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EVALUATION OF THE POLYPHENOLIC CONTENT, ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITIES OF TOTAL EXTRACT FROM *EUGENIA PYRIFORMES* CAMBESS (UVAIA) FRUITS

MARIA ROSANA RAMIREZ[1,3]*, CARLOS EDUARDO SCHNORR[2], LUCAS B. FEISTAUER[2], MIRIAM APEL[1], AMÉLIA TERESINHA HENRIQUES[1], JOSE CLAUDIO FONSECA MOREIRA[2] and JOSE ÂNGELO S. ZUANAZZI[1]*

[1]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000, Brazil
[2]Departamento de Bioquimica, ICBS, Universidade Federal de Rio Grande do Sul, Porto Alegre (RS), Brazil

[3]Corresponding author. TEL: 55 (51) 3308 5243; FAX: 55 (51) 3308 5437; EMAIL: mariarosanar@yahoo.com.br

*These authors contributed equally to this work.

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ABSTRACT

Eugenia pyriformes Cambess is a plant native to Brazil, where it is known as "uvaia." Their fruits are used in juice or are eaten raw. In this study, fruits from nine different genotypes were investigated for their *in vitro* antichemotactic activity, their antioxidant activity and their total polyphenolic content. The extract showed similar antichemotactic and antioxidant activities and had substantially different total polyphenolic contents. The samples were also investigated for antiedematogenic and antioxidant activity in animal models. The total extract of *uvaia* exhibited significant dose-related anti-inflammatory and antioxidant activity at 0.5 and 1.0 g/kg b.w.

PRACTICAL APPLICATION

The species *Eugenia pyriformes* Cambess is one of the most important species of the *Eugenia* genus. It is a small bushy tree (5–15 m tall) that is cultivated in the southern part of Brazil. In order to find a potential application for this native fruit, the antioxidant and anti-inflammatory effects were investigated. These results obtained showed that *E. pyriformes* possess antioxidant/anti-inflammatory property (*in vivo*/*in vitro*). Therefore, our results showed the possibility to use the uvaia extracts as harmless and economical antioxidant and anti-inflammatory compounds. The structure function rationale of this research can also provide the information for better design of animal studies for more precise dietary recommendations.

INTRODUCTION

Eugenia pyriformes Cambess is a plant belonging to the Myrtaceae family. This species is native to Brazil and is widely used in reforestation programs and in metropolitan areas (Cronquist 1981). *E. pyriformes* is commonly known in Brazil by diverse popular names, including "uvaia, uvalha, uvaia-do-mato and uvalheira." The fruit is yellow and spherical with a bitter and juicy pulp. It contains 39.5 mg of vitamin C per 100 g of fruit (Maiochi 2008). This native fruit has potential use for processing to make jelly, juice, jams and ice cream (Romagnolo and Souza 2006; Roesler *et al.* 2009).

A survey of the relevant literature revealed that the leaves of the *Eugenia* genus exhibit antioxidant, antimicrobial, anti-inflammatory, analgesic, antipyretic, spasmolytic and central nervous system depressant activities and are used in traditional folk medicine (Schapoval *et al.* 1994; Auricchio and Bacchi 2003). Thus, it can be assumed that as a member of the

Eugenia genus, uvaia may have antioxidant and anti-inflammatory activities in addition to its other useful bioactivities. However, there are no data in the literature concerning the possible pharmacological effects and the chemical characteristics of this fruit.

The objective of this study was to evaluate and compare the bioactive effects of nine *E. pyriformes* Cambess genotypes in an effort to find functional native fruits. The evaluation of the antioxidant activity was performed using multiple assays including 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), thiobarbituric acid reactive species (TBARS), total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR). The antichemotactic effect was also evaluated in all genotypes using the cell model (Boyden 1962). The anti-inflammatory activity of the ethanolic extract of *E. pyriformes* was evaluated by the carrageenan-induced rat paw edema method (Winter *et al*. 1962). Furthermore, polyphenolic content of uvaia extract was also determined.

MATERIALS AND METHODS

Drugs and Reagents

Glycogen from oyster (type II) lipopolysaccharide (LPS) is from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

E. pyriformes Cambess Samples

Uvaia genotypes were produced by EMBRAPA DE CLIMA TEMPERADO (Pelotas, RS, Brazil), and the fruits were kept at −20C. Pesticide analysis was previously carried out, and no sign of these substances was found, assuring no interference of pesticides on our results. The *E. pyriformes* Cambess fruits were triturated mechanically and later lyophilized and kept sheltered from light.

Determination of Total Polyphenols

Total polyphenolic constituents of ethanol extract were performed employing the literature methods involving Folin–Ciocalteu reagent (Brazilian Pharmacopoeia 2003), using gallic acid as a standard. Lyophilized fruits of *E. pyriformes* Cambess (1 g) were extracted with 95% ethanol at room temperature for 24 h. The ethanolic extract (20 mL) were filtered and concentrated in vacuum to obtain dry ethanolic extract (500 mg, 50%). This extract was resuspended in distilled water (150 mL), and polyphenolic compounds were extracted in a water bath for 30 min at 90–95C. A volume of 2 mL aliquot of this diluted extract was added to a test tube along with 1 mL of Folin–Ciocalteu reagent (Sigma Chemical Co.) and 10 mL of distilled water. After vortexing, 12 mL of aqueous sodium carbonate solution (290 g/L) was added to the mixture, mixed and left for 30 min in the dark. Absorbance of the blue-colored solution was recorded and was read at 760 nm, and results were converted to total polyphenols and expressed as mg equivalents of gallic acid per 100 g of lyophilized fruit. All analyses were performed in triplicate, and the results were averaged.

Measurement of DPPH Radical Scavenging Activity

The method was performed as described by Ramirez *et al*. (2010). Each uvaia extract was dissolved in ethanol at concentrations of 2.0, 1.0, 0.5 and 0.25 mg/mL of total polyphenols. A volume of 150 μL of extracts was added to 2.850 mL of DPPH (Sigma Chemical Co.) ethanol solution (100 μM). The samples were vortexed, and after 30 min, their absorbance was measured at 517 nm. The DPPH radical scavenging activity obtained by each *uvaia* genotypes was compared with that of Trolox (2.0, 1.0, 0.5, 0.25 mg/mL) an analog of vitamin E (97%; Aldrich Chem. Co., Sao Paulo, Brazil). The antioxidant activity of the uvaia genotypes was expressed as percent inhibition of DPPH radical formation after 30 min of reaction time.

Animals

Experiments were carried out using male Wistar rats. The animals were housed in plastic cages, with *ad libitum* access to water and food, under a 12 h light/ 12 h dark cycle (lights on at 7:00 a.m.) at a constant temperature of 22C. Experiments were performed after protocol approval by the Institutional Ethics Committee (protocol number 196/96-2004346, UFRGS) and were carried out according to the current guidelines for laboratory animal care and ethical guidelines for investigations of experimental pain in conscious animals. The number of animals and intensity of noxious stimuli used were the minimum necessary for demonstrating the consistent effects of the treatments. Each animal was used only once.

Antichemotactic Assay

The chemotaxis test was performed as described by Boyden (1962), with the same modifications introduced by Zigmond and Hirsch (1981). All uvaia genotypes were tested in this assay.

Rat Neutrophil Isolation

To obtain the rat polymorphonuclear neutrophils, 20 mL of sterile 1% glycogen (w/v) was injected into the peritoneum of a Wistar rat. Four hours later, the rat was killed by decapitation, and the leukocytes were collected after the injection of 60 mL of Hanks' balanced salt solution (HBSS), pH 7.4 con-

taining 1 μL/mL heparin. The leukocyte-rich supernatant was centrifuged at 4C for 10 min. The cell pellets were washed twice, suspended in HBSS in order to obtain a leukocyte density of about 4×10^6 cells/mL. The cell suspension was transferred to different tubes, treated separately with each *uvaia* extract at a distinct final concentration (25–5 μg/μL of total polyphenols). The cells were then incubated at 37C for 1 h and manually stirred 10 times every 5 min.

Collection of Plasma and Chemotactic Stimulation

Five rats were lightly anesthetized with ether, and a median incision was made in the ventral abdominal wall in order to expose the posterior vena cava. Blood was collected from this vessel into a syringe previously containing 1 μL/mL heparin. The blood samples from all animals were pooled and centrifuged (3,000 rpm for 30 min). The plasma was finally removed and incubated at 37C for 30 min with a 65 μg mL solution of LPS from *Escherichia coli* and then diluted in HBSS (1:5).

Neutrophil Chemotaxis

The leukocyte/*uvaia* suspension was placed in the upper wells of a modified Boyden chamber, separated from the chemotactic stimulant in HBSS present in the lower compartment by an 8.0-μm nitrocellulose filter (Millipore, Sao Paulo, Brazil). The positive and the negative controls contained cell suspension in the upper wells in LPS/HBSS and plasma in the lower wells, respectively. The chemotaxis chamber was incubated at 37C in a humidified atmosphere for 1 h. Thereafter, the filters were removed, fixed in absolute ethanol and stained with hematoxylin. Chemotactic migration of leukocytes through the nitrocellulose filter, toward the chemotactic stimulant (lipopolysaccharide-treated plasma), was measured using the micrometer on the fine-focus knob of a Nikon Alphaphot-2 YS2 microscope. The distance from the top of the filter to the farthest plane of focus containing two cells, in ten microscopic fields, allowed the evaluation of leukocyte migration.

Carrageenan-Induced Paw Edema in Rats

Experimental Design. Rats were acclimatized to the laboratory environment and to the investigator who handled them. They were subsequently divided into a control group that drank water *ad libitum* and two groups that drank water supplemented with different concentrations of total uvaia extract. The extract was administered orally for 21 days ($n = 10$ animals/group), and indomethacin (5 mg/kg, p.o.) was used as the reference drug. For the *in vivo* experiments, lyophilized uvaia fruits were homogenized in 96% ethanol, agitated for 90 min and centrifuged at 3,000× *g* for 15 min. The supernatants were filtered and concentrated by rotary evaporation at 30C. After, extracts were stored until the time of administration to the rats, when they were redissolved in distilled water. The daily quantity of uvaia extracts offered to the animals was calculated to provide 1.000, 500 and 250 mg/kg/day of fruit lyophilized. The volume of juice provided was 30 mL/rat/day in all cases. Food was available *ad libitum*. Juice intake and weight were recorded daily. These doses of fruit lyophilized are based on previous work and pilot studies in our laboratory (data not shown).

Paw Edema Test. The antiedematogenic effects of *uvaia* were evaluated using the carrageenan-induced paw edema test in rats, according to the method of Winter *et al*. (1962). Male Wistar rats (weighing 250–300 g) were briefly anesthetized with pentobarbital and injected subplantarly into the right hind paw with 0.1 mL of carrageenan suspension (0.5 mg/mL) in isotonic saline. The left hind paw was injected with 0.1 mL of saline and used as a control. Paw volume was measured prior to and 1, 2, 3 and 4 h after carrageenan administration using a mercury plethysmograph (Ugo Basile, Comerio, Italy). The difference between the readings at time 0 h and different time interval was taken as the thickness of edema. The results are expressed in mL as the difference between the right and left paws.

Biochemical Analysis

Preparations of the Samples. The animals were sacrificed by decapitation after the carrageenan-induced paw edema test. Blood samples from all animals were collected for separation of plasma or serum and used for biochemical assays. Serum cholinesterase was quantified according to the Doles enzymatic technique (Dietz *et al*. 1973). The liver, kidney, heart and hippocampus were dissected out immediately after the rats were sacrificed and weighed for posterior toxicological and/or biochemical analyses.

The hippocampus, cerebral cortex and striatum were stored at 70C for posterior analyses. Samples were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) using a Potter–Elvehjem-type glass homogenizer. For biochemical assays, the tissue was homogenized in phosphate buffer for samples (PBS, 0.1 M), pH 7.4, so the homogenate was centrifuged at 700× *g* to remove debris, and the resulting supernatant was used as the mother solution. Results were normalized by the protein content using bovine serum albumin as standard (Lowry *et al*. 1951).

TBARS. As an index of lipoperoxidation, we detected TBARS formation through a hot and acidic reaction. This is widely

adopted as a method for measurement of lipid redox state, as previously described (Draper and Hadley 1990). Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined by absorbance in a spectrophotometer at 532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane (TMP) as standard (2 nm/mL), which was subjected to the same treatment as that applied to the supernatants of the samples. The standard curve was made with 10, 20, 40, 60, 80 and 100 μL of the standard TMP solution. Results are expressed as nanomoles TBARS per milligram protein (Lowry *et al*. 1951; Da Rocha *et al*. 2010).

Nonenzymatic Antioxidant Potential. The total reactive antioxidant potential (TRAP) has been used as an index of the nonenzymatic antioxidant capacity, based on the peroxyl radical (generated by AAPH solution, 2,2′-azobis[2-amidinopropane], with luminol) quenching by sample compounds. The TRAP/TAR protocol was performed as described by Lissi *et al*. (1992). AAPH or ABAP ([2,2′-azobis-2-methyl-propanimidamide] dihydrochloride) 10 mM solution was prepared in glycine buffer (pH 8.4), in the experimental day, and was light protected. Luminol (50 mM) solution was prepared in NaOH (0.1 N), light protected and stored in refrigerator at 4C. In the experimental day, a Luminol (4 mM) solution was prepared in glycine buffer (pH 8.4) and was also light protected. The reading is done by chemiluminescence emission.

TRAP/TAR system was prepared in the experimental day with 4 mL of the AAPH (10 mM) solution and 10 μL of the Luminol (4 mM) solution per tube and stabilized in 2 h, and then 10 μg of the sample was added for the assay. After the sample addition, we analyzed the readings at the lumineter counter for 96-well microplates for nearly 30 min. The results were transformed in percentual, and the area under curve (AUC) was calculated by GraphPad® software (version 5.00; GraphPad Software Inc., San Diego, CA) as described. For the TRAP, it is important to note how much lower the AUC is and higher the antioxidant potential is, playing an inversely proportional relation.

The total antioxidant reactivity (TAR) was also analyzed, and it is based on the same technical principles of TRAP. The TAR results were calculated as the ratio of light in absence of samples (I_0)/light intensity right after sample addition (I). For the TAR, the values play a directly proportional relation to the antioxidant capacity. Although TAR and TRAP evaluations are obtained in the same experiment, they represent different observations, because the TAR is more related to the antioxidant quality (reactivity, the scavenging capacity in a short-term period), and the TRAP is more related to the antioxidants amount (Lissi *et al*. 1992, 1995).

Statistical Analysis. The total polyphenolic content was calculated as mean ± SD ($n = 4$). For the antichemotactic assay, data are expressed as mean and deviation (SD) and were expressed as a percentage of the maximal chemotaxis toward reference chemoattractant (LPS) (ANOVA, analysis of variance). Group's comparisons were performed using a paired samples *t* test. For paw edema, data are expressed as the mean ± SEM. Results were calculated, and the data were submitted to statistical analysis by ANOVA, followed by Student's *t* test or Newman–Keuls test, using the confidence intervals of 95% ($P < 0.05$).

Biochemical results are expressed as mean ± SD, and *P* values were considered significant when $P < 0.05$. Differences in each experimental group were determined by the one-way ANOVA. Comparison between means was carried out using the post hoc Tukey's test. The statistical analysis and the graph making were conducted with GraphPad® Software (version 5.00).

RESULTS AND DISCUSSION

The antioxidant capacity of the fruits is associated to their bioactive compounds, mainly antioxidant polyphenols, because of their ability to scavenge free radicals (Mandic *et al*. 2008). As shown in Table 1, the total polyphenolic (TP) content differed between uvaia samples. Genotypes 9 and 11 had the highest polyphenols content in ethanol extracts (651–652 mg/100 g fruit lyophilized); genotypes 2 and 12 had the lowest total polyphenols content (375 mg TP/100 g fruit lyophilized). TP content of some *Eugenia* species, including *Eugenia polyantha*, *Eugenia umbelliflora*, *Eugenia uniflora*, *Eugenia jambolana* and *Eugenia dysenterica*, ranged from 897 to 136.96 mg gallic acid equivalent (GAE)/g

TABLE 1. TOTAL POLYPHENOLS CONTENTS IN *EUGENIA PYRIFORMES* CAMBESS

Genotypes	Total polyphenols (mg/100 g fruit lyophilized)
S1	460 ± 1.01
S2	375 ± 1.50
S3	450 ± 0.98
S4	390 ± 0.85
S5	480 ± 0.85
S8	481 ± 0.88
S9	652 ± 0.96
S11	651 ± 0.56
S12	373 ± 0.97

Note: Total polyphenol content of the samples was determined using the Folin–Ciocalteu method and is expressed per 100 g fruit lyophilized as gallic acid equivalent. Values are means of three measurements ± standard deviation. Results were processed by using one-way variance analysis (ANOVA). Differences at $P < 0.05$ were considered to be significant.

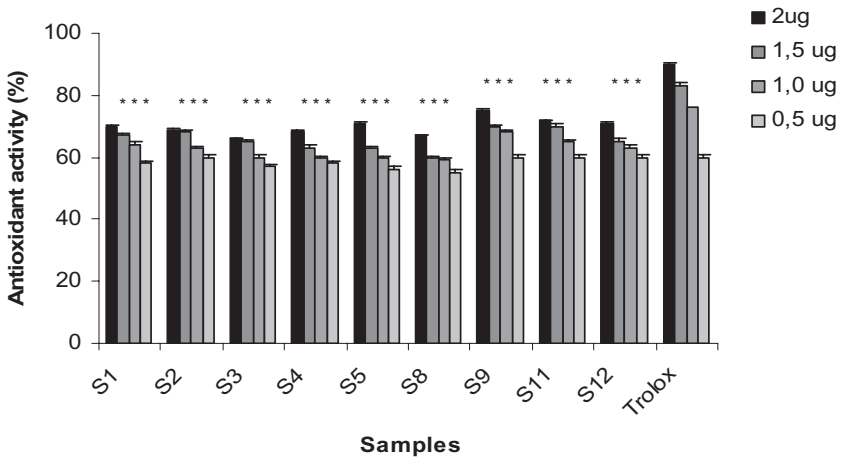

FIG. 1. RADICAL SCAVENGING ACTIVITIES OF *EUGENIA PYRIFORMES* CAMBESS FRUITS
The fruits extracts were incubated with DPPH for 30 min, and the absorbance at 517 nm because of DPPH radical was determined. Asterisks indicate a significant difference in relation to Trolox ($P < 0.05$). Results were processed by using one-way variance analysis (ANOVA). Differences at $P < 0.05$ were considered to be significant. DPPH, 2,2-diphenyl-1-picrylhydrazyl.

according to the literature values (Kuskoski *et al*. 2006; Melo *et al*. 2008; Itoh *et al*. 2009). When our results are compared with these values, it is seen that the average TP content of *E. pyriformes* are in good agreement with values in the other *Eugenia* species.

The DPPH scavenging activity of the ethanolic extracts from uvaia genotypes are given in Fig. 1. The antioxidant activity as measured by the DPPH radical scavenging assay was high for all uvaia samples; however, the activity of S9 was the highest and that of S3 was slightly low. Extracts of S1 and S12 showed nearly identical levels of radical scavenging activity to S11 extract; nevertheless, total amounts of polyphenols in S1 or S12 were nearly half those of S11, implying that antioxidant activity is not necessarily parallel with the amount of polyphenols.

The chemotaxis inhibitory tests are used to measure the antichemotactic property of phytocompounds, and the assay is based on the measurement of a chemotactic response from leukocytes by applying a chemoattractant source in the lower compartment of the chamber that evokes the physiological response of inflammation. As far as the antichemotactic effect is concerned, all the evaluated genotypes were effective in reducing the chemotaxis migration (Fig. 2). Likewise, chemotaxis inhibition by uvaia extracts did not appear to be associated to total polyphenols but was linked with antioxidant activity.

In this work, the *in vivo* anti-inflammatory effect of *E. pyriformes* extract was investigated in rats by the carrageenan-induced paw edema procedure using indomethacin (5 mg/kg, p.o.) as a reference compound. It is well known that carrageenan-induced paw edema is characterized by biphasic event with involvement of several inflammatory mediators. In the first phase (2 h after carrageenan injection), chemical mediators such as histamine and serotonin play role, while in second phase (3–4 h after carrageenan injection), kinin and prostaglandins are involved (Griffiths 1999).

Our results clearly demonstrated that administration of *E. pyriformes* extract inhibited the edema starting from the first hour and during all phases of inflammation, which is probably inhibition of different aspects and chemical mediators of inflammation (Table 2). The inhibition of the paw edema was dose-dependent, and the efficacy of the

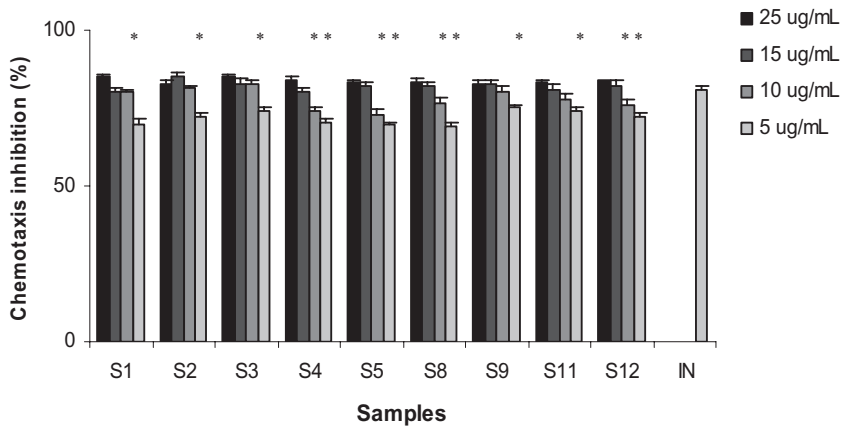

FIG. 2. EFFECTS OF *EUGENIA PYRIFORMES* CAMBESS TESTED ON THE *IN VITRO* CHEMOTAXIS OF POLYMORPHONUCLEAR NEUTROPHILS TOWARD LIPOPOLYSACCHARIDE (LPS)
Chemotaxis in the presence of test compounds is expressed as the percentage of the maximal chemotaxis to LPS in the same experiment. Results were processed by using one-way variance analysis (ANOVA). Differences at $P < 0.05$ were considered to be significant. Positive control: indomethacin (IN).

TABLE 2. EXTRACTS TESTED ON CARRAGEENAN PAW EDEMA TEST

Groups	Edema dm (cm)			
Dose	1 h	2 h	3 h	4 h
NS	1.07 ± 0.23	1.30 ± 0.27	1.87 ± 0.10	1.75 ± 0.17
IN	0.73 ± 0.23 (19%)	0.99 ± 0.22 (24%)	0.86 ± 0.22 (51%)	0.60 ± 0.13 (66%)
(1.0 g/kg b.w.)	0.47 ± 0.09* (53%)	0.61 ± 0.23* (52%)	0.78 ± 0.15* (58%)	0.86 ± 0.12* (51%)
(0.5 g/kg b.w.)	0.62 ± 0.13* (38%)	0.84 ± 0.19* (35%)	0.95 ± 0.17* (49%)	1.00 ± 0.18* (43%)

Note: Antiedematogenic effect of *Eugenia pyriformes* Cambess fruit on 1% carrageenan-induced rat paw edema. Extract (doses indicated) were administered orally 21 days before subplantar carrageenan injection. Control animals were treated with indomethacin (IN, 5 mg/kg, p.o.) or saline (NS). The values represent the mean ± SE of the variation in the paw volume of 8–10 animals for each group. Results were processed by using ANOVA (analysis of variance), followed by Student's *t* test or Newman–Keuls test, $P < 0.05$.

* Different from control.

highest dose of the uvaia extract (1.0 and 0.5 g/kg b.w.) was inferior to indomethacin (5 mg/kg b.w.). Percentage of inhibition of the edema was about 51% and 43% for total extracts and 66% for the indomethacin. Anti-inflammatory activity of *E. pyriformes* has not been reported before, although a potent activity was described for *E. uniflora* leaves (Schapoval *et al.* 1994).

The effect of treatment on plasma antioxidant capacity was investigated using TAR and TRAP assays, as seen in Fig. 3. Our results showed that the TRAP measurement, which reflects tissue nonenzymatic antioxidant defenses, was significantly reduced in a concentration-dependent manner in rat supplemented, after paw edema. In contrast, TAR that represents the quality of tissue antioxidants and corresponds to the capacity of a given tissue to modulate the damage related with an enhanced production of free radicals was significantly increased in the same animals (Da Rocha *et al.* 2010). It may be presumed that this increase corresponds to the hydrophilic antioxidants, which suggests that dietary *E. pyriformes* or their metabolic products present in plasma contribute significantly to TAR.

Evaluation of TBARS is habitually used to measure plasma and tissue concentrations of malondialdehyde (MDA), a decomposition product of oxidized lipids and as an index of lipid peroxidation. Numerous animal investigations that have measured plasma or tissue TBARS have reported significant decreases with polyphenol administration. However, in this study, plasma TBARS did not significantly change when the subjects drank uvaia or water (not shown). Similar results were described by others studying the effect of polyphenol-rich extracts (Lambert *et al.* 2007).

A number of authors have provided evidence that oxygen radical plays an important role in the maintenance of carrageenan paw edema (Yam *et al.* 2010). Phytocompounds that inhibit the prostaglandin synthesis or scavenge the oxygen radical can decrease the inflammation (Chi *et al.* 2001; Yang *et al.* 2008). Because the antioxidant property of *E. pyriformes* extracts is a well-established phenomenon, we cannot rule out the possibility that uvaia extract also exerts antiedematogenic activity through the inhibition of oxidative stress.

Under the conditions of the present study, nonsignificant ($P > 0.05$) differences were found in serum cholinesterase levels, average body mass in all groups throughout the study.

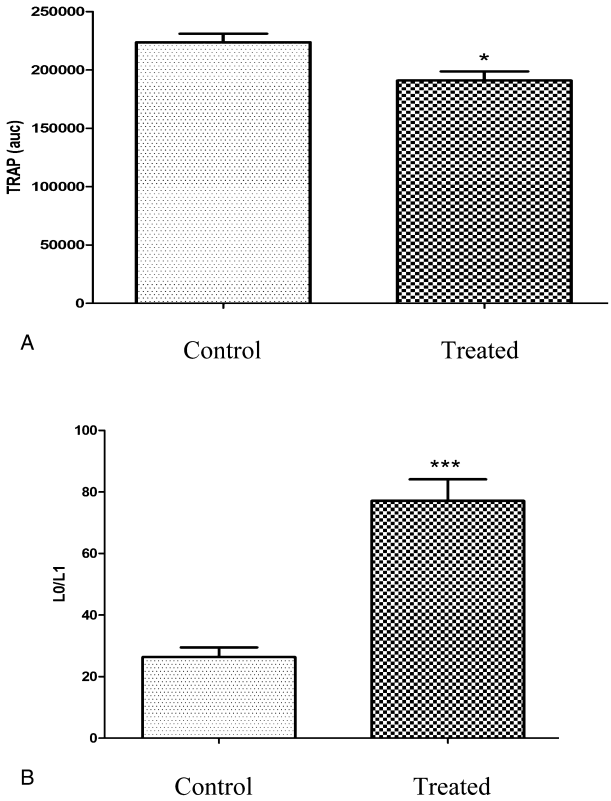

FIG. 3. EFFECT OF *EUGENIA PYRIFORMES* CAMBESS FRUITS ON (A) TOTAL REACTIVE ANTIOXIDANT POTENTIAL (TRAP) AND (B) TOTAL ANTIOXIDANT REACTIVITY (TAR) OF RAT PLASMA
Results were processed by using one-way variance analysis (ANOVA). Comparison between means was carried out using the post hoc Tukey's test. Differences at $P < 0.05$ were considered to be significant. $n = 10$ per group (1 g/kg b.w. day fruit lyophilized). *Different from control.

The results obtained in the present study provide evidence that the mixed compounds present in the uvaia extract have no toxic effects in rats. Results similar to ours have been reported for *E. uniflora* leaves (Auricchio and Bacchi 2003).

In summary, to the best of our knowledge, this is the first report to demonstrate that *E. pyriformes* total extract possess antioxidant/anti-inflammatory property (*in vivo*/ *in vitro*). The anti-inflammatory ability makes it a suitable candidate for consideration as a dietary supplement to reduce stress oxidative and inflammation. Identification of the bioactive constituents of the *E. pyriformes* fruit are in progress.

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