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Jaboticaba peel: Antioxidant compounds, antiproliferative and antimutagenic activities

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ABSTRACT

This paper reports on the anthocyanin and antioxidant contents, an the 'in vitro' antiproliferative and 'in vivo' mutagenic/antimutagenic activities of freeze-dried jaboticaba peel (JP). According to the proximate composition, JP showed a high dietary fiber content. The identification and quantification of the JP anthocyanins was carried out by HPLC-PDA and LC–MS/MS, which revealed the presence of two compounds: delphinidin 3-glucoside and cyanidin 3-glucoside (634.75 and 1963.57 mg 100 g^{-1} d. w., respectively). JP showed a strong antioxidant potential: 25,514.24 ± 3037 μM TE g^{-1}, 45.38 ± 0.50 μg mL^{-1} and 9458 ± 97 μM TEAC g^{-1}, for ORAC, DPPH and ABTS, respectively. The polar JP extract demonstrated antiproliferative effects against leukemia (K-562), and the non-polar extract was the most active against prostate cancer cell (PC-3), according to the antiproliferative assay. The micronucleus test in mice demonstrated that the polar JP extract induced no DNA damage and hence it showed no cytotoxic properties on mice bone marrow cells and caused no mutagenic effects.

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1. Introduction

There is increasing interest in the antioxidant activity of the phytochemicals present in the diet, since they play a very important role in the organism's defense system against reactive oxygen species (ROS), which are mainly generated during the regular energy metabolism of aerobic cells (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). It is known that excessive concentrations of ROS and RNS (reactive nitrogen species) can cause biological damage to proteins, lipids and DNA and the human body depends on a series of antioxidant systems to eliminate free radicals from the organism, although such systems are not 100% efficient. Thus fruits, nuts and vegetables, considered excellent sources of antioxidants, could play a crucial role in the antioxidant status (Clerici & Carvalho-Silva, 2011; Haytowitz et al., 2007, Leite et al., 2011).

Thus some foods are currently consumed not only for their sensory properties and personal preference, but as a source of nutrients and bioactive compounds (Roosen, Marette, Blanchemanche, & Verger, 2007). Besides the essential nutrients, most fruits contain considerable quantities of micronutrients, such as minerals, fibers, vitamins and phenolic compounds. Several studies have shown the importance of these micronutrients to human health (Rufino et al., 2010).

Anthocyanins belong to a class of phenolic compounds, which have attracted increasing attention because of their antioxidant activity. Several anthocyanins from plant extracts have been tested since they could be active in the reduction of oxidative stress, prevention of some inflammatory diseases; prevention of heart diseases, protection against obesity and hypoglycemia; enhancement of memory and the protection of fetal cerebral tissue (Brito et al., 2007; Dai, Gupte, Gates, & Mumper, 2009).

Jaboticaba is known as one of the richest Brazilian sources of anthocyanins. Commonly found in Brazilian markets, fresh jaboticaba fruit is widely consumed, and its popularity has been compared to that of grapes in the United States. The species/varieties *Myrciaria cauliflora* (DC) Berg., popularly known as *jabuticaba assú* and *Myrciaria jaboticaba* (Vell.) Berg., popularly known as *jabuticaba sabará*, are the most important ones. The fruit grows directly on the main trunk and branches, has a diameter of 3–4 cm, 1–4 big seeds, and a thick purple peel that covers a sweet jelly-like white pulp (Reynertson, Yang, Jiang, Basile, & Kennelly, 2008). The fruit has a sweet and subacid taste

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probably due to its sugar, organic acid and terpene contents (Plagemann, Krings, Berger, & Maróstica Júnior, 2012).

Recent studies by the same authors have shown that the addition of 1 and 2% of freeze-dried jaboticaba peel to normal diets improves the antioxidant status of healthy rats (Leite et al., 2011), and this effect could be attributed to the amount of anthocyanins identified in this byproduct. However, no previous results have been reported on the *in vitro* antiproliferative activity on tumor cells or *in vivo* mutagenic tests of jaboticaba peel (JP). Thus, the aim of this study was to identify and quantify the jaboticaba anthocyanins, and determine the different anthocyanin forms (total, monomeric), total phenolic compounds and antioxidant capacity using different methods. An additional purpose was to evaluate the *in vitro* antiproliferative activity and mutagenic and antimutagenic effects of the jaboticaba peel extract on the bone marrow cells of mice using the micronucleus test.

2. Material and methods

2.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH); 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX); 2,2′-azobis(2-methylpropionamidine) dihydrochloride (APPH) and gallic acid were all obtained from Sigma-Aldrich (São Paulo, Brazil). Fluorescein sodium salt was purchased from Vetec Química Fina (São Paulo, Brazil). Myrtillin chloride, ideain chloride and kuromanin chloride standards were purchased from Extrasynthèse (Genay, France). Doxorubicin chloridrate and cyclophosphamide were purchased from Europharma (São Paulo, Brazil).

2.2. Determination of macronutrients in the samples

Jaboticaba fruits (*M. jaboticaba* (Vell.) *Berg.*) were bought at the local market in Campinas, São Paulo State, Brazil, in September, 2008. The fruits were washed, manually peeled and frozen at −18 °C. The peels were dried in a freeze-dryer (LP1010, Liobras, São Carlos, São Paulo, Brazil) at 30 °C, 300 μm Hg for 95 h, and the freeze-dried product (JP powder) stored at −80 °C.

The total N content was determined according to the Kjeldahl method (AOAC, 1975), and the moisture, ash (Instituto Adolfo Lutz, 1985) and lipid contents (Bligh & Dyer, 1959) also determined. The soluble and insoluble fiber contents were determined according to Asp, Johanson, Hallmer, and Siljestrom (1983).

2.3. Determination of anthocyanins and polyphenols by spectrophotometry

The Lees and Francis (1972) was used to determine total anthocyanin contents, with some adaptations. One hundred milligrams of freeze-dried JP powder were homogenized in 25 mL of the extractor solution (95% ethanol: 1.5 N HCl, 85: 15 v v^{-1}) and stored for 12 h at 4 °C. The samples were percolated and the residues exhaustively rinsed with solvent until complete removal of the pigments, and then maintained in the dark for 2 h at 25 °C. The absorbance was measured at 535 nm in a Beckman®, DU640 spectrophotometer (Corona, USA), and the determinations being made in triplicate.

The total anthocyanins were also quantified according to the method described by Wrolstad (1976) and adapted by Abe, Da Mota, Lajolo, and Genovese (2007). The freeze-dried sample (0.2 g) was triturated in an Ultra-Turrax (Polytron®-Kinematica GnbH, Kriens-Luzern, Switzerland) with 20 mL of methanol: hydrochloric acid (99.9: 0.1 v v^{-1}) and centrifuged at 2000 *g* for 15 min (4 °C). Part of the supernatant was diluted from 5 to 25 times using 0.025 M potassium chloride buffer, pH = 1.0, according to the sample color. The absorbance was read at 510 and 700 nm using a Beckman® DU640 spectrophotometer (Corona, USA). Another part of the supernatant was diluted in the same proportions in 0.4 M sodium acetate buffer, pH 4.5, and the absorbance read at the same wavelengths. The absorbance (according to the referred method) was then calculated using Eq. (1):

$$A = [(A_{510nm}–A_{700nm})pH = 1.0−(A_{510nm}–A_{700nm})pH = 4.5] \quad (1)$$

The anthocyanin content (mg 100 g^{-1}) was calculated as cyanidin 3-glucoside (PM = 449.2) using Eq. (2):

$$C\left(\text{mg } 100 \text{ g}^{-1}\right) = A.MW.DF \div \xi.1 \quad (2)$$

where ξ = molar absorptivity (26,900 mol L^{-1}), 1 = tray thickness (cm), MW = molecular weight and DF = dilution factor.

The phenolic compounds were quantified according to the method described by Swain and Hillis (1959), adapted by Roesler et al. (2007), Roesler, Malta, Carrasco, and Pastore (2007) using the Folin–Ciocalteau reagent. Freeze-dried JP powder was dissolved in methanol to give a 0.5 mg mL^{-1} solution. In the same way, the fresh whole fruit and peels were triturated in an Ultra-Turrax (Polytron®-Kinematica GnbH, Kriens-Luzern, Switzerland) and dissolved in methanol as described above. This solution was placed in an ultrasound for 2 h and then percolated through a cellulose acetate membrane. A 0.5 mL portion of the methanol extract (0.5 mg mL^{-1}) was added to 2.5 mL of aqueous 10% Folin–Ciocalteau solution and 2.0 mL of 7.5% sodium carbonate. The mixture was incubated for 5 min in a water bath at 50 °C and the absorbance measured at 760 nm. The blank was prepared by replacing the sample with water in the reaction mixture. The results were expressed in gallic acid equivalents (g GAE 100 g^{-1}) using a standard curve of gallic acid dissolved in distilled water at concentrations from 0.02 to 0.12 mg mL^{-1}. The phenolic compounds were quantified in the extracts in triplicate.

2.4. Identification and quantification of anthocyanins (HPLC-MS)

One gram of freeze-dried JP was weighed into a centrifuge tube and extracted with 15 mL of methanol/water/acetic acid (85:15:0.5, v v^{-1}). The sample was shaken in a vortex for 30 s and then placed in an ultrasound bath for 5 min. After 10 min at room temperature it was mixed for 30 s, and after a further 5 min centrifuged at 4550 *g* for 10 min and the supernatant discarded. The sample was extracted a second time with 10 mL of methanol/H_2O/acetic acid using the same procedure, and the supernatants combined. The solution was finally filtered through a 0.22 μm Teflon filter (Wu, Gu, Prior, & McKay, 2004).

Five microliters of the extract were diluted in 995 μL of methanol: H_2O (1: 1) and 0.1% formic acid, and then submitted to ESI (electrospray ionization) using a Q-TOF mass spectrometer (Micromass, Manchester, United Kingdom). The general conditions were: source temperature of 100 °C, capillary voltage of 3 kV and cone voltage of 35 kV. The measurements were made by direct infusion with a flow rate of 10 μL min^{-1} and a syringe pump (Harvard Apparatus). Mass spectra were acquired and accumulated during 60 s before and scanning in the 100–1000 *m/z* range (Leite et al., 2011).

The HPLC method for quantification of the anthocyanin content of the freeze-dried JP was based on Favaro (2007), with some modifications (Leite et al., 2011). The analysis was carried out in a HPLC (Waters®, model 515, Massachussetts, USA) system equipped with a photodiode array detector (Waters®, model 2996, Massachussetts, USA) and Empower software. The anthocyanins were separated using a C18 column (ODS2, length = 25.0 cm, internal diameter = 4.6 mm, particle size = 5 μm and pore size = 100 Å, Varian, Microsorb MV). With a mobile phase of deionized water: acetonitrile: formic acid (81:9:10 v/v/v). The elution was isocratic with a mobile phase flow volume of 1.0 mL min^{-1}; injection volume of 20 μL;

elution time of 40 min and detection at 520, 525 and 540 nm. Each anthocyanin peak was identified based on a comparison of the relative retention time (RT), peak area percentage and spectra data of the anthocyanin standards. The standards cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, and delphinidin-3-*O*-glucoside were used to quantify the individual anthocyanidins. The standard curves (10, 20, 30, 40, 50 and 60 μg mL^{-1} concentrations) for all the anthocyanins were determined using the same chromatographic conditions described above. For quantification of anthocyanins in the whole fruit, the samples were extracted as described in the HPLC procedure above.

2.5. Determination of the free radical sequestering capacity

The freeze-dried powder was dissolved in ethanol to obtain a concentration of 10 mg mL^{-1}, placed in the ultrasound bath for 30 min and then filtered. The same extract was used for all the antioxidant assays. To ORAC assay, the whole fresh fruit and fresh peel were extracted as the freeze-dried powder.

The ORAC (Oxygen Radical Absorbance Capacity) test was carried out according to the method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004). The reagents were placed in microplate wells where the reaction occurred. Twenty microliters of extract (final concentration of 0.6 mg mL^{-1}), 120 μL of fluorescein in potassium phosphate buffer (concentration of 0.378 μg mL^{-1}, pH 7.4) and 60 μL of AAPH (108 mg mL^{-1}) were mixed. The AAPH solution was prepared just before using and for the blank, the extract was substituted by potassium phosphate buffer. As the standard, Trolox was used in potassium phosphate buffer (0.1; 1.0; 10; 25; 50; 80; and 100 μM). Fluorescence was measured every minute, for 80 min. With the following filters: emission 520 nm and excitation 485 nm. The extracts were determined in triplicate. The results were expressed as equivalents in μmol of Trolox per gram of sample and were based on the area under the curve for the decline in fluorescence time (AUC) and on the Net Area (NAUC), calculated in approximate values, as described in Eqs. (3) and (4):

$$AUC = 1 + \sum_{i=1}^{i=80} fi/fo \quad (3)$$

where f_0 is the initial fluorescence reading at 0 min and fi is the fluorescence reading at time *i*.

$$NAUC = AUC_{sample} - AUC_{blank} \quad (4)$$

The NAUC was plotted against sample concentration and the results compared to the standard curve (NAUC versus Trolox concentration).

The DPPH test was carried out according to Roesler, Malta, Carrasco, and Pastore (2007) with some adaptations. 250 μL of DPPH solution (0.004% $m\ v^{-1}$) and 50 μL of extract at different concentrations were placed on a microplate and the plate incubated for 30 min at room temperature in the dark. The same procedure was adopted for the gallic acid. The control assay (DPPH reagent) was prepared according to the procedure above, without the addition of extract and ethanol was used to correct the baseline. The DPPH solution was prepared daily, stored in dark flasks and kept in the dark at 4 °C until used. The percentile decrease in absorbance was determined at 517 nm using a microplate reader (NOVOstar®, BMG Labtech—Offenburg, Germany) and the free radical quenching capacity was calculated as follows (Eq. (5)):

$$\%\,\text{Inhibition} = (\text{ADPPH} - \text{AExtract})/\text{ADPPH}) * 100 \quad (5)$$

where ADPPH is the absorbance of DPPH solution absorbance and AExtract that of the sample.

The IC_{50} value is the final concentration in mg mL^{-1} of the dry extract required to decrease or inhibit 50% of the initial DPPH concentration, and was determined by linear regression.

The TEAC assay was applied to the JP based on the reports of Rufino et al. (2007) and Le, Chiu, and Ng (2007) with modifications. The ABTS solution was prepared using 5 mL of a 7.0 mM ABTS solution and 88 μL of a 145 mM potassium persulfate solution, and leaving it to react for 12 h at room temperature in the dark. Ultra pure water was added to the solution until reaching an absorbance of 0.700 ± 0.05 at 734 nm using a microplate reader (NOVOstar®, BMG Labtech—Offenburg, Germany). Using a 96-well transparent microplate, 50 μL of sample solution and 250 μL of ABTS solution were added. Absorbance measurements were carried out at room temperature after 6 min of reaction and the Trolox was used as the antioxidant standard.

2.6. In vitro antiproliferative activity in tumor cell lines

Freeze-dried JP was extracted by successive maceration (1: 10 sample: solvent, 3×1 h) with dichloromethane at room temperature to obtain a non-polar extract, after solvent evaporation. The same process was repeated, but using ethanol, to get the polar extract. The polar and non-polar JP extract concentrations used in the assays were 0.25, 2.5, 25 and 250 μg mL^{-1}.

Human and other tumor cell lines, U251 (glioma, central nervous system); UACC-62 (melanoma), MCF7 (breast), NCI-ADR/RES (adriamycin-resistant ovarian cancer); 786–0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovary), HT29 (colon), and K-562 (leukemia) and VERO (a non-tumoral cell line, green monkey kidney) were kindly provided by the NCI (National Cancer Institute). Stock cultures were grown in a medium containing 5 mL RPMI 1640 (GIBCO BRL) supplemented with 5% fetal bovine serum. Gentamicin (50 μg mL^{-1}) was added to the experimental cultures. The cells were placed in 96-well plates (100 μL cells $well^{-1}$) and exposed to different sample concentrations in DMSO/RPMI (0.25, 2.5, 25 and 250 μg mL^{-1}) at 37 °C, with 5% of CO_2 in the air for 48 h. The maximal final concentration of DMSO was 0.2% and did not affect cell viability. The cells were then fixed with 50% trichloroacetic acid and cell proliferation determined by spectrophotometric quantification (540 nm) of the cell protein content using the sulforhodamine B assay. Doxorubicin chloridrate (0.025 to 25 μg mL^{-1}) was adopted as the positive control. Using the concentration–response curve for each cell line, the GI_{50} (concentration of JP producing 50% growth inhibition) was determined using a nonlinear regression analysis (Table 3) with the ORIGIN 7.5 software (OriginLab Corporation) (Shoemaker, 2006).

2.7. In vivo mutagenic/antimutagenic assay

The animals were housed in wire-topped opaque polycarbonate cages and maintained under constant room conditions on a 12 h light/ dark schedule. The temperature was 20 ± 2 °C and the air humidity was maintained at 50%. Three-week-old male Swiss mice weighing 25 g were obtained from the Central Animal Facility of the Federal University of Alfenas. Commercial food pellets and water were provided *ad libitum*. The animals were allowed to adapt in the housing facilities for at least 1 week before the experiments began. All experiments were carried out in accordance with the Declaration of Helsinki on the welfare of experimental animals and with the approval of the Ethics Committee of the Federal University of Alfenas (#239/2011).

The animals were segregated into 8 groups. Group 1: JP 30 mg kg^{-1} body weight (b. w.) + cyclophosphamide (CP); Group 2: JP 30 mg kg^{-1} b. w. + NaCl; Group 3: JP 100 mg kg^{-1} b. w. + CP; Group 4: JP 100 mg kg^{-1} b. w. + NaCl; Group 5: JP 300 mg kg^{-1} b. w. + CP; Group 6: JP 300 mg kg^{-1} b. w. + NaCl; Group 7: CP

(negative control) and Group 8: NaCl (negative control). The test substances were dissolved in water and administered by gavage daily during 15 days in 150 μL doses at concentrations of 30, 100 and 300 mg kg^{-1} b. w. The negative and positive controls only received the vehicle. Half the groups received intraperitoneal injections of 50 mg kg^{-1} b. w. of CP 24 h before euthanasia and the other half received injections of NaCl. Both femur bones were excised and their bone marrows flushed into test tubes using a syringe containing bovine fetal serum. The percentage reduction in the frequency of CP-induced DNA damage was calculated as follows (Eq. (6)):

$$\%\,\text{Reduction} = \left[\left(\bar{y}\,A\right)-\left(\bar{y}\,B\right)/\left(\bar{y}\,A\right)-\left(\bar{y}\,C\right)\right]\times 100 \quad (6)$$

where A = positive control group treated with CP; B = group fed with the polar JP extract + CP; and C = negative control group.

For the conventional assessment of micronucleus frequencies, two slides were prepared for each animal according to the method of MacGregor et al. (1987). The cells were stained with the Leishman stain, centrifuged at 2000 rpm for 5 min, and the supernatant removed. The slides were coded and the cells scored blindly by light microscopy at ×1000 magnification. The frequency of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with the variability (standard deviation) based on differences amongst the animals in the same group. The polychromatic erythrocytes/ normochromatic erythrocytes (PCE/NCE) ratio was also determined from a total of 1000 erythrocytes counted.

To analyze the JP extract antimutagenicity, the MNPCE frequencies observed in the treated groups were compared with those of the positive control group compared. To evaluate the cytotoxicity of JP, the polychromatic erythrocytes/normochromatic erythrocytes ratios (PCE/NCE) obtained for all the treated groups were compared to both the results obtained in the mutagenic effect evaluation for the negative control group and those obtained in the antimutagenic effect evaluation for the positive control.

2.8. Statistical analysis

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

In order to analyze the mutagenic activity, ANOVA followed by Tukey were used to evaluate the MNPCE frequencies; EHP and MI antimutagenicity. The cui-squared test (χ^2) at $P<0.05$ was used to evaluate the cytotoxity of EHP, MI and PCE/NCE.

3. Results and discussion

3.1. Fruit and extract characterization

The average weight percentages of peel and pulp + seeds in relation to the whole fruit were 28.9 ± 4.92% (m m^{-1}) and 66.82 ± 4.92% (m m^{-1}), respectively. The average gross weight of each fruit was 5.63 ± 1.00 g, and the moisture content of fresh jaboticaba peel was 79.5%.

According to the proximate composition of the freeze-dried JP, the powder was constituted mostly of soluble and insoluble fibers (Table 1). JP could be highlighted as a food rich in dietary fibers and a high content of ash has already been found in *M. jaboticaba* peel (Lima, Corrêa, Alves, Abreu, & Dantas-Barros, 2008).

Table 1
Proximate composition of freeze-dried jaboticaba peel powder.

Components (%m m^{-1})	Content (%)	SD
Moisture	15.33	0.19
Lipids	1.72	0.02
Ash	3.52	0.02
Crude protein[a]	4.89	0.10
Insoluble fibers	20.00	2.00
Soluble fibers	5.00	0.50

Each value was obtained from the average ± standard deviation of at least three replicates. Conversion factor: N = 6.25.
[a] Nitrogen value = 0.78.

3.2. Determinations of bioactive compounds present in the freeze-dried jaboticaba peels and its antioxidant activity

The high total phenolic compound contents (556.3 g GAE kg^{-1}) found in the dried JP powder in this study were also reported by a few other authors (Abe, Lajolo, & Genovese, 2012; Lima et al., 2008; Silva, Constant, Figueiredo, & Moura, 2010), since this Brazilian fruit has not been widely studied. The phenolic compounds found in the whole fruits and peel of *M. cauliflora* have also been published (Reynertson et al., 2008; Santos & Meireles, 2011; Santos, Veggi, & Meireles, 2010). In the present study, the fresh JP and whole fruit of *M. jaboticaba* was shown to contain 114 and 32.15 g GAE kg^{-1}, respectively (Table 2).

Reynertson (2007) showed that whole *M. cauliflora* fruits contained 31.63 ± 0.1 g GAE kg^{-1} of dry weight (d. w.), which is a high value compared to the freeze-dried powder of several blueberry species (12.00 ± 0.77 to 14.81 ± 1.58 g GAE kg^{-1}) (Dai, Gupte, Gates, & Mumper, 2009). The phenolic compound content (determined using the Folin–Ciocalteau method) of two varieties of açaí, a berry produced in Brazil and Bolivia, were 31.2 and 73.0 mg GAE kg^{-1} d. w. (Kang et al., 2012). These values were comparable to those found in the whole jaboticaba fruit in the present work. Interestingly, recent data showed that the main contribution to the total phenolic contents of the whole fruit in *M. jaboticaba* came from the peel (Abe et al., 2012), which corroborates the present data.

The cyanidin 3-glucoside content found in the freeze-dried JP powder (1514.82 ± 45.51 mg 100 g^{-1}) was expressive. It has been reported that the monomeric anthocyanin contents in jaboticaba *Sabará* peel were about 205 and 66 times higher than those in the pulp and seeds, respectively (Lima, Corrêa, Saczk, Martins, & Castilho, 2011). Thus, it was confirmed that the anthocyanins of jaboticaba fruits were more concentrated in the peel.

The Lees and Francis (1972) differential pH method, showed a lower content of total anthocyanins (732.77 ± 22.42 mg 100 g^{-1}) in comparison to the Wrolstad (1976) method (1514.82 ± 45.51 mg 100 g^{-1}). This difference could be due to the different solvents used in each method. The solvent used in the first method (95% ethanol/ 1.5 N HCl, 85: 15) could have failed to properly extract the

Table 2
Bioactive compounds and anti-radical activity found in the freeze-dried jaboticaba peel powder.

Polyphenols and antioxidant capacity[a]	Values
Total phenolic compounds	556.3 g GAE kg^{-1}[b]
Total anthocyanins	732.77 ± 22.42 mg 100 g^{-1}
Monomeric anthocyanins	1514.82 ± 45.51 mg cyd 3-glu 100 g^{-1}[c]
DPPH anti-radical activity	45.38 ± 0.50 μg mL^{-1}
ABTS anti-radical activity	9458 ± 97 μM TEAC g^{-1}
ORAC anti-radical activity	25,514.24 ± 3037.24 μM TE g^{-1}[d]

[a] Average of triplicate analyses.
[b] Values expressed in gallic acid equivalents.
[c] Cyanidin 3-glucoside.
[d] Trolox equivalents.

anthocyanins in full. An alternative to improve the extraction of anthocyanins from *M. jaboticaba* could be the application of the efficient extraction procedure used for *M. cauliflora* peels, employing high pressure CO_2 (Santos & Meireles, 2011; Santos et al., 2010). In this case, the optimum extraction conditions showed total anthocyanin and phenolic compound contents of 2.237 ± 0.307 mg g^{-1} cyanidin 3-glucoside and 12.895 ± 1.460 mg GAE g^{-1} d. w., respectively (Santos & Meireles, 2011).

The IC_{50} for freeze-dried JP found in the DPPH test was 45.38 μg mL^{-1}. The regression analysis of the DPPH results showed a high dependence on the total phenolics concentration. The best adjustment correlation was obtained using a linear model ($y = 0.9858x + 5.2665$; $R^2 = 0.9926$). Reynertson (2007) found a value for $IC_{50} = 35$ μg mL^{-1} for a methanol extract of jaboticaba in the DPPH test. Einbond, Reynertson, Luo, Basile, and Kennelly (2004), working with purified methanol jaboticaba extract, found a value for $IC_{50} = 6.2 \pm 0.7$ μg mL^{-1}. Low IC_{50} values indicate a higher antioxidant potential and hence the value found in the present study indicates a lower antioxidant potential when compared to the other studies, although the differences in extraction methods and species could explain such differences.

The ABTS assay gave a value of 9458 μM TEAC g^{-1} d. w. for the freeze-dried JP sample. Silva et al. (2010), working with jaboticaba peel (*Myrciaria* ssp.), found an ABTS value of 723.84 ± 37.00 μM TEAC g^{-1} and Rufino et al. (2010) reported 138.0 ± 3.1 μM TEAC g^{-1} d. w. for whole jaboticaba fruits. Therefore, the present results are higher than those found in previous reports, but this could be explained by the high anthocyanin and total phenolic compound content found in the peel.

The ORAC test result found for the freeze-dried powdered extract was 25,514.24 μM TE g^{-1} d. w. with ORAC values of 5230.4 μM TE g^{-1} and 1511 μM TE g^{-1} for the fresh fruit peel and whole fresh fruit, respectively. A United States Department of Agriculture publication (Haytowitz et al., 2007) presented a list of the antioxidant powers of 277 foods based on ORAC determinations. The foods listed as anthocyanin sources: blackberries, blueberries, cranberries and elderberries, had lower ORAC values than the freeze-dried JP powder (5245 μM TE g^{-1}, 6520 μM TE g^{-1}, 9382 μM TE g^{-1}, 14,500 μM TE g^{-1}, and respectively).

Ou, Hampsch-Woodill, and Prior (2001) found ORAC values of 2792 μM TE g^{-1} for blueberry extract and 15,675 μM TE g^{-1} for the freeze-dried 50% acetone extract of grape peels. Schauss et al. (2006), using the same solvent, found an ORAC value of 997 μM TE g^{-1} for the freeze-dried powder of the Brazilian palm berry named açaí. Recently, Kang et al. (2012) also showed values ranging from 985.9 to 1792.3 μM TE g^{-1} d. w. for two species of açaí. However, the jaboticaba fruit has not been extensively studied and there are no results from tests previously carried out with freeze-dried jaboticaba powder.

Chromatographic analyses of the anthocyanins in the freeze-dried jaboticaba peel revealed the presence of two major compounds: delphinidin 3-glucoside and cyanidin 3-glucoside, confirmed by comparing their retention times with those of authentic standards, and also by chromatography coupled to mass spectrometer (LC–MS/MS) using ESI ionization in a Q-ToF mass spectrometer (Fig. 1). The quantifications were based on standard curves obtained from the HPLC analysis. The curves had the following equations for cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside: $y = 30{,}685x + 22{,}740$; $R^2 = 0.9987$; and $y = 29{,}887x – 198{,}467$; $R^2 = 0.9842$, respectively.

The total anthocyanins content as determined by HPLC was 2598.32 mg 100 g^{-1} of freeze-dried JP powder; and the LCESI-MS/MS analysis confirmed the identity of the compounds. Cyanidin-3-*O*-glucoside (peak 2; $[M]^+$ *m/z* 449; MS/MS *m/z* 287) was the dominant anthocyanin with 75.6% of the total anthocyanins (1963.57 ± 52.72 mg 100 g^{-1}). Delphinidin 3-*O*-glucoside (peak 1, $[M]^+$ *m/z* 465; MS/MS *m/z*) represented 634.75 ± 1.83 mg 100 g^{-1} of the freeze-dried JP (Leite et al., 2011). Reynertson et al. (2006) identified cyanidin 3-glucoside and peonidin 3-glucoside in the fruit. The scientific literature supports the present data, as cyanidin and delphinidin 3-glucosides have already been described in the fruit and peel of *M. cauliflora* and *M. jaboticaba* (Abe et al., 2012; Lima et al., 2011; Santos et al., 2010).

As already suggested, jaboticaba could be considered to be a Brazilian berry since its anthocyanin composition is very similar to that of the berries, more specifically, blackberries, in which delphinidin 3-glucoside and cyanidin 3-glucoside were identified (Dugo, Mondello, Errante, Zappia, & Dugo, 2001).

Reynertson (2007) found 433 mg 100 g^{-1} d. w. for cyanidin 3-glucoside, and 81 mg 100 g^{-1} d. w. for delphinidin 3-glucoside in the whole jaboticaba fruits. The present results for the whole fruit were slightly higher: 567 mg 100 g^{-1} for cyanidin 3-glucoside and 186.6 mg 100 g^{-1} for delphinidin 3-glucoside. Using a different extraction method, Favaro (2007) found 25 mg of anthocyanins in each 100 g of fresh whole jaboticaba fruit; 20 times lower than that found in the freeze-dried powder.

3.3. In vitro antiproliferative activity against tumor cells

This is the first work exploring the antiproliferative effect of JP (*M. jaboticaba*) against a panel of ten human cancer cell lines and normal monkey cells (VERO). A range of JP extract concentration (from 0.25 μg mL^{-1} to 250 μg mL^{-1}) was used to investigate the relative degree of growth inhibition against the following cell lines: glioma (U251), melanoma (UACC-62), breast cancer (MCF7), adriamycin-resistant ovarian cancer (NCI-ADR/RES), kidney (786–0), non-small lung cancer (NCI-H460), ovarian cancer (OVCAR-3), prostate cancer (PC-3), colon cancer (HT29), leukemia (K-562) and normal green monkey kidney cells (VERO). The concentrations of JP that generated a 50% (GI_{50}) inhibition of cell growth are summarized in Table 3.

In order to correlate chemical composition with antiproliferative effects, freeze-dried jaboticaba peels were submitted to a partition on dichlorometane and ethanol affording apolar JP and polar JP extracts. It was observed that only K-562 ($GI_{50} = 1.9$ μg mL^{-1}), NCI-ADR/RES ($GI_{50} = 29.4$ μg mL^{-1}) and UACC-62 ($GI_{50} = 48.6$ μg mL^{-1}) cell lines were sensitive to polar JP extract. On the other hand, non-polar JP extract was active against the PC-3 ($GI_{50} = 13.8$ μg mL^{-1}), K-562 ($GI_{50} = 15.8$ μg mL^{-1}), NCI-H460 ($GI_{50} = 28.0$ μg mL^{-1}), U251cells ($GI_{50} = 28.5$ μg mL^{-1}) and NCI-ADR/RES ($GI_{50} = 30.5$ μg mL^{-1}) cell lines. It was interesting to notice that both JP extracts have presented a GI_{50} value for VERO cell line (non-tumoral cell line) higher than that observed for the most sensitive cells, which might suggest a low toxicity to normal cells (Table 3).

There is evidence that some flavonoid compounds extracted from *M. cauliflora* exhibited antiproliferative effects against HT29 and HCT116 colon cell lines (Reynertson et al., 2006). Moreover, it has been reported that the deep purple *M. jaboticaba* peel contains high concentrations of total (22.5 g kg^{-1} d. w.) and free (0.145 g kg^{-1} d. w.) ellagic acid (Abe et al., 2012), a polyphenol that has a powerful potential to suppress the proliferation of leukemia cells (Mertens-Talcott, Talcott, & Percival, 2003). Jaboticaba also contains cyanidin and cyanidin 3-glucoside (Table 2), anthocyanins capable of suppressing neoplastic cell lines (Song et al., 2012). Thus, the probable presence of these compounds could be related to antiproliferative effect against leukemia (Tables 2 and 3).

Volatile terpenes may have a synergistic cytostatic effect together with the aforementioned phenolic compounds, against the tumor cell lines. They could generate additional radicals after penetrating the cells by interactions with reactive oxygen species and act as pro-oxidants, which could lead to cytotoxic effects (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). Indeed, some findings concerning bioflavors have shown that limonene could show antiproliferative activity against UACC-62 melanoma and HT-29 colon cells; and α-terpineol could have good inhibitory effects against OVCAR-03 (ovarian) and K-562 (leukemia) cells (Bicas, Neri-Numa, Ruiz, Carvalho, &

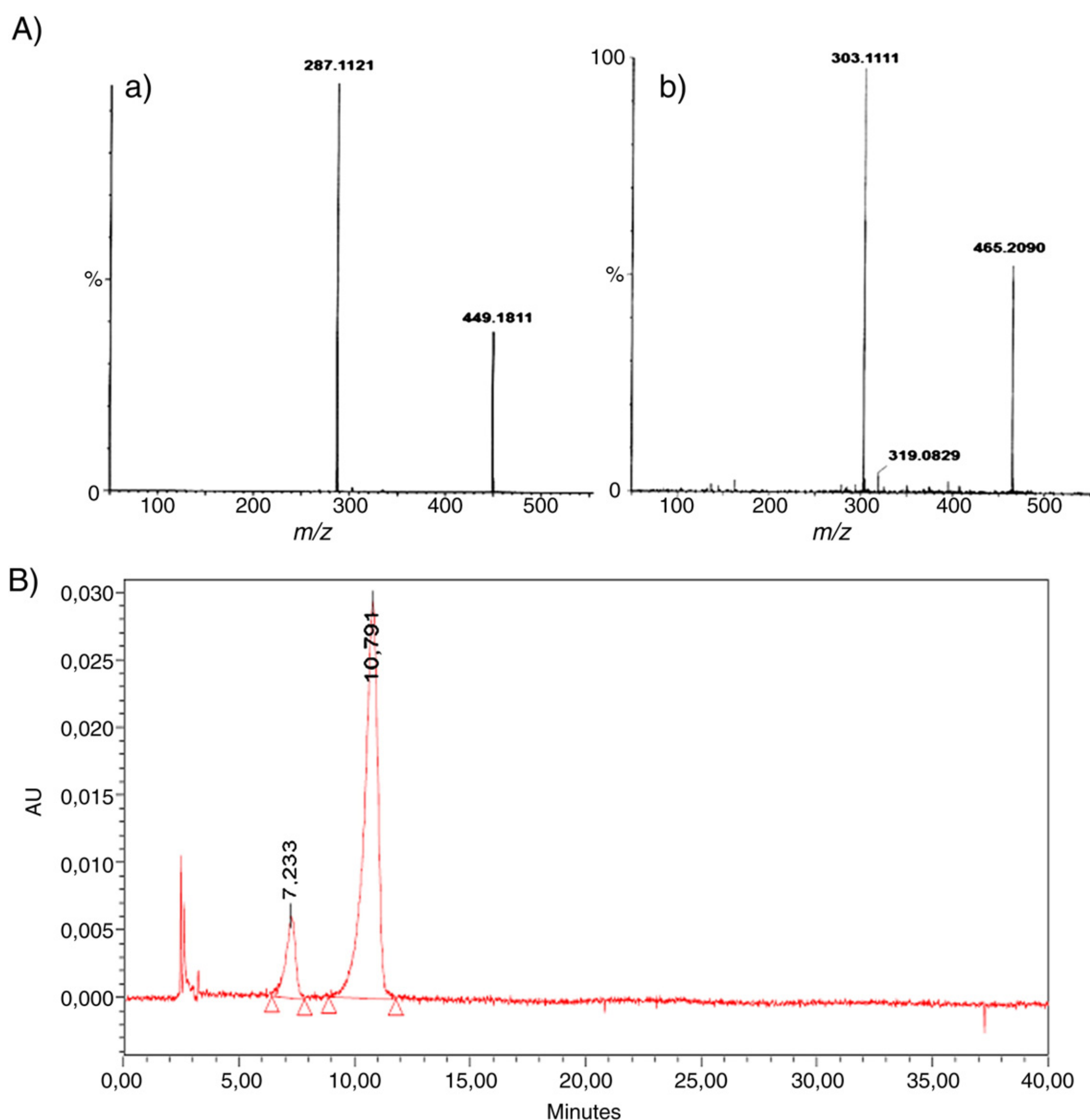

Fig. 1. Identification and quantification of anthocyanins in the freeze-dried jaboticaba peel powder. A) ESI-MS/MS of the two anthocyanins (*m/z* 449, cyanidin and m/z 465, delphinidin) indentified in freeze-dried the jaboticaba peel powder. B) Chromatogram obtained from freeze-dried jaboticaba peel powder extract. Chromatographic conditions: column C18; mobile phase deionized water: acetonitrile: formic acid 81:9:10 (v/v/v); injection volume 20 μL; and spectrophotometer detection at 520, 525, and 540 nm. Peak 1 refers to delphinidin-3-*O*-glucoside and peak 2 to cyanidin-3-*O*-glucoside.

Pastore, 2011). Another study of the present research group identified limonene and other terpenes in jaboticaba fruit (Plagemann et al., 2012). However, further investigations about volatile compounds in jaboticaba peel could contribute to the hypothetical role of volatile compounds in the suppression of *in vitro* tumor cells.

3.4. In vivo mutagenic test in mice

Some studies have reported that a high consumption of fruits is associated with a low incidence of degenerative diseases, including cancer (Carvalho-Silva et al., 2012; Clerici & Carvalho-Silva, 2011; Gerber et al., 2002; Rufino et al., 2010). However, few studies have demonstrated such effects using exotic fruits. To the best of the authors' knowledge, this is the first study that reports on the 'in vivo' antimutagenic properties of *M. jaboticaba*.

There was no significant variation in body weight or food intake amongst the experimental groups ($P<0.05$) during the study period (Table 4). These results indicated that different concentrations of JP extract administration did not interfere with animal development or growth.

CP is a drug commonly used to treat cancer malignancies and as an immunosuppressive agent and has been shown to produce gene mutations, chromosome aberrations, micronuclei and sister chromatid exchanges in rats, mice and Chinese hamsters. This explains its wide use as a positive control in studies about the protective effect of a given compound. The activation process begins with hydroxylation in the liver and further reactions lead to the formation of phosphoramide mustard and acrolein as the main active metabolites (De Vitta, Hellman, & Rosenberg, 1999; Zhang, Wu, Duan, & Yang, 2008).

Table 5 shows the micronuclei (MN) frequency in polychromatic erythrocytes (MNPCEs) of bone marrow in mice after administration of JPi. The PCE:NCE ratio in the CP and treated groups was not significantly different from that of the negative control group ($P>0.05$), indicating that JP did not present cytotoxic properties in the presence of CP in mice bone marrow cells at any of the doses tested (Table 5). Also, The PCE:NCE ratio in the JP + 0.9% NaCl groups (G2, G4 and G6) was not significantly different from that of the negative control group (0.9% NaCl) ($P>0.05$), again corroborating that JP did not present cytotoxic properties in mice bone marrow cells at the doses tested (Table 5).

Table 3
Antiproliferative activity of doxorubicin, and the non-polar and polar jaboticaba extracts against tumor cell lines.

Cell lines	GI_{50} (μg mL^{-1})[a]		
	DOX[b]	Non-polar extract[c]	Polar extract[d]
Glioma, Central Nervous System (U251)	0.025	28.5	132.3
Melanoma (UACC-62)	0.025	66.9	48.6
Breast (MCF7)	0.078	132.4	181.2
Kidney (786–0)	0.069	116.2	>250
Drug resistant ovarian (NCI-ADR/RES)	0.20	30.5	29.4
Lung (NCI-H460)	<0.025	28.0	209.1
Prostate (PC-3)	0.18	13.8	>250
Ovary (OVCAR-3)	0.35	40.3	>250
Colon (HT29)	0.19	144.0	180.9
Leukemia (K-562)	<0.025	15.8	1.9
Green monkey kidney (VERO)	0.20	78.2	162.6

[a] GI_{50} values (concentration of JP that produces 50% of growth inhibition) were obtained using the concentration-response curve for each cell line, in triplicate. The values were determined from a nonlinear regression analysis using the ORIGIN 7.5® software (OriginLab Corporation). Dose range tested: 0.25 to 250 μg mL^{-1}.
[b] Doxorubicin (positive control).
[c] Extraction with dichloromethane.
[d] Extraction with 80% ethanol.

The micronucleus test detects genomic alterations and/or damage during mitosis. Although genetic toxicity is not a carcinogenicity measurement, it is often associated with the appearance of cancer, since there is a positive correlation between the enhanced frequency of micronuclei and the appearance of tumors in mice and humans (Ramírez, Surralles, Puerto, Creus, & Marcos, 1999; Vinod, Tiwari, & Meshram, 2011).

Cyclophosphamide, a mutagenic substance in the bone marrow, has been used as a positive control substance in many assay systems since CP and its metabolites can bind to DNA, causing damage that may result in chromosome breaks, micronucleus formation and cell death (Ahmadi, Hosseinimehr, Naghshvar, Hajir, & Ghahremani, 2008). In the present experiment, CP positive control had shown a statistically significant induction of chromosomal damage in immature erythrocytes (MNPCE) in comparison to that of negative control (0.9% NaCl). On the other hand, JP powder extract had not been able to reduce CP-induced chromosomal damage despites had shown antioxidant activities. However, according to literature relates the antioxidant activity cannot predict antimutagenic effects and a product with low antioxidant activity could have antimutagenic effects. Carvalho-Silva et al. (2012) working with *Rheedia brasiliensis*, an exotic Brazilian fruit, investigated the cytotoxic and antioxidant activities and *in vivo* mutagenic/antimutagenic potential of different concentrations of the hexane extract (EHP) and isolated the molecule

Table 4
Means* and standard deviations for the body weight and weight gain in mice during experiment (n = 8 per group).[a]

Group/ Treatment	Initial body weight (g)[b]	Final body weight (g)[b]	Weight gain (g)[b,c]	Food intake (g)
(G1) JP 30 + CPA	23.40 ± 1.29	27.52 ± 2.03	4.12 ± 2.44	622.72
(G2) JP 30 + NaCl	22.33 ± 1.98	27.19 ± 2.03	4.86 ± 2.02	614.29
(G3) JP 100 + CPA	22.97 ± 2.10	27.54 ± 2.22	4.57 ± 2.81	611.65
(G4) JP 100 + NaCl	23.76 ± 1.87	28.03 ± 2.58	4.27 ± 2.04	629.31
(G5) JP 300 + CPA	22.46 ± 2.03	27.83 ± 2.01	5.37 ± 2.72	622.01
(G6) JP 300 + NaCl	22.79 ± 2.10	28.41 ± 2.23	5.62 ± 2.79	632.67
(G7) CPA	23.93 ± 2.65	28.28 ± 2.48	4.35 ± 2.46	618.30
(G8) NaCl	22.90 ± 2.30	28.59 ± 2.55	5.69 ± 2.88	632.33

[a] Abbreviations used: JP. jaboticaba peel extract; CPA. Cyclophosphamide.
[b] Means ± standard deviation of three determinations.
[c] Relative the two experimental weeks.
* There was no significant variation in body weight and ration consumption between the experimental groups according to the Tukey test ($P<0.05$).

Table 5
Frequency of erythrocytes with polychromatic micronuclei (MNPCEs) in the bone marrow cells of Swiss mice in the experimental groups treated with JP extract.

Groups/ Treatments	Number of PCEs analyzed	MNPCEs			Relation PCE/NCE
		No.	Mean + SD (%) *	% Reduction	
(G1) JP 30 + CP	8000	143	2.05 ± 0.12[b]	12	0.76 ± 0.06
(G2) JP 30 + NaCl	8000	058	0.84 ± 0.13[d]	25	1.07 ± 0.08
(G3) JP 100 + CP	8000	142	1.87 ± 0.09[b]	25	0.86 ± 0.05
(G4) JP 100 + NaCl	8000	064	0.80 ± 0.12[d]	26	1.54 ± 0.08
(G5) JP 300 + CP	8000	158	2.70 ± 0.08[a]	–	0.84 ± 0.06
(G6) JP 300 + NaCl	8000	073	1.04 ± 0.09[d]	12	1.17 ± 0.09
(G7) CP	8000	180	2.25 ± 0.08[b]	–	1.12 ± 0.08
(G8) NaCl	8000	041	1.08 ± 0.10[d]	–	1.23 ± 0.05

* Abbreviations used: JP—Jaboticaba peel extract; CP—Cyclophosphamide. Equal letters in the same column are statistically equals if $P<0.05$ according to the Tukey test. JP extract dissolved in water and administered daily by gavage during 15 days.

7-epi-clusianone (MI). In all the extracts evaluated, *R. brasiliensis* showed low antioxidant activity. The EHP and MI showed no mutagenic effects in mice, and in fact, both EHP (50 and 100 mg kg^{-1} b. w.) and MI (15 mg kg^{-1} b. w.) had demonstrated protection against mutagenic effects.

4. Conclusion

This work reported that freeze-dried *jaboticaba* peels were rich in fiber and anthocyanins (delphinidin and cyanidin 3-glucoside) and showed high antioxidant activity. The polar JP extract showed antiproliferative effects against leukemia (K-562) and the non-polar extract was active against prostate (PC-3). The micronucleus test in mice showed that the polar JP extract induced no DNA damage and caused no mutagenic effects.

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