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Phytochemical Characterization and Biological Evaluation of the Aqueous and Supercritical Fluid Extracts from *Salvia sclareoides* Brot.

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Abstract: Plants belonging to the genus *Salvia* (Lamiaceae) are known to have a wide range of biological properties. In this work, extracts obtained from the aerial parts of *Salvia sclareoides* Brot. were evaluated to investigate their chemical composition, toxicity, bioactivity, and stability under *in vitro* gastrointestinal conditions. The composition of the supercritical fluid extract was determined by GC and GC-MS, while the identification of the infusion constituents was performed by HPLC-DAD and LC-MS. The *in vitro* cytotoxicity of both extracts (0-2 mg/mL) was evaluated in Caco-2 cell lines by the MTT assay. The anti-inflammatory and anticholinesterase activities were determined through the inhibition of cyclooxygenase-1 and acetylcholinesterase enzymes, while β-carotene/linoleic acid bleaching test and the DPPH assays were used to evaluate the antioxidant activity. The infusion inhibited cyclooxygenase-1 (IC_{50} = 271.0 μg/mL), and acetylcholinesterase (IC_{50} = 487.7 μg/mL) enzymes, also demonstrated significant antioxidant properties, as evaluated by the DPPH (IC_{50} = 10.4 μg/mL) and β-carotene/linoleic acid (IC_{50} = 30.0 μg/mL) assays. No remarkable alterations in the composition or in the bioactivities of the infusion were observed after *in vitro* digestion, which supports the potential of *S. sclareoides* as a source of bioactive ingredients with neuroprotective, anti-inflammatory and antioxidant properties.

Keywords: *Salvia sclareoides*, GC-MS, HPLC, bioactivity, *in vitro* digestion.

1 Introduction

Phytochemicals ingested daily or natural extracts used to develop new functional ingredients have become an important area of human nutritional and therapeutic research. *Salvia* species (Lamiaceae) have a long history of culinary and medicinal uses, so there is a remarkable interest concerning their chemistry and biological properties [1]. The industrial and economic importance of this genus has increased in recent years due to evidence that some of its secondary metabolites, mainly terpenes and polyphenols, have valuable pharmaceutical and nutraceutical properties [2]. Several clinical trials have been developed to investigate *Salvia* extracts, and promising results were obtained [3]. Ethnobotanical treatment strategies against Alzheimer´s disease (AD) and brain aging included the use of *Salvia* genus as a source of bioactive compounds [4-6]. We have focused our studies on *Salvia sclareoides* Brot., an Iberian endemic species, whose butanol extract revealed a promising inhibitory activity of AChE, an enzyme related to AD [7]. The phenolic profile, toxicity, antioxidant activity and prion binding properties were also studied [8]. More recently, NMR spectroscopy experiments were used to screen *S. sclareoides* extracts for their content in AChE ligands [9] and also for molecules that bind to Aβ 1-42 oligomers, recognized as highly toxic to AD [10].

Pursuing our studies on this plant, we report here, for the first time, the chemical composition of *S. sclareoides* supercritical fluid extracts as well as its cytotoxicity on Caco-2 cell line as a model of the intestinal epithelium.

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Because the traditional use of this plant relies on the intake of the infusion, it was also evaluated on cell assays. Since no toxicity was denoted by the infusion, it was subjected to more detailed *in vitro* studies involving its anti-inflammatory, anticholinesterase and antioxidant properties. In addition, the infusion was submitted to a simulated *in vitro* digestive process, where the antioxidant activity was monitored by the DPPH assay, and HPLC-DAD-MS/MS was used to analyze eventual alterations on chemical profile after gastric and pancreatic digestions. It is well known that inflammation and oxidative imbalance play a role in neurodegenerative impairments and so, our main goal is to explore multitarget and non-toxic plant constituents that maintain their bioactivities even after they are submitted to simulated gastrointestinal conditions. Overall, the aim of the present study is the valorization of *S. sclareoides* as a source of different chemical constituents that can be obtained by two complementary extraction methods. Non-polar compounds, present in volatile oils obtained by supercritical fluid extraction (SFE), and hydrophilic molecules, found in the aqueous extract, can be explored regarding their potential applications in pharmaceutical, agrochemical or food industries, giving an add-value to this natural resource.

2 Experimental Procedure

2.1 Chemicals

All chemicals were of analytical grade. MTT (thiazolyl blue tetrazolium bromide) acetylcholinesterase from electric eel, pepsin, pancreatin, COX-1 (cyclooxygenase 1) from sheep, and chemicals used to evaluate the anti-inflammatory and antioxidant activities were supplied by Sigma-Aldrich (Barcelona, Spain). DMEM (Dulbecco´s modified Eagle's medium), HBSS (Hanks´ balanced salt solution) with and without phenol red, glutamine, Pen-Strep (penicillin and streptomycin mixture), PBS (phosphate buffered saline) and FBS (foetal bovine serum) were bought from Lonza (Verviers, Belgium). HPLC grade water, methanol and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany). CO_2 (99.995% purity) was supplied by Air Liquide (Lisbon, Portugal).

2.2 Plant material

Plant flowering aerial parts were collected at Serra dos Candeeiros, Portugal, and identified by Prof. Ana Isabel Correia, from the Herbarium of Jardim Botânico da Universidade de Lisboa, where a voucher specimen (LISU 233279) was deposited.

2.3 Extracts preparation

2.3.1 Supercritical fluid extract

The volatile oil from the aerial parts of *S. sclareoides* was obtained by SFE, using 79.05 g of plant material. SFE apparatus was described in detail in previous work [11-13], and the operational conditions of pressure, temperature, and mean particle size of plant material, CO_2 flow rate, and amount of CO_2 consumed were as follows: 90 bar/40 °C/0.6 mm/1.1 kg h^{-1}/4.6 kg, respectively.

In order to obtain a pure volatile oil free of waxes, a fractional separation was carried out at 80 bar and -8 °C (first separator), and 20 bar and -15 °C (second separator). The amount of volatile oil collected in the second separator corresponds to the combination of eight fractions collected at t = 9.95 min, t = 29.85 min, t = 58.28 min, t = 98.01 min, t = 147.18 min, t = 208.00 min, t = 237.82 min and t = 252.83 min. Extraction procedure was stopped when no more oil was obtained.

2.3.2 Aqueous extract

To prepare the infusion, boiling water (100 mL) was poured over dried plant (2.5 g) staying in infusion for 30 min. Then, it was filtered on Whatman® Grade 1 filter paper and cooled at room temperature. For HPLC-DAD analysis, the infusion (5 mL) was filtered through a 0.45 µm membrane filter (VWR) and immediately analyzed. The remaining infusion was lyophilized in a Heto Power Dry 3000 apparatus, giving the dried extract (60.24 mg), which was kept at 4 °C for further assays. Extract concentrations ranging from 0.0 to 2.0 mg/mL were prepared for bioactivity assays.

2.4 Phytochemical analysis

2.4.1 SFE extract

SFE volatile oil was analyzed by gas chromatography (GC), for component quantification, and by gas chromatography coupled to mass spectrometry (GC-MS) for component identification, as detailed in Mota *et al.* (2013) [14]. The identity of the components was assigned

by comparison of their retention indices, relative to C_9-C_{16} *n*-alkane indices and GC-MS spectra from a lab-made library (Plant Biotechnology Center, Faculty of Sciences, Lisbon University), constructed based on the analyses of reference oils, laboratory-synthesized components and commercial available standards [15].

2.4.2 Aqueous extract

HPLC-DAD and LC-MS/MS techniques were applied to the identification of infusion constituents, as previously described [16]. For HPLC-DAD analysis, *S. sclareoides* infusion (25 µL, 1mg/mL), was injected with an auto injector, and elution performed with a gradient composed of solution A (0.05% trifluoroacetic acid), and solution B (methanol) as following: 0 min, 80% A, 20% B; 20 min, 20% A, 80% B; 25 min, 20% A, 80% B. Regarding LC–MS and LC–MS^n, the sample was analyzed by using a linear gradient composed of solution A (1.0% formic acid), and solution B (methanol) as following: 0 min, 80% A, 20% B; 20 min, 20% A, 80% B; 25 min, 20% A, 80% B. The mass spectrometer was operated in both positive and negative ion modes in the range *m/z* 120–1000, and the parameters were adjusted in order to optimize the signal-to-noise ratios (S/N) for the ions of interest [16].

2.5 Cytotoxicity studies with Caco-2 cells

Caco-2 cells (ATCC#HTB37), a human colorectal epithelial adenocarcinoma cell line, were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine, at 37 °C, in an atmosphere with 5% CO_2 [16]. The culture medium was changed every 48 to 72 h, and the cells were passaged before reaching confluence. Cytotoxicity studies were performed in 96-well plates, exposing the cells for 24 h to several concentrations (0.1 to 2.0 mg/mL) of the plant extracts in culture medium, and using the MTT viability test [17].

2.6 Anti-inflammatory activity

To study the *in vitro* anti-inflammatory activity, the lyophilized infusion was dissolved in Tris-buffer, pH 8, in concentrations ranging from 0.1 to 2.0 mg/mL. COX-1 activity was performed as described in previous work [18].

2.7 Acetylcholinesterase inhibition

Anti-acetylcholinesterase activity of the infusion was measured according the methodology previously reported [16], using adaptations of the method described by Ingkaninan et al. (2003) [19].

2.8 Antioxidant activity

The ability of the extract to inhibit the bleaching of the β-carotene-linoleic acid emulsion was determined using the method described by Khadri et al. (2010) [20] with a slight modification. In brief, β-carotene dissolved in chloroform (0.5 mg/mL), linoleic acid (25 µL) and Tween 40 (200 mg) were added and transferred into a round-bottom flask. When the chloroform was removed under vacuum, distilled water (50 mL) saturated with oxygen for 2 h was added to the flask with vigorous stirring. Then, 2.5 mL aliquots of this emulsion were transferred to a series of tubes containing the dissolved extract (100 µL) of each concentration and were further shaken. Absorbance of each sample was recorded at 470 nm immediately after sample preparation (t = 0 min). After that, the samples were placed in the dark for 18 h, at room temperature, together with two blanks, one containing the antioxidant BHT (butylated hydroxytoluene) as a positive control, and the other with the same volume of water instead of the extract. With the later tube a complete oxidation is obtained with the initial yellow color vanishing, while in the test tube with BHT, the yellow color is maintained during the incubation period. Tests were carried out in triplicate and the absorbance of each sample after incubation time was measured [18, 20]. Radical scavenging activity was evaluated by the DPPH method, as previously described [21].

2.9 *In vitro* digestions

In vitro digestions by the gastric and pancreatic juices were performed according the methodology described in [16], which was adapted from Yamamoto and co-workers (1999) [22].

2.10 Statistical analysis

All the bioassays were carried out in triplicate. The software used for data analysis was Microsoft ® Excel 2010 and the results were expressed as means ± standard deviation.

Additional analysis of variance (ANOVA) was performed with $P = 0.05$ and $P = 0.01$.

3 Results and Discussion

3.1 Phytochemical analysis

3.1.1 SFE extract

The extraction yield of the volatile oil isolated by SFE from the dried flowering aerial parts of *S. sclareoides* was 0.4% (g/g dry weight), which is in the same range as those reported for the hydrodistilled essential oils obtained from some other species of the genus *Salvia* [23-25], but lower than that of *S. officinalis* [26]. Regarding SFE, the yields reported in the literature for *Salvia* species are more variable and depend on the pressure and temperature conditions used for the extractions, and are higher for higher pressures [27-28]. At pressures above 90/100 bar, other compounds may contaminate the volatile oil inducing the increase of the extraction yield [12]. Therefore, a pressure/temperature set of 90 bar/40 °C was selected in this work to guarantee that the chemical composition of the volatile oil was free of heavier compounds, namely the waxes present in plants.

The chemical composition of the volatile oil obtained by SFE from *S. sclareoides* was analyzed by GC-MS. As can be seen in Table 1, it was dominated by *trans*-β-caryophyllene oxide, hexadecanoic acid, phytol acetate 2, linoleic acid ethyl ester and hexadecanoic acid methyl ester. On the other hand, monoterpene hydrocarbons were present in trace amounts and oxygen containing monoterpenes were absent.

The chemical variability among different species of *Salvia* is well known. These results contrast with those described for other *Salvia* species submitted to SFE, such as *S. lavandulifolia* Vahl. [29], and *S. officinalis* L. [30-31], which were richer in monoterpene hydrocarbons and the main compounds were oxygen containing monoterpenes [1,8-cineole, (*E*)-ocimenone and camphor]. Moreover, hydrodistilled essential oils of several species of *Salvia* are mainly composed by monoterpene hydrocarbons (α- and β-pinene, limonene) or oxygen containing monoterpenes (1,8-cineole, camphor, α-thujone, α-terpinenyl acetate, linalool), depending on the species, and less commonly, sesquiterpenes are the major compounds (β-caryophyllene, caryophyllene oxide) [23-26]. On the other hand, the hydrodistilled essential oils of some less studied *Salvia* species contains fatty acids and their esters as well as diterpenoids in considerable amounts, such as, *S. hierosolymitana* (21% of fatty acids and 4.8% of phytol) [32], *S. sclarea* (12.77% of phytol) [33], *S. splendens* Scarlet Sage Red (10.99% of fatty acids and 41.46% of phytol) [34], and *S. dorisiana* (3.99% of fatty acids and 10.81% of phytol) [34]. *S. ceratophylla* (29.0% of fatty acids and 14% of phytol) [35], *S. miltiorrhiza* (28.8% of fatty acids and aliphatic esters and 8.9% of phytol) [36], *S. glutinosa* (13.7% of phytol) [37], and *S. reflexa* (28% of fatty acids and 3% of phytol isomer) [37]. In addition, SFE of *S. desoleana* obtained at 90 bar and 50 °C was rich in the diterpenoid sclareol (74.6%), a compound also found in the hydrodistilled oil (5%) [38].

Apart from the current study on the apolar fraction of *S. sclareoides*, only one study assessing the composition of its essential oil obtained by hydrodistillation has been published. As shown by Sepahvand et al. (2014) [39], the essential oil of *S. sclareoides* obtained by hydrodistillation was a complex mixture mainly composed by linalool (27.6%), *trans*-caryophyllene (16.6%), β-*trans*-ocimene (11.8%), germacrene-D (10%), bicyclogermacrene (3.3%), caryophyllene oxide (2.9%), linalyl acetate (2.7%), 1-octanol (2.0%), spathulenol (1.6%) and *cis*-ocimene (1.4%). Lower amounts of β-pinene, β-elemene, α-humulene, phytol and α-farnesene were also found, among other minor compounds. Comparing this composition with that obtained through using SFE, eight compounds were in common, and in both cases the presence of high amounts of sesquiterpenes was verified, conversely to the amount of monoterpenes.

The evolution of the volatile oil composition with the extraction time was also evaluated in this study and, as it can be seen in Table 1, changes were observed. Sesquiterpene hydrocarbons were only extracted in the first hour of extraction, while oxygen-containing sesquiterpenes were present in the fractions collected until 147.18 min (2.45 h). On the other hand, fatty acids and their derivatives as well as the diterpenoid phytol acetate 2 were extracted since the beginning of the extraction but corresponded to 98% of the volatile oil at the end of the extraction. Similar behavior was observed by other authors [29, 30] who described that monoterpene hydrocarbons and oxygen containing monoterpenes were mainly extracted at the beginning, with the percentage of lighter components decreasing with the extraction time. Conversely, higher-molecular-weight compounds, such as sesquiterpene hydrocarbons and oxygenated sesquiterpenes showed a continuous percentage increase at increasing extraction times. The authors explained this behavior by considering that lighter compounds have shorter internal diffusion times than heavier ones and are,

Table 1: Composition of the volatiles extracted by supercritical fluid extraction (SFE) from the dried flowering aerial parts of *Salvia sclareoides*.

Components	RI	SFE§							
		1	2	3	4	5	6	7	8
Benzaldehyde	927	t	t	t	t	t	t	t	t
α-Pinene	930	t	t	t	t	t	t	t	t
1-Octen-3-ol	961	t	t	t	t	t	t	t	t
β-Pinene	963	t	t	t	t	t	t	t	t
Benzene acetaldehyde	1002	t	t	t	t	t	t	t	t
Limonene	1009	t	t	t	t	t	t	t	t
n-Nonanal	1073	0.5	0.3	t	t	t	t	t	t
cis-Theaspirane	1286	t	t	t	t	t	t	t	t
trans-Theaspirane	1300	t	t	t	t	t	t	t	t
n-Tridecane	1300	t	t	t	t	t	t	t	t
Bicycloelemene	1544	t	t	t	t	t	t	t	t
δ-Elemene	1332	t	t	t	t	t	t	t	t
β-Elemene	1388	t	t	t	t	t	t	t	t
trans-β-Caryophyllene	1414	2.7	1.5	t	t	t	t	t	t
Guaia-6,9-diene*	1447	5.2	2.9	t	t	t	t	t	t
α-Humulene	1447	0.5	0.3	t	t	t	t	t	t
trans-β-Farnesene	1455	0.5	t	t	t	t	t	t	t
β-Ionone	1456	0.4	t	t	t	t	t	t	t
Bicyclogermacrene	1487	3.1	2.8	0.6	t	t	t	t	t
α-*trans*-*trans*-Farnesene	1500	t	t	t	t	t	t	t	t
Elemol	1530	4.5	0.3	t	t	t	t	t	t
trans-Nerolidol	1549	0.4	t	t	t	t	t	t	t
Spathulenol	1551	t	1.0	t	t	t	t	t	t
trans-β-Caryophyllene oxide	1561	9.4	5.5	1.5	3.1	0.7	t	t	t
Globulol	1566	2.2	3.0	0.8	0.6	t	t	t	t
α-Bisabolol	1656	t	t	t	t	t	t	t	t
α-*trans*-Bergamotol	1669	t	t	t	t	t	t	t	t
Hexadecanoic acid (= palmitic acid)	1908	14.6	12.6	17.1	21.3	32.1	35.5	20.9	27.6
Phytol acetate 2	2047	15.4	27.0	40.8	28.9	18.1	14.5	19.2	15.1
Linoleic acid ethyl ester	2137	15.0	11.0	14.0	19.5	19.0	14.3	19.6	18.3
Hexadecanoic acid methyl ester	2137	15.1	14.4	20.5	20.5	27.5	30.9	39.1	37.0
% of Identification		89.5	82.6	95.3	93.9	97.4	95.2	98.8	98.0
Grouped Components									
Monoterpene hydrocarbons		t	t	t	t	t	t	t	t
Sesquiterpene hydrocarbons		12.0	7.5	0.6	t	t	t	t	t
Oxygen-containing sesquiterpenes		16.5	9.8	2.3	3.7	0.7	t	t	t
C13 components		0.4	t	t	t	t	t	t	t
Others		60.6	65.3	92.4	90.2	96.7	95.2	98.8	98.0

RI = Retention index relative to C_9-C_{22} *n*-alkanes on the DB-1 column; t = trace (<0.05%), * based on mass spectra only.
§ Eight fractions of volatile oil collected in the second separator at different times.

thus, more readily available. To the best of our knowledge this is the first report on the chemical composition of the SFE-volatile oil, free of waxes, from *S. sclareoides*, contributing to the extent of knowledge on the chemical composition of the genus *Salvia*.

3.1.2 Aqueous extract

The infusion of *S. sclareoides* was analyzed by HPLC-DAD (Fig.1) and its main components were tentatively identified by LC-MS/MS (Table 2).

The identification of each peak (Fig. 1 and Table 2) was based on the fragmentation patterns in MS^2 and MS^3 experiments, and comparison with literature and Mass Bank data base [40].

Rosmarinic acid was identified as the major component of the infusion, and its fragmentation pattern is in full agreement with the previously reported data [9, 41]. In addition, this compound has also been appointed as the major constituent of other *Salvia* species [1, 42]. Luteolin derivatives, like luteolin diglucuronide [1], and luteolin-7-*O*-(6´´*O*-acetylglucoside) [9], as well as sagerinic acid [43], were also detected in minor amounts in the *S. sclareoides* infusion.

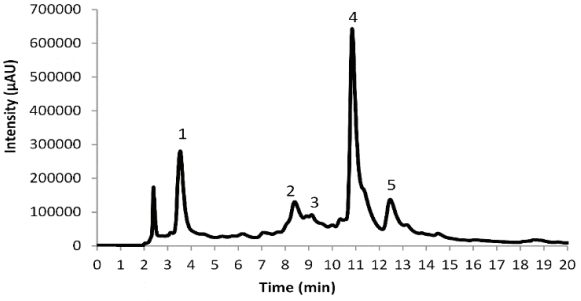

Figure 1: HPLC-DAD chromatogram of *Salvia sclareoides* infusion: (**1**) unknown, (**2**) luteolin diglucuronide, (**3**) sagerinic acid, (**4**) rosmarinic acid, (**5**) luteolin-7-*O*-(6´´- *O*-acetylglucoside).

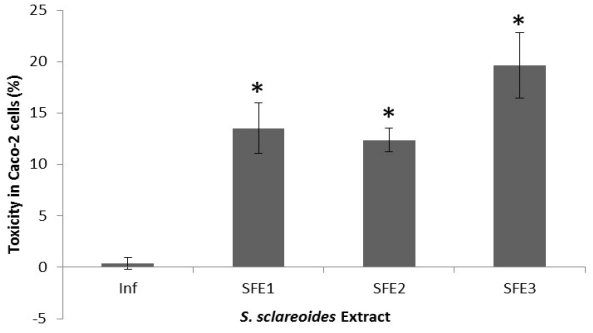

Figure 2: Toxicity of the infusion (Inf.) and of the first three fractions (SFE1-3) of the supercritical fluid extract from *Salvia sclareoides* for Caco-2 cells, presented as % of decrease in cell viability.
* Values statistically different from the control ($P < 0.05$).

Table 2: Putative identification of compounds present in *Salvia sclareoides* infusion by LC-MS.

Peak	RT (min)	$[M-H]^-$	Main product ions (*m/z*)	Compound
1	3.5	223	178, 118, 113, 89	Unknown
2	8.6	637	381, 351, 285	Luteolin diglucuronide
3	9.1	719	539, 521, 359, 341	Sagerinic acid
4	11.1	359	197, 179, 161	Rosmarinic acid (Massbank score 0.732, Massbank IDPR100686)
5	12.5	489	285, 241, 199, 175, 151	Luteolin acetylglucoside (Massbank score for the aglycone 0.941, Massbank ID BML00144)

3.2 Toxicity evaluation in Caco-2 cells

Extracts´ cytotoxicity was evaluated by the MTT (methylthiazolyldiphenyl-tetrazolium bromide) method. It is based on the cellular reduction of tetrazolium salts to dyed formazan-based products by mitochondrial dehydrogenases, providing information on cell metabolism [44]. Despite some limitations of this method, when applied to plant extracts [45-46], it is a rapid and inexpensive assay for a preliminary cell viability evaluation. Although infusions of *S. sclareoides* are taken traditionally by people, the cytotoxicity of its SFE extracts remains unknown. The extracts were analyzed concerning their toxicity towards Caco-2 cell line, which is an accepted model for intestinal cells [47]. The cells were incubated 24 h with different concentrations (0.1-2 mg/mL) of the extracts and, even with the highest concentration (2 mg/mL), the inhibitions were below 50% for all extracts (Fig. 2) and therefore IC_{50} values could not be reached.

None of the extracts tested can be considered toxic as generally IC_{50} values above 0.1 mg/mL are referred as non-toxic [48]. However, with 2 mg/mL, the three first fractions of the SFE extract presented toxicities significantly different from 0% (the control, untreated cells), respectively, SFE1 (13.5 ± 2.4%), SFE2 (12.4 ± 1.1%) and SFE3 (19.7 ± 3.2%), in opposition to the infusion that showed no significant toxicity (0.39 ± 0.55%) even with

2 mg/mL (Fig. 2). Therefore, caution must be taken when ingesting the SFE extracts, as the toxic values may be more easily reached and cause gastrointestinal problems, when compared to the infusion. These results also show that the traditional preparation of *S. sclareoides* extracts as an infusion may be the safest way of ingesting the plant extract, and so, we pursued our research work on bioactivities of the lyophilized infusion.

3.3 Anti-inflammatory activity

Non-steroid anti-inflammatory drugs have important therapeutic interest in the treatment of inflammatory diseases, since they are widely used for the treatment of pain, inflammation and fever. It is assumed that the main mechanism of action of these drugs consists in the inhibition of the cyclooxygenase enzymes: the constitutive cyclooxygenase-1 (COX-1) and the inducible cyclooxygenase-2 (COX-2) isoenzymes that catalyze the conversion of arachidonic acid into prostaglandins (PGs), whose contribution to the process of inflammation is well known [49]. Recently, COX-1 isoenzyme has been receiving increased attention, also because of its involvement in many health problems [50]. In brain, COX-1, predominantly localized in microglia, is an important player in neuroinflammation [51], being involved in several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and traumatic brain injury, among others [50]. In order to investigate the potential of COX-1 as therapeutic target, selective COX-1 inhibitors have been developed [52]. These findings encouraged us to evaluate the ability of *S. sclareoides* infusion to inhibit COX-1 and we found that it inhibited the enzyme (IC_{50} = 271.0 ± 4.0 μg/mL) in a dose dependent manner, evidencing a moderate inhibitory activity.

3.4 Acetylcholinesterase inhibition

The infusion of this plant is traditionally used for the prevention of cognitive detrimental status. Once acetylcholinesterase is an enzyme involved in the depletion of the neurotransmitter acetylcholine, its inhibition may be a target to reverse cognitive disorders. *S. sclareoides* infusion significantly inhibited the enzyme AChE (IC_{50} = 487.7 ± 14.8 μg/mL). Rosmarinic acid, the major phenolic component of the infusion, has shown to be an inhibitor of the acetylcholinesterase activity [53], and so we can assume that this phenolic compound has an important role on the anticholinesterase effect of the herbal tea. To corroborate this assumption, our previous studies on NMR spectroscopy binding experiments of *S. sclareoides* crude extracts in the presence of AChE, determined rosmarinic acid as the only explicit binder to this enzyme [9].

3.5 Antioxidant capacity

Antioxidant activity was evaluated through two different assays: β- Carotene/linoleic acid bleaching test and DPPH free radical-scavenging activity. In the first, a model system made of β-carotene and linoleic acid undergoes a rapid discoloration in the absence of an antioxidant. The free linoleic acid radical formed upon the abstraction of a hydrogen atom from one of its methylene groups attacked the β-carotene molecules, which lost the double bonds and therefore its characteristic orange color [54]. Regarding the DPPH test, it measures the capacity of the extracts to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl formed in solution. If the extracts have the capacity to scavenge the DPPH free radical, the initial blue/purple solution will change to a yellow color, due to the formation of diphenylpicrylhydrazine [55].

When evaluated by the β-carotene assay, *S. sclareoides* infusion presented an IC_{50} of 30.0 ± 5.9 μg/mL, indicating that the extract has a significant antioxidant activity, when compared to the synthetic antioxidant BHT (IC_{50} = 4.5 ± 0.8 μg/mL). Regarding the DPPH assay, the obtained IC_{50} (10.4 ± 0.4 μg/mL) evidences a good radical scavenging activity. When compared to other extracts from the same plant species, also studied by our group [8], the infusion evidences a better antioxidant activity in both assays. Regarding other *Salvia* species, although their chemical variability and the different method of extract preparation influence bioactivities, our results add further support to the well documented antioxidant and anticholinesterase properties of extracts from this genus [56-60], evidencing their potential for therapeutic exploitation.

3.6 *In vitro* digestion by gastric and pancreatic juices and remaining bioactivities

In traditional medicine, *S. sclareoides* is taken orally, meaning that infusion components are subjected to gastrointestinal conditions. An *in vitro* simulation of such conditions allows to predict absorption and distribution of its components assuring that they can exert their bioactivities. The *in vitro* digestions were made with artificial gastric and pancreatic juices, mimicking the

digestion in the gastrointestinal tract, and the infusion was analyzed, in the beginning and after digestion, by HPLC. Moreover, the antioxidant and anti-acetylcholinesterase activities were also evaluated.

The composition of the plant extract was not significantly altered neither in gastric nor in pancreatic digestion, which is in accordance with previous studies showing the stability of rosmarinic acid, the main component, under *in vitro* digestive conditions [61-63]. Concerning the extract bioactivities, no significant alterations were observed in the antioxidant activity after the digestion with gastric juice (99.3 ± 9.4%), which agrees with the HPLC analysis that showed no remarkable changes in the extract composition. After pancreatic digestion, the final antioxidant activity (100.5 ± 8.3%) remained similar to the initial one, indicating that the main antioxidant components of the infusion persisted intact. It is known that AChE inhibitors stimulate gastrointestinal motility [61], enhancing the potential purgative properties of *S. sclareoides* infusion. Although a slight decrease in the acetylcholinesterase inhibition was observed after pancreatic digestion (82.4 ± 8.1%), the inhibitory capacity of the extract increased to 105.3 ± 10.9% after gastric digestion. This demonstrates that the plant infusion is not only active, but also that its bioactive components may reach the intestine.

Dietary intervention studies in several mammalian species, using phenolic rich extracts, have indicated an ability to improve memory, by protecting vulnerable neurons, or by stimulating neuronal regeneration [64]. In addition, the anti-inflammatory properties of phenolic compounds [65], contribute to their neuroprotective effects through their capacity to reduce oxidative imbalance. Based on these findings, *S. sclareoides* can be regarded as an important source of multitarget bioactive molecules with potential to the prevention of neurodegenerative and age-related chronic diseases.

4 Conclusions

SFE can be regarded as an adequate methodology to obtain volatiles from *S. sclareoides* in good yields. Although, at high concentrations, the SFE extract has denoted some cytotoxicity to Caco-2 cells, when compared to the infusion, its potential to be used for agricultural and pharmaceutical formulations with antimicrobial properties needs to be explored. Regarding the non-toxic aqueous extract, a simulated *in vitro* digestion, evidenced no remarkable alterations in the composition or in the bioactivities, suggesting that the infusion can pass through the gastrointestinal tract, keeping its composition and biological properties. The present work reinforces the results of our previous research on *S. sclareoides* extracts and adds further support to the use of the infusion as a source of multitarget bioactive molecules with potential application on the prevention and treatment of neurodegenerative diseases.

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