Evaluation of bioactive properties and phenolic compounds in different extracts prepared from *Salvia officinalis* L.

Natália Martins,[1,2] Lillian Barros,[1] Celestino Santos-Buelga,[3] Mariana Henriques,[2] Sónia Silva,[2] Isabel C.F.R. Ferreira[1,*]

[1]Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.

[2]CEB, Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal.

[3]GIP-USAL, Faculty of Pharmacy, University of Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain

* Author to whom correspondence should be addressed e-mail: iferreira@ipb.pt; telephone +351-273-303219; fax +351-273-325405.

Abstract

The therapeutic benefits of medicinal plants are well known. Nevertheless, essential oils have been the main focus of antioxidant and antimicrobial studies, remaining scarce the reports with hydrophilic extracts. Thus, the antioxidant and antifungal activities of aqueous (prepared by infusion and decoction) and methanol/water (80:20, *v/v*) extracts of sage (*Salvia officinalis* L.) were evaluated and characterized in terms of phenolic compounds. Decoction and methanol/water extract gave the most pronounced antioxidant and antifungal properties, being positively related with their phenolic composition. The highest concentration of phenolic compounds was observed in the decoction, followed by methanol/water extract and infusion. Fungicidal and/or fungi static effects proved to be dependent on the extracts concentration. Overall, the incorporation of sage decoction in the daily diet or its use as a complement for antifungal therapies, could provide considerable benefits, also being an alternative to sage essential oils that can display some toxic effects.

Keywords: antifungal activity; antioxidant activity; hydrophilic extracts; *Salvia officinalis* L.

1. Introduction

At the present time, opportunistic fungal infections constitute a serious threat to human health and wellbeing (Lott, Fundyga, Kuykendall, & Arnold, 2005). Indeed, the frequency and severity of diagnosed fungal infections is growing and the consequences of this are exacerbated by a concomitant increase in resistance to traditional antifungal agents (Oberoi et al., 2012). Within the *Candida* genus, *Candida albicans* has been considered the main agent responsible for those opportunistic pathogenic infections. Recently however, other non-*albicans Candida* species have also been described. The balance between *C. albicans* and non-*albicans Candida* species determine the profiles associated with virulence (Oberoi et al., 2012). In this context, and considering the increasing number of microorganisms with drug resistance, the identification of efficient alternative therapies to the current antifungal agents is crucial.

The use of medicinal plants to improve health is an ancient practice (Longe, 2005). However, in recent years, it has been observed an increasing interest of scientific researchers for the study of plants biological properties and active principles responsible for their therapeutic effects (Silva & Fernandes Júnior, 2010; Junio et al., 2011).

Salvia officinalis L. commonly known as sage, is considered the queen of herbs and belongs to the *Lamiaceae* (Labiatae) family. It is widely used both in culinary and medicinal preparations (Longe, 2005; Khan & Abourashed, 2010) as an antispasmodic, antimicrobial, anti-inflammatory, carminative and mucolytic agent; as well as a hormonal regulator and to control mild to moderate states of Alzheimer's disease, reducing patients agitation (Longe, 2005; Scholey et al., 2008; Khan & Abourashed, 2010; Albano & Miguel, 2011).

Some studies report antioxidant properties of sage essential oils, methanol and aqueous extracts, namely obtained by infusion (Wang et al., 1998, 1999; Zimmermann, Walch,

Tinzoh, Stühlinger, & Lachenmeier, 2011; Abu-Darwish et al., 2013). Nevertheless, as far as we know there are no reports available with sage aqueous extracts obtained by decoction. Regarding antimicrobial properties, a few articles report results on aqueous extracts (Jasim & Al-khaliq, 2011; Velickovic et al., 2011), being essential oils the most studied against bacteria strains and filamentous fungi, such as *Candida* species (*C. albicans, C. parapsilosis, C. krusei* and *C. tropicalis*), *Aspergillus* species and *Dermatophytes* (Khalil & Li, 2011; Abu-Darwish et al., 2013). Due to some neurotoxic and convulsing effects of essential oils, mainly in children, they should be used very careful avoiding exceeding the recommended doses (Longe, 2005; Khan & Abourashed, 2010). Thus, other therapeutic alternatives are safer than essential oils. Several studies reported a direct relation between antioxidant potential and antimicrobial activity; both associated to the phenolic composition, especially flavonoids and phenolic acids (Caturla, Vera-Samper, Villalain, Mateo, & Micol, 2003; Araújo et al., 2012).

The aim of the present work was to assess the antifungal effects of aqueous (prepared by infusion and decoction) and methanol/water (80:20, *v/v*) extracts of *S. officinalis* against *Candida* species, to determine their antioxidant activity and to identify the main bioactive molecules (*e.g.*, phenolic compounds) present in the extracts.

2. Materials and methods

2.1. Sample

Flowering aerial parts (leaves, separated from branches) of *Salvia officinalis* L., previously dried, supplied by Soria Natural (Garray - Soria, Spain), were obtained in April-May 2012. The samples were clean products with monitored parameters of pesticides, herbicides, heavy metals and radioactivity.

2.2. Standards and reagents

Methanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, France). The phenolic compound standards were from Extrasynthese (Genay, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and RPMI 1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA) were from Merck (Darmstadt, Germany). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Preparation of the extracts

Aqueous extracts were obtained by infusion and decoction processes. In the infusion process, each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. In the decoction process, each sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The aqueous extracts were frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

Methanol/water (80:20, *v/v*) extract was obtained by stirring each sample (1 g) with 30 mL of the solvents mixture at 25 ºC and 150 rpm for 1 h, and filtered through Whatman No. 4 paper. The residue was then extracted with additional 30 mL portion of the methanol/water mixture. The combined extracts were evaporated at 35 ºC under reduced

pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then further lyophilized.

The lyophilized methanol/water extracts were re-dissolved in methanol/water (80:20, *v/v*), while the aqueous extracts were re-dissolved in water. Stock solutions were prepared at a concentration of 20 mg/mL.

2.4. Evaluation of bioactivity

2.4.1. Antioxidant activity

Four different *in vitro* assays were performed using solutions prepared by serial dilution of the stock solutions: scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, reducing power (measured by ferricyanide Prussian blue assay), inhibition of β-carotene bleaching and inhibition of lipid peroxidation in brain cell homogenates by TBARS (thiobarbituric acid reactive substances) assay (Barros, Carvalho, Morais, & Ferreira, 2010).

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_E)/A_{DPPH}] \times 100$, where A_E is the absorbance at 515 nm of the solution containing the extract, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate Reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which was measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in pig (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances

(TBARS); the color intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) abduct was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the extracts solution, respectively. The results were expressed in EC_{50} values, *i.e.* extract concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay (Dias et al., 2013).

2.4.2. Antifungal activity

Nineteen *Candida* strains were tested (**Table 2**), four from the American Type Culture Collection (ATCC), and fifteen clinical isolates from vaginal tract and oral cavity. The clinical isolates were obtained from the archive collection of the biofilm group of the Centre of Biological Engineering, University of Minho, Braga, Portugal. Before each experiment, all the strains were grown in SDA for 24h at 37 ºC. After that time, one loop of each colony of cells was transferred to an Erlenmeyer, containing 30 mL of SDB liquid medium and incubated under stirring at 37 ºC during 24h. An aliquot of each species (300 µL), containing approximately $1x10^5$ cells/mL was spread in SDA Petri dishes. Then, an aliquot (25 µL) of each sage extract (20 mg/mL) was placed on a sterile blank disc by using a sterile micropipette. Sterile water was used as negative control. The plates were incubated at 37 ºC, during 24-48h (NCCLS/CLSI & ANVISA, 2003). The evaluation of inhibitory properties was performed using a qualitative method based on the disc diffusion assay and corresponding zones of inhibition. The qualitative results, expressed as the presence/absence of the halo, were converted in a semi-quantitative scale being classified the distinctness of the halo as: (-) absence of halo; (+) weak halo – 3.0-7.0 mm; (++) moderate halo – 8.0-10.0 mm; (+++) strong halo - higher than 11.0 mm.

Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined for the extracts that demonstrated positive results, according with the guidelines from the Nature Protocols (Wiegand, Hilpert, & Hancock, 2008), with some modifications. Afterwards, an aliquot of yeast cells (1 mm diameter), from the colonies in SDA Petri dishes, were suspended in 5 mL of sterile saline solution (0.85% NaCl) and mixed for 15s, with a vortex. The resulting suspension was adjusted by a spectrophotometric method, adding saline solution to reach the value of the 0.5 McFarland scale. Serial dilutions of each sage preparation (0.625, 1.25, 1.75 and 2.5 mg/mL) were prepared in RPMI 1640 medium at pH 7. Aliquots of each extract (100 µL), at a threefold final concentration, were dispensed into the 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium). Furthermore, the plates were also incubated with aliquots (100 µL) at a threefold concentration of the *Candida* species. Sample-free and yeast controls were also included. The 96-well plates were incubated at 37 ºC for 48 h. After visualization of the resultant plate, the MIC values were correspondent to the antifungal concentration where there was no growth or even fungistatic effect, by comparison with the control (cells grown without extracts). Then, the number of viable cells was assessed by the determination of number of colony forming units (CFUs), through several dilutions. After 24h of incubation at 37 ºC, the number of colonies formed was counted. The results were presented as the total of CFUs (Log CFUs) and the experiments repeated in triplicate on three different occasions. MFC correspond to the lowest extract concentration in which no visible macroscopic growth was found on the agar plates after the incubation period.

2.5. Analysis of phenolic compounds

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Dias et al., 2013). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve (1-100 μg/mL) for each available phenolic standard was constructed based on the UV signal: apigenin 7-*O*-glucoside (y=159.62x+7.5025; R^2=0.999); caffeic acid (y=611.9x-4.5733; R^2=0.999); isorhamnetin 3-*O*-rutinoside (y= 327.42x + 313.78; R^2=0.999); luteolin 7-*O*-glucoside (y=80.829x-21.291; R^2=0.999); quercetin-3-*O*-glucoside (y=363.45x+117.86; R^2=0.999), rosmarinic acid (y=336.03x+170.39; R^2=0.999). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in mg per g of dried extract.

2.6. Statistical analysis

Three samples were used and all the extracts were prepared and analyzed in triplicate. The results, expressed as mean values and standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$, performed with SPSS v. 20.0 program.

3. Results and discussion

3.1. Evaluation of antioxidant activity

The results of antioxidant activity evaluation are shown in **Table 1**. In general, all the extracts revealed high antioxidant activity, including free radicals scavenging activity (RSA), reducing power (RP), β-carotene bleaching inhibition (CBI) and lipid peroxidation inhibition (LPI) in brain cell homogenates. For all the assays, the order regarding antioxidant properties was: methanol/water extract, aqueous extract obtained by decoction, aqueous extract obtained by infusion. Thus, compounds with stronger antioxidant activity seem to have higher solubility in methanol/water mixtures than only in water. LPI proved to be the most significant activity, conducting to the lowest EC_{50} values in all the extracts. Lipid peroxidation is recognized as one of the main predisposing factors for neurodegenerative and mental disorders (Singh, Sharad, & Kapur, 2004; Chaturvedi & Beal, 2013).

Grzegorczyk, Matkowski and Wysokińska (2007) have previously reported the antioxidant potential of methanol and acetone extracts prepared from organs (shoots and hairy roots) and undifferentiated (cell and callus) *in vitro* cultures of *S. officinalis*. These authors reported higher RSA for methanolic extracts than for acetone extracts. The RSA EC_{50} value obtained in the present work for the methanol/water extract (32.97 µg/mL; **Table 1**) is comprised in the range found by the mentioned authors for methanolic extracts (18.4 - 81.7 µg/mL) and higher that the ones described for acetone extracts (61.8 - >5000 µg/mL). It was also higher than RSA described by Kontogianni et al. (2013) for sage hexane/ethyl acetate extract (EC_{50} = 78 µg/mL). Otherwise, Albano and Miguel (2011) reported a higher RSA (EC_{50} =2.8 µg/mL) in a water extract of sage in comparison with the values reported in the present work for aqueous extracts (73.53 and 95.96 µg/mL for the extracts obtained by decoction and infusion, respectively; **Table 1**). It should be highlighted that the water extract obtained by the mentioned authors result

from a fractionation procedure using different solvents (diethyl ether, ethyl acetate and n-butanol), after an extraction with ethanol/water (70:30, *v/v*).

3.2. Evaluation of antifungal activity

Table 2 shows the results obtained in the screening of antifungal activity of sage extracts performed by the disc diffusion halo assay. In general, the tested extracts did not show antifungal activity or showed a weak potential against the majority of *Candida* species. Moderate to high effects were observed against *C. parapsilosis* (AD) and *C. tropicalis* (ATCC 750); aqueous extract obtained by decoction showed a strong effect against the last mentioned strain. *C. albicans* 575541 was only susceptible to aqueous extracts. Furthermore, no effects were observed against any of the three tested *C. glabrata* strains.

The aqueous extract obtained by decoction (activity against 5 strains) and the methanol/water extract (activity against 4 strains) presented higher antifungal potential than the aqueous extract obtained by infusion (activity against 3 strains) (**Table 2**). Variable effects were observed among strains of the same species; the extraction solvent seems to influence sage bioactivity.

Similarly to our study, Unver, Arslan, Cetynkaya and Ozcan (2008), using a disc diffusion assay, also observed a weak *in vitro* antifungal effect of sage methanol/water (90:10, *v/v*) extract against clinical isolates of eleven species of yeasts (*C. krusei, C. clus, Rhodotorula rubra, C. albicans, C. tropicalis, C. glabrata, C. parasilosis, C. insane, C. rhodotonla, C. holmii* and *C. glabrata*). In contrast, Jasim and Al-khaliq (2011) described an inhibitory effect of aqueous extracts from *S. officinalis* leaves at different concentrations, on the growth of *C. albicans* in infected women with vaginal candidiasis; a total inhibitory effect (100%) was observed for the extract at 25 mg/mL,

while the minimal inhibitory effect was observed at 15 mg/mL (170 mm diameter). This is in agreement with the results observed herein, where sage aqueous extracts presented also a positive effect against *C. albicans*. Velickovic et al. (2011) reported antimicrobial activity of *S. officinalis* methanolic extracts against *C. albicans*.

MIC and, in some cases, MFC values were determined for the most susceptible strains: *C. tropicalis* ATCC 750 (MIC = 1.25 mg/mL and MFC = 2.5 mg/mL for all the extracts), *C. parasilosis* AD (MIC = 2.5 mg/mL for all the extracts) and *C. parasilosis* ATCC 22019 (MIC = 2.5 mg/mL for aqueous extracts). In the case of *C. tropicalis* ATCC 750, all the sage extracts can be considered promising antifungal agents, since aqueous and methanol/water extracts, at 2.5 mg/mL, completely inhibited the growth of the mentioned *Candida* species.

Figure 1 shows the logarithm of the number of colony forming units (CFUs) by the most susceptible *Candida* species treated with the different sage extracts. In general, the aqueous extract obtained by decoction presented the most pronounced fungicidal and/or fungistatic effect against *C. parapsilosis* (**Figure 1a**) and *C. tropicalis* **(Figure 1b)**, varying directly with the sample concentration. The aqueous extract obtained by decoction and the methanol/water extract presented similar potential against *C. parapsilosis* (**Figure 1a**). The aqueous extract obtained by decoction was the most relevant against *C. tropicalis*, followed by the aqueous extract obtained by infusion and methanol/water extract (**Figure 1b**).

3.3. Identification and quantification of phenolic compounds

The phenolic profile of *S. officinalis* obtained after methanol/water extraction, and recorded at 370 nm is shown in **Figure 2**; peak characteristics and tentative identities are presented in **Table 3**. Twenty-one compounds were detected. Ten of which were

phenolic acid derivatives (mainly rosmarinic acid derivatives) and twelve flavonoids (mainly luteolin derivatives).

As for the phenolic acids, caffeic acid (compound 4) and *trans* rosmarinic acid (compound 16) were positively identified according to their retention, mass spectra and UV-vis characteristics in comparison with commercial standards. Caffeic acid and rosmarinic acid have been extensively reported in *S. officinalis* (Lu, Foo, & Wong, 1999; Lu & Foo, 1999, 2001, 2002; Hossain, Brunton, Martin-Diana, & Barry-Ryan, 2010; Zimmermann et al., 2011; Roby, Sarhan, Selim, & Khalel, 2013). The latter one being reported as the main phenolic acid, as it was also observed in the present study. Compound 15, with similar characteristics as compound 1, was tentatively iden tified as *cis* isomer of rosmarinic acid. Hydroxycinnamoyl *cis* derivatives would be expected to elute before the corresponding *trans* ones, as observed after UV irradiation (366 nm, 24 h) of hydroxycinnamic acids in our laboratory. Furthermore, based on their mass spectra, compounds 2 ($[M-H]^-$ at *m/z* 341) and 9 ($[M-H]^-$ at *m/z* 521) were identified as hexoside derivatives of caffeic acid and rosmarinic acid, respectively. A caffeic acid hexoside has been previously described to occur in sage (Hossain et al., 2010), whereas the presence of rosmarinic acid 3'-glucoside (salviaflaside) was reported in different *Salvia* spp. (Kasimu et al., 1998; Lu & Foo, 2002).

A compound with the same pseudomolecular ion ($[M-H]^-$ at *m/z* 503) as compound 1 was previously reported in *Salvia* spp. by different authors (Wang et al., 1999; Ho et al., 2000; Zimmermann et al., 2011; Cvetkovikj et al., 2013) and identified as 6-*O*-caffeoyl-fructofuranosyl-(2-->1)-glucopyranoside.

The pseudomolecular ion of compound 5 ($[M-H]^-$ at *m/z* 537) matched the caffeic acid trimers salvianolic acids H/I and lithospermic acid A. Salvianolic acids H and lithospermic acid A were, however, discarded as possible identities because they present

quite a different fragmentation pattern (Ruan et al., 2012) to the one observed in our sample. Thus, the peak was tentatively assigned as salvianolic acid I (i.e., 3'-*O*-(8''-*Z*-caffeoyl) rosmarinic acid), which was already described in *S. officinalis* (Lu et al., 1999; Lu & Foo, 2001) *Salvia* spp. (Cvetkovikj et al., 2013) and sage infusions (Zimmermann et al., 2011). Compound 14 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 717 and a fragmentation pattern with successive losses of 198 mu ((3-(3,4-dihydroxyphenyl)lactic acid, danshensu) or 180 mu (caffeic acid) units, coherent with salvianolic acid B (also known as lithospermic acid B) found in different *Salvia* spp. (Kasimu et al., 1998; Lu & Foo, 2002; Chen et al., 2011) and also reported in infusions of sage leaves by Zimmermann et al. (2011).

Compound 13 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 719 and an MS^2 majority fragment at *m/z* 359 corresponding to $[M-2H]^{2-}$. These mass characteristics coincided with those of sagerinic acid, a rosmarinic acid dimer (caffeic acid tetramer) isolated from sage by Lu and Foo (1999). Finally, compound 8 ($[M-H]^-$ at *m/z* 535) showed the same molecular weight, fragmentation pattern and UV spectra as sagecoumarin, a caffeic acid trimer identified in sage infusions by Zimmermann et al. (2011), and previously reported in *S. officinalis* by Lu et al. (1999) and Lu and Foo (2001).

Flavonoids detected in the analyzed sample mainly belonged to the group of flavones. Luteolin 7-*O*-glucoside (compound 12) and apigenin-7-*O*-glucoside (compound 17) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Luteolin 7-*O*-glucoside was previously identified in *S. officinalis* (Wang et al., 1998; Lu & Foo, 2000 and 2001), and found in sage tea bags and infusions by Zimmermann et al. (2011). The presence of both compounds has been reported in leaves and aerial parts of *S. officinalis* by different authors (Hossain et al., 2010; Cvetkovikj et al., 2013).

Peaks 6, 10 and 11 were identified as luteolin derivatives according to their UV and mass spectra. The pseudomolecular ion of compound 11 ($[M-H]^-$ at *m/z* 461) was coherent with a luteolin glucuronide. Different glucuronide derivatives of luteolin have been previously reported to occur in *Salvia* spp., namely luteolin 7-*O*-glucuronide (Lu & Foo, 2000 and 2001; Zimmermann et al., 2011; Cvetkovikj et al., 2013), luteolin 3'-*O*-glucuronide (Lu & Foo, 2000) and luteolin 3-*O*-glucuronide (Hossain et al., 2010). Although the location of the sugar moiety cannot be established with the available information, peak 11 was tentatively assigned as luteolin 7-*O*-glucuronide, as it was the that the compound most consistently reported in *Salvia officinalis*. Peak 10 ($[M-H]^-$ at *m/z* 593) could be assigned as a luteolin rutinoside. The presence of luteolin 7-*O*-rutinoside in samples of *S. officinalis* was described by different authors (Hossain et al., 2010; Zimmermann et al., 2011; Cvetkovikj et al., 2013; Roby et al., 2013), so that this identity was tentatively assumed for the compound. Peak 6 ($[M-H]^-$ at *m/z* 637) was identified as a luteolin diglucuronide, also described in *Salvia* spp. by Cvetkovikj et al. (2013).

Compound 18 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 489, releasing two MS^2 fragment ions at *m/z* 447 (-42 mu, acetyl residue) and 285 (further loss of a hexosyl residue), which allowed its tentative identification as a luteolin acetylglucoside.

Compound 19 ($[M-H]^-$ at *m/z* 475) yielded MS^2 fragment ions at *m/z* 299 (-176 mu; loss of a glucuronide residue) and 284 (further loss of $-CH_3$). Although the information obtained in our case was not sufficient to conclude about the actual identity of the compound, it might be tentatively assigned as hispidulin glucuronide, previously reported in *Salvia* spp. by Cvetkovikj et al. (2013). Compound 21 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 299 releasing an MS^2 fragment ion at *m/z* 284 (loss of $-CH_3$). A compound with the same pseudomolecular ion was reported in *Salvia* spp.

by Cvetkovikj et al. (2013) and assigned as cirsiliol (i.e., 3',4',5-trihydoxy-6,7-dimethoxyflavone), although that identity is clearly wrong as the molecular weight of such compound is 330 Da. Instead, a tentative identity as hispidulin (4',5,7-trihydoxy-6-methoxyflavone) might be proposed, owing to the description of various hispidulin glycosides in different populations of *Salvia* by the same authors.

Compounds 3 ($[M-H]^-$ at *m/z* 445) and 20 ($[M-H]^-$ at *m/z* 473) were tentatively assigned as apigenin *O*-pentoside and acetyl apigenin *O*-acetylglucoside respectively based on their pseudomolecular ions and the release of an MS^2 fragment ion at *m/z* 269. UV spectrum (λ_{max} at 348 nm) and mass characteristics of peak 7 ($[M-H]^-$ at *m/z* 477 yielding an MS^2 fragment ion at *m/z* 301) were coherent with those of 6-hydroxyluteolin 7-*O*-glucuronide, a compound identified in *Salvia officinalis* by Lu and Foo (2000) and further reported in several populations of *Salvia* spp. by Cvetkovikj et al. (2013).

From the twenty-one phenolic compounds identified: rosmarinic acid and luteolin 7-*O*-glucuronide were the most abundant phenolic acid and flavonoid respectively found in all the samples. The aqueous extract obtained by decoction presented the highest concentration in phenolic compounds (either phenolic acids or flavonoids), followed by methanol/water extract and aqueous extract obtained by infusion.

There are various publications reporting the phenolic composition of *S. officinalis* from different origins and using different extraction methodologies (Lu et al., 1999; Lu & Foo, 1999; Wang et al., 1999; Lu & Foo, 2001, 2002; Hossain et al., 2010; Zimmermann et al., 2011; Cvetkovikj et al., 2013; Kontogianni et al., 2013; Roby et al., 2013). There are some differences in the phenolic composition described, but some similarities are also observed. Mainly, rosmarinic acid being the main phenolic compound found. These differences could also be explained by different environmental factors, such as: growing conditions (soil, climate, rainfall, altitude), harvesting,

processing, among other factors that can directly interfere with the levels of phenolic compounds (Farhat, Chaouch-Hamada, Sotomayor, Landoulsi & Jordán, 2014). Regarding the quantitative results reported by the above mentioned authors, Zimmermann et al. (2011) performed a quantification of all the different phenolic compounds, however these results can not be compared to the levels obtained in this work due to the units used to express results (mg/L). The same occurs with results reported by Roby et al. (2013) that were expressed in relative percentage of the phenolic compounds identified. Kontogianni et al. (2013) described lower concentrations, and this can be noticed in rosmarinic acid levels (10 mg/g of dry extract), which can also be explained by the different solvents used in the extraction procedure (successive extractions with ethyl acetate and hexane). The other cited authors did not quantify the phenolic compounds that they were able to identify.

Considering all the results obtained, it is feasible to refer that aqueous extracts obtained by decoction and methanol/water extracts of *S. officinalis* provide the most significant bioactivities, which are positively related to their phenolic composition. Regarding antifungal activity, the aqueous extract obtained by decoction presented the most pronounced effect in the majority of *Candida* strains, followed by methanol/water extract and/or the aqueous extract obtained by infusion and, in some cases, varying directly according to the extract concentration. The aqueous extract obtained by decoction also showed the highest concentration of phenolic compounds (either phenolic acids or flavonoids), including rosmarinic acid and luteolin 7-*O*-glucuronide, the main phenolic acid and flavonoid, respectively, found in the extracts. Luteolin derivatives, such as luteolin diglucuronide and 6-hydroxyluteolin-7-*O*-glucuronide,

which were found in higher levels in the aqueous extract obtained by decoction, and could also contribute for the mentioned properties.

Concerning antioxidant activity, higher potential was observed for the methanol/water extract, followed by aqueous extracts obtained by decoction and, lastly by infusion. The higher antioxidant activity presented by the methanol/water extract could be related to higher levels of specific phenolic compounds, such as caffeic acid, luteolin-7-*O*-glucoside, apigenin acetylglucoside and hispidulin. Furthermore, other molecules besides phenolic compounds are present in aqueous extracts (water is an extraction solvent less selective than methanol/water mixtures), and might exert some antagonistic effects in the antioxidant activity.

All in all, it could be anticipated that the use of sage aqueous extract obtained by decoction might provide antimycotic benefits, either incorporated as a dietary supplement, or used as a coadjuvant in antifungal therapies. This is very interesting because it provides an alternative to sage essential oils that can display some toxic effects. Notwithstanding, more studies should be performed in order to establish *in vivo* antifungal activity and, even to test other *C. albicans* and non-*albicans Candida* species, commonly related to opportunistic fungal infections.

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for the N. Martins grant (SFRH/BD/87658/2012), L. Barros researcher contract under "Programa Compromisso com Ciência – 2008" and financial support to the research center CIMO (strategic project PEst-OE/AGR/UI0690/2011). This work was also supported by the Programa Operacional, Fatores de competitividade – COMPETE and by national funds through FCT – Fundação para a Ciência e a Tecnologia on the scope

of the projects FCT PTDC/EBB-EBI/120495/2010, RECI/EBB-EBI/0179/2012 and PEst-OE/EQB/LA0023/2013. The authors thank the Project "BioHealth - Biotechnology and Bioengineering approaches to improve health quality", Ref. NORTE-07-0124-FEDER-000027, co-founded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER. Author CSB is thankful to the Spanish MINECO for financial support through the project BFU2012-35228.

References

Abu-Darwish, M.S., Cabral, C., Ferreira, I.V., Gonçalves, M.J., Cavaleiro, C., Cruz, M. T., Al-bdour, T.H. & Salgueiro, L. (2013). Essential oil of common sage (*Salvia officinalis* L.) from Jordan: assessment of safety in mammalian cells and its antifungal and anti-inflammatory potential. *BioMed Research International*. doi: 10.1155/2013/538940.

Albano, S.M., & Miguel, M.G. (2011). Biological activities of extracts of plants grown in Portugal. *Industrial Crops and Products*, *33*, 338–343.

Araújo, M.G.F., Hilário, F., Vilegas, W., Santos, L.C. dos, Brunetti, I.L., Sotomayor, C.E., & Bauab, T.M. (2012). Correlation among antioxidant, antimicrobial, hemolytic, and antiproliferative properties of *Leiothrix spiralis* leaves extract. *International Journal of Molecular Sciences*, *13*, 9260–77.

Barros, L., Carvalho, A.M., Morais, J.S., & Ferreira, I.C.F.R. (2010). Strawberry-tree, blackthorn and rose fruits: Detailed characterisation in nutrients and phytochemicals with antioxidant properties. *Food Chemistry*, *120*, 247-254.

Caturla, N., Vera-Samper, E., Villalain, J., Mateo, C.R., & Micol, V. (2003). The relationship between the antioxidant and the antibacterial properties of galloylated

catechins and the structure of phospholipid model membranes. *Free Radical Biology and Medicine*, *34*, 648–662.

Chaturvedi, R.K., & Beal, M.F. (2013). Mitochondrial diseases of the brain. *Free Radical Biology & Medicine*, *63*, 1–29.

Chen, H., Zhang, Q., Wang, X., Yang, J., & Wang, Q. (2011). Qualitative analysis and simultaneous quantification of phenolic compounds in the aerial parts of *Salvia miltiorrhiza* by HPLCDAD and ESI/MSn. *Phytochemical Analysis*, 22, 247–257.

Cvetkovikj, I., Stefkov, G., Acevska, J., Stanoeva, J.P., Karapandzova, M., Stefova, M., Dimitrovska, A., & Kulevanova, S. (2013). Polyphenolic characterization and chromatographic methods for fast assessment of culinary Salvia species from South East Europe. *Journal of Chromatography* A, 1282, 38-45.

Dias, M.I., Barros, L., Dueñas, M., Pereira, E., Carvalho, A.M., Alves, R.C., Oliveira, M.B.P.P., Santos-Buelga, C., & Ferreira, I.C.F.R (2013). Chemical composition of wild and commercial *Achillea millefolium* L. and bioactivity of the methanolic extract, infusion and decoction. *Food Chemistry*, *141*, 4152-4160.

Grzegorczyk, I., Matkowski, A., & Wysokińska, H. (2007). Antioxidant activity of extracts from in vitro cultures of *Salvia officinalis* L. *Food Chemistry*, *104*, 536-541.

Farhat, M.B., Chaouch-Hamada, R., Sotomayor, J.A., Landoulsi, A., & Jordán, M.J. (2014). Antioxidant potential of *Salvia officinalis* L. residues as affected by the harvesting time. *Industrial Crops and Products*, *54*, 78-85.

Ho, C.T., Wang, M.F., Wei, G.J., Huang, T.C., & Huang, M.T. (2000). Chemistry and antioxidative factors in rosemary and sage. *Biofactors*, *13*, 161-166.

Hossain, M.B., Rai, D.K., Brunton, N.P., Martin-Diana, A.B., & Barry-Ryan, C. (2010). Characterization of phenolic composition in Lamiaceae spices by LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry*, *58*, 10576-10581.

Jasim, R.M., & Al-khaliq, I.M.A. (2011). Inhibitory effect of aqueous *Salvia officinalis's* leaves in the growth of *Candida albicans* from infected women with vaginal candidiasis. *Al-Kindy College Medical Journal*, *6*, 47–49.

Junio, H.A., Sy-Cordero, A.A., Ettefagh, K.A., Burns, J.T., Micko, K.T., Graf, T.N., Richter, S.J., Cannon, R.E., Oberlies, N.H. & Cech, N.B. (2011). Synergy-directed fractionation of botanical medicines: a case study with goldenseal (*Hydrastis canadensis*). *Journal of Natural Products*, *74*, 1621–1629.

Kasimu, R., Tanaka, K., Tezuka, Y., Gong, Z.-N., Li, J.-X., Basnet, P., Namba, T., & Kadota, S. (1998). Comparative study of seventeen Salvia plants: aldose reductase inhibitory activity of water and methanol extracts and liquid chromatography-mass spectrometry (LC–MS) analysis of water extracts. *Chemical and Pharmaceutical Bulletin*, *46*, 500–504.

Khalil, R., & Li, Z. (2011). Antimicrobial activity of essential oil of *Salvia officinalis* L. collected in Syria. *African Journal of Biotechnology*, *10*, 8397–8402.

Khan, I.A., & Abourashed, E.A. (2010). *Leung's Encyclopedia of Common Natural Ingredients: Used in Food, Drugs and Cosmetics*. (Hoboken, Ed.) (3rd ed., p. 845). N.J. : John Wiley & Sons, Inc.

Kontogianni, V.G., Tomic, G., Nikolic, I., Nerantzaki, A.A., Sayyad, N., Stosic-Grujicic, S., Stojanovic, I., Gerothanassis, I.P. & Tzakos, A.G. (2013). Phytochemical profile of *Rosmarinus officinalis* and *Salvia officinalis* extracts and correlation to their antioxidant and anti-proliferative activity. *Food Chemistry*, *136*, 120-129.

Longe, J.L. (2005). *The Gale Encyclopedia of Alternative Medicine*. (D.S. Blanchfield, F. Laurie, & E. Watts, Eds.) (2nd ed.). Thomson GALE.

Lott, T.J., Fundyga, R.E., Kuykendall, R.J., & Arnold, J. (2005). The human commensal yeast, *Candida albicans*, has an ancient origin. *Fungal Genetics and Biology*, *42*, 444–451.

Lu, Y., & Foo, L.Y. (1999). Rosmarinic acid derivatives from *Salvia officinalis*. *Phytochemistry*, *51*, 91–94.

Lu, Y., & Foo, L.Y. (2000). Flavonoid and phenolic glycosides from *Salvia officinalis*. *Phytochemistry*, *55*, 263–267.

Lu, Y., & Foo, L.Y. (2001). Antioxidant activities of polyphenols from sage (*Salvia officinalis*). *Food Chemistry*, *75*, 197–202.

Lu, Y., & Foo, L.Y. (2002). Polyphenolics from Salvia: a review. *Phytochemistry*, *59*, 117-140.

Lu, Y., Foo, L.Y., & Wong, H. (1999). Sagecoumarin, a novel caffeic acid trimer from *Salvia officinalis*. *Phytochemistry*, *52*, 1149–1152.

NCCLS/CLSI, & ANVISA. (2003). Standardization of the antimicrobial susceptibility tests for disc-diffusion: Approved Standard - Eighth Edition.

Oberoi, J.K., Wattal, C., Goel, N., Raveendran, R., Datta, S., & Prasad, K. (2012). Non-*albicans Candida* species in blood stream infections in a tertiary care hospital at New Delhi, India. *The Indian Journal of Medical Research*, *136*, 997–1003.

Roby, M.H.H., Sarhan, M.A., Selim, K.A.-H., & Khalel, K.I. (2013). Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts. *Industrial Crops and Products*, *43*, 827–831.

Ruan, M., Li, Y., Li, X., Luo, J., & Kong, L. (2012). Qualitative and quantitative analysis of the major constituents in Chinese medicinal preparation Guan-Xin-Ning injection by HPLC-DAD-ESI-MSn. *Journal of Pharmaceutical and Biomedical Analysis*, *59*, 184-189.

Scholey, A.B., Tildesley, N.T.J., Ballard, C.G., Wesnes, K.A., Tasker, A., Perry, E.K., & Kennedy, D.O. (2008). An extract of *Salvia* (sage) with anticholinesterase properties improves memory and attention in healthy older volunteers. *Psychopharmacology*, *198*, 127–139.

Silva, N.C.C., & Fernandes Júnior, A. (2010). Biological properties of medicinal plants: a review of their antimicrobial activity. *The Journal of Venomous Animals and Toxins Including Tropical Diseases*, *16*, 402–413.

Singh, R.P., Sharad, S., & Kapur, S. (2004). Free radicals and oxidative stress in neurodegenerative diseases: relevance of dietary antioxidants. *Journal Indian Academy of Clinical Medicine*, *5*, 218–225.

Unver, A., Arslan, D., Cetynkaya, Z., & Ozcan, M.M. (2008). Antimycotic activity of methanol extracts of sage (*Salvia officinalis* L.), laurel (*Laurus nobilis* L.) and thyme (*Thymbra spicata* L.). *Journal of Essential Oil Bearing Plants*, *11*, 90–95.

Velickovic, D.T., Karabegovic, I.T., Stojicevic, S.S., Lazic, M.L., Marinkovic, V.D., & Veljkovic, V.B. (2011). Comparison of antioxidant and antimicrobial activities of extracts obtained from *Salvia glutinosa* L. and *Salvia officinalis* L. *Hemijska Industrija*, *65*, 599–605.

Wang, M.F., Li, J., Rangarajan, M., Shao, Y., LaVoie, E.J., Ho, C.T., & Huang, T.C. (1998). Antioxidative phenolic compounds from sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry*, *46*, 4869-4873.

Wang, M.F., Shao, Y., Li, J., Zhu, N., Rangarajan, M., LaVoie, E.J., & Ho, C.T. (1999), Antioxidative phenolic glycosides from sage (*Salvia officinalis*). *Journal of Natural Products*, *62*, 454-456.

Wiegand, I., Hilpert, K., & Hancock, R.E.W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*, 163–175.

Zimmermann, B.F., Walch, S.G., Tinzoh, L.N., Stühlinger, W., & Lachenmeier, D.W. (2011). Rapid UHPLC determination of polyphenols in aqueous infusions of *Salvia officinalis* L. (sage tea). *Journal of Chromatography. B*, *879*, 2459-2464.

Figure Legends

Figure 1. Logarithm of number of colony forming units (CFUs) of different strains of *C. parapsilosis* (a) and *C. tropicalis* (b) cultured within different concentrations of the aqueous (obtained by infusion or decoction) and methanol/water (80:20, *v/v*) extracts of *Salvia officinalis* L. Error bars represent standard deviation (SD).

Figure 2. Phenolic profile of *Salvia officinalis* L. methanol/water (80:20, *v/v*) extract at 370 nm (A) and 280 nm (B).

Table 1. Antioxidant activity of different extracts of *Salvia officinalis* L. (mean ± SD).

	EC_{50} values		
	Aqueous (Infusion)	Aqueous (Decoction)	Methanol/water
DPPH scavenging activity (RSA)	95.96±3.44[a]	75.53±4.08[b]	32.97±2.30[c]
Reducing power (RP)	83.62±1.89[a]	66.50±1.40[b]	24.79±0.13[c]
β-carotene bleaching inhibition (CBI)	138.95±11.20[a]	50.87±3.73[b]	6.62±0.30[c]
TBARS inhibition (LPI)	18.01±0.89[a]	10.40±0.91[b]	2.06±0.10[c]

EC_{50} values (μg/mL) correspond to the sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the RP assay. In each row different letters mean significant differences ($p<0.05$).

Table 2. Antifungal activity of different extracts of *Salvia officinalis* L. against several Candida species, evaluated by disc diffusion assay.

Strains	Origin	Aqueous (Infusion)	Aqueous (Decoction)	Methanol/water
C. albicans ATCC 90028	Reference	-	-	-
C. albicans 575541	Urinary	+	+	-
C. albicans 557834	Vaginal	-	-	-
C. albicans 558234	Vaginal	-	-	-
C. glabrata ATCC 2001	Reference	-	-	-
C. glabrata D1	Oral	-	-	-
C. glabrata 513100	Urinary	-	-	-
C. parapsilosis ATCC 22019	Reference	+	+	-
C. parapsilosis AM2	Oral	-	-	-
C. parapsilosis AD	Oral	-	-	++
C. parapsilosis 491861	Vaginal	-	+	+
C. parapsilosis 513143	Vaginal	-	-	-
C. tropicalis ATCC 750	Reference	++	+++	++
C. tropicalis AG1	Oral	-	-	-
C. tropicalis 75	Vaginal	-	-	-
C. tropicalis 12	Vaginal	-	-	-
C. tropicalis 544123	Urinary	-	-	-
C. tropicalis 519468	Urinary	-	-	-
C. tropicalis T2.2	Oral	-	+	+

(-) absence of halo, 0.0 mm; (+) weak halo, 3.0-7.0 mm; (++) moderate halo, 8.0-10.0 mm; (+++) stronger halo, greater than 11.0 mm.

Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification of phenolic compounds in different extracts of *Salvia officinalis* L. (mean ± SD).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g dried extract)		
						Aqueous (Infusion)	Aqueous (Decoction)	Methanol/water
1	6.46	326	503	341(17),281(15),221(8),179(34),161(38),135(5)	6-*O*-caffeoyl-fructosyl-glucoside	0.78 ± 0.04	0.94 ± 0.06	0.86 ± 0.02
2	7.26	324	341	179(100),161(51),149(8),135(47)	Caffeic acid hexoside	0.48 ± 0.03	0.65 ± 0.05	0.47 ±0.02
3	8.54	340	401	269(100)	Apigenin *O*-pentoside	0.43 ± 0.01	0.59 ± 0.01	0.48 ± 0.01
4	11.55	328	179	135(100)	Caffeic acid	0.50 ± 0.01	0.76 ± 0.01	2.00 ± 0.01
5	13.84	328	537	519(84),341(10),179(32),161(48),135(10)	Salvianolic acid I	0.19 ± 0.01	0.41 ± 0.04	0.32 ± 0.05
6	15.43	350	637	285(100)	Luteolin diglucuronide	11.89 ± 0.15	16.82 ± 0.05	4.94 ± 0.01
7	17.21	348	477	301(100)	6-Hydroxyluteolin 7-O-glucuronide	2.53 ± 0.08	3.60 ± 0.07	1.72 ± 0.09
8	17.33	332	535	359(68),197(21),179(49),161(94)	Sagecoumarin	1.11 ± 0.05	1.85 ± 0.10	0.76 ± 0.09
9	18.91	328	521	359(100),197(22),179(34),161(74)	Rosmarinic acid hexoside	0.40 ± 0.01	0.66 ± 0.04	0.59 ± 0.01
10	19.87	350	593	285(100)	Luteolin 7-*O*-rutinoside	9.35 ± 0.20	13.74 ± 0.26	12.57 ± 0.03
11	20.60	348	461	285(100)	Luteolin 7-*O*-glucuronide	88.12 ± 0.36	129.82 ± 1.04	94.73 ± 2.55
12	20.80	348	447	285(100)	Luteolin 7-*O*-glucoside	37.41 ± 0.65	52.20 ± 1.59	56.09 ± 3.45
13	21.43	284	719	539(21),521(12),359(89),197(18),179(30),161(82)	Sagerinic acid	2.92 ± 0.08	3.79 ± 0.30	3.35 ± 0.31
14	22.23	284/340	717	537(78),519(100),493(53),339(27),321(45)295(62	Salvianolic acid B	1.78 ± 0.14	2.87 ± 0.07	2.10 ± 0.13
15	23.24	326	359	359(47),197(73),179(71),161(100)	*cis* Rosmarinic acid	0.97 ± 0.07	1.62 ± 0.12	1.20 ± 0.01
16	24.05	328	359	359(66),197(93),179(89),161(100)	*trans* Rosmarinic acid	73.97 ± 0.15	93.46 ± 0.64	93.22 ± 0.12
17	25.37	336	431	269(100)	Apigenin 7-*O*-glucoside	5.40 ± 0.01	7.93 ± 0.37	7.47 ± 0.06
18	26.14	346	489	447(5),285(42)	Luteolin acetylglucoside	15.56 ± 0.33	21.13 ± 0.71	21.73 ± 0.78

19	26.78	346	475	299(100),284(40)	Hispidulin glucuronide	10.53 ± 0.25	15.54 ± 0.83	15.08 ± 0.14
20	30.29	340	473	311(7),269(20)	Apigenin acetylglucoside	0.53 ± 0.05	0.40 ± 0.05	1.54 ± 0.5
21	31.05	346	299	284(100)	Hispidulin	1.01 ± 0.03	1.55 ± 0.04	2.24 ± 0.13
					Total phenolic acids	83.10 ± 0.45[c]	107.00 ± 0.15[a]	104.88 ± 0.68[b]
					Total flavonoids	182.77 ± 1.20[c]	263.32 ± 2.70[a]	218.59 ± 1.51[b]
					Total phenolic compounds	265.87 ± 0.75[c]	370.32 ± 2.55[a]	323.47 ± 2.19[b]

For the last three rows, in each row different letters mean significant differences (p<0.05).