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Original Article

The influence of leaf age on methylxanthines, total phenolic content, and free radical scavenging capacity of *Ilex paraguariensis* aqueous extracts

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ABSTRACT

Yerba-mate (*Ilex paraguariensis* A. St. Hil., Aquifoliaceae) is a South American native species that is widely used for its industrial potential in the preparation of drinks, teas and cosmetics. Its properties are directly related to the presence of its chemical constituents, such as saponins, methylxanthines and phenolic compounds. This study aimed to investigate the influence of leaf age on methylxanthine and total phenolic contents by High Performance Liquid Chromatography and Ultraviolet Spectroscopy, as well as on free radical scavenging capacity, of aqueous extracts of *I. paraguariensis* leaves. The results showed great variability in all the metabolites measured. Leaf ageing significantly increased the methylxanthine content and total phenolic content of the extracts. Free radical scavenging capacity was also significantly affected ($p < 0.05$) by leaf age. A positive correlation was observed, between the antioxidant activity and total phenolic content.

Introduction

Ilex paraguariensis A. St. Hil., Aquifoliaceae, is a South American native perennial tree that is popularly known as “yerba-mate” or “mate”. It is one of the most popular and widely-consumed beverages in southern Brazil, Argentina, Paraguay, and Uruguay, where it is used as a decoction or infusion. Mate is used for its central nervous system stimulant properties, which are due to the presence of the methylxanthines caffeine and theobromine (Blumenthal and Brinckmann, 2000; Dermarderosian, 2001; Filip et al., 1998). Additionally, yerba-mate is also considered a functional food, because of its nutritional and medicinal properties, such as hypocholesterolemic, hepatoprotective, diuretic, and antioxidant properties (Bixby et al., 2005; Filip et al., 2000; Gugliucci and Stahl, 1995; Heck and De Mejia, 2007; Rivelli et al., 2007; Valerga et al., 2012), which can protect against the harmful effect of free radicals, thereby increasing the defense system of the organism. It can also help prevent atherosclerosis and coronary heart disease (Heck and De Mejia, 2007; Miranda et al., 2008; Puangpraphant and de Mejia, 2009; Boaventura et al., 2012).

These health benefits have been attributed to phenolic compounds, which are major constituents of *I. paraguariensis* (Heck and De Mejia, 2007). The main polyphenols present in “mate” are caffeoyl derivatives (chlorogenic, 3,5-dicaffeoylquinic, 4,5-dicaffeoylquinic and 3,4-dicaffeoylquinic acids), and caffeic acid. Moreover, yerba-mate also contains high methylxanthines, saponins, and a minor content of flavonoids, such as quercetin, rutin and kaempferol (De Souza et al., 2011; Coelho et al., 2010; Filip et al., 2001; Reginatto et al., 1999; Gosmann et al., 1995).

It is widely known in natural product chemistry that the growth conditions play a role in the production of phytochemicals in the plant (Gobbo-Neto and Lopes, 2007; Meyer et al., 2006). In regard to age of the leaves, there have been few reports showing its influence on metabolite content. Such as Esmelindro et al. (2004) showed that young leaves of *I. paraguariensis* contain a high production of methylxanthines, and Dartora et al. (2011) reported no significant differences between phenolic and methylxanthine contents in leaves at 1 and 6 months of growth. In addition, these reports suggest that intrapopulation genetic conditions, such as age of the leaves, play an important role in the distribution of these compounds in *I. paraguariensis*. Finally, knowledge about this chemical composition is important for our understanding of the changes in potential biological activities of *I. paraguariensis*. The present work therefore assesses the influence of leaf age on the phytochemical

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composition of *I. paraguariensis*, and on its free radical scavenging activity.

Materials and methods

Plant material

The leaves of 11 trees of *I. paraguariensis* A. St. Hil., Aquifoliaceae, from a native population were collected at Chapecó, in the State of Santa Catarina, Brazil (27°08′48″ S; 52°37′01″ W). The plant samples were cultivated under natural sunlight conditions. The plant material (RSPF 11074) was harvested in October 2010 and the leaves were separated according to age, as defined by embranchment; leaves at one month (first to third leaf pairs from the branch tips), at two months (forth to eighth leaf pairs) and at six months (ninth to fifteenth leaf pairs). All the samples were immediately frozen, lyophilized, crushed separately, and stored at −20 °C until tested.

Extraction

The extracts of each sample were prepared by aqueous infusion. Briefly, five grams of each dried leaf sample was mixed with 100 ml of distilled water (90 ± 2 °C) for 20 min. The extracts were filtered, the volumes adjusted to 100 ml with water, and the samples frozen.

HPLC-DAD analysis of methylxanthines

The quantitative analyses of caffeine and theobromine in the extracts were carried out in a PerkinElmer Series 200 High Performance Liquid Chromatography (HPLC) equipped with a Diode Array Detector (DAD), quaternary pump, online degasser and autosampler. Chromera® Workstation software was used for the data acquisition. The injection volume was 20 μl and the baseline resolution was obtained at room temperature (24 ± 2 °C). For the methylxanthine analysis, separation was performed on a Perkin Elmer Brownlee Choice C_8 column (150 mm × 4.6 mm i.d.; 5 μm) and a mixture of methanol/ammonium hydroxide 0.2% (20:80 v/v) as the mobile phase, with constant flow rate at 0.9 ml min^{-1}. The mobile phase was prepared daily and degassed by sonication before use. The chromatograms were recorded at 280 nm, while the UV spectra were monitored over a range of 200–450 nm. Peaks were characterized by comparing the retention time and UV spectra with the reference standards, and by co-injection of the authentic samples. The standard solutions were prepared in different ranges: 0.625–400 μg ml^{-1} for the caffeine (Sigma-Aldrich®) and 0.3125–75 μg ml^{-1} for the theobromine (Fluka®). The extracts were analyzed at a concentration of 2.00 mg ml^{-1}. Quantification of caffeine and theobromine was performed using seven-point regression curves ($r^2 > 0.999$). The regression equations were "$y = 16478x + 11339$" for caffeine and "$y = 26525x + 1930$" for the theobromine. All analyses were performed in triplicate, and the peak average areas were measured. The results were expressed as milligrams of compound per g of extract (mg compound g^{-1} E).

Total phenolic content

The determination of total phenolic content (TPC) was performed as described by Medina (2011a,b) based on the direct interactions of polyphenols with Fast Blue BB diazonium salt (Sigma-Aldrich®). Seven chlorogenic acid (Fluka®) calibration standard points ($r^2 = 0.999$) were prepared within the range of 10–150 μg ml^{-1} in distilled water and 1.0 ml of each was transferred to a borosilicate tube. A 0.1 ml aliquot of 0.1% Fast Blue BB reagent was added to all the chlorogenic acid standard tubes, mixed for 1 min, and then 0.1 ml 5% NaOH was added. The reaction was allowed to complete at room temperature (24 ± 2 °C) for 90 min and the optical density was measured at 420 nm. The TPC of the *I. paraguariensis* extracts were determined as described above, except that each sample was analyzed with a blank containing only the sample, to measure natural non-phenolic interferences at 420 nm. The results were expressed as milligrams of chlorogenic acid equivalents per g of extract (mg CA g^{-1} E).

Free radical scavenging capacity

The free radical scavenging capacity was determined as previously described Brandwilliams et al. (1995). Briefly, 0.1 ml of each sample extract at four different concentrations was added to 3.9 ml of a methanolic solution of 2,2-diphenyl-1-picrylhydrazyl [DPPH (60 μM)]. The absorbances were measured at 515 nm (Lambda25 UV/Vis, PerkinElmer®). The percentage of remaining DPPH (Sigma-Aldrich®) was calculated and plotted against the sample concentration, in order to obtain the EC_{50}, which was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. Chlorogenic acid was used as positive control.

Validation of HPLC-DAD analysis of methylxanthines

The analytical procedures were validated according to Cass and Degani (2001) and the ICH guidelines (ICH). The validated parameters were specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ) and limit of optical detection (LOD).

Data analysis

Data were expressed as mean values ± S.E.M. from three independent measurements. For the determination of EC_{50} values, linear regressions of concentration–response curves were used. Differences between treatments were compared by ANOVA analysis of variance followed by Tukey's test adopting $\alpha = 0.05$.

Results and discussion

Validation of HPLC-DAD analysis of methylxanthines

The analytical curves of both authentic standards showed good linearity ($r^2 > 0.999$). Linear regression equation for the calibration curve were "$y = 16482x + 10373$" for caffeine and "$y = 26495x + 2422.7$" for theobromine (Fig. 1).

The observed values of validation parameters are summarized in Table 1.

The HPLC-DAD quantifications showed good linear relationships between peak area and concentration ($r^2 > 0.999$) for all standard solutions in both methods. The limit of quantification (LOQ) and limit of detection (LOD) were defined by relative standard deviation (RSD < 5%) and by a signal:noise ratio of 3:1, respectively. The precision was determined by repeatability (intra-day assay) and intermediate precision (inter-day assay) (Cass and Degani, 2001; ICH, 2005). The intra-day assay was performed by triplicate analysis of three different concentrations of standard solutions, and expressed as relative standard deviation. Good repeatability was obtained from lower, medium and higher concentrations of the curve, with an RSD ≤ 3.96% for all standard analyses. The inter-day assay was determined by the analysis of a medium concentration in the curve, three times a day, on three different days. As in repeatability, the intermediate precision RSD value did not exceed the limits recommended in the literature (Cass and Degani, 2001; ICH, 2005). In relation to accuracy, good recovery was observed in the extract for all the standards.