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Quantitation of Methylxanthinic Alkaloids and Phenolic Compounds in Mate (*Ilex paraguariensis*) and Their Effects on Blood Vessel Formation in Chick Embryos

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Methylxanthinic alkaloids and phenolic compounds are related to the therapeutic properties of *Ilex paraguariensis* infusions. Considering the known vascular tropism of xanthines, an aqueous extract (mate) and caffeine were evaluated on blood vessel formation, in connection with the analysis of those secondary metabolites, which was performed in young and mature leaf samples collected in three cultivation systems located in the southern region in Brazil (Santa Catarina State). Samples of young and mature leaves from a monoculture cultivation system (MC) showed the highest content of phenolic compounds (149.68 μg/mL, young leaves; 135.50 μg/mL, mature leaves) and caffeine (young leaves, 148.07 μg/mL; mature leaves, 244.63 μg/mL) as compared to samples from agroforesty (AF) and shaded-native (NT) cultures. Theophylline was not detected in samples by reverse phase-high performance liquid chromatography, and mature leaves showed lower theobromine amounts (11.46 μg/mL). Treatments performed with mate aqueous extract and caffeine (1.03−4.12 μM/disk) in the yolk sac vascular membranes of 2-day-old chick embryos revealed pro-vasculo- and angiogenic properties as well as embryonic growth enhancement. These findings, uncoupled from any detectable embryotoxic effect, suggest a potential therapeutic and/or prophylactic use in cardiovascular disorders for caffeine and related constituents of mate plant extracts, an issue that waits further studies.

KEYWORDS: *Ilex paraguariensis*; metabolite profiling; phenolic compounds; caffeine; vasculogenesis; angiogenesis

INTRODUCTION

The mate plant (*Ilex paraguariensis* St. Hill-Aquifoliaceae) has a wide distribution in South America and is of large economical importance. Its biomass, leaves and twigs, is marketed as a fresh product, sold to yerba mate processing companies (also known as "ervateiras" in Southern Brazil) that dry, grind, and pack it. This processed product, referred to as erva mate is sold for local, regional (*1*), and more recently to US markets (*2*). *I. paraguariensis* has been produced in different cultivation systems such as open plantation (monoculture) or associated with other species consisting of the agroforestry system with varying field conditions such as, for instance, the incidence of light on the plants (*1, 3, 4*).

Mostly in Brazil, Paraguay, Argentina, and Uruguay, native populations use the dried and ground aerial parts of the mate plant to prepare, by means of infusion, a beverage named *mate* (Spanish-speaking) or *chimarrão* (Portuguese-speaking populations) (*2*). Daily consumption ranges from 1.5 to 6 L (*5*), and interesting biological effects of *I. paraguariensis* aqueous extract have been reported, such as diuretic (*5*), choleretic (*6*), glycogenolytic, lipolytic, antioxidant (*7*−*9*), neural stimulant, hypocholesterolemic, hepatoprotective, vasorelaxant, and atherosclerosis inhibition (*10*). Actually, it is included in the 1996 British Herbal Pharmacopeia, Complete German Commission and Monographs, Martindale, and in the Argentine Food Code (*6, 11*). However, to the best of our knowledge, no reports have shown the provascular effects of mate so far.

However, caffeine which is a major mate constituent is possibly the most consumed substance worldwide, and it was already associated with the occurrence of congenital malformations in mice when administered in high concentrations (*12*).

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Mate tea might present meaningful differences in its sensorial characteristics, especially with regard to its bitter and astringent taste, due to changes in the chemical composition of raw materials as polyphenolic and caffeine contents (*13*), with direct economical effect on the market price. Accordingly, it is also possible to envisage that the content of pharmacologically active compounds, polyphenols and methylxanthinic alkaloids, for example, also varies over the harvests (*14*) and among the cultivation systems because of the influence of field variables and agronomic management (*3, 4*). Indeed, the genotype, the cultivation system adopted, and the climatic factors (e.g., temperature, luminosity, and relative humidity) have been pointed out as being of great importance on the variation observed in the chemical constituents of the biomass of *I. paraguariensis* (*14*). Thus, to produce *I. paraguariensis* raw material with superior quality, the quantitative analysis of polyphenol and alkaloid compounds seems to be needed. This is because the content of such secondary metabolites might be used as a reference to evaluate and improve the production systems of that crop (*1, 4*).

The first aim of this study was to quantify the methylxanthinic alkaloids caffeine, theobromine, and theophylline as well as the phenolic compounds in the aqueous extract of young and mature leaves produced in three cultivation systems of *I. paraguariensis*, that is, monoculture (open plantation; MC), agroforestry (AF), and shaded-native (NT) cultures. Moreover, due to vascular tropism of xanthines (*15*) and promising prophylactic/therapeutic properties of mate in dyslipidemias (e.g., LDL/VLDL), as evidenced by ethnopharmacological data, the second aim of our work was to evaluate the effect of the aqueous extract of mate and caffeine in the process of early blood vessel formation and growth of chick embryos.

The first/primordial blood vessels develop in early embryonic period by vasculogenesis. In that process, progenitor endothelial cells (angioblasts) are recruited from the mesoderm (intermediate germinal layer adjacent to embryo) or originated by local cell division and differentiate in endothelial cells (*16*). Afterward, these precursors organize cell clusters (blood islands) that lay down/establish a primary vasculature network/primary vascular plexus (*17*). A subsequent remodeling of these preexisting vessels (angiogenesis) gives rise to a more refined microvasculature that accompanies the growth and shaping of the body (*16, 18*).

Our hypothesis assumes that *I. paraguariensis* leaves have distinct contents of methylxanthinic alkaloids and phenolic compounds according to their maturation stage and cultivation system, with direct effect on the biological activities of mate tea, that is, blood vessel formation.

MATERIALS AND METHODS

Plant Material and Aqueous Extracts. Leaf samples of *I. paraguariensis* were collected in three cultivation systems, located in Canoinhas county (Santa Catarina State, Brazil, 26°10′ S; 50°18′ W), hereafter referred to as monoculture (MC), a typically *I. paraguariensis* open plantation, agroforestry (AF), and shaded-native culture (NT). These cultures differ mainly in accordance with the incidence of light radiation that ranges from 100% (MC) to 52.78% (AF) and 37.81% (NT) of sunlight irradiance. These radiation levels are mean values determined at the beginning of the experiments (November, 2003) by means of quantitation of the sunlight irradiance with a global solar radiation analyzer (Kipp&Zonen) recording from 10 a.m. to 2 p.m., at 1.5 m from the ground around the treetop of five-year-old plants with similar height. The soil where the cultivation systems were located was classified as dystrophic red latosol (i.e., Oxisol (*19*)), and agronomic management was similarly made.

Young leaves (<than one-year-old) and mature leaves (>than one-year-old) were collected from adult mate plants (about 5-year-old) in November 2003. Twenty leaves/replication (4 replications/treatment) were collected and dried at 55 °C, powdered, and arranged into 6 groups in accordance with the developmental stage (i.e., young and mature leaves) and the production system (MC, AF, and NT). In order to obtain the aqueous extract, each sample (5 g, leaves dry weight) was added to 50 mL of distilled−deionized water, followed by boiling (20 min) in a Soxhlet apparatus. Aqueous extract (about 30 mg/mL) was recovered by filtration on a cellulose support and concentrated under low pressure.

Quantitative Assay of Phenolic Compounds. Phenolic compounds were quantified in the aqueous extract as previously described (*20*) using the Folin−Ciocalteau reagent (Sigma, St. Louis, MO, USA), by means of the absorbance determination (725 nm; Shimadzu UV 1203 spectrophotometer). The calculation of the total content of phenolic compounds was performed with the use of a standard curve of gallic acid (10−160 μg/mL; $r^2 = 0.99$).

Extraction and Chromatographic Analysis of the Xanthinic Alkaloids. The aqueous extract (15 mL) of the mate leaves was incubated for 1 h in 60 mL of dichloromethane, the organosolvent extract was recovered, concentrated to 2 mL under reduced pressure, and stored at −18 °C. Aliquots (10 μL/sample) were injected into a liquid chromatograph (Shimadzu LC-10) equipped with a reverse-phase column (Shim-pack C_{18}, 4.6 mm ID × 250 mm long) thermostatized at 30 °C, and an UV−visible detector (Shimadzu SPD 10A, $\lambda = 272$ nm). An isocratic mobile phase of acetonitrile/0.1% formic acid (15:85 (*21*)) was used with a flow-rate at 1.0 mL/min. Previous to injection, all of the samples were centrifuged (5.000 rpm/10 min) and filtrated (0.22 μm). For purpose of quantitative analysis, a standard calibration curve was obtained by plotting the area of peaks against different concentrations (1.0 to 100.0 μg/mL; $r^2 = 0.99$) of caffeine (Sigma, St. Louis, MO, USA). Similarly, for each sample the final concentration of the compounds was determined by averaging the content after three consecutive injections.

Activity of Mate Aqueous Extract and Caffeine on Embryonic Vessel Formation. The ability of the substances to modulate early vessel formation (vasculo and angiogenesis) was determined by the yolk-sac membrane (YSM) *in vivo* assay. This method was adapted from the chorioallantoic membrane (CAM) assay adopted to evaluate ongoing-developmental angiogenesis (*22*).

Chicken fertilized eggs (*Gallus domesticus*) supplied by a poultry producer (Macedo Koerich S.A.) were incubated at 37 °C and 30% relative humidity. After a preliminary period of 48 h of incubation (E2 embryonic day; stage 13-HH), the eggs were removed from the incubator, and a window (10 mm diameter) was opened in the eggshell, at a position adjacent to the embryo.

The treatments ($n = 8$) were administered by implanting disk-shaped methylcellulose supports (10 μL volume, 2 mm diameter; one disk per embryo) impregnated with the aqueous caffeine solution (1.03 μM, 2.06 μM, and 4.12 μM caffeine/disk; pH 7.2) or mate aqueous extracts of MC-mature leaves (concentrations adjusted to 1.03 μM, 2.06 μM, and 4.12 μM caffeine/disk; pH 7.2). Each substance under investigation was adsorbed on the methylcellulose (0.45%, p/v) disk, and the solution was air-dried on a Teflon-coated metal tray before being applied to the developing extra embryonic membranes for testing of vessel formation. Blank methylcellulose disks (water ultrafiltered as solvent; pH 7.2) were used as negative control.

Disk implantation was performed in the outer one-third surface of the 1.5-day yolk-sac membrane, near the embryo, where blood islands were present together with early capillaries. Afterward, the shell windows were closed with black binding cellophane tape, and the eggs were returned to the incubator. Two days after implantation (fourth day, 96 h, i.e., E4 embryonic day; stage 23-HH), the zone around the methylcellulose disk was examined under a stereoscopic microscope (30×, Olympus, Tokyo, Japan).

The effects in vessel formation were determined by the increase or decrease in vessel number on the methylcellulose disk limit as compared to the control. Subsequently, embryos ($n = 6$) were dissected from the

Table 1. Content (μg/mL) of Total Phenolic Compounds, Caffeine, and Theobromine in Mate Aqueous Extracts Derived from Monoculture (MC), Agroforestry (AF), and Shaded-Native (NT) Cultivation Systems, According to the Leaf Maturation Stage

plant material	total phenolics (μg/mL)[a]	caffeine (μg/mL)[b]	theobromine (μg/mL)[b]
MC_{young}	149.68 ± 0.04	148.07 ± 0.99	10.69 ± 0.09
MC_{mature}	135.50 ± 0.02	244.63 ± 2.01	8.99 ± 0.07
AF_{young}	131.82 ± 0.05	60.90 ± 0.77	5.27 ± 0.07
AF_{mature}	69.82 ± 0.04	144.96 ± 0.82	5.20 ± 0.04
NT_{young}	64.08 ± 0.03	145.50 ± 1.15	15.58 ± 0.12
NT_{mature}	59.30 ± 0.03	77.68 ± 1.01	11.46 ± 0.02

[a] Each sample was performed in triplicate. [b] Mean ± SEM for three consecutive injections.

egg membranes, and the total body length was determined through the sum of measures on the body segments (axes) defined as head-flexure to cervical flexure and from this last one to curled tail-edge.

Statistical Analysis and Ethics. The results were summarized, expressed as the mean and standard error of mean (S.E.M.), and compared through one-way analysis of variance (ANOVA). The differences between treatments were grouped by the least-squares difference method (LSD). P values <0.05 were considered statistically significant.

All animal studies were carried out in accordance with the procedures outlined by NIH guidelines and defined in protocol number 256/proc.23080.028649/2003-07/CEUA/UFSC, approved by the local committee for Care and Ethical Use of Animals on Research (CEUA/UFSC, Florianópolis, SC, Brazil).

RESULTS AND DISCUSSION

For our first aim, the experimental area consisted of three agricultural cultivation systems named monoculture (MC), a typical mate-plant cultivation thoroughly exposed to sunlight incidence, agroforestry cultivation (AF), and shaded-native cultivation (NT). Despite the fact that the MC system usually affords higher yield, leaf biomass with superior quality has been found in AF and NT cultivation systems (*13*).

The occurrence of phenolic compounds in foods and beverages has been investigated more intensively over the last years, mainly due to their known beneficial effects to human health. Antioxidant activity is probably the most prominent effect of the aqueous infusion of *I. paraguariensis* (*8, 23*). **Table 1** shows the values of total phenolic content in leaf aqueous extracts of the samples in the study according to the cultivation system. Leaves from the MC system presented the highest amount of total phenolic compounds in both young and mature leaves (149.68 μg/mL and 135.50 μg/mL, respectively), while the lowest contents were found in samples from the NT system, 64.08 μg/mL (young leaves) and 59.30 μg/mL (mature leaves; **Table 1**). It is noticeable that in spite of the cultivation system, the young leaves were characterized for their highest contents of those secondary metabolites. These findings are in accordance with the literature (*24*), relating the presence of prominent polyphenol amounts in young plant tissues as a chemical defense strategy to protect them against biotic (pathogens and predators) or abiotic stressor factors.

With respect to xanthinic alkaloids, a comparison among the cultivation systems revealed that the highest concentration of caffeine occurred in MC samples (**Table 1**). Indeed, the mature leaves presented the highest values (244.63 μg/mL), while young leaves showed a concentration 1.6 orders of magnitude lower (148.07 μg/mL). It also should be noted that a direct correlation between the stages of leaf maturation and alkaloid content in the samples was not detected. In all of the experiments, the concentration of theobromine was significantly lower than caffeine, and theophylline was not detected in the aqueous extracts. These findings are in accordance with other reports (*11, 14, 25*).

Taken as a whole, the results evidenced biochemical differences revealed by the chemical composition of the leaf aqueous extracts, i.e., polyphenolic and methylxanthinic compounds, mainly between MC and NT samples. These findings suggest that the presence of phenolic compounds and xanthinic alkaloids in leaf tissues of *I. paraguariensis* is somehow positively regulated by the intensity of sunlight radiation to which the crops are exposed (*14*), notably in the MC-system. However, possible relevant influences of other abiotic and also biotic factors in these results should not be overlooked.

Many pharmacological properties related to phenolic acids and their derivatives found in *I. paraguariensis* leaf extracts have been reported, such as the inhibition *ex vivo* of low density lipoprotein oxidation and lipid peroxidation by caffeoil-derivates (*8, 26*). Similarly, methylxanthines have also shown biological activities of clinical interest as, for example, stimulant and diuretic effects (*5, 14*), and more recently the induction of peroxidase secretion on submandibulary glands (*27*). Interestingly, such effects are mainly related to the content of caffeine in mate. Notwithstanding, studies focusing on the effect of mate on the development of blood vessels are lacking. In order to examine whether the mate aqueous extract and caffeine alone exert modulatory activity in blood islands and primordial capillaries (vessel formation), we performed the *in vivo* chick embryo–YSM assay.

As shown in **Figure 1**, whereas control YSMs treated with vehicle developed an average of 100 ± 5 vessels on the disk limit, those membranes treated with the mate aqueous extract consistently exhibited more vessels on the disk limit. The group treated with 2.06 μM caffeine/disk promoted 91% more vascularity around the disk (191 ± 8 vessels) as compared to control, which confirms the pro-vasculo/angiogenic effect of that extract.

The effect promoted by administrating caffeine alone (1.03−4.12 μM/disk) to chick embryos was a significant ($P < 0.01$) increase in vessel number on disk limit as compared to control group as well. Indeed, a prominent effect was detected for the highest concentration of that alkaloid (4.12 μM/disk−146 ± 10 vessels), similar to that observed with mate aqueous extract treatment at the same concentration (146 ± 9 vessels).

In spite of the significant effect found for caffeine treatments as compared to the control, methylxanthine, as an isolated compound, was a little less effective in promoting blood vessel formation in the YSM comparatively to mate aqueous extract at 1.03 and 2.06 μM/disk. This effect may probably occur because of the presence of other mate active compounds in the extract, which would be acting synergistically on the vessels (*28, 29*); **Figure 1**.

Another important finding of the current study was the change in the pattern of embryonic growth, determined by body length measures, in response to treatments. In order to assess the pro-vascular activity of mate aqueous extract and caffeine on embryonic growth, we first determined the effect of that extract by measuring the total body length of the treated 4-day chick embryos (**Figure 2**). As demonstrated, the mate extract (1.03−4.12 μM caffeine/disk), significantly ($P < 0.05$) and concomitantly to the increase in vessel number, promoted an increase in 15% the body length (ca. 13.8 mm) as compared to the control group (11.8 mm). We next also examined the effect of increasing concentrations of caffeine alone in embryonic total length. As shown in **Figure 2**, the treatment with caffeine (1.03,

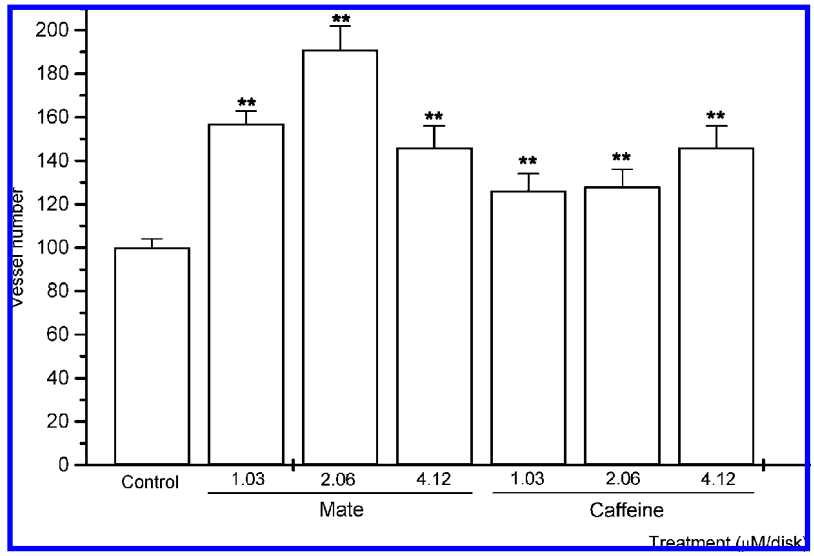

Figure 1. Number of vitelline vessels in the yolk sac of chick embryos (2−4 days of incubation) in response to treatments performed with mate aqueous extract or caffeine (1.03−4.12 μM caffeine/disk). Methylcellulose disks containing only ultrafiltered water (vehicle) were used as the control. Each column and vertical bar represents the mean ± SEM of 8 embryos, and the asterisks denote significance at the level of $^{**}P < 0.01$ (ANOVA, followed by LSD method) as compared to the control.

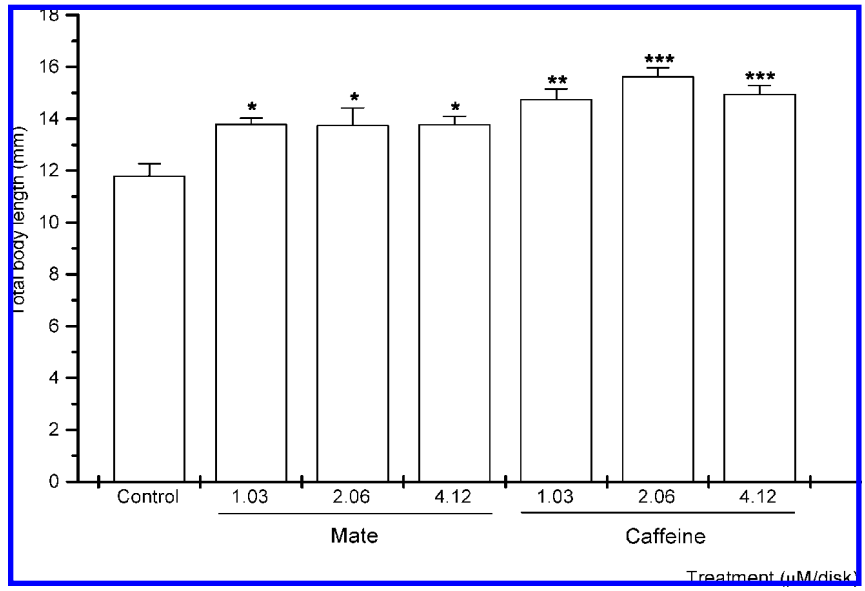

Figure 2. Total body length (mm) of 4-day-old chick embryos (*G. domesticus*) in response to treatments performed with mate aqueous extract or caffeine (1.03−4.12 μM caffeine/disk) in the yolk sac blood islands. Disks containing only ultrafiltered water (vehicle) were used as the control. Each column and vertical bar represents the mean ± SEM of 6 embryos, and the asterisks denote significance at the level of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (ANOVA, followed by LSD method) as compared to the control (vehicle).

2.06, and 4.12 μM/disk) markedly increased body length to 14.8 mm ($P < 0.01$), 15.6 mm, and 14.9 mm ($P < 0.001$), respectively.

The increase in body length by treatment with caffeine indicates that this alkaloid exhibits an effect more related to the embryonic development (regarding the growth) as compared to treatment with the mate aqueous extract, which relatively more directly improved the process of vessel formation (**Figures 1** and **2**). With this in mind, it is possible to speculate that the alkaloid effects on embryo growth, as well as on the vascularity, can be due to the direct or specific influences in cell signaling involving calcium and/or protein kinase C (PKC) pathways and possibly up-regulation of amino acid intake and transport, which are crucial for embryonic development and growth (*29*). As suggested, such consequent increase on embryonic metabolism also would be in accordance with the well-known alkaloid-stimulating action on the muscarinic receptors of cardiac muscles (*30*), for instance. It is also plausible to consider that the effect observed in embryonic growth reflects a positive impact by the improvement on nourishment (on nutritional status), in the morphogenetic process, due to the increase in vessel number. Moreover, these effects afforded by treatments with mate aqueous extract and caffeine were uncoupled from apparent dimorphic features or any detectable embryotoxic effect, which represents an additional advantage. Nonetheless, the mechanisms underlying the actions promoted by mate and alkaloid treatments remain to be characterized.

The results described above might be useful as a strategy to adequate the use of *I. paraguariensis* raw material, originated from any specific cultivation system, to its final destination in the market, that is, different applications to distinct markets. Thus, one could envisage the use of leaf biomass with higher

content in secondary metabolites (MC system) as more suitable for the production of phytomedicines and/or food supplements (nutraceutical products), whereas the biomass obtained from AG and NT cultivation systems seem to be more interesting to the beverage market providing superior sensorial characteristics to mate. The former approach might contribute to increase the market value of leaf biomass originated from the MC system, especially because of its highest content of pharmacological compounds of interest to human health. However, these findings are also of interest to small farmers since the biomass from the MC cultivation system has found, in the Brazilian market, prices 50% lower in comparison to raw material from the NT system. Furthermore, some of its chemical constituents, that is, phenolic compounds and caffeine, could be directed to the market as a factor of differentiation among the currently available prophylactic and therapeutic drugs. Additionally, due to the inducer effect in the early processes of vessel formation, mate tea appears to be an interesting complex matrix for pharmacological studies on the bioavailability of secondary metabolites as targets related to the prevention or therapeutics of ischemia and related vascular system disorders.

ABBREVIATIONS USED

AF, agroforestry cultivation system; ANOVA, one-way analysis of variance; CAM, chorioallantoic membrane assay; CEUA, care and ethical use of animals on research committee; LSD, least-squares difference method; MC, monoculture cultivation system; NT, native-shaded cultivation system; SEM, standard error of mean; YSM, yolk sac membrane assay.

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