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Article

Methylxanthines Accumulation in *Ilex* Species - Caffeine and Theobromine in Erva-Mate (*Ilex paraguariensis*) and Other *Ilex* Species

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Ilex paraguariensis St.Hil. é uma espécie de importância econômica tendo em vista sua utilização na região meridional da América do Sul no preparo do chimarrão, uma bebida estimulante, preparada pela infusão de suas folhas e talos moídos. Este trabalho apresenta a análise de metilxantinas em folhas de *I. paraguariensis* e outras espécies de *Ilex* freqüentemente relatadas como adulterantes. A determinação do teor de metilxantinas realizada por CLAE constatou a presença de cafeína (0,65%) e teobromina (0,12%) em *I. paraguariensis* var. *paraguariensis*, cafeína (0,003%) e teobromina (aprox. 0,22%) em *I. paraguariensis* var. *vestita*, e ausência de metilxantinas em *I. brevicuspis*, *I. dumosa* e *I. microdonta*. Os resultados indicam que a acumulação de cafeína e teobromina é, até o momento, uma característica única de *I. paraguariensis*. Além do interesse taxonômico, esses resultados sugerem a possibilidade de identificar adulterações em erva-mate utilizando a metodologia descrita.

Ilex paraguariensis St.Hil. is an important crop used commonly at the meridional South America as a source of a stimulant beverage, called maté, prepared by infusion of its processed leaves and twigs. We describe herein the methylxanthines analyses in the leaves of *I. paraguariensis* and other *Ilex* species reported as maté adulterants. The methylxanthines content determined by HPLC were 0.65% for caffeine and 0.12% for theobromine from *I. paraguariensis* var. *paraguariensis*, and 0.003% for caffeine and 0.22% (estimated) for theobromine from *I. paraguariensis* var. *vestita*. It was not detected any methylxanthines from *I. brevicuspis*, *I. dumosa* and *I. microdonta*. Considering the results, simultaneous caffeine and theobromine accumulation seems hitherto to be a particular characteristic of *I. paraguariensis*. In addition to taxonomical significance, these data suggest that it should be possible to trace adulterations of the genuine *I. paraguariensis* using the methodology described herein.

Keywords: Ilex, *methylxanthines, HPLC of methylxanthines*

Introduction

Ilex paraguariensis St. Hil. is a South American native perennial tree belonging to the "holly" family (Aquifoliaceae). It has been used historically as a source of a mildly stimulant beverage, called maté ("erva-mate" or "yerba-mate"), prepared by infusion of its dried leaves and twigs. Although the production of maté is becoming industrialized, its adulteration by variable quantities of leaves of other South American *Ilex* species is still frequent[1,2]. These added leaves might modify its physiological and pharmacological activities, as some of these species do not contain caffeine[3,4]. Despite many publications concerning the determination of purine alkaloids in maté leaves and commercial samples[5-10], the knowledge of xanthine content of other South American *Ilex* species remained rather poor until recently[4,11,12]. Additionally, in our systematic investigation of *Ilex* species, we found chemical differences in the saponins structures[13,14]. Continuing our efforts to develop methodologies for the quality control of maté products based on its chemical composition, we describe herein the analyses of *I. brevicuspis* Reissek, *I. dumosa* Reissek, *I. microdonta* Reissek, *I. paraguariensis* St. Hil. var. *paraguariensis*, and *I. paraguariensis* St. Hil. var. *vestita* (Reiss.) Loes. by TLC and HPLC determination of methylxanthines in their leaves.

Experimental

Plant material

Leaves of *I. paraguariensis* var. *vestita* were collected in August 1996 in the city of Ivaí, PR, Brazil, and mature leaves of other *Ilex* species were collected in February 1997 at the Botanic Garden of Porto Alegre, RS. Herbarium specimens are on deposit in the Botany Department Herbarium of Rio Grande do Sul Federal University, Porto Alegre, Brazil.

Methylxanthines extract

15 g of dried ground leaves were boiled for 10 min in a 20% (v/v) sulfuric acid aqueous solution (150 mL), then filtered. The filtrate, after being neutralized with a 25% (v/v) ammonium hydroxide aqueous solution, was extracted four times using 50 mL of a chloroform:isopropanol mixture (3:1, v/v). The dried organic phase was concentrated yielding the methylxanthines extract (for yields see Table 1). This residue was submitted to HPLC analyses by dilution in the mobile phase, then evaporated to dryness for TLC analyses. The final extract concentrations used for HPLC analyses were 477.6 μg/mL for *I. brevicuspis*, 569 μg/mL for *I. dumosa*, 684 μg/mL for *I. microdonta*, 51.56 μg/mL for *I. paraguariensis* var. *paraguariensis*, 7.93 μg/mL and 635 μg/mL for theobromine and caffeine detection, respectively, for *I. paraguariensis* var. *vestita*.

TLC

The methylxanthines extract was diluted in chloroform:methanol (40:60, v/v) to obtain a solution with 250 μg/mL. 30 μl of the extract were applied on the plates using an automatic dispenser. Chromatographic conditions were modified from literature[15] as follows: Silica gel 60 F_{254} (Merck aluminium sheets), eluent CH_2Cl_2:EtOH (90:5, v/v), in a saturated chamber using small dishes with 30% (v/v) aqueous NH_4OH, detection by quenching of the fluorescence in UV_{254}.

HPLC

A liquid chromatograph (WATERS, model 600E) with a Rheodyne injection valve fitted with a 20 μL injection loop, a variable ultraviolet detector (WATERS, model 486), and an integrator (WATERS, model 747) were used. Chromatographic separation was accomplished using column NovaPack® RPC8 (3.9 x 150 mm I.D., 5 μm), precolumn RPC18 (3.0 x 39 mm I.D., 50 μm).

An isocratic system using methanol:water (25:75, v/v) as mobile phase was used at a flow rate of 0.5 mL/min at room temperature (21 °C). Detection was at 280 nm at 0.05 AUFS. Sample solutions were injected in triplicate and their peak areas were compared with the calibration curve. Methanol was of HPLC grade (Merck).

Calibration curve for HPLC

Standard solutions of caffeine (5.0, 10.0, 15.0, 20.0 and 25.0 μg/mL) and theobromine (1.25, 2.5, 5.0, 7.5 and 10.0 μg/mL) were prepared using the mobile phase as solvent. The standard solutions were injected in triplicate and the peak area measured. The linearity was evaluated by linear regression and the precision and accuracy were determined by coefficient of variation (CV). Caffeine and theobromine were purchased from Merck as analytical grade.

Results and Discussion

The results of TLC analyses of methylxanthines extracts from leaves of *Ilex* species are shown in Table 2. Theophylline was not detected in any sample whereas caffeine and theobromine were only detected in the two *I. paraguariensis* varieties. It is important to remark that for *I. brevicuspis* and *I. microdonta* other substances probably related to purine alkaloids were detected and, on the contrary, *I. dumosa* did not present any methylxanthine in TLC.

Table 1. Yields of extracts and caffeine and theobromine contents determined by HPLC from leaves of *Ilex* species[a]:

Ilex species	Yield of methylxanthines extract	Caffeine (m/m)	Theobromine (m/m)
I. brevicuspis	59.7 mg (0.40%)	not detected	not detected
I. dumosa	56.9 mg (0.39%)	not detected	not detected
I. microdonta	85.5 mg (0.57%)	not detected	not detected
I. paraguariensis var. *paraguariensis*	257.8 mg (1.72%)	0.646% CV 0.38%	0.12% CV 0.27%
I. paraguariensis var. *vestita*	48.0 mg (0.32%)	0.003% CV 0.77%	b

a) Standard and sample solutions were injected in triplicate.
b) Theobromine was eluted with another substance, the resolution of both peaks was not adequate for quantitative determination.

Table 2. R_f values for standard methylxanthines and for detected substances by TLC in methylxanthines extracts from leaves of *Ilex* species:

Standard compound or sample	R_f
caffeine	0.79
theobromine	0.29
theophylline	0.07
I. brevicuspis	0.96, 0.69, 0.21
I. dumosa	no methylxanthine detected
I. microdonta	0.96, 0.69, 0.21
I. paraguariensis var. *paraguariensis*	0.79, 0.69, 0.29
I. paraguariensis var. *vestita*	0.69, 0.29, 0.21

Typical HPLC chromatograms of caffeine and theobromine standards, and methylxanthines extract of *I. paraguariensis* var. *paraguariensis* are shown in Fig. 1. The regression equations ($y = ax + b$) and their correlation coefficients (r) were calculated as follows: $y = -45846.568 + 425376.534x$ ($r = 0.99997$) for caffeine and, $y = -545.51549 + 210297.36x$ ($r = 0.99987$) for theobromine. The retention time (rt) of caffeine was 8.24 min and that of theobromine was 4.03 min. Theophylline (rt = 5.30 min) was not detected by HPLC assay, even using extracts on higher concentration. The sharp and symmetrical peaks were obtained with good baseline resolution and minimal tailing, thus facilitating accurate measurement of the peak area ratio. A HPLC mobile phase with 75% of water was idealized which is better than the usual buffered ones[7,8,10,12], as it does not damage the equipment and is less expensive and easy to prepare. In this study, the HPLC procedure used had advantages of simplicity, precision, and ruggedness.

Quantitative data for methylxanthines in different *Ilex* samples are presented in Table 1. Caffeine and theobromine were detected only in the two *I. paraguariensis* varieties, and surprisingly, in opposite proportions. The methylxanthines values determined were 0.65% for caffeine and 0.12% for theobromine from mature leaves of *I. paraguariensis* var. *paraguariensis*. It is reported that leaves of this *I. paraguariensis* variety have caffeine levels varying from 0.16%, in old leaves, to 1.4%, in young leaves, and theobromine levels varying from 0.02%, in old leaves, to 0.27%, in young leaves[9,10,16]. In this study, the minute quantities of caffeine observed for the *vestita* variety, comparing to the *paraguariensis* variety, together with the presence of theobromine are very important, and may explain some intriguing results observed for commercial samples for which significant amounts of theobromine were reported[8].

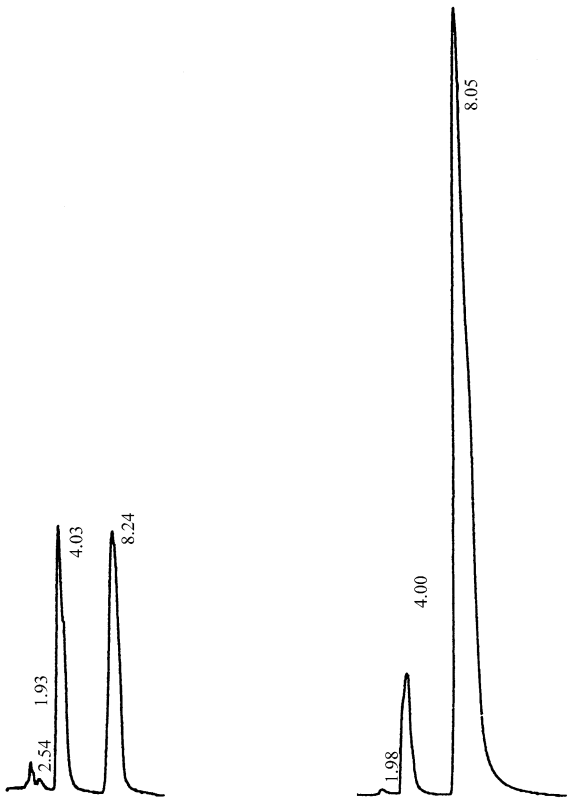

Figure 1. Chromatograms of theobromine (rt = 4.03 min) and caffeine (rt = 8.24 min) standard solutions [**A**] and methylxanthines extract from leaves of *Ilex paraguariensis* var. *paraguariensis* [**B**]. Chromatographic conditions: Column: WATERS Nova-Pack® RPC8 (3.9 x 150 mm I.D., 5 μm); mobile phase: methanol:water (25:75, v/v); flow rate: 0.5 mL/min; detection 280 nm (0.05 AUFS).

The methylxanthines values reported herein should be taken carefully as the influence of different factors on maté chemical composition is known, such as the genetic variability, the environmental conditions, harvest period, and industrial treatment of the raw material[9,10,16,17]. Notwithstanding this variability, the literature data cited herein allowed to indicate a minimal caffeine content for maté, presently established in Brazilian legislation as 0.5% (m/m)[18] for commercial maté. Recent publication also showed that only in *I. paraguariensis* the simultaneous presence of caffeine and theobromine could be detected considering the species commonly used as maté adulterants[12]. Considering this report and our results, the use of congeneric *Ilex* species to adulterate maté would affect significantly the methylxanthines content.

I. paraguariensis var. *paraguariensis* is the genuine maté, and has a large area of native dispersion, on the contrary, *I. paraguariensis* var. *vestita*, the pubescens variety, has a small area of native dispersion[19]. We are

currently investigating the variability of the methylxanthines content in populations of *I. paraguariensis* var. *vestita*.

Conclusions

Considering the results obtained, simultaneous caffeine and theobromine accumulation seems hitherto to be a particular characteristic of *I. paraguariensis*. In addition to taxonomical significance, these data suggest that it should be possible to trace adulterations of the genuine *I. paraguariensis* using the methodology described herein. To our knowledge, this is the first report of methylxanthines analyses in *I. paraguariensis* var. *vestita*.

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