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# FITOKEMIJSKI SASTAV I BIOLOŠKI UČINCI ODABRANIH VRSTA RODA *SALVIA* L. IZ HRVATSKE FLORE

DOKTORSKI RAD

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# PHYTOCHEMICAL COMPOSITION AND BIOLOGICAL EFFECTS OF SELECTED *SALVIA* SPECIES FROM THE CROATIAN FLORA

DOCTORAL DISSERTATION

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## Sažetak

U okviru ovog doktorskog rada po prvi put su usporedno istraženi fitokemijski sastav i biološki učinci sedam odabranih biljnih vrsta roda *Salvia* L. koje samoniklo rastu na području Republike Hrvatske: *S. fruticosa* Mill., *S. glutinosa* L., *S. nemorosa* L., *S. officinalis* L., *S. pratensis* L., *S. sclarea* L. i *S. verticillata* L. Ekstrakcija biološki aktivnih sastavnica odabranih biljnih vrsta roda *Salvia* provedena je različitim organskim otapalima s i bez primjene ultrazvuka, niskotemperaturnim eutektičkim otapalima, ugljikovim(IV) oksidom u superkritičnom obliku te destilacijom pomoću vodene pare. Kvalitativna i kvantitativna analiza terpenskih i polifenolnih sastavnica provedena je primjenom spektrofotometrijskih metoda te različitih kromatografskih tehnika kao što su HPTLC, HPLC-DAD i GC-MS. Ružmarinska kiselina bila je najzastupljenija fenolna kiselina u svim ispitanim vrstama, dok su glikozidni derivati luteolina i apigenina otkriveni kao najzastupljeniji flavonoidi. Vrste *S. fruticosa* i *S. officinalis* istaknule su se kao mediteranske vrste izrazito bogate eteričnim uljem, dok je sadržaj eteričnog ulja u ostalih ispitanih vrsta bio značajno manji. U sastavu eteričnog ulja vrste *S. fruticosa* najzastupljenija terpenska sastavnica bio je 1,8-cineol, dok je kariofilen-oksid prevladavao u eteričnom ulju vrste *S. sclarea*. Značajni udjeli α- i β-tujona određeni su u eteričnom ulju vrste *S. officinalis*, a germakren D je bio glavna sastavnica eteričnih ulja vrsta *S. glutinosa* i *S. verticillata*. Antioksidacijski učinci etanolnih ekstrakata, eteričnih ulja i odabranih terpenskih i polifenolnih sastavnica te njihova sposobnost inhibicije odabranih enzima koji se smatraju važnim terapijskim metama ispitani su spektrofotometrijskim metodama u prikladnim eksperimentalnim sustavima. Etanolni ekstrakti ispitanih biljnih vrsta pokazali su snažna antioksidacijska svojstva na temelju sposobnosti hvatanja slobodnih radikala, keliranja i redukcije iona željeza te inhibicije lipidne peroksidacije. Ispitani ekstrakti su također imali sposobnost inhibicije enzima elastaze i tirozinaze, a neki su ekstrakti inhibirali aktivnost enzima acetilkolinesteraze i α-glukozidaze. Rezultati provedenih fitokemijskih i bioloških ispitivanja odabranih vrsta roda *Salvia* L. iz hrvatske flore ukazali su na njihov biomedicinski potencijal i mogućnosti primjene u sprječavanju i liječenju kroničnih bolesti vezanih uz starenje.



**Ključne riječi:** *Salvia,* polifenoli, ružmarinska kiselina, eterično ulje, zelena ekstrakcija, antioksidacijski učinak, inhibicija enzima, acetilkolinesteraza, α-glukozidaza, elastaza, tirozinaza

## Summary

### *Background*

Recently, scientific research on various medicinal plants as a source of leading bioactive compounds for the discovery of new drugs or potential new phytotherapeutics has increased. In Croatia, there are many plant species rich in bioactive natural compounds. The genus *Salvia* from the Lamiaceae family is one of the most remarkable medicinal plant genera in the Croatian flora. Many *Salvia* species have traditionally been used for the treatment of sore throats and skin inflammations, mild dyspeptic complaints, excessive sweating, headaches, hyperglycemia and cognitive disorders. In addition, numerous studies have shown a wide range of pharmacological effects of *Salvia* species, such as antioxidant, antimicrobial, antinociceptive, anti-inflammatory, antimutagenic, anticancer, hypoglycemic, hypolipidemic, and memory enhancing effects. The most representative *Salvia* species growing wild in Croatia, *S. fruticosa* Mill., *S. glutinosa* L., *S. nemorosa* L., *S. officinalis* L., *S. pratensis* L., *S. sclarea* L. and *S. verticillata* L., were studied in this doctoral thesis.

### *Aims*

The main objective of this research was a comparative characterization of the bioactive components and an evaluation of the biomedical potential of insufficiently studied *Salvia* species growing wild in Croatia. The specific objectives were as follows: (i) qualitative and quantitative analysis of polyphenolic and terpenic components by UV-Vis spectrophotometry and various chromatographic techniques (HPTLC, HPLC-DAD, GC-MS); (ii) determination of antioxidant activity and ability to inhibit enzymes representing important therapeutic targets (acetylcholinesterase, α-glucosidase, α-amylase, tyrosinase, elastase); (iii) contribution to the understanding of the mechanisms of action of polyphenolic and terpenic bioactive components of *Salvia* species.

### *Methods*

Various organic solvents, with and without sonication, as well as green extraction techniques such as supercritical carbon (IV) oxide ($CO_2$) and deep eutectic solvents were used for the extraction of the polyphenolic and terpenoid compounds. In addition, the essential oils were isolated by appropriate distillation procedures. High-performance thin-layer chromatography (HPTLC) was used to detect various subclasses of polyphenols in methanolic extracts and

terpenoids in essential oils of selected *Salvia* species. The total amounts of phenolic acids, flavonoids, tannins, and anthocyanins in the leaves of *Salvia* species were determined using appropriate spectrophotometric methods. In addition, polyphenolic compounds were identified and quantified using high-performance liquid chromatography with diode array detection (HPLC-DAD). The hyphenated gas chromatography/mass spectrometry technique was used to characterise the terpenoid compounds from the respective essential oils of selected *Salvia* species. The biological effects of *Salvia* ethanolic extracts, essential oils, and selected polyphenolic and terpenoid constituents were studied using appropriate spectrophotometric methods. The antioxidant properties of selected *Salvia* species were tested using five different assays to determine their free radical scavenging ability, metal chelating activity, reducing power, and inhibitory effect on lipid peroxidation. Various colorimetric methods were used to determine the inhibitory effect of the tested *Salvia* species on acetylcholinesterase, α-glucosidase, α-amylase, elastase, and tyrosinase. The resulting data were subjected to a statistical test ANOVA using Tukey's post-hoc test and principal component analysis.

***Results***

The presence of phenolic acids, flavonoids, tannins, anthocyanins, and terpenoids in the studied *Salvia* species was confirmed by HPTLC. The contents of total phenolic acids (3.55-12.44 %), flavonoids (0.31-1.07 %), tannins (1.37-2.60 %) and anthocyanins (0.02-0.08 %) were determined in the leaves of *Salvia* species. Total phenolic acids and flavonoids were also determined spectrophotometrically in sonicated deep eutectic solvent extracts of leaves of selected *Salvia* species. The deep eutectic solvent used in this study contained two biodegradable components, choline chloride and ethylene-glycol in different molar ratios (1:3, 1:4, and 1:5). The extraction process was optimized by varying two parameters, temperature and duration of the extraction process. The total phenolic acid content ranged from 0.31 to 0.41 % in *S. officinalis* and 0.40 to 0.53 % in *S. fruticosa*, while the total flavonoid content ranged from 0.003 to 0.01 % and from 0.01 to 0.05 % in these species, respectively. Further quantification of polyphenolic compounds was performed using HPLC-DAD. Rosmarinic acid was the most abundant component in all samples and its content ranged from 9.4 to 38.8 mg/g. The contents of rosmarinic acid in the studied extracts were present in the following order: *S. officinalis* > *S. verticillata* > *S. fruticosa* > *S. pratensis* > *S. sclarea* > *S. nemorosa* > *S. glutinosa*. Of the other hydroxycinnamic acid derivatives, caffeic acid, chlorogenic acid and *p*-coumaric acid were present in all samples studied, while ferulic acid was determined in *S. fruticosa* and *S. verticillata*. Flavonoids were significantly less represented in all samples

compared to phenolic acids. Luteolin-7-O-glucoside was present in all samples and its content was the highest in *S. officinalis* (656.61 μg/g). Apigenin-7-O-glucoside was also detected in all of the tested *Salvia* species. *S. officinalis* contained the highest amount of said glycosidic derivative (478.11 μg/g). The extraction yields (V/m) of the essential oils isolated by hydrodistillation from the leaves of the studied *Salvia* species ranged from 0.25 mL/kg in *S. verticillata* to 27.61 mL/kg in *S. fruticosa*. According to the results of the GC-MS analysis, 146 different compounds were identified in all samples, accounting from 87.34 % of the total oil content for *S. glutinosa* to 99.62 % of the total oil content for *S. officinalis*. The highest total oil content was found in the essential oil of *S. officinalis*, while the lowest content was determined in the essential oil of *S. glutinosa*. The most abundant compound in the *S. fruticosa* essential oil was 1,8-cineole (53,78 %). The essential oil of *S. officinalis* contained significant amounts of α-thujone (12.23 %), camphor (17.51 %), and γ-gurjunene (14.68 %). The essential oil of *S. sclarea* contained caryophyllene oxide as the major terpenoid constituent (27.61 %), while germacrene D was highlighted as the main constituent of the essential oil of *S. glutinosa* and *S. verticillata* (25.10 % and 43.13 %, respectively). The terpenoid contents in the extracts of *S. officinalis* obtained with supercritical $CO_2$ were also determined using GC-MS. During the optimization process of supercritical $CO_2$ extraction, the influence of pressure and temperature on the extraction yield and terpenoid content was tested. The process was tested at temperatures from 40 °C to 60 °C and at pressures from 100 bar to 220 bar. The calculated extraction yields varied from 0.60 to 4.51 %. The temperature and pressure changes affected the content of the most abundant terpenoids in the obtained $CO_2$ extracts: α-and β-thujone (20.43-35.40 %), 1,8-cineole (6.34-10.63 %), manool (20.10-35.50 %), γ-gurjunene (6.57-10.88 %) and camphor (7.18-11.46 %).

The antioxidant activity of ethanolic extracts of *Salvia* species was determined by five different spectrophotometric methods in comparison with rosmarinic acid and reference antioxidants. All tested extracts showed significant ability to scavenge DPPH ($IC_{50}$=2.49-7.71 μg/mL) and nitric oxide ($IC_{50}$=26.96-101.73 μg/mL). The essential oil of *S. officinalis* also expressed significant free radical scavenging activity. In addition, the ethanolic extracts possessed strong reducing properties ($IC_{50}$=7.14-17.51 μg/mL) and inhibited lipid peroxidation ($IC_{50}$=53.18-327.23 μg/mL). $IC_{50}$ values for metal chelating activity varied from 163.02 to 1592.53 μg/mL. In addition to *S. officinalis, S. fruticosa, S. verticillata* and *S. glutinosa* showed the strongest antioxidant potential, except for metal chelating activity. *S. sclarea* was highlighted as the strongest metal chelator among the species tested. The results indicated that rosmarinic acid is

an important contributor to the antioxidant capabilities of the *Salvia* species. The enzyme inhibitory activity of the ethanolic extracts of *Salvia* species, rosmarinic acid and reference inhibitors was also evaluated using appropriate colorimetric assays. *S. officinalis*, *S. fruticosa* and *S. verticillata* showed anti-acetylcholinesterase activity ($IC_{50}$=268.45-1607.87 μg/mL). There was no significant difference between the anti-acetylcholinesterase activity of *S. officinalis* and *S. fruticosa*, while *S. verticillata* showed lower activity. The essential oils of *S. fruticosa* and *S. officinalis* also showed strong anti-acetylcholinesterase activity. *S. officinalis*, *S. glutinosa* and *S. fruticosa* possessed inhibitory properties against α-glucosidase ($IC_{50}$=4451.85-5291.51 μg/mL) that were four to five times higher than the activity of the reference antidiabetic drug acarbose. *S. officinalis* and *S. glutinosa* expressed stronger anti-α-glucosidase activity than *S. fruticosa*. None of the extracts tested was active against α-amylase, whereas all showed antielastase and antityrosinase activity. The $IC_{50}$ values of antielastase activity of *Salvia* species ranged from 799.90 to 2273.04 μg/mL, while the $IC_{50}$ value of antityrosinase activity ranged from 933.07 to 1471.87 μg/mL. *S. officinalis* was highlighted as the strongest elastase and tyrosinase inhibitor, while *S. glutinosa* showed the weakest enzyme inhibitory activity.

***Conclusions***

The research conducted within the framework of this doctoral thesis has provided new insights into the phytochemical composition and biological effects of selected *Salvia* species growing wild in Croatia. The results showed that *Salvia* species are a rich source of biologically active compounds with antioxidant, neuroprotective, hypoglycemic and wound-healing properties. As such, they have biomedical potential for treatment and prevention, and as a source of lead compounds for the discovery of new drugs against various age-related chronic diseases.

**Key words:** *Salvia*, polyphenols, rosmarinic acid, essential oil, green extraction, antioxidant activity, enzyme inhibition, acetylcholinesterase, α-glucosidase, elastase, tyrosinase