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ANTIOXIDANT, ANTIMICROBIAL, AND SPASMOLYTIC EFFECTS OF THE CLARY SAGE (*SALVIA SCLAREA* L.) HYDROETHANOLIC EXTRACTS PREPARED BY DIGESTION

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In traditional medicine, clary sage (*Salvia sclarea* L.) is known as an aromatic and medicinal plant used in the treatment of respiratory, digestive, and menstrual issues, as well as various inflammatory processes such as gingivitis, stomatitis, canker sores, and arthritis. This study examined the antioxidant, antimicrobial, and spasmolytic effects of the hydroethanolic extracts from the aerial parts *of S. sclarea* collected in Malča, near Niš, Serbia. Two extracts were prepared using the digestion method with 80% and 60% ethanol (HE80D and HE60D, respectively). The extracts were chemically characterized, revealing significant amounts of phenolic compounds, with rosmarinic acid being the most dominant. The tested extracts showed considerable antioxidant potentials in the 2,2-diphenyl-1-picrylhydrazyl and *β*-carotene/linoleic acid systems. The results indicated that the extracts were particularly effective as antilipoperoxidant agents. The most pronounced antimicrobial effects of the extracts were observed against *Staphylococcus aureus*, with moderate effects against *Bacillus cereus* and *Listeria monocytogenes*. The extracts demonstrated significant inhibition of rat ileum spontaneous smooth muscle contractions. At the maximum concentration of 1.5 mg/ml, the HE80D and HE60D extracts reduced ileum contractions by 40.45±2.06% and 40.60±1.22%, respectively. Further research on *S. sclarea* extracts should be directed towards more detailed *in vivo* and clinical studies to assess their potential use in rational phytotherapy.

Keywords: *Salvia sclarea* L., hydroethanolic extracts, antioxidant effects, antimicrobial effects, spasmolytic effects

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ANTIOKSIDATIVNI, ANTIMIKROBNI, I SPAZMOLITIČNI EFEKTI HIDROETANOLNIH EKSTRAKATA MUSKATNE ŽALFIJE (*SALVIA SCLAREA* L.) PRIPREMLJENI METODOM DIGESTIJE

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U tradicionalnoj medicini muskatna žalfija (*Salvia sclarea* L.) je poznata kao aromatična i lekovita biljka koja se koristi u tretmanu respiratornih, digestivnih i menstrualnih tegoba, i raznih inflamatornih procesa poput gingivitisa, stomatitisa, afti, artritisa. Cilj rada je ispitati antioksidativne, antimikrobne i spazmolitične efekte vodeno-etanolnih ekstrakata nadzemnih delova *S. sclarea* koji su sakupljeni u Malči, u okolini Niša, Srbija. Pripremljena su dva ekstrakta tehnikom digestije sa 80% i 60% etanolom (HE80D i HE60D, redom). Ekstrakti su hemijski okarakterisani pri čemu je utvrđeno da sadrže značajne količine fenolnih jedinjenja od kojih je ruzmarinska kiselina bila najdominantnija. Ispitivani ekstrakti su ispoljili značajne antioksidativne potencijale u 2,2-difenil-1-pikrilhidrazil i *β*-karoten/linolenska kiselina sistemima. Rezultati su pokazali da su ekstrakti naročito efikasni kao antilipoperoksidativni agensi. Najizraženiji antimikrobni efekti ekstrakata zabeleženi su prema *Staphyloccocus aureus*, a umereni prema *Bacillus cereus* i *Listeria monocytogenes*. Ekstrakti su pokazali značajnu inhibiciju spontanih kontrakcija glatkih mišića ileuma pacova. Pri maksimalnoj koncentraciji HE80D i HE60D ekstrakata od 1.5 mg/ml kontrakcije ileuma su smanjene za 40.45±2.06% i 40.60±1.22%, respektivno. Dalja istraživanja ekstrakata *S. sclarea* treba usmeriti na detaljnija *in vivo* ispitivanja i kliničke studije kako bi se sagledala njihova moguća upotreba u racionalnoj fitotetapiji.

Ključne reči: *Salvia sclarea* L., vodeno-etanolni ekstrakti, antioksidativni efekti, antimikrobni efekti, spazmolitični efekti

Introduction

Traditional medicine has historically been vital in both the prevention and treatment of diseases (1). It is based on the action of various pharmacologically active compounds with therapeutic effects that are confirmed through the experience and knowledge gained during the long history of disease treatment, and can also be approved by clinical trials (2). Numerous plant species from the Lamiaceae family are commonly utilized in traditional medicine, and their pharmacological effects have been validated through various studies (3). The most prevalent species in this family are from the sage genus, *Salvia* L., which is represented in many of the world's pharmacopeias. The range of traditional uses for *Salvia* species in folk medicine is remarkably extensive. They are most often used as antiseptics, spasmolytics, carminatives, and agents for rinsing the mucous membranes of the oral cavity and throat in inflammatory processes, healing skin wounds, and skin care. Alongside *Salvia officinalis* L., the most frequently used and researched species, there has been growing interest in other species of this genus, including *Salvia sclarea* L. known as clary sage (4). It is employed as a treatment for digestive and respiratory conditions, such as dyspepsia, painful spasms, flatulence, colds, and coughs, in addition to gingivitis, polyarthritis, rheumatism, and menstrual issues (5-14).

Recently, there has been considerable interest in the use of natural antioxidant agents, prompted by studies suggesting potential adverse effects associated with synthetic antioxidants. A variety of plant materials are recognized as natural sources of antioxidants, and the growing interest in these natural components stems from their biological value and economic impact (15). Furthermore, natural antioxidants are crucial for antioxidant defense and redox regulation in living cells, serving as potential protectors against diseases such as atherosclerosis, diabetes, neurodegenerative disorders, cancer, infections, chronic inflammatory diseases, and autoimmune conditions (16). *Salvia* species are sources of various compounds with powerful antioxidant activity finding a promising place in industry and healthcare products (17).

The increasing prevalence of drug-resistant pathogens underscores the need to identify and isolate new substances from medicinal plants, offering innovative and effective strategies to combat pathogenic bacteria. Many medicinal plants have been historically recognized and extensively used as antiviral, antifungal, antibacterial, or antiparasitic agents (18). *Salvia* species, similar to many other plants, tend to inhibit the growth of Gram-positive bacteria more effectively than Gram-

negative bacteria, a difference attributed to the synergistic action of phytocompounds, primarily flavonoids, phenolic acids, tannins, and volatiles (1,19-21).

Most aromatic drugs are excellent spasmolytic and carminative agents used for treating many gastrointestinal complaints such as colics, flatulence, irritable bowel syndrome, congestive dyspepsia, slow digestion, chronic inflammations, and infections. These medicinal plants and their herbal products have been used for generations due to their multiple nutritional and therapeutic benefits, which are attributed to their active chemicals (22). *S. officinalis* is traditionally used in herbal medicinal products to alleviate mild dyspeptic complaints, including heartburn and bloating (23). Nowadays, other *Salvia* species are currently being studied extensively for their effects on gastrointestinal complaints, particularly on digestive motor function.

With all this in mind, this study intended to demonstrate the pharmacological properties of a less-studied species within the *Salvia* genus, *S. sclarea*, growing in the area of Niš, Serbia. We sought to show the antioxidant, antimicrobial, and spasmolytic activities of its extracts prepared with hydroethanolic solvents by the digestion technique, estimating their possible application in functional foods and dietary supplements.

Material and methods

Plant material and extraction

The aerial parts of *S. sclarea* (including the stem, leaves, and flowers) were collected in Malča, near Niš, in July during full bloom. Dr. Bojan Zlatković (the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš) identified the plant material. The herbarium specimen was submitted to the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac" and assigned collection number 17077.

The material was dried in a dark, well-ventilated space and stored in paper bags until it was analysed. The dried plant material was powdered on a mechanical mill and extracted using the digestion (D) method and two hydroethanolic (HE) solvents (ethanol:water (v/v) ratio - 80:20 and 60:40), in a drug:solvent ratio of 1:10. Thus, two extracts were obtained, HE80D and HE60D. The digestion method involved extracting the powdered plant material for three hours in a water bath at 40°C. After filtration, the resulting liquid extracts were completely evaporated using a vacuum evaporator. All dry extracts were stored in sealed glass vials, at 4°C, until analysis.

Chemical characterization of the extracts

Determination of the content of total phenols

The content of total polyphenols was determined by the colorimetric method according to Hagerman et al. (2000) (24). The method involves the use of the commercially available Folin-Ciocalteu reagent, which is diluted with the same volume of distilled water immediately before the analysis. Distilled water (480 µl), Folin-Ciocalteu reagent (250 µl), and Na_2CO_3 x $10H_2O$ solution (20%, 1250 µl) were added to the diluted extract samples (20 µl) in a test tube. The contents of the test tubes were thoroughly mixed and then allowed to stand at room temperature for 40 minutes. The absorbance was measured at 725 nm, using a UV-Vis spectrophotometer (Evolution 60 Thermo Scientific) with distilled water serving as a blank instead of the diluted extract solution. Prior to analysing the extracts, a calibration curve was established using various dilutions of gallic acid following the same procedure. The results are reported as milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

Determination of the content of total tannins

Total tannin contents in the extracts were assessed as a continuation of the previous experiment using the Folin-Ciocalteu solution (24). Polyvinyl polypyrrolidone (PVPP, 100 mg), which binds the tannins from the extract, was added to a test tube containing 1 ml of distilled water and 1 ml of the diluted sample. The mixture was shaken thoroughly and cooled for 15 minutes at 4°C. It was then centrifuged for 10 minutes at 3000 revolutions per minute. The supernatant, containing all phenolic compounds except tannins, was used to determine non-tannic polyphenols using the previously described method. The tannin content in the extracts was calculated as the difference between the total phenols and the non-tannic polyphenols.

Determination of the content of total flavonoids

The total flavonoid contents in the extracts were determined using the colorimetric $AlCl_3$ method (25,26). The extract solution (0.5 ml, 5 mg/ml) was mixed with 1.5 ml of concentrated ethanol, 0.1 ml of $AlCl_3 \cdot 6H_2O$ solution (10%), 0.1 ml of a 1M sodium acetate solution, and 2.8 ml of water. After 30 minutes, the solutions were transferred to microtiter plates (200 µl), and their

absorbance was measured at 405 nm using an ELISA reader (Multiskan Ascent No354, Thermo Labsystems, Finland). The blank consisted of a mixture without the $AlCl_3$ solution, replaced by an equivalent volume of water. The results are presented as micrograms of luteolin equivalents per milligram of extract (mg LE/g). Before analysing the extracts, a calibration curve was established using various dilutions of luteolin following the same procedure.

Determination of the content of total hydroxycinnamic acid derivatives (THAD)

The content of total hydroxycinnamic acid derivatives was determined according to the prescription from the 11th European Pharmacopoeia (Ph. Eur. 11.0, 2023), which is described in the monograph of the drug *Rosmarini folium* (27).

HPLC analysis of the extracts

The analysis of the chemical composition of the *S. sclarea* extracts using HPLC was performed according to the procedure previously described by Kostić et al. (2017) (28).

Determination of the antioxidant activity of the extracts

Antiradical activity in the DPPH (2,2-diphenyl-1-picrylhydrazyl) system

The ability of the extract to neutralize and "capture" free radicals was estimated in the DPPH system, which was carried out according to the method reported by Kostić et al. (2017) (28). Increasing concentrations of dissolved extracts (40 µl) were added to the wells of a 96-microtiter plate that already contained 120 µl of methanol. Next, 40 µl of a methanol solution of DPPH (0.2 mg/ml) were added to each well. The microtiter plates were briefly mixed on a shaker and then kept in the dark for 30 minutes. The absorbances of the mixtures in the wells were measured at 550 nm using an ELISA reader (Multiskan Ascent No354, Thermo Labsystems, Finland). The percentage of free radical inhibition was calculated using the following formula:

$$\% \text{ inhibition of DPPH} = ((AK-AA)/(AK-AS))\times 100$$

In the equation, AK represents the absorbance of the solvent (methanol) + DPPH solution, AA is the absorbance of the analysed extract solution + methanol + DPPH solution, and AS is the absorbance of methanol. The concentration of the extract that neutralizes 50% (IC_{50}) of free radicals

was determined based on a curve constructed with the inhibition percentages and the extract concentrations. Vitamin E (*α*-tocopherol) and vitamin C (ascorbic acid) were used as the standards.

Antilipoperoxidant activity in the β-carotene-linoleic acid system

The capacity of the extracts to inhibit the process of lipid peroxidation was investigated according to the method of Kostić et al. (2017) (28). This method assesses the extract's effectiveness in protecting linoleic acid molecules from lipid peroxidation, thereby reducing the reliance on *β*-carotene, which would otherwise function as an antioxidant in the absence of the extract. *β*-Carotene (2 mg) was dissolved in 10 ml of chloroform, and one milliliter of this solution was transferred to a round-bottom flask along with linoleic acid (25 µl) and Tween 20 (180 mg). After the complete evaporation of chloroform, 50 ml of oxygenated water was added to the flask with shaking to form an emulsion. The emulsion without *β*-carotene was used as a blank. This aqueous emulsion (200 µl) was pipetted into each well of a microtiter plate, and mixed with 25 µl of the tested extracts with increased dilutions. The plates were then incubated for 2h at 55ºC. The absorbances of the obtained mixtures in the wells of the plates were measured with an ELISA reader (Multiskan Ascent No354, Thermo Labsystems, Finland) at 450 nm before incubation (A0) and after 2h (120 minutes) (A120). The contents of the microtiter plates were mixed on a shaker before measuring the absorbances. The percentage of inhibition was determined according to the formula:

$$\% \text{ inhibition} = (A120/A0)\times 100\%$$

The concentration of the extract that neutralizes 50% of free radicals (IC_{50}) was calculated based on a curve created from the inhibition percentages and extract concentrations. Ascorbic acid and *α*-tocopherol served as the standards.

Spasmolytic effects of the extracts on rat ileum spontaneous contractions

Experimental animals

The experimental procedures adhered to the guidelines outlined in the European Directive 2010/63/EU for animal experiments and received approval from the Veterinary Directorate of the Ministry of Agriculture and Environmental Protection (decision number 323-07-00073/2017-05/04). A male Wistar albino rat, weighing about 200 g and aged 10 weeks, was obtained from the vivarium of the Faculty of Medicine at the University of Niš. One week before the experiments, the rat was

housed individually in stainless steel cage with a room temperature of 20 to 24°C and a 12-hour light-dark cycle. Throughout the study, the rat had ad libitum access to food and water, except for a 24-hour fasting period before the experiments.

Isolation and preparation of rat ileum

Following anesthesia, thoracic and aortic dissection was performed to isolate the rat's ileum. The ileal sections were carefully separated from the mesentery and connective tissue. These segments were then placed in an organ bath containing 20 mL of Tyrode's solution, maintained at 37°C with a continuous supply of a gas mixture composed of 95% O_2 and 5% CO_2. A stabilization period of thirty minutes was allowed for the ileum in the organ bath (29). Contractile changes were measured using a transducer (Transducer-TSZ-04-E, Experimetria Ltd., Budapest, Hungary), and the data were analysed with the SPEL Advanced ISOSYS Data Acquisition System software.

Experimental design with rat ileum

This experiment aimed to evaluate the impact of the hydroethanolic *S. sclarea* extracts on the spontaneous contractions of isolated rat ileum. Following an adaptation period, the extracts were added in incremental doses (ranging from 0.005 to 1.5 mg/mL), and the dose-dependent response was determined from the resulting curve. The spasmolytic effect of each extract concentration was expressed as a percentage relative to the initial spontaneous activity of the isolated ileum without the presence of the tested extract (29). Papaverine, an opium alkaloid known for its spasmolytic activity, served as the positive control (0.01 - 3 µg/mL).

Antimicrobial effects of the extracts

The antimicrobial efficacy of the tested *S. sclarea* extracts was assessed using laboratory control strains sourced from the American Type Culture Collection (ATCC). The assay encompassed Gram-positive and Gram-negative bacteria, and a fungus. *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 9433, *Streptococcus pneumoniae* ATCC 6301, *Streptococcus pyogenes* ATCC19615, *Bacillus cereus* ATCC 11778, and *Lysteria monocytogenes* ATCC 15313 were used as the representatives of Gram-positive bacteria. *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 12453, *Salmonela entertidis* ATCC 13076, *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048, and *Klebsiella pneumoniae* ATCC 10031 were used for the testing of

antimicrobial properties against Gram-negative bacteria. Antifungal effects of the extracts were assessed against a yeast, *Candida albicans* ATCC 24433.

The antibacterial and antifungal activities were evaluated using the microdilution method following the Clinical & Laboratory Standards Institute (30). Overnight cultures of the tested microorganisms were prepared to achieve a suspension with a standard turbidity of 0.5 McFarland, equivalent to $1.5x10^8$ colony forming units (CFU)/mL for bacteria and $1.5x10^7$ CFU/mL for *C. albicans*. The extracts were dissolved in a 10% aqueous solution of dimethyl sulfoxide, and serial double dilutions (ranging from 0.1 to 100 mg/mL) were prepared in 96-well microtiter plates containing inoculated nutrient broth. After incubation at 37°C for 24 hours for bacteria or 25°C for 48 hours for *C. albicans*, microbial growth was detected by adding 20 µL of a 0.5% aqueous solution of 2,3,5-triphenyl tetrazolium chloride to each well (31). The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no visible growth of microorganisms was observed. The minimum bactericidal concentration (MBC or MFC) was determined as the lowest concentration of the extract capable of killing 99.9% of the investigated bacteria or fungus. To ascertain the MBC/MFC, the broth from each well with no visible growth was inoculated into Mueller-Hinton agar at 37°C for 24 hours for bacteria or Sabouraud-dextrose agar at 25°C for 48 hours for *C. albicans*. Positive controls containing chloramphenicol, streptomycin, or nystatin (0.008 - 16 µg/mL) were employed.

Statistical analysis

The collected data were analysed statistically, and the results were presented as mean values from three measurements for chemical composition or six measurements for spasmolytic activity, along with standard deviations (SD), except for antimicrobial activity and extraction yields. An effective concentration EC_{50} and an inhibitory concentration IC_{50}, representing the concentrations causing 50% of maximal response or inhibition, were calculated via regression analysis. Statistical significance between/among the means was determined using Student's t-test/one-way ANOVA followed by Duncan's post hoc test ($p<0.05$ or $p<0.01$). The statistical analyses were conducted using the SPSS 20.0 statistical package (SPSS, Inc., Chicago, IL, USA).

Results

Plant extraction and chemical characterization of the extracts

The yields of extractions in the present study were high and very similar, exceeding 20% (Table 1). Further, the *S. sclarea* extracts were chemically characterized by the determination of total phenolics, tannins, flavonoids, and hydroxycinnamic acid derivatives (Table 1), and by HPLC analysis which was used for the precise definition of present phytocompounds (Table 2). A significant amounts of total phenols and tannins were quantified in the extracts, whereby HE60D contained their higher values, 125.07±3.88 mg GAE/g and 31.85±0.88 mg GAE/g, respectively. Hydroxycinnamic acid derivatives were more dominant in HE80D with a value of 7.47±0.30% of rosmarinic acid, while flavonoids were extracted with both hydroethanolic solvents in a similar quantity.

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Table 1. Yields of extractions (%), the contents of total phenols and tannins (mg GAE/g), hydroxycinnamic acid derivatives (% of rosmarinic acid), and flavonoids (mg LE/g) in *Salvia sclarea* L. hydroethanolic extracts

EXTRACT	Y	TP	TT	THAD	TF
	%	mg GAE/g	mg GAE/g	%RA	mg LE/g
HE80D	21.76	107.22±1.72^a	27.30±1.72^a	7.47±0.30^a	27.13±0.99^a
HE60D	22.01	125.07±3.88^b	31.85±0.88^b	5.82±0.08^b	26.49±1.17^a

Results show the mean of three measurements ± standard deviation. Different lowercase letters in the columns indicate a statistically significant difference in the content of total phenols, tannins, hydroxycinnamic acid derivatives and flavonoids between the extracts (Student's t-test, $p<0.05$).

EXTRACT	RA	CA	L	A	S	LG	AG
	µg/mg						
HE80D	167.81±1.10^a	1.32±0.06^a	1.21±0.03^a	0.97±0.01^a	1.86±0.02^a	6.30±0.26^b	6.68±0.20^c
HE60D	136.16±0.11^b	0.96±0.03^b	0.78±0.04^b	0.69±0.01^b	1.16±0.05^b	6.82±0.43^b	6.70±0.22^c

Table 2. The contents of phenolic acids and flavonoids (µg/mg) in *Salvia sclarea* L. hydroethanolic extracts

RA – rosmarinic acid; CA – caffeic acid; L – luteolin; A – apigenin; S – salvigenin; LG – luteolin-7-*O*-glucoside; AG – apigenin-7-*O*-glucoside. Results show the mean of three measurements ± standard deviation. Different lowercase letters in columns indicate a statistically significant difference in compound content between the extracts (Student's t-test, $p<0.05$).

All tested extracts were chemically characterized using a precise HPLC technique. The recorded chromatograms show the dominance of rosmarinic acid in relation to all other compounds. Caffeic acid, flavonoid aglycones (apigenin, luteolin and salvigenin), and flavonoid heterosides

(apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside) were identified and then quantified (Table 2). Retention times (RT) were as follows: caffeic acid 20.5 min, luteolin-7-*O*-glucoside 29 min, apigenin-7-*O*-glucoside 32.5 min, rosmarinic acid 33.5 min, luteolin 39.3 min, apigenin 42.7 min, and salvigenin 53 min. Figures 1 and 2 present HPLC chromatograms of each of the extracts. The highest amounts of rosmarinic and caffeic acid were recorded in HE80D with the values of 167.81±1.10 μg/mg and 1.32±0.06 μg/mg, respectively. All three flavonoids aglycons (apigenin, luteolin and salvigenin) were better isolated using 80% ethanol. Flavonoids heterosides, luteolin- and apigenin-7-*O*-glucoside, were more present in HE60D.

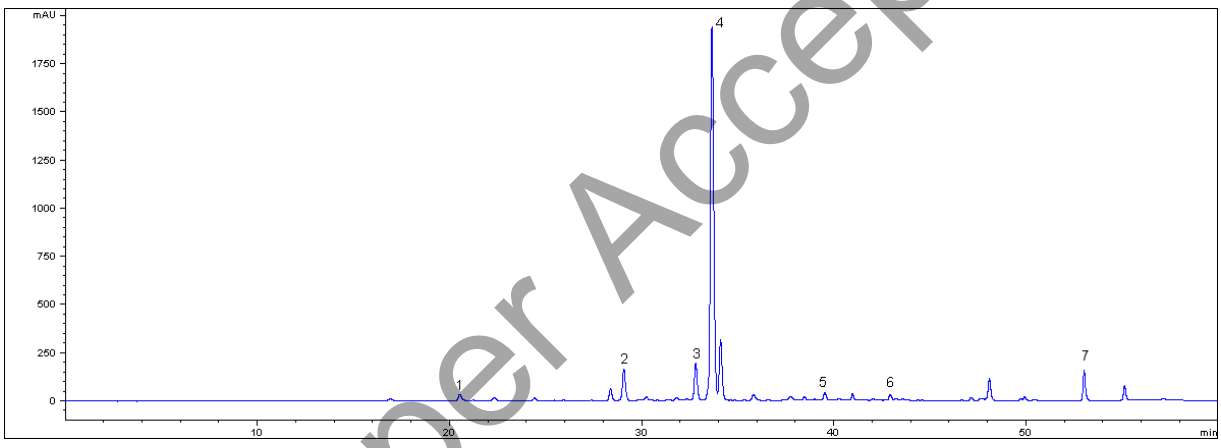

Figure 1. HPLC chromatogram of the *Salvia sclarea* L. extract HE80D (330 nm). 1. caffeic acid, 2. luteolin-7-*O*-glucoside, 3. apigenin-7-*O*-glucoside, 4. rosmarinic acid, 5. luteolin, 6. apigenin, 7. salvigenin

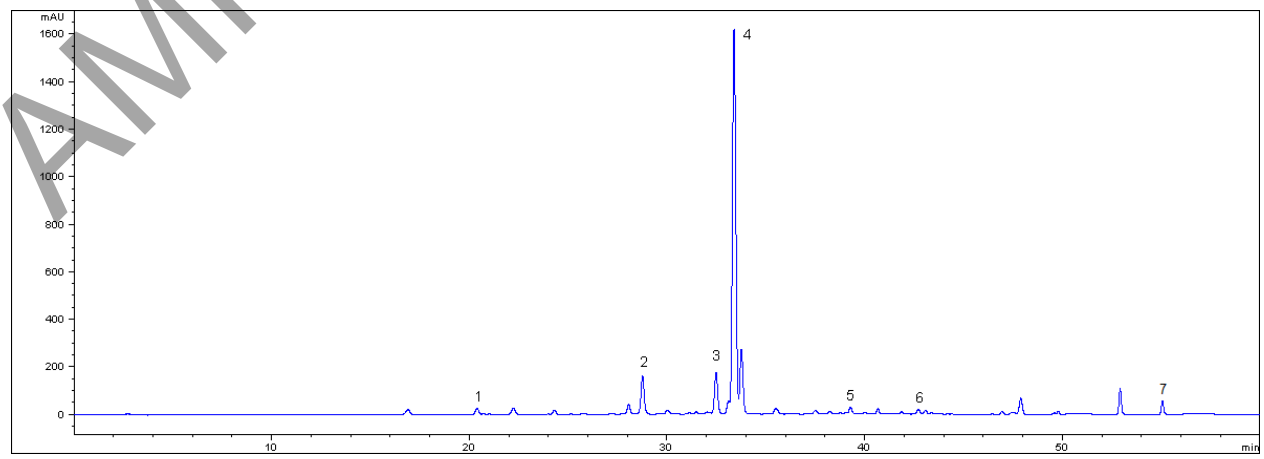

Figure 2. HPLC chromatogram of the *Salvia sclarea* L. extract HE60D (330 nm). 1. caffeic acid, 2. luteolin-7-*O*-glucoside, 3. apigenin-7-*O*-glucoside, 4. rosmarinic acid, 5. luteolin, 6. apigenin, 7. salvigenin

Antioxidant activity of the extracts

The antioxidant activity of the extracts was investigated in two complementary test systems, DPPH and *β*-carotene/linoleic acid (BCL). Table 3. displays the IC_{50} (μg/ml) values of the extracts and positive controls. The extracts produced very similar effects in the antiradical activity assay, while the HE60D extract was the stronger antioxidant agent in the BCL system with the IC_{50} value of 13.36±0.00 μg/ml. This antilipoperoxidant effect was even better than the ones of ascorbic acid, which was, on the other hand, unassailable as free radical scavenger. .

Table 3. Antioxidant activity of *Salvia sclarea* L. hydroethanolic extracts in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *β*-carotene/linoleic acid (BCL) assay

EXTRACT	DPPH	BCL
	IC_{50} (μg/ml)	
HE80D	35.13±1.01[a]	24.78±1.88[a]
HE60D	36.07±1.17[a]	13.36±0.00[b]
AA	4.74±0.34[b]	22.95±1.52[c]
***α*-TOC**	10.40±1.73[c]	0.15±0.00[d]

AA-ascorbic acid; α-TOC- *α*-tocopherol.The results show the mean values of three measurements ± standard deviation. Different lowercase letters in columns indicate a statistically significant difference in IC_{50} values among extracts and standards (Duncan's t- test, $p<0.05$).

Antimicrobial effects of the extracts

Gram-positive bacteria exhibited greater sensitivity to the tested extracts compared to Gram-negative bacteria. While inhibitory concentrations were notable, bactericidal concentrations of all extracts were not significant, reaching 100 mg/ml or higher (Table 4). The most remarkable antibacterial effects were observed against *S. aureus*, with minimum inhibitory concentrations of 6.25

mg/ml. Moderate activity was observed against *B. cereus* and *L. monocytogenes*, while weaker effects were noted against *E. faecalis*, *S. pneumoniae*, and *S. pyogenes*. These clary sage extracts showed poor effects against Gram-negative bacteria. A more significant antimicrobial effect can be attributed to the HE80D extract, which was active against *P. aeruginosa* and *E. aerogenes* with MIC values of 50 mg/ml. Against *C. albicans*, the extracts showed insignificant effects due to high MIC and MFC values.

Table 4. Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal (MBC/MFC) concentrations of the *Salvia sclarea* L. extracts bacterial Gram-positive and Gram-negative strains and yeast

EXTRACTS	**HE80D**	**HE60D**	**S**
Bacterial strains	**MIC/MBC (mg/ml)**		**MIC/MBC (µg/ml)**
Gram (+)			Chloramphenicol
Staphylococcus aureus	6.25/>100	6.25/100	7.81/15.61
Enterococcus faecalis	100/>100	50/100	3.91/7.81
Streptococcus pneumoniae	>100/>100	>100/>100	0.06/0.12
Streptococcus pyogenes	100/>100	100/>100	0.25/0.49
Bacillus cereus	12.5/>100	12.5/>100	7.81/15.61
Listeria monocytogenes	12.5/100	25/100	0.25/0.49
Gram (-)			Streptomycin
Pseudomonas aeruginosa	50/>100	100/>100	0.60/0.60
Proteus mirabilis	>100/>100	>100/>100	0.30/0.30
Salmonella enteritidis	>100/>100	100/>100	0.30/0.30
Escherichia coli	>100/>100	>100/>100	0.16/0.16
Enterobacter aerogenes	50/>100	100/>100	0.60/0.60
Klebsiella pneumoniae	100/>100	100/>100	0.30/0.30
Fungal strain	**MIC/MFC (mg/ml)**		**MIC/MBC (µg/ml)**
Yeast			Nystatin
Candida albicans	>100/>100	100/>100	3.91/7.81

Spasmolytic effects of the extracts on spontaneous rats ileal contractions

Extracts HE80D and HE60D demonstrated notable dose-dependent relaxation of ileal smooth muscle contractions (Figure 3). At the concentrations of 1.5 mg/ml, they decreased the ileal contractions by 40.45±2.06% and 40.60±1.22%, respectively (Table 5). Although the spasmolytic effects of the extracts were significant, they were less potent in comparison to the non-specific muscle relaxant, papaverine. Papaverine, at the concentration of 0.003 mg/ml, inhibited 97.00±0.02% of contractions, with the EC_{50} value of $1.2 \cdot 10^{-4} \pm 0.1 \cdot 10^{-4}$ mg/ml.

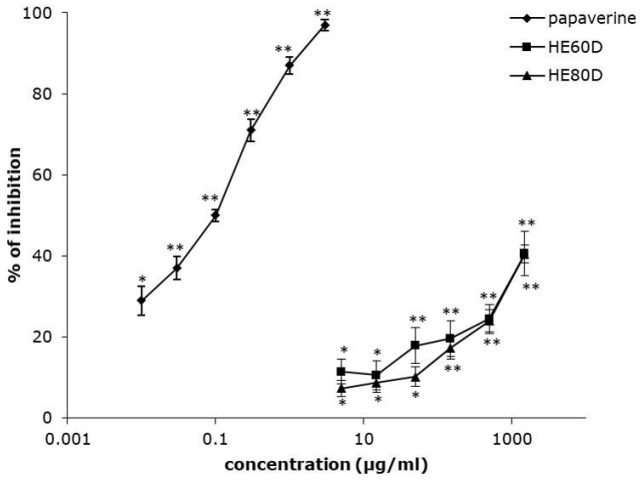

Figure 3. Relaxant effects of the *Salvia sclarea* L. extracts (HE80D and HE60D) and papaverine on spontaneous contractions of isolated rat ileum. Each point represents the average percentage of relaxation relative to spontaneous contractions in Tyrode solution (control) ± standard deviation (Student's t-test, *p<0.05, **p<0.01 vs. control)

Table 5. Spasmolytic effects of the *Salvia sclarea* L. extracts and positive control (papaverine) on the spontaneous contractions

EXTRACT	EC_{50}	max response
	mg/ml	%
HE80D	1.89 ± 0.12^{b}	40.45 ± 2.06^{a}
HE60D	1.97 ± 0.11^{b}	40.60 ± 1.22^{a}
papaverine	$1.2\cdot10^{-4}\pm0.1\cdot10^{-4c}$	97.00 ± 0.02^{b}

The results represent the mean of the six measurement ± the standard deviation. Different lower case letters in the columns indicate a statistically significant difference among EC_{50} values of the extracts and the control (Duncan test, $p < 0.05$)

Discussion

Many factors play important roles in extraction efficiency, such as extraction technique, type of solvent, temperature, time, pH, and particle size (32). Water, as a solvent, has a great extraction capacity, but it also has certain disadvantages. It can cause hydrolytic and fermentative decomposition, lead to the adsorption of the active substance on the swollen drug, dissolve ballast

substances, and enable the development of microorganisms. Due to these shortcomings of water, ethanol-water mixtures are used more often. Ethanol is a much more selective solvent because it perfectly isolates active and less ballast substances (33). Increasing the water proportion in the solvent system causes swelling of the plant material and results in an increase in the contact between the plant matrix and the solvent, as well as an increase in the yield of extraction (34). Considering this fact, the ethanol-water mixture of 60% was a more effective solvent in this study. In addition, the selection of the appropriate extraction technique is very important for the isolation of the desired active ingredients. Changing the temperature can improve the extraction process and reduce the required amount of solvent for extraction (17,35). Therefore, we chose to use the digestion method, presenting one-time extraction that uses heat at up to 50°C for the extraction of phytocompounds (36).

The investigated *S. sclarea* extracts contained high amounts of total phenols and tannins with a preference for extraction using a more polar solvent, 60% ethanol. Phenols are widely found in the plant kingdom and constitute the most abundant group of secondary metabolites, featuring over 8,000 phenolic structures. These range from simple molecules like phenolic acids to complex, highly polymerized compounds such as tannins (37). Polyphenols, as compounds with more than one phenolic hydroxyl group, are gaining special importance because of their possible roles in the prevention of various diseases such as cancer, cardiovascular or neurodegenerative diseases (38). Hydroxycinnamic acid derivatives form a sizable category of simple phenolic acids, acting also as precursor molecules for various compounds including stilbenes, chalcones, flavonoids, lignans, and anthocyanins (39). Hydroxycinnamic acid derivatives were found to be more abundant in the extract prepared with 80% ethanol, whereas flavonoids were extracted in similar quantities using both hydroethanolic solvents.

HPLC analysis of the *S. sclarea* extracts identified two phenolic acids, rosmarinic and caffeic, along with three flavonoid aglycones of the flavone type: apigenin, luteolin, and salvigenin, and two heterosides: apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside. Among all these compounds, rosmarinic acid is identified as the most abundant. The extraction of rosmarinic and caffeic acid was significantly more efficient with 80% ethanol compared to 60%. The importance of the high content of rosmarinic acid is reflected in potential pharmacological activities such as antioxidant, antibacterial, antiviral, antimutagenic, anti-inflammatory, anti-thrombotic, anti-aggregation, anxyolitic,

spasmolytic, renoprotective, etc. (40,41-43). Our previous study of the chemical profiles of *S. sclarea* methanol extracts prepared from the same plant material revealed significant quantities of rosmarinic acid. In four methanolic extracts, its values were even larger and ranged from 171.99±1.88 to 197.48±2.00 μg/mg (44). In our another investigation, concentrate ethanol (96%, v/v) was shown to be weak solvent for the extraction of rosmarinic acid which was present in the concentration of 85.65±0.38 μg/mg (42). According to the literature data other phenolic acids were also identified in *S. sclarea* extracts such as gallic, chlorogenic, ferulic, ellagic, *p*-coumaric, *o*-coumaric, fumaric, vanillic, syringic, quinic, tannic, salvianolic B and K, salvianolic A, lithospermic B, caffeoylmalic, cinnamic, benzoic, 3-hydroxybenzoic, 3,4-dihydroxybenzoic, and *p*-hydroxybenzoic acid (45-55).

Less polar forms of flavonoids, aglycones, were better extracted from *S. sclarea* with the solvent with lower water fraction (80% ethanol). In addition, flavonoid glucosides were more present in the HE60D extract prepared with more polar solvent and higher water fraction (60% ethanol). Considering these and the results from our previous studies (42,44), it can be concluded that concentrated ethanol (96% ethanol) is the optimal solvent for isolating apigenin, luteolin and salvigenin from this plant material, while hydroalcoholic mixtures are suitable for isolating glucosides. According to previous literature data, flavones are mostly present in *S. sclarea*. Flavones such as 4'-methylapigenin, 6-hydroxyluteolin-6,7,3',4'-tetramethyl ether, 6-hydroxyapigenin-7,4'-dimethyl ether, genkwanin, chrysin, acacetin, jaceosidin, viscosine, cirsimaritin, eupatorin, hispidulin and chrysoeriol were identified in different *S. sclarea* extracts, along with apigenin, luteolin and salvigenin. In addition, other types of flavonoids can be found in *S. sclarea* such as kaempferol, myricetin, quercetin, isoquercitrin, rutin, pinocembrin, naringenin, hesperidin genistein and catechin (40,45,48,50-52,54-57).

It is widely recognized that sages contain polyphenolic and terpenic compounds that contribute to their strong antioxidant effects (1). While the antioxidant activity of polyphenols is linked to various mechanisms, their heightened reactivity towards free radicals is regarded as the most significant one (58). Rosmarinic acid, caffeic acid, apigenin, luteolin, and salvigenin have been proven to be excellent antioxidants (59-62) contributing to the same activity of plant extracts. This study assessed the antioxidant activity of *S. sclarea* extracts using two *in vitro* test systems. The DPPH test was used to measure the free radical scavenging capacity of the extracts. During the experiment, the phenolics contained in the extracts reacted with the stable purple DPPH• radical,

which after receiving a proton (hydrogen) was converted into the DPPH reduced form, followed by a color change from purple to yellow (63). The investigated *S. sclarea* extracts showed a strong activity in this test system with similar IC_{50} values, but they were slightly weaker antioxidant agents in comparison to the extract prepared from the same plant material, with 80% ethanol and ultrasound method, from our previous study (IC_{50}=27.82±2.05 µg/ml). The second method of testing the antioxidant efficiency of clary sage extracts was related to examining the antilipoperoxidant activity in the BCL system. This type of antioxidant activity test relies on the competitive decolorization of *β*-carotene during the oxidation of linoleic acid in the presence of oxygenated water. As an effective antioxidant, this carotenoid prevents the formation of linoleic acid peroxide by discoloring itself. The addition of potential antioxidants, which are being evaluated for their effectiveness, halts the degradation of *β*-carotene and maintains the yellow color of the system (58). The HE60D extract, which contained higher amounts of luteolin- and apigenin-7-*O*-glucoside, demonstrated a stronger antilipoperoxidant activity. Its effect was also better than the effect of the extract from our previous study made by the ultrasound method and 80% ethanol (IC_{50}=19.13±1.70 µg/ml).

The antimicrobial activities of *Salvia* species are primarily ascribed to the synergistic actions of their volatile compounds, flavonoids, phenolic acids, and tannins (64-66). The examined *S. sclarea* extracts inhibited the growth of Gram-positive bacteria more effectively than that of Gram-negative bacteria, demonstrating greater bacteriostatic activity compared to bactericidal activity. Considering the high prevalence and growing resistance of staphylococci (67), good antibacterial properties against *S. aureus* (MIC=6.25 mg/mL) are very important. The extracts were also effective against *B. cereus* and *L. monocytogenes*, which is noteworthy considering their role as significant causative agents of gastrointestinal infections (68). Although the effects against Gram-negative bacteria are weak, moderate bacteriostatic activity against *P. aeruginosa* and *E. aerogenes* can be distinguished for HE80D. These pathogens belong to the group of nosocomial and opportunistic bacteria in medical centers, and their treatments are quite difficult due to their multi-resistant nature. *C. albicans* was found to be resistant to the *S. sclarea* extracts in our study, indicating that they cannot be considered effective antifungal agents. Similar antimicrobial effects were reported for other ethanolic and methanolic *S. sclarea* extracts from our previous studies (42,44). Polyphenols are known for their strong antimicrobial properties, and their presence in extracts plays a significant role in inhibiting the growth and destroying microorganisms (69). Numerous *in vitro* studies have shown that the

polyphenols identified in the tested extracts exhibit these effects, particularly against Gram-positive bacteria (42,70-72).

Salvia species have a long history of use in treating various gastrointestinal complaints, thanks to their exceptional spasmolytic properties (73). Todorov et al. (1984) (74) were the first to demonstrate the *in vitro* spasmolytic properties of *Salvia* species by showing their ability to inhibit ileum contractions induced by acetylcholine, histamine, serotonin, and barium chloride. The investigated *S. sclarea* extract significantly reduced spontaneous rats ileum contractions. The isolated intestinal preparation contraction is induced by an increase in the concentration of free calcium ions in the cytoplasm, which activates the contractile elements. This rise in intracellular calcium concentration occurs through voltage-dependent L-type calcium channels or through release from the sarcoplasmic reticulum. Periodic depolarization governs spontaneous movements, and at the peak of depolarization, an action potential is generated due to a rapid influx of calcium ions through these channels (75,76). The first evidence of the spasmolytic activity of *S. sclarea* was presented by researchers Lis Balchin and Hart (1997) (77), who found that its essential oil significantly reduces the contractions of the small intestine of guinea pigs. Furthermore, the spasmolytic actions of the *S. sclarea* extracts were revealed in our two studies. Namely, the ethanolic and methanolic extracts prepared by maceration and ultrasound extraction expressed relaxant effects on spontaneous, but also on KCl- and acetylcholine contractions. The extract made of concentrated ethanol showed the best spasmolytic effects on spontaneous contractions with EC_{50} of 1.33±0.09 mg/ml (42). In addition, docking analysis indicated that apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside exhibited the highest binding affinity to voltage-gated calcium channels (44).

Conclusion

The investigated hydroethanolic *Salvia sclarea* extracts are significant sources of polyphenolic compounds, and are particularly notable for their high content of rosmarinic acid. In addition to it, caffeic acid and flavonoids of the flavone type were also determined. These extracts can be characterized as excellent antioxidant agents because they successfully suppressed free radical production in both model systems used. In addition, they exhibited moderate antibacterial effects, acting more as bacteriostatic than bactericidal agents and could, therefore, serve as supportive agents in managing some infective disorders. It is of great importance that the extracts successfully

reduced spontaneous rat ileal contractions in *in vitro* conditions, and could be beneficial in managing associated gastrointestinal complaints. Hence, *S. sclarea* and its extracts hold promise as potential herbal remedies in modern phytotherapy. Nevertheless, additional studies are necessary to validate their efficacy in human subjects.

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