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ORIGINAL CONTRIBUTION

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Researching on new species of "Mate": *Ilex brevicuspis*

Phytochemical and pharmacology study

Summary *Background Ilex paraguariensis* St. Hilaire (Aquifoliaceae) ("Mate" or "Yerba mate") is one of the most commercialized plants of South America which grows naturally in NE Argentina, Uruguay, SE Brazil and E Paraguay, where it is also cultivated. It is used to prepare a tea-like beverage (infusions or decoctions) appreciated for its peculiar flavor, stimulation and nutritional properties. *Ilex brevicuspis* Reisseck grows in the same habitat and is widely used as a substitute or adulterant of *Ilex paraguariensis*. In a previous work, methylxanthines (caffeine, theobromine and theophylline) were not detected in it by HPLC. *Aim of the study* This study was undertaken in order to isolate, identify and quantify the polyphenolic compounds (caffeoyl derivatives and flavonoids) and to investigate some of the pharmacological activities of *I. brevicuspis*, related with the traditional use of the "Mate" (choleretic, intestinal propulsion and antioxidant activities). Acute toxicity was also investigated. *Methods* Decoctions, like extracts, were prepared in order to compare the results with preparations commonly used by the local people. For the phytochemical analysis, the extracts were analyzed by HPLC with a diode array detector. Choleretic and intestinal propulsion activities were assayed in rats. Sodium dehydrocholate (DHC) was used as a choleretic reference standard. Antioxidant activity was tested in liposomes that were oxidized by the free radical generator 2,2'-azobis [amidinopropane] chloride (AAPH). *Results* For the first time in *I. brevicuspis* the following compounds were isolated and quantified: A) caffeoyl derivative compounds (chlorogenic acid; caffeic acid; 3,4-dicaffeoylquinic acid; 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. B) flavonoids (rutin, quercetin and kaempferol). Biological activity assays demonstrated that *I. brevicuspis* extracts produced a significant increase of bile flow (BF) in rats in the first 30 min period and in the percentage of BF increase accumulated at 120 min. It also produced an increase in the intestinal propulsion activity. Moreover, this species showed a high antioxidant activity. The acute toxicity test showed that *Ilex brevicuspis* did not produce any sign of toxicity at the analyzed doses. *Conclusions* An Argentinean *Ilex* specie *(I. brevicuspis)* has choleretic, intestinal propulsion, antioxidant activities and these results may lead to the potential development of a new "Yerba Mate" and/or phytopharmaceutical products, without central nervous system (CNS) stimulant activity.

Key words *Ilex brevicuspis* – HPLC – antioxidant – choleretic – acute toxicity

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Introduction

"Mate" or "Yerba Mate" (*Ilex paraguariensis* St. Hil., Aquifoliaceae) is used in north-eastern Argentina, southern Brazil and eastern Paraguay [1] to prepare a tea-like beverage (infusions or decoctions) appreciated for its peculiar flavor, stimulation (due to the xanthines: caffeine and theobromine content) [2] and also nutritional properties [3]. It is one of the most commercial-

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ized plants of South America where approximately 30 % population drink more that 1 L/day of this beverage [4]. It is also traditionally used in gastrointestinal disorders as eupeptic and choleretic [5]. It is included in the Argentine Food Code, Latin-American Food Code and in the main Pharmacopoeias (Martindale, British Herbal Pharmacopoeia, German Commission E Monographs). Caffeoyl derivative compounds have been isolated from this specie [6]. Phenylpropanoids compounds (caffeoyl derivatives and flavonoids) are usually recognized as compounds responsible for the antioxidant and choleretic activities of plant extracts [7–9]. Recent works support the role of these compounds as protective agents against cardiovascular disease, breast, esophageal, gastrointestinal, lung and skin cancer [10].

Ilex brevicuspis Reisseck grows in the same habitat and is locally used as a substitute or adulterant of *Ilex paraguariensis*. Some authors suggested the use of this specie in order to enhance the quality of yerba mate [11]. In a previous work, methylxanthines (caffeine, theobromine and theophylline) were not detected in it by HPLC (detection limit: 0.2 ppm) [2].

Given the lack of research on this specie, this study was undertaken in order to isolate, identify and quantify the polyphenol compounds (caffeoyl derivatives and flavonoids) by HPLC with a diode array detector, and to investigate the pharmacological activities of *I. brevicuspis* extracts, related with the traditional use of the "Mate" (choleretic, intestinal propulsion and antioxidant activities). Acute toxicity was also investigated.

Material and methods

Plant material

I. brevicuspis Reisseck was collected in the original habitat (Misiones, Argentina) and identified in comparison with voucher-specimen of herbarium standards. A sample was deposited in the CEFYBO Herbarium (Buenos Aires) under the number BACP Nº: 94.

Preparation of plant extracts

Dried leaves were ground to fine powder. Decoctions extracts were prepared according to Farmacopea Nacional Argentina [12] in order to compare with preparations commonly used by local people. Three grams was boiled with 30 ml of water during 20 min, then it was cooled to 40–45 °C, filtered and the volume adjusted to 10 ml.

Determination of caffeoyl derivative compounds and flavonoids

High performance liquid chromatography

Caffeoyl derivative compounds and flavonoids were resolved with a reverse phase column applying a gradient, using as the mobil phase: Solvent A: water:acetic acid (98:2); solvent B: methanol:acetic acid (98:2). Gradient: 15 % B to 40 % B, 30 min; 40 % B to 75 % B, 10 min; 75 % B to 85 % B, 5 min. Flow rate: 1.2 ml/min. The separation column was IB-SIL RP 18 (5 µm, 250 x 4.6 mm I. D.) Phenomenex. Detection was carried out by UV Varian 9050 UV Detector and Varian 9065 Photodiode-Array Detector. UV: 325 nm (caffeoyl derivatives); 255 nm (rutin); 254 nm (quercetin); 263 nm (kaempferol). The equipment had a Rheodyne injector, fitted with a 100 µl loop.

Quantification was achieved by the external standard method using Carl Roth chlorogenic acid, caffeic acid, rutin, quercetin and kaempferol standards. The amount of 3,4-dicaffeoylquinic and 3,5-dicaffeoylquinic isomer acids were calculated and expressed as the 4,5-dicaffeoylquinic acid, which was isolated from *Pterocaulon virgatum* (Asteraceae) [13].

Validation of quantitative analysis

Suitable quantities of each standard compound were weighed and mixed in a volumetric flask, dissolved and diluted with water:methanol (3:7). Curves showed a linear relationship between the amount of each compound and its peak area and followed Beer's Law. The regression equations of these curves and their correlation coefficients were calculated as follows: $Y = 1.15 \times 10^4 X - 2.12430$ ($r^2 = 0.9995$) for chlorogenic acid. $Y = 2.23 \times 10^4 X - 5.8470$ ($r^2 = 0.9982$) for caffeic acid. $Y = 1.18 \times 10^4 X - 27.4065$ ($r^2 = 0.9993$) for 4,5-dicaffeoylquinic acid. $Y = 2.08 \times 10^3 X - 1425$ ($r^2 = 0.9992$) for rutin. $Y = 2.35 \times 10^4 X - 2325$ ($r^2 = 0.9983$) for quercetin. $Y = 1.95 \times 10^4 X - 4028$ ($r^2 = 0.9994$) for kaempferol, where X is the amount of each compound expressed as mg/ml and Y is the peak area given by the data processor. Analyses were carried out in triplicate and the intraassay variation coefficient was below 2 %, with a quantification limit of 1 ppm. Recovery studies were carried out in order to assess the efficiency of the quantification, extraction procedure and HPLC assay. The mean recoveries were up to 95 %, thus, validating the outlined method.

Antioxidant capacity assay

Liposomes were oxidized in the presence of 2,2'-azobis [amidinopropane] chloride (AAPH), which generates free radicals initially in the aqueous phase [14]. Lipo-

somes (500 µl, 0.5 mg/mL) were incubated at 37 °C, for 60 min, with 20 µL of AAPH (10 mM final concentration) in the absence (maximum oxidation) or the presence of 20 µL of 1/100–1/1000 dilution of the extracts in water (equivalent to the addition of 20–200 nL of the original extract). Lipid damage to liposomes was evaluated by the measurement of a final product of lipid oxidation [2-thio-barbituric acid reactant substances (TBARS) assay] [15, 16]. The antioxidant capacity of the extracts was calculated by the following expression:

[1- (value of the sample/value of maximum oxidation)] x 100

and expressed as % of inhibition.

Preparation of liposomes

Liposomes were prepared as described by Oteiza [17].

TBARS assay

After incubating with AAPH, 100 µL butylated hydroxyanisole (4 % in ethanol), 250 µL of 3 % (w/v) sodium dodecyl sulfate, and 500 µL of 1 % (w/v) thiobarbituric acid were added, then and acidified with 500 µL of 7 mM hydrochloric acid [18]. The samples were heated for 15 min at 95 °C, and extracted with 2 mL 1-butanol. TBARS were measured fluorometrically (excitation/emission: 515/555 nm).

The antioxidant activity is defined as the volume of the plant extract that produced a 50 % inhibition of TBARS formation and is expressed as the concentration of trolox (a reference antioxidant) that produced the inhibition. A sample of red wine was also included in the assay.

Choleretic and intestinal propulsion activity

Animals

Female Wistar rats (180–210 g) and male Swiss mice were used in this study. They were housed under standard conditions (23 ± 1°C, humidity 60 ± 5 %, 12 h light/dark cycle), and fed with standard diet and tap water ad libitum for one week prior to treatment.

Bile flow

Rats were starved for 18 h before the experiment with free access to water. Animals were anesthetized with urethane (1.2 g/kg imp.). Tracheotomy and femoral vein cannulation were performed. The abdomen was opened by a midline incision and the common bile duct exposed and cannulated just before the hepatic hilus in order to avoid contamination with pancreatic juice. Rectal temperature was monitored and maintained at 37 ± 0.5 °C throughout the experiment, using a warming lamp. Bile was collected by gravity in pretared vials at 30 min intervals for 120 min. Bile flow was determined by weight assuming that the specific gravity of rat bile is 1.0 and was expressed as mg/min/100 g body weight. After a constant basal flow, bile was collected during 30 min (basal value). One group of five rats was then treated with the *Ilex brevicuspis* (plant decoctions) dissolved in 0.9 % NaCl solution at a dose of 250 mg/kg. A reference group of five rats received 20 mg/kg of sodium dehydrocholate (DHC), and a control group received saline solution. All substances were injected in the femoral vein at 0.2 ml/100 g. Every 30 min after the infusion, DHC or saline injection and over a total 120 min, variation of basal bile flow (BF) for each animal was calculated using the following formula:

(BF-Basal BF)/basal BF x 100 = %BF

Results are also expressed in accumulated percentage of bile flow for extracts, DHC and control. In all groups the variation of basal bile flow (%BF) for each animal and for every 30 min period was calculated and then accumulated for 120 min [19].

Bile acids

The concentrations of bile acids were measured from 30 min before to 30 min after extract administration. The method of Carducci et al. [20] was followed for quantitative determination of bile acids by HPLC.

Intestinal transit

Mice were fasted for 24 h before use, with access only to tap water. Animals were orally given 0.3 ml of an aqueous suspension of 10 % charcoal in 1 % carboxymethylcellulose [21]. Twenty minutes later, mice were killed by cervical dislocation, and the stomach and intestines excised from the gastroesophageal junction to the ileocecal junction. The distance the charcoal had traveled from the pylorus was measured, and expressed as the percentage of the total length of the small intestine from the gastropyloric junction to the ileocecal junction (intestinal transit). For studying the effects of *I. brevicuspis* on intestinal transit, decoctions were administered orally at a dose of 500 mg/kg, 30 min before charcoal.

Data analysis

Results are expressed as means ± SEM. Differences between control and treated batches in these experiments

were tested for significance using a one-way analysis of variance (ANOVA), followed by Dunnett's t-test, taking $p < 0.05$ as significant.

Acute toxicity test

Groups of 10 CF-1 mice, 5 male and 5 female weighing 18–23 g each, were used. The animals had free access to a standard diet and water *ad libitum* in a 12 h light-dark cycle at 22 °C. Aqueous extract of *I. brevicuspis* was administered orally by means of a gastric catheter at a dose of 3 g/kg (0.5 ml/25 g body weight). Food was withdrawn 16 h before the day of the experiment. Toxicity was evaluated over 30 days in terms of weight loss, autonomic and neurobehavioral profile and animals were killed by cervical dislocation. Vital organs were observed.

Results and discussion

Dietary components contribute to the antioxidant activity in human tissues. There is evidence that plant-derived compounds may have beneficial effects on human health and that some of them (caffeoyl derivatives and flavonoids) exert antioxidant activity [14–15, 22].

Identification of constituents was carried out by comparison of retention times and spectra obtained on the diode array detector with the standards. The following compounds were isolated and quantified and the results are expressed as % on dried weight: A) caffeoyl derivative compounds (chlorogenic acid: 0.915 ± 0.064; caffeic acid: 0.005 ± 0.001; 3,4-dicaffeoylquinic acid: 0.130 ± 0.010; 3,5-dicaffeoylquinic acid: 0.360 ± 0.060 and 4,5-dicaffeoylquinic acid: 0.490 ± 0.040); B) flavonoids (rutin: 0.0022 ± 0.0003; quercetin: 0.0006 ± 0.0002 and kaempferol: 0.0003 ± 0.0001).

The antioxidant activity, expressed as equivalents of a well-recognized antioxidant (trolox) for the 50 % inhibition of TBARS was 7.78 (mM trolox). On the basis of previous research, it has been established that the antioxidant activity of 150 mL of red wine is equivalent to 300 mL of green or black tea [23]. According to the ability to prevent liposomes oxidation showed here, the antioxidant activity of the extracts of *I. brevicuspis* was slightly lower compared with red wine, in equivalents of trolox (7.78 vs 8.85 mM, respectively).

The effect on bile excretion showed that control rats presented a slight regular decrease in bile flow level during the experiment. The administration of DHC at 20 mg/kg induced marked though transient stimulation of bile flow during the first 30 min (55 %), which decreased during the following 30 min to reach a non-significant hypercholeresis at 90 min. Significant increases in bile flow were obtained with the extracts of *I. brevicuspis* in the first 30 min period (38 %) in doses of 250 mg/kg and non-significant hypercholeresis at 90 min (Fig. 1).

The increase in bile flow produced by *I. brevicuspis* extract was not associated with any significant change in biliary excretion of bile acids. A similar effect was seen with DHC. This probably indicates that *I. brevicuspis* is a hydrocholeretic-like agent. When decoctions were assayed for intestinal propulsion, *I. brevicuspis* was found to induce a 22 % increase versus the control group, at a 500 mg/kg dose (Table 1), but no effect was discernible at lower doses.

We also found that the aqueous *I. brevicuspis* extract did not produce any sign of toxicity at the analyzed doses. No mortality was observed during the study period (30 days) and at necropsy, no macroscopic changes in viscera could be detected in the treated groups.

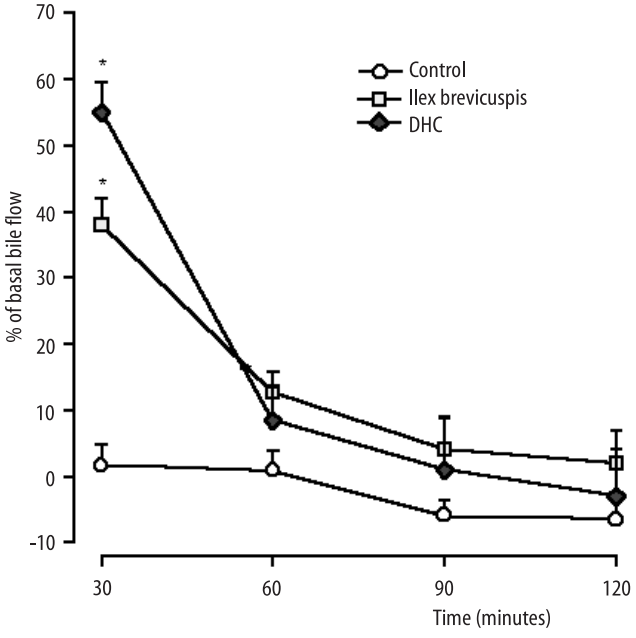

Fig. 1 Effect of *Ilex brevicuspis* decoctions (250 mg/kg) sodium dehydrocholate (DHC) (20 mg/kg) and saline solution (control) on bile flow in the rat. Results are expressed as means ± SEM. * $p < 0.01$ vs control

Table 1 Effect of *I. brevicuspis* decoctions (250 mg/kg) on bile acids secretion and intestinal transit[a]

Treatment	Bile acids output (nmol/min/kg)	Intestinal transit (%)[b]
Control	10.49 ± 3.74	47.03 ± 1.25
I. brevicuspis	9.51 ± 2.85	61.34 ± 3.09*
Sodium dehydrocholate	7.54 ± 2.24	–

* $p < 0.01$ vs control
[a] Values are means ± SEM
[b] Intestinal transit is expressed as the percentage of the distance traveled by the charcoal with respect to the total length of the small intestine

Conclusions

According to the results obtained, *I. brevicuspis* extracts have a high content of the most important caffeoyl derivative compounds and flavonoids in comparison with other *Ilex* spp. used as substitutes or adulterants of "Yerba Mate" [24] and this fact could explain the antioxidant and choleretic activities demonstrated in this work. It also induced an increase in intestinal transit in the experimental conditions employed and did not produce any sign of toxicity at the analyzed doses.

The above-mentioned results lead to the potential development of a new "Yerba Mate" and/or phytopharmaceutical products, without CNS stimulant substances due to the absence of xanthines in this species.

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