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Evaluation of the Antioxidant Activity and Polyphenols Content of *Ilex paraguariensis* (Mate) During Industrialization

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Abstract: The fresh leaves and stems of *Ilex paraguariensis* (Aquifoliaceae) (green maté) are industrialized to prepare the commercial product named "yerba mate" used in South America as a tea-like beverage. It is exported to the US, Europe and Asia as vegetal drug or extracts used in complementary and alternative medicine and in formulations for functional foods. The aims of this study were: a) to evaluate if the antioxidant activity (DPPH free radical scavenging activity and the prevention of lipid peroxidation) of *I. paraguariensis* is affected by the industrial processing and if so, b) to determine which stages of the industrial process could provide the most useful material with antioxidant properties to be used in food and pharmaceutical industries. Leaves at different stages of the industrial process (green, zapecado, dried and forced aging) were used to prepare aqueous extracts. The total polyphenols content was determined by spectrophotometry (Folin Ciocalteu´s method). The main bioactive compounds were identified and quantified by HPLC-DAD. The results obtained in this work demonstrated that maté green leaves could provide the most useful material with antioxidant properties to be used in formulations for food and pharmaceutical industries.

Key words: Antioxidant activity, *Ilex paraguariensis*, mate, polyphenols

INTRODUCTION

The imbalance between production and consumption of reactive oxygen species, leading to oxidative stress, is implicated in the pathophysiology of a plethora of genetic and acquired disorders, such as cancer, arteriosclerosis, malaria and rheumatoid arthritis, as well as neurodegenerative diseases and aging processes (Halliwell and Gutteridge, 2007; Vasconcelos *et al*., 2007). Epidemiology studies have shown an inverse association between the daily consumption of fruits and vegetables and the risk to suffer from degenerative and chronic diseases (John *et al*., 2002; Zibadi *et al*., 2007). The protective effects of fruits and vegetables have long been attributed to their antioxidant compounds, such as polyphenols, carotenoids, and vitamins C and E. Antioxidants act in various ways, which include the complexation of redox-catalytic metal ions, scavenging of free radicals and decomposition of peroxides.

The fresh leaves and stems of *Ilex paraguariensis* (Aquifoliaceae) (maté) are employed to prepare the commercial product named "yerba mate" used in North-Eastern Argentina, Southern Brazil and Eastern Paraguay to prepare a tea-like beverage named "mate" (infusions and decoctions) that is consumed by 30% of the population at a rate of 1 L/day (Rosovsky, 1983). *I. paraguariensis* is populary used as antirheumatic and to treat gastrointestinal disorders for its eupeptic and choleretic properties (Alonso Paz *et al*., 1992). Our previous studies have demonstrated that *I. paraguariensis* has choleretic (Gorzalczany *et al*., 2001) and antioxidant activities (Anesini *et al*., 2006; Filip *et al*., 2000). There are an increasing number of maté products patents, as well as a growing interest in this product by countries whose population do not traditionally consume maté beverages (Bastos *et al*., 2007). Nowadays it is exported to the United States, Europe and Asia as vegetal drug or extracts used in different phytopharmaceutical, food and cosmeceutic preparations (De Mejía *et al*., 2010).

The industrial processing of *I. paraguariensis* involves different stages and could be summarized as follows:

- Harvesting: green leaves and small stems are cut manually or mechanically and then carried to the processing plant. In this work this material was named "green".
- "Zapecado": the green yerba is exposed to direct fire at temperatures between 250ºC and 550ºC during 2-4 min. Approximately 25% of moisture is lost in this process.

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- Drying: the product is exposed to a current of hot air during 3 to 6 h. until a 3% of moisture is reached. In this work the plant material obtained after this stage was named "dried".
- Aging: the dried material is stored under controlled temperature, humidity and air circulation conditions during 30-60 days in order to acquire the desired taste, aroma and color. This is a forced aging process and is named "FA" in this work. Previous investigations have demonstrated the presence of methylxanthines, caffeoyl derivatives and flavonoids in *I. paraguariensis* (Filip *et al*., 1998, 2001). It was also found that the industrial processing can alter the amount of bioactive compounds (Isolabella *et al*., 2010). However, no literature data is available on the impact that each one of the stages of the industrialization process has on the antioxidant activities of *I. paraguariensis.*

It is known that polyphenols (caffeoyl derivatives and flavonoids) have antioxidant activity (Lee, 2000; Wang *et al*., 1999). Recent findings suggest the role of these compounds as protective agents against cardiovascular diseases and breast, gastrointestinal and skin cancers (Carbonaro *et al*., 2001). Polyphenols have been shown to be potent antioxidants interfering with the celular oxidative/antioxidative potential or to act as free radicals scavengers (Lodovici *et al*., 2001).

Many phytotherapeutic and herbal food supplements products available on the market, which formulated with green or processed *I. paraguariensis* or its extracts. These products are claimed to aid weight loss, to have CNS stimulant activity, to be diuretic, antioxidant and antihipercholesterolemic among other properties (Ruxton *et al*., 2007). However, the origin of the plant material used in such formulations is sometimes unknown. The growing market in maté products makes manufacturers require high quality material and to provide scientific research-based products.

Polyphenols (caffeoyl derivatives and flavonoids) present in this species have been associated with its antioxidant activities (Filip *et al*., 2000). Caffeine is the compound responsible for the stimulant properties of this plant (Filip *et al*., 1998).

The assessment of the ability of an antioxidant to scavenge DPPH radical and to inhibit lipid peroxidation has been a method traditionally accepted to evaluate the antioxidant capacity of a compound.

Taking into account the reported antioxidant effect of *I. paraguariensis*, the aims of this work were: a) to evaluate if the antioxidant activity (DPPH free radical scavenging activity and the prevention of lipid peroxidation) of *I. paraguariensis* is affected by the industrial processing and if so, b) to determine which stages of the industrial process could provide the most useful material with antioxidant properties to be used in food and pharmaceutical industries.

MATERIALS AND METHODS

Plant material and extracts: For this study, the leaves of *Ilex paraguariensis* were provided by a factory of "yerba mate" located in the province of Corrientes, Argentina. The plant material belonged to the same lot at different stages of the industrial process: 1) "green", 2) "zapecado" 3) "dried" and 4) "forced aging" (FA). The green leaves were dried in a stove with hot air circulation and thermostatized at 40°C.

The plant material was ground to a fine powder in an electric mill to obtain uniform size particles (1 mm pore mesh). Aqueous extracts were prepared with this material in order to compare to the preparation used by people. Ten grams of each sample were boiled with 200 mL of water during 20 min and cooled at room temperature to 40-45ºC. After filtration (Whatman Nº.1), extracts were lyophilized. This study was conducted in Buenos Aires in March 2009.

Chemicals and reagents: For the determination of the Total Phenolic Content (TPC) the Folin-Ciocalteu's phenol reagent (Merck Chemicals Argentina, Buenos Aires), gallic acid (99% purity, Sigma Argentina), anhydrous sodium carbonate (99% purity, Anedra Argentina) were used. For the determination of the antioxidant activity 1,1-diphenyl-2-picrylhydrazyl, linoleic acid, ascorbic acid, trizma base (Sigma Argentina); $FeCl_2$ and ammonium thiocyanate (Merck chemicals Argentina) were employed.

Total polyphenol determination: The total polyphenols content was determined by spectrophotometry (Shimadzu UV 2101) according to the Folin-Ciocalteu`s method (Peschel *et al*., 2006). Briefly, 10,0 mg of lyophilized extract were solubilized in 100 mL of deionized distilled water. One mL of the sample extract was transferred in duplicate to separate tubes containing 7 mL distilled water and then 0.5. mL of Folin-Ciocalteu's reagent were added. After 2 min, 1.5 mL of a sodium carbonate anhydrous solution (20% w/v) were added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The TPC was expressed as g of gallic acid equivalents (GAE) /100 g material. The concentration of polyphenols in samples were derived from a standard curve of gallic acid ranging from 10 to 50 mg/mL (Pearson´s correlation coefficient: $r^2 = 0.9996$).

High performance liquid chromatography: A Varian™ series 9000 chromatography device equipped with a

binary pump Varian 9012 was used. The quantitation of caffeoyl derivatives and caffeine was carried out using validated HPLC external standard methods. Pure standards were obtained from Carl Roth. A reverse phase IB-SIL RP 18 (5 μm, 250 x 4.6 mm I.D.) Phenomenex column and a gradient consisting of solvent A: water: acetic acid (98:2); solvent B: methanol: acetic acid (98:2) were used. For caffeoyl derivatives a gradient range from 15% B to 40% B in 30 min; 40% B to 75% B in 10 min. and 75% B to 85% B in 5 min was employed. Flow rate was set at 1.2 mL/min. For caffeine a gradient range was from 17% B to 20 m % in 10 min; 20% B (isocratic) for 5 min; 20% B to 23% B in 10 min and 23% B to 100% B in 5 min with a flow rate of 1.0 mL/min was used. Identification and quantitation were carried out by simultaneous analysis of retention times and detection with a Varian 9050 UV detector and Varian 9065 Photodiode-Array Detector at 325 nm for caffeoyl derivatives, 273 nm for caffeine and 254 nm for rutine. Samples were injected with a Rheodyne injector fitted with a 100 μL loop (Filip *et al*., 1998, 2001).

Determination of the antioxidant activity by the ferric thiocyanate method: The antioxidant capacity was determined by the ferric thiocyanate method (FTC) (Osawa and Namiki, 1981). A volume of 0.8 mL of each concentration of extracts was mixed with 0.05 M phosphate buffer pH 7 and 2.5% linoleic acid in ethanol to obtain 4 mL of solution. The resulting solutions were incubated at 38.5ºC in a glass flask. Aliquots were taken at regular intervals and a $FeCl_2$/ammonium thiocyanate solution was added in order to allow any peroxides resulting from the oxidation of linoleic acid to react, forming a complex that can be detected spectrophotometrically at 500 nm (Shimatzu UV 2101). This step was repeated every 24 h until the control (phosphate buffer plus linoleic acid) reached its maximum absorbance value. Therefore, high absorbance values indicated high levels of lino1eic acid oxidation. Phosphate buffer was used as reaction blank. The total antioxidant activity was expressed as the average of three independent determinations carried out in duplicate. The percentage inhibition of lipid peroxidation of linoleic acid was ca1culated applying the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - [(As/Ao) \times 100]$$

where Ao is the absorbance of the control reaction (linoleic acid alone, 100% peroxidation) and *As* is the absorbance obtained in the presence of the sample extract or positive control of antioxidant activity (1 mg/mL ascorbic acid). The effective concentration 50 (CE50) values were calculated from data obtained graphically, using a mathematical method based on the principle of the right-angled triangle:

$$CE50 = D - [(A - 50\ \%\ \text{max response}).\ X]/Y$$

in which A is the immediately higher response of 50% max response; B is the immediately lower response of 50% max response; D = log concentration corresponding to A response; C = log concentration corresponding to B response; X = D-C; Y = A-B (Alexander *et al*., 1999).

Determination of the free radical scavenging activity by the 1,1-diphenyl-2-picrylhydrazyl free-radical scavenging assay: Scavenging activities of the extracts on the stable free radical DPPH were assayed using the modified Bloi´s method in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence of the sample (Blois, 1958). A volume of 0.1 mL of an aqueous dilution of the extracts were mixed with 0.5 ml of a 500 μM DPPH solution in absolute ethanol and 0.4 mL of a 0.1 M Tris-ClH buffer pH 7.4. The mixture was kept for 20 min in the darkness and then the absorbance was read at 517 nm. The percentage of decrease of DPPH bleaching was calculated by measuring the absorbance of the sample and applying the following equation:

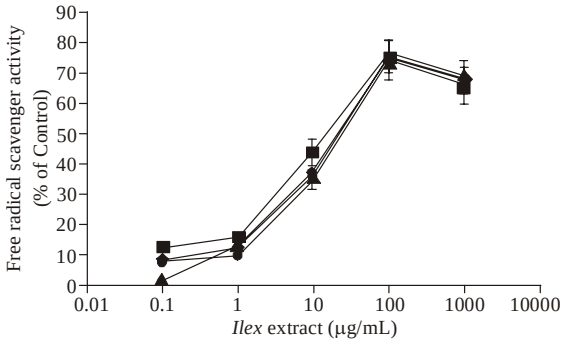
$$\text{Inhibition (\%)} = [1 - (As/A0)] \times 100$$

where *As* is absorbance of sample (i.e, extracts) and *Ao* is the absorbance of the DPPH solution. Ascorbic acid solutions of different concentrations were used as positive controls for antioxidant activity.

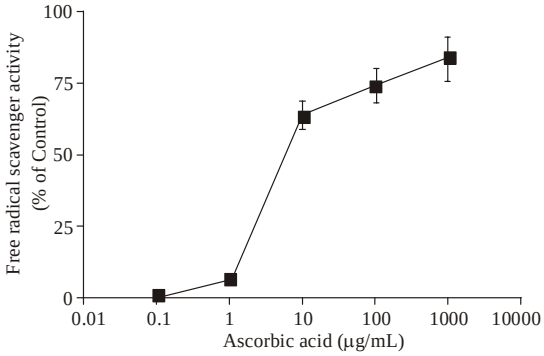
Statistical analysis: Data were expressed as means±SD or SEM of three independent experiments carried out in duplicate. A one-way ANOVA with the a *posteriori* Student-Newman-Keuls test or Dunnett´s test was used to evaluate the significancy of results. A probability $p<0.05$ was considered significant.

RESULTS

Antioxidant activity of crude extracts: Firstly, the antioxidant effect of the extracts from *I. paraguariensis* during different stages of the industrial process: green, zapecado, dried and Forced Aging (FA) was analyzed. All the studied extracts presented scavenging activity on free radical DPPH in a concentration-dependent manner. Nevertheless, the green extract was the most potent (Fig. 1a and c and Table). No significant differences were observed between the other extracts. It is noteworthy that, the maximum scavenging effect exerted by all the extracts was similar to that exerted by the antioxidant control containing ascorbic acid (Fig. 1b). Moreover, the extracts displayed a preventive effect on lipid peroxidation in a dose-dependent fashion (Fig. 2a and c). The green extract



(a)



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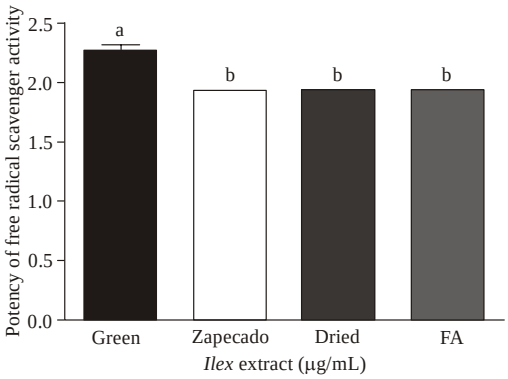
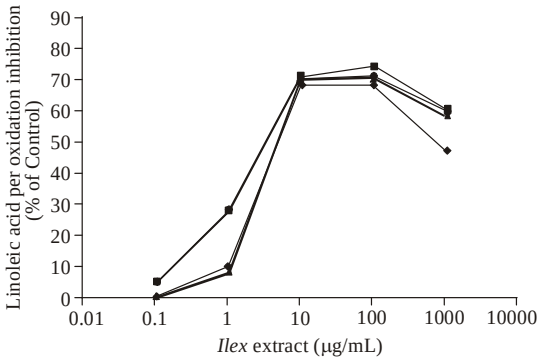


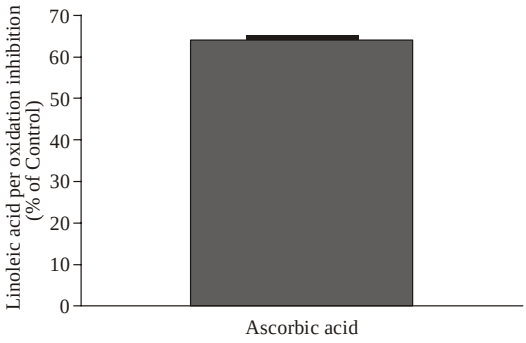
(c)

Extract	EC_{50} (µg/mL)	Dried	11,7±1,1
Green	5,88 ±0,6	FA	11,5± 1,1
Zapecado	11,0± 1,0		

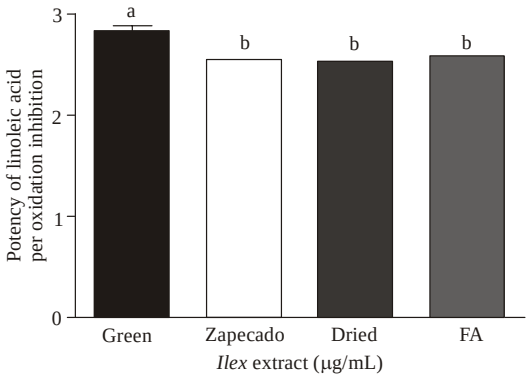
Fig. 1: Scavenging activity on the free radical DPPH of *Ilex paraguariensis* extracts during the different stages of industrial process and (a) ascorbic acid; (b) Potency; (c) was calculated as follows: P = -log EC50. Table inserted: Free radicals scavenger activity of each extract expressed as EC50.. Data represent the means ± SEM of three independent experiments carried out in duplicate. ■ green; ▲ zapecado; ▼ dried; ♦ Forced aging (FA). Letters a and b indicate significant differences between samples (one way ANOVA and Student-Newman-Keuls test, $p<0.05$)



(a)



(b)



(c)

Extract	EC_{50} (µg/mL)	Dried	2.88 ± 0.25
Green	1.62 ± 0.1	FA	2.63 ± 0.2
Zapecado	2.75 ± 0.2		

Fig. 2: Inhibition of linoleic acid peroxidation by *Ilex paraguariensis* extracts, during the different stages of the industrial process, and (a) ascorbic acid; (b) Potency; (c) was calculated as follows: P = -log EC50. Table inserted: Inhibition of linoleic acid peroxidation of each extract expressed as EC50. Data represent the means ± SEM of three independent experiments carried out in duplicate. ■ green; ▲ zapecado; ▼ dried; ♦ Forced aging (FA). Letters a and b indicate significant differences between samples (one way ANOVA and Student-Newman- Keuls test, $p<0.05$)

Table 1: Total polyphenols content in *I. paraguariensis* at the different stages of the industrial process

	Green	Zapecado	Dried	FA
Polyphenols %	7.59 [a] ± 0.09	8.71 [b] ± 0.08	8.85 [b] ± 0.55	8.64 [b] ± 0.38

Values were determined by spectrophotometry (Folin-Ciocalteu's method) and represent the means ± SEM of three independent experiments carried out in duplicate; Results are expressed as gallic acid equivalents (GAE; g/100 g plant material) on dried weight; Different letters indicate significant differences between samples (one way ANOVA and Student-Newman- Keuls test $p<0.05$)

Table 2: Caffeoyl derivatives, methylxantines and rutin content in *Ilex paraguariensis* at the different stages industrial process

Compound %	Green	Zapecado	Dried	FA
Chlorogenic acid	1.84 [a] ± 0.04	2.04[b] ± 0.03	2.06[b] ± 0.05	2.03[b] ± 0.05
Caffeic acid	0.033[a] ± 0.002	0.033[a] ± 0.003	0.035[a] ± 0.003	0.035[a] ± 0.002
Caffeine	0.91[a] ± 0.03	1.49[b] ± 0.04	1.36[c] ± 0.04	1.39[c] ± 0.02
Rutin	0.98[a] ± 0.05	1.25[b] ± 0.01	1.35[c] ± 0.03	1.30[c] ± 0.03

Letters indicate significant differences ($p<0.05$ one way ANOVA and Student-Newman- Keuls test); Values represent the means±SEM. Results are expressed as % of dried plant material; FA: forced aging

exerted by the antioxidant control containing ascorbic acid (Fig. 2b). No significant differences were observed in the antioxidant action among the other extracts (zapecado, dried and FA) (Fig. 2c).

Phytochemical analysis of aqueous extracts:

Total polyphenols content: The processed *I. paraguariensis* extracts (zapecado, dried and FA) showed higher polyphenols content than green samples. No significant differences were observed among the processed extracts (Table 1).

Quantitation of bioactive compounds by HPLC-DAD: All the extracts were analyzed by HPLC with DAD detector and the main bioactive compounds: caffeoyl derivatives (caffeic acid and chlorogenic acid), methylxantine: (caffeine) as well as the majoritary flavonoid (rutin) were identified and quantified. The flavonoids quercetin and kaempferol were found in low concentrations (below the limit of quantification: 1 ppm) because of this they were not quantified. Results are shown in Table 2.

DISCUSSION

This study was focused to investigate the impact of the industrial process on the antioxidant capacity of *I. paraguariensis* and to provide an overall profile of its quality in terms of the antioxidant activities related to the phytochemical composition of the extracts. The antioxidant activity of *I. paraguariensis* extracts during the different stages of the industrial process was demonstrated. All the extracts presented antioxidant activity, demonstrated by the scavenging effect on the DPPH radical and on the prevention on lipid peroxidation. The extract prepared with green leaves presented the highest antioxidant potency, as demonstrated by the EC_{50} value, which was between 1.80 to 1.99 times lower in the case of DPPH scavenging activity and was between 1.62 to 1.78 times lower in the case of the prevention of lipid peroxidation, when compared to the EC_{50} values obtained for the extracts subjected to industrial treatment (zapecado, dried and FA). Nevertheless it is noteworthy that all the extracts displayed an antioxidant activity comparable to the antioxidant activity obtained with the ascorbic acid used as control (Fig. 1).

The DPPH is a relatively stable radical species (actually one of the few stable and commercially available organic nitrogen-centred radicals). In addition, DPPH is insensitive to side reactions mediated by polyphenols, such as metal ion chelation and enzyme inhibition. For this reason, the DPPH scavenging activity assay has been widely used. A freshly prepared DPPH solution displays a deep purple colour (λmax = 516 nm) that gradually vanishes in the presence of a good hydrogen donor, i.e., a potent antioxidant. The antioxidant effect of *Ilex* species has previously been reported by other authors. It has been reported that the leaves of *Ilex* species (Aquifoliaceae) i.e., *I. brevicuspis* and especially *I. paraguariensis*, have antioxidant activity. It has been demonstrated that *I. brevicuspis* has antioxidant action in liposomes treated with the free radical generator 2,2'-azobis [amidinopropane] chloride (AAPH) (Filip and Ferraro, 2003). In '*in vivo*' studies, it has been demonstrated that the antioxidant compounds present in *I. paraguariensis* are absorbed to reach sufficiently high levels in plasma to inhibit the copper-induced LDL autoxidation by increasing the aqueous-phase antioxidant capacity (Gugliucci, 1996). Furthermore, the peroxynitrite and lipoxygenase-induced human LDL oxidation are inhibited by *I. paraguariensis* extracts in a potent, dose-dependent fashion (Bracesco *et al*., 2003).

Phytochemical investigations have revealed that the major constituents of the *Ilex* spp. are polyphenols such as caffeic acid derivatives, flavonoids, triterpenoids, and triterpenoid saponins (Heck and de Mejia, 2007). Polyphenols are abundant antioxidants present in fruits and vegetables and are involved in the inhibition of endogenous production of free radicals and progression of oxyradical-mediated degenerative pathologies in humans (Mattei *et al*., 2001). It is well known that phenolic compounds have a wide impact on the biological system

being their antioxidant property, their most interesting feature (Karakaya, 2004). On the other hand, different *in vitro* assays have demonstrated a high correlation between the polyphenols content (mainly caffeic acid, chlorogenic acid and rutin) and the antioxidant activity of different extracts (Abu-Amsha *et al*., 1996; Gulcin, 2006; Kono *et al*., 1997). Similar results have also been observed in a comparative study of *Ilex* spp and commercial "yerba mate" (Filip *et al*., 2000).

The extracts obtained at the different stages of the industrial process were analyzed spectrophotometrically to determine the total polyphenols content. The results showed that the samples subjected to the industrial process ("zapecado", "dried" and "FA") displayed higher total polyphenols content than that obtained from "green" leaves (Table 1). Accordingly, the polyphenols present in each extract were identified and quantified by HPLC with DAD detector. The chromatographic analysis demonstrated that all the samples analyzed proved to have a similar qualitative-quantitative pattern. The caffeic acid content was similar in all extracts. Chlorogenic acid content was slightly lower in "green" samples when compared to the ones subjected "zapecado", "dried" and "FA" and no significant differences were found among these three stages. The major differences were found in the caffeine and rutin contents. Samples prepared with green leaves presented a less significant content in caffeine and rutin than the samples subjected to industrial processing (Table 2). These results are in line with those reported by other authors. Thus, the temperature and humidity conditions employed during the industrial process in combination with the cell disruption and mechanical impact during the processing stages could increase the availability of compounds to be extracted from the plant tissues (Esmelindro *et al*., 2002; Isolabella *et al*., 2010).

Numerous protective effects have been observed with caffeoyl derivatives such as the hepatoprotective (Basnet *et al*., 1996; Xiang *et al*., 2001), analgesic and anti-inflammatory activities as well as the prevention of LDL oxidation (Hung *et al*., 2006).

Chlorogenic acid is one of the most naturally existing phenolic compounds found in numerous plant species. Chlorogenic acid and related compounds are well known antioxidants (Kono *et al*., 1997; Ohnishi *et al*., 1994). Furthermore, the antioxidant effect of caffeic acid has also been reported. This compound can inhibit low density lipoprotein oxidation *in vitro* (Abu-Amsha *et al*., 1996) and has an effective $ABTS^{+}$, DPPH and superoxide anion radical scavenging activity. The total reducing power and metal chelating on ferrous ions activities has also been described for caffeic acid (Gulcin, 2006). Furthermore, the antioxidant activity of the flavonoid rutin has been well documented. Rutin can prevent the Cu^{2+}-induced oxidation of HDL (Meng *et al*., 2004). It has also been demonstrated that the oral administration of rutin to streptozotocin-induced diabetes in rats significantly decreased the levels of thiobarbituric acid (TBA) reactive substances and lipid hydroperoxides and increased the non-enzymatic antioxidants (Narasimhanaidu and Mainzen, 2006).

The results obtained in this work indicated that a possible antagonistic interaction among the antioxidant compounds in the food mixture may take place. Further studies are needed to better understand these phenomena and to predict *in vivo* effects.

CONCLUSION

In this study, the antioxidant activity of extracts from *I. paraguariensis* during the different stages of the industrial process was characterized for the first time by two different methodologies, that is, the DPPH free radical scavenging assay and the ferric thiocyanate assay. The results demonstrated that the industrialization process decreases the total antioxidant activity of the extracts. For this reason, maté green leaves could provide the most useful material with antioxidant properties to be used in formulations for food and pharmaceutical industries.

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