Determination of phenolic compounds and antioxidant activity of green, black and white teas of *Camellia sinensis* (L.) Kuntze, Theaceae

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ABSTRACT: Green, black and white teas are all produced from leaves and shoots of *Camellia sinensis*, the only difference is how they are processed. The aim of this study was to compare the total phenols and flavonoid contents and antioxidant capacity of green, black and white tea bags of different brands. The morphodiagnosis of leaves was used to identification of plant material. HPLC-DAD fingerprinting coupled with Principal Component Analysis (PCA) was applied to analyze similarities of the tea samples. The results showed considerable variability between tea brands in both total phenols (30.55 to 60.85 mg of pyrogallol/g) and flavonoids (6.35 to 8.92 mg of quercetin/g). Green and white teas demonstrated the highest ABTS and DPPH radical scavenging activities.

Keywords: infusions, total phenols, flavonoids, free radical and chemometry.

RESUMO: Determinação de compostos fenólicos e atividade antioxidante dos chás, verde, preto, e branco, de *Camellia sinensis* (L.) Kuntze, Theaceae. Os chás verde, preto e branco são produzidos de folhas e brotos de *Camellia sinensis*, diferenciando-se pelo processamento. O objetivo deste estudo foi comparar o conteúdo de fenóis totais, flavonoides e capacidade antioxidante dos chás verde, preto e branco de diferentes marcas na forma de sachês. A análise morfo-anatômica das folhas foi realizada para a identificação do material vegetal. Os perfis químicos (*fingerprints*) obtidos por Cromatografia Líquida de Alta Eficiência (CLAE - DAD) foram analisados por ferramentas quimiométricas de análise exploratória (PCA) para análise comparativa entre as amostras. Os resultados evidenciaram considerável variação entre as marcas de chás, tanto para fenóis totais (30,55 a 60,85 mg de pirogalol/g), quanto para flavonoides (6,35 a 8,92 mg de quercetina/g). As amostras de chá verde e de chá branco apresentaram maior atividade antioxidante contra os radicais ABTS e DPPH.

Palavras-chave: infusões, fenóis totais, flavonoides, radical livre e quimiometria.

INTRODUCTION

Camellia sinensis (L.) Kuntze is a very branched tree belonging to the Theaceae family (Duarte & Menarim, 2006). Although it is originated in China, Tibet and Northern India, today is widely cultivated throughout the world. In Brazil, the tea plant has been cultivated in the Ribeira Valley, State of São Paulo (Lima et al., 2009).

The leaves and shoots are utilized in the production of different types of tea, available to consumers as tea bag, ready-to-drink tea, flavoured tea, and organic tea. The tea plant is often packaged in individual bags which contain approximately 1 g of plant material.

The types of tea produced from *Camellia sinensis* (white, green and black) differ in the processing of the plant material (Coggon et al., 1973). While black tea undergo a fermentation stage before drying, green tea consists in leaves heated immediately after harvesting, mechanically wound and compressed, and then the leaves are dry to ensure the preservation of color and natural constituents (Cabrera et al., 2003; Hilal & Engelhardt, 2007) .

The white tea has a particular method of post-harvest processing, containing a greater proportion of sprouts, which are covered with a thin layer of silvered hair characterizing the tea coloring (Karori et al., 2007). Unlike black and green teas, white tea is not rolled or crushed, but is slightly fermented, cooked quickly and its leaves are dried

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naturally in air to preserve the most polyphenols (Cheng, 2006).

There are lot of chemical constituents in the *Camellia sinensis* tea, such as polyphenols, methylxanthines (caffeine, theophylline and theobromine), vitamins, amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, fluoride, minerals, trace elements, and other undefined compounds (Cabrera et al., 2003). Polyphenols are compounds of great interest because they present potent antioxidant activity both in vitro and in vivo due to its reducing properties (Wu & Wei, 2002).

Phenolics compounds are characterized by having one or more aromatic nuclei containing hydroxylated substituents and/or its functional derivates; a group of phenolic compounds are flavonoids called catechins (Lima et al., 2009; Ashihara et al., 2010). Monomeric primary catechins that are present in high quantity in the teas of *Camellia sinensis* are: (-)epigallocatechin gallate, (-) epigallocatechin, (-)epicatechin gallate, epicatechin and catechin (Ho et al., 1992; Nagle et al., 2006).

The fermentation method used for obtaining the black tea results in the oxidation or condensation of primary catechins, giving origin to dimers or polymers, called as theaflavins, theasinensins and thearubigins, which provides peculiar and unique organoleptic characteristics, besides of aroma and color to the teas (Chan et al., 2007; Barcirova, 2010).

Flavonoids as quercetin and myricetin are also observed in considerable quantities in tea of *Camellia sinensis* (Saito & Miyata, 2000). Both catechins and other flavonoids can capture reactive oxygen species such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), all considered extremely harmful to lipids, proteins and DNA (Senger et al., 2010).

The teas obtained from *Camellia sinensis* have been considered as beneficial to human health due to its high contents of phenolic antioxidant compounds. There are two principal varieties of *Camellia sinensis*: var. *sinensis* (Chinese tea) and var. *assamica* (Indian tea) and the levels of phenolics can be significantly affected depending on variety, environmental factors, post-harvest handling and storage conditions, leaf and shoots composition, and degree of fermentation (Yanagimoto et al., 2003; Graham, 1992).

Although many studies relating to the medicinal properties of *Camellia sinensis* has been made, very few studies been directed towards comparative analysis of commonly consumed teas. In this paper, and for the first time, the Brazilian teas from different types and brands, were studied regarding their total phenols, flavonoid contents and antioxidant activities.

MATERIAL AND METHOD

Samples

Three commercial tea bags of white tea, green tea and black tea were purchased at supermarkets in the region of Ponta Grossa – Paraná State, Brazil. For each commercial sample, three bags were opened and the contents homogenized. Then, 1 g of the powder was placed back in original bag and resealed.

Morpho-anatomical analysis

The tea bags samples were examined for their microscopical characters. Powders of tea bags were cleared by boiling with chloral hydrated solution 10% (w/v) for 3 min., washed with distilled water and mounted with glycerinated gelatin. Photomicrographs were taken using a Photomicroscope Olympus CX 31, with the help of Olympus Camedia C-7070 digital camera.

Tea infusions preparation

Samples were prepared in a similar manner to that recommended in the packaging. A beaker containing 100 mL of distilled water was heated in a microwave oven Consul (model Practice) for 50 seconds at full power. Then a tea bag (containing 1g) was added into the beaker and slowly stirred, manually for 3 min. and then removed from the infusion. After cooling, the infusions were filtered through a filter paper into a 100 mL volumetric flasks and the volume completed with distilled water. These solutions were used for determinations of total phenols, flavonoids and evaluation of antioxidant activity immediately after preparation.

HPLC instrumentation and chemicals

Fingerprinting analysis was performed on a Waters 2695 Alliance HPLC system (Milford, MA, USA) with a Photodiode Array Detector (Waters 2998). The analytical column was a Luna phenyl-hexyl (15 x 0.46 cm I.D, 10 µm particle size and 100 A pore size). Acetonitrile (ACN) was HPLC-grade (J.T. Baker, Philipsburg, PA, USA); water (H_2O) was purified with a Millipore Milli-Q system (Millipore, São Paulo, SP, Brazil) and it was used for all experiments.

The filtered tea infusions were analyzed by gradient elution with ACN (B) in water (A) - (5 to 30% in 16 min., 30 to 50% in 4 min., 50 to 100% in 5 min. an isocratic run at 100% of B was maintained for 5 min before doing the reverse gradient to 5% of B (10 min)). The injected volume was 40 µL and the flow rate was 1 mL/min.

Determination of total phenols

The total phenols content was determined by adapting the method of Folin-Denis described in

the Brazilian Pharmacopoeia (2003). In test tubes was added 500 µL of diluted tea infusions (1:10) and 300 µL of Folin-Denis and the volume was made to 5 mL with sodium carbonate 10.6% (w/v). The absorbance reading was done after 10 minutes at 715 nm using a spectrophotometer Genesys 10. The calibration curve was obtained in a similar manner to that described for samples using standards solutions of pyrogallol (1, 2, 4, 5, 6 and 8 µg/mL). The results were expressed as mg of pyrogallol equivalents per gram of dry material.

Determination of flavonoid content

The concentration of flavonoids was determined by adapting the spectrophotometric procedure described in Brazilian Pharmacopoeia (2003). In a 10 mL volumetric flask, 5 mL aliquots of infusions and 500 µL of methanol solution of aluminum chloride 2% (w/v) were added and the volume was completed with a solution of acetic acid 5% (v/v). After 30 minutes, the absorbance was read at a wavelength of 425 nm using a spectrophotometer Genesys 10. The calibration curve was performed with standard solutions of quercetin (2, 4, 8, 10 and 14 µg/mL). The results were expressed as mg of quercetin equivalents per gram of dry material.

DPPH radical scavenging of tea infusions

The antioxidant activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) was performed by adapting the method described by Duarte-Almeida et al. (2006). A DPPH solution (20 mg/mL) was prepared in ethanol and the infusions were diluted (1:1, 1:10 and 1:100) with distilled water. The tests were performed by adding three different volumes of each diluted sample (10, 25, and 50 µL), DPPH solution (500 µL) and ethanol to a final volume of 1 mL. The absorbance readings were made after 15 minutes at 531 nm and the antioxidant activity was expressed by IC_{50} values.

ABTS radical scavenging of tea infusions

The antioxidant capacity on ABTS [2,2 '-azino-bis (3-ethylbenzthiazoline) 6-sulfonic acid], was performed by adapting the method described by Re et al. (1999). A solution of 55 µM ABTS was prepared in 10 mM phosphate buffer (pH 7,0) and infusions were diluted (1:1, 1:10 and 1:100) with water. The tests were performed by adding three different volumes of each diluted sample (10, 25, and 50 µL), ABTS solution (500 µL) and phosphate buffer to a final volume of 1 mL. The absorbance readings were taken after 30 minutes at 734 nm and antioxidant activity was expressed by IC_{50} values.

PCA in HPLC fingerprints

Chromatographic data acquisition, analysis and reporting were performed using Empower chromatography software (Waters, Milford, MA, USA). The resulting data matrix was imported into Excel® software (Microsoft, USA).

PCA was applied to separate the samples (n = 9) according to their HPLC chromatogram data (0 – 26 minutes, 280 nm). For this purpose, the results obtained for each time were adopted as columns and the green, black and white tea samples as rows. Mean-Center was used as a pre-treatment of the results.

PCA in total phenol, flavonoid content and antioxidant activity

PCA was applied to separate the samples (n = 9) according to their values of total phenol, total flavonoids and antioxidant capacity (ABTS and DPPH). For this purpose, the results obtained for each parameter were adopted as columns and the tea samples as rows. Autoscaling was used as a pre-treatment of the results. The purpose of this procedure was to equalize the statistical importance of all the variables.

Statistical analysis

All tests were performed in triplicate. Analysis of variance - ANOVA - was held using the GraphPad Prism® software to comparing of the mean content of total phenols, flavonoids and antioxidant activity.

Principal component analysis (PCA) implemented in the Pirouette® v.4.0 software (Infometrix, Bothell, WA, USA), was the chemometric method used to analyze the results.

Result and discussion

The samples analyzed presented consistent characters as described for the *Camellia sinensis* species (Figure 1A), as the presence of anomocytic stomata located only on the abaxial surface (Duarte & Menarim, 2006). In the Theaceae family the leaf is usually dorsiventral, where the spongy parenchyma occupies almost two thirds of mesophyll (Figure 1B) and contains calcium oxalate druses crystals (Figures 1B and 1C) frequently encountered in *Camellia sinensis* (Gilg et al., 1926).

Unicellular and thick-walled trichomes on abaxial side of leaves (Figure 1D) are described as typical anatomical markers of the species (Pereira et al., 2009) and were also found in all samples, confirming the identity of the plant material.

Tea infusions were analyzed by gradient HPLC procedure with the purpose of to obtain the *fingerprinting* chromatograms. The UV spectra of some chromatographic bands presented in the *fingerprinting* of the samples obtained from HPLC-DAD analysis (Figure 1) indicated the presence of

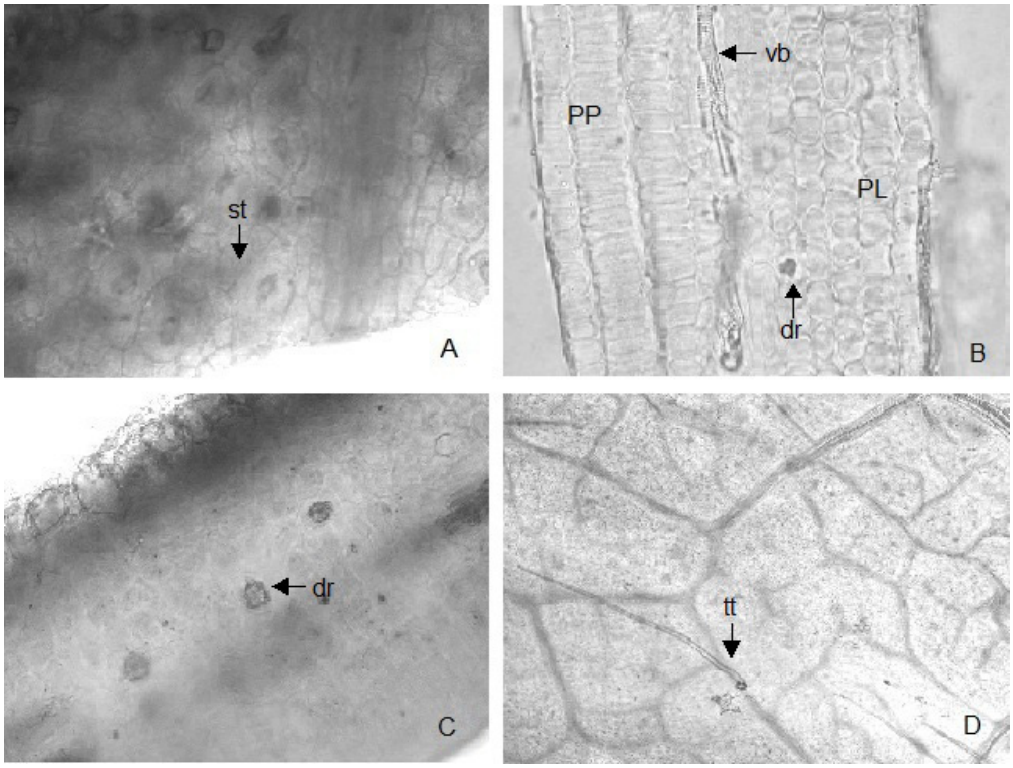

Figure 1. A) Front view of the fragment of leaf showing anomocytic stomata (st); B) Cross section of leaf showing vascular bundles (vb) and drusen (dr); C) Detail of druse (dr) in the leaf cross section; D) Spotlight tector trichomes (tt) unicellular in front view. PP = palisade chlorenchyma, PL = spongy chlorenchyma.

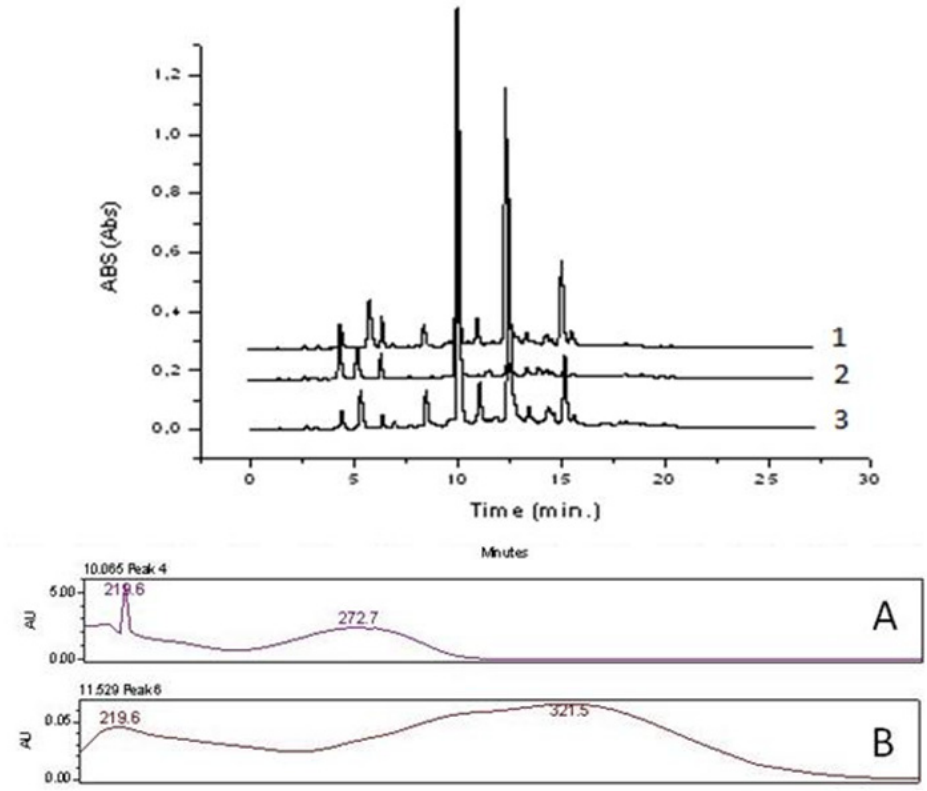

Figure 2. Overlay of fingerprint chromatograms of black (1), green (2) and white (3) tea infusions and UV-DAD spectra (200-400 nm) using HPLC method. (A) rt = 10 min., (B) rt = 11.5 min.

phenolic compounds. The UV spectra with maxima on 254 nm (band II/ring A) and another on 321 nm (band I/ring B) confirm the aromatic character of the compounds, correlated with the benzopirone skeleton indicating characteristics of flavonoids to the compounds (Figure 1B). In the same way some bands presented the UV spectra with bands in 219 nm and 270 nm confirming the presence of organic phenolic acids presents in all the samples studied. Zeraik & Yariwake (2010) report that the UV spectra of the chromatographic bands allows the selection of interested peaks (flavonoid and phenolic organic acids) for qualitative and quantitative analysis.

The visual analysis of the chromatograms did not show clear differences between some samples analyzed, although a variation in the intensities of the same signals could be observed and different peaks were observed in some samples. According Beltrame et al. (2012) the fingerprinting chromatogram is complex to evaluate thus a possible approach is to observe the metabolite profile as a whole using multivariate methods, for example, by application of Principal Component Analysis (PCA).

Chemometric evaluation (PCA) was applied to identify the similarities and the differences between the samples of black, green and white teas, as described by Cass et al. (2011). The results demonstrated that the PCA score plot (Figure 3) showed a similar distribution for the majority of samples tested.

As can be seen in the Figure 3, the difference observed in the PCA classification of two samples (black 3 and white 1) could be attributed to the fact the chemical composition is not exactly the same for samples from different varieties of tea. However, considering the overall chromatographic information, a phytochemical profile is observed for *Camellia sinensis* teas.

The determination of total phenols was accomplished by Folin-Denis method. Results were expressed in mg of pyrogallol/g of dry matter and are shown in Figure 4.

Statistical analysis of the mean reveals that green tea showed a higher content of total phenols (55.40 mg/g), differing significantly ($p < 0.05$) from the white tea (46.91 mg/g) and black tea (36.28 mg/g). In the comparative analysis of the brands of the same type of tea, it was observed that there is a significant difference ($p < 0.05$) between most of them.

The levels of total phenols obtained in this study are in agreement with those reported in the literature. Sakanaka *et al.* (1989) found about 50 to 100 mg of polyphenols in a cup (100 mL) of green tea, while Dalluge & Nelson (2000) reported an average value of 60 mg/g (ranging from 9 to 117 mg/g), depending on the origin of tea.

The flavonoid contents were determined by spectrophotometric method based on the complexation with aluminum chloride. Results were expressed in mg of quercetin/g of dry matter and are shown in Figure 5.

The values of flavonoids in green tea and

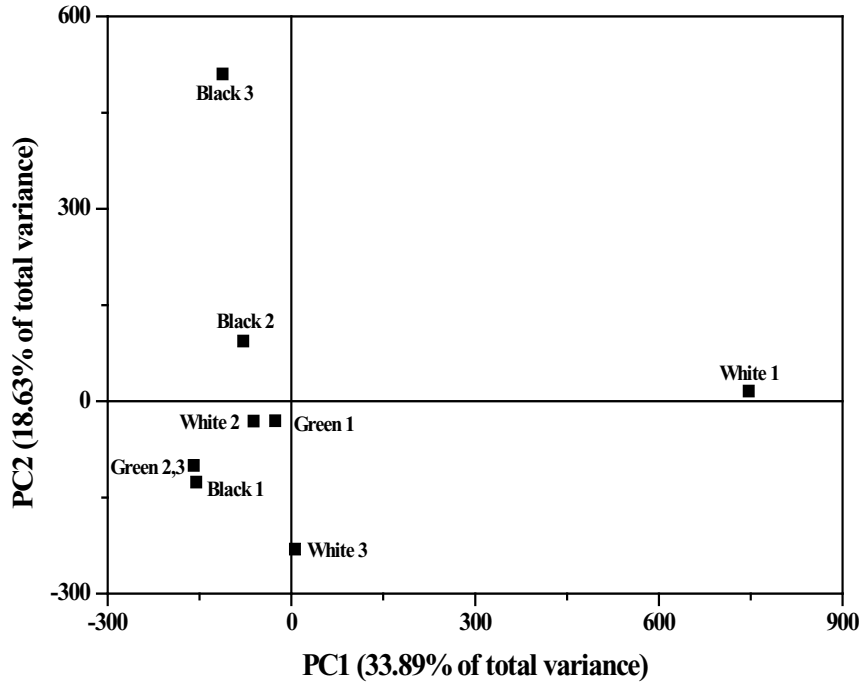

Figure 3. PC 1 vs. PC 2 scores plot of the chromatograms obtained by HPLC-UV analysis of black, green and white teas. PC 1 and PC 2 account for 33.89 and 18.63% of the variance, respectively.

white tea varied between 7.31 and 9.58 mg/g and were significantly lower in black tea (6.11 - 8.75 mg/g). The average values of flavonoids of green tea (8.30 mg/g) and white tea (8.31 mg/g) showed no significant difference ($p < 0.05$). Unlike what was observed in the analysis of total phenols, a lower number of brands were significantly different ($p < 0.05$) when considering the same type of tea.

Rusak et al. (2008) using HPLC showed that the catechins are predominant phenolic compounds in samples of *Camellia sinensis* and green tea is so rich in phenols as white tea, but the efficiency of the extraction depends on the infusion time and the solvent used. Astill et al. (2001) and Chen et al. (2001) also reported the influence of these factors and temperature on the extraction of phenolics and antioxidant activity.

The processing of the leaves prior to drying, geographic location of the planting and cultivation conditions may influence the catechin contents (Yanagimot et al., 2003). Graham (1992) reports that the season, climate and leaf age also influence the composition of the teas. Pereira et al. (2009) and Nishyama et al. (2010) suggest that to obtain the total antioxidant capacity of *Camellia sinensis* tea, the infusion time must be at least 5 minutes under mild agitation. However, in general, tea infusions are prepared in much less than 5 min. In this work, the selected infusion time was 3 min. as the manufacturers' recommendation contained in packaging.

The tea particle size influences in the extraction process, since the smaller particles lead to greater surface area exposed to hot water,

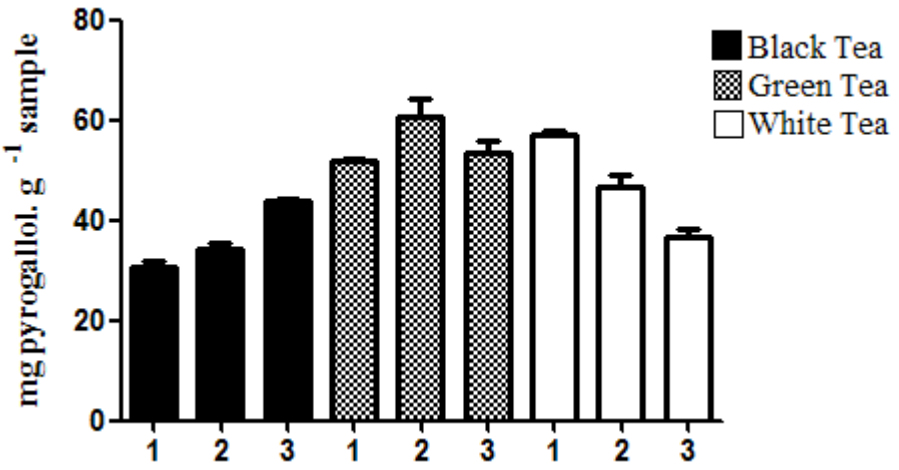

Figure 4. Total phenols in *Camellia sinensis* teas.

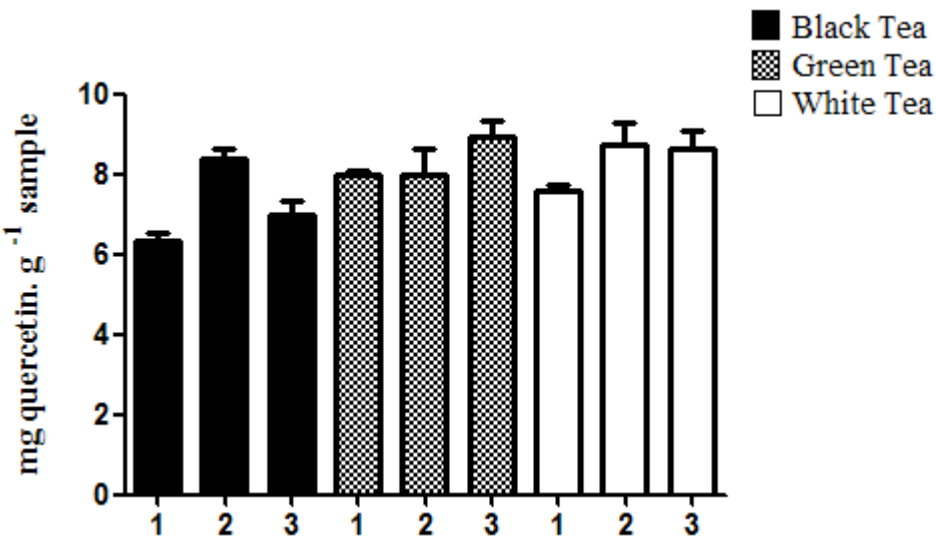

Figure 5. Flavonoid contents in *Camellia sinensis* teas.

increasing the extraction efficiency. The difference in the particle size of the selected tea bag samples possibly influenced the results of this study.

In some types of deeply fermented tea (black tea), monomeric catechins are oxidized or condensed to form theaflavins and thearubigins (Chan et al., 2007; Barcirova, 2010). The results of this study suggest that black tea has lower contents of total phenols and flavonoids, which may have been degraded during the fermentation process.

Antioxidant activity was measured DPPH and ABTS assays. IC_{50} values for DPPH and ABTS representing a sample concentration required to inhibit radical formation by 50% and are presented in Table 1 and 2, respectively. Samples that have lower IC_{50} values correspond to those that have stronger antioxidant activity.

The results showed high correlation coefficient (R^2=0.969) of measured values between DPPH and ABTS assays for tea bags analyzed and that green tea and white tea exhibited more pronounced antioxidant activity than black tea.

Khalaf *et al.* (2008) using methanol extracts and DPPH assay observed that green tea has higher antioxidant activity than black tea. Almajano *et al.* (2008) found that all types of tea (green, white, black and red) showed antioxidant activity, although green and white teas showed the highest radical-scavenging activity against trolox.

The results of this study show in general, a relatively low direct correlation between the free radical scavenging activity and the total phenols or flavonoid contents in tea samples. However, when considering the average values of all brands (1, 2 and 3), the results show that the antioxidant activity on both radicals (DPPH and ABTS) is most pronounced in green tea and white tea, agreeing with the amount of flavonoids.

Green tea has antioxidant activity mainly attributed to catechins, while black tea is dependent on theaflavins and thearubigins (Yashin et al., 2011). This fact could explain individual differences between phenolic contents and antioxidant activity observed in this study.

Multivariate statistical techniques, including PCA, have been used to monitor the difference between phenols contents and antioxidant activity in many types of products, including medicinal plants (Komes et al., 2011) and teas (Deetae et al., 2012). PCA also were applied in order to evaluate the data of total phenols, flavonoids and antioxidant capacity. PC1 explained up to 68.63% of total variance and PC2 explained 18.49%, totalizing 87.12%. First, considering the relative position of the samples it was possible to verify the separation into two groups (Figure 6).

For black teas, a group was observed with lowest content of total phenols and flavonoids and highest values of IC_{50} (ABTS and DPPH), indicating a lower antioxidant activity. Another group is formed by white and green teas with highest levels of total phenols and flavonoids and lowest values of IC_{50} (ABTS and DPPH), indicating a higher antioxidant activity.

Table 1. IC_{50} values for DPPH radical scavenging of tea infusions.

	$IC_{50} \pm s$		
	Brand 1	Brand 2	Brand 3
Black Tea	45.10 ± 0.01 a*	52.65 ± 1.28 b*	37.30 ± 2.34 c*
Green Tea	18.79 ± 0.26 a□	35.10 ± 1.34 b□	13.51 ± 0.98 c□
White Tea	21.14 ± 0.45 a□	27.55 ± 1.93 b•	22.09 ± 0.09 a•

The results are expressed in μg.mL^{-1} followed the standard deviation. Lowercase letters are for comparison of brands (lines) and symbols to type (columns). Different letters or symbols show a significant difference ($p < 0,05$).

Table 2. IC_{50} values for ABTS radical scavenging of tea infusions

$IC_{50} \pm s$			
	Brand 1	Brand 2	Brand 3
Black Tea	11.85 ± 0.99 a*	12.99 ± 1.32 a*	10.36 ± 0.61 b*
Green Tea	6.81 ± 0.36 a□	9.87 ± 0.37 b□	5.61 ± 0.01 a□
White Tea	6.32 ± 0.05 a□	9.30 ± 0.08 b□	7.12 ± 0.08 a•

The results are expressed in μg.mL^{-1} followed the standard deviation. Lowercase letters are for comparison of brands (lines) and symbols to type (columns).

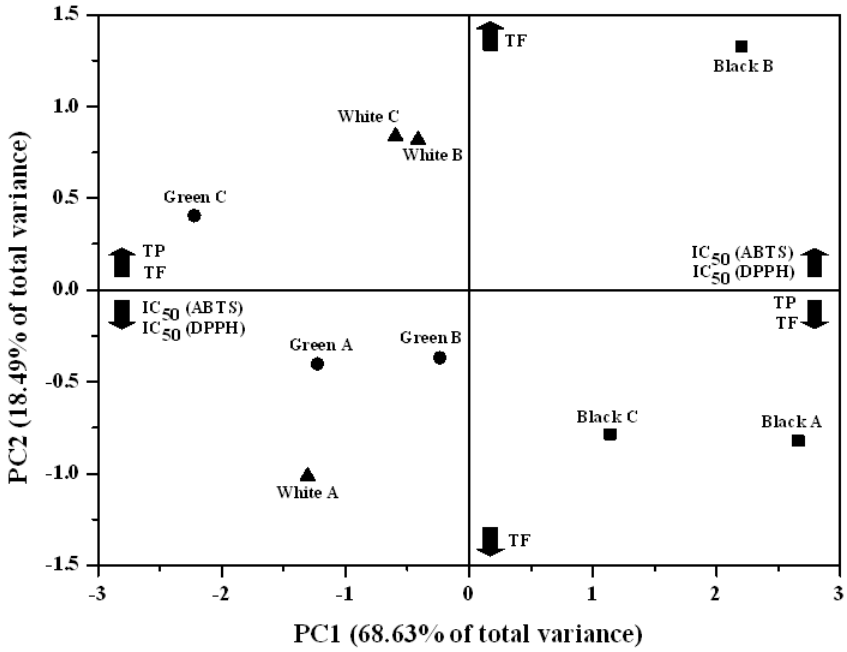

Figure 6. A scatter plot PC1 vs. PC2 on the main sources of variability between the black, green and white teas.

In this study through anatomical markers characteristic of the species *Camellia sinensis* was possible to prove the identity of the plant material contained in the tea bags. The HPLC-DAD fingerprints and chemometric analysis (PCA) proved usefulness in differentiating among tea samples of *Camellia sinensis* and that the majority of samples presented very similar phytochemical profiles. The results demonstrate that total phenols and flavonoid levels are very variable among different brands of tea bags and that green and white teas presented higher levels of total phenols, flavonoids and presents better free radical scavenging results on DPPH and ABTS than black teas.

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