaves from *Allophylus edulis* (A. St.-Hil., A. Juss. Cambess &) Hieron. ex Niederl

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*Allophylus edulis* is a Brazilian plant commonly used in the mid-west region of Brazil for treatment of disorders related to oxidative stress such as diabetes, inflammation, hypertension and digestive diseases. The aims of the present study were to quantify flavonoids and phenolic compounds, evaluate the antioxidant activity, antimicrobial effect and acute toxicity of leaves of *A. edulis*. Ethanolic (EEAE) and aqueous (AEAE) extracts of *A. edulis* were prepared. The antioxidant activity was determined by 2,2-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, lipid peroxidation and oxidative hemolysis induced by 2,2'-azobis (2-amidinopropane). The antimicrobial assays of diffusion on solid media and broth microdilution were performed against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The acute toxicity was assessed in Wistar rats treated with doses of 2 and 5 g/kg of body weight. The EEAE presented higher concentrations of flavonoids and phenolic compounds, and higher activity of scavenge DPPH free radicals. In addition, it was more effective against *S. aureus* compared to AEAE. The extracts were unsuccessful against *E. coli* and *C. albicans*. The EEAE prevented the lipid peroxidation in human erythrocytes and inhibited oxidative hemolysis in all the concentrations assessed. During the evaluation of acute toxicity, the dose of 5 g/kg of body weight increased hepatic mass. Together, these results demonstrated that the EEAE of leaves of *A. edulis* is more effective than the AEAE, showing antioxidant activity and antimicrobial effect against *S. aureus*, as well as low toxicity.

**Key words:** Cocum, native Brazilian plant, lipid peroxidation, DPPH, AAPH, thiobarbituric acid reactive substances (TBARS), malondialdehyde, Sapindaceae.

## INTRODUCTION

Medicinal plants have been used for centuries to treat numerous diseases around the world (Garg et al., 2012). The biological activity of extracts from medicinal plants is associated with the presence of phytochemical components, many of which have been studied to develop new drugs (Calixto, 2005). The ethnopharmacological approach is an important method of investigating the properties of medicinal plants. Indeed, it is reported that almost 80% of the compounds isolated from medicinal plants by the pharmaceutical industry were obtained from information from folk medicine (McClatchey et al., 2009).

There is a growing interest in substances derived from medicinal plants with antioxidant capacity, such as tannins, flavonoids and other phenolic compounds that can eliminate free radicals (Burda and Oleszek, 2001; Casagrande et al., 2014). Free radicals are highly reactive substances that can induce the oxidation of molecules, leading to cellular and tissue damage. The presence of these substances increases the risk of developing several diseases (as diabetes, cancer, inflammatory and cardiovascular diseases) and promotes the premature aging process (Burton and Jauniaux, 2011). Antioxidant compounds can protect the organism through different mechanisms, such as reducing the lipid peroxidation of cell membranes and the damage to proteins and DNA (Farber, 1994; Halliwell, 1992).

Although antioxidant activity has been extensively studied (Raposo et al., 2014), the scientific community's interest in medicinal plants has also increased due to their antimicrobial properties. Indeed, it is known that various components of plants are potential antimicrobial agents (Cowan, 1999). However, in order to ensure the safety of the medicinal use of any plant species, its toxicity should first be investigated. *Allophylus edulis* is a native Brazilian plant that is popularly known as "chalchal", "cocum", "vacum" and "fruto do pombo" it also occurs in the Uruguay, Bolivia, Argentina and the Guayanas (Díaz et al., 2014). Its leaves are used for their antihypertensive, digestive, anti-inflammatory and healing purposes, particularly in the Midwest region of Brazil (Abreu et al., 2005; Alves et al., 2008). Although this plant has been frequently used by the population, there are no existing reports on their biological properties. Therefore, the aim of the present study was to evaluate the antioxidant and antimicrobial properties, as well as the toxicological risk, of the extract of leaves of *A. edulis*.

## MATERIALS AND METHODS

### Reagents

Folin-Ciocalteu and ethanol PA (Chemical Dynamic®); sodium carbonate, butyric alcohol, gallic acid, aluminum chloride, trichloroacetic acid (Vetec®); quercetin (Sigma-Aldrich®); malondialdehyde and thiobarbituric acid (Merck®); 2,2'-azobis (2-amidinopropane) (AAPH) and and 2,2-diphenil-1-picrylhydrazil (DPPH<sup>•</sup>) (Sigma-Aldrich®); ascorbic acid (Proquímios®) and butyl hydroxy toluene (BHT) (Via farma®); Mueller Hinton agar and Mueller Hinton broth (Merck Brasil®).

### Plant material and preparation of extracts

The leaves of *A. edulis* were collected in April, 2011, in native Cerrado located at 424 m altitude, latitude 22° 05' 545" and longitude 055° 20' 746", in the countryside of Dourados, Mato Grosso do Sul/Brazil. The species was identified by botanist and deposited in the Herbarium of the Federal University of Grande Dourados (UFGD). The species has been cataloged in the voucher specimen number 4676. After drying in air-circulation oven (40°C) for seven days, the leaves were pulverized in a Wiley mill. For each gram of powder 10 ml of 80% ethanol or distilled water in the ratio of 1:10 was added. The ethanolic extract from *A. edulis* was kept on maceration, under constant stirring and at room temperature, for four weeks; the aqueous extract of *A. edulis* kept macerating under constant stirring at 4°C for one week. After that, the extracts were filtered through filter paper, rotaevaporated and lyophilized. The yield of extracts (in percentage) was calculated by the expression: R (%): (dry extract mass/dry plant material mass) × 100. The dried extracts were kept in a freezer (-20°C) for subsequent studies. The dry extract obtained from the ethanol solvent was named EEAE and the one obtained from water was called AEAE.

### Determination of total polyphenols

The content of total polyphenols was determined by Folin-Ciocalteu method described by Meda et al. (2005). An aliquot of 0.5 ml (200 µg/ml of EEAE and AEAE) was mixed with 2.5 ml of Folin-Ciocalteu reagent prepared in water (1:10). After 5 min incubation, 2 ml of aqueous solution of sodium carbonate (14%) was added to the solution. After 2 h at room temperature, the reading on spectrophotometer (T 70 UV/VIS spectrometer PG Instruments®) was performed at a wavelength of 760 nm. The quantification was performed using a calibration curve with gallic acid standard (0.4 to 11 µg/ml). Ethanol was used as a blank. The analytical curve was plotted using GraphPad Prism 3.0 software being implemented as the linear regression and the equation of the straight line was obtained ( $y = a + b.x$ ) by correlating the concentration of gallic acid and the absorbance of each sample. The results were expressed in mg of gallic acid equivalents (GAE) per 100 mg of extract. The assay was performed in triplicate.

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### Determination of total flavonoids

To determine the levels of total flavonoids, the methodology was kept by using the aluminum chloride described by Liberio et al. (2011), with some modifications. An aliquot of 0.5 ml (200 µg/ml of EEAE and AEAE) solubilized was added to 4.5 ml of a solution of hexahydrated aluminum chloride solution (2%). After 30 min rest at room temperature, the absorbances were read on spectrophotometer (T 70 UV/VIS spectrometer PG Instruments®) at a wavelength of 415 nm. To determine the concentration of flavonoids, a calibration curve was prepared using quercetin as standard (0.4 to 11 µg/ml). Methanol was used as a blank. The analytical curve was plotted using GraphPad Prism 3.0 software being implemented as the linear regression and the equation of the straight line was obtained ( $y = a + bx$ ) by correlating the concentration of quercetin and the absorbance of each sample. The results were expressed in mg of quercetin equivalents (QE) per 100 mg of extract. The assay was performed in triplicate.

### Determination of the presence of saponins

For this test, 10 mg of each extract (EEAE and AEAE) was solubilized in 2 ml of 80% ethanol, and then 5 ml of boiling water was added to the mixture. After cooling, it was vigorously stirred, and was followed by a rest period of 20 min. The presence or absence of foam was visually observed. The presence of foam indicates the presence of saponins in the extracts (Barbosa et al., 2004).

### Antioxidant assays

#### DPPH free radical scavenging activity

The method of scavenging of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to determine the antioxidant activity, as described by Gupta and Gupta (2011), with some modifications. A volume of 0.2 ml of the extracts (EEAE and AEAE), solubilized in 80% ethanol at different concentrations (0.1 to 1000 g/ml), was homogenized in 1.8 ml of a DPPH (0.11 mM) solution. After 30 min, at room temperature and protected from light, the reading on spectrophotometer at wavelength of 517 nm was performed. Antioxidants, ascorbic acid and butylated hydroxytoluene (BHT), were used as standard in the same concentrations of the extracts. 80% ethanol was used as a blank. To determine the percentage of antioxidant activity, the following equation was used:

$$\text{Inhibition of DPPH free radical (\%)} = (1 - \text{Absorbance sample/Absorbance control}) \times 100$$

For the absorbance control DPPH (0.1 mM) was used. Three independent experiments were performed in duplicate. The  $IC_{50}$ , concentration capable of reducing by 50% the initial concentration of DPPH, was calculated by nonlinear regression after determining the antioxidant activity curve.

#### Inhibition of lipid peroxidation assay

After approval by the Ethics Committee of the University Center of Grande Dourados (Unigran), Brazil (CEP No. 123/12), 5 ml of blood

from nonsmoking healthy adults was collected, and lipid peroxidation was determined by measurement of malondialdehyde (MDA) formed as described in Campos et al. (2014). Erythrocytes induced to lipid peroxidation by AAPH were used to assess the protective effects of EEAE and of ascorbic acid. The erythrocytes were washed three times with saline (0.9% NaCl). A suspension of these erythrocytes was prepared (5% final hematocrit) and an aliquot of 0.25 ml was homogenized with 0.25 ml extract (EEAE) and ascorbic acid at different concentrations (100 to 175 µg/ml). After 30 min in water bath at 37°C, 0.5 ml of AAPH solution (50 mM) was added. After 3 h in water bath at 37°C with constant stirring, an aliquot of 0.5 ml of supernatant was added to 0.5 ml of trichloroacetic acid (20%). Then, 0.5 ml of the solution was added to 1 ml of thiobarbituric acid (TBA) (10 nM) solution. The homogenate was kept in a water bath at 94°C for 45 min. After 45 min the samples were kept at room temperature for 15 min for cooling, followed by addition of 4 ml of butane with subsequent stirring and centrifugation. The reading of the supernatant absorbance was performed on spectrophotometer (532 nm). Lipid peroxidation was determined by quantification of MDA. The calculation for the amount of MDA in the sample was obtained by the formula:

$$\text{MDA} = \text{Absorbance of samples} \times (20 \times 220.32 / \text{Absorbance } 1,1,3,3\text{-tetrahydroxipropane standard})$$

The results were expressed in nmol/ml. The experiment was accomplished in duplicate.

#### Inhibition oxidative hemolysis induced by 2,2- diphenyl-2-picryl hydrazyl assay

For this, the method for inducing hemolysis by 2,2- diphenyl-2-picryl hydrazyl (AAPH), described by Valente et al. (2011), with some modifications was used. We used an erythrocyte suspension with a final hematocrit of 2.5%. Erythrocytes were preincubated at 37°C for 30 min in the presence of different concentrations of EEAE and ascorbic acid (100 to 175 µg/ml). After this period, 0.5 ml of AAPH solution (50 mM) was added. The mixture was incubated for 240 min in a water bath at 37°C, with periodic stirring. The hemolysis was determined spectrophotometrically at 540 nm, and aliquots for the determination of hemolysis were taken every 60 min of incubation, diluted in saline and centrifuged at 3600 rpm for 10 min. The percentage of hemolysis was determined using the formula:  $A/B \times 100$  (A) abs. of the sample and (B) total hemolysis (erythrocytes with distilled water). All experiments included: negative control (erythrocytes in 0.9% saline), control of extracts and ascorbic acid (erythrocytes in 0.9% saline solution with EEAE and ascorbic acid in different concentrations in the presence and absence of AAPH), solvent control (erythrocytes in 0.9% saline solution with 1% ethanol solvent). Two independent experiments were performed in duplicate.

#### Antimicrobial assay

For the antimicrobial activity of the extracts (EEAE and AEAE) the diffusion assay method was kept in a solid medium from the hole, and also the broth microdilution method, as described by Mokale et al. (2011) with some modifications and observing the recommendations of the standard M100 S5 of the National Committee for Clinical Laboratory Standards (NCCLS/CLSI) (2005).

All strains were purchased from the American Type Culture Collection (ATCC). The identification of microorganisms was confirmed by the Laboratory of Mycology, Department of Mycology of the University Center of Grande Dourados, Unigran, Dourados, MS, Brazil. Three microorganisms were used: gram-positive bacterium *Staphylococcus aureus* (ATCC: 25923), a gram-negative bacterium *Escherichia coli* (ATCC: 8739) and the fungus *Candida albicans* (ATCC: 10231). Microbial inocula were prepared in 0.9% physiological solution and its density was adjusted according to McFarland turbidity standard scale 0.5 ( $5 \times 10^5$  CFU/ml). Fungal and bacterial suspensions were homogenized and sown with a sterile swab and on the surface of Petri plates containing the culture medium agar Mueller Hinton (AMH). With the aid of sterile stainless steel tubes, holes of 6 mm diameter were made in culture medium. The holes were filled with 0.1 ml of the extract (300 mg/ml). The plates were kept for 1 h at room temperature for diffusion of the extracts. Then the plates were incubated in an oven maintained at 37°C for 24 and 48 h. As positive controls, we used tetracycline for bacteria and ketoconazole for fungus, both at a concentration of 4 mg/m. The solvent ethanol 80% was used as a negative control. The experiment was performed in triplicate.

#### **Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

The extracts were submitted to a broth microdilution assay for determining the minimum inhibitory concentration and minimum bactericidal concentration according to Bussmann et al. (2010), with some modifications and observing the recommendations of standard M100 S5 of the National Committee for Clinical Laboratory Standards (NCCLS/CLSI) (2005). Sterile plastic microplates containing 96 wells with 100 µl of the culture medium AMH were used. The initial wells of the microtiter plate received a 100 µl aliquot of the extracts (300 mg/ml). Then, a serial dilution of the extract was performed, resulting in concentrations achieved from 1.50 to 1.56 mg/ml. At the end, 100 µl of the bacterial inoculum in the concentration  $5 \times 10^5$  CFU/ml (0.5 McFarland scale) was added to all wells except to the sterility control of the medium (HAM only). In each microplate was used a negative control (80% ethanol). The microplates were incubated in greenhouse at 37°C for 24 h. After this period of time, the resulting turbidity was evaluated in a microplate reader (TP Reader NM, Thermo Plate®) at 620 nm. The CIM<sub>100%</sub> was defined as the lowest concentration of the extract able to inhibit microbial growth. Two independent experiments were performed in triplicate. To determine the MBC, an aliquot of 20 µl was removed from the wells of the determined concentration as MIC and, at least, two upper levels to it, and transferred to Petri plates with HAM medium. The plates were incubated for 24 h at 37°C. MBC was defined as the lowest concentration that produced negative subculture. This method determined bacteriostatic (lowest concentration that inhibit of bacterial growth) and bactericidal (lowest concentration that kills a bacteria) effects of the antimicrobial agents (Bakker-Woudenberg et al., 2005).

#### **Acute toxicity assay**

##### **Animals**

After approval by the Ethics Committee on the use of animals (CEUA) of Unigran, number 015/12, this study followed international protocols of the guide for animal testing of chemical substances of the Organization for Economic Co-operation and

Development (OECD) number 425 (2008). The animals were obtained from School of Environmental and Biological Sciences of UFGD, and were kept under conditions of controlled temperature and humidity, fed with ration (purine-Labina®) and water *ad libitum*.

#### **Experimental model**

Twelve Wistar rats weighing an average of  $226.7 \pm 4.6$  g were used. The animals were randomly divided into 4 experimental groups, each of them containing 3 animals. The experimental groups were: C = control group that received water; TC = control group that received a solution of Tween 80 (20%); LD = experimental group that received 2 g/kg and HD = experimental group that received 5 g/kg of EEAE solubilized in Tween 80 (20%). A single dose by gavage was administered. After the administration, and daily throughout the experimental period, the presence or absence of clinical toxicity signs (piloerection, tremors, excitability, irritability, muscle contraction, salivation, and death) was observed. The variation in body weight and food and water consumption was evaluated three times a week. On the 15th day, after fasting for 12 h, euthanasia was performed. Organs and tissues were macroscopically examined and stored at -20°C. Blood samples were obtained for haematological and biochemical analysis (Asare et al., 2011).

#### **Statistical analysis**

Data were expressed as mean  $\pm$  standard error of the mean (SEM). Analyses were performed using variance analysis (ANOVA) followed by Tukey's test or Student *t* test. The level of significance was set at  $p < 0.05$ . GraphPad Prism 3.0 software was used.

## **RESULTS**

#### **Preparation of the extracts, determination of phenolic compounds, flavonoids and saponins**

The EEAE and AEAE presented a yield of 8.8 and 10.0%, respectively, after extraction with the solvent. The concentrations of phenolic compounds were  $17.6 \pm 0.6$  and  $9.0 \pm 0.2$  mg GAE/100 mg, respectively. Conversely, the concentrations of flavonoids were  $2.0 \pm 0.3$  and  $1.0 \pm 0.2$  mg QE/100 mg, respectively. Saponins were not detected in any of the extracts.

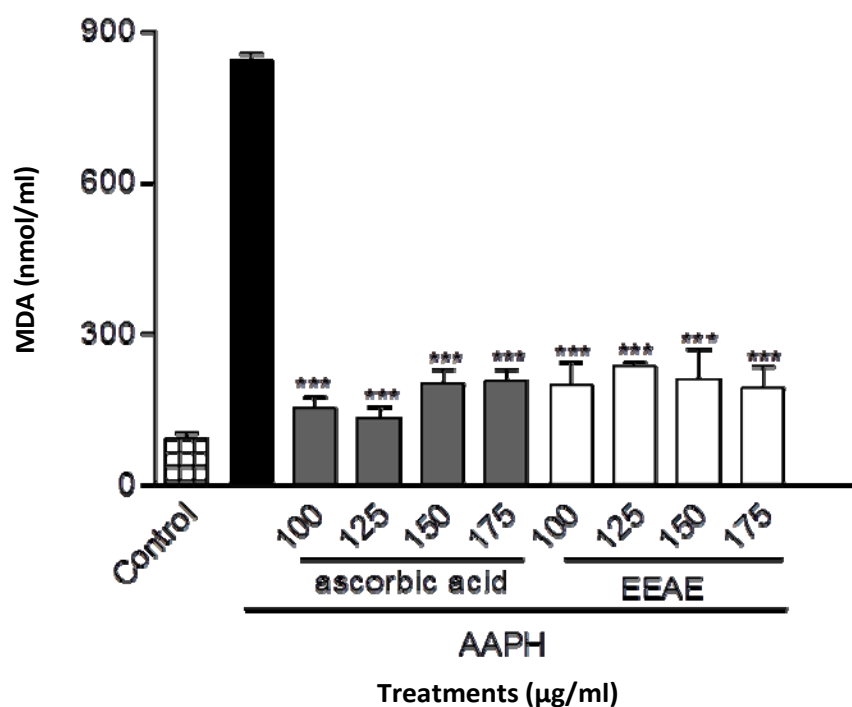
#### **DPPH free radical scavenging activity**

The extracts and controls were assessed at different concentrations (0.1 to 1000 µg/ml). The 50% inhibitory concentration (IC<sub>50</sub>) and the maximal activity of DPPH free radical scavenging are shown in Table 1. These data showing that the EEAE exhibited an antioxidant capacity 4.5 times lower than that of ascorbic acid and 0.6 times higher than that of BHT. On the other hand, the AEAE presented an antioxidant capacity 1.4 times lower than

**Table 1.** DPPH radical scavenging activity (%) of ethanolic (EEAE) and aqueous extracts (AEAE) of *A. edulis* at different concentrations ( $\mu\text{g/ml}$ ) and  $\text{IC}_{50}$  values.

Treatments	0.1	1	5	10	50	100	500	1000	$\text{IC}_{50}$
Ascorbic acid	1.8 $\pm$ 1.2	10.2 $\pm$ 4.2	48.5 $\pm$ 3.7	94.2 $\pm$ 2.3	95.2 $\pm$ 1.3	97.7 $\pm$ 0.3	96.0 $\pm$ 0.9	96.3 $\pm$ 0.8	3.9 $\pm$ 0.8
BHT	6.0 $\pm$ 3.6	8.2 $\pm$ 2.4	19.3 $\pm$ 4.6	31.7 $\pm$ 7.4	69.3 $\pm$ 9.5	81.0 $\pm$ 5.3	95.2 $\pm$ 0.7	95.0 $\pm$ 0.6	31.5 $\pm$ 1.9
EEAE	6.2 $\pm$ 3.0	6.8 $\pm$ 2.3	13.8 $\pm$ 3.2	27.8 $\pm$ 2.3	91.8 $\pm$ 1.9	96.0 $\pm$ 0.8	91.3 $\pm$ 1.8	94.5 $\pm$ 2.5	17.7 $\pm$ 2.6
AEAE	2.8 $\pm$ 1.0	6.0 $\pm$ 1.4	10.0 $\pm$ 4.9	13.8 $\pm$ 1.4	47.2 $\pm$ 2.5	78.3 $\pm$ 2.9	91.8 $\pm$ 2.7	89.0 $\pm$ 1.3	45.8 $\pm$ 4.6

The results were expressed as mean  $\pm$  standard error of the mean (SEM),  $n = 3$ , duplicate.  $\text{IC}_{50}$  represents the half-maximal inhibitory concentration and the values obtained were different between all samples.



**Figure 1.** Effects of ethanolic extract of *A. edulis* (EEAE) on the AAPH-induced lipid peroxidation of erythrocytes. Control group (erythrocytes incubated with saline solution) and AAPH-treated group (erythrocytes incubated with 50 mM of AAPH). Values are expressed as the mean  $\pm$  SEM. \* $p < 0.001$ , compared with AAPH at respective time.

that of ascorbic acid and 11.7 times lower than that BHT. The EEAE exhibited an antioxidant activity 0.4 times higher than that of AEAE, reaching maximal activity at the concentration of 50  $\mu\text{g/ml}$ , which is 10 times higher than the AEAE concentration (500  $\mu\text{g/ml}$ ) (Table 1).

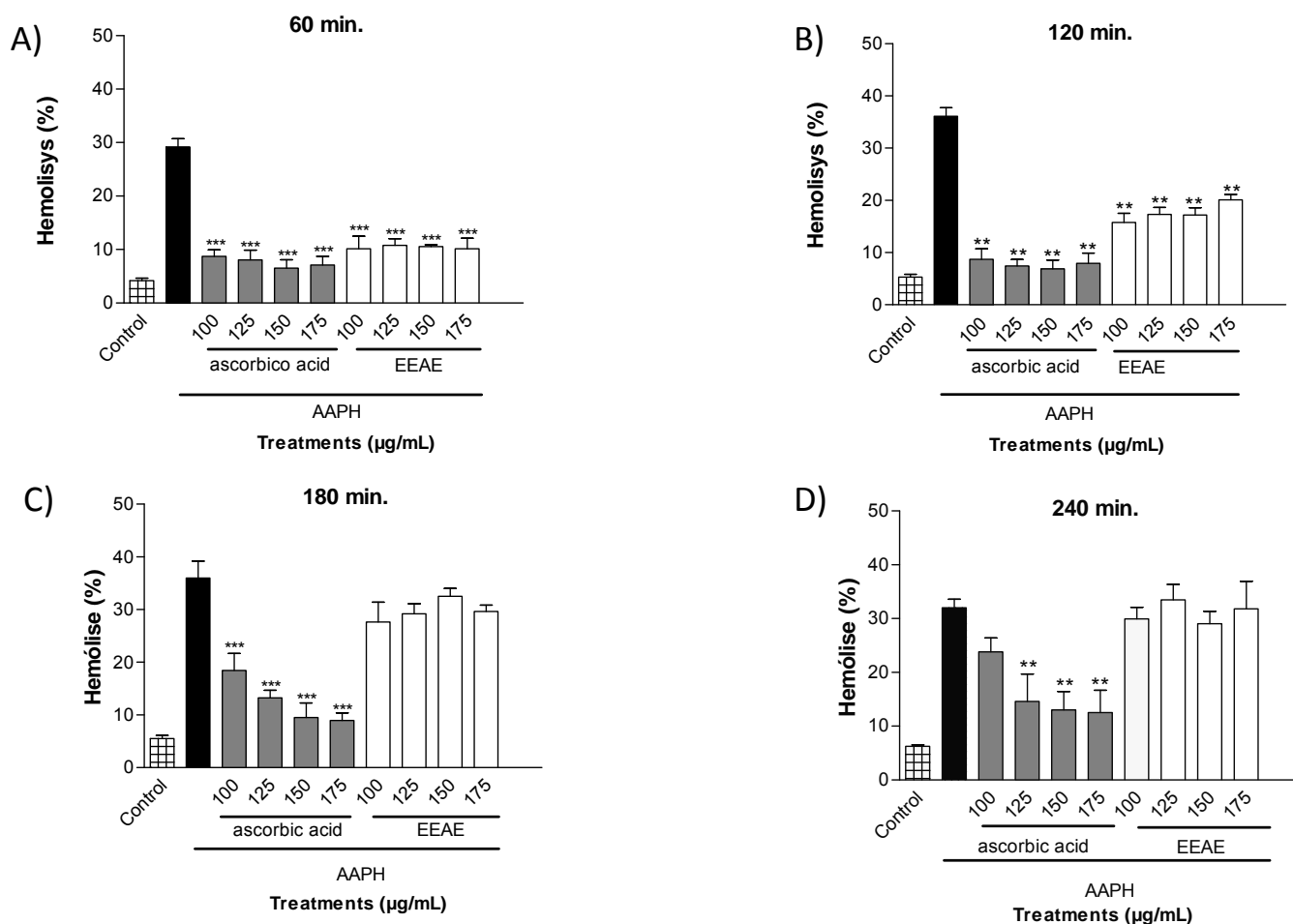
#### Inhibition of lipid peroxidation

In this test, the EEAE was able to prevent lipid peroxidation of the erythrocytes induced by the AAPH in all of the concentrations assessed (100 to 175  $\mu\text{g/ml}$ ).

Levels of MDA were between 72 and 77% lower than those in the group of erythrocytes with AAPH alone. This result was similar to that of ascorbic acid, with levels of MDA between 76 and 84%, which were lower than the control group (Figure 1).

#### Inhibition of hemolysis induced by AAPH

All concentrations of the extract tested were able to protect against hemolysis at 60 and 120 min after the beginning of the test. Despite this, none of the concentra-



**Figure 2.** Effects of ethanolic extract of *A. edulis* (EEAE) on the AAPH- induced hemolysis of erythrocytes. Control group (erythrocytes incubated with saline solution) and AAPH-treated group (erythrocytes incubated with 50 mM of AAPH). A) Erythrocytes incubated for 60 min (B) 120 min, (C) 180 min and (D) 240 min. The results were expressed as mean  $\pm$  standard error of the mean (SEM),  $n = 2$ , duplicate. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  versus. AAPH at respective time.

tions of the extract were able to protect against hemolysis at 180 and 240 min (Figure 2). The ascorbic acid was able to provide protection at almost all of the concentrations and time-points analyzed, with the exception of the 100 µg/ml concentration at 240 min. None of the concentrations of EEAE, ascorbic acid or solvent significantly affected the rate of basal hemolysis throughout the study period.

#### Antimicrobial activity and MIC

The extracts were effective against the bacterium *S. aureus*. The EEAE exhibited an inhibition zone of  $20.3 \pm$

0.3 mm. In addition, bactericidal activity was detected at the concentration of 150 mg/ml. Similarly, the AEAE presented an inhibition zone of  $17.3 \pm 1.2$  mm, but only bacteriostatic activity at the same concentration of 150 mg/ml. None of the extracts were effective against *E. coli* and *C. albicans* (Table 2).

#### Test of acute toxicity

In this test, no clinical signs of toxicity, macroscopic changes in the organs or death were observed throughout the study. In addition, no changes in the water/food intake and body weight of the animals were

**Table 2.** Diameter of inhibition zones, MIC and MBC/MFC of ethanolic (EEAE) and aqueous (AEAE) extracts of *A. edulis*.

Treatments		<i>Staphylococcus aureus</i>					<i>Escherichia coli</i>			<i>Candida albicans</i>		
		DI	MIC	MBC	Activity	F. effectiveness (%)	DI	MIC	MBC	DI	MIC	MBC
Ketoconazole	-	-	-	-	-	-	-	-	-	24±1.2	-	-
Tetracycline	-	24.0±1.0	-	-	-	100	30.0±2.0	-	-	-	-	-
<i>A. edulis</i>	EEAE	20.3±0.3	150	150	bactericidal	85	0	-	-	0	-	-
	AEAE	17.3±1.2*	150	>150	bacteriostatic	72	0	-	-	0	-	-

DI: diameter of inhibition zone. Ketoconazole and tetracycline (4 mg/ml), F: effectiveness: samples / Tetracycline × 100, EEAE and AEAE (300 mg/ml), MIC and MBC (150 to 1.56 mg/ml). -: not tested. The results were expressed as mean ± standard error of the mean (SEM), n = 2, triplicate. \*p<0.05 versus tetracycline treatment.

**Table 3.** Body weight, food/water intake, hematological and biochemical indices of the Control group (C) and the Tween control group (TC). Low dose group (LD = 2 g/kg BW) and High dose group (HD = 5 g/kg BW) on day 15 after the administration of ethanolic extract (EEAE) of *A. edulis* in Wistar rats.

Variable	C	TC	LD	HD
Food intake (g/24 h)	22.0±1.0	22.0±1.0	20.0±0.6	20.0±1.7
Water intake (ml/24 h)	44.0±3.1	38.0±1.4	42.0±1.3	42.0±2.2
B.w 1° day (g)	226.7±4.6	231.4±7.1	232.0±7.8	220.2±7.5
B.w 15° day (g)	246.3±3.8	250.0±8.1	251.7±8.4	233.8±12.7
Liver (g/100 g)	3.2±0.1	3.0±0.1	3.30±0.1	3.6±0.1*
Kidney (g/100 g BW)	0.66±0.1	0.69±0.1	0.66±0.1	0.70±0.0
Heart (g/100 g BW)	0.38±0.1	0.39±0.0	0.41±0.1	0.41±0.0
Lung (g/100 g BW)	0.58±0.0	0.57±0.1	0.54±0.1	0.61±0.1
RBC (10 <sup>6</sup> /ml)	8.9±0.1	8.6±0.1	8.1±0.6	8.5±0.1
WBC (10 <sup>3</sup> /ml)	7.4±0.5	5.9±1.4	6.3±1.4	6.1±0.7
HGB (g/dl)	13.8±0.1	13.5±0.2	13.5±0.3	13.8±0.2
HCT (%)	47.1±0.4	45.6±0.4	44.3±1.7	47.0±0.3
PLT (10 <sup>3</sup> /ml)	977.7±60.2	996.0±100.3	902.3±68.2	790.5±46.5
URE (mg/dl)	31.6±4.5	30.2±0.9	31.9±0.4	31.5±1.1
CR (mg/dl)	0.2±0.03	0.3±0.06	0.3±0.03	0.3±0.03
γ-GT (U/L)	0.7±0.3	0.7±0.3	0.5±0.5	0.7±0.3
ALT (U/L)	57.0±8.2	60.0±2.9	56.0±3.1	57.0±4.5
AST (U/L)	152.7±1.8	271±32.9*	183.3±20.3	198.3±22.4

BW = body weight WBC = White Blood Cells; RBC = Red Blood Cells; HGB = Hemoglobin; HCT = Hematocrit; PLT = Platelet; URE = urea; CR = creatinine; γ-GT = γ-glutamyltranspeptidase; ALT = alanine aminotransferase; AST = aspartate aminotransferase. p<0.05 versus C group.

detected. At a dose of 2 g/kg, the extract did not change any of the parameters assessed. However, the dose of 5 g/kg caused an increase in the liver weight of treated animals, when compared with the control group. Finally, the control group treated with tween exhibited an increase in serum levels of aspartate aminotransferase, when compared with the control group treated with water

(Table 3).

## DISCUSSION

In recent years, the number of studies on alternative therapies for several diseases has increased, with

medicinal plants as the main target for scientific research seeking to develop more efficient new drugs and reduce side effects. In Brazil, several species of plants with medicinal properties are used, although less than 5% of plants have been phytochemically and biologically studied (Calixto, 2005; Simões et al., 2004). Among these plant species, *A. edulis* contains compounds with anti-insect activity (Diaz et al., 2014), flavonoids and phenolic molecules, which are directly associated with their biological activities. Previous studies have demonstrated that essential oils, alkaloids (Bandoni et al., 1972; Yajia et al., 1999) and polyol L-quebrachitol are the main constituents of this vegetal (Diaz et al., 2008). The presence of flavonoids in *A. edulis* extract could be associated with DPPH free radical scavenging and antioxidant activities.

Flavonoids present a chemical structure which favors the inactivation of free radicals, since free hydroxyl groups are able to donate hydrogen and electrons which will neutralize the free radicals (Burda and Oleszek, 2001). Several phenolic and flavonoid compounds with antioxidant activity have been identified and isolated from plant extracts (Lee et al., 1998). These constituents are represented by various molecules which are considered natural antioxidants (Dryden et al., 2006; Middleton, 1998).

The higher antioxidant activity exhibited by EEAE compared to AEAE could be related to higher concentrations in the ethanol extract of phenolic and flavonoid compounds. The antioxidant activity demonstrated by EEAE was 2.7 times higher than that reported in a previous study using fruit of the same species (Umeo et al., 2011). Other antioxidant properties of EEAE have been demonstrated by its ability to prevent the lipid peroxidation of the membranes of human erythrocyte, as evidenced by the reduction of malondialdehyde production, and its ability to prevent oxidative hemolysis.

These activities could be attributed to the phenolic compounds of EEAE, which are able to eliminate the peroxy radicals produced by the thermal decomposition of AAPH. This reaction may occur before the action on the lipid molecules of the erythrocyte membrane, breaking the chain reaction of free radicals, which inhibits lipid peroxidation and consequently, hemolysis (Silva et al., 2011). Previous studies have reported that phenolic compounds are the main components associated with the anti-hemolytic ability of natural products (Valente et al., 2011; Campos et al., 2014; Casagrande et al., 2014).

Besides the presence of phenolic compounds, other metabolites have been described in terms of their protective ability against oxidative hemolysis. For example, polyol L-quebrachitol, previously described for this species (Diaz et al., 2008), could be responsible for

the antioxidant activity observed. A previous study demonstrated the antioxidant activity of L-quebrachitol in other plant species belonging to the same family of the *A. edulis* (Nobre Junior et al., 2006). These protective activities are of great importance, since the effects of oxidative stress on the organism include damage to the cell membrane by lipid peroxidation (Halliwell, 1992). This oxidative process is present in various pathologies, such as diabetes, cancer, cardiovascular and inflammatory diseases (Burton and Jauniaux, 2011).

Antimicrobial activity is another significant biological property. Indeed, it is known that there is an increase in the number of new multi-resistant strains to conventional drugs, which cause high morbidity and mortality rates among patients and pose a threat to public health (Kamicker et al., 2008). The antimicrobial activity of EEAE and AEAE was assessed against *S. aureus*, *E. coli* and *C. albicans*, with effective results associated with the treatment of *S. aureus*. This is an important finding, since *S. aureus* has been reported as a multi-resistant bacterium of medical concern (Russell, 2002). This agent is responsible for several syndromes, such as food poisoning, toxic shock syndrome, skin lesions and atopic dermatitis (Guay, 2003). Among the molecules related to the antimicrobial activity of natural products, flavonoids are one of the most important due to their ability to interfere in the synthesis of nucleic acid and the energetic metabolism of microorganisms. In addition, they can also bind to the proteins of cell membranes, causing their death (Cowan, 1999; Cushnie and Lamb, 2005, 2011). However, the extracts were not able to inhibit the growth of the gram-negative bacterium *E. coli* and the fungus *C. albicans*. Gram-negative bacteria and fungi exhibit a complex cell membrane, which is difficult to penetrate (Braun, 2009). Most likely, the different composition of the cell membrane of these organisms is the cause of the resistance observed herein. The resistance of these microorganisms to plant extracts has already been reported (Engels et al., 2011; Hendra et al., 2011).

Toxicological studies of medicinal plants are important to understand the eventual toxic effects that could reduce its medicinal value. Many studies are performed in Wistar rats to evaluate the toxicity of the leaves extract from medicinal plants. In these animals the most important signs of toxicity are characterized by reduction of body weight, hind limb paralysis, increase in creatinine, aspartate aminotransferase, sodium and potassium serum levels, reduction of urea and albumin, leucopenia and small alteration in color and consistency of viscera (Félix-Silva et al., 2014). Considering the popular use of *A. edulis* as a medicinal plant, the knowledge of its toxicological effects is essential. In the test of acute toxicity, only the highest dose of 5 g/kg of EEAE caused an increase in liver weight, suggesting hepatotoxicity at



this dose. However, in the group treated with EEAE (2 g/kg), no alterations were observed in the organs or in the haematological, biochemical and toxic parameters assessed. Therefore, based on the guidelines of the Organization for Economic Co-operation and Development, the lethal dose (LD<sub>50</sub>) of *A. edulis* is higher than 5 g/kg and EEAE can be considered an extract of low toxicity.

## Conclusion

The results of the present study show that the extract of leaves of *A. edulis* has antioxidant activity *in vitro* by scavenging free radicals and inhibiting hemolysis and lipid peroxidation in human erythrocytes incubated with an oxidizing agent. It was active against the bacterium *S. aureus*, as well as showed low toxicity. The antioxidant and antimicrobial activities of this extract can be attributed to the presence of flavonoids and phenolic compounds. Therefore, these results suggest that this natural product may be used for the treatment and/or prevention of various diseases related to microorganisms and oxidative stress.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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