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Pouteria ramiflora (Mart.) Radlk. extract: Flavonoids quantification and chemopreventive effect on HepG2 cells

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ABSTRACT

Pouteria ramiflora (Mart.) Radlk., popularly known as curriola, is commonly used in Brazil as medicinal plant to treat worm infections, dysentery, pain, inflammation, hyperlipidemia, and obesity. At present the safety of this extract when used therapeutically in human remains to be determined. Thus, the aim of this study was to examine cytotoxicity, antiproliferative, and antimutagenic actions of this extract. The hydroalcoholic extract from *P. ramiflora* leaves consisted of flavonoids identified and quantified as myricetin-3-O-β-D-galactopyranoside (13.55 mg/g) and myricetin-3-O-α-L-rhamnopyranoside (9.61 mg/g). The extract exhibited cytotoxicity at concentrations higher than 1.5 µg/ml in human hepatocarcinoma (HepG2) and 2.5 µg/ml in non-tumoral primary gastric (GAS) cells using the MTT assay, and at concentrations higher than 3 µg/ml in HepG2 and 3.5 µg/ml in GAS cells by the neutral red assay. The extract did not show antiproliferative effect as evidenced by the nuclear division index (NDI). However, in the presence of benzo [a]pyrene (BaP) (positive control), an enhanced cytostatic effect in the NDI and flow cytometry was noted. It is of interest that when the extract was co-incubated with BaP a significant decrease in DNA damage was observed indicating an antimutagenic action. This protective effect might be attributed to myricetin and gallic acid found in *P. ramiflora* extract. The low cytotoxicity action and protective effect observed in the present study encourage further studies regarding other biological effects of *P. ramiflora*, as well as its potential use as a chemopreventive agent.

ARTICLE HISTORY

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Introduction

The genus *Pouteria*, which belongs to family Sapotaceae, is found in tropical and subtropical areas worldwide (Swenson and Anderberg 2005) and comprises approximately 430 species in Brazil (Almeida et al. 1998). Many of these species have been used in folk medicine to treat fever, inflammation, diabetes, diarrhea, nausea, and vomiting (Silva, Simeoni, and Silveira 2009).

Pouteria ramiflora (Mart.) Radlk., popularly known as curriola, is a highly lactescent tree (15–30 m tall) found in semi-deciduous forests and in “cerradões” in Southern-central Brazil and in the Amazon region. The timber from such tree is used

in construction sites and the fruit pulp is used in sweets and beverages. *P. ramiflora* is also employed as medicinal plant to treat worm infections, dysentery, pain, inflammation, hyperlipidemia, and obesity (Fontes Jr et al. 2009; Lorenzi 1998; Silva et al. 2010).

Phytochemical analysis demonstrated that triterpenes and flavonoids are the main chemical constituents belonging to genus *Pouteria* (Silva, Simeoni, and Silveira 2009). According to Silva, Simeoni, and Silveira (2009), the presence of these compounds in the extracts from species, such as *Pouteria caimito*, *P. cambodiana*, *P. campechiana*, *P. ramiflora*, *P. torta*, *P. venosa*, and *P. viridis* has been associated with antioxidant

activity. Although several extracts of different *Pouteria* species were previously chemically characterized, only the extract of *P. torta* was evaluated for its possible mutagenic activity, where Costa et al. (2014) demonstrated mutagenicity for *Salmonella thyphimurium* and in rats. Thus far, there are no apparent data available regarding the assessment of *P. ramiflora* extract for mutagenic and antimutagenic actions in mammalian cells *in vitro*. The presence of flavonoids in species belonging to genus *Pouteria* and the neuroprotective effect of *P. ramiflora* extract against oxidative damage in rats suggested a potential chemopreventive effect of this extract (Costa et al. 2013).

Chemoprevention comprises the use of compounds to disrupt tumor onset, promotion or progression in order to prevent DNA damage that may induce mutations (Steward and Brown 2013). Anticancer drugs are classical cytotoxic agents and are classified in different categories according to their mechanism of action, such as DNA-interactive agents (Nussbaumer et al. 2011). According to Blagosklonny (2005), chemopreventive agents, anticancer drugs, and carcinogens overlap. The same agent may produce and/or treat cancer. Similarly, chemopreventive agents might induce cancer in one setting and prevent it in other setting. Therefore, it is essential to determine the mutagenicity/antimutagenicity of plant extracts, since plants exhibiting mutagenic/antimutagenic activity have the potential to be used as anticarcinogens or chemopreventive agents (Acésio et al. 2017; Carmona et al. 2017; Ribeiro et al. 2018; Serpeloni et al. 2015).

Using high performance liquid chromatography-photodiode array detector (HPLC-DAD) to quantify compounds in plant extracts is an economical, simple, and reliable alternative to the standardization of plant extracts (Costa et al. 2014; Saldanha, Vilegas, and Dokkedal 2013). The plant profile traced through the use of HPLC containing one or more plant-related components may be considered the fingerprint of the species, since it enables identification of biological markers and other compounds (Drašar and Moravcova 2004; Liu et al. 2007).

Thus, the aim of the current study was to (1) identify and quantify major constituents in the hydroalcoholic extract from *P. ramiflora* leaves

though HPLC-DAD; (2) assess possible cytotoxic activity through different cellular parameters (mitochondrial activity, lysosomal integrity and plasma membrane integrity) in human hepatocarcinoma (HepG2) and non-tumoral primary gastric (GAS) cell lines; (3) investigate the proliferative, mutagenic, oxidant, and protective activities in HepG2 cells, in order to select dosages for the safe use of *P. ramiflora* for therapeutic purposes; and (4) indicate *P. ramiflora* as a possible new herbal medicine to be used in the future.

Materials and methods

Plant material and extract obtainment

Pouteria ramiflora (Mart.) Radlk. leaves were collected and identified by Prof. Morais of Mato Grosso State University in September 2011 in Cáceres County (MT); an exsiccata was deposited in the herbarium of São Paulo State University "Júlio de Mesquita Filho" (UNESP) in Rio Claro County (SP); record number HRCB 58166. The leaves were dried and pulverized; part of the material was percolated using 70% ethanol, and this provided a lyophilized hydroalcoholic extract with 15% yield.

For the HPLC-DAD analysis, the hydroethanolic crude extract (60 mg) was dissolved in 2 ml methanol:H₂O (5:95, v/v) using an ultrasonic bath for 5 min. A clean-up step was performed to remove possible contaminants, wherein the solution was purified by solid phase extraction (SPE) using Phenomenex Strata C18 cartridges (500 mg of stationary phase). The extract was eluted from cartridges using 3 ml methanol:H₂O (5:95, v/v) to obtain a final volume of 5 ml of a 12 mg/ml extract solution. The samples were then filtered through a 0.22 µm GHP filter and aliquots of 20 µl were directly injected into the HPLC. The concentrations of *P. ramiflora* extract utilized in the entire cell assays were diluted with 70% ethanol from a stock solution at 50 mg/ml prepared through the dilution of 100 mg of extract in 2 ml 70% ethanol.

Chromatographic conditions and quantification

The HPLC system used in this study was a JASCO 2010 HPLC (Jasco, Tokyo, Japan), equipped with a

PU-2089S Plus pump, a MD-2018 Plus Photodiode Array Detector (DAD), an AS-2055 Plus autosampler, and a column oven (CO-2065 plus). The Chrom Nav (Workstation JASCOChrom Nav 1.18.03) software was used to control the analytical system and perform data collection and processing. Liquid chromatography was performed according to Costa et al. (2014).

Quantification and validation of HPLC-DAD method

The quantification method and its validation were conducted according to Costa et al. (2014). Recovery assays were conducted to verify the method's accuracy. Known amounts of myricetin were added to *P. ramiflora* hydroalcoholic extracts, and peak areas attributed to myricetins calculated through the analytical curve equation. Myricetin was used as standard because its electronic UV spectra are practically superimposable with those of the analyzed substances at the monitored wavelength.

The specificity of the method was evaluated by co-injections assays. The extract co-injection assays were performed using 10% standard samples (standard: extract, m/m) of gallic acid, myricetin-3-*O*-galactopyranoside (M-3-*O*-GAL), myricetin-3-*O*-arabinopyranoside (M-3-*O*-ARA) and myricetin-3-*O*-rhamnopyranoside (M-3-*O*-RHA).

Nine chromatographic runs with three runs every 24 hr were performed in order to quantify the studied substances. Limits of quantification (LOQ) and limits of detection (LOD) were calculated according to ANVISA (Brasil 2003).

Cell lines

The human metabolizing cell line HepG2, isolated from a hepatocarcinoma, was kindly provided by Nutrigenomics Laboratory (FCFRP/USP-Brazil), and human non-metabolizing primary gastric cell line (GAS), obtained through the biopsy of a single healthy individual, was kindly provided by Human Cytogenetics Laboratory (UFPA-Brazil) to the Laboratory of Mutagenesis and Oncogenetics of State University of Londrina. The DMEM low glucose (Dulbecco Modified

Eagle Medium (DMEM) – Gibco) culture medium supplemented with 15% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) was utilized to grow the HepG2 line. The DMEM high glucose (Gibco) culture medium supplemented with 10% FBS (Gibco) was employed to grow the GAS line. Both cell lines were treated with 0.06 g/l penicillin and 0.1 g/l streptomycin (Sigma-Aldrich, St-Louis, MO, USA) and cultivated at 37°C in atmosphere contained 5% CO₂ and 95% relative humidity. The assays were performed using HepG2 and GAS cell cultures between the third and eighth passages.

Chemical agents

Benzo[a]pyrene – B[a]P (CAS: 50–32-8, Sigma-Aldrich) carcinogen was used as DNA damage inducer in HepG2 cells at concentration 20 µM according to the results presented by Ribeiro et al. (2016). The MTT (3-[4,5-dimethyl-thiazol-2-yl] –2,5-diphenyl-tetrazolium bromide – CAS: 298–93-1, Sigma-Aldrich) was employed to measure cell viability. Cytochalasin B (CAS: 14930–96-2, Sigma-Aldrich) that blocks cytokinesis by inhibiting the polymerization of actin filaments and by generating binucleated cells (Carter 1967) was used in the micronucleus (MN) assay at the final concentration of 3 µg/ml. Trypsin-EDTA (CAS: 9002–07-7, Gibco) was added to the culture at 0.05% concentration.

Cell viability assays

With respect to the viability assays, 1×10^4 cells from both lines (HepG2 and GAS) were seeded (per well) in 96-well culture plates and stabilized in complete culture medium for 24 h, according to Specian et al. (2016). Subsequently, the culture medium was removed, cells washed in PBS and treatments added to minimal culture medium for 24 h. Based upon the solubility limit, nine extract concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, or 4.5 µg/ml) were selected to be assessed through cell viability assays. At least four replications were performed in each assay. The results of the MTT and neutral red (NR) assays are expressed in cell viability rate by considering the negative control as 100%, whereas the results of the LDH assay are presented as LDH enzyme activity expressed in U/l.

MTT assay (mitochondrial activity)

The MTT assay (CAS: 298–93-1, Sigma-Aldrich) was performed according to Mosmann (1983). After the treatment period was completed, cells were washed in PBS and incubated in MTT solution (0.5 mg/ml) for 4 h at 37°C. After the MTT solution was removed, 200 µl DMSO were added in each well to solubilize formazan crystals. The absorbance reading was performed in a spectrophotometer (Biotek Eon, Winooski, VT, USA) at 570 nm.

Neutral red assay (lysosomal integrity)

The NR assay was performed according to Repetto, Peso, and Zurita (2008). After treatment was terminated the NR solution (33 µg/ml) was added and incubated for 3 h at 37°C. Then, 0.2 ml acetic acid solution was added to ethanol (1% acetic acid to 50% ethanol) in order to enable reading in the Biotek Eon spectrophotometer (540 nm).

Lactate dehydrogenase assay – LDH (membrane integrity)

The LDH Liquiform kit (LDH – CAS: 86–2-30 – Labtest, Lagoa Santa, MG, BR) was used in the LDH assay, according to the manufacturer's instructions. An aliquot of the culture medium was withdrawn after 24 h treatment and solutions in the kit added to medium. Two absorbance readings (340 nm) were conducted in the Biotek Eon spectrophotometer, with 2-min interval between them, in order to calculate the enzyme activity. Cells treated with 1% Triton 100-X (CAS: 9002–93-1) constituted the positive control group.

Cell treatment and cytogenetic assays

Three *P. ramiflora* extract concentrations (0.5, 1, or 2 µg/ml) were selected based upon results obtained in the cell viability assays in order to be assessed in the MN assay using the HepG2 cell line. A negative-control group (treated with PBS), a positive-control group (20 µM B[a]P) and a solvent-group (70% ethanol) were added.

The extract concentration 1 µg/ml was selected based upon the mutagenicity results in order to assess the possible protective effect of the extract through three different protocols: (1) pretreatment (treatment with the extract for 24 h and then B[a]P for 24 h); (2) simultaneous treatment (treatment with both the extract and B[a]P for 24 h); or (3) post-treatment (treatment with B[a]P for 24 h followed by the extract for 24 h). Three repetitions of each experiment were performed in all cases. The results were compared to the respective positive (B[a]P) and negative (PBS) controls. All these treatments were performed according to OECD test number 487 (OECD 2016).

Cytokinesis-block micronucleus assay and nuclear division index (NDI)

The MN assay *in vitro* was performed using HepG2 cells according to the protocol of Fenech (2007), with modifications by Ribeiro et al. (2016). HepG2 were seeded in culture flask containing 5 ml complete medium and stabilized for 24 h. Subsequently, cells were incubated for 24 h with *P. ramiflora* extract (0.5, 1, or 2 µg/ml) to assess mutagenicity. To determine possible antimutagenicity, cells were treated following the protocols described in *Cell Treatment and Cytogenetic Assays* section (pretreatment, simultaneous and post-treatment). After these treatments, cells were incubated for 30 h in presence of cytochalasin B (3 µg/ml) and fixed with Carnoy's fixative (3methanol:1acetic acid). The slides were stained with acridine orange (100 µg/ml) and analyzed in a Nikon fluorescence microscope (Tokyo, Japan) equipped with 40X objective lens. Three thousand (3000) binucleated cells were assessed per treatment and MN frequency recorded according to the criteria described by Fenech (1993). Fifteen hundred cells were analyzed per treatment and the number of cells containing 1, 2, 3, or 4 nuclei was recorded in order to perform the NDI assessment. The NDI was calculated according to the formula of Eastmond and Tucker (1989). The damage reduction rate in the pre-, simultaneous and post-treatment protocols was calculated according to Waters et al. (1990):

$$\text{Damagereduction (\%)} = \frac{[(\text{NBMN}_{\text{CP}} - \text{NBMN}_{\text{T}}) / (\text{NBMN}_{\text{CP}} - \text{NBMN}_{\text{CN}})]}{\times 100} \quad (1)$$

where:

NBMN = number of binucleate cells with micronucleus

CP (treated with B[a]P – positive control)

T (treated with extract of *P. ramiflora* and B[a]P)

CN (treated with PBS – negative control)

According to OECD test number 487 (OECD 2016), the treatments should not present cytotoxicity greater than 50% because high cytotoxicity levels may induce DNA damages as side effect. Cytotoxicity is determined through cytostasis rate higher than or equal to 55 ± 5 .

$$\% \text{Cytostasis} = 100 - 100[(\text{CBPI}_{\text{T}} - 1) / (\text{CBPI}_{\text{C}} - 1)] \quad (2)$$

where:

CBPI = $[(\text{N}^{\circ} \text{ mononucleate cells}) + (2 \times \text{N}^{\circ} \text{ binucleate cells}) + (3 \times \text{N}^{\circ} \text{ multinucleate cells})] / (\text{Total number of cells})$

CBPI_T = Cytokinesis-Block Proliferation Index of treatment culture

CBPI_C = Cytokinesis-Block Proliferation Index of negative control culture

Flow cytometry

HepG2 cells (2.5×10^5) were seeded in 24-well plates in order to assess cell cycle kinetics. Cells were washed in PBS and trypsinized after the treatments according to the pre-, simultaneous and post-treatment protocols. The suspensions were centrifuged at 174 g/min for 5 min. The supernatant was removed and the pellet washed and centrifuged again. The pellet was fixed in 2 ml 70% ice-cold ethanol, centrifuged, resuspended in 0.5 ml PBS, then 25 µl RNase and 25 µl propidium iodide (both at 1 mg/ml) added. The suspension was analyzed in the FACSVantage cell sorter flow cytometer (Becton Dickinson – Franklin Lakes, NJ, USA) after 10 min incubation. Data were analyzed in the Cell Quest software (Becton Dickinson – Franklin Lakes, NJ, USA) and cell cycle profiles were identified using Flow Jo software (Tree Star

Incorporation, Ashland, OR, USA). The results were expressed as cell rate at different cell cycle stages, namely: subG1 (cell death), G1, S, and G2/M.

Oxidative stress

The CM-H₂DCFDA probe (Life Technologies, Eugene, OR, USA) was used in the 1, 6, 12, or 24-h incubations to assess oxidant/antioxidant activity of the extract. A pre-treatment protocol comprising 1 mM hydrogen peroxide (H₂O₂) (CAS: 7722-84-1, Synth) was used to assess anti-oxidant activity. The positive control group (H₂O₂) was also included in the assay, which was conducted according to the manufacturer's instructions. The values presented correspond to oxidation rate taking positive control as 100%.

Statistical analysis

The results of all biological assays were analyzed using the GraphPad Prism 5 statistical software version 5.01. The analysis of variance (ANOVA) was applied and followed by Tukey's test. The Combination Index (CI) was used to determine possible synergistic effects when the combination of chemicals was employed in antimutagenic protocols. According to Foucquier and Guedj (2015) a CI < 1 indicates synergy; CI = 1 indicates simple additivity and a CI > 1 indicates antagonism.

Results

Extract identification and quantification

The chromatographic profile of the hydroethanolic extract of *P. ramiflora* leaves indicates the presence of gallic acid and three derivatives of myricetin (Figure 1). Figure 2 shows the chromatograms obtained through the co-injection of gallic acid, M-3-O-GAL, M-3-O-ARA and M-3-O-RHA standards in the *P. ramiflora* extract, which were used to identify the peaks in Figure 1. The *P. ramiflora* hydroalcoholic extract was standardized through the quantification of the glycosylated myricetins, except for M-3-O-ARA, whose peak exhibited no correlation coefficient with the standard substance peak (Table 1). Simultaneous with quantification,

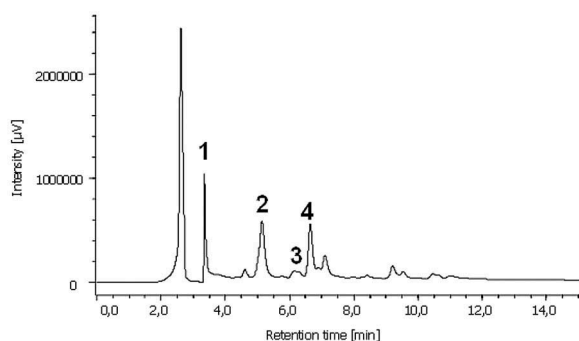


Figure 1. HPLC-DAD chromatogram of the hydroalcoholic extract from *P. ramiflora* leaves (b). Identified peaks: gallic acid (1), M-3-O-GAL (2), M-3-O-ARA (3) and M-3-O-RHA (4). Phenomenex® Synergi® Hydro-RP (250 × 4.6 mm, 5 μm) column, 20–42% B in 30 min (Solvent A: H₂O acidified with 0.1% TFA; Solvent B: ACN acidified with 0.1% TFA, 40°C, DAD (254 nm), flow 1 ml/min.

the method was validated to determine the contents of the M-3-O-GAL and M-3-O-RHA markers in *P. ramiflora* hydroalcoholic extracts (70% EtOH, v/v). The quantification results are shown in Table 2, along with the relative standard deviations (RSD).

Cell viability assays

The cell lines incubated with varying *P. ramiflora* extract concentrations demonstrated differing responses depending upon the assay used to assess cytotoxicity. Cell viability was significantly decreased at extract concentrations higher than or equal to 2 μg/ml in HepG2 cells and 3 μg/ml in

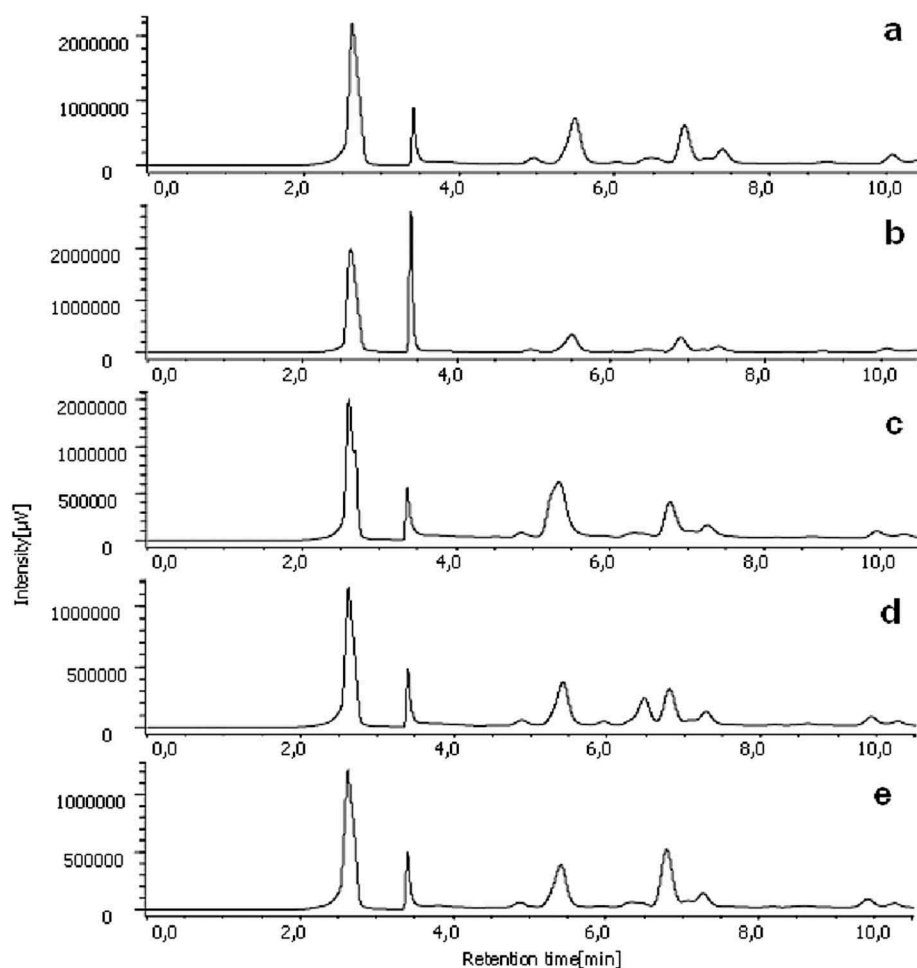


Figure 2. Chromatograms of the hydroalcoholic extract from *P. ramiflora* leaves (a) and of its co-injection with gallic acid (b), M-3-O-GAL (c), M-3-O-ARA (d), M-3-O-RHA (e). Phenomenex® Synergi® Hydro-RP (250 × 4.6 mm, 5 μm) column, 20–42% B in 30 min (Solvent A: H₂O acidified with 0.1% TFA; Solvent B: ACN acidified with 0.1% TFA, 40°C, DAD (254 nm), flow 1 ml/min.

Table 1. Peak Purity, Retention Times (t_R) and Correlation Coefficient Between UV Spectra of Myricetin Derivatives and Their Respective Standards.

Standard	Standards t_R (min)	Purity	Correlation coefficient	Extract t_R (min)
M-3-O-GAL	5.14	93%	0.989	5.48
M-3-O-ARA	6.36		ND	6.46
M-3-O-RHA	6.65	96%	0.984	6.89

ND: No spectrum with similarity to the peak with $t_R = 6.46$ min was detected in the library of standards.

Table 2. Myricetin Derivative Contents Quantified by HPLC-DAD in *P. ramiflora* Hydroalcoholic Extract.

Substance	mg/g extract	mg/g leaves	RSD (%)
M-3-O-GAL	13.55	2.04	2.23
M-3-O-RHA	9.61	1.44	2.33

The concentration data of flavonoids are shown as means ($n = 3$) in mg of flavonoid per gram of hydroethanolic crude extract and leaves. RSD = relative standard deviation.

GAS cells by the MTT assay (Figure 3A1 and A2). The cell viability was significantly reduced at concentrations higher than or equal to 3.5 $\mu\text{g/ml}$ in HepG2 cells and 4 $\mu\text{g/ml}$ in GAS cells using the NR assay (Figure 3B1 and B2). In the case of LDH assay no *P. ramiflora* extract concentration exerted a marked cytotoxic effect in both cell lines (Figure 3C1 and C2).

Micronucleus (MN) assay

P. ramiflora extract concentrations 0.5, 1, or 2 $\mu\text{g/ml}$ did not induce significant affect on the frequency of binucleated cells with MN in HepG2 cells. The treatment protocols used to assess anti-mutagenesis found the protective effect of the extract at 1 $\mu\text{g/ml}$ against DNA damage induced by B[a]P in the pre- and post-treatments (Table 3). A 72.73% and 81.77% damage reduction respectively was noted. The NDI did not indicate any apparent marked cell proliferation change when the three extract concentrations were utilized. It was not possible to reverse or prevent the B[a]P-mediated cytostatic effect in the pre-, simultaneous and post-treatment protocols.

Flow cytometry

The results of flow cytometry demonstrated that *P. ramiflora* extract and B[a]P altered the cell cycle by diminishing and increasing respectively, G1 phase

cell and S-phase cell populations relative to negative control (Figure 4A). When the extract was combined with B[a]P, it was not able to prevent or reverse the cell cycle delay produced by this carcinogen. However, this combination elevated the cell population in the subG1 phase and B[a]P cytotoxicity in the pre-treatment protocol indicating potentiation. The S-phase cycle delay was synergistically enhanced in the simultaneous ($\text{CI} = -5.12$) and post-treatment protocols ($\text{CI} = -6.7$) (Figure 4B).

Oxidative stress

The extract failed to exhibit any significant oxidizing activity at any of the concentrations tested in the oxidative stress assessment. The co-incubation of extract and H_2O_2 in the pre-treatment protocol did not detect alterations in reactive oxygen species (ROS) compared to the positive control (H_2O_2) alone. Therefore, data indicated that *P. ramiflora* extract exerted no marked antioxidant effect at these three concentrations tested in the present study using the CM- H_2DCFDA probe (Supplemental material).

Discussion

The Brazilian population has a long tradition in the use of medicinal plants to treat several diseases. However, these medicinal plants need to be registered in order to prove the derived extracts are safe, effective and maintain quality in order to be utilized as medicinal products (Dutra et al. 2016). Despite the use of *P. ramiflora* to treat several diseases, there are no apparent comprehensive studies proving the efficacy and safety of using this plant therapeutically. Thus, it was necessary to conduct studies to determine the biological properties *P. ramiflora* extract and identify active components.

Analysis of the hydroethanolic extract of the leaves from *P. ramiflora* by HPLC-DAD showed the presence of three flavonoids. Although the chromatographic profile of the hydroethanolic extract of *P. ramiflora* leaves was similar to that of *P. torta* (Costa et al. 2014), the glycosylated myricetin derivatives content found in *P. ramiflora* extract (Table 2) were quantitatively higher than that detected by Costa et al. (2014) in *P. torta*

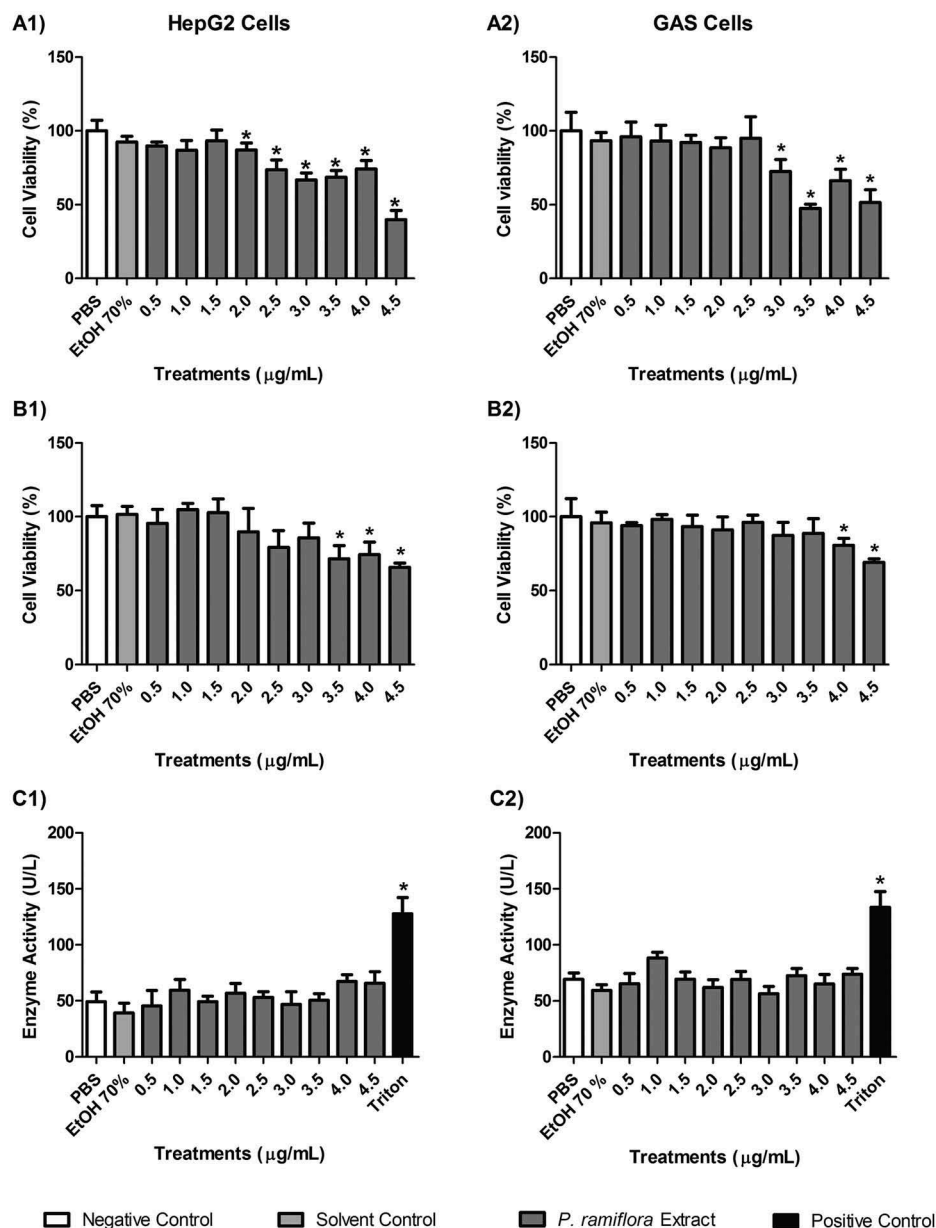


Figure 3. Data obtained (mean $\pm$ standard deviation) in cell viability assays performed in HepG2 (1) and GAS (2) cells after the treatment at different *P. ramiflora* hydroalcoholic extract concentrations. (A) MTT; (B) Neutral red; (C) LDH. PBS = phosphate-buffered saline, 70% EtOH = 70% ethanol.

*Values statistically different from the negative control (PBS), $p < 0.05$. ANOVA followed by Tukey test.

extract (M-3-O-GAL, 2.7 mg/g; M-3-O-ARA, 1.4 mg/g and M-3-O-RHA, 2.1 mg/g).

Previously, Matsubara and Rodriguez-Amaya (2006) found myricetin levels of 0.7 mg/g in dry green tea leaves (*Camellia sinensis*) and 0.5 mg/g in dry black tea leaves (*C. sinensis*), which are considered high-flavonoid content teas. Data suggested that *P. ramiflora* leaves contained a significant amount of glycosylated myricetin derivatives, since the sum of myricetin derivative levels quantified in the *P.*

ramiflora leaves reached 3.48 mg/g in this study. Due to *P. ramiflora* extract containing significant myricetin derivatives content, the study of a potential chemopreventive effect is important since myricetin was found to exert anticancer effects (Devi et al. 2015).

Cytotoxicity assays were conducted in order to identify safe therapeutic concentrations. Data demonstrated that the extract decreased cell viability in the MTT and NR assays at the tested concentrations and that the MTT assay appeared to be

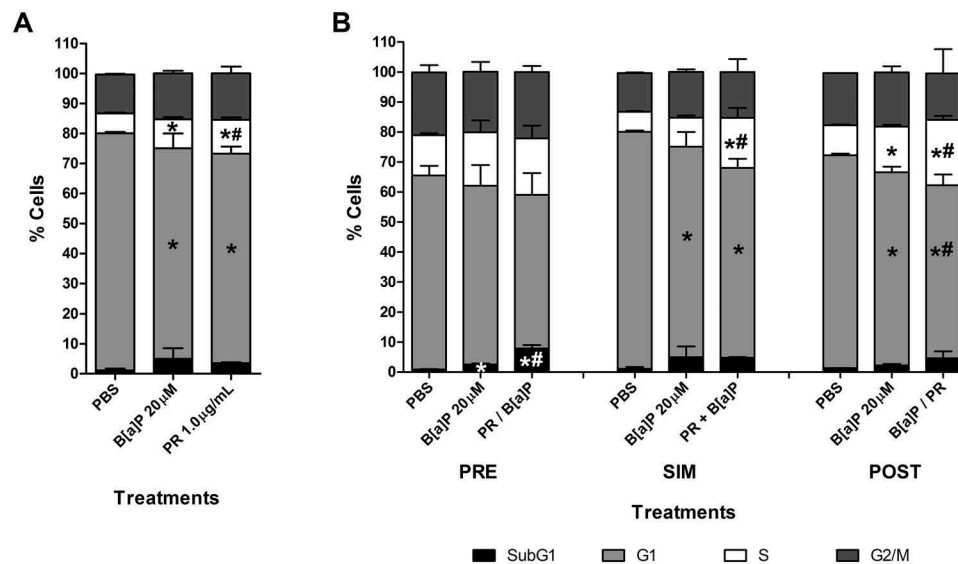
Table 3. Frequency of Binucleated Cells with Micronucleus (BNMN), DNA Damage Reduction Rate, Nuclear Division Index (NDI) and Cytostasis after Treating HepG2 Cells Using *P. ramiflora* Extract and the Extract Associated with Benzo[a]pyrene in Pre-, Simultaneous (SIM) and Post-Treatment Protocols.

Treatments	BNMN	Damage reduction (%)	NDI	Cytostasis (%)
PBS	15.33 ± 2.89		1.69 ± 0.07	
70% EtOH	16.33 ± 4.04		1.59 ± 0.06	14.49
B[a]P	30.67 ± 3.21*		1.52 ± 0.07*	24.64
PR 0.5 µg/ml	22.33 ± 3.79		1.63 ± 0.07	8.67
PR 1 µg/ml	22.33 ± 5.69		1.65 ± 0.04	5.80
PR 2 µg/ml	16.33 ± 5.69		1.64 ± 0.02	7.25
PRE				
PBS	20.00 ± 4.00		1.70 ± 0.03	
70% EtOH/B[a]P	28.33 ± 2.52*		1.53 ± 0.05*	24.29
B[a]P	31.00 ± 2.65*		1.58 ± 0.03*	17.14
PR 1 µg/ml/B[a]P	23.00 ± 2.65 [#]	72.73	1.50 ± 0.06*	28.57
SIM				
PBS	18.67 ± 2.31		1.69 ± 0.02	
EtOH 70% + B[a]P	25.67 ± 2.08*		1.49 ± 0.02*	28.98
B[a]P	29.00 ± 1.73*		1.47 ± 0.05*	31.88
PR 1 µg/ml + B[a]P	25.67 ± 2.31*		1.44 ± 0.04*	36.23
POST				
PBS	23.33 ± 1.53		1.68 ± 0.06	
B[a]P/70% EtOH	31.33 ± 2.08*		1.49 ± 0.06*	27.94
B[a]P	36.00 ± 2.65*		1.54 ± 0.03*	20.59
B[a]P/PR 1 µg/ml	25.67 ± 1.53 [#]	81.77	1.41 ± 0.04 [#]	39.71

PBS = Phosphate-buffered saline (negative control), 70% EtOH = 70% ethanol (solvent control), B[a]P = Benzo[a]pyrene 20 µM (positive control), PR 0.5, 1, and 2 µg/ml = *P. ramiflora* extract at 0.5, 1, and 2 µg/ml. Values are given as mean ± standard deviation.

*Values statistically different from the negative control (PBS).

[#]Values statistically different from the positive control (B[a]P), $p < 0.05$. ANOVA followed by Tukey test.

**Figure 4.** Mean rate of HepG2 cells obtained through flow cytometry after treatment with *P. ramiflora* extract alone (A) and pre-, simultaneous and post-treatment protocols of *P. ramiflora* extract associated with B[a]P (B).

PBS = phosphate-buffered saline (negative control), EtOH = 70% ethanol (solvent control), B[a]P = Benzo[a]pyrene 20 µM (positive control), PR = *P. ramiflora* extract at 1 µg/ml. *Values statistically different from the negative control (PBS). #Values statistically different from the positive control (B[a]P), $p < 0.05$. ANOVA followed by Tukey test.

more sensitive than NR. The extract failed to exert any marked cytotoxicity in the LDH assay. These observations suggest that the cell membranes were

not damaged and that cell death detected by the other assays might not involve necrosis. The different cytotoxicity levels found after conducting

three different assays reinforce the importance of using more than one assay to assess cell viability. Our results are in agreement with Fotakis and Timbrell (2006), who considered the MTT and NR assays more sensitive than LDH assay when they assessed cadmium chloride cytotoxicity in liver tumor cells (HTC and HepG2). The results indicated that the two cell types (metabolizing cells – HepG2 and non-metabolizing cells – GAS) used in the present study did not differ markedly from each other and thus cytotoxicity may not depend upon metabolic factors of extract components. Thus, the other assays were conducted using HepG2 cells only, since this cell line appeared to be more sensitive than GAS.

Since *P. ramiflora* extract exerted no marked antiproliferative effect (NDI), the possible protective activity against the B[a]P-mediated antiproliferation was assessed in HepG2 cells. Metabolism of B[a]P by the cytochrome enzyme P450 1A1 generates B[a]P epoxide, which forms adducts and produces DNA damage (Divi et al. 2014). In addition, B[a]P promotes S-phase cycle arrest in HepG2 cells indicating that DNA synthesis was inhibited. This consequence enables cellular mechanisms to recognize the damaged DNA and initiate repair during the replication process (Hockley et al. 2006; Stellas et al. 2014).

In the post-treatment protocol a potentiation of the cytostatic effect of B[a]P was observed; however, no significant NDI changes occurred when the extract concentration 1 µg/ml was co-incubated with B[a]P. The data obtained with flow cytometry confirmed this illustrating that the effects generated by B[a]P were not prevented or reversed by the extract. Surprisingly, this combination increased the B[a]P antiproliferative effect with accumulation of cells in S phase indicating a synergism (CI = -6.7). The expressive presence of phenolic compounds, such as gallic acid and myricetins, identified in the *P. ramiflora* extract may be responsible for the synergism observed between B[a]P and the extract. Tan et al. (2015) found dosage- and time-dependent cell proliferation reduction produced by gallic acid in Hela and HepG2 cells. Quercetin, myricetin and apigenin exhibited anti-proliferative effects and were proapoptotic in HepG2 cells (Lee et al. 2015; Shukla and Gupta 2010).

The lack of extract mutagenicity in the MN assay indicated the need to assess possible antimutagenic effects. The expressive protective effect of the extract at 1 µg/ml against the mutagenicity induced by B[a]P in HepG2 cells under pre- (72.73%) and post-treatment (81.77%) suggested that the extract provided such protection by more than one route by inactivating the mutagen prior to entry into the cell or by activating different mechanisms, such as detoxification, antioxidant and DNA repair (Ślarczyńska et al. 2014).

The same extract components that may have potentiated the antiproliferative effect produced by B[a]P may have also acted on the antimutagenic mechanism observed in the MN assay, since two glycosylated myricetins (M-3-O-GAL and M-3-O-RHA) were identified as secondary metabolites in the *P. ramiflora* hydroalcoholic extract. These two myricetins exerted antigenotoxic activity in K562 leukemic cells through modulation of repair genes including *XPC*, *LIG4*, *DDIT3*, *TDG*, *ERCC5*, and *RPA3* (Hayder et al. 2008). In addition, Delgado et al. (2008) demonstrated that myricetin and quercetin reduced the DNA damage induced by B[a]P in HepG2 cells using the comet assay. Thus, in the present study, the mechanisms involved in the protective effect attributed to the extract against B[a]P-initiated DNA damage appear to be associated with inhibition of cell proliferation such that repair mechanisms are able to act.

Costa et al. (2013) reported that *P. ramiflora* extract exerted antioxidant activity in the lipid peroxidation reduction assay conducted in rats. Hayder et al. (2008) noted antioxidant activity of myricetins in three assays *in vitro* (DPPH radical sequestration, xanthine oxidase inhibition and lipid peroxidation inhibition). These results, along with the fact that no oxidative activity was found to with *P. ramiflora* extract in the present study and that flavonoids such as myricetins exhibit antioxidant and anticancer activity (Shukla and Gupta 2010), suggested the need to assess the extract antioxidant activity using the CM-H₂DCFDA probe in HepG2 cells. However, the results did not indicate any marked antioxidant effect of the *P. ramiflora* extract in HepG2 cells. At present it is difficult to reconcile the basis for a lack of antioxidant activity using *P. ramiflora* extract in HepG2 cells

Conclusions

The adoption of the HPLC-DAD methodology enabled identification and quantification of glycosylated myricetins in the hydroalcoholic extract from *P. ramiflora* leaves. The *P. ramiflora* extract appeared to display safety for therapeutic use at dosages lower than 2 µg/ml attributed to lack of significant antiproliferative, cytotoxic, mutagenic and oxidizing effects in human HepG2 cells. Myricetins, as well as the gallic acid found in the extract may be predominantly responsible for the extract protective effect found in the MN assay. Moreover, when the extract was co-incubated with BaP, the plant extract potentiated the antiproliferative effect of this carcinogen which may have contributed to the observed protective effect.

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Conflicts of interest

All the authors stated that there are no conflicts of interest.

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