



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.

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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 (fourth 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 ($90 \pm 2^{\circ}\text{C}$) 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 ($24 \pm 2^{\circ}\text{C}$). For the methylxanthine analysis, separation was performed on a Perkin Elmer Brownlee Choice C₈ column (150 mm \times 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 $\mu\text{g ml}^{-1}$ for the caffeine (Sigma-Aldrich[®]) and 0.3125–75 $\mu\text{g ml}^{-1}$ 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 $\mu\text{g ml}^{-1}$ 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 ($24 \pm 2^{\circ}\text{C}$) 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 EC₅₀, 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 \pm S.E.M. from three independent measurements. For the determination of EC₅₀ 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 $\leq 3.96\%$ 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.

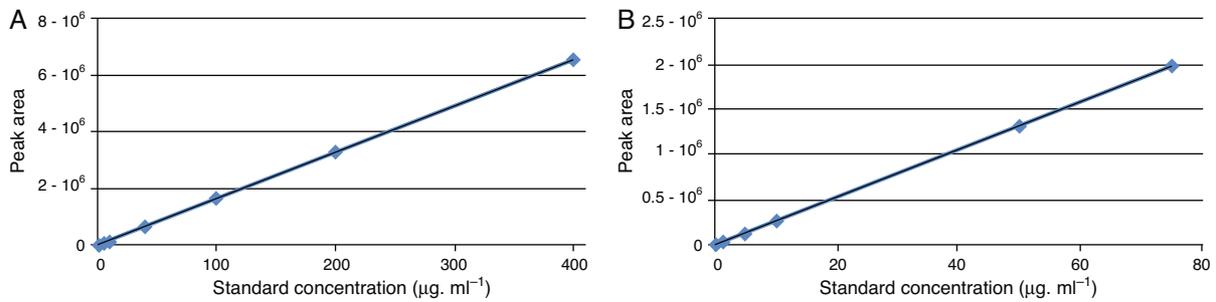


Fig. 1. CLAE-DAD calibration curves for caffeine (A) and theobromine (B).

Table 1

Validated analytical parameters for the HPLC-DAD quantification of caffeine and theobromine in aqueous infusions of *Ilex paraguariensis*.

Compound	Precision ^a				Accuracy ^b (recovery)		LOQ ^c ($\mu\text{g ml}^{-1}$)	LOD ^c ($\mu\text{g ml}^{-1}$)
	Repeatability		Intermediate precision		Mean (%)	R.S.D. (%)		
	Mean ($\mu\text{g ml}^{-1}$)	R.S.D. (%)	Mean ($\mu\text{g ml}^{-1}$)	R.S.D. (%)				
Caffeine	0.625	3.96	100.00	1.52	99.8	1.74	0.625	0.10
	100.00	0.97						
	400.00	1.35						
Theobromine	0.3125	4.08	15.00	0.87	101.3	1.33	0.3125	0.10
	15.00	1.26						
	75.00	1.85						

^a Limit: R.S.D. < 5%.

^b Recovery was determined by injection of spiked samples, in triplicate, with standard solution.

^c LOQ = limit of quantification; LOD = limit of detection.

Methylxanthines content

In the chromatographic analysis of methylxanthines, theobromine and caffeine presented retention times of 3.1 and 9.1 min, respectively. Fig. 2 shows the methylxanthine chromatogram of aqueous extract from *I. paraguariensis* leaves of sample 2. Theophylline was not detected in any sample analyzed, a finding that is corroborated by several studies in the literature (Athayde et al., 2000; Clifford and Ramirez-Martinez, 1990; Coelho et al., 2007; Filip et al., 1998; Reginatto et al., 1999).

The data analysis showed that there is great variability in caffeine and theobromine contents in this population of *I. paraguariensis*. This is clearly showed by the variation in contents shown in Table 2.

Athayde et al. (2007) also observed significant differences in methylxanthine content among yerba-mate plants within the same population, as well as between different populations. In some cases, the caffeine content detected by the authors, within the same plant population, was more than 100 times higher.

The statistical analysis (Table 3) showed that leaf age has a significant influence on caffeine, theobromine and total methylxanthine contents of the extracts ($p < 0.05$). Analyzing the mean caffeine content of the total set of samples, it is evident that there is a tendency for total caffeine and methylxanthine production to decrease over time. Theobromine does not follow exactly the same pattern, as higher levels were found in the leaves at one and two months.

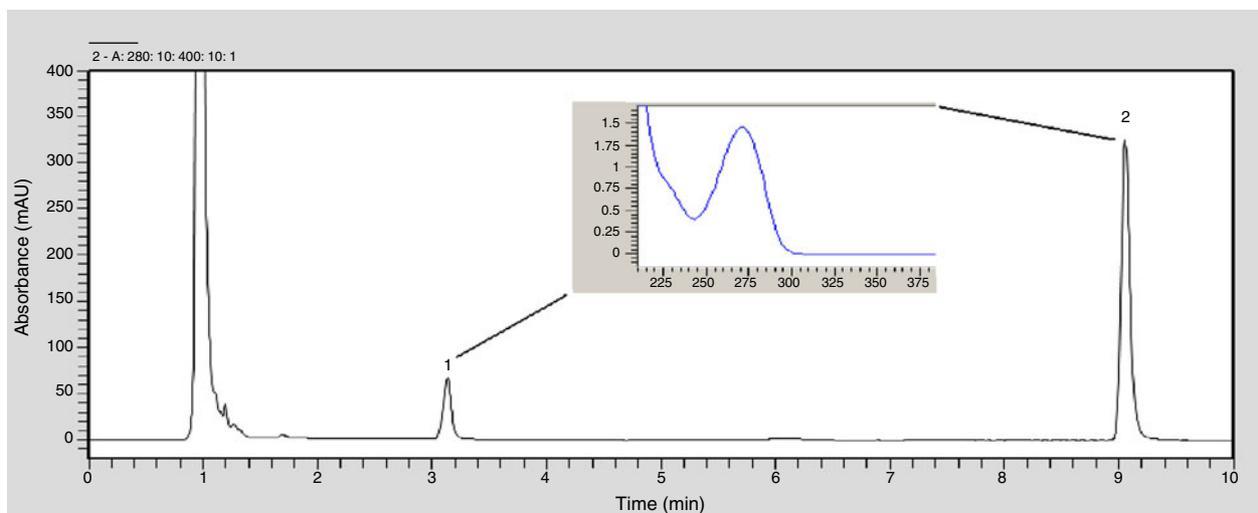


Fig. 2. CLAE-DAD chromatogram of the sample 2 of *I. paraguariensis* leaves aqueous extract (2.0 mg ml^{-1}) with detection at 280 nm. 1. Theobromine; 2. Caffeine. For chromatographic conditions, see Section "Materials and methods".

Table 2Variation in contents of methylxanthines and total phenolics in aqueous extracts of *Ilex paraguariensis* leaves.

Age of leaves	Extent of variation	Caffeine ^a	Theobromine ^a	Methylxanthines ^a	Total phenolics ^{**}
1 month	Minimum	46.46 ± 0.15	3.07 ± 0.01	50.86 ± 0.16	230.97 ± 7.78
	Maximum	133.98 ± 0.41	24.93 ± 0.14	137.05 ± 0.42	437.81 ± 15.49
2 months	Minimum	38.76 ± 0.24	5.80 ± 0.13	44.73 ± 0.26	172.32 ± 3.95
	Maximum	90.50 ± 0.12	30.88 ± 0.13	101.80 ± 0.17	391.66 ± 0.99
6 months	Minimum	5.68 ± 0.08	0.26 ± 0.01	6.20 ± 0.09	75.27 ± 5.54
	Maximum	81.57 ± 0.18	14.25 ± 0.06	84.49 ± 0.19	257.22 ± 3.43

^a Caffeine, theobromine and total methylxanthines contents were determined by CLAE-DAD and the data are mean ± S.D. values expressed as mg compound/g extract ($n = 11$).

^{**} Total phenolic content was determined by the *Fast Blue BB* method and the data are mean ± S.D. values expressed as mg chlorogenic acid/g extract ($n = 11$).

The higher caffeine levels in relation to theobromine could be explained by the biosynthesis pathway of these compounds. Theobromine is the direct precursor of caffeine biosynthesis by the methylation of xanthosine by *S*-adenosylmethionine (SAM) action (Kato et al., 2000; Ogawa et al., 2001; Uefuji et al., 2003). Although this information has been obtained from coffee (*Coffea arabica*) and tea (*Camellia sinensis*), the available evidence indicates that the pathway is essentially the same in other purine alkaloid-forming plants, such as *I. paraguariensis* (Ashihara, 1993).

Caffeine catabolism usually begins with its conversion to theophylline. Theophylline is degraded to CO₂ far more rapidly than caffeine, indicating that the conversion of caffeine to theophylline is the major rate-limiting step of caffeine catabolism and the reason why caffeine accumulates in high concentrations in tissues of *C. sinensis* and *C. arabica* (Ashihara et al., 1996; Ito et al., 1997).

There is only one published report, in which the purine alkaloid biosynthesis of mate by different aged leaves was investigated. Young leaves, but not mature dark-green leaves, incorporated each precursor into theobromine and caffeine, and no significant degradation of caffeine was detected by Ashihara (1993). These results are in agreement with the decrease in caffeine levels with ageing detected by our research.

The influence of age and plant development on secondary metabolite contents, and the relative proportions of these chemical components, has been demonstrated by several authors for different plant species (Bowers and Stamp, 1993; Doan et al., 2004; Hendriks et al., 1997; Höft et al., 1998). Hartmann (1996) states that young tissues generally have higher rates of metabolites' biosynthesis. Although the influence of leaf age has been described for other species, there is no general conclusion applicable to all plant species. Also, there are insufficient studies on this subject for *I. paraguariensis*. Notwithstanding, some authors have demonstrated the influence of leaf age on mate. Mazzafera (1994) found higher caffeine contents in younger leaves, and Esmelindro et al. (2004) found a significantly higher content of caffeine and theobromine in leaves at six months than in older leaves. Recently, Dartora et al. (2011) evaluated the methylxanthine content in *I. paraguariensis* samples under different growth conditions, treatment and age. Their results did not show significant differences in methylxanthine content in leaves between one and six months. Some studies with yerba-mate in the literature have evaluated the influence of harvest time on methylxanthine content. Schubert et al. (2006) found

higher methylxanthine content in the spring and early summer. Similar results were obtained by Coelho et al. (2001) analyzing samples of *I. paraguariensis* collected in the State of Paraná, Brazil, in two different periods. Coelho and Mariath (1996) found that the main sprouting in *I. paraguariensis* occurs in late September and October, and in some plants, sprouting can also occur between February and March. The results obtained by Athayde et al. (2000), Coelho et al. (2001) and Schubert et al. (2006) can be explained partly by the age of the leaves, since experimental results indicate that the biosynthesis of caffeine in *Ilex* only occurs in young leaves (Ashihara, 1993). Thus, the high methylxanthine contents identified in the summer can be attributed to the development of the young leaves, while the results for late fall and winter may indicate older, more mature leaves with low biosynthetic activity. The data (Table 3) presented in our study confirm this theory, since the caffeine and total methylxanthine contents showed a significant decrease with ageing of the leaves. Thus, the age of leaves may affect the characteristics of the raw material and therefore, their processed products.

Total phenolic content

The obtained results showed a high concentration of phenolic compounds in the samples of yerba-mate, corroborating the numerous literature data that report high levels of these compounds, mainly caffeoylquinic derivatives. Filip et al. (2001) found higher levels of these phenolic compounds in *I. paraguariensis*, when compared to the other seven *Ilex* species, detecting a concentration of 9.6% phenol derivatives on dry extract and 0.06% flavonoids. Marques and Farah (2009) described that yerba-mate contains, on average, 55% caffeoylquinic derivatives in green leaves and 73% in toasted leaves. Additionally, Bracesco et al. (2003) found that phenolic compounds are three times higher than the content of these compounds in green tea. When evaluating the mean of all the samples (Table 3), it was noted that there is a decrease in the production of phenolic compounds over time, since the contents are higher in leaves at one month, intermediate at two months and lower at six months. Thus, the age of the leaves has a significant influence in TPC. However, Dartora et al. (2011) revealed no significant differences in levels of phenolic compounds comparing leaves at one and six months.

Table 3Leaf age effects on the quantitative contents of caffeine, theobromine, methylxanthines and total phenolics in the aqueous extracts of *Ilex paraguariensis* leaves.

Age of leaves	Caffeine	Theobromine	Methylxanthines	Total phenolics
1 month	75.63 ± 16.24 ^a	9.22 ± 3.11 ^a	84.85 ± 27.49 ^a	286.7 ± 46.1 ^a
2 months	60.19 ± 13.4 ^{a,b}	12.4 ± 4.19 ^{a,b}	72.6 ± 20.14 ^{a,b}	252.6 ± 50.85 ^{a,b}
6 months	31.94 ± 14.6 ^c	4.95 ± 2.85 ^{a,c}	36.89 ± 16.52 ^c	166.6 ± 35.13 ^c

Caffeine, theobromine and total methylxanthine contents were determined by CLAE-DAD and the data are mean ± S.D. values expressed as mg compound/g extract ($n = 11$). Total phenolics' content was determined by the *Fast Blue BB* method and the data are mean ± S.D. values expressed as mg chlorogenic acid/g extract ($n = 11$). Different letters indicate significant differences (ANOVA and Tukey's post test, $p < 0.05$).

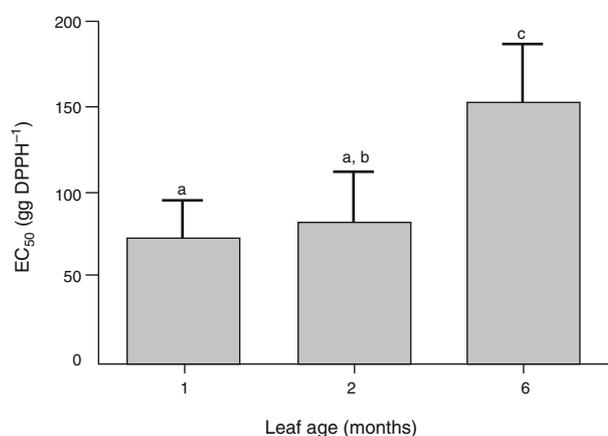


Fig. 3. Leaf age effects on the free radical scavenging capacity of *Ilex paraguariensis* aqueous extracts. The EC₅₀ were determined by the DPPH method and the values are mean ± S.D. expressed as g extract/g DPPH ($n = 11$). Different letters indicate significant differences (ANOVA and Tukey's post test, $p < 0.05$).

Table 4

Correlation analysis.

Correlation	r^2 value	p value
TPC [*] × EC ₅₀ ^{***} (all samples) ^a	0.2720	0.0019
TPC [*] × EC ₅₀ ^{***} (by leaf age groups) ^b	0.9246	0.0022
Caffeine ^{***} × EC ₅₀ ^{***} (all samples) ^a	0.2728	0.0018
Caffeine ^{***} × EC ₅₀ ^{***} (by leaf age groups) ^b	0.8885	0.0048
Caffeine ^{***} × TPC [*] (all samples) ^a	0.1978	0.0095
Caffeine ^{***} × TPC [*] (by leaf age groups) ^b	0.9529	0.0008

^a Correlation of all samples individually considered.

^b Correlation of the mean of each leaf age group.

^{*} Total phenolic content was determined by the *Fast Blue BB* method.

^{**} The free radical scavenging capacity (EC₅₀) of *Ilex paraguariensis* aqueous extracts was determined by the DPPH method.

^{***} Caffeine content was determined by CLAE-DAD. More details: see Section "Materials and methods".

Free radical scavenging capacity

It was observed that leaf age significantly affects the ability to scavenge free radicals, and there is a significant decrease in this capacity over time (Fig. 3). It is possible to observe an inverse correlation between TPC and EC₅₀ values (Table 4), because the higher the concentration of phenolic compounds, the lower the amount of extract required to reduce the DPPH.

The potent antioxidant activity of yerba-mate extracts has been demonstrated by several authors, as well as their correlation with phenolic compounds. Using different free radical generators, Schinella et al. (2000) concluded that the aqueous extract of *I. paraguariensis* was able to inhibit lipid peroxidation in enzymatic and nonenzymatic rat liver microsomes in a dose-dependent manner. The extract exhibited radical scavenging properties in relation to the superoxide anion and DPPH radical. Using the same DPPH methodology, Bixby et al. (2005) found a direct positive correlation between antioxidant activity and phenolic compounds, with the highest activity being obtained for the aqueous extracts of yerba-mate compared to green tea and black tea (*C. sinensis* (L.) Kuntze), marcela (*Achyrocline satureioides* (Lam.) DC.) and some types of red and white wines. Grujic et al. (2012) also found a correlation between the DPPH free radical scavenging activity and the total phenolic compounds of mate tea. Likewise, Bassani et al. (2014) showed that the total content of phenolic compounds was significantly correlated with the free radical scavenging activity towards DPPH radicals.

Anesini et al. (2012) demonstrated that chlorogenic and caffeic acids and the flavonoid rutin present in aqueous extracts of *I.*

paraguariensis samples contribute directly to the antioxidant activity detected, by preventing lipid peroxidation. Oxidation of low density lipoprotein (LDL) induced by free radicals, for example, plays an important role in atherosclerosis. In this context, aqueous extracts of *I. paraguariensis* have demonstrated the ability to inhibit LDL oxidation, thereby inhibiting lipid peroxidation, and hence the oxidation of DNA (Bracesco et al., 2011; Gugliucci, 1996; Gugliucci and Stahl, 1995). Anesini et al. (2012) also found that the methylxanthine caffeine has no free radical scavenging activity, and induces lipid peroxidation of linoleic acid, acting as a pro-oxidant compound.

Although DPPH free radical scavenging capacity of isolated caffeine was not tested in our study, our data revealed a significant and direct correlation between the detected caffeine and the antioxidant activities (Table 4). This correlation can be explained by TPC, which is directly correlated with the caffeine contents. Therefore, the detected activity is probably associated with phenolic compounds and not with caffeine. The prevention of lipid peroxidation, in which caffeine is identified as a pro-oxidant compound, remains unexplained in our work, since the antioxidant activity has not been evaluated by the ferric thiocyanate method, but by the DPPH method. Thus, we cannot say what is the role of caffeine present in the samples evaluated in the prevention of lipid peroxidation.

From the point of view of chemical ecology, it is interesting to evaluate the individuals in their entirety, in order to better understand how plants individually respond to these factors. In this approach, a significant correlation is observed in Table 4, but with a low coefficient of linear correlation. On the other hand, from the pharmaceutical and industrial point of view, it is necessary to have an approach that takes into account the consistency of the raw plant material to be used in the preparation of food products, intermediate pharmaceutical forms and cosmetics properties. Therefore, when plants are grouped by leaf age, this correlation is shown to be as effective as the previous approach, but more linear (Table 4).

In summary, leaf age showed a significant influence on methylxanthines and total phenolic contents of the evaluated extracts, with a decrease in these contents over time. Additionally, the free radical scavenging capacity was also significantly affected by leaf age, with a direct positive correlation between antioxidant activity and total phenolic content.

Authors' contributions

C.H.B.S. (PhD student) helped in the running of the laboratory work, analysis of the data, and drafted the paper. G.C.C. contributed to the collection of plants sample and their identification, and analysis of the data. F.H.R. and E.P.S. designed the study, supervised the laboratory work, and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflict of interest

The authors declare no conflict of interest.

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References

- Anesini, C., Turner, S., Cogoi, L., Filip, R., 2012. Study of the participation of caffeine and polyphenols on the overall antioxidant activity of mate (*Ilex paraguariensis*). *LWT – Food Sci. Technol.* 45, 299–304.
- Ashihara, H., 1993. Purine metabolism and the biosynthesis of caffeine in maté leaves. *Phytochemistry* 33, 1427–1430.
- Ashihara, H., Monteiro, A.M., Moritz, T., Gillies, F.M., Crozier, A., 1996. Catabolism of caffeine and related purine alkaloids in leaves of *Coffea arabica* L. *Planta* 198, 334–339.
- Athayde, M.L., Coelho, G.C., Schenkel, E.P., 2000. Caffeine and theobromine in epicuticular wax of *Ilex paraguariensis* A. St. Hil. *Phytochemistry* 55, 853–857.
- Athayde, M.L., Coelho, G.C., Schenkel, E.P., 2007. Populational diversity on methylxanthines content of mate (*Ilex paraguariensis* A. St. Hil., Aquifoliaceae). *Lat. Am. J. Pharm.* 26, 275–279.
- Bassani, D.C., Nunes, D.S., Granato, D., 2014. Optimization of phenolics and flavonoids extraction conditions and antioxidant activity of roasted yerba-mate leaves (*Ilex paraguariensis* A. St. Hil., Aquifoliaceae) using response surface methodology. *Anais Acad. Bras. Cienc.* 86, 923–934.
- Bixby, M., Spieler, L., Menini, T., Gugliucci, A., 2005. *Ilex paraguariensis* extracts are potent inhibitors of nitrosative stress: a comparative study with green tea and wines using a protein nitration model and mammalian cell cytotoxicity. *Life Sci.* 77, 345–358.
- Blumenthal, M., Goldberg, A., Brinckmann, J., 2000. *Herbal Medicine: Expanded Commission and Monographs*. American Botanical Council.
- Boaventura, B.C.B., Di Pietro, P.F., Stefanuto, A., Klein, G.A., de Moraes, E.C., de Andrade, F., Wazlawik, E., da Silva, E.L., 2012. Association of mate tea (*Ilex paraguariensis*) intake and dietary intervention and effects on oxidative stress biomarkers of dyslipidemic subjects. *Nutrition* 28, 657–664.
- Bowers, M.D., Stamp, N.E., 1993. Effects of plant age, genotype and herbivory on *Plantago* performance and chemistry. *Ecology* 74, 1778–1791.
- Bracesso, N., Dell, M., Rocha, A., Behtash, S., Menini, T., Gugliucci, A., Nunes, E., 2003. Antioxidant activity of a botanical extract preparation of *Ilex paraguariensis*: prevention of DNA double-strand breaks in *Saccharomyces cerevisiae* and human low-density lipoprotein oxidation. *J. Alt. Complement. Med.* 9, 379–387.
- Bracesso, N., Sanchez, A.G., Contreras, V., Menini, T., Gugliucci, A., 2011. Recent advances on *Ilex paraguariensis* research: minireview. *J. Ethnopharmacol.* 136, 378–384.
- Brandwilliams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free-radical method to evaluate antioxidant activity. *Food Sci. Technol. Leb.* 28, 25–30.
- Cass, Q.B., Degani, A.L.G., 2001. Desenvolvimento de métodos por HPLC: fundamentos, estratégias e validação. EdUFSCar, São Carlos.
- Clifford, M.N., Ramirez-Martinez, J.R., 1990. Chlorogenic acids and purine alkaloids contents of Maté (*Ilex paraguariensis*) leaf and beverage. *Food Chem.* 35, 13–21.
- Coelho, G.C., Mariath, J.E.A., 1996. Inflorescences morphology of *Ilex* L. (Aquifoliaceae) species from Rio Grande do Sul, Brazil. *Feddes Repert.* 107, 19–30.
- Coelho, G.C., Athayde, M.L., Schenkel, E.P., 2001. Methylxanthines of *Ilex paraguariensis* A. St. Hil. var. *vestita* Loes. and var. *paraguariensis*. *Braz. J. Pharm. Sci.* 37, 153–158.
- Coelho, G.C., Rachwal, M.F.G., Dedecek, R.A., Curcio, G.R., Nietsche, K., Schenkel, E.P., 2007. Effect of light intensity on methylxanthine contents of *Ilex paraguariensis* A. St. Hil. *Biochem. Syst. Ecol.* 35, 75–80.
- Coelho, G.C., Gnoatto, S.B., Bassani, V.L., Schenkel, E.P., 2010. Quantification of saponins in extractive solution of mate leaves (*Ilex paraguariensis* A. St. Hil.). *J. Med. Food* 13, 439–443.
- Dartora, N., de Souza, L.M., Santana, A.P., Iacomini, M., Valduga, A.T., Gorin, P.A.J., Sasaki, G.L., 2011. UPLC-PDA-MS evaluation of bioactive compounds from leaves of *Ilex paraguariensis* with different growth conditions, treatments and ageing. *Food Chem.* 129, 1453–1461.
- De Souza, L.M., Dartora, N., Scoparo, C.T., Cipriani, T.R., Gorin, P.A.J., Iacomini, M., Sasaki, G.L., 2011. Comprehensive analysis of maté (*Ilex paraguariensis*) compounds: development of chemical strategies for matesaponin analysis by mass spectrometry. *J. Chromatogr. A* 1218, 7307–7315.
- Dermarderosian, A., 2001. *The Review of Natural Products. Facts & Comparisons*, St. Louis.
- Doan, A.T., Ervin, G., Felton, G., 2004. Temporal effects on jasmonate induction of anti-herbivore defense in *Physalis angulata*: seasonal and ontogenetic gradients. *Biochem. Syst. Ecol.* 32, 117–126.
- Esmelindro, A.A., Girardi, J.D., Mossi, A., Jacques, R.A., Dariva, C., 2004. Influence of agronomic variables on the composition of mate tea leaves (*Ilex paraguariensis*) extracts obtained from CO₂ extraction at 30 °C and 175 bar. *J. Agric. Food Chem.* 52, 1990–1995.
- Filip, R., Lopez, P., Coussio, J., Ferraro, G., 1998. Mate substitutes or adulterants: study of xanthine content. *Phytother. Res.* 12, 129–131.
- Filip, R., Lotito, S.B., Ferraro, G., Fraga, C.G., 2000. Antioxidant activity of *Ilex paraguariensis* and related species. *Nutr. Res.* 20, 1437–1446.
- Filip, R., López, P., Giberti, G., Coussio, J., Ferraro, G., 2001. Phenolic compounds in seven South American *Ilex* species. *Fitoterapia* 72, 774–778.
- Gobbo-Neto, L., Lopes, N.P., 2007. Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. *Quím. Nova* 30, 374–381.
- Gosmann, G., Guillaume, D., Taketa, A.T.C., Schenkel, E.P., 1995. Triterpenoid saponins from *Ilex paraguariensis*. *J. Nat. Prod.* 58, 438–441.
- Grujic, N., Lepojevic, Z., Srdjenovic, B., Vlastic, J., Sudji, J., 2012. Effects of different extraction methods and conditions on the phenolic composition of mate tea extracts. *Molecules* 17, 2518–2528.
- Gugliucci, A., 1996. Antioxidant effects of *Ilex paraguariensis*: induction of decreased oxidability of human LDL *in vivo*. *Biochem. Biophys. Res. Commun.* 224, 338–344.
- Gugliucci, A., Stahl, A.J.C., 1995. Low density lipoprotein oxidation is inhibited by extracts of *Ilex paraguariensis*. *Biochem. Mol. Biol. Int.* 35, 47–56.
- Hartmann, T., 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. *Entomol. Exp. Appl.* 80, 177–188.
- Heck, C.I., De Mejia, E.G., 2007. Yerba mate tea (*Ilex paraguariensis*): a comprehensive review on chemistry, health implications, and technological considerations. *J. Food Sci.* 72, R138–R151.
- Hendriks, H., AndersonWildeboer, Y., Engels, G., Bos, R., Woerdenbag, H.J., 1997. The content of parthenolide and its yield per plant during the growth of *Tanacetum parthenium*. *Planta Med.* 63, 356–359.
- Höft, M., Verpoorte, R., Beck, E., 1998. Leaf alkaloid contents of *Tabernaemontana pachysiphon* as influenced by endogenous and environmental factors in the natural habitat. *Planta Med.* 64, 148–152.
- ICH, I.C.o.H., 2005. *Validation of Analytical Procedures: Text and Methodology – Q2(R1)*. ICH, I.C.o.H., London.
- Ito, E., Crozier, A., Ashihara, H., 1997. Theophylline metabolism in higher plants. *BBA – Gen. Subj.* 1336, 323–330.
- Kato, M., Mizuno, K., Crozier, A., Fujimura, T., Ashihara, H., 2000. Plant biotechnology: caffeine synthase gene from tea leaves. *Nature* 406, 956–957.
- Marques, V., Farah, A., 2009. Chlorogenic acids and related compounds in medicinal plants and infusions. *Food Chem.* 113, 1370–1376.
- Mazzafera, P., 1994. Caffeine, theobromine and theophylline distribution in *Ilex paraguariensis*. *Rev. Bras. Fisiol. Veg.* 6, 149–151.
- Medina, M.B., 2011a. Determination of the total phenolics in juices and superfruits by a novel chemical method. *J. Funct. Foods* 3, 79–87.
- Medina, M.B., 2011b. Simple and rapid method for the analysis of phenolic compounds in beverages and grains. *J. Agric. Food Chem.* 59, 1565–1571.
- Meyer, S., Cerovic, Z.G., Goulas, Y., Montpied, P., Demotes-Mainard, S., Bidet, L.P.R., Moya, I., Dreyer, E., 2006. Relationships between optically assessed polyphenols and chlorophyll contents, and leaf mass per area ratio in woody plants: a signature of the carbon–nitrogen balance within leaves? *Plant Cell Environ.* 29, 1338–1348.
- Miranda, D.D.C., Arçari, D.P., Pedrazzoli, J., Carvalho, P.d.O., Cerutti, S.M., Bastos, D.H.M., Ribeiro, M.L., 2008. Protective effects of mate tea (*Ilex paraguariensis*) on H₂O₂-induced DNA damage and DNA repair in mice. *Mutagenesis* 23, 261–265.
- Ogawa, M., Herai, Y., Koizumi, N., Kusano, T., Sano, H., 2001. 7-Methylxanthine methyltransferase of coffee plants: gene isolation and enzymatic properties. *J. Biol. Chem.* 276, 8213–8218.
- Puangpraphant, S., de Mejia, E.G., 2009. Saponins in yerba mate tea (*Ilex paraguariensis* A. St. Hil.) and quercetin synergistically inhibit iNOS and COX-2 in lipopolysaccharide-induced macrophages through NFκB pathways. *J. Agric. Food Chem.* 57, 8873–8883.
- Reginato, F.H., Athayde, M.L., Gosmann, G., Schenkel, E.P., 1999. Methylxanthines accumulation in *Ilex* species – caffeine and theobromine in erva-mate (*Ilex paraguariensis*) and other *Ilex* species. *J. Braz. Chem. Soc.* 10, 443–446.
- Rivelli, D.P., Silva, V.V.d., Ropke, C.D., Miranda, D.V., Almeida, R.L., Sawada, T.C.H., Barros, S.B.d.M., 2007. Simultaneous determination of chlorogenic acid, caffeic acid and caffeine in hydroalcoholic and aqueous extracts of *Ilex paraguariensis* by HPLC and correlation with antioxidant capacity of the extracts by DPPH-reduction. *Rev. Bras. Cienc. Farm.* 43, 215–222.
- Schinella, G.R., Troiani, G., Davila, V., de Buschiazzo, P.M., Tournier, H.A., 2000. Antioxidant effects of an aqueous extract of *Ilex paraguariensis*. *Biochem. Biophys. Res. Commun.* 269, 357–360.
- Schubert, A., Zanin, F.F., Pereira, D.F., Athayde, M.L., 2006. Variação anual de metilxantinas totais em amostras de *Ilex paraguariensis* A. St. Hil. (erva-mate) em Ijuí e Santa Maria. *Estado do Rio Grande do Sul. Quím. Nova* 29, 1233–1236.
- Uefuji, H., Ogita, S., Yamaguchi, Y., Koizumi, N., Sano, H., 2003. Molecular cloning and functional characterization of three distinct N-methyltransferases involved in the caffeine biosynthetic pathway in coffee plants. *Plant Physiol.* 132, 372–380.
- Valerga, J., Retat, M., Lanari, M.C., 2012. Polyphenol input to the antioxidant activity of yerba mate (*Ilex paraguariensis*) extracts. *Food Sci. Technol.* 45, 28–35.