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Hydrolysis Influence on Phytochemical Composition, Antioxidant Activity, Plasma Concentration, and Tissue Distribution of Hydroethanolic *Ilex paraguariensis* Extract Components

Diogo P. Rivelli,* Rebeca L. Almeida, Cristina D. Ropke, and Silvia B. M. Barros

School of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes 580, bloco 17, sala 114, Cidade Universitária, São Paulo SP, CEP 05508-000, Brazil

ABSTRACT: The infusion of aerial parts of *Ilex paraguariensis* is widely consumed. Its antioxidant activity suggests an important role of this plant in the treatment/prevention of oxidative stress related diseases. Plant extract active compounds are frequently found in esterified form that may be poorly absorbed. Hydrolysis of the extract is a possible approach to increase its bioavailability. The aim of this study was to perform a phytochemical analysis and evaluate in rats the plasma concentration and tissue distribution of antioxidant compounds in the hydroethanolic extract of *Ilex paraguariensis*, before and after enzymatic hydrolysis. Both extracts presented high antioxidant activity and phenolic content. Rats given single or repeated doses of the hydrolyzed extract showed increased plasma antioxidant activity and higher plasma levels of caffeic acid. However, no changes of endogenous antioxidants were observed. In conclusion, hydrolysis of the extract of *Ilex paraguariensis* is a strategy to improve its bioavailability and in vivo antioxidant activity.

KEYWORDS: *Ilex paraguariensis*, antioxidant activity, plasma concentration, tissue distribution, enzymatic hydrolysis

INTRODUCTION

Imbalance between the rates of production and elimination of reactive oxygen species (ROS) has been implicated in numerous pathophysiological conditions such as rheumatism, infections, type 2 diabetes, and cardiovascular diseases.[1]

ROS levels are controlled by enzymatic systems, such as superoxide dismutase, catalase, and glutathione peroxidase/reductase, and by nonenzymatic compounds such as tocopherols, carotenes, ascorbic acid, and glutathione. Also, the consumption of polyphenols may decrease ROS levels.[1]

Some epidemiological studies have suggested that the intake of natural products containing polyphenols and other antioxidant compounds may reduce the incidence of aging related diseases.[2] But it is important to keep in mind that some hazardous effects of supplementation are not well described yet.[3]

A decrease of free radical formation after UVA exposure and a decrease in the amount and size of skin tumor formation after UVB chronic exposure were observed in mice treated orally and topically with caffeic acid[4] and with brown algae polyphenols,[5] respectively.

Ilex paraguariensis St. Hilaire (Aquifoliaceae), known as yerba mate, is a South American plant widely cultivated in Argentina, Brazil, and Paraguay. The aerial parts are used to prepare an infusion beverage with peculiar flavor and stimulating properties.[6]

Along with caffeine, sucrose, fructose, folic acid, trigonelline, choline, thiamine, and riboflavin, numerous phenolic compounds, such as caffeic acid derivates and flavonoids, have been identified in the aqueous extract.[7]

The antioxidant activity of *Ilex paraguariensis* was assayed in vitro and in vivo. The aqueous extract was shown to be effective in the inhibition of LDL peroxidation when ingested by healthy subjects.[8] This extract was also able to inhibit lipid peroxidation induced by Fe^{2+}/ascorbate and CCl_4/NADPH systems.[9]

In a comparative study between aqueous and ethanolic extracts, it was described that the ethanolic extract was 40 times less active against the inhibition of LDL oxidation than the aqueous extract.[7] In another assay comparing aqueous and hydroethanolic extracts (50% v/v by percolation), it was demonstrated that they are similar concerning in vitro antioxidant activity as measured by the DPPH reduction method.[10] The antioxidant activity of the aqueous extract of *Ilex paraguariensis* leaves was attributed to its high concentration in hydroxycinnamic acid derivatives, such as caffeoylquinic and dicaffeoylquinic acids.[6]

Phenolic acids are rarely found in plants in the free form. Frequently, they occur as esters or glycosides that can be released after acidic or alkaline hydrolysis. The major compound of the hydroxycinnamic family is caffeic acid, which mainly occurs in plants in esterified forms, such as caffeoylquinic acids.[11]

The phenolic compounds metabolism and bioavailability in foods as well as their biological activity have recently attracted scientific interest. Many of these compounds may either not be absorbed or may experience metabolic degradation. Therefore, to assess their biological function it is important to know whether they reach the tissues where they could exert their activities.[12]

Differences in the composition, activity, and bioavailability of hydrolyzed and nonhydrolyzed plant extracts have been reported. Increased amounts of free components, such as caffeic acid, and a decrease of esterified components, such as caffeoylquinic acids, were demonstrated after the hydrolysis of *Trichilia emetica* and coffee extract, respectively.[13,14] Also in vitro antioxidant

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activity and bioavailability of phenolic compounds were higher for the hydrolyzed extracts and for free acids.

Most of the papers in the scientific literature related to *Ilex paraguariensis* use aqueous extracts obtained by decoction. In this work, hydroethanolic percolation was chosen in order to increase the amount of less polar phenolic compounds, such as caffeoyl derivates and flavonoids, usually found in very low concentrations in aqueous extracts.[6]

The objectives of this study were to compare the phytochemical composition of a hydroethanolic extract of *Ilex paraguariensis*, before and after hydrolysis, investigate the effects of hydrolysis over antioxidant activity of the extract, and evaluate the plasma concentration and tissue distribution of some antioxidant compounds in rats.

MATERIAL AND METHODS

Chemicals. Acetonitrile and methanol were of chromatographic grade from Merck (Darmstadt, Germany). Analytical grade reagents were as follows: caffeic acid (CAS number 331-39-5), 5-caffeoylquinic acid (CAS number 327-97-9), ascorbic acid (CAS number 50-81-7), quercetin (CAS number 117-39-5), kaempferol (CAS number 520-18-3), rutin (CAS number 153-18-4) caffeine (CAS number 58-08-2), metaphosphoric acid, lithium perchloride, Folin−Ciocalteu reagent, gallic acid (CAS number 149-91-7), uric acid (CAS number 69-93-2) naringenin (CAS number 480-41-1), Trolox (CAS number 53188-07-1), AAPH (2,2-azobis(2-amidinopropane) dihydrochloride, CAS number 2997-92-4), DPPH (2,2-diphenylpicrylhydrazyl radical, CAS number 1898-66-4), and β-glucuronidase type HP-2 (*Helix pomatia*) from Sigma-Aldrich (St. Louis, MO, USA); acetic acid, potassium chloride, aluminum chloride, DNPH (2,4-dinitrophenylhydrazine), sulfuric acid, acetone, hexane, and ethanol from Merck (Darmstadt, Germany); sodium carbonate and sodium acetate from J.T. Baker (Phillipsburg, NJ, USA); sodium fluorescein from Riedel-de Haën (Seelze, Germany); randomly methylated β-cyclodextrin (RMCD) from Cyclodextrin Technologies (High Springs, FL, USA); perchloric acid from QM (Cotia, SP, Brazil); potassium hydroxide from Berzog (São Paulo, Brazil); and chlorogenate esterase 15U/g from Kikkoman Corporation (Noda, Japan).

Extract Preparation. Dried leaves of *Ilex paraguariensis* (295 g, code ETMP124356, batch 2.0607.0215 donated by Anidro do Brasil S/A, Botucatu, SP, Brazil) were ground, sieved through a 40 mesh, and percolated with 1.1 L of ethanol/water (50% v/v) at room temperature for one week. The extract was vacuum-concentrated and freeze-dried.

Extract Characterization. *Total Phenolic Compounds.* Fifty microliters of Folin−Ciocalteu reagent (0.4 N) was added to 50 μL of standard (caffeic acid) or sample in a 96 well microplate. Next, 100 μL of sodium carbonate (700 mmol/L) was added, the reaction mixture was incubated for 1 h in the absence of light at room temperature, and its absorbance was read at 760 nm.[15] Because ascorbic acid can interfere with the method, the quantification of this compound was performed in the extract prior to the determination of phenolic compounds.[16]

Total Flavonoid Content. The most common procedure for determining the total content of flavonoids is a spectrophotometric assay based on the formation of a complex between Al^{3+} ion and the carbonyl group. However, flavonoids (flavonols and flavanones) can interact with Al^{3+}, depending on their molecular structure, generating complexes which absorb light at different wavelengths. There is a significant change in the absorbance profile in the absence of double bonds between carbons 2−3 of the C-ring. Therefore, two methods, one for flavonols/flavones and another for flavanones, were used.[17]

The determination of total flavonol content was performed as follows: 100 μL of water, 60 μL of ethanol, 10 μL of aluminum chloride (300 mmol/L aqueous solution), 10 μL of sodium acetate (400 mmol/L aqueous solution), and 20 μL of the extract diluted in water or standard (quercetin 82.7 to 992 μmol/L) were added to a 96 well microplate. The reaction mixture was incubated at room temperature for 30 min, and its absorbance was read at 415 nm.[17,18]

The determination of total flavanone content was performed as follows: 0.5 mL of the extract diluted in water or standard (naringenin 459 to 7346 μmol/L) was mixed with 1 mL of 2,4-dinitrophenylhydrazine (DNPH) solution and 1 mL of methanol. The mixture was heated at 50 °C for 50 min. After that, 2.5 mL of potassium hydroxide (10% p/v in methanol 70% v/v) was added followed by, after 2 min, 2.5 mL of methanol. The final mixture was centrifuged at 1000*g* for 10 min and the supernatant read at 495 nm.[19]

DNPH solution was prepared by dissolving 1 g of 2,4-dinitrophenylhydrazine in 2 mL of 96% sulfuric acid and the addition of 98 mL of methanol.[19]

Chromatographic Analysis. The HPLC determinations were carried out on a system consisting of a Constametric Pump, model 3500 (Fremont, CA, USA), a Rheodyne 7125 manual injector (Contati, CA, USA) equipped with a 20 μL loop, and a Lab Alliance Model 525 UV detector (San Jose, CA, USA).

The system for caffeic acid, 5-caffeoylquinic acid, and caffeine determination was composed of a 10 μm Phenomenex Bondclone C18 column (300 × 4.6 mm), a mobile phase of 0.2% aqueous metaphosphoric acid solution/acetonitrile (80:20 v/v), a flow rate of 1.0 mL/min, and detection at 330 nm for caffeic acid and 5-caffeoylquinic acid and 275 nm for caffeine.[10]

The quercetin, rutin, and kaempferol determination system included a 10 μm Phenomenex Luna C18 column (250 × 4.6 mm) (Torrance, CA, USA), methanol/acetonitrile/water (40:15;45 v/v/v) with 1% of acetic acid as mobile phase, a flow rate of 1.0 mL/min, and detection at 368 nm.[20]

Ascorbic acid analyses was performed with a 10 μm Phenomenex Bondclone C18 column (300 × 4.6 mm), 0.2% metaphosphoric acid aqueous solution as the mobile phase, a flow rate of 0.7 mL/min, and detection at 254 nm.[21]

Limit of detection of the methods were 2.8 μmol/L, 1.0 μmol/L, 1.3 μmol/L, 0.6 μmol/L, and 1.1 μmol/L, for caffeic acid, caffeine, quercetin, kaempferol, and ascorbic acid, respectively.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The extract was diluted to 1 mg/mL in acetone/water (50% v/v). Further dilutions were prepared in phosphate buffer (75 mmol/L, pH 7.0). An aliquot of 25 μL of the diluted sample or of the different concentrations of standard (Trolox) was combined with 150 μL of fluorescein solution (40 nmol/L in phosphate buffer 75 mmol/L, pH 7.0) in a 96 well microplate and incubated for 30 min at 37 °C. The reaction was initiated by the addition of 25 μL of AAPH (153 mmol/L in phosphate buffer 75 mmol/L, pH 7.0)/ followed by shaking at maximum intensity for 10 s. A Synergy HT Multi-Detection Microplate Reader (Winooski, VT, USA) equipped with a 485 nm/20 nm bandpass excitation filter and a 528 nm/20 nm bandpass emission filter was used. Fluorescence was measured from the bottom at a sensitivity setting at 60 and kinetically monitored every 60 s.[22]

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. The extract was diluted in methanol 80% (v/v). Then 50 μL of the dilution was added to 150 μL of DPPH (100 μmol/L in 80% methanol). The microplate (96 wells) was kept in the absence of light for 2 h, and the absorbance was read at 517 nm. Trolox was used as the standard.[23]

Hydrolysis. The hydrolysis of 5-caffeoylquinic acid was performed using chlorogenate esterase, a commercially available enzyme, isolated from *Aspergillus japonicus*.[24]

In order to determine the optimum incubation time, 0.5 mL of a 5-caffeoylquinic acid solution (1411 μmol/L) diluted in phosphate buffer (60 mmol/L pH 6.5) was added to 30 μL of the enzyme (0.6 U/mL) diluted in the same buffer. The solution was incubated at 30 °C,

and the product/substrate ratio was measured at different times. The extract was hydrolyzed under the same conditions as those described for 5-caffeoylquinic acid.

In Vivo Experiments. The in vivo experiments were approved by the Animal Experimentation Ethics Committee of the School of Pharmaceutical Sciences, University of São Paulo under protocol CEEA number 131 (12/11/2006). The animals (male Wistar rats with approximately 200 g) were obtained from and maintained in an animal housing facility at the School of Pharmaceutical Sciences, University of São Paulo. Water and food (standard rat chow (Nuvital Nutrientes S/A, Colombo, PR, Brazil)) were provided ad libitum and the animals kept in a 12 h light−dark regimen, temperature at 22 °C ± 2 °C and a relative humidity of air at 55% ± 10%.

Plasma Concentration of Caffeic Acid and 5-Caffeoylquinic Acid after Oral Administration. Animals were dosed by gavage with 200 mg of caffeic acid[4] or 393.33 mg of 5-caffeoylquinic acid/kg body weight (bw) (1.11 mmol/kg bw) dissolved in 1 mL of distilled water. The animals were intramuscularly anesthetized with xylazine/ketamine (15 mg/kg and 100 mg/kg, respectively), and blood was collected by cardiac puncture at different times (3 animals per time point (10, 20, and 30 min for caffeic acid and 10, 20, 30, 45, 60, and 120 min for 5-caffeoylquinic acid). After 10 min of centrifugation at 3000 rpm, plasma was separated, and 10% metaphosphoric acid (1:1 v/v) was added. Then the resulting solution was vortexed for 2 min, centrifuged for 4 min at 10000 rpm, filtered through a membrane with a pore size of 0.22 μm, and injected into an HPLC system.

The determinations of caffeic and 5-caffeoylquinic acid were carried out on a system consisting of a Waters/Millipore model 510 pump (Milford, MA, USA) and a Rheodyne 7125 (Contati, CA, USA) manual injector equipped with a 20 μL loop and a HP 1049A electrochemical detector (Waldbronn, Germany) with a glassy carbon electrode. The separation was achieved on a 3 μm Phenomenex Luna C8 column (75 × 4.6 mm) (Torrance, CA, USA). The mobile phase consisted of 0.2% aqueous metaphosphoric acid solution/acetonitrile (90:10 v/v) containing 20 mmol/L $LiClO_4$ and 2 mmol/L KCl with a flow rate of 0.7 mL/min. The potential was +0.6 V on amperometric mode.

Single Dose (SD) Administration of Hydroethanolic Extract of *Ilex paraguariensis*. Animals (10 treated with hydrolyzed or nonhydrolyzed extract and 5 control) were treated by gavage with 200 mg equiv of caffeic acid/kg bw of the extract (dissolved in distilled water) or 1 mL of distilled water for controls. They were intramuscularly anesthetized with xylazine/ketamine (15 mg/kg and 100 mg/kg, respectively), and blood was collected by cardiac puncture at the time of highest plasma concentration, which was experimentally determined above. After 10 min of centrifugation at 3000 rpm, plasma was separated, and 10% metaphosphoric acid (1:1 v/v) was added. Then the resulting solution was vortexed for 2 min, centrifuged for 4 min at 10000 rpm, filtered through a membrane with a pore size 0.22 μm, and injected into an HPLC system.

The determinations of free caffeic and 5-caffeoylquinic acid followed the method described in the Plasma Concentration of Caffeic Acid and 5-Caffeoylquinic Acid after Oral Administration section, and for the quantification of caffeine and ascorbic acid, the methodology described for the extract characterization was used. Uric acid determination was performed in the same system developed for ascorbic acid analyses, with detection at 292 nm. Results were expressed in μmol/L of plasma.

The amount of caffeic acid conjugated with glucuronic acid present in the plasma was determined after β-glucuronidase treatment.[25,26] Briefly, 100 μL of plasma was added to 300 μL of ethanol and vortexed. The mixture was centrifuged at 4 °C, 10000 rpm for 5 min. A 300 μL aliquot of the supernatant was dried under nitrogen flow. The residue was resuspended in 0.5 mL of acetate buffer (100 mmol/L pH 5.0), and 500 U of β-glucuronidase was added. The tube was incubated at 37 °C overnight in a Girotory Water Bath Shaker G76, New Brunswick (Edison, NJ, USA), at speed 3. The mixture was filtered through a membrane with a pore size of 0.22 μm and injected into an HPLC system.

Multiple Dose (MD) Administration of a Hydroethanolic Extract of *Ilex paraguariensis*. Animals (10 treated with hydrolyzed or nonhydrolyzed extract and 5 controls) were orally treated by gavage with 200 mg equiv of caffeic acid/kg bw of the extract (dissolved in distilled water) or 1 mL of distilled water for controls during 30 days between 3 and 4 p.m. After the period of treatment, the animals were intramuscularly anesthetized with xylazine/ketamine (15 mg/kg and 100 mg/kg respectively), and blood (cardiac puncture), liver, brain, and skin were collected at the time of highest plasma concentration determined in the Plasma Concentration of Caffeic Acid and 5-Caffeoylquinic Acid after Oral Administration section.

Phosphate buffer, 140 mmol/L pH 7.4 (1:2 w/v), was added to the tissues. Then the mixture was triturated in UltraTurrax, homogenized with a Potter-Elvehjem system, and centrifuged at 1000*g* and 4 °C for 20 min. Metaphosphoric acid 10% (1:1 v/v) was added to the supernatant. The mixture was vortexed for 2 min, centrifuged for 4 min at 10000 rpm, filtered through 0.22 μm pore size membrane, and injected into an HPLC system as described above for the analysis in plasma in the single dose administration. Results were expressed as nmol/g of protein in the supernatant.

The determination of the amount of caffeic acid conjugated with glucuronic acid in the plasma and liver followed the same procedure as that for the analysis in plasma in the single dose administration.

Antioxidant Activity of Plasma, Skin, Liver, and Brain Homogenate (ORAC Assay). Samples (100 μL) were added to 200 μL of ethanol, 100 μL of water, and mixed, followed by 400 μL of hexane. After vortexing, the mixture was centrifuged for 5 min at 10000 rpm. The hexane layer (350 μL) was removed and placed in an amber tube. An additional 400 μL of hexane was added to the original tube, vortexed, and centrifuged for 5 min at 10000 rpm. The second hexane layer (350 μL) was removed and combined with the first one.

The combined hexane extracts were dried under nitrogen in preparation for lipophilic ORAC analysis. The dried hexane extract was dissolved in 250 μL (for the plasma or liver homogenate) or 125 μL (for the skin or brain homogenate) of acetone and then added to 750 μL (for the plasma or liver homogenate) or 375 μL (for skin or brain homogenate) of a 7% RMCD (randomly methylated β-cyclodextrin) solution (50% acetone/50% water (v/v)). Further dilutions were prepared using the 7% RMCD solution which was also used as a blank and to dissolve Trolox standards for the lipophilic assay.[27] The oxygen radical absorbance capacity (ORAC) assay protocol was followed as described above.

For the hydrophilic assay, 400 μL of 0.5 mol/L perchloric acid was added to the aqueous residue in order to precipitate the proteins. After centrifugation at 10000 rpm for 5 min, the supernatant was diluted in phosphate buffer (75 mmol/L pH 7.0) and used for the ORAC assay as described above.

Statistical Analysis. Data were statistically analyzed by factorial analysis of variance (ANOVA). The 0.05 significance level was used as minimum statistical relevance.

RESULTS

Hydrolysis of 5-Caffeoylquinic Acid and the Hydroethanolic Extract of *Ilex paraguariensis*. Figure 1 shows the kinetic assay of the hydrolysis of 5-caffeoylquinic acid by chlorogenate esterase and the formation of caffeic acid indicating that the hydrolysis was completed after 30 min of incubation. K_m (Michaelis−Menten constant) and V_{max} (maximum rate) for the enzymatic reaction were determined corresponding to 1597 μmol and 1667 nmol/s, respectively.

The hydrolysis of the hydroethanolic extract of *Ilex paraguariensis* was, therefore, carried out for 30 min. All 5-caffeoylquinic

acid in the crude extract was converted into caffeic acid. Two other major compounds present in the extract were detected in very low concentrations in the hydrolyzed extract (data not shown).

Phytochemical Characterization of the Crude Hydroethanolic Extract of *Ilex paraguariensis*. HPLC analysis of the hydroethanolic crude extract showed the presence of 5-caffeoylquinic acid (7.93%) and caffeine (1.48%). Caffeic acid, quercetin, kaempferol, rutin, and ascorbic acid levels were below the limit of detection.

Total flavonol/flavone content was 162 ± 5 μmol equiv quercetin/g extract, and flavanones were not detected.

Antioxidant Activity and Phenolic Content of the Hydrolyzed and Nonhydrolyzed Hydroethanolic Extract of *Ilex paraguariensis*. Table 1 shows the antioxidant activity and total phenolic content of the hydrolyzed and nonhydrolyzed hydroethanolic extracts of *Ilex paraguariensis*.

There was no need to perform a correction of the Folin values because ascorbic acid was not detected in the crude extract.

In Vivo Determinations. The maximum plasma concentrations determined in our experiments were achieved after 10 and 20 min of administration of 5-caffeoylquinic and caffeic acids, respectively. Therefore, in rats treated with hydrolyzed and nonhydrolyzed extracts, the plasma levels of these compounds were determined at these times.

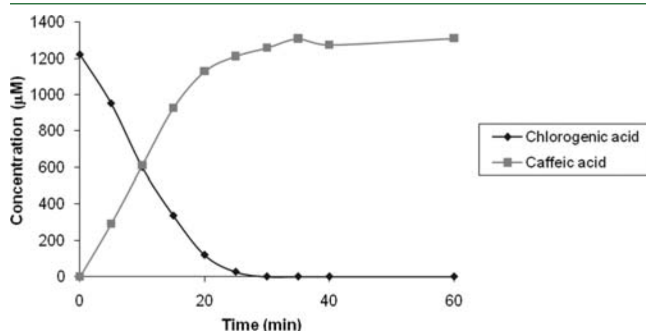

Figure 1. Relationship between enzyme incubation time, 5-caffeoylquinic acid consumption, and caffeic acid production during hydrolysis.

Table 2 shows the concentrations of 5-caffeoylquinic acid, caffeic acid, and caffeine in plasma after single and multiple oral doses of the hydrolyzed or nonhydrolyzed hydroethanolic extracts of *Ilex paraguariensis.* These compounds were not detected in skin and brain homogenates. However caffeic acid was present in the liver of the animals treated with multiple doses of the hydrolyzed extract (Table 3).

Treatment with the extracts (hydrolyzed or nonhydrolyzed) had no effect on uric acid and ascorbic acid levels (Table 2).

The oral administration of the hydrolyzed extract increased the ORAC values of plasma hydrophilic fraction by 71% for single dose and 61% for multiple dose regimens (Table 4). Antioxidant activity of skin, liver, and brain homogenates were not affected by the treatment with both the extracts (hydrolyzed or nonhydrolyzed) (Table 5).

DISCUSSION

The results of this article indicate that the hydroethanolic extract of *Ilex paraguariensis* has a high phenolic content with a small contribution of flavonoids and high in vitro antioxidant activity when compared with that of other extracts presented in the literature.[22] The difference between ORAC and DPPH values can be related to the principle of each method. The DPPH assay makes use of a nitrogen radical in organic medium, whereas the ORAC method employs a peroxyl radical in buffered aqueous medium.[28] Probably, the antioxidants compounds present in the *Ilex paraguariensis* extract interact better with the free radical used in the ORAC assay. However, it is important to have in mind that in vitro antioxidant assays do not yield absolute values but are usually used in screening experiments to select molecules, extracts, or mixtures for *in vivo* assays.

Table 1. Antioxidant Activity (ORAC and DPPH Assays) and Total Phenolic Content (Folin Assay) of the Hydrolyzed and Nonhydrolyzed Hydroethanolic Extracts of *Ilex paraguariensis*

	nonhydrolyzed extract	hydrolyzed extract
ORAC[a]	4221 ± 319 μmol eq.Trolox/g	4287 ± 456 μmol eq. Trolox/g
DPPH	1492 ± 83 μmol eq.Trolox/g[b]	2161 ± 133 μmol eq. Trolox/g[b]
Folin[a]	1157 ± 69 μmol eq. caffeic acid/g	1120 ± 64 μmol eq. caffeic acid/g

[a] ORAC and Folin assays presented no statistical difference. [b] $p < 0.0001$.

Table 2. Plasma Concentration of Caffeic and 5-Caffeoylquinic Acid and Caffeine after Single (S) and Multiple (M) Oral Doses of the Hydrolyzed (H) or Nonhydrolyzed (NH) Hydroethanolic Extract of *Ilex paraguariensis*[a]

plasma	S-NH	S-H	M-NH	M-H
5-caffeoylquinic acid	-	N/E	*	N/E
free caffeic acid	-	13.99 ± 10.27●	*	26.25 ± 14.26○
total caffeic acid	-	97.08 ± 18.76●	5.61 ± 7.60	100.52 ± 35.41○
caffeine	22.04 ± 5.15	17.92 ± 4.43	25.93 ± 6.69	17.40 ± 5.05
ascorbic acid control	58.99 ± 14.99	58.71 ± 1.99	40.31 ± 7.95	41.87 ± 9.08
ascorbic acid treated	47.86 ± 4.09	50.53 ± 5.56	35.49 ± 14.44	45.99 ± 7.61
uric acid control	51.90 ± 18.57	22.02 ± 3.63	28.10 ± 7.50	46.37 ± 24.28
uric acid treated	46.96 ± 9.11	23.69 ± 13.81	41.07 ± 17.32	32.74 ± 8.63

[a] values expressed as μmol/L ± standard deviation (n = 10 for treated animals and n = 5 for control animals). *, below the limit of quantitation; -, below the limit of detection; N/E, not evaluated; ●,○, $p < 0.005$ comparing free and total caffeic acid concentration in the same dose regimen. No statistical difference was observed between other groups.

Table 3. Caffeic Acid Concentration in the Liver of Rats Treated with Multiple (M) Oral Doses of the Hydrolyzed (H) and Nonhydrolyzed (NH) Hydroethanolic Extract of *Ilex paraguariensis*[a]

	M-NH	M-H
free caffeic acid	-	289.6 ± 203.9
total caffeic acid	-	393.2 ± 312.8

[a] -, below the limit of detection; values expressed as nmol/g protein ± standard deviation of 10 determinations. The difference between free and total caffeic acid was not considered statistically relevant.

Table 4. Plasma Antioxidant Activity Evaluated by ORAC (Lipophilic and Hydrophilic Method) after Single (S) and Multiple (M) Oral Doses of the Hydrolyzed (H) or Nonhydrolyzed (NH) Hydroethanolic Extract of *Ilex paraguariensis*[a]

	S-NH	control-NH	S-H	control-H
		Single Dose		
hydrophilic	1730 ± 211	1952 ± 378	2019 ± 336*	1176 ± 149
lipophilic	269 ± 112	194 ± 23	132 ± 27	130 ± 32
		Multiple Doses		
hydrophilic	2312 ± 474	1979 ± 338	2361 ± 268*	1467 ± 379
lipophilic	230 ± 58	171 ± 60	182 ± 90	157 ± 51

[a] $^*p < 0.0002$ relative to the control. No statistical difference between other groups. Values expressed as μmol equiv Trolox/L plasma ± standard deviation (n = 10 for treated animals, and n = 5 for control animals).

The evaluation of antioxidant activity and total phenolic content showed that the hydrolyzed extract had its antioxidant capacity significantly increased in the DPPH assay when compared with that in the nonhydrolyzed extract. This result can be explained by the higher antioxidant activity of caffeic acid (approximately three times) compared with that of 5-caffeoylquinic acid when assayed by this method.[29,30]

The hydrolysis of the extract revealed that two other peaks (besides 5-caffeoylquinic acid) were present in the HPLC chromatogram of the crude extract. The observed reduction in the size of these peaks after hydrolysis suggests the presence of caffeoyl derivatives, such as di- and tricaffeoylquinic acids in the crude extract. Given the stoichiometry of the reaction, we should expect that 1 mol of 5-caffeoylquinic acid would produce 1 mol of caffeic acid. However, the concentrations of 5-caffeoylquinic acid before hydrolysis and caffeic acid after hydrolysis were 8.01 ± 0.30 μmol/L and 26.53 ± 1.32 μmol/L, respectively, corroborating the presence of other hydrolyzable compounds in the crude extract. This was previously described[13] verifying an overexpected increase of caffeic acid concentration after hydrolysis.

Concerning the in vivo assays, in the present study the highest measured caffeic acid plasma concentration in animals that received 200 mg/kg bw by gavage was 36.8 μmol/L, lower than that previously reported (305.3 μmol/L).[4] Also in our experiments, the time to reach this concentration was 20 min against only 5 min in the referred work.[4] This difference may be explained by the fact that mice were used as the experimental model in the latter, whereas rats were used in our study.

Plasma levels of caffeic acid at a maximum value of 0.36 μmol/L were observed in rats treated with caffeic acid solution (11.25 μmol/kg bw).[31] We could speculate that if the plasma concentration of caffeic acid follows dose-independent absorption rate, and 200 mg/kg bw were administered to the rats (as in our experiments), the plasma concentration would be 35.5 μmol/L, which is consistent with our results.

Table 5. Liver, Brain, and Skin Antioxidant Activity Evaluated by ORAC after Multiple (M) Oral Doses of the Hydrolyzed (H) or Nonhydrolyzed (NH) Hydroethanolic Extract of *Ilex paraguariensis*[a]

	NH	control-NH	H	control-H
skin hydrophilic	130 ± 22	147 ± 17	178 ± 32	180 ± 53
skin lipophilic	40 ± 6	40 ± 9	71 ± 9	85 ± 16
brain hydrophilic	97 ± 14	80 ± 23	62 ± 9	69 ± 20
brain lipophilic	25 ± 6	26 ± 8	39 ± 5	39 ± 4
liver hydrophilic	44 ± 14	50 ± 7	48 ± 13	51 ± 13
liver lipophilic	21 ± 3	20 ± 6	11 ± 2	13 ± 3

[a] Values expressed as μmol equiv Trolox/g protein ± standard deviation (n = 10 for treated animals and n = 5 for control animals. No statistical difference was observed between the treated and control groups.

In an assay involving humans receiving 400 mg of green coffee (about 170 mg eq. caffeic acid) via oral methods, caffeic acid plasma concentration was 1.1 μmol/L. Considering the volunteers' average weight, if 200 mg equiv caffeic acid/kg was used then the plasma concentration would be 82.9 μmol/L, which is consistent with our results. However, the time required to reach this level was 3.6 h, indicating that there are important differences in the absorption profile between rats and humans.[32]

The hydrolysis of the extract improved the plasma concentration of caffeic acid in both administration regimens, and it is mainly conjugated with glucuronic acid (more than 75% according to table 2) and the antioxidant activity of plasma hydrophilic fraction in agreement with the high content of phenolic compounds in *Ilex paraguariensis* extract. Since the concentrations of ascorbic and uric acids, the main hydrophilic antioxidants in plasma,[33] remained unaltered after hydrolysis, the increase in plasma antioxidant activity may be attributed to the increase in caffeic acid levels. Furthermore, caffeic acid was also found in the liver of the animals that received multiple doses of the hydrolyzed extract, which corroborates the hypothesis that hydrolysis of the extract can improve the *in vivo* distribution of this compound. However, the levels of caffeic acid in the liver were not able to increase the antioxidant activity of this tissue.

The antioxidant activity of the skin and brain was not altered by any treatment, suggesting that antioxidant compounds in the extract were not effectively distributed to these tissues or that the time to reach measurable concentrations was different from that in the sample collection in our study. Plasma caffeine concentration remained constant during all treatments, showing that hydrolysis and the different regimens had no effect on its absorption profile.

Another important fact is that, in disagreement with that previously reported,[4] in our experiments none of the measured compounds could be detected in the skin. An explanation could be that the author used pure caffeic acid and not a plant extract. It is well known that the bioavailability of natural compounds can be different if administered as isolated or in mixtures as in plant extracts. It was demonstrated that pure ferulic acid is more rapidly absorbed than that in mixture form.[34] As already mentioned, the difference in animal species between our experiments

(rats) and the one reported (mice)[4] could also explain these results, considering the differences in the plasma concentration profile of the studied compounds.

In this article, we were able to demonstrate that enzymatic hydrolysis can be used as a tool to improve antioxidant activity of the hydroethanolic extract of *Ilex paraguariensis*. This treatment of the extract can also increase the plasma concentration of antioxidant compounds, such as caffeic acid, consequently leading to an improvement in plasma antioxidant capacity and an increase in the liver concentration.

AUTHOR INFORMATION

Corresponding Author

*Phone: 55 11 30913631. Fax: 55 11 38132197. E-mail: diogopineda@gmail.com.

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