ORIGINAL ARTICLE

IN VITRO – IN VIVO CORRELATION OF THE ANTIOXIDANT CAPACITY OF *SALVIAE AETHEROLEUM* ESSENTIAL OIL

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Abstract

Both free radicals and oxygen reactive species have been involved as mediators to cell and tissue injury, inflammation, ischemia, neurodegeneration and dementia. Therefore, a great amount of evidence-based studies tried to explain the mechanisms and structure-relation activity. Most of these studies are related to polyphenols, renowned compounds for their powerful antioxidant properties. Our research aimed to evaluate the antioxidant capacity of the essential oil extracted from *Salvia officinalis* L. from biologic cultures. We used *in vitro* and *in vivo* (the determination of superoxide dismutase activity and acetylcholinesterase inhibitory activity) studies to fully assess its potential. The GC-MS analysis indicated the presence of 45 compounds and the principal components of the essential oil, in addition to thujone, were 1,8-cineol and camphor. The results of *in vitro* tests indicated that *Salviae aetheroleum* is a powerful inhibitor against 15-lipoxygenase (IC_{50} 0.064 µL/mL) and cholinesterase (IC_{50} 0.478 µL/mL) and a good scavenger of free radicals (IC_{50} 10.5 µL/mL). For the *in vivo* testing, the essential oil was administered by inhalation to rats with induced Alzheimer disease and brain tissue samples were analysed. Daily exposures for one week to sage essential oil increased antioxidant enzymes activity, suggesting that the main mechanism to prevent neurodegeneration is related to antioxidant properties.

Rezumat

Atât radicalii liberi, cât şi speciile oxigen reactive sunt implicaţi ca mediatori celulari în leziuni tisulare, inflamaţie, ischemie, neurodegenerare şi demenţă. Prin urmare, numeroase cercetări experimentale au încercat să explice mecanismele şi interrelaţia structură-acţiune. Polifenolii, compuşi renumiţi pentru proprietăţile lor antioxidante, au stat la baza celor mai multe dintre cercetări. Scopul studiului de faţă a fost de a evalua capacitatea antioxidantă a uleiului esenţial izolat din *Salvia officinalis* L provenită din culturi biologice. Am încercat să evidenţiem potenţialul acestui ulei prin teste *in vitro* şi *in vivo* (superoxid dismutază şi activitate de inhibare a acetilcolinesterazei). Analiza GC-MS a indicat prezenţa a 45 de compuşi, iar pe lângă tuionă, componentele principale ale uleiului au fost 1,8-cineolul şi camforul. Rezultatele testelor *in vitro* au indicat că *Salviae aetheroleum* este un inhibitor puternic al 15-lipooxigenazei (IC_{50} 0.064 µL/mL) şi colinesterazei (IC_{50} 0.478 µL/mL) şi un bun scavenger de radicali liberi (IC_{50} 10.5 µL/mL). Pentru testarea *in vivo*, uleiul esenţial a fost administrat prin inhalare, la şobolani cu boală Alzheimer indusă, de la care au fost recoltate pentru analiză probe de ţesut cerebral. Expunerea zilnică timp de o săptămână la ulei volatil de salvie a indus creşterea activităţii enzimelor antioxidante, sugerând că mecanismul principal anti-neurodegenerativ este legat de proprietăţile antioxidante.

Keywords: sage essential oil; antioxidant, *in vitro*, *in vivo*

Introduction

The antioxidant capacity of many medicinal plants has been the main goal of different studies. Such properties play an important role in preventing mutagenesis, tumorigenesis and degenerative disorders, induced by oxidative stress [6, 11]. Considerable attention has been devoted to genus *Salvia*, for example hydro-alcoholic leaf extracts from *S. officinalis, S. sclarea, S. triloba* and *S. lavandulifolia* were investigated as radical scavengers against 1,1-diphenyl-2-picrylhydrazyl (DPPH), among which *S. officinalis* extracts were the most active (IC_{50} 41 µg/mL) [10]. There is much literature to support the high antioxidant activity of hydroxycinnamic acid derivatives (notably rosmarinic acid), caffeic acid, as well as phenolic diterpenes (carnosic acid, carnosol and derivatives) [12, 16, 18]. Also, in 2007 Grzegorczyk et al. proved that extracts of sage roots from *in vitro* cultures exhibited strong antioxidant activities, dependent on the type of the solvent used for extraction, when low polarity extracts in acetone (containing mainly carnosic acid and carnosol)

showed a strong activity in the lipid peroxidation assay, whereas the methanolic extract (characterized by the presence of both rosmarinic acid and diterpenes derivatives) had better radical scavenging properties against DPPH [5]. Nevertheless, sage is mostly known for its essential oil that confers soothing, carminative, tonic and antimicrobial effects, being used as a medicinal remedy and as a food and cosmetic preservative. In aromatherapy, the essential oil of sage is known for its antiviral, antibacterial, antifungal, secretolytic, febrifuge, carminative, dermal healing, regenerating and oestrogen-like effects. At CNS level it induces relaxation, enhances memory and concentration. Therefore, *Salvia* essential oil is recommended in aromatherapy as a remedy for cough and bronchitis, thrush wounds, herpes and zoster herpes, hyperhidrosis, climacteric syndrome, impaired concentration. Due to its known high content in thujone and camphor, the maximum level of inclusion of *Salviae aetheroleum* in foods (*Regulatory Status on Thujone-Scientific Commitee on Food*) and perfumes is 0.013% and 0.8% respectively. However, there is no published data on the *in vitro* - *in vivo* comparison of the antioxidant potential of the essential oil extracted from *Salviae folium*. Therefore, the goal of this study was to evaluate both *in vitro* and *in vivo* the antioxidant effects of sage essential oil isolated from dried leaves of *Salvia officinalis* of certified origin, cultivated in ecological environmental conditions.

Materials and Methods

Vegetal material and isolation of the essential oil

The plant material was acquired from well-established sources (Biological Research Centre from Piatra Neamt, Romania) to ensure a certified origin and a proper identification of the investigated species: *Salvia officinalis* leaves. The dry plant material was sorted and weighed, then extracted by distillation with water vapours according to the method specified in the European Pharmacopoeia 7th edition. The extraction was carried out in a Neo-Clevenger type apparatus for 3 hours, after which the essential oil volume was measured and compared to the 100 g dry plant product. The extraction yield was 2.9 mL to 100g dry plant [14].

GC-MS analysis

The gas-chromatographic analysis was performed using an Agilent 6890 coupled with a mass spectrometer / flame ionization detector, using the following coordinates: chromatographic column DB-5MS (30m x 0.25mm, 0.25µm), column temperature (from 40°C to 280°C), injector/detector temperature 250°C, split ratio 100:1, mobile phase: Helium (1mL/min), injection volume 0.3 µL volatile oil. Retention indices (RI) were calculated using n-alkanes (C8-C22) injected in similar conditions to the samples. Identification of components of essential oil was based on RI relative to n-alkanes and computer matching of the mass spectra with the Wiley libraries and literature data (Adams).

In vitro antioxidant assays

DPPH radical scavenging. Free radical scavenger activity was assessed by measuring the ability to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the transformation of the reduced form by the analysed plant extracts [15]. Due to unshared pair of electrons of the two nitrogen atoms, DPPH is a stable radical that strongly absorbs at 517nm. By reaction with a scavenger of free radicals, the DPPH solution changes its colour from purple to yellow due to the formation diphenyl-picrylhydrazyne (DPPH-H), a compound with low absorbance at 517nm. The stock solution (60 mg/mL) of the essential oil was diluted in methanol to obtain concentrations ranging from 10 µL/mL to 40 µL/mL. Diluted solutions (1 mL each) were mixed with 1 mL of a freshly prepared 4% DPPH radical methanol solution and allowed to incubate for 30 min, in the dark, at room temperature. The absorbance was recorded on an ultraviolet- visible (UV–Vis) spectrometer (ABL&E JASCO) at 517 nm using a blank containing the same concentration of oil without DPPH solution. Inhibitions (%) of DPPH radical were calculated with the following formula:

$$\mathbf{I\% = 100 \times scavenger\ activity \times (A_{control} - A_{sample}) / A_{control},}$$

where: **Acontrol** = absorbance of DPPH solution before adding the test solution; **Asample**= absorbance of DPPH solution, 5 minutes after the addition of the test solution. In parallel, for each sample, the $\mathbf{IC_{50}}$ value (sample concentration providing 50% inhibition) was calculated expressed in µL/mL final solution. All values were reported as means ± SD of triplicates.

15-lipoxygenase inhibition (amended Malterud method). Active compounds found in the essential oil have the ability to block the activity of lipoxygenase which catalyses the oxidation of linoleic acid, thus reducing the absorbance at 234 nm. 0.05 mL of lipoxygenase solution in borate buffer was treated with 0.05 mL of test solution in dimethyl sulfoxide (DMSO) and left to stand for 10 minutes at room temperature, after which 2 mL of linoleic acid in borate buffer were added. The absorbance of each sample at 234 nm was recorded for up to 120 seconds [13]. Essential oil dilutions in DMSO ranged from 0.78 to 25 µL/mL. Lipoxygenase inhibitory capacity was calculated according to the formula:

$$\mathbf{I\% = (A_{EFI} - A_{ECI}) \times 100 / A_{EFI},}$$

where: $\mathbf{A_{EFI}}$ – is the difference between the absorbance of the enzyme solution without inhibitor at 90 and 30 seconds; $\mathbf{A_{ECI}}$ - the difference between the absorbance of the enzyme solution treated with inhibitor at 90 and 30 seconds. NOTE: for both tests linalool was used a control, considering that it is a monoterpene alcohol and that the literature states it as a good antioxidant.

Acetylcholinesterase inhibition (Ellman's method). Acetylcholinesterase (AChE) from *Electrophorus electrics* with acetylthiocholine iodide (ATCI) as a substrate, were used for this test. The inhibitory potential of sage essential oil dilution was assessed by Ellman's method with minor modifications [15, 17]. Briefly, in the reaction medium, containing the enzyme (0.2 U/mL in phosphate buffer), 0.2 M Ellman solution and the sample (sage essential oil diluted in DMSO) were added. After 15 minutes at 25ºC, ATCI 0.2 M is added to the mixture and the absorbance is recorded at 412 for 5 minutes. The ability to inhibit acetylcholinesterase was calculated according to the formula:

$$\mathbf{I\% = (A_{EFI} - A_{ECI}) \times 100 / A_{EFI}},$$

where: $\mathbf{A_{EFI}}$ - the difference between the absorbance of the enzyme solution with inhibitor at after 5 minutes and the absorbance of the same solution at time 0; $\mathbf{A_{ECI}}$ - is the difference between the absorbance of the inhibitor treated enzyme solution after 5 minutes and the absorbance of the same solution at the beginning (time 0).

In vivo antioxidant assays

Animals. 40 male Wistar rats (200 ± 30 g, housed in a temperature and light-controlled room, fed and allowed to drink water *ad libitum*) were divided into 4 groups (10 animals per group): (1) control group with saline treatment (0.9% NaCl); (2) beta-amyloid peptide 1-42 (Aβ(1-42)) alone-treated group (by intracerebroventricular injection of 400 pmol of beta-amyloid peptide 1-42, Rat, Sigma-Aldrich, Germany), 20 days prior to testing [3, 4]; (3) Aβ(1-42)-treated group received by inhalation *Salvia officinalis* essential oil 1% ((SO 1%) + Aβ(1-42)); and (4) Aβ(1-42)-treated group received by inhalation *Salvia officinalis* essential oil 3% ((SO 3%) + Aβ(1-42)). The first two groups of animals were caged in similar conditions and did not receive *Salviae aetheroleum*. Tested animals were treated in accordance with the guidelines of animal bioethics in compliance with the European Council Directive of 24 November 1986 (86/609/EEC) with the approval of the local Ethics Committee.

Essential oil administration. The sage essential oils (1% and respectively 3%) were administered *via* an electronic vaporizer placed at the bottom of a Plexiglas chamber (50 cm×40 cm×28 cm), but out of the reach of the animals, daily, for 7 continuous days for 60 minutes. This period of exposure (60 min) to vapours is a suitable inhalation period to induce biological effects [11].

Biochemical parameters assay. One week after the exposure to sage vapours, animals were sacrificed under anaesthesia and the temporal lobes were collected, weight and homogenized (1:10) in a Potter Homogenizer coupled with a Cole-Parmer Servodyne Mixer in ice-cold 0.1 M potassium phosphate buffer (pH 7.4), 1.15% KCl. The homogenate was centrifuged (15 min at 3000 rpm) and the supernatant was used for the biochemical assays [3, 4].

Determination of SOD activity. The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 1.5 mL reaction mixture contained 100 mM Tris/HCl (pH 7.8), 75 mM NBT, 2 M riboflavin, 6 mM EDTA, and 200 µL of supernatant. Monitoring the increase in absorbance at 560 nm followed the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% as previously described [3]. The enzyme activity is expressed as units/mg protein.

Acetylcholinesterase assay. For this assay we used the Ellman's method as described previously in this article (for *in vitro* testing), but instead of using the enzyme we used the brain homogenates (in phosphate buffer) of the animals included in the study. The activity of the enzyme was calculated with the same equation used for the *in vitro* assays.

Results and Discussion

During the GC-MS/FID analysis we identified 45 peaks out of which the majority (84.48%) represented monoterpenes (α- and β-pinene 4.64%, camphene 3.50%, sabinene, myrcene, limonene 1.39%, 1,8-cineole 13.94%, thujone, camphor 12.17%, 4-terpineole, borneol 2.42%, bornyl acetate 1.94%) and only a small amount were sesquiterpenes (3.10% cariophyllene, 3.49% humulene, 2.11 % gurjumene). This chemical composition is somewhat similar to *Salviae aetheroleum* for aromatherapeutic use that should contain: 30-60% monoterpene ketones (thujone), 8-15% oxides (1,8-cineole), 5-15% monoterpenes (pinene, camphene), 5-15% sesquiterpenes, 5-10% monoterpenols (borneol), 1-4% sesquiterpenols and traces of diterpenes such as salviol [1, 2]. Since the total amount of thujone was 30.86%, which indicates a low neurotoxicity considering that there are samples of *Salvia officinalis* with amounts above 60%, we concluded that we could use the isolated essential oil for animal testing.

For the *in vitro* testing we chose linalool as a standard, but in most of the assays its activity was lower than the diluted essential oil, therefore we believe that the actual biologic activity is due to the phytocomplex, maybe by synergistic effects, and is less due to a single compound.

Regarding the DPPH assay we noted that the intensity of the scavenger activity is directly proportional and depends on the concentration of the sample. The IC_{50} value for sage oil was 10.5±0.2µL/mL. This value, by comparison with other essential oils tested in a similar manner is higher [8, 9], which signifies a moderate scavenger activity. Nevertheless, a team of researchers from Iran noted that the essential oil of *Salvia eremofila* had no effect as a DPPH radical scavenger, whereas the methanolic extract had a significant potency [9].

15-lipoxygenase is an enzyme from the oxidoreductases group that catalyses the oxidation reaction of unsaturated fatty acids to form lipid peroxides [7]. Due to its wide distribution in the brain, it has been demonstrated that its overexpression could cause Alzheimer's disease and neuropsychiatric anxiety disorders. Thus, the inhibition of this enzyme in the brain may lead to reduced oxidation of protein post-ischemia and impaired function of blood-brain barrier [13, 19]. The sage essential oil tested in this research had a protective action against lipoxygenase because concentrations of 25 µL/mL inhibit the enzyme by over 75% (Table I). In addition, low IC_{50} values (0.064 ± 0.03) encourage the use of the essential oil in therapy, such low doses being safe and non-toxic.

Table I

15-lipoxygenase inhibition capacity

Sample	Concentration / %Activity						IC_{50}
	25 µL/mL	12.5 µL/mL	6.25 µL/mL	3.125 µL/mL	1.5625 µL/mL	0.78125 µL/mL	
Sage oil	91.31 ± 0.70	84.73 ± 0.63	56.72 ± 0.71	47.01 ± 0.81	29.18 ± 0.73	15.83 ± 0.68	0.064 ± 0.03
Linalool	76.03 ± 0.14	68.57 ± 0.71	52.15 ± 0.25	28.21 ± 0.37	18.14 ± 0.34	13.56 ± 0.25	0.097 ± 0.0005

However, a valid conclusion can be outlined only afters correlating the *in vitro* results with animal model outputs.

The therapy with cholinesterase inhibitors in Alzheimer's disease has been shown to induce beneficial effects with clinical significance in patients with incipient forms of the disease. Compounds of this class may lead to an improvement of a patient's behaviour and daily activities or of the cognitive functions [6]. Therefore, our research investigated also the capacity of AChE inhibition for sage essential oil (Table II). Due to certain limitations imposed by the enzymatic environment the maximum concentration of essential oil diluted in DMSO was 12,5µL/mL.

Table II

AChE inhibition capacity of *Salviae aetheroleum*

Sample	Sample concentration / % Activity					IC_{50}
	12.5 µL/mL	6.25 µL/mL	3.125 µL/mL	1.5625 µL/mL	0.78125 µL/mL	
Sage oil	57.00 ± 2.37	34.61 ± 1.75	16.10 ± 0.68	14.21 ± 0.91	7.92 ± 0.20	0.478 ± 0.032
Linalool	13.87 ± 0.78	11.10 ± 0.21	9.46 ± 0.51	6.73 ± 0.35	5.11 ± 0.08	-

There are few scientific publications on the essential oils capacity to inhibit AChE, most studies being conducted on alcoholic or hydro-alcoholic plant extracts with high content, of polyphenol-carboxylic acids [9, 15, 16, 17]. Our results prove that the essential oil has definite an AChE inhibitory potential, the IC_{50} value being suggestive. Even though there are certain differences between our results and the literature data, these are most probably influenced by the environmental conditions from where the plants have been harvested. Also, the extraction process is of great impact on the chemical composition of the essential oil.

The *in vivo* tests confirm the antioxidant potential of the essential oil included in our study, although no correlation between the concentration of the sample and the intensity of enzymes activity was noted (Figure 1).

Among other endogenous enzymes SOD is part of the first line of defence against free radicals. In our research we noted that the intensity of its activity depended on the concentration of the administered essential oil, SO3% induced a strong activity of the fore mentioned enzyme. Still, the lower concentration sage essential oil (1%) stimulated also the SOD activity.

In AChE assay, SO 1% showed the highest activity, whereas SO 3% has a lower inhibitory potential, but still statistically significant. Such a discrepancy could signify either that there is a competition for the same receptors or it may be due to toxicity, since the concentration is three times higher than the maximum admitted for aromatherapy. Thus, we confirmed once more that a concentration of 1% essential oil is sufficient to induce pharmacological effects both *in vitro* and *in vivo*.

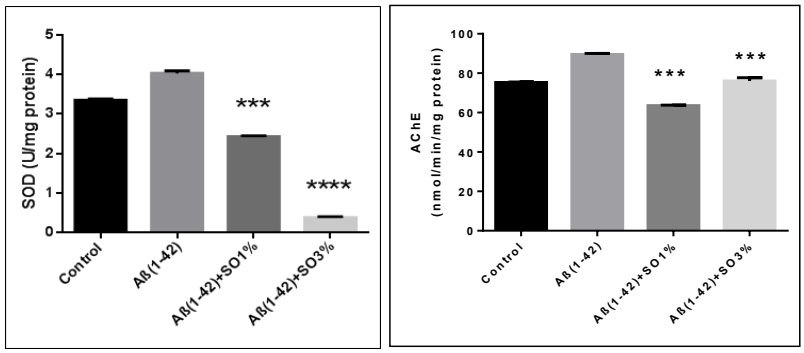

Figure 1.
Diagrams of *in vivo* assays: SOD activity and AChE inhibition ($p < 0.001$)

Conclusions

In the present study we confirmed that the essential oil extracted from dry leaves of *Salvia officinalis* L., cultivated in an ecological environment, has a significant antioxidant activity. Moreover, its biological effects are present both *in vitro* and *in vivo*, in direct correlation to the concentration of the sample and to its composition rich in oxygenated monoterpenes. Also, *Salviae aetheroleum* exhibited an anticholinesterase effect that signifies that this essential oil might be used for aromatherapy in patients with Alzheimer dementia in order to improve the oxidative status or as an adjuvant to regular therapy. Nevertheless, it remains to identify the distribution of the aromatic compounds and their impact on certain types of receptors to better establish their mechanism of action.

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