Flavonoid Detection in Hydroethanolic Extract of *Pouteria torta* (Sapotaceae) Leaves by HPLC-DAD and the Determination of Its Mutagenic Activity

Daryne L.M.G. Costa,^{1,2} Daniel Rinaldo,³ Eliana A. Varanda,⁴ Juliana F. de Sousa,⁴ Ana L.M. Nasser,⁴ Ana C.Z. Silva,¹ Débora C. Baldoqui,⁵ Wagner Vilegas,⁶ and Lourdes Campaner dos Santos¹

¹Organic Chemistry Department, Institute of Chemistry, UNESP, São Paulo State University, Araraquara, São Paulo, Brazil.

²IFMT, Mato Grosso Federal Institute, Cuiaba, Mato Grosso, Brazil.

³Chemistry Department, Faculty of Sciences, UNESP, São Paulo State University, Bauru, São Paulo, Brazil.

⁴Department of Biological Sciences, Faculty of Pharmaceutical Sciences, UNESP, São Paulo State University, Araraquara, São Paulo, Brazil.

⁵Chemistry Department, UEM, State University of Maringa, Maringa, Parana, Brazil.

⁶UNESP, São Paulo State University, São Vicente, São Paulo, Brazil.

ABSTRACT It is well known that phytotherapy has grown in popularity in recent years. Because a drug cannot be administered without ensuring its effectiveness and safety, the standardization and regulation of phytotherapeutic drugs are required by the global market and governmental authorities. This article describes a simple and reliable high-performance liquid chromatography–diode array detection analysis method for the simultaneous detection of myricetin-3-*O*- β -D-galactopyranoside, myricetin-3-*O*- α -L-arabinopyranoside, and myricetin-3-*O*- α -L-rhaminopyranoside present in the hydroethanolic extract (ethanol/H₂O, 7:3, v/v) of *Pouteria torta*. The mutagenic activity of the extract was evaluated on *Salmonella typhimurium* and by an *in vivo* micronucleus test on the peripheral blood cells of Swiss mice. The linearity, sensitivity, selectivity, repeatability, accuracy, and precision of the assay were evaluated. The analytical curves were linear and exhibited good repeatability (with a deviation of less than 5%) and demonstrated good recovery (within the 83–107% range). The results demonstrate that the hydroethanolic extract exhibited a mutagenic activity in both assays, suggesting caution in the use of this plant in folk medicine.

KEY WORDS: • flavonoids • liquid chromatography • mutagenicity • *Pouteria torta* • quantification

INTRODUCTION

THE SAPOTACEAE family is subdivided into five tribes with 53 genera and ~1250 species that are mainly found in the tropical and subtropical regions of Asia and South America.¹ The genus *Pouteria* Aublet is a pantropical group consisting of 9 sections and 325 species.² Many of these species produce high-quality timber and edible fruits, and several species have been used in folk medicine for a variety of ailments.

The biological activity has been attributed to some species of this genus, including antioxidant,³ antibacterial, and antifungal properties.^{4,5} However, the real potential of this genus as a source of new drugs or phytomedicine remains unknown.

Several *Pouteria* species have been used in folk medicine to treat fever, inflammation, skin eruptions, ulcers, diabetes,^{6,7} diarrhea,⁸ nausea, vomiting, and back pain and to promote lactation in milk feeding mothers.³ However, there is little scientific evidence for most of the purported biological activity.

Triterpenes, such as α - and β -amyirin, α - and β -amyirin acetate, lupeol, lupeol acetate, betulinic and ursolic acid, and flavonoids, such as myricetin, myricetin-3-*O*- α -L-rhaminopyranoside, galic acid, (+)-galocatechin, (+)-catechin, (–)-epicatechin, (+)-catechin-3-*O*-gallate, and dihydromyricetin, are the main constituents of this genus.^{6,7,9}

Pouteria torta (Mart.) Radlk is a perennial tree that is widespread in the Brazilian Cerrado, popularly called “guapeva,” “curiola,” “acá ferro,” “abi do cerrado,” and “grão de galo.” The fruit and bark of this tree are used in folk medicine for anti-dysentery purposes.

P. torta branches contain α -amyirin acetate, β -amyirin acetate, betulinic acid, and ursolic acid, which have been isolated through a methanol extract.⁹ Three flavonoids were identified and isolated from the methanolic extract of

Manuscript received 9 May 2013. Revision accepted 14 May 2014.

Address correspondence to: Lourdes Campaner dos Santos, PhD, Organic Chemistry Department, Institute of Chemistry, UNESP, São Paulo State University, Professor Francisco Degni St., 55, CEP 14800-900, Araraquara, São Paulo, Brazil. E-mail: loursant@gmail.com

P. torta leaves: myricetin-3-*O*- β -D-galactopyranoside, myricetin-3-*O*- α -L-arabinopyranoside, and myricetin-3-*O*- α -L-rhaminopyranoside.¹⁰ Hexane extracts from the leaves contain lupeol acetate.⁸

Recently, it was reported that the methanol extract of *P. torta* leaves exhibited an antimicrobial activity against *Cladosporium sphaerospermum*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa*.¹¹

One therapeutic approach for treating diabetes involves the inhibition of α -amylase and α -glucosidase activities to reduce the postprandial blood glucose levels. The *P. torta* extract exhibited a strong α -amylase and α -glucosidase inhibitory activity.¹²

Many plants containing mutagenic compounds have been reported in the literature, such as furocoumarins, tannins, anthraquinones, and flavonoids.^{13–15} Mutation events are involved in several degenerative diseases, such as cancer and arteriosclerosis.¹⁶ Therefore, research on the mutagenicity of medicinal plants is fundamental because the presence of mutagens can be dangerous to human health.

Once flavonoids may act as mutagens at high doses,¹⁷ their quantification in plant extracts becomes an important issue. In this study, we report a simple and reliable analytical high-performance liquid chromatography–diode array detection (HPLC-DAD) method for simultaneous determination of myricetin-3-*O*- β -D-galactopyranoside, myricetin-3-*O*- α -L-arabinopyranoside, and myricetin-3-*O*- α -L-rhaminopyranoside present in the hydroethanolic extract (ethanol/H₂O, 7:3, v/v) of *P. torta* leaves.

HPLC occupies a very important position in the analysis of flavonoids. Traditional HPLC is most frequently coupled with simple ultraviolet (UV) or DAD, and HPLC-DAD is widely used due to its rapidity, simplicity, and convenience. Several works describe the use of HPLC-DAD to quantify phenolic compounds in plant extracts.^{18–20}

The sensitivity, specificity, linearity, accuracy, and interday precision of the proposed method were evaluated. The efficiency of the analytical procedure was determined by calculating the recovery values. Furthermore, due to the use of these species in folk medicinal treatments, it is important to assess the risk of consuming this plant. Thus, the mutagenic activity of the hydroethanolic extract from leaves of *P. torta* was evaluated using a *Salmonella*/microsome assay and the micronucleus test, wherein the peripheral blood cells of mice were treated *in vivo*.

MATERIALS AND METHODS

Materials

Trifluoroacetic acid (TFA), acetic acid, acetonitrile, and methanol were HPLC grade and purchased from the Tedia Company (Fairfield, OH, USA). The myricetin standard (98% of purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Billerica, MA, USA). All solutions prepared for HPLC were filtered through a 0.22- μ m GHP filter (Waters, Milford, MA, USA) before use. Myricetin-

3-*O*- β -D-galactopyranoside and myricetin-3-*O*- α -L-rhaminopyranoside were isolated and identified by Rodrigues *et al.*²¹ The purity of compounds, as determined by NMR and by HPLC, was found to be higher than 90%. Myricetin-3-*O*- α -L-arabinopyranoside was isolated and identified from a methanolic extract of *P. torta* aerial components by Silva *et al.*¹⁰

Plant material

The leaves of *P. torta* (voucher BOTU 27806) were obtained from the city of Botucatu, São Paulo State, Brazil and authenticated by Professor Dr. Luiz Fernando Rolim from the Institute of Biosciences, UNESP, Botucatu city, São Paulo State, Brazil. A voucher specimen was deposited at the Herbarium of São Paulo State University (BOTU). The leaves of *P. torta* were obtained from adult specimens, shadow dried, and stored away from light at room temperature.

Hydroethanolic extract preparation

This study is inserted into a project called Natural Products—Standardization of Phytotherapeutic Drugs for the Treatment of Chronic Diseases. In this project, all plants extracts studied must be prepared by percolation at room temperature using a mixture of ethanol:H₂O (7:3, v/v). The hydroethanolic extract was prepared by the extraction of dried and powdered *P. torta* leaves (500 g). The solvents were evaporated to dryness under a low pressure to afford 152 g of crude hydroethanolic extract (30.4%).

Instrumentation

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 JASCO-Chrom Nav 1.18.03) software was used to control the analytical system and perform the data collection and processing.

High-performance liquid chromatography–electrospray ionization-ion trap mass spectrometry (HPLC-ESI-IT-MS²) was performed on a HPLC coupled to a mass spectrometer LCQ Fleet (Thermo Scientific®, Madison, WI, USA) and equipped with a direct insertion deposition flow injection analyzer. The studied matrix was analyzed by using the ESI technique. The multistage (MS²) and the fragmentation in multiple stages (MS²) were performed at an IT interface. The Xcalibur version 1.3 (Thermo Finigan®, San Jose, CA, USA) software was used to acquire and process data.

Chromatographic conditions

For both HPLC-ESI-IT-MS and HPLC-DAD, liquid chromatography was performed using a Hydro RP18 (25 cm $\times$ 4.6 mm $\times$ 5 μ m) reversed-phase column protected by a Hydro guard column (2.5 cm $\times$ 3 mm) from Phenomenex, Inc. (Torrance, CA, USA). The sample injection volume was 20 μ L. The signal was monitored at 254 nm. For the HPLC-ESI-IT-MS assay, a binary exploratory gradient elution system was employed, using solvent A (0.1% acetic

acid in H₂O) and solvent B (0.1% acetic acid in methanol), wherein the amount of solvent B increased linearly from 5% to 100% in 60 min. The flow rate was 0.8 mL min⁻¹.

The elution system used for the HPLC-DAD assay was a binary gradient elution system with solvent A (0.1% TFA in H₂O) and solvent B (0.1% TFA in acetonitrile) eluted at an initial linear gradient of 80:20 (A:B), which was changed to 58:42 (A:B) after 30 min. The flow rate was 1.0 mL min⁻¹.

Standard solutions

Myricetin stock solutions were prepared in ethanol at a concentration of 980.0 µg mL⁻¹ in the presence of 0.1% TFA. The stock solutions were stored at 4°C in the dark. The purity of the standard was taken into account when calculating the concentration.

Sample preparation

For the HPLC-ESI-IT-MS² assay, 1 mg of the hydroethanolic crude extract was dissolved in 1 mL of methanol:H₂O (5:95, v/v) in an ultrasonic bath for 5 min. The samples were then filtered through a 0.22-µm GHP filter, and 20-µL aliquots were directly injected into the HPLC-ESI-IT-MS² system.

For the HPLC-DAD analysis, the hydroethanolic crude extract (60 mg) was dissolved in 2 mL of 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 using Phenomenex Strata C₁₈ cartridges (500 mg of stationary phase). Before analysis, these columns were activated with 5 mL of methanol and equilibrated with 5 mL of methanol:H₂O (5:95, v/v). Flavonoids were eluted from cartridges using 3 mL of methanol:H₂O (5:95, v/v) to obtain a final volume of 5 mL. The samples were then filtered through a 0.22-µm GHP filter, and aliquots of 20 µL were directly injected into the HPLC.

Identification and quantification

The identification of the flavonoids in the hydroethanolic extract of *P. torta* leaves was performed by both HPLC-ESI-IT-MS² and HPLC-DAD employing the conditions described in the Chromatographic Conditions section.

The identification of the compounds by HPLC-ESI-IT-MS² was mainly performed on the basis of their fragmentation patterns in the MS² experiments. The negative mode was selected for the generation and analysis of mass spectra for the first order (MS), and the remaining experiments were conducted in multiple stages under the following conditions: capillary voltage of -25 V, voltage spray of -5 kV, capillary temperature of 275°C, nitrogen (N₂) carrier gas with a flow rate of 8 arbitrary units (A.U.), helium (He) collision gas, and a track acquisition of 100–2000 *m/z*. The collision energy for MS/MS was adjusted to 20–25%.

These extracts were also analyzed using HPLC-DAD (λ = 254 nm) by coinjecting the pure standards at a 4:1 (extract:standard) ratio to confirm the different flavonoids

identified in the chromatographic profile according to their retention times (*t_R*). The UV spectra of these compounds (pure standard × hydroethanolic extract) were compared and the indices of spectral similarity were calculated using the EZChrom Elite 3.1.7 software. Finally, HPLC-DAD was employed to quantify each identified flavonoid.

The method of external standard was applied to quantify each compound. Quantification of analytes was performed using a regression curve, each point in triplicate.

The amount of analytes in the sample was determined by measuring the peak areas and comparing them with those obtained for the reference myricetin standard solution. The myricetin-3-*O*-β-D-galactopyranoside, myricetin-3-*O*-α-L-arabinopyranoside, and myricetin-3-*O*-α-L-rhaminopyranoside identified in the samples were quantified using the myricetin calibration curves. This analysis could be performed because the electronic UV spectra of the standards are practically superimposable with those of the analyzed substances at the monitored wavelength.

Mutagenicity assays

Salmonella microsome assay. The mutagenic activity was evaluated using the *Salmonella*/microsome assay with *Salmonella typhimurium* tester strains TA98, TA100, TA97a, and TA102, which were kindly provided by Dr. B.N. Ames (Berkeley, CA, USA). The assay was performed in the presence and absence of metabolism by the pre-incubation method.²² The strains were grown from frozen cultures overnight for 12–14 h in the Oxoid Nutrient Broth No. 2. The S9 fraction, prepared from the livers of Sprague Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254, was purchased from Molecular Toxicology, Inc. (Boone, NC, USA). The metabolic activation system consisted of 4% of the S9 fraction, 1% of 0.4 M MgCl₂, 1% of 1.65 M KCl, 0.5% of 1 M D-glucose-6-phosphate disodium, 4% of 0.1 M NADP, 50% of 0.2 M phosphate buffer, and 39.5% of sterile distilled water. The metabolic activation mixture (S9) was freshly prepared before each test. Five different doses of test extract were diluted in DMSO, the concentrations of which were selected based on a preliminary toxicity test. In all subsequent assays, the upper limit of the dose range was either the highest nontoxic dose or the lowest toxic dose determined in the preliminary assay. The toxicity was apparent either as a reduction in the number of His⁺ revertants or as an alteration in the auxotrophic background (*i.e.*, the background lawn). Various concentrations (0.62, 1.25, 2.5, 5.0, and 7.5 mg/plate) of hydroethanolic extract were added to 0.5 mL of 0.2 M phosphate buffer (pH 7.4) or 0.5 mL of a 4% S9 mixture with 0.1 mL of bacterial culture and then incubated at 37°C for 20–30 min. Subsequently, 2 mL of top agar was added to the mixture and poured onto a plate containing minimal agar. The plates were incubated at 37°C for 48 h and the His⁺ revertant colonies were counted manually. All experiments were performed in triplicate. The results were analyzed using the Salanal statistical software package (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, USA;

version 1.0, from Research Triangle Institute, Research Triangle Park, NC, USA) and adopting the Bernstein *et al.* model.²³ The data (revertants/plate) were assessed by performing an analysis of variance (ANOVA) followed by a linear regression. The mutagenic index (MI) was calculated for each concentration as an average number of revertants per plate, and this value was compared with that of a test compound divided by the average number of revertants per plate with a negative (solvent) control. A sample was considered mutagenic when a dose–response relationship was detected and a twofold increase in the number of mutants ($MI \geq 2$) was observed for at least one concentration.²⁴ The standard mutagens used as positive controls in experiments in the absence of the S9 mix were 4-nitro-*o*-phenylenediamine (10 $\mu\text{g}/\text{plate}$) for TA98 and TA97a, sodium azide (2.5 $\mu\text{g}/\text{plate}$) for TA100, and mitomycin (0.5 $\mu\text{g}/\text{plate}$) for TA102. 2-Anthramine (1.25 $\mu\text{g}/\text{plate}$) was used with TA98, TA97a, and TA100, whereas 2-aminofluorene (10 $\mu\text{g}/\text{plate}$) was used with TA102 in the experiments with metabolic activation. DMSO served as the negative (solvent) control (75 $\mu\text{L}/\text{plate}$).

Micronucleus assay of peripheral blood cells. To perform the micronucleus assay, blood samples from 5- to 6-week-old albino Swiss mice (*Mus musculus*) weighing ~ 30 g from the Central Animal Facility of the São Paulo State University (São Paulo, Brazil) were used. Each animal was kept in a single polypropylene cage, according to the conditions for animal care recommended by the Canadian Council on Animal Care.²⁵ All the procedures were approved by the Ethics Research Committee of the São Paulo State University in Araraquara (UNESP, SP, Brazil - process number 22/2010). The mice were divided into five groups of 10 animals (5 males and 5 females) for the evaluation of the hydroethanolic extract: a negative control group that received distilled water; a positive control group treated with cyclophosphamide (50 mg kg^{-1} body weight [b.w.]); and the three groups that received the extract. The extract was assessed at three different doses: 15.0, 11.2, and 7.5 mg kg^{-1} b.w. These doses were based on the solubility limit of the extract in distilled water. In addition, the doses tested in this study were not toxic to the animals. Each animal in these groups was treated with 0.1 mL of each test solution per 10 g of body weight. The animals received water and food *ad libitum* between the treatment and the blood sampling. The treatments with plant extract and distilled water (negative control) were performed through gavage. The treatment with cyclophosphamide was performed intraperitoneally. The micronucleus test on the peripheral blood cells was conducted according to the method of Hayashi *et al.*²⁶ using slides that were prestained with acridine orange. The staining was performed by heating glass slides to 70°C on a hot-plate and then adding an aqueous solution of the dye (10 μL , 1 mg mL^{-1}) to each slide. The solution was then spread evenly over the surface with one end of another clean slide. After drying, the slides were kept in the dark at room temperature for at least 24 h. Blood samples were obtained from the tail veins of mice before treatment (T0) and 30 h (T1) after treatment by perforating the caudal vein of the mouse

with a needle and collecting 5 μL aliquots of blood. Each aliquot was placed in the center of a slide prestained with acridine orange and covered with a coverslip (24 $\times$ 60 mm). These slides were then maintained in the dark at -20°C for a minimum of 24 h before performing the cytological examination of the blood cells. The cell preparations were examined under a fluorescence microscope (Nikon) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter using an immersion objective. Reticulocytes (2000 per treated animal) were analyzed and the proportion of micronucleated cells was determined. The results were analyzed using parametric (ANOVA/Tukey) or nonparametric tests (Kruskal–Wallis/Dunn's *post hoc*), according to the nature of the data distribution.

RESULTS AND DISCUSSION

Identification and quantification

Figure 1 shows the chromatogram of the hydroethanolic extract from *P. torta* leaves by HPLC-ESI-IT-MS². Peak 1 (R_t 27.23 min, Fig. 1) reveals the signal for the deprotonated molecule $[\text{M-H}]^-$ with a m/z 479, peak 2 (R_t 28.56 min, Fig. 1) has a m/z 449, and peak 3 (R_t 28.86 min, Fig. 1) has a m/z 463. The second order fragmentation (MS/MS) of the most representative ions led to the identification of secondary metabolites, which confirmed the presence of O-glycoside flavonoids (Fig. 2 and Table 1). In the second order fragmentation pattern, a signal for the deprotonated molecule $[\text{M-H-162}]^-$ is observed with a m/z 317 for peak 1, suggesting the loss of a hexose. Similarly, peak 2 contains the signal for a deprotonated molecule $[\text{M-H-132}]^-$ at m/z 317 suggesting the loss of a pentose. Second order fragmentation generated the ion at m/z 317 $[\text{M-H-146}]^-$ in peak 3 suggesting the loss of a deoxyhexose.

All MS² spectra obtained for the O-glycoside flavonoids (Fig. 2) reveal a signal with a m/z 316. This signal can be attributed to the radical aglycone ions $[\text{M-162-H}]^{\bullet-}$, $[\text{M-146-H}]^{\bullet-}$, and $[\text{M-132-H}]^{\bullet-}$ besides the regulars $[\text{M-162-H}]^-$, $[\text{M-146-H}]^-$, and $[\text{M-132-H}]^-$ ion, corresponding to the deprotonated aglycones. The $[\text{M-162-H}]^{\bullet-}$, $[\text{M-146-H}]^{\bullet-}$, and $[\text{M-132-H}]^{\bullet-}$ ions are formed by a hemolytic cleavage of the glycoside bond.²⁷ According to Cuyckens and Claeys,²⁷ the radical aglycone ion:deprotonated aglycone ion ratio suggests a certain glycosylation position; this is especially the case for flavonol 3-O-glycosides where this ratio is very high. A number of preliminary HPLC experiments employing samples were performed to establish the optimal conditions for the analysis of myricetin, myricetin-3-O- β -D-galactopyranoside, myricetin-3-O- α -L-arabinopyranoside, and myricetin-3-O- α -L-rhaminopyranoside, structures of which can be seen in Figure 3.

The identification of flavonoids from the hydroethanolic extract from the leaves of *P. torta* was performed as described in the Identification and Quantification section.

The indices of spectral similarity between the UV spectra of the flavonoid standards and those of the chromatographic profile were within the 0.9999 and 1.0000 range (Table 2).

FIG. 1. Negative ion HPLC-ESI-IT-MS² analysis of hydroethanolic extract of *P. torta* leaves. Total ion chromatogram (a); extracted ion chromatograms for m/z 479 (b); m/z 449 (c); and m/z 463 (d). HPLC-ESI-IT-MS², high-performance liquid chromatography–electrospray ionization–ion trap mass spectrometry.

FIG. 2. MS² spectra of (a) m/z 479 at $t_R = 27.23$ min corresponding to myricetin-3-O- β -D-galactopyranoside; (b) m/z 449 at $t_R = 28.56$ min corresponding to myricetin-3-O- α -L-arabinopyranoside; and (c) m/z 463 at $t_R = 28.86$ min corresponding to myricetin-3-O- α -L-rhaminopyranoside.

TABLE 1. FLAVONOIDS IDENTIFIED IN *P. TORTA* LEAF HYDROETHANOLIC EXTRACT BY HPLC-ESI-IT-MS²

Peak	<i>t_R</i> (min)	[M-H] ⁻	Product ion scan fragments of [M-H] ⁻	Compound
1	27.23	479	317 [M-162-H] ⁻ 316 [M-162-H] ^{-•}	Myricetin-3- <i>O</i> -β-D-galactopyranoside
2	28.56	449	317 [M-132-H] ⁻ 316 [M-162-H] ^{-•}	Myricetin-3- <i>O</i> -α-D-arabinopyranoside.
3	28.86	463	317 [M-146-H] ⁻ 316 [M-162-H] ^{-•}	Myricetin-3- <i>O</i> -α-L-rhaminopyranoside

HPLC-ESI-IT-MS², high-performance liquid chromatography–electrospray ionization-ion trap mass spectrometry.

As shown in Figure 4, the HPLC-DAD analysis provided excellent separation of the compounds of interest, which could be analyzed in a satisfactory amount of time (myricetin-3-*O*-β-D-galactopyranoside, *R_t* = 5.89 min, myricetin-3-*O*-α-L-arabinopyranoside, *R_t* = 6.89 min, and myricetin-3-*O*-α-L-rhaminopyranoside, *R_t* = 7.20 min). Under the optimized sample preparation conditions, the intervals at which the compounds were eluted were free of interference in all tested samples.

The results of the quantification assay are provided in Table 3.

Linearity and sensitivity

The linearity of the DAD detector was investigated at the $\lambda = 254$ nm for myricetin by selecting the calibration range according to the amount of the flavonoid analytes in the samples. The experimental results for the curves were analyzed using the Huber Test to reject anomalous results; by using $k = 1$, three of the seven available curves were rejected to achieve a correlation coefficient (R^2) of less than 0.9993. The limits of detection and quantification were $0.81 \mu\text{g mL}^{-1}$ and $2.44 \mu\text{g mL}^{-1}$, respectively. The linear range was within 15.63 – $125.0 \mu\text{g mL}^{-1}$. Three injections were made for each solution, and the peak area (response, y) of the analyte was plotted as a function of the concentration (c) in $\mu\text{g mL}^{-1}$ to obtain a calibration curve with four data points (15.63 , 31.25 , 62.5 , and $125 \mu\text{g mL}^{-1}$; $y = 52550 - 217866.47c$).

Selectivity

Selectivity was evaluated by comparing the *R_t* of each standard reference compound with that of the peaks obtained by analyzing the *P. torta* extract. Further confirma-

tion of peak identities was obtained by performing spiking experiments (Fig. 4) and comparing the results with the recorded UV spectra (Fig. 4 and Table 2).

Repeatability and precision

The interday and intraday precision of the *R_t* was determined with five injections of the sample on 3 different days. The interday variation was 5%, whereas the intraday variation was between 1.0 and 3.0% for one of the three flavonoids.

Accuracy

The accuracy of the method was investigated by performing recovery studies. Recovery was calculated by comparing the amount of flavonoids in standard solutions subjected to the extraction procedure described in the Sample Preparation section and the amount of flavonoids in standard solutions that were not subjected to the extraction procedure. Three sample replicates were prepared for each case, and percentage of recovery was calculated for each flavonoid. The recovery values were 90%, 107%, and 83% for myricetin-3-*O*-β-D-galactopyranoside, myricetin-3-*O*-α-L-arabinopyranoside, and myricetin-3-*O*-α-L-rhaminopyranoside, respectively.

Mutagenicity assays

In Table 4, the mutagenic activity was evaluated using the Ames test. The crude hydroethanolic extract was mutagenic to the TA97a and TA98 strains in the absence of metabolism, inducing a significant increase in the frequency of revertants per plate. Following metabolic activation with S9, the mutagenic effect was not observed. Therefore, these results reveal that the compounds present in the extract

FIG. 3. Chemical structures of the flavonoids. Myricetin ($R = \text{OH}$); myricetin-3-*O*-β-D-galactopyranoside ($R = \text{O-gal}$); myricetin-3-*O*-α-L-arabinopyranoside ($R = \text{O-ara}$); and myricetin-3-*O*-α-L-rhaminopyranoside ($R = \text{O-rha}$).

TABLE 2. INDICES OF SPECTRAL SIMILARITY BETWEEN THE ULTRA-VIOLET SPECTRA OF THE FLAVONOID STANDARDS AND THOSE OF THE CHROMATOGRAPHIC PROFILE OBTAINED BY HPLC-DAD

Standard	Peak	Similarity index
myricetin-3- <i>O</i> -β-D-galactopyranoside	<i>t_R</i> = 5.89 min	1.0000
myricetin-3- <i>O</i> -α-L-arabinopyranoside	<i>t_R</i> = 6.89 min	0.9999
myricetin-3- <i>O</i> -α-L-rhaminopyranoside	<i>t_R</i> = 7.20 min	1.0000

DAD, diode array detection.

FIG. 4. HPLC-DAD profiles obtained from the hydroethanolic extract of the leaves of *P. torta* employing the conditions described in the Chromatographic Conditions section. The chromatograms were recorded using the timed wavelength program registered at 254 nm. (a) Refers to the crude extract, while (b), (c), and (d) were spiked with myricetin-3-*O*-β-D-galactopyranoside (1), myricetin-3-*O*-α-L-arabinopyranoside (2), and myricetin-3-*O*-α-L-rhaminopyranoside (3), respectively. DAD, diode array detection.

cause a frameshift-type mutation, which directly affects the DNA. The data in Table 4 indicate that following the metabolic activation, compounds that cause mutations in the TA98 and TA97a strains lose their activity. In Table 4, it was also observed that the highest nontoxic dose for TA98 strain (–S9) is 5.0 mg/plate. The highest dose used (7.5 mg/plate) has lower MI than the dose of 5.0 mg/plate in TA98

TABLE 3. CONTENT OF MYRICETIN HETEROSIDES IN HYDROETHANOLIC EXTRACT OF *P. TORTA*

Analytes (mg g ⁻¹) ^a ± RSD%	
Miricetin-3- <i>O</i> -β-D-galactopyranoside	2.7 ± 0.6
Miricetin-3- <i>O</i> -α-L-rhaminopyranoside	1.4 ± 1.8
Miricetin-3- <i>O</i> -α-D-arabinopyranoside	2.1 ± 2.8

^aThe concentration data of flavonoids are shown as means (*n* = 3) in mg of flavonoid per gram of hydroethanolic crude extract.

RSD, relative standard deviation.

(–S9) indicating signs of cytotoxicity. For the TA100 and TA102 strains, the mutagenic effect was not observed.

Because the *in vitro* Ames test did not completely represent the conditions found in a more complex *in vivo* system, we evaluated the mutagenic potential of the hydroethanolic extract *in vivo*.

In vivo micronucleus tests were performed on albino Swiss mice, which were treated with three different doses of the extract using a gavage. As shown in Table 5, all tested doses induced a significant proliferation of micronuclei after the acute treatment, which confirmed the mutagenic potential of the extract. This result indicates that compounds present in the hydroethanolic extract of *P. torta* may cause breaks in the DNA or aneuploidy. In this manner, these results confirm the *in vitro* assay results, which also indicated mutagenicity.

Therefore, we used *in vitro* (Ames test) and *in vivo* (micronucleus test) assays to evaluate the genotoxic properties of extract from the leaves of *P. torta* and both assays are considered to be effective tests for this kind of evaluation.^{28,29}

TABLE 4. MUTAGENIC ACTIVITY EXPRESSED AS THE MEAN AND STANDARD DEVIATION OF THE NUMBER OF REVERTANTS/PLATE AND THE MUTAGENIC INDEX IN BACTERIAL STRAINS TA98, TA100, TA97a, AND TA102 EXPOSED TO THE HYDROETHANOLIC EXTRACT OF *P. TORTA*, AT VARIOUS DOSES, WITH (+S9) OR WITHOUT (–S9) METABOLIC ACTIVATION

Treatments (mg/plate)	Number of revertants His +/plate in <i>S. typhimurium</i> (MI)							
	TA98		TA100		TA97a		TA102	
	–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
DMSO	18 ± 7	30 ± 5	142 ± 6	201 ± 14	150 ± 6	170 ± 12	121 ± 13	241 ± 14
0.62	19 ± 2 (1.0)	24 ± 3 (0.8)	147 ± 7 (1.0)	197 ± 20 (0.9)	159 ± 25 (1.0)	155 ± 11 (0.9)	136 ± 18 (1.1)	266 ± 15 (1.1)
1.25	28 ± 4 (1.5)	28 ± 5 (0.9)	180 ± 26 (1.3)	193 ± 26 (0.9)	181 ± 12 (1.2)	181 ± 12 (1.0)	128 ± 8 (1.0)	261 ± 17 (1.0)
2.5	32 ± 5 (1.7)	22 ± 2 (0.7)	228 ± 37 (1.6)	201 ± 8 (1.0)	208 ± 14 (1.4)	153 ± 19 (0.9)	118 ± 15 (1.0)	258 ± 10 (1.0)
5.0	49 ± 1** (2.7)	22 ± 3 (0.7)	218 ± 14 (1.5)	198 ± 4 (0.9)	344 ± 30** (2.3)	193 ± 23 (1.1)	129 ± 6 (1.1)	247 ± 16 (1.0)
7.5	25 ± 4 (1.3)	22 ± 5 (0.7)	155 ± 26 (1.0)	176 ± 15 (0.8)	363 ± 30** (2.4)	199 ± 26 (1.1)	131 ± 7 (1.1)	232 ± 22 (0.9)
Control +	492 ± 69 ^a	540 ± 51 ^b	1629 ± 332 ^c	2119 ± 413 ^b	1255 ± 291 ^a	1330 ± 70 ^b	835 ± 22 ^d	969 ± 57 ^e

DMSO, 75 µL/plate; control + : positive control.

^a4-nitro-*o*-phenylenediamine (10.0 µg/plate).

^b2-Anthramine (1.25 µg/plate).

^cSodium azide (2.5 µg/plate).

^dMitomycin (0.5 µg/plate).

^e2-aminofluorene (10.0 µg/plate).

***P* ≤ .01 analysis of variance.

MI, mutagenic index.

Ames³⁰ pointed out that many elements found in the human diet could have noxious effects. Other studies have demonstrated that natural products can represent serious risks to the DNA integrity of different organisms.^{31,32}

The methodology employed herein affirms that the extract of *P. torta* is mutagenic *in vivo* and *in vitro* in the absence of metabolic activation. The *in vivo* test would reflect the metabolism of the active constituents and the metabolization is more complex. However, the micronucleus assay identifies agents that cause DNA breaks (clastogenesis) or mitotic fails that result in chromosome loss (aneuploidy), and the *S. typhimurium* assay identifies agents that cause point mutations.

The phytochemical analysis of *P. torta* reveals the presence of α -amyrin acetate, β -amyrin acetate, betulinic acid, ursolic acid, and lupeol acetate.^{8,9} In addition, this work has demonstrated the presence of myricetin glycosides in *P. torta*.

Myricetin exhibits a more pronounced mutagenic activity than quercetin.³³ Even though it has the same molecular

characteristics as quercetin, the addition of a sixth hydroxyl on the 5' carbon of the B ring results in a higher reactivity. Myricetin is a flavonol found in a wide variety of edible plants and although it has been proven to be genotoxic to bacteria and can induce a significant concentration-dependent nuclear DNA degradation concurrent with lipid peroxidation, very little is known about the mechanisms of its genotoxicity.³⁴

Myricetin derivatives were identified in extracts of *P. torta* leaves. These secondary metabolites are flavonols, which are the most abundant subclass of flavonoids. Rietjens *et al.*¹⁵ reported that the mutagenic activity of flavonols is related to its quinone/quinone methide chemistry and is dependent on specific structural features. Based on these requirements, a metabolic pathway for the flavonol activation of DNA-reactive species was proposed, which included an enzymatic and/or chemical oxidation to *O*-quinone, followed by isomerization of the *O*-quinone to quinone methides. These quinone methides are expected to be the active alkylating

TABLE 5. MICRONUCLEATED RETICULOCYTES NUMBER, PER 2000 ANALYZED CELLS, 30 H AFTER THE ACUTE TREATMENT WITH THE DIFFERENT DOSES OF THE HYDROETHANOLIC EXTRACT OF *P. TORTA*

Treatments	Control <i>T</i> ₀			30 h		
	Number of animals	MNRETs	$x \pm SD/\text{animal}$	Number of animals	MNRETs SD/animal	$x \pm SD/\text{animal}$
Water	10	20	2.0 ± 1.36	10	21	2.1 ± 1.27
Cyclophosphamide	10	14	1.4 ± 1.22	10	988	98.8 ± 3.74*
Extract of <i>P. torta</i> (mg kg ^{−1} b.w.)						
15.0	10	14	1.4 ± 0.88	10	111	11.10 ± 2.9*
11.2	10	17	1.7 ± 1.20	10	88	8.78 ± 1.41*
7.5	10	13	1.3 ± 0.60	10	72	7.20 ± 2.68*

Positive control: cyclophosphamide-50 mg kg^{−1} b.w.; negative control: water.

*Statistically different of the negative control. (*P* < .001).

b.w., body weight; MNRET, micronucleated reticulocytes; SD, standard deviation.

DNA-reactive intermediates. Thus, it appears that flavonols present in *P. torta* leaves contribute to the induced mutations.

These types of mutation events may represent serious risks to human health. Mutations are involved in the initial steps of degenerative diseases, such as cancer. Thus, the results reported herein underline the risks of indiscriminate use of natural compounds and the importance of research on the mutagenicity of compounds obtained from plants, especially those used in popular medicines or foods.

In conclusion, a simple method was developed for the simultaneous determination of myricetin-3-*O*- β -D-galactopyranoside, myricetin-3-*O*- α -L-arabinopyranoside, and myricetin-3-*O*- α -L-rhaminopyranoside, which did not require tedious procedures to eliminate interfering materials and can be used to standardize the polar extracts of *P. torta*. The method is sensitive enough for the analysis of the three myricetin derivatives, with adequate limits of quantification, as defined by the Eurachem and ANVISA (Brazil) Legislation. Acceptable levels of accuracy and precision were obtained for the three myricetin heterosides. In addition, good results were obtained with respect to repeatability (a relative standard deviation of <5%) and recovery (90%, 83%, and 107%).

Based on the results presented in this article, we also conclude that the compounds present in the extract cause frameshift mutations *in vitro* (Ames assay) as well as breaks in and/or loss of entire chromosomes *in vivo* (micronucleus assay). The likely mutagenic agent that contributes to this activity are myricetin derivatives, which is present in *P. torta*.

ACKNOWLEDGMENTS

The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial aid to L.C.S. and W.V. They also thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for grants to L.C.S., E.A.V., and W.V.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

- Swenson U, Anderberg AA: Phylogeny, character evolution, and classification of Sapotaceae (Ericales). *Cladistics* 2005;21:101–130.
- Triono T, Brown AHD, West JG, Crisp MD: A phylogeny of *Pouteria* (Sapotaceae) from Malesia and Australasia. *Aust Syst Bot* 2007;20:107–118.
- Manosroi A, Saraphanchotiwithaya A, Manosroi J: *In vitro* immunomodulatory effect of *Pouteria cambodiana* (Pierre ex Dubard) Baehni extract. *J Ethnopharmacol* 2005;101:90–94.
- Agripino DG, Lima MEL, Silva MR, Meda CI, Bolzani VS, Cordeiro I, Young MCM, Moreno PRH: Screening of Brazilian plants for antimicrobial and DNA-damaging activities. I. Atlantic rain forest-ecological station juréia-itatins. *Biota Neotrop* 2004;4:1–15.
- Boleti AP, Freire MG, Coelho MB, Silva W, Baldasso PA, Gomes VM, Marangoni S, Novello JC, Macedo ML: Insecticidal and antifungal activity of a protein from *Pouteria torta* seeds with lectin-like properties. *J Agric Food Chem* 2007;55:2653–2658.
- Ma J, Yang H, Basile MJ, Kennelly EJ: Analysis of polyphenolic antioxidants from the fruits of three *Pouteria* species by selected ion monitoring liquid chromatography-mass spectrometry. *J Agric Food Chem* 2004;52:5873–5878.
- Montenegro LHM, Oliveira PES, Conserva LM, Rocham EMM, Brito AC, Araújo RM, Trevisan MTS, Lemos RPL: Triterpenóides e avaliação do potencial antimalárico, larvívica, anti-radicalar e anticolinesterástico de *Pouteria venosa* L (Sapotaceae). *Rev Bras Farmacogn* 2006;16:611–617.
- Perfeito JP, Santos ML, Lopez KSE, Paula JE, Silveira D: Characterization and biological properties of *Pouteria torta* extracts: a preliminary study. *Rev Bras Farmacogn* 2005;15:183–186.
- Che CT, Koike K, Cordell GA, Fong HHS, Dobbshstetn RH: Triterpenes of *Pouteria torta* (Sapotaceae). *J Nat Prod (Lloydia)* 2005;43:420–421.
- Silva ACZ, Vilegas W, Nasser ALM, Stefanini M: Isolamento e Identificação de Flavonóides em *Pouteria torta*. Presented at XXI Congresso de Iniciação Científica da UNESP. Sao Jose do Rio Preto, Brazil, November, 3–7, 2009.
- Alves TMA, Silva AF, Brandão MGL, Grandi TSM, Smânia EF, Zani CL: Biological screening of Brazilian medicinal plants. *Mem Inst Oswaldo Cruz* 2000;95:367–373.
- Souza PM, Sales PM, Simeoni LA, Silva EC, Silveira D, Magalhães PO: Inhibitory activity of α -amylase and α -glucosidase by plant extracts from the Brazilian Cerrado. *Planta Med* 2012;78:393–399.
- Varanda EA, Pozetti GL, Lourenço MV, Vilegas W, Raddi MSG: Genotoxicity of *Brosimum gaudichaudii* measured by the *Salmonella*/microsome assay and chromosomal aberrations in CHO cells. *J Ethnopharmacol* 2002;81:257–264.
- Ferreira ICDF, Vargas VMF: Mutagenicity of medicinal plant extracts in *Salmonella*/microsome assay. *Phytother Res* 1999;13:397–400.
- Rietjens IMCM, Boersma MG, Woude H, Jeurissen SMF, Schutte ME, Alink GM: Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk. *Mutat Res* 2005;574:124–178.
- De Flora S: Mechanisms of inhibitors of mutagenesis and carcinogenesis. *Mutat Res* 1998;402:151–158.
- Skibola CF, Smith MT: Potencial health impacts of excessive flavonoid intake. *Free Radic Biol Med* 2000;29:375–383.
- Irakli MN, Samanidou VF, Biliaderis CG, Papadoyannis IN: Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. *J Sep Sci* 2012;35:1603–1611.
- Saldanha LL, Vilegas W, Dokkedal AL: Characterization of flavonoids and phenolic acids in *Myrcia bella* Cambess. Using FIA-ESI-IT-MSⁿ and HPLC-PAD-ESI-IT-MS combined with NMR. *Molecules* 2013;18:8402–8416.
- Zehl M, Braunberger C, Conrad J, Crnogorac M, Krasteva S, Vogler B, Beifuss U, Krenn L: Identification and quantification of flavonoids and ellagic acid derivatives in therapeutically important *Drosera* species by LC–DAD, LC–NMR, NMR, and LC–MS. *Anal Bioanal Chem* 2011;400:2565–2576.

21. Rodrigues CL, Rinaldo D, Sannomiya M, Santos LC, Montoro P, Piacente S, Vilegas W: High-performance liquid chromatographic separation and identification of polyphenolic compounds from the infusion of *Davilla elliptica* St. Hill. *Phytochem Anal* 2008;19:17–24.
22. Maron DM, Ames BN: Revised methods for the *Salmonella* mutagenicity test. *Mutat Res* 1983;113:173–215.
23. Bernstein L, Kaldor J, McCann J, Pike MC: An empirical approach to the statistical analysis of mutagenesis data from the *Salmonella* test. *Mutat Res* 1982;97:267–281.
24. Santos FV, Colus IMS, Silva MA, Vilegas W, Varanda EA: Assessment of DNA damage induced by extracts and fractions of *Strychnos pseudoquina*, a Brazilian medicinal plant with anti-ulcerogenic activity. *Food Chem Toxicol* 2006;44:1585–1589.
25. Olfert ED, Cross BM, Mcwillan AA: *The Guide to the Care and Use of Experimental Animals*. Canadian Council on Animal Care, Toronto, Canada, 1993.
26. Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate MJR: The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutat Res* 1990;245:245–249.
27. Cuyckens F, Claeys M: Determination of the glycosylation site in flavonoid mono-O-glycosides by collision-induced dissociation of electrospray-generated deprotonated and sodiated molecules. *J Mass Spectrom* 2005;40:364–372.
28. Mortelmans K, Zeiger E: The Ames *Salmonella*/microsome mutagenicity assay. *Mutat Res* 2000;455:29–60.
29. Krishna G, Hayashi M: *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. *Mutat Res* 2000;455:155–166.
30. Ames BN: Dietary carcinogens and anticarcinogens. *Science* 1983;221:1256–1264.
31. Santos FV, Calvo TR, Colus IMS, Vilegas W, Varanda EA: Mutagenicity of two species of the genus *Alchornea* measured by *Salmonella* microsome assay and micronucleus test. *Rev Bras Farmacogn* 2010;20:382–389.
32. Resende FA, Vilegas W, Santos LC, Varanda EA: Mutagenicity of flavonoids assayed by bacterial reverse mutation (Ames) test. *Molecules* 2012;17:5255–5268.
33. Yoshino M, Haneda M, Naruse M, Murakami K: Prooxidant activity of flavonoids: copper-dependent strand breaks and the formation of 8-hydroxy-2'-deoxyguanosine in DNA. *Mol Genet Metab* 1999;68:468–472.
34. Silva ID, Gaspar J, Rodrigues A, Costa GG, Laires A, Rueff J: Mechanisms of myricetin mutagenicity in V79 cells: involvement of radicalar species. *Teratog Carcinog Mutagen* 1996;16:253–268.