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Evaluation of the antioxidant, antiproliferative and antimutagenic potential of araçá-boi fruit (*Eugenia stipitata* Mc Vaugh — Myrtaceae) of the Brazilian Amazon Forest

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ABSTRACT

Eugenia stipitata is a fruit from Amazonia rich in terpene, volatile compounds, fiber, and vitamin C. The fruit is recognized for its high antioxidant activity and has attracted much attention due to their potential health benefits to humans. The total polyphenols, antioxidant, antiproliferative, antimutagenic and antigenotoxic activities of *E. stipitata* ethanolic extract were investigated. Total polyphenols were determined by the Folin–Ciocalteu method and showed 184.05 ± 8.25 mg GAE/100 g. The radical scavenging activity was DPPH $_{IC50}$ 0.69 ± 0.23 μg/mL and TAC-ORAC$_{FL}$ 371.98 μmol.TE/100 g. The extract was evaluated for its ability to inhibit the growth of tumor cell lines and had not complete cystostatic effect against any of the tested cell lines. Antimutagenic and antigenotoxic activities were investigated by micronucleus test and comet assay in mice, respectively. Ethanolic extract of *E. stipitata* showed higher antimutagenic and antigenotoxic properties at the highest concentration tested (300 mg/kg of body weight). In conclusion, these results suggest that this fruit could be used as a preventive agent against cancer.

1. Introduction

The Amazon rainforest hosts about 50% of the planet's biodiversity, and nearly 70% of the forest area is located inside Brazilian Amazon. This territory is recognized as an important biological hotspot of global mega-diversity. Brazil is one of the countries with the biggest biodiversity on the planet, where about a third of the world's remaining tropical forests is located (Conservation International of Brazil - Amazônia, 2012).

Particularly, this country has a wide variety of native, wild and non-commercially cultivated fruits, which are excellent sources of bioactive compounds that are vital substances possessing the ability to protect the body from damage caused by free radical, induced oxidative stress. It is believed that the compounds largely responsible for those protective effects are vitamins C, E and phytochemicals, which have antioxidant properties (Barreto, Benassi, & Mercadante, 2009; Carvalho-Silva et al., 2012; Clerici & Carvalho-Silva, 2011; Leite et al., 2011; Oliveira, Yamada, Fagg, & Brandão, 2012).

Many species of Myrtaceae (*Myrciaria jaboticaba* (*Vell*) *Berg*, *Campomanesia cambessedeana Berg*, *Eugenia uniflora*) are used as traditional medicines in divergent practices for inflammatory conditions, intestinal disorders, high blood pressure and diabetes (Leite-Legatti et al., 2012; Malta et al., 2012; Reynertson, Yang, Jiang, Basile, & Kennelly, 2008).

Araçá-boi (*Eugenia stipitata* ssp. Sororia Mc Vaugh — Myrtaceae) is a fruit tree native to western Amazonia, rich in terpenes volatiles, fiber and mainly vitamin C. Preliminary studies showed a good antioxidant activity and high phenolic content differing among araçá genotypes. The few investigations about this fruit suggest nutritional and functional potential (Medina et al., 2012). In a recent study, high contents of glycosylated quercetin derivatives were found on the fruit, and their potency as inhibitors of enzymes of carbohydrate metabolism seems to be related to the pattern of glycosylation (Carvalho-Silva et al., 2012; Clerici & Carvalho-Silva, 2011; Gonçalves, Lajolo, & Genovese, 2010; Oliveira et al., 2012).

Many techniques have been proposed to assess antioxidant activity against Reactive Oxygen Species (ROS) in biologic systems because of ROS involvement in disorders development, such as cancer, cardiovascular disease, inflammation, neurodegenerative diseases, and aging process (Bicas, Neri-Numa, Ruiz, De Carvalho, & Pastore,

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2011). The current study evaluated the phenolic contents, antioxidant, antiproliferative, mutagenic and antimutagenic effects of *E. stipitata*.

2. Materials and methods

2.1. Plant material and chemicals

The *E. stipitata* fruit was obtained at "Feira da Panair" in Manaus city, state of Amazonas, Brazil, in January 2010. The identification of botanical araçá-boi was performed according to the classification system proposed by APG II (2003) published by the Angiosperm Phylogeny Group. Undamaged fruits were selected, washed and only the edible portion of the fruits was used for antioxidants extraction and flavonoid extraction/hydrolysis.

The standards of myricetin, quercetin and kaempferol and the reagents 2,2-diphenyl-1-picrylhydrazil (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,20-azobis(2-methyl-amidinopropane)-dihydrochloride (AAPH), sodium fluorescein, cyclophosphamide monohydrate, gentamicin and doxorubicin were purchased from Sigma-Aldrich Co. (St. Louis, USA). Water was purified with a Milli-Q water purification system (Millipore, Bedford, USA). Methanol (HPLC-grade) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, USA). Reagent grade formic acid and ascorbic acid were from Merck (Frankfurt, Germany) and Labsynth Ltda. (Diadema, Brazil) respectively. All other chemicals and solvents in this study were of analytical grade. Fetal bovine serum and RPMI 1640 were purchased from Gibco-Invitrogen (New York, USA).

2.2. Extraction method

To obtain the ethanolic extract, the fruits were homogenized with aqueous ethanol (5:95 v/v, water, ethanol) for 20 min, according to Roesler, Malta, Carrasco, & Pastore, 2006. The sample was concentrated using a vacuum rotary evaporator under reduced pressure at 40 °C, lyophilized, pulverized and stored at −20 °C in dark glass bottle. For the lipophilic extract, sample was homogenized with hexane (1:3 w/v) using a blender for 20 min. Residual hexane was evaporated and the residue was pulverized and kept in the dark at −20 °C until analysis.

2.3. Determination of flavonoid contents and total polyphenol

2.3.1. Flavonoid extraction and hydrolysis

The simultaneous extraction and hydrolysis were based of the method described by Hoffmann-Ribani and Rodriguez-Amaya (2008). To the 15 g homogenized composite sample, 25 mL of methanol and 10 mL of HCL were added (0.6 M HCl) and extracted for 60 min. After refluxing, the extract was cooled and passed through a 130 mesh sieve. The volume was made up to 50 mL with methanol and an aliquot of about 5 mL was filtered through a 0.4 μm polytetrafluoroethylene (PTFE) filter (Millipore Ltd., Bedford, USA) prior to HPLC analysis.

2.3.2. Determination of total polyphenols

Total polyphenols were determined by the Folin–Ciocalteu method proposed by Swain and Hillis (1959), with some modifications. Ethanolic and aqueous extracts were dissolved in methanol to get a 0.5 mg /mL solution. A 500μL aliquot of homogenate was passed through a 0.45 μm membrane filter and mixed with 2.5 mL of 10-fold diluted Folin–Ciocalteu reagent and 2.0 mL of 7.5% sodium carbonate solution. After 5 min at 50 °C, absorbance at 750 nm was read in spectrophotometer (DU-640™, Beckamn-Coulter – Brea, CA, USA). Results were expressed as milligrams of gallic acid equivalents (GAE/100 g) of fruit dry weight. Estimation of the phenolic compounds was carried out in triplicate and averaged.

2.4. Antioxidant capacity

2.4.1. DPPH (2,2-difenil-1-picrilhidrazila) — free radical-scavenging activity

Free radical-scavenging activity was measured using a method adapted by Brand-Williams, Cuvelier, and Berset (1995). Experiments were performed on freshly prepared solutions of DPPH. Ethanolic solutions in different concentrations were prepared by adding 1000 μL of DPPH (0.004% w/v), and the final volume completed to 1200μL with ethanol. Samples were incubated in dark at room temperature for 30 min. After, the absorbance of the remaining DPPH was measured at 517 nm against pure ethanol (blank). Free radical-scavenging activity was expressed as a percentage of the absorbance of the control DPPH solution, obtained from the following equation: $\%Activity = [(A_{DPPH} - A_{Extr})/A_{DPPH})] \times 100$, where A_{DPPH} is the absorbance value of the $DPPH_{blank}$ sample, and A_{Extr} is the absorbance value of the test solution. A_{Extr} was evaluated as the difference between the absorbance value of the test solution and the absorbance value of its blank. The IC_{50} values are reported as final concentration of extract in the cuvettes defined as g/mL of dried extracts required to decrease the initial DPPH concentration by 50%. Results were expressed as percentage activity. Index IC_{50} (concentration which results in a 50% reduction, or inhibition, of DPPH) was determined by linear regression.

2.4.2. Oxygen radical absorbance capacity ($ORAC_{FL}$) assay

The assays were based in the method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) with some modifications. Reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant sample (20 μL) and fluorescein (120 μL; 70 μM, final concentration) solutions were mixed in one of the 96 wells of a black microplate. Then, 60μL of an AAPH solution (final concentration 12 mM) was added and fluorescence was checked every cycle of 60 s for 80 cycles in a microplate reader (NOVOstar®, BMG Labtech — Offenburg, Germany) at 37 °C. A blank experiment, without antioxidant (20μL of phosphate buffer, 120 μL of fluorescein and 60μL of AAPH) was also performed. The results, expressed as equivalent for μmol of Trolox (standard antioxidant) per μmol of sample, are based on the Area under the Curve of fluorescence decay over time (AUC) and on the Net Area (NAUC), calculated in approximate values, as described in the following Equations:

$$AUC\,1 + \sum f_i/f_0.$$

In which f_0 is the initial fluorescence (t = 0) and fi is the fluorescence obtained at t = i (minutes).

$$NAUC = AUC_{sample} - AUC_{blank}$$

NAUC was plotted against sample concentration and results were compared to the standard curve (NAUC *versus* Trolox concentration). The equivalence of Trolox was given by the angular coefficient of Trolox curve concentration (μM) *versus* Sample concentration (μM). All assays were performed in three independent replicates.

2.5. HPLC flavanol analysis

This analysis was performed according to the method proposed by Hoffmann-Ribani and Rodriguez-Amaya (2008) in Waters system, equipped with a Rheodyne injection valve with a 5 μl fixed loop, a quaternary pump (Waters model 600) and a UV–vis photodiode array detector (Waters model 996), controlled by a Millenium workstation (version 32). A Symmetry C-18 (2.1 mm × 150 mm, 3.5 μm) water column was used, the mobile phase consisted of methanol and water, both acidified with 0.3% formic acid, and the flow rate was 0.2 ml per minute. A multilinear gradient was applied from

20:80 to 48:52 in 6 min, this proportion was maintained until 29 min, and then changed to 28:72 in 2 min, and this proportion was maintained until 40 min. Finally, the mobile phase was bought back to the initial proportion of 20:80 in 3 min, maintaining this proportion until 60 min for column reequilibration before subsequent injection. Analytes were monitored from 200 to 600 nm and detection at 370 nm was used for quantification, which was done by external standardization.

The linearity of the HPLC method was checked for flavonoids in the 0.03–4.8 mg/100 mL range (0.06–2.3, 0.03–40.8, 0.018–3.7 mg/100 mL for myricetin, quercetin and kaempherol, respectively) (Fig. 1). Calibration was performed by injecting the standard working solution in triplicate at five different concentrations for each flavonoid, based on the expected flavonoid content ranges in the samples. All standard curves passed through the origin, were linear in the concentration ranges expected in the samples, with coefficients of determination ranging from 0.99 for quercetin.

2.6. Evaluation of antiproliferative activity in vitro

Antiproliferative activity of the extracts was tested against nine cell lines of different human tissues kindly provide by the National Cancer Institute — Frederick, MA, USA: Human tumor cell lines U251 (glioma, CNS) UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (breast expressing phenotype multiple drug resistance), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon) and Vero (kidney, non cancer cell line, African green monkey).

Stock cultures were grown in a medium containing 5 mL of RPMI 1640 (Gibco BRL) supplemented with 5% of fetal bovine serum. Gentamicin (50 mg/mL) was added to experimental cultures. Cells in 96 well plates (100 μL cells/well) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25, and 250 μg/mL) at 37 °C, 5% of CO_2 in air for 48 h. Final DMSO concentration did not affect cell viability. Afterwards, cells were fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content, employing sulforhodamine B assay (Monks, Scudiero, & Skehan, 1991). Using the concentration–response curve for each cell line, TGI (concentration that produces total growth inhibition or cytostatic effect) was determined through non-linear regression analysis, utilizing software ORIGIN 7.5 (OriginLab Corporation).

2.7. In vivo studies

2.7.1. Animals

Newly weaned male Swiss albino mice were obtained and maintained under controlled conditions of temperature (22–24 °C), light (12 h light/12 h dark), and humidity (45–65%), with food and water *ad libitum*. All mice used for experimental research had body weight between 25–35 g. The UNICAMP Animal Bioethical Committee approved this study under protocol number 2362-1 in accordance with the Brazilian society of science in laboratory animals.

2.7.2. Acute toxicity study

The acute toxicity (LD_{50}) study was done to determine which concentrations could cause the potential therapeutic activity but not kill the animals, as described by Litchfield and Wilcoxon (1953). Three groups of five animals were treated with the following doses (i.p.) of dietary crude extract (DCE): 100, 300 and 1000 mg/kg, and were observed and scored daily for behavior and clinical conditions

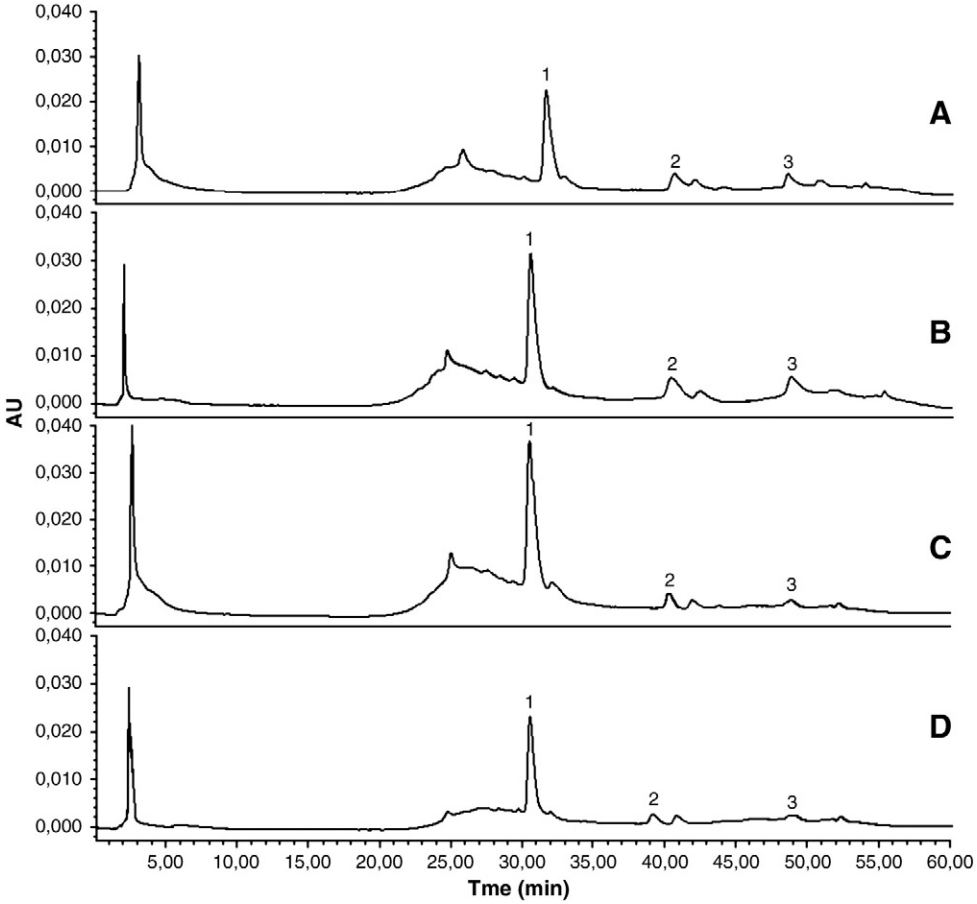

Fig. 1. Separation of aglygon flavonoids of araçá-boi (*Eugenia stipitata*) by HPLC with diode array detection (370 nm). 1: myricetin, 2: quercetin and 3: kaempferol after hydrolysis to obtain the flavonoid aglycone forms in A) 40.0 min of hydrolysis; B) 47.5 min of hydrolysis; C) 55.0 min of hydrolysis; D) 62.5 min of hydrolysis.

according to Ullman-Cullere and Foltz (1999) during the sequence 15 days, when an animal received a score of 16 or more, according to these criteria, the animal was sacrificed and that day was considered to be the day of its death. The (LD_{50}) was calculated by a linear regression.

2.7.3. Micronucleus (MN) test

The MN test was performed according to the guidelines of MacGregor et al. (1987) in order to investigate the protective effect of *E. stipitata* extract against the clastogenicity induced by cyclophosphamide (CP). Fruit extract was administered at dose of 100 μL by orogastric gavage for 15 consecutive days, at concentrations of 30, 100 and 300 mg/kg body weight (bw), selected on the basis of our acute toxicity studies in mice, which was higher than 1000 mg/kg. The negative control group received 0.9% NaCl. The positive control group received (CP) at a dose of 50 mg/kg bw. Experiments were performed according distribution groups: G1 = NaCl 0.9% + CP (positive control); G2 = NaCl 0.9% + NaCl 0.9% (negative control); G3 = DCE 30 mg/kg bw + CP; G4 = DCE 100 mg/kg bw + CP; G5 = DCE 300 mg/kg bw + CP; G6 = DCE 30 mg/kg bw + NaCl 0.9%; G7 = DCE 100 mg/kg bw + NaCl 0.9%; G8 = DCE 300 mg/kg + NaCl 0.9%. Half of the groups received intraperitoneal injections of 50 mg/kg body weight of CPA 24 h before the euthanasia, and the other half received injections of NaCl 0.9%. Both femur bones were excised, and their bone marrow was flushed into test tubes using a syringe containing bovine fetal serum. The percentage of reduction in the frequency of CP-induced DNA damage was calculated as follows: % reduction = $[(\bar{y}A) - (\bar{y}B)/(\bar{y}A) - (\bar{y}C)] \times 100$ where A = positive control group treated with CP; B = group fed with DCE + CP; and C = negative control group.

All animals were euthanized 24 h after treatment by cervical dislocations under ether anesthesia.

For the conventional assessment of micronucleus frequencies, two slides for each animal were prepared according to the method of MacGregor et al. (1987). Briefly, femurs were dissected and cleaned of any adhering muscle, and bone marrow cells were flushed with fetal calf serum into a centrifuge tube. The cells were stained with Leishman stain and centrifuged at 2000 rpm for 5 min, and the supernatant was removed. The slides were coded, and the cells were blindly scored by light microscopy at 1000 magnification. The frequency of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit with variability (standard deviation) based on differences among animals within the same group. The polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) ratio was also determined on a total of 1000 erythrocytes counted.

2.7.4. Evaluation of the genotoxic potential by comet assay (SCGE)

The alkaline procedure described by Singh, McCoy, Tice, and Schneider (1988) was used with modifications required by the material. The SCGE assay was done after the MN test, using liver tissue obtained at the sacrifice same of mice used in the MN test. The liver samples were placed in 3 mL of cold Hanks buffer and finely minced in order to obtain a cell suspension. The slides were covered with 1.5% normal melting point agarose, allowed to solidify at 25 °C and stored at 4 °C. A 30 μL aliquot of the liver cell suspension was embedded in 120 μL of 0.5% low melting point agarose, spread on agarose-precoated microscope slides and allowed to solidify at 4 °C for 10 min. After, removal of the coverslip, the slides were placed in freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 Mm Tris, pH 10, with 1% Triton X-100 and 10% DMSO) for 24 h at 4 °C. Then, slides were subsequently incubated in electrophoresis solution (1 mL EDTA and 300 mM NaOH, pH 13 at 4 °C) for 30 min to allow for DNA unwinding and expression of alkali-labile damage before electrophoresis. Electrophoresis was done at 25 V and 300 mA (0.90 V/cm) for 25 min at 4 °C. Slides were then neutralized in Tris buffer (0.4 M Tris/HCl pH 7.5), and stained with 30 μL of an aqueous solution containing 20 mg/mL ethidium bromide. Nucleoids were evaluated visually in a blind test (Collins, Ma, & Duthie, 1995) using an Eclipse E600 microscope (Nikon) equipped for fluorescence microscopy (excitation filter, λ 516–560 nm; barrier filter λ 590 nm). Comets were classified and assigned to five categories (0–4) according to the extent of DNA migration. Bright nucleoids with no apparent tails were assigned to class O (no detectable damage), while comets with very small heads and long diffused tails were assigned to class 4. Comets showing intermediary features between classes 0 and 4 were assigned class 1, 2 or 3 test (Collins et al., 1995).

Table 2
Identification and contents of flavonoids in *Eugenia stipitata*.

Sample	Concentration (mg/100 g)*		
	Myricetin	Quercetin	kaempherol
Eugenia stipitata	17.0 ± 0.50	5.16 ± 1.40	3.70 ± 3.30

Range (mean ± standard deviation), in mg/100 g for the fruit pulp analyzed in triplicate.

2.8. Statistical analysis

SPSS for Windows version 15.0 was used for data analysis (Norussis, 2006). Statistically significant differences between groups were calculated by the application of the one way analysis of variance (ANOVA) for unpaired observations between controls and experimental samples. Tukey test was used for multiple comparisons; *p* values of 0.05 or less were considered statistically significant. All tests were performed in triplicate.

In order to analyze the mutagenic activity of *E. stipitata* extract, the MNPCE frequencies obtained for the treated groups were compared to the negative control group using ANOVA, followed by a multiple comparison procedure (Tukey test). The MNPCE frequencies observed in the treated groups and in the positive control group were compared in order to analyze the *E. stipitata* extract antimutagenicity, using ANOVA followed by the Tukey test. To evaluate the cytotoxicity of *E. stipitata* extract, the polychromatic erythrocytes/normochromatic

Table 1
Total phenolic content and antioxidant capacity of *Eugenia stipitata* and antioxidant standard (ascorbic acid, gallic acid, myricetin, quercetin, kaempferol).

Sample	TP (mg GAE/g)	$DPPH_{IC50}$ (μg/mL) (m/v)	H-$ORAC_{FL}$ (μmol TE/100 g)	L-$ORAC_{FL}$ (μmol TE/100 g)	TAC-$ORAC_{FL}$ (μmol TE/100 g)
Eugenia stipitata	184.08 ± 8.25	0.69 ± 0.23[c]	362.18 ± 6.39	9.80 ± 9.64	371.98 ± 11.50
Ascorbic acid	n.a	11.34 ± 0.17[a]	7.42	n.a	n.a
Gallic acid	n.a.	4.74 ± 0.26 [b]	9.98	n.a.	n.a
Myricetin	n.a	5.26 ± 0.58 [b]	10.72	n.a	n.a
Quercetin	n.a	4.48 ± 0.28 [b]	16.69	n.a	n.a
Kaempferol	n.a	n.a	16.01	n.a	n.a

Data was expressed as mean of triplicate ± standard deviation measurements. n.a = not available; GAE = gallic acid equivalents; TP = total phenols; DPPHIC50 = radical scavenging activity expressed as final concentration of extract in the cuvettes defined as μg/mL of dried extract required to decrease the initial DPPH concentration by 50%; hydrophilic $ORAC_{FL}$; lipophilic $ORAC_{FL}$, total antioxidant capacity, calculated as the sum of H-$ORAC_{FL}$ and L-$ORAC_{FL}$. Equal letters in the same column are statistically equals if $p<0.05$ by Tukey test.

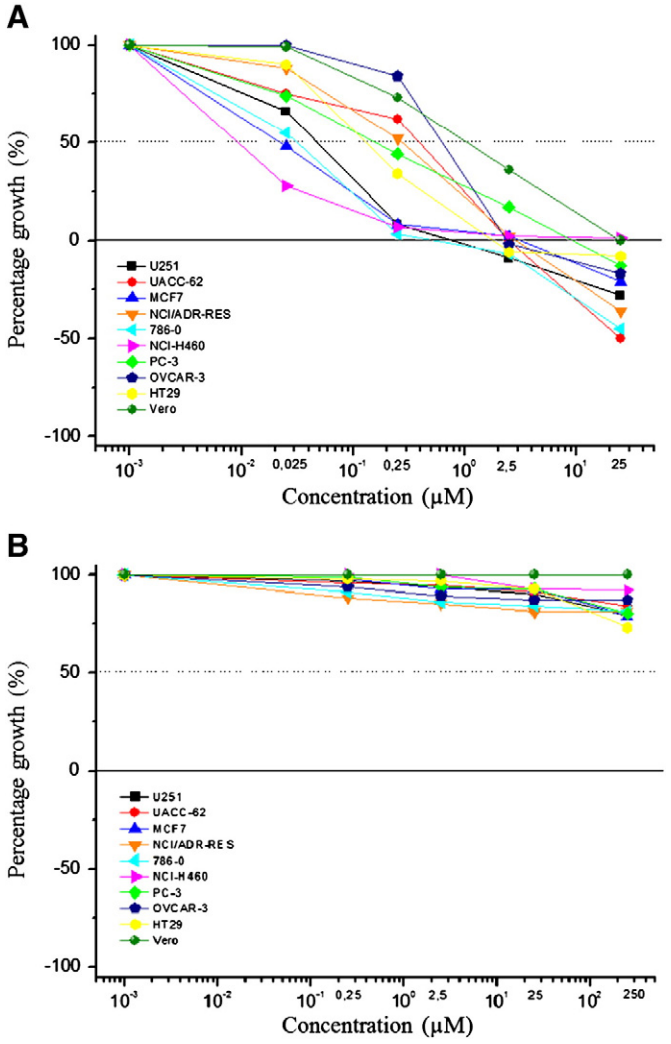

Fig. 2. Antiproliferative activity of doxorubicin (A) and (B) *Eugenia stipitata* against nince cancerous cell lines: 2 = U251 (glioma, SNC); u = UACC-62 (melanoma); m = MCF-7 (breast adenocarcinoma); a = NCI-ADR/RES (ovary, multidrug resistance phenotype); 7 = 786-0 (kidney); 4 = NCI-H460 (lung, non-small cell adenocarcinoma); p = PC-3 (prostate); o = OVCAR-3 (ovary); h = HT-29 (colon); v = VERO (kidney, normal cell of green monkey).

erythrocytes ratio (PCE/NCE) of all treated groups was compared to the result obtained in the mutagenic effect evaluation for the negative control group, and the results found in the antimutagenic effect evaluation for the positive control, using qui-square test (χ^2); p values of 0.05 or less were considered statistically significant.

3. Results and discussion

3.1. Total phenolic content and antioxidant capacity

Total phenolic content and antioxidant capacity are reported in Table 1.

Total phenolic of *E. stipitata* ethanolic extract was 184.08 ± 8.25 mg GAE/g. Vasco, Ruales, and Kamal-Eldin (2008) and Rufino et al. (2010), who studied the total phenolic content of several fruits, classified the fruits into three categories: low (<100 mg GAE/100 g), medium (100–500 mg GAE/100 g) and high (>500 mg GAE/100 g) for samples based on fresh matter. According to this classification *E. stipitata* fits in the medium category.

The radical scavenging activity was $DPPH_{IC50}$ 0.69 ± 0.23 μg/mL. It was found a good correlation between total phenolics and DPPH in *E. stipitata* and the standard, whereas the ORAC method is based on the transference of hydrogen atoms from the antioxidant to the radical. Phenolic compounds presented higher DPPH scavenging capacity, indicating a positive correlation between phenolic content and antioxidant potential, which has also been seen in other fruit, plants, juices and wine (Medina et al., 2011; Rusak, Komes, Likic, Horzic, & Kovac, 2008; Zhao & Hall, 2008).

For comparison, the tests were also performed for some reference standards, as ascorbic acid, gallic acid, myricetin, quercetin and kaempferol. The value TAC-$ORAC_{FL}$ 371.98 μmol.TE/100 g was observed. Previous studies reported similar results in fruits of Myrtaceae family (Patthamakanokporn, Puwastien, Nitithamyong, & Sirichakwal, 2008). However, camu-camu is considered to contain high levels of ascorbic acid which have been shown to interfere with the TP measurement by the same oxidation/reduction reaction that detects phenolics.

3.1.1. Flavonoid level in E. stipitata

The levels of myricetin, quercetin and kaempferol in *E. stipitata* obtained in the present study were 17.0 mg/100 g, 5.1 mg/100 g, and 3.7 mg/100 g, respectively (Table 2). Gonçalves et al. (2010) evaluated the composition of native fruit pulp, among them the araçá (guava). It was found 14.4 mg of quercetin and 2.5 mg of kaempferol in 100 g of fresh pulp of *E. stipitata*. In a study with 14 fruits from the Myrtaceae family, it was observed, in 5 species, significant amounts of myricetin, ranging from 1 to 4 g/100 g, and amounts of quercetin between 1 and 28 g/100 g in 13 out of the 14 species analyzed (Reynertson et al., 2008).

3.1.2. Antiproliferative activity in vitro (E. stipitata)

Fig. 2 shows the percentage of growth for each cell in the presence of different concentrations of positive control doxorubicin (A) and *E. stipitata* (B). A horizontal line at 0% was traced to visualize total growth inhibition (TGI), *i.e.* the concentration required to completely inhibit cell growth (total cytostatic effect), which is show in Table 3. The negative values in Fig. 2 represent the percentage of cell death. It can be seen that great part of the cell lines are sensitive to doxorubicin, with TGI in a concentration range of <0.025 – 0.3 μM.

In order to consider the extract as active, *i.e.*, antiproliferative, it must present growth inhibition higher than 50% and show a dose-dependent pattern, showing selectivity for the cell types. Doxorubicin has growth inhibition higher than 50% and is dose-dependent (Fig. 2). At the concentration tested (250 mg/L), *E. stipitata* had no complete cytostatic effect against any of the tested cell lines. A similar result was found in the study done by Malta (2011), who analyzed three fruits from Brazilian Cerrado (guapeva, gabiroba, and murici), in which none of those fruits showed antiproliferative activity.

Table 3
Comparative total growth inhibition (TGI) in human tumoral cell lines.

TGI_{50} (mg/m)										
	2	u	m	a	7	4	p	o	h	v
Doxorrubicin	0.040	0.30	0.025	0.24	0.028	<0.025	0.15	0.31	0.14	1.0
Eugenia stipitata	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250

2 = U251 (glioma. SNC); u = UACC-62 (melanoma); m = MCF-7 (breast adenocarcinoma); a = NCI-ADR/RES (ovary, multidrug resistance phenotype); 7 = 786-0 (kidney); 4 = NCI-H460 (lung. non-small cell adenocarcinoma); p = PC-3 (prostate); o = OVCAR-3 (ovary); h = HT-29 (colon); v = VERO (kidney, normal cell of green monkey).

Table 4
Body weight and food consumption in the different experimental groups of mice used in the micronucleus test and comet assay.

Group/treatment	Body weight (g)		Total feed consumption (g)
	Initial	Final	
G1	30.34 ± 1.01	30.94 ± 0.84	534.45
G2	30.14 ± 1.13	31.13 ± 1.17	510.11
G3	28.85 ± 1.19	26.31 ± 1.07	503.98
G4	30.02 ± 1.73	27.43 ± 1.89	513.49
G5	30.11 ± 2.85	26.89 ± 1.95	522.61
G6	31.85 ± 1.87	28.40 ± 1.60	507.55
G7	30.18 ± 1.63	27.33 ± 1.55	530.12
G8	30.78 ± 0.87	28.44 ± 1.29	502.03

There was no significant variation of body weight and ration consumption between experimental groups by Tukey test ($p<0.05$).

Table 5
Frequency of erythrocytes polychromatics micronucleus (MNPCEs) of bone marrow cells of Swiss mice in experimental groups treated with *Eugenia stipitata*.

Eugenia stipitata/ group	Treatment	Number of analyzed PCEs	MNPCEs		% reduction
			No.	%*	
G1	NaCl + CP	12,000	122	1.02	–
G2	NaCl + NaCl	12,000	20	0.17	
G3	Extract 30 mg/kg bw + CP	12,000	36	0.30	84.31
G6	Extract 30 mg/kg bw + NaCl	12,000	20	0.17	
G4	Extract 100 mg/kg bw + CP	12,000	41	0.34	79.41
G7	Extract 100 mg/kg bw + NaCl	12,000	24	0.20	
G5	Extract 300 mg/kg bw + CP	12,000	35	0.29	85.29
G8	Extract 300 mg/kg bw + NaCl	10,000	14	0.14	

CP — cyclophosphamide. There was no significant variation between experimental groups (G3–G8) by Tukey test ($p<0.05$).

3.1.3. Micronucleus (MN) test

During the experiment, the animals were weighed, and the consumption of feed was controlled. The results are shown in Table 4, suggesting that there was no significant variation of body weight and ration consumption between experimental groups ($p>0.05$) during the study period.

Positive control (CP + NaCl) caused a significant ($p<0.05$) increase in MNPCE compared to the negative control, confirming the test sensitivity. Groups that received NaCl in the last day were compared with negative control, and groups that received CP in the last day were compared with positive control. These data indicate that *E. stipitata* extract did not show mutagenic activity at any concentration tested. All the concentrations of the extracts showed antimutagenic activity when compared with positive control ($p<0.05$). The animals treated with the 300 mg/kg extracts presented higher reduction, which is shown in Table 5, (85.29%) followed by the 30 mg/kg extract with 84.31% and the 100 mg/kg extract with 79.41%.

Swiss mice treated with *Eugenia desinterica* ethanolic leaf extract, a plant from the same family of *E. stipitata* (Myrtaceae), indicated that this plant protected mice cells against the genotoxic and cytotoxic activities of CP at all doses analyzed (50, 100, 150, and 200 mg/kg), which suggests the presence of antigenotoxic and anticytotoxic compounds in this plant species (Vieira, Veronezi, Silva, & Chen-Chen, 2012). Costa, Vieira, Andrade, and Maistro (2008) studied the antimutagenic effect of *Psidium cattleianum* Sabine, another plant from Myrtaceae family, by using the micronucleus test. At all concentrations evaluated, no mutagenic effects were found.

The MN test detects genetic alterations arising from chromosomal damage and/or damage to the mitotic apparatus caused by clastogenic or aneugenic agents. The MNs are indicative of irreversible DNA loss, their frequency may be used as a mutation index. It is known that there is a positive correlation between increased frequency of MNs and the appearance of tumors in rodents and humans (Hayashi et al., 2000; Vieira et al., 2012).

3.1.4. Single-cell gel electrophoresis (SCGE) assay

During the last few years, there has been a growing interest in the search for food and food compounds with the capacity to prevent damage to genetic material, thereby acting as chemoprotective agents. The rationale for using chemoprotective compounds is based on their efficiency as well as on the understanding of their mechanism of action (Miadoková et al., 2005; Angeli, Ribeiro, Camelini, Mendonça, & Mantovani, 2009). Several mechanisms may be involved, such as antigenotoxic effects evaluated by single-cell gel electrophoresis assay.

The antigenotoxic effect (Table 6) was verified in groups G3 (extract 30 mg/kg bw + CP), G4 (extract 100 mg/kg bw + CP), and G5 (extract 300 mg/kg bw + CP). It was observed a significant reduction in cell damage in G5. In the other groups it was not observed any antigenotoxic effect. Malta (2011) also observed a reduction in the DNA damage in a study with native fruits from the Brazilian Cerrado; however, in this study, the concentration of fruits used (100, 200, and 400 mg/bw) did not interfere with the reduction in damage.

In both tests, MN and comet assay, it can be observed that the extract from *E. stipitata* at the concentration of 300 mg/kg bw showed protective effects in DNA damage.

4. Conclusions

According to the results obtained, it can be observed that the extract of the pulp of *E. stipitata* showed a total phenolic amount rated as medium and with good antioxidant activity. No cytostatic effects were found, however, antimutagenic and antigenotoxic activities were observed at 300 mg of extract/kg bw.

Possibly, the content of total phenolics and antioxidant activity shown by *E. stipitata* might contribute to antimutagenic and antigenotoxic activities. Since the best effects were observed in the highest extract dosage, it is necessary that antimutagenic and antigenotoxic activities are studied at different concentrations of the *E. stipitata* extract.

Table 6
Evaluation of the genotoxic potential by comet assay–single-cell gel electrophoresis assay.

Eugenia stipitata group	Treatment	Comet class (mean ± SD)					Mean ± SD
		0	1	2	3	4	
G1	NaCl + CP	0.8 ± 1.21	4.4 ± 2.94	18.8 ± 9.98	60.4 ± 8.85^{a}	15.6 ± 3.71	90.8 ± 12.96^{a}
G2	NaCl + NaCl	69.0^{a} ± 4.08	23.2 ± 3.06^{b}	3.2 ± 1.50 ^{d}	1.8 ± 1.36^{d}	2.8 ± 1.32^{a}	34.3 ± 14.30 ^{b}
G3	30 mg/kg bw + CP	0.0 ± 0.00	3.8 ± 6.17^{d}	37.0 ± 8.15^{a}	55.6 ± 8.80^{a}	3.6 ± 1.96^{a}	107.42 ± 22.26^{a}
G4	100 mg/kg bw + CP	0.0 ± 0.00	17.8 ± 2,50^{c}	24.6 ± 4.40^{a}	53.2 ± 7.06^{a}	4.4 ± 3.66^{a}	105.47 ± 19.32^{a}
G5	300 mg/kg bw + CP	1.2 ± 1.37^{d}	24.0 ± 9.13^{b}	37.4 ± 11.96 ^{a}	34.4 ± 9.18^{b}	3.0 ± 2.58^{a}	112.3 ± 31.21^{a}
G6	30 mg/kg bw + NaCl	28.6 ± 2.42 ^{c}	30.6 ± 4.95^{b}	26.6 ± 9.04^{a}	13.4 ± 5.11^{c}	0.8 ± 1.47^{a}	84.8 ± 31.36^{a}
G7	100 mg/kg bw + NaCl	36.8 ± 10.07 ^{b}	22.0 ± 12.9^{b}	28.8 ± 12.3^{a}	9.4 ± 10.38^{c}	3.0 ± 3.98^{a}	75.41 ± 25.0^{a}
G8	300 mg/kg bw + NaCl	49.0 ± 4.89 ^{b}	38.6 ± 2.71^{a}	9.2 ± 5.29 ^{c}	3.2 ± 0.98^{d}	0.0 ± 0.00	62.16 ± 25.58^{a}

CP — cyclophosphamide. Significantly different from the negative control ($p<0.001$). [1]Total number of damaged cells (class 1 + 2 + 3 + 4). Equal letters in the same column are statistically equals if $p<0.05$ by Tukey test.

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