

Comparison Between the Phytochemical and Antioxidant Properties of Plants Used in Plant Infusions for Medicinal Purposes

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Abstract

It has already been acknowledged among the medical community that plant based treatments represent an interesting contribution to modern therapeutics due to the presence in their composition of molecules with pharmacological and antioxidant action. The aim of this study was to evaluate the contents of total phenolics, flavonoids, and caffeine in six plants used traditionally by healers in Portugal and usually consumed as tea or infusion namely: *Camellia sinensis*, *Melissa officinalis*, *Lippia citriodora*, *Cymbopogon citratus*, *Matricaria chamomilla*, and *Tilia cordata*. Total phenolics ranged from 32.05 mg GAE/100g for aqueous extracts obtained from leaves of *L. citriodora* to 145.28 mg GAE/100g for aqueous extracts of *C. sinensis*. Significant variations in the flavonoid content were also found among analyzed plants and depending on the nature of the extract, with *C. sinensis* standing out again with the highest values (78.31 mg CE/100g) and the ethanolic extract obtained from the flowers of *T. cordata* exhibiting the lowest content (25.15 mg CE/100g). The concentration of caffeine was also very diverse and followed the sequence *M. officinalis* < *T. cordata* < *C. citratus* < *M. chamomilla* < *L. citriodora* < *C. sinensis*. The antioxidant activity of each plant was evaluated *in vitro* using a standard model system, the DPPH assay, and was found to vary according to *C. citratus* (90.9%) > *C. sinensis* (87.8%) > *M. officinalis* (50.7%) > *M. chamomilla* (45.3%) > *T. cordata* (32.2%) > *L. citriodora* (28.0%). The aqueous extracts presented lower antioxidant activity than the corresponding ethanolic ones.

Keywords: medicinal plants, caffeine; phenolics, flavonoids, antioxidant activity

1. Introduction

The health benefits associated with the consumption of tea and herbal infusions have resulted in their recognition as botanical dietary supplements, which are widely consumed nowadays as adjuvants for complementary and alternative medicines (Monbaliu et al., 2010). Many of these health benefits are due to the action of antioxidant molecules such as ascorbic acid (vitamin C), α -tocopherol (vitamin E), carotenoids, polyphenols (a wide class of components including phenolic acids, catechins, flavonols and anthocyanins) and caffeine, in which plants are naturally rich (Giovanelli & Buratti, 2009; Jehad, 2009). The current state of medical knowledge attributes to antioxidant molecules a crucial role in counteracting the effects of oxidative stress caused by Reactive Oxygen Species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl, nitric oxide and peroxynitrite radicals. It is now recognized that oxidative stress is related to the pathogenesis of several diseases such as metabolic syndrome, cancer and degenerative disorders (Alfadda & Sallam, 2012; Brieger et al., 2012; Finkel, 2000). The oxidation of lipids, proteins, DNA, carbohydrates, and other biological molecules by toxic ROS may cause DNA mutations and/or damage target cells and tissues, and this often results in cell senescence and death. This lead many clinicians and researchers to advocate as a strategy for inhibiting, delaying or even reversing the carcinogenesis process and preventing cardiovascular disease the chemoprevention through the intake of natural antioxidants (Ferri & Grassi, 2010; Grassi et al., 2009; Valko et al., 2007). The antioxidants obtained from plants do not induce the negative side effects provoked by synthetic ones (Ndhlala, Moyo, & Staden, 2010). For example one of the most frequently used synthetic antioxidants in food industry at high doses, butylated hydroxyanisole

(BHA), is known to exhibit genotoxic and carcinogenic effects, while butylated hydroxytoluene (BHT) was proven to cause hemorrhaging (Ndhlala et al., 2010; Kahl & Kappus, 1993; Chen, Pearson & Gray, 1992; Ito et al., 1986). These facts have prompted many researchers to quest for potent and cost-effective antioxidants from various plant sources.

The aim of this study was to determine the contents of phenolics, flavonoids and caffeine in extracts obtained from six plants, usually consumed in Portugal as tea or infusions for medicinal purposes namely: *C. sinensis*, *M. officinalis*, *L. citriodora*, *C. citratus*, and flowers of *M. chamomilla*, and *T. cordata* (Table 1).

The *in vitro* antioxidant activity of each plant extracts was also determined. These plant infusions are recommended by traditional Portuguese healers for the treatment of different ills namely: fever (*T. cordata*, *C. citratus*), pain (*C. citratus*), hypertension and sore stomach (*C. sinensis*), anxiety problems (*T. cordata*, *C. citratus*, *M. officinalis*, *M. chamomilla*), respiratory problems (*T. cordata*), fungal infections (*C. citratus*), inflammatory conditions (*C. citratus*, *M. chamomilla*) and digestive track spasms (*L. citriodora*, *M. chamomilla*) (Vinha et al., 2013; Moradkhani et al., 2012; Mosavi, 2012; Namita et al., 2012; Shah et al., 2011; Singh et al., 2011; Cotrim et al., 1999).

2. Materials and Methods

2.1 Plant Material

Fresh herbal plant materials were purchased from a reputable medicinal plant producer located in the North of Portugal (41° 52' 26" N; 8° 50' 26" W, average altitude: 16 m), characterized by a Mediterranean climate. Six medicinal plants were studied: *Camellia sinensis*, *Melissa officinalis*, *Matricaria chamomilla*, *Tilia cordata*, *Cymbopogon citratus* and *Lippia citriodora* (Table 1). The collected plant material was air-dried at room temperature (20±2°C) and protected from light. Dried plant material was cut and stored in tightly sealed dark containers until needed.

Table 1. Botanical designation and popular Portuguese name of the plants studied, together with the part of the plant used for medicinal purposes

Plant	Family	Common name	Organ used
<i>Camellia sinensis</i>	Theaceae	Green tea	Leaves
<i>Melissa officinalis</i>	Lamiaceae	Lemon balm	Leaves
<i>Lippia citriodora</i>	Verbenaceae	Lemon verbena	Leaves
<i>Cymbopogon citratus</i>	Poaceae	Lemon grass	Leaves
<i>Matricaria chamomilla</i>	Asteraceae	Chamomilla	Flowers
<i>Tilia cordata</i>	Tiliaceae	Tilia	Flowers

2.2 Preparation of Plant Extracts

Medicinal plants can be used fresh or dried. Drying is the most common method for post-harvest preservation, and must be accomplished as soon as possible after harvesting, to maintain the quality and to prevent any contamination and phytochemical deterioration. The dried plant material was coarsely crushed in small pieces of 2-5 mm using a cylindrical crusher and extracted with water or ethanol. Each extract was obtained by mixing 5.0 g of dried plant in 200.0 ml of water or ethanol at approximately 100°C for 10 min. The extracts were filtered through a paper filter (Whatman, No. 1) and stored under refrigeration in glass flasks tapered with screw plastic lid. This procedure has been described by several authors as efficient conditions to extract phytochemicals, such as phenolic and flavonoid compounds from plant material (Katalinic et al., 2006; Su et al., 2006).

2.3 Determination of Caffeine by UV/Vis Spectrophotometry

Total caffeine content was determined using a portion of 0.25 g of each plant that was dissolved in distilled water. 20 mL of sample solution were pipetted to a 250 mL flask to which 10 mL of 0.01 mol/L hydrochloric acid and 2 mL lead acetate solution were added together with distilled water to complete the volume. The flask contents were shaken up and filtered. 50 mL of filtered solution were then mixed with 0.2 mL of 4.5 mol sulfuric acid and the necessary volume of distilled water to complete the volume of a 100 mL flask. The resultant solution was shaken and filtered. The absorbance of the working standards and samples were measured on a UV/Vis spectrophotometer (Shimadzu, model UV-1800) at 274 nm using a 10 mm quartz cuvette. The caffeine

concentration in the samples was calculated from the calibration curve obtained.

2.4 Total Phenolic Compounds Analysis

Total phenolics were determined colorimetrically using the Folin-Ciocalteu reagent (Velioglu et al., 1998) procedure with minor modifications. Briefly, 200 μL of each extract (aqueous and ethanolic, respectively) was mixed with 1.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water), and left to stand at $22\pm 1^\circ\text{C}$ for five minutes. 1.5 mL sodium bicarbonate solution (8%) was added to the mixture. After 90 min of incubation, the absorbance was measured at 725 nm. Total phenolics were quantified from the calibration curve obtained from measuring the absorbance of a known concentration of gallic acid standards (15-300 mg/ml). The concentrations were expressed as mg of gallic acid equivalents (GAE) per 100g of dry weight. The total phenolic assay was measured six times for each plant extract.

2.5 Total Flavonoids Assay

Total flavonoid content was evaluated by the aluminum chloride assay, previously described (Zhishen et al., 1999). An aliquot (1 mL) of aqueous, ethanolic extract or standard solution of catechin [50, 100, 150, 200, 250 and 300 mg/mL] was added to a 10 ml volumetric flask containing 4 mL of distilled water. To this flask was also added 0.3 mL of NaNO_3 (5%). After 5 min of incubation, 0.3 mL of 10% AlCl_3 was added, and at the 6th min, 2 mL of NaOH (1 M). The mixture was completed up to 10 mL. The final solution was mixed and the absorbance was measured against a reagent blank at 510 nm. Total flavonoid content was expressed as mg catechin equivalents (CE)/100g dry weight (DW). The total flavonoid assay was measured six times for each plant extract.

2.6 Evaluation of Antioxidant Activity

When the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) reacts with an antioxidant compound, capable of donating hydrogen, it is reduced. The extent of this reaction is a measure of the antioxidant capacity. The reaction mixture in each one of the 96-wells of an ELX80 Microplate Reader (Bio-Tek) consisted of one of the different concentration plant extracts (20 μL) and an aqueous/methanolic solution (80:20 v/v, 280 μL) containing DPPH radicals (6×10^{-5} mol/L). The changes in color (from deep—violet to light—yellow) were measured at 517 nm, according to the method previously reported (Shah et al., 2011). Results are expressed as percentage of radical scavenging activity (% RSA), which is defined by the following expression: % RSA = $[(A_{\text{DPPH}} - A_s) / A_{\text{DPPH}}] \times 100$, where, A_s is the absorbance of the solution with the sample extracts and A_{DPPH} the absorbance of the DPPH[•] solution. All determinations were performed in triplicate. The EC₅₀, i.e., the concentration necessary to inhibit 50% of DPPH[•] radical, was determined according to published procedures (Kulisic et al., 2006; Cheel et al., 2005). Sample concentration corresponding to 50% of DPPH[•] absorbance was calculated graphically. Trolox was used as standard.

2.7 Statistical Analysis

Data are reported as mean \pm standard deviation obtained from six measurements. Statistical analyses were performed using the statistical package SPSS v 20.0 (SPSS for Windows; SPSS Inc., Chicago, IL). One-way ANOVA was employed to compare three or more groups using post-hoc Dunnett's test for multiple comparisons. Independent samples t-test was used to compare the extracts obtained from the same plant. Differences at $p < 0.05$ (95% confidence level) were considered to be significant.

3. Results and Discussion

Table 2. Caffeine concentration (mg/100g dry weight) of the six plants studied

Plant	Organ used for medicinal purposes	Caffeine content
<i>Camellia sinensis</i>	Leaves	3195.47 \pm 4.89 ^a
<i>Melissa officinalis</i>	Leaves	717.79 \pm 2.69 ^d
<i>Lippia citriodora</i>	Leaves	2340.01 \pm 1.46 ^b
<i>Cymbopogon citratus</i>	Leaves	768.43 \pm 3.08 ^d
<i>Matricaria chamomilla</i>	Flowers	1571.47 \pm 2.54 ^c
<i>Tilia cordata</i>	Flowers	738.19 \pm 2.53 ^d

All values are expressed as means \pm S.D. obtained from six measurements. Different superscript letters indicate statistically different caffeine contents ($p < 0.05$).

Herbal medicine or phytotherapy, referring to the medical use of plant organs (leaves, stems, roots, flowers, fruits and seeds) for curative purposes helped mankind since immemorial times. It is now time to use the scientific and technical knowledge at our disposal, to unravel the scientific basis of the therapeutic action of plants, especially its relation to their chemical composition.

The caffeine content, in the part used for medicinal purposes, of the six plants studied is presented in Table 2.

Caffeine, an alkaloid of the methylxanthine family, is mainly found in plant leaves as a secondary metabolite, it is a pharmacologically active substance and, depending on the consumed dose, may be a mild central nervous system stimulant because it acts as an adenosine-receptor antagonist (Schellack, 2012). In particular, caffeine is a nonspecific, competitive blocker of adenosine A1 and A2A receptors, distributed throughout the central nervous system (Cauli & Morelli, 2005). Binding of adenosine to target receptors has general depressant effects, slowing heart rate and lowering blood pressure. A recent study revealed a new face to caffeine: its potential as antioxidant, by demonstrating that caffeine has the ability to directly binding to the hydroxyl radical thus neutralizing it (Carmona & Galano, 2011). This means that caffeine is not only a metabolic stimulant but also a protective antioxidant. The results presented in Table 2 reveal that caffeine amounts are quite diverse among the six plants consumed as infusions, ranging from 717.79 mg/100g (*M. officinalis*) to 3195.47 mg/100g (*C. sinensis*). The fact that *C. sinensis* presented the highest caffeine content, agrees with the data previously published by some investigators (Schellack, 2012; Friedman et al., 2005; Henning et al., 2003) on commercial teas consumed in the United States. The lower caffeine values exhibited by *M. officinalis*, *T. cordata* and *C. citratus* may explain why these are traditionally used as calmative infusions (Shah et al., 2011; Moradkhani et al., 2010; Cotrim et al., 1999). The variability in the levels of caffeine among the teas was, however, much narrower than that of catechins found in the generality of teas obtained from medicinal plants (Dadáková et al., 2010; Velayutham et al., 2008).

Total phenolic and flavonoid contents of the six Portuguese medicinal infusions and teas are shown in Table 3. In our experiments two different extracts were studied: aqueous and ethanolic.

Table 3. Phytochemicals contents determined in aqueous and ethanolic extracts obtained from the six medicinal plants: The phenolics concentration is expressed in (mg GAE/100g sample) and that of flavonoids in (mg CE/100g sample)

Medicinal Plant	Solvent	Phenolics	Flavonoids
<i>Camellia sinensis</i>	Water	145.28±2.87 ^A	78.31±4.50 ^A
	Ethanol	57.87±1.22 ^a	54.58±7.02 ^a
<i>Melissa officinalis</i>	Water	69.66±1.08 ^A	57.28±4.02 ^B
	Ethanol	56.45±2.08 ^a	51.87±1.98 ^a
<i>Lippia citriodora</i>	Water	32.05±2.99 ^E	30.24±2.97 ^D
	Ethanol	41.56±4.08 ^c	22.38±1.54 ^c
<i>Cymbopogon citratus</i>	Water	87.25±3.21 ^C	43.38±0.38 ^C
	Ethanol	48.04±2.89 ^b	31.01±3.99 ^b
<i>Matricaria chamomilla</i>	Water	43.35±4.71 ^D	41.32±0.87 ^C
	Ethanol	47.78±1.98 ^c	24.18±1.71 ^c
<i>Tilia cordata</i>	Water	41.69±3.01 ^D	25.15±0.18 ^E
	Ethanol	28.74±4.03 ^d	22.01±3.74 ^c

All values are expressed as means ± S.D. obtained from six measurements. Means in the same column having identical superscripts are not significantly different ($p > 0.05$).

The tea or infusion may be considered a sort of water extract; however, in order to quantify the liposoluble components it is necessary to use organic solvents. The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals in preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical and cosmetic products (Vinha et al., 2013; Dai & Mumper, 2010).

The first aspect that stands out from the results presented in Table 3 is the richness in phenolics and flavonoids exhibited by the plants studied, superior or comparable to many autochthonous fruits and vegetables for example

apples (*Malus pumila*), cherries (*Prunus avium*), figs (*Ficus carica*) or kohlrabi (*Brassica oleracea* var. *caulorapa*) just to name a few (Vinha et al., 2012; Marinova et al., 2005; Rodrigues et al., 2011). The second aspect is the wide diversity of contents between the different plants. *C. sinensis* is the plant whose extracts present the higher contents of total phenolics (145.28 mg GAE/100g (aqueous) and 57.87 mg GAE/100g (ethanolic extract) followed by *C. Citratus*, *M. officinalis*, *M. chamomilla*, *T. cordata* and *L. citriodora* that presents the lower total phenolic contents, only 32.05 mg GAE/100g (aqueous) and 41.56 mg GAE/100g (ethanolic extract). This sequence is essentially the same for the flavonoid contents: *C. sinensis* > *M. officinalis* > *C. citratus* ~ *M. chamomilla* > *L. citriodora* > *T. cordata*. Interestingly, we found no statistical difference in terms of flavonoid contents between the ethanolic extracts of *L. citriodora*, *M. chamomilla*, and *T. cordata* ($p > 0.05$), but significant differences between their aqueous extracts ($p < 0.05$). It is also noted that, generally, the aqueous extracts possess higher levels of total phenolics and flavonoids than the corresponding ethanolic ones. This may reflect the hydrosoluble character of the majority of the compounds. These results are consistent with those obtained by other authors who found a similar distribution on total phenolic compounds for different plants used as infusions (Al-Othman et al., 2012; Hussain et al., 2011; Katalinic et al., 2006; Atoui et al., 2005). It is worth referring that several factors might affect the concentration of phenolic and flavonoid compounds in medicinal infusions of the same plant, such as preparation method (plant processing, concentration, time and temperature of infusion), organ used, stage of development, cultivation characteristics (soil, climate, stresses), and method of analysis (Al-Othman et al., 2012; Vinha et al., 2012; Hussain et al., 2011; Dai et al., 2010; Atoui et al., 2005). Given the high content of phenolic compounds and flavonoids, it is legitimate to assume that the therapeutic action of these plants is also due to the action of these compounds. It has been recognized that phenolic compounds have potent anti-inflammatory and antimicrobial action while flavonoids have been reported to act as anti-inflammatory, antibacterial, antiviral, antiallergenic, cytotoxic antitumour, neurodegenerative disease prophylactic and vasodilatory (Brieger et al., 2012; Vinha et al., 2012; Hussain et al., 2011; Cai et al., 2004; Kessler, 2003; Finkel, 2000). In addition, phenolics and flavonoids are known to act synergically with other molecules like ascorbic acid, α -tocopherol and carotenoids, as antioxidants, capturing free radicals and chelating divalent cations. This prevents oxidative cell damage and carcinogenesis (Alfadda & Sallam, 2012; Giovanelli & Buratti, 2009; Jehad, 2009). In this regard, the ability of the tested plant extracts (aqueous and ethanolic), to scavenge the free radical DPPH was evaluated and is presented in Figure 1. Catechin, a major phenolic compound described in medicinal plants, was employed as reference.

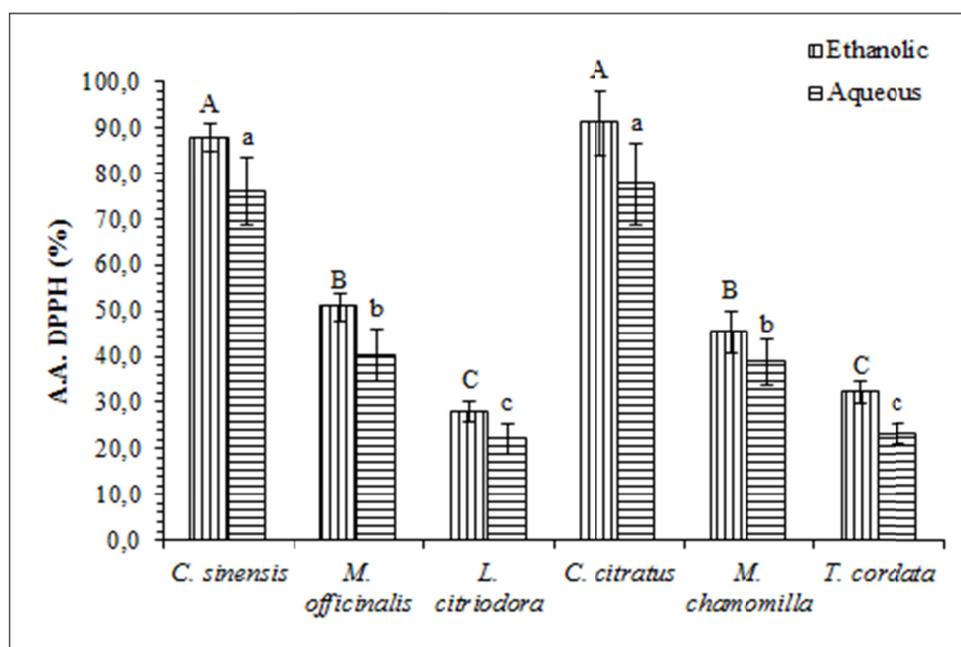


Figure 1. Antioxidant activity (A.A.) of ethanolic and aqueous extracts obtained from the various medicinal plants on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The symbol “*” indicates the existence of significant statistical differences ($p < 0.05$) among the antioxidant activity exhibited by the ethanolic and aqueous extract of the same plant. Identical capital letters signalize ethanolic extracts that exhibit the same antioxidant activity. Lowercase letters are used to the same purpose in case of aqueous extracts

The results show that the ethanolic extracts of *C. citratus* (90.9%) and *C. sinensis* (87.8%) presented the highest activity, followed by *M. officinalis* ~ *M. Chamomilla* > *T. cordata* ~ *L. citriodora*. Aqueous extracts presented lower antioxidant activity, but in the same order: 77.6% > 75.9% > 40.2% > 38.6% > 23.1% > 22.1% (*C. citratus* > *C. sinensis* > *M. officinalis* > *M. chamomilla* > *T. cordata* > *L. citriodora*, respectively) (Figure 1). Despite the fact that the antioxidant activity results from the action of several classes of molecules, the ordering of the plants according to their antioxidant activity is the same as was observed above for the phenolic compounds contents.

In accordance with the antioxidant activity displayed in Figure 1, the EC₅₀ values presented in Figure 2 are lower for *C. sinensis*, *C. citratus* and *M. officinalis* (148.87, 232.04 and 270.33 µg/mL, respectively), while *M. chamomilla*, *T. cordata* and *L. citriodora* require the highest concentrations (549.57, 1431.04, and 2098.59 µg/mL, respectively) (Figure 2).

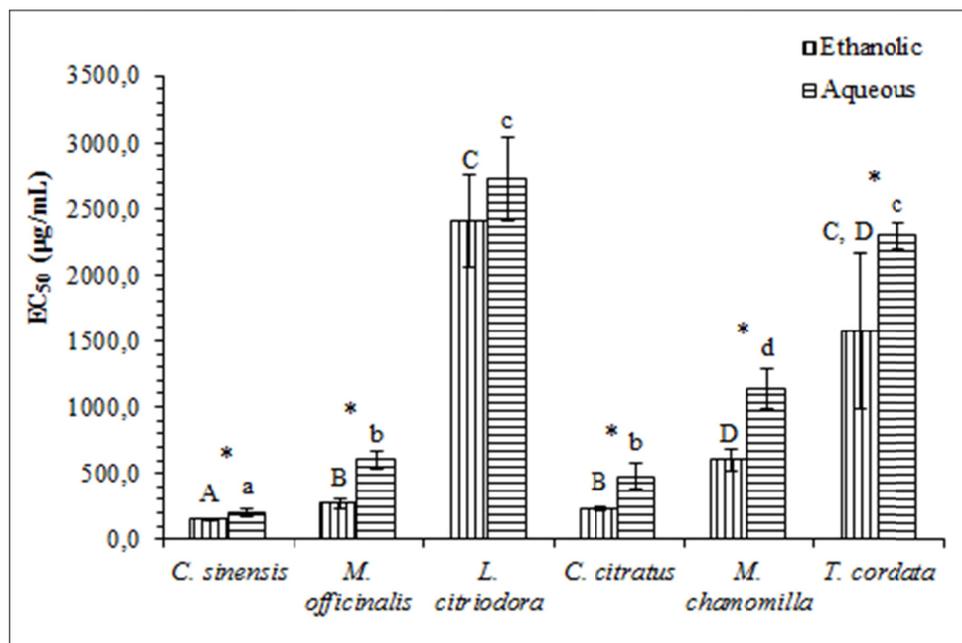


Figure 2. Amount of extract sample (µg/mL) needed for 50% decrease in the initial DPPH free radical concentration (EC₅₀). The symbol “*” and letters have the same interpretation as in Figure 1

For each plant, the EC₅₀ values of the aqueous extracts are significantly higher than those of ethanolic nature, corresponding to inferior antioxidant activity (Figure 2). These results were consistent to those reported by Atoui and colleagues (2005). *L. citriodora* was the only plant for which the difference among the two extracts wasn't statistically significant. Each plant presented different phenolic and flavonoid profiles, which explains the different inhibition percentages (Kulicic et al., 2006). Similar results were obtained by others researchers, demonstrating that there is a correlation between phenolic amount and antioxidant activity in herbal infusions (Vinha et al., 2012; Kumar, 2011; Katsube et al., 2004).

4. Conclusion

Camellia sinensis, *Melissa officinalis*, *Matricaria chamomilla*, *Tilia cordata*, *Cymbopogon citratus* and *Lippia citriodora*, traditionally used for medicinal purposes as tea or infusions in Portugal, were found to contain high levels of total phenolics, flavonoids and caffeine. The leaves of *C. sinensis* exhibited the highest total phenolics and flavonoids contents (145.28 mg GAE/100g and 78.31 mg CE/100g, respectively) while those of *L. citriodora* the lowest (32.05 mg GAE/100g, 22.38 mg CE/100g). The other plants had values in between according to the sequences *C. Citratus*, *M. officinalis*, *M. chamomilla*, *T. cordata* for total phenolics and *C. sinensis* > *M. officinalis* > *C. Citratus* ~ *M. chamomilla* > *L. citriodora* > *T. cordata* for flavonoid contents. The concentration of caffeine was also very diverse and followed the sequence *M. officinalis* < *T. cordata* < *C. citratus* < *M. chamomilla* < *L. citriodora* < *C. sinensis*. All these classes of compounds have proved pharmacological action. Phenolic compounds and flavonoids have been associated with a wide range of benefic effects including: anti-inflammatory, antibacterial, antiviral, antitumoral, anti-allergic, neurodegenerative diseases prevention and vasodilators.

Caffeine is a central nervous system and metabolic stimulant and also an antioxidant. Therefore, it is fair to assume that part of the beneficial effects, traditionally attributed to the plants studied here, result from the action of these compounds. Accordingly the plant extracts demonstrated a high capacity to scavenge free radicals, i.e. antioxidant capacity. This ability followed the sequence *C. citratus* > *C. sinensis* > *M. officinalis* > *M. chamomilla* > *T. cordata* > *L. citriodora*. To the best of our knowledge, this is the first study demonstrating the antioxidant potential of these medicinal plants cultivated in Portugal. The results indicate that, to a certain extent, plant composition fits the medical purposes for which it is traditionally used. Interestingly the plants presenting higher contents of phytochemicals (*C. sinensis*, *C. citratus* and *M. officinalis*) are the ones that are traditionally used to treat the widest range of ailments.

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