

EFFECTS OF ORAL DOSAGE FORMS AND STORAGE PERIOD IN THE ANTIOXIDANT
PROPERTIES OF FOUR SPECIES USED IN TRADITIONAL HERBAL MEDICINE

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Running Head: Effect of oral dosage forms and storage period in herbs antioxidants

ABSTRACT

Herbal infusions and decoctions in water are some of the most commonly consumed beverages in the world. Although water is not a good solvent for many of the active components in herbs, liquid preparations are rich in several bioactive compounds. Most of them have powerful antioxidant activity and have been related to medicinal herbs' properties. Herein, decoctions and infusions in water of lemon-verbena (*Aloysia citrodora*) aerial parts and leaves, fennel (*Foeniculum vulgare*), pennyroyal (*Mentha pulegium*) and spearmint (*Mentha spicata*) aerial parts with different periods of storage (0, 30, 60 and 120 days), were prepared. The effects of the method of preparation and storage period in their antioxidant properties were analysed. For all the analysed species, infusions gave better results than the corresponding decoctions. Spearmint infusions showed the highest antioxidant properties, at all the storage periods, probably due to the highest levels and synergy between phenolics, flavonoids and ascorbic acid found in this sample. Linear discriminant analysis confirmed that the length of storage period has a significant influence in antioxidant activity and antioxidants content. Flavonoids and reducing sugars proved to be the parameters that most highly contribute to cluster individual groups according to different periods of storage.

Keywords: Herbal infusions and decoctions; Antioxidant activity; Storage period; Linear discriminant analysis.

INTRODUCTION

Reactive oxygen species (ROS), such as singlet oxygen, hydroxyl radical, superoxide anion and peroxy radical can be generated from normal metabolism in the human body, and can cause DNA damage, cancer, cardiovascular disease and aging. Antioxidants can reduce the damage of ROS to the human body (Haliwell, 1996). A powerful tool of contemporary medicine is the use of plant-derived phytochemicals to balance the antioxidant/pro-oxidant status for the prevention and treatment of diseases. Regular consumption of foods and drinks containing antioxidants is a good alternative for health prophylaxis (Prior, 2003; Kiselova *et al.*, 2006).

All over the world many plants are widely used to prepare beverages that are drunk after meals or applied in folk therapy. In Portugal as well as in Spain, some of the most popular medicinal plants have been traditionally gathered for preparing herbal infusions or decoctions, locally known as *chá* or *té* (respectively the Portuguese and the Spanish word for tea) (Pardo de Santayana *et al.*, 2005). Such is the case of the four species studied herein: lemon-verbena, fennel, pennyroyal and spearmint (**Table 1**) that are often mentioned and used in the Portuguese pharmacopoeia and usually drunk as herbal teas, for the pleasure of their flavour and digestive effects (Camejo *et al.*, 2003; Novais *et al.*, 2004; Salgueiro, 2004; Carvalho, 2005; Cunha *et al.*, 2007; Neves *et al.*, 2009). Lemon-verbena, an introduced deciduous shrub, is widely cultivated in Portuguese homegardens. Its leaves or aerial parts (shoots), depending on informants' opinion, are mainly used in infusions for its stomachic, sedative, febrifuge and antispasmodic effects (Camejo *et al.*, 2003; Salgueiro, 2004; Carvalho, 2005; Cunha *et al.*, 2007). Besides the common use for seasoning, infusions and decoctions of fennel and pennyroyal aerial parts are prepared for the respiratory, gastrointestinal and genitourinary systems. They are claimed to have depurative, diuretic, bechic, antiseptic, digestive, carminative,

galactagogue, emmenagogue and stimulant properties (Camejo *et al.*, 2003; Novais *et al.*, 2004; Salgueiro, 2004; Carvalho, 2005; Cunha *et al.*, 2007). Spearmint tea is considered a digestive beverage and has traditionally been used in the treatment of headaches and respiratory and digestive disorders. The species is anticatarrhal, antiemetic, antispasmodic, carminative, diuretic, restorative, stimulant, stomachic and antihelmintic (Carvalho, 2005; Cunha, 2007).

Most of the studies on bioactive compounds and antioxidant activity of aerial parts of lemon-verbena (Mothana *et al.*, 2008; Yoo *et al.*, 2008), fennel (Schaffer, 2005; Mata *et al.*, 2007; Barros *et al.*, 2009), pennyroyal (López *et al.*, 2007; López *et al.*, 2009) and spearmint (Dorman *et al.*, 2003; Arumugam *et al.*, 2006; Choudhury *et al.*, 2006) were performed in the extracts and not in tisanes or decoctions prepared according to folk recipes/formulations (including decoctions and infusions). Only a few studies have evaluated antioxidant activity in infusions of lemon-verbena (Valentão *et al.*, 2002; Vaquero *et al.*, 2010) and spearmint (Kiselova *et al.*, 2006). As far as we know, this is the first evaluation of the effects of preparation methods (infusion and decoction) and storage period in free radical scavenging activity, reducing power, lipid peroxidation inhibition and in antioxidants content of herbal oral dosage forms in water.

MATERIALS AND METHODS

Standards and reagents. All the solvents were of analytical grade purity; methanol was supplied by Lab-Scan (Lisbon, Portugal). The standards used in the antioxidant activity assays: trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid, α -tocopherol, gallic acid and (+)-catechin were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa

Aesar (Ward Hill, MA, USA). The standard butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Plant material and samples. Aerial parts of the four studied species (**Table 1**) were gathered in June 2009, in Bragança, Trás-os-Montes, north-eastern Portugal. The selected sites and gathering practices took into account local consumers criteria and the optimal growth stage preferences for preparing herbal beverages, such as infusion and decoction. Thus, fennel and pennyroyal flowering shoots (stems, leaves and flower buds) were collected in half shade sites at the edges of woods. Shoots (stems and leaves) from lemon-verbena and spearmint were picked up in two homegardens with informants' agreement. Morphological key characters from the Flora Iberica ([Castroviejo coord., 2003 and 2010](#)) were used for plant identification. Voucher specimens are deposited in the Herbarium at the Escola Superior Agraria de Bragança.

As lemon-verbena use-reports ([Carvalho, 2005](#)) were not consensual about which part of the plant (only leaves or semi-woody stems with leaves) should be use, two different samples of this species were prepared in order to respect the informants' practices.

All four species and the respective five samples (two of lemon-verbena) were used fresh, immediately after being collected, and shade-dried, after being stored in a dark, dry and room temperature place, for 30, 60 and 120 days, simulating informants usual conditions.

Preparation of the samples. According to informants' practices ([Carvalho, 2005](#)), preparing half a litter of an infusion or decoction requires a handful of fresh plant

material. Therefore, a handful of each fresh sample has been weighted and the correspondent dried weight calculated. Herbal dosage forms were prepared by decoction and infusion using samples with different storage times. For time zero, fresh samples were used (15.1 g of lemon-verbena aerial parts; 10.0 g of lemon-verbena leaves; 18.0 g of fennel aerial parts; 15.2 g of pennyroyal aerial parts; ~10.1 g of spearmint aerial parts). In the subsequent times of storage (30, 60 and 120 days) dry weight corresponding to the mentioned fresh weights were used (6.3 g of lemon-verbena aerial parts; 3.8 g of lemon-verbena leaves; 6.3 g of fennel aerial parts; 5.3 g of pennyroyal aerial parts; 2.3 g of spearmint aerial parts). The codes used to identify each sample are shown in **Table 2**.

Decoctions. The sample was added to 500 mL of distilled water, and heated (heating plate, VELP scientific) until boiling. The mixture was left stand at boiling temperature for 5 min and at room temperature for 5 minutes more, and then filtered under reduced pressure. The obtained decoction was frozen, lyophilized (Ly-8-FM-ULE, Snijders, Holland) and redissolved in water at a concentration of 2.5 mg/mL.

Infusions. The sample was added to 500 mL of boiling distilled water and left to stand at room temperature for 5 minutes, and then filtered under reduced pressure. The obtained infusion was frozen, lyophilized and redissolved in water at a concentration of 2.5 mg/mL.

Evaluation of antioxidant activity

Radical scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture in each one of the 96-wells consisted of sample solution (30 μ L) and aqueous methanolic solution (80:20 v/v, 270 μ L) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand

for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Guimarães *et al.*, 2010). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{\text{DPPH}} - A_S) / A_{\text{DPPH}}] \times 100$, where A_S is the absorbance of the solution when the sample has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against sample concentration. Trolox was used as standard.

Reducing power. This methodology was performed using the microplate reader described above. The sample solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm (Guimarães *et al.*, 2010). The concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against sample concentration. Trolox was used as standard.

β -carotene bleaching inhibition. The antioxidant activity of the samples was evaluated by the β -carotene linoleate model system, as described previously by us (Guimarães *et al.*, 2010). A solution of β -carotene was prepared by dissolving β -carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40°C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask

with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50°C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer (Analytikjena Specord 200-2004 spectrophotometer). A blank, devoid of β -carotene, was prepared for background subtraction. β -Carotene bleaching inhibition was calculated using the following equation: (β -carotene content after 2h of assay/initial β -carotene content) \times 100. The concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β -carotene bleaching inhibition percentage against sample concentration. Trolox was used as standard.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances

(TBARS). Brains were obtained from pig (*Sus scrofa*) of body weight ~150 Kg, dissected and homogenized with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (0.1 ml) of the supernatant was incubated with the samples solutions (0.2 mL) in the presence of $FeSO_4$ (10 μ M; 0.1 ml) and ascorbic acid (0.1 mM; 0.1 ml) at 37°C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm (Guimarães *et al.*, 2010). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] \times 100%, where A and B were the absorbance of the control and the compound solution, respectively. The concentration providing 50% lipid

peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition percentage against sample concentration. Trolox was used as standard.

Evaluation of antioxidants

Phenolics

Total phenolics were estimated by a colorimetric assay (Barros *et al.*, 2009). An aliquot of the sample solution was mixed with *Folin-Ciocalteu* reagent (5 ml, previously diluted with water 1:10 v/v) and sodium carbonate (75 g/l, 4 ml). The tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm. Gallic acid was used to calculate the standard curve (0.05-0.8 mM), and the results were expressed as mg of gallic acid equivalents (GAEs) per g of decoction/infusion.

Flavonoids. Total flavonoids were determined spectrophotometrically using a method based on the formation of a complex flavonoid-aluminum, with some modifications (Barros *et al.*, 2009). An aliquot (0.5 ml) of the sample solution was mixed with distilled water (2 ml) and subsequently with $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further 6 min, thereafter, NaOH solution (4%, 2 ml) was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.0156-1.0 mM) and the results were expressed as mg of (+)-catechin equivalents (CEs) per g of decoction/infusion.

Ascorbic acid. A fine powder (20 mesh) of sample (150 mg) was extracted with metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through Whatman N° 4 filter paper. The filtrate (1 ml) was mixed with 2,6-dichloroindophenol (9 ml) and the absorbance was measured within 30 min at 515 nm against a blank (Guimarães *et al.*, 2010). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.006-0.1 mg/ml), and the results were expressed as mg of ascorbic acid per g of decoction/infusion.

Reducing sugars. Reducing sugars were determined by the DNS (dinitrosalicylic acid) method according to a procedure previously described by us (Guimarães *et al.*, 2010). Briefly, the decoction/infusion (~1 mL) was mixed with DNS (1 mL) and distilled water (2 mL), and boiled for 5 min. The mixture was put on ice for 5 min to stop the reaction, and the absorbance was measured at 540 nm. Glucose was used to calculate the standard curve (250-1500 µg/mL); the results were expressed as mg of reducing sugars per g of decoction/infusion.

Statistical analysis. All the assays were carried out in triplicate in three different samples of each single herb. The results are expressed as mean values \pm standard error (SE) or standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic.

Linear discriminant function analysis was done following stepwise method, in order to determine which variables discriminate better the five naturally occurring groups, according with the values of F to enter (3.84) and F to remove (2.71), the guidelines of

the stepwise procedure. The *F*-value for a variable indicates its statistical significance in the discrimination between groups. Discriminant analysis defines an optimal combination of variables in a way that the first function furnishes the most general discrimination between groups, the second provides the second most, and so on (Benitez *et al.*, 2006).

Leave-one-out classification method was performed in order to validate the obtained results. These treatments were carried out using SPSS v. 16.0 program.

RESULTS AND DISCUSSION

Herbal beverages were prepared according to folk recipes used in Trás-os-Montes (North-eastern Portugal). The antioxidant properties of decoctions and infusions were evaluated by four different tests as there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively: DPPH radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β -carotene-linoleate model system in liposomes and TBARS assay in brain homogenates. Spearmint revealed the highest antioxidant properties (significantly lower EC_{50} values; $p < 0.05$), while fennel and lemon-verbena leaves gave the lowest antioxidant potential (Table 3). A lemon-verbena lyophilized infusion (prepared from leaves collected in Vila da Feira, Portugal) showed potent antioxidant activity, achieved by the scavenging abilities observed against superoxide, hydroxyl radicals and hypochlorous acid, but a pro-oxidant effect for higher concentrations of the lyophilized infusion. The authors attributed the protective effects to the presence of phenolic compounds, namely verbascoside and luteolin derivatives (Valentão *et al.*, 2002). Moreover, infusions of aerial parts of lemon-verbena collected in Argentina revealed DPPH radical scavenging activity of 73.0% (Vaquero *et al.*, 2010). Infusions of spearmint from Bulgaria were

reported as having high antioxidant activity measured by the ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) cation radical decolorization assay (Kiselova *et al.*, 2006). As far as we know, there are no reports on antioxidant properties of fennel and pennyroyal infusions, or on decoctions of the four plants studied herein. For all the herbal species and storage periods, the method of infusion gave better results than the corresponding decoction, probably due to the thermal shock verified in that procedure. Furthermore, herbal infusions and decoctions in water seem to have higher antioxidant properties than methanolic or ethanolic extracts. In particular for DPPH scavenging activity of fennel, EC₅₀ values obtained with these herbal beverages (0.53 mg/ml for decoction and 0.44 mg/ml for infusion at time zero) were lower than the ones obtained with methanolic extracts of different parts of fennel (1.34 mg/ml- shoots; 6.88 mg/ml- leaves; 12.16 mg/ml- stems; 7.72 mg/ml- inflorescences; Barros *et al.*, 2009). The same was observed for pennyroyal infusion and decoction (0.18 mg/ml for decoction and 0.13 mg/ml for infusion at time zero) who revealed higher antioxidant activity than aerial parts methanolic extracts (0.56 mg/ml; data not shown). Also in the case of spearmint beverages, EC₅₀ values (0.14 mg/ml for decoction and 0.11 mg/ml for infusion at time zero) were lower than the ones obtained with extracts prepared by hydrodistillation (0.2 mg/ml; Dorman *et al.*, 2003) and with methanolic leaves extracts (Choudhury *et al.*, 2006). EC₅₀ values obtained for lemon-verbena beverages (0.25 mg/ml for decoction and 0.24 mg/ml for infusion at time zero) were lower than the ones obtained for hot aqueous extracts (> 1 mg/ml; Mothana *et al.*, 2008), but higher than the values obtained with methanolic extracts (0.03 mg/ml- Mothana *et al.*, 2008 or < 0.1 mg/ml- Yoo *et al.*, 2008).

The composition in antioxidant compounds, including phenolics, flavonoids, ascorbic acid and reducing sugars (**Table 4**) was investigated. Phenolics and flavonoids were the

main antioxidant compounds found in all the herbal beverages. The better scavenging activity, reducing power and lipid peroxidation inhibition (with the lowest EC₅₀ values) showed by spearmint infusions (**Table 3**) might be due to the highest levels and synergy between phenolics, flavonoids and ascorbic acid found in this sample (**Table 4**). Also, other authors correlate the antioxidant activity of spearmint ([Kiselova *et al.*, 2006](#)) and lemon-verbena ([Vaquero *et al.*, 2010](#)) infusions with phenolic content.

The results were evaluated through linear discriminant analysis (LDA) to evaluate differences among storage periods (0, 30, 60 and 120 days). All independent variables were selected by the stepwise procedure with the tolerance level of $1-R^2 > 0.52$ and were statistically significant according to the Wilks' λ test ($P < 0.001$). The LDA defined seven functions for all the assayed herbs, from which the first three were plotted, as it can be seen in **Figure 1**.

Regarding fennel (F), 94.8% of the observed variance was explained by the first three functions (**Figure 1a**). The first function separates primarily FI30, FD30 and FD120 (means of the canonical variance, MCV: FI30 = 39.076; FD30 = 21.535; FD120 = -39.000), and revealed to be more powerfully correlated with flavonoids, reducing sugars and reducing power. The second function separates mainly FD30, FI120, FD60 and FI60 (MCV: FD30 = -9.315; FI120 = 1.214; FD60 = -26.262; FI60 = -27.097) and proved to be more correlated with phenolics and flavonoids. The third function was able to separate FD60 and FI60 (FD60 = -8.534; FI60 = -4.501), presenting higher correlations with reducing sugars.

Considering lemon-verbena aerial parts (La), the first three functions included 95.7% of the observed variance (**Figure 1b**). The first function separates clearly LaD30, LaI30, LaD60 and LaI60) (MCV: LaD30 = 44.901; LaI30 = 72.328; LaD60 = -62.457; LaI60 =

-46.205), and revealed to be more powerfully correlated with flavonoids. The second function separates mostly LaI30, LaD60 and LaI60 (MCV: LaI30 = 42.204; LaD60 = 13.949; LaI60 = 62.469) being more correlated with reducing sugars. The third function was needed to separate adequately LaD120 and LaI120 (LaD120 = 20.817; LaI120 = 6.543), presenting stronger correlations with reducing power and TBARS inhibition.

In the case of lemon-verbena leaves (L), the first three functions included 99.3%, of the observed variance (**Figure 1c**). The first function separates mainly LII30, LID120, LID0, LII0 and LID30 (MCV: LII30 = 258.686; LID120 = -165.349; LID0 = 62.818; LII0 = 86.140; LID30 = 93.252), showing higher correlation with flavonoids. The second function separates predominantly LID120, LII30 and LID0 (MCV: LID120 = 88.858; LII30 = 16.874; LID0 = 6.665), presenting greater correlation with reducing power. The third function separated effectively LID60 and LII120 (LID60 = 38.251; LII120 = -33.003), being more correlated with reducing sugars.

Regarding pennyroyal (P), the first three functions explained 98.8%, of the observed variance (**Figure 1d**). The first function separates primarily PD0, PI0 and PD30 (MCV: PD0 = 142.289; PI0 = 122.933; PD30 = -16.588), showing major correlation with TBARS. The second function separates mostly PD0, PI60, PD120 and PI120 (MCV: PD0 = -2.869; PI60 = 11.576; PD120 = -15.864; PI120 = -29.132), presenting better correlation with reducing sugars. The third function permitted a good separation between PD60, PI60 and PD30 (PD60 = 23.274; PI60 = 7.503; PD30 = -9.871), having a strong correlation with β -carotene bleaching inhibition.

Taking spearmint (S) in consideration, the first three functions justified 98.9%, of the observed variance (**Figure 1e**). The first function separates primarily SD0, SD60 and SI60 (MCV: SD0 = 5.076; SD60 = -29.981; SI60 = -17.261), demonstrating higher correlation with flavonoids. The second function separates predominantly SI30, SD120

and SI120 (MCV: SI30 = -0.841; SD120 = 20.535; SI120 = 27.269), proving to be more correlated with reducing sugars. The third function was useful to separate SD0, SI0, SD30 and SI30 (SD0 = 15.920; SI0 = 2.079; SD30 = -7.343; SI30 = -3.756), showing a powerful correlation with TBARS inhibition.

The different samples were clustered in individual groups when the algorithm was applied for selecting variables according with antioxidant activity assays and bioactive compounds contents in different storage periods. LDA confirmed that storage period has a significant influence in antioxidant activity and antioxidants content. Flavonoids and reducing sugars proved to be the parameters with higher discriminant power. The obtained classification was 100% correct either to original and cross-validated grouped cases. The results were validated according with the leave-one-out classification method.

In the analysis of storage period effects on antioxidant properties, the 30 days' period gave the best results, closely followed by the 60 days' period. Moreover, significantly negative linear correlations were established between the phenolics and flavonoids content after 30 days of storage, and EC₅₀ values of DPPH scavenging activity ($y = -1807x + 737.6$; $R^2 0.794$ for phenolics and $y = -867.5x + 366.2$; $R^2 0.751$ for flavonoids; $p < 0.001$), reducing power ($y = -2707x + 673.0$; $R^2 0.829$ for phenolics and $y = -1245x + 328.0$; $R^2 0.721$ for flavonoids; $p < 0.001$). The worst results were obtained for 120 days, probably due to eventual compound losses in this relatively long period of time. It must be reminded that, despite the herbs were stored protected from the light, they were exposed to a normal atmosphere, that may have oxidized some of the antioxidant compounds. This statement could point out that the formulations prepared at 0 days time should present the best results, as no compound would have been lost, but the drying process could transform the compounds into more powerful antioxidants up to a

limit of 60 days according to the present study. After that, other factors such as the air exposition may be the cause of the observed compounds lost. Although, dried branches are traditionally suspended in the cellars for subsequent use, several people usually keep the vegetal material, after being dried for 30 days, in closed bottles or flax and cotton bags.

CONCLUSION

Infusions gave better results than the corresponding decoctions at the same storage period. The highest scavenging activity, reducing power and lipid peroxidation inhibition was observed for spearmint infusions, probably due to the highest levels and synergy between phenolics, flavonoids and ascorbic acid found in this sample. It's also notorious that the storage time had higher influence than the preparation method. Considering all the parameters assayed, herbal "teas" (infusion=I; decoction=D) presented best results in the following order: I 30 days>I 60 days>I 0 days>D 30 days>D 60 days>D 0 days>I 120 days>D 120 days.

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Table 1. Medicinal uses of four herbal decoctions/infusions reported in Portuguese ethnobotanical studies.

Samples	English name	Local name	Portuguese region	Body parts treated
<i>Aloysia citrodora</i> Palau	Lemon-verbena	Limonete, erva-Luísa, lucialima	Minho and Trás-os-Montes (north), Beira (center) and Alentejo (south),	Respiratory, digestive and nervous systems
<i>Foeniculum vulgare</i> Mill.	Fennel	Funcho, fiolho, fionho, erva-doce		Respiratory, digestive and genitourinary systems
<i>Mentha pulegium</i> L.	Pennyroyal	Poejo, mangerico-do-rio		Respiratory and digestive systems
<i>Mentha spicata</i> L.	Spearmint	Hortelã-pimenta		Trás-os-Montes Digestive and nervous systems

Table 2. Identification of the samples.

Time of storage	Samples	Tea	Code
0 days	Lemon-verbena aerial parts	Decoction	LaD0
		Infusion	LaI0
	Lemon-verbena leaves	Decoction	LID0
		Infusion	LII0
	Fennel	Decoction	FD0
		Infusion	FI0
	Pennyroyal	Decoction	PD0
		Infusion	PI0
	Spearmint	Decoction	SD0
		Infusion	SI0
30 days	Lemon-verbena aerial parts	Decoction	LaD30
		Infusion	LaI30
	Lemon-verbena leaves	Decoction	LID30
		Infusion	LII30
	Fennel	Decoction	FD30
		Infusion	FI30
	Pennyroyal	Decoction	PD30
		Infusion	PI30
	Spearmint	Decoction	SD30
		Infusion	SI30
60 days	Lemon-verbena aerial parts	Decoction	LaD60
		Infusion	LaI60
	Lemon-verbena leaves	Decoction	LID60
		Infusion	LII60
	Fennel	Decoction	FD60
		Infusion	FI60
	Pennyroyal	Decoction	PD60
		Infusion	PI60
	Spearmint	Decoction	SD60
		Infusion	SI60
120 days	Lemon-verbena aerial parts	Decoction	LaD120
		Infusion	LaI120
	Lemon-verbena leaves	Decoction	LID120
		Infusion	LII120
	Fennel	Decoction	FD120
		Infusion	FI120
	Pennyroyal	Decoction	PD120
		Infusion	PI120
	Spearmint	Decoction	SD120
		Infusion	SI120

Table 3. Antioxidant activity (EC₅₀ values; mg/mL) of decoctions/infusions obtained from medicinal plants after different times of storage. The results are expressed as mean \pm SD (n=9). In each column different letters mean significant differences ($p < 0.05$).

Samples	DPPH Scavenging activity	Reducing power	β -carotene bleaching inhibition	TBARS inhibition
LaD0	0.25 \pm 0.01 c	0.18 \pm 0.00 bc	0.55 \pm 0.02 b	0.59 \pm 0.07 b
LaI0	0.24 \pm 0.00 c	0.15 \pm 0.00 de	0.23 \pm 0.01 f	0.26 \pm 0.00 ef
LID0	0.25 \pm 0.01 c	0.17 \pm 0.02 cd	0.27 \pm 0.00 e	0.49 \pm 0.02 c
LII0	0.21 \pm 0.01 d	0.13 \pm 0.03 ef	0.15 \pm 0.02 h	0.22 \pm 0.32 f
FD0	0.53 \pm 0.01 b	0.37 \pm 0.05 a	0.88 \pm 0.01 a	0.72 \pm 0.02 a
FI0	0.44 \pm 0.02 a	0.20 \pm 0.00 b	0.55 \pm 0.02 b	0.60 \pm 0.01 b
PD0	0.18 \pm 0.00 e	0.12 \pm 0.00 fg	0.46 \pm 0.00 c	0.42 \pm 0.00 d
PI0	0.13 \pm 0.00 fg	0.11 \pm 0.00 g	0.40 \pm 0.00 d	0.46 \pm 0.00 cd
SD0	0.14 \pm 0.00 f	0.06 \pm 0.00 h	0.19 \pm 0.00 g	0.28 \pm 0.05 e
SI0	0.11 \pm 0.00 g	0.04 \pm 0.00 h	0.05 \pm 0.00 i	0.14 \pm 0.02 g
LaD30	0.37 \pm 0.03 a	0.19 \pm 0.00 c	0.46 \pm 0.01 b	0.27 \pm 0.02 b
LaI30	0.24 \pm 0.01 de	0.12 \pm 0.00 e	0.19 \pm 0.02 c	0.22 \pm 0.01 c
LID30	0.26 \pm 0.01 bc	0.15 \pm 0.00 d	0.06 \pm 0.00 f	0.15 \pm 0.00 d
LII30	0.25 \pm 0.01 cd	0.15 \pm 0.00 d	0.05 \pm 0.00 f	0.10 \pm 0.01 e
FD30	0.35 \pm 0.02 a	0.22 \pm 0.00 b	0.48 \pm 0.01 a	0.47 \pm 0.04 a
FI30	0.27 \pm 0.01 b	0.22 \pm 0.00 a	0.45 \pm 0.0 b	0.24 \pm 0.02 c
PD30	0.22 \pm 0.01 e	0.11 \pm 0.01 f	0.11 \pm 0.01 d	0.15 \pm 0.00 d
PI30	0.19 \pm 0.01 f	0.09 \pm 0.00 g	0.09 \pm 0.01 e	0.11 \pm 0.00 e
SD30	0.09 \pm 0.00 g	0.04 \pm 0.00 h	0.09 \pm 0.00 e	0.10 \pm 0.01 e
SI30	0.09 \pm 0.00 g	0.04 \pm 0.00 h	0.05 \pm 0.00 f	0.06 \pm 0.00 f
LaD60	0.22 \pm 0.00 b	0.13 \pm 0.00 e	0.64 \pm 0.06 c	0.14 \pm 0.01 c
LaI60	0.21 \pm 0.02 b	0.12 \pm 0.00 e	0.48 \pm 0.00 d	0.09 \pm 0.00 d
LID60	0.41 \pm 0.00 a	0.25 \pm 0.01 a	0.84 \pm 0.03 a	0.09 \pm 0.01 d
LII60	0.22 \pm 0.00 b	0.15 \pm 0.01 d	0.46 \pm 0.03 de	0.07 \pm 0.00 de
FD60	0.20 \pm 0.01 c	0.20 \pm 0.01 c	0.86 \pm 0.02 a	0.54 \pm 0.02 a
FI60	0.18 \pm 0.01 d	0.21 \pm 0.01 b	0.74 \pm 0.04 b	0.43 \pm 0.08 b
PD60	0.19 \pm 0.01 cd	0.09 \pm 0.00 f	0.42 \pm 0.03 e	0.07 \pm 0.00 de
PI60	0.17 \pm 0.01 e	0.08 \pm 0.00 g	0.35 \pm 0.01 f	0.08 \pm 0.00 de
SD60	0.09 \pm 0.00 f	0.05 \pm 0.00 h	0.30 \pm 0.00 g	0.05 \pm 0.00 e
SI60	0.09 \pm 0.00 f	0.05 \pm 0.00 h	0.17 \pm 0.01 h	0.04 \pm 0.00 e
LaD120	0.40 \pm 0.03 e	0.31 \pm 0.01 e	0.12 \pm 0.01 e	0.12 \pm 0.00 f
LaI120	0.35 \pm 0.01 e	0.25 \pm 0.01 f	0.15 \pm 0.03 d	0.22 \pm 0.00 e
LID120	2.37 \pm 0.19 a	1.18 \pm 0.03 a	0.44 \pm 0.01 a	0.81 \pm 0.05 a
LII120	0.60 \pm 0.03 d	0.34 \pm 0.01 d	0.24 \pm 0.04 c	0.27 \pm 0.00 d
FD120	1.90 \pm 0.15 b	0.91 \pm 0.02 b	0.44 \pm 0.04 a	0.39 \pm 0.02 b
FI120	1.14 \pm 0.02 c	0.67 \pm 0.02 c	0.30 \pm 0.01 b	0.35 \pm 0.01 c
PD120	0.30 \pm 0.01 e	0.13 \pm 0.00 h	0.24 \pm 0.00 c	0.08 \pm 0.00 g
PI120	0.36 \pm 0.01 e	0.16 \pm 0.00 g	0.25 \pm 0.00 c	0.10 \pm 0.00 fg
SD120	0.15 \pm 0.00 f	0.07 \pm 0.00 h	0.04 \pm 0.00 f	0.09 \pm 0.00 g
SI120	0.16 \pm 0.01 f	0.08 \pm 0.00 h	0.17 \pm 0.04 d	0.09 \pm 0.00 g

Table 4. Antioxidant compounds present in decoctions/infusions obtained from medicinal plants after different times of storage. The results are expressed as mean \pm SD (n=9). In each column different letters mean significant differences ($p < 0.05$).

Samples	Phenolics (mg GAE/g)	Flavonoids (mg CE/g)	Ascorbic acid (mg/g)	Reducing sugars (mg/g)
LaD0	125.08 \pm 0.26 h	73.69 \pm 0.78 g	7.63 \pm 0.09 bc	1.05 \pm 0.04 ef
LaI0	163.96 \pm 0.29 g	75.11 \pm 0.16 g	3.01 \pm 0.47 g	1.09 \pm 0.03 e
LID0	221.90 \pm 0.21 f	95.44 \pm 0.63 f	6.80 \pm 0.49 cd	1.01 \pm 0.01 f
LII0	445.04 \pm 0.17 c	107.42 \pm 0.55 e	8.05 \pm 0.65 b	0.91 \pm 0.01 g
FD0	90.31 \pm 7.68 i	73.96 \pm 0.24 g	6.17 \pm 0.12 de	0.58 \pm 0.02 h
FI0	52.62 \pm 0.62 j	50.82 \pm 1.26 h	5.21 \pm 0.79 f	0.49 \pm 0.02 i
PD0	329.63 \pm 0.39 d	201.38 \pm 0.32 c	5.27 \pm 0.95 ef	1.57 \pm 0.01 c
PI0	318.43 \pm 0.25 e	180.94 \pm 7.76 d	4.84 \pm 0.66 f	2.21 \pm 0.05 a
SD0	520.87 \pm 5.55 b	259.80 \pm 3.88 b	3.73 \pm 0.77 g	1.99 \pm 0.10 b
SI0	670.21 \pm 6.44 a	373.79 \pm 6.40 a	9.35 \pm 0.62 a	1.41 \pm 0.07 d
LaD30	185.22 \pm 8.62 g	107.72 \pm 3.44 f	1.58 \pm 0.23 f	1.17 \pm 0.03 e
LaI30	297.71 \pm 3.05 cd	172.06 \pm 0.35 d	7.46 \pm 0.27 b	2.13 \pm 0.05 b
LID30	230.07 \pm 7.30 f	106.72 \pm 0.68 f	0.83 \pm 0.10 g	0.99 \pm 0.08 f
LII30	286.19 \pm 19.99 de	182.91 \pm 0.91 c	6.51 \pm 0.54 c	1.21 \pm 0.09 e
FD30	138.68 \pm 3.79 h	58.52 \pm 2.39 h	2.47 \pm 0.78 e	1.48 \pm 0.08 d
FI30	112.12 \pm 0.42 i	91.40 \pm 1.30 g	2.75 \pm 0.09 e	1.74 \pm 0.26 c
PD30	272.87 \pm 6.89 e	106.21 \pm 3.44 f	3.46 \pm 0.33 d	3.28 \pm 0.06 a
PI30	311.40 \pm 6.43 c	148.24 \pm 0.45 e	3.99 \pm 0.27 d	2.14 \pm 0.17 b
SD30	620.58 \pm 26.33 b	318.62 \pm 13.48 b	7.83 \pm 0.49 b	1.17 \pm 0.07 e
SI30	684.90 \pm 11.73 a	336.15 \pm 0.30 a	8.59 \pm 0.44 a	1.53 \pm 0.01 d
LaD60	295.32 \pm 5.09 e	11.31 \pm 0.44 de	2.44 \pm 0.09 d	1.36 \pm 0.03 d
LaI60	296.45 \pm 0.72 e	12.88 \pm 2.21 d	4.45 \pm 0.25 c	3.69 \pm 0.07 a
LID60	194.61 \pm 4.62 f	7.41 \pm 0.31 e	2.14 \pm 0.08 d	2.65 \pm 0.07 c
LII60	293.00 \pm 18.19 e	11.77 \pm 0.01 de	2.03 \pm 0.16 d	1.37 \pm 0.04 d
FD60	154.43 \pm 4.27 h	9.63 \pm 0.69 de	0.66 \pm 0.04 e	0.39 \pm 0.01 e
FI60	168.81 \pm 3.71 g	20.96 \pm 4.18 c	4.40 \pm 0.67 c	0.42 \pm 0.01 e
PD60	337.82 \pm 0.65 d	19.51 \pm 0.86 c	4.51 \pm 0.66 c	3.22 \pm 0.18 b
PI60	373.96 \pm 3.01 c	75.14 \pm 2.15 a	2.04 \pm 0.83 d	3.59 \pm 0.14 a
SD60	630.04 \pm 2.11 b	35.84 \pm 2.39 b	6.32 \pm 0.10 b	2.63 \pm 0.02 c
SI60	651.39 \pm 5.33 a	79.12 \pm 8.04 a	7.05 \pm 0.22 a	2.66 \pm 0.07 c
LaD120	214.58 \pm 1.38 e	20.13 \pm 1.71 ef	4.18 \pm 0.11 d	0.24 \pm 0.01 e
LaI120	185.57 \pm 1.62 f	19.67 \pm 2.09 ef	4.23 \pm 0.20 d	0.14 \pm 0.01 g
LID120	55.63 \pm 0.55 h	11.78 \pm 0.24 g	4.55 \pm 0.08 c	0.09 \pm 0.00 h
LII120	179.80 \pm 0.80 f	18.96 \pm 0.15 f	4.01 \pm 0.10 d	0.23 \pm 0.02 e
FD120	73.23 \pm 0.74 g	22.46 \pm 2.02 e	4.05 \pm 0.19 d	0.17 \pm 0.00 f
FI120	76.35 \pm 0.03 g	11.11 \pm 0.67 g	4.75 \pm 0.08 bc	0.15 \pm 0.00 fg
PD120	308.71 \pm 6.39 c	55.77 \pm 3.95 c	1.58 \pm 0.19 e	0.54 \pm 0.02 c
PI120	248.60 \pm 0.33 d	36.72 \pm 4.22 d	4.88 \pm 0.21 b	0.38 \pm 0.01 d
SD120	409.16 \pm 0.58 a	70.35 \pm 1.79 a	4.81 \pm 0.37 bc	0.80 \pm 0.02 b
SI120	368.90 \pm 17.95 b	64.35 \pm 0.34 b	7.59 \pm 0.13 a	0.84 \pm 0.03 a

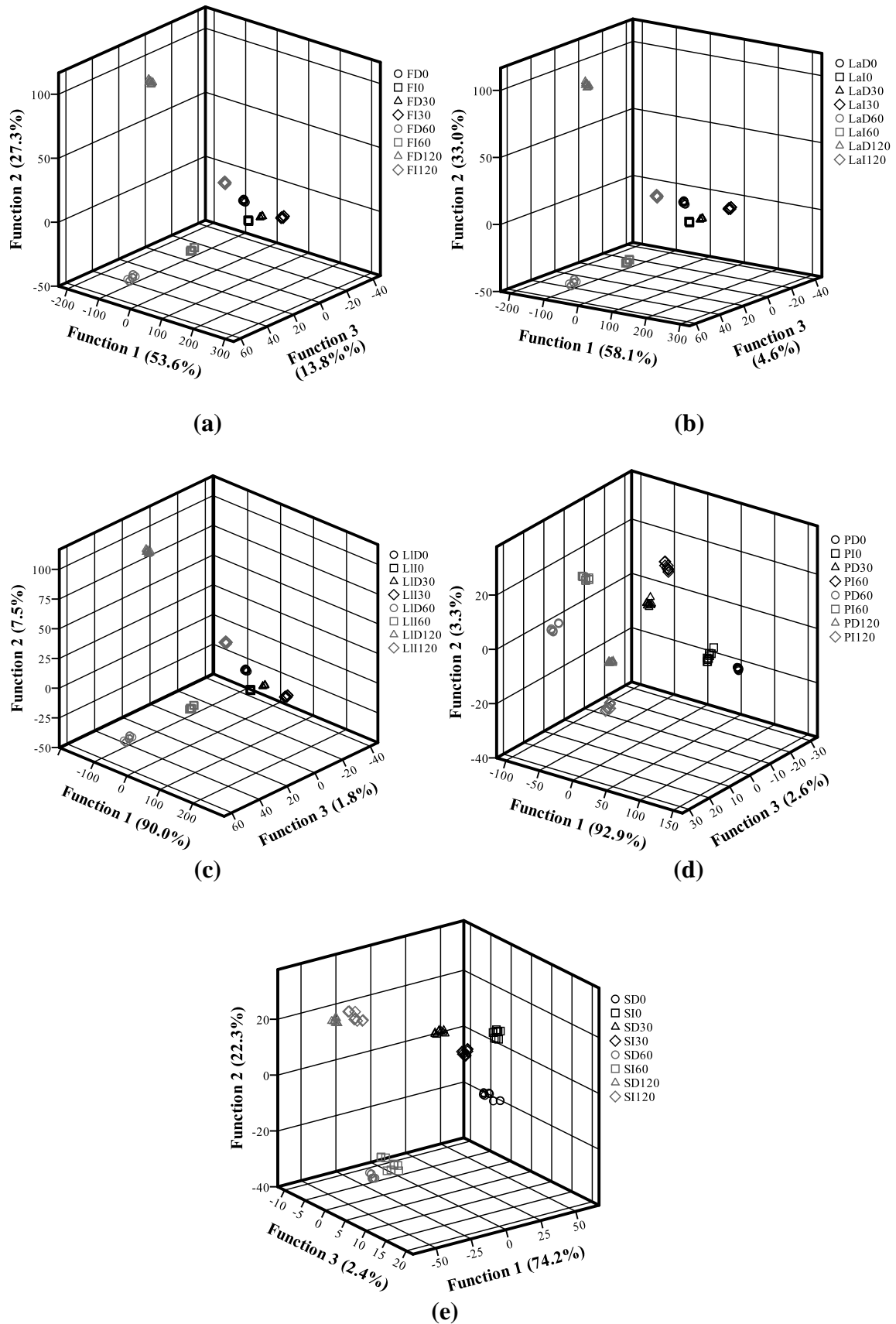


Figure 1. Canonical analysis of fennel (a), lemon-verbena aerial parts (b), lemon-verbena leaves (c), pennyroyal (d) and spearmint (e) samples.