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ROSMARINIC AND CAFFEIC ACID CONTENT AND ANTIOXIDANT POTENTIAL OF THE *SALVIA AETHIOPIS* L. EXTRACTS

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Aromatic plants are the source of pharmacologically active compounds with high antioxidant effects. Among them, *Salvia* L. species, sages, have been known world-wide as spices, aromas, medicines, natural preservatives and antioxidant agents since ancient times. Literature data have shown that *Salvia aethiopis* L. expresses various biological effects. The aim of this research was to determine the quantity of rosmarinic and caffeic acid, usually present in sages, in *S. aethiopis* extracts prepared with different solvents and to estimate their antioxidant effects. The above-ground parts of S. *aethiopis* were collected in the period of full blossom in the surrounding area of Niš, Ploče, Serbia. The plant material was air-dried, pulverized and extracted with absolute and 80% methanol, 96%, 80% and 60% ethanol and ethyl acetate (M, M80, E, E80, E60 and EA, respectively) in an ultrasonic bath. The phenolic acids were quantified by High Performance Liquid Chromatography and antioxidant effect was estimated by two complementary in vitro methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene/linoleic acid (BC) models. Extract E80 contained the highest amount of rosmarinic acid (231.09±4.11 μg/mg) and E60 was the richest in caffeic acid (4.39±0.80 μg/mg). M80 was the most efficient in DPPH antioxidant assay, while E60 expressed the best antilipoperoxidant activity in BC method. The presence of significant amount of rosmarinic acid along with caffeic acid and excellent antioxidant activity of the extracts may be contributable to their potential usage in different pathological conditions, especially in the modulation of oxidative stress. *Acta Medica Medianae 2017;56(3):121-128.*

Key words: *Salvia aethiopis L., extracts, rosmarinic acid, caffeic acid, antioxidant activity*

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Introduction

The recent upsurge of natural antioxidants usage has raised the importance of aromatic plants due to their safety and benefits that they can offer as extracts, essential oils and spices (1). These plant preparations are the source of pharmacologically active compounds with high antioxidant potentials such as phenolic acids, flavonoids, diterpenes and tannins which can act as reducing agents, hydrogen donators or singlet oxygen quenchers (2, 3). It is well-established that diets rich in the antioxidants have a protective role with anticarcinogenic or cardioprotective effects (3).

Salvia L. species have been known for their medicinal properties and world-wide use as spices, aromas, natural preservatives and antioxidant agents since ancient times (4). Among them, *Salvia officinalis* L. is most used in traditional medicine and it is attributed with a large number of pharmacological effects such as antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-nociceptive, antimutagenic, hypoglycemic and hypolipidemic. This herb is traditionally and mainly used for the treatment of mild dyspepsia, hyperhidrosis, age-related cognitive disorders, and throat and skin inflammations (5). S. officinalis and other sages are rich in polyphenols, especially in phenolic acids and flavonoids (4). The majority of phenolic acids in these species are caffeic acid derivatives. Caffeic acid is the structural basis of different plant metabolites, including simple monomers and a variety of oligomers (6). This phenolic acid manifests a variety of pharmacological effects such as antioxidant, antidepressive, antimetastatic, anti-tumor, hepatoprotective, anti-

inflammatory, anti-coagulatory, antimicrobial and photoprotective (7-13). The most important compound in sages is rosmarinic acid, a caffeic acid ester, which has been increasingly studied lately in terms of its pharmacological activities (14). Rosmarinic acid has been proven to express antioxidant, anti-inflammatory, antimutagenic, hepatoprotective, antithrombotic, antiplatelet, anti-angiogenic, antibacterial, antiviral (especially against *Herpes simplex* infections), cancer hemopreventive activity, anxiolytic and astringent effects (15-17).

Salvia aethiopis L. is biennial, rarely perennial plant species that occurs sporadically in Serbia inhabiting dry places, meadows and sandy substrates (18). There is not much information on its traditional use except its use as antiflatulent and reconstituent (19). Scientific literature data show that S. *aethiopis* extracts express antimicrobial, antifungal, antimycobacterial, cytotoxic, antioxidant, anti-cholinesterase and muscarinic receptor activity (20-25). In addition, aethiopinone, an o-naphthoquinone diterpenoid, isolated from roots manifested significant antinociceptive, anti-inflammatory and the bleeding time increasing effects (26).

All these facts point to a great therapeutic potential of this plant species, its extracts and isolated compounds. There is a lack of data on rosmarinic and caffeic acid content in S. *aethiopis* extracts, especially from Serbia, which are certainly, in a high percentage, responsible for their activities. Therefore, the aim of this research is directed to the determination of these compounds in S. *aethiopis* extracts prepared with different solvents. Furthermore, the study defines antioxidant effects of the extracts including the determination of the rosmarinic and caffeic acid impact on their manifestation.

Material and methods

Plant material

The above-ground parts of S. *aethiopis*, Lamiaceae family were collected in the period of full blossom in the wider surrounding area of Niš, Ploče, Serbia. A voucher specimen has been deposited at the Faculty of Science and Mathematics, University of Niš, Department of Biology and Ecology (No. 13198).

Extracts preparation

The plant material was dried at well-ventilated place protected from direct sunlight and then ground to powder. The powdered material was extracted with absolute and 80% methanol, 96%, 80% and 60% ethanol and ethyl acetate (M, M80, E, E80, E60 and EA, respectively), in 1:10 ratio, in an ultrasonic bath for 40 minutes. Final extracts were obtained after filtration and total removal of the solvents in a rotary vacuum evaporator. The ex-tracts were kept in a sealed bottle in a dark place at 4°C before the experiments.

HPLC (High-performance liquid chromatography) determination of rosmarinic and caffeic acid

HPLC analysis was conducted using a method described by Kostic et al. (2015) (1) with some modifications. The compounds were quantified on Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, Calif., U.S.A.) with a diode array detector (DAD) using the analytical column - Purospher STAR RP-18e (150×4.6 mm) with the particle size of 5 μm. The extracts were dissolved in HPLC methanol to a concentration of 10 mg/mL. The obtained methanolic solutions (10 μL) were injected into the system with column flow of 0.7 mL/min. Operating temperature was adjusted and kept at 30ºC. A mobile phase consisted of 0.1% water-trifluoroacetic acid solution (A) and acetonitrile (B) with linear gradient: 0-3 min 5-5% B, 3-32 min 5-28% B, 32-44 min 25-50% B, 44-52 min 50-80% B, 52-54 min 80-90% B, 54-59 min 90-5% B, and 59-60 min 5% B. The quantification of rosmarinic and caffeic acid was performed using calibration curves made with the standards and the final results are expressed as μg of the compound in mg of the extract.

Determination of antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assessment

The antiradical activity of the extracts was evaluated with DPPH method reported by Miladinović et al. (2014) (27). The extracts were initially dissolved in several decreasing concentrations. Forty microliters of the dissolved extract were added to the microtitre plate's well which contained 120 μL of ethanol, immediately followed by 40 μL of DPPH solution (0.2 mg/mL). The plate was placed in a dark location for 30 minutes after short shaking. The absorbances of the wells' contents were measured using ELISA reader (Multiskan Ascent No354, Thermo Labsystems, Finland) at 540 nm. The inhibition of free-radical generation (%) was calculated by the equation:

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

AA is the absorbance of the analyte, AK is the absorbance of the control made up of the solvent and DPPH solution and AS is the absorbance of the solvent. The inhibitory concentration of the extract which neutralized 50% of DPPH radicals (IC_{50}) was calculated from a curve constructed with the percentage of inhibition and the concentration of the extract. Ascorbic acid and α-tocopherol served as positive controls.

β-Carotene/linoleic acid method for anti-lipoper-oxidation assessment

The ability of the extract to reduce lipid peroxidation was assessed with the β-carotene/

linoleic acid method described by Miladinovic et al. (2014) (27). Two milligrams of crystalline β-carotene was dissolved in 10 mL of chloroform and then 2 mL of this solution were pipetted into a round-bottom flask, followed by 364 μL of Tween 20 and 50 μL of linoleic acid. After the total evaporation of chloroform in a vacuum evaporator, 100 mL of oxigenated water was added with shaking. The resulting emulsion was transferred (200 μL) into the wells of microtitre plates which had already contained 25 μL of the extracts dissolved in several decreasing concentrations. A solution without β-carotene was prepared as a blank control. The absorbances of the solution in microtitre plates were immediately measured by ELISA reader (Multiskan Ascent No354, Thermo Labsystems, Finland) at 450 nm after gentle shaking (A_0). Microtitre plates were then placed at 55°C in an incubator for 2 hours. After incubation the absorbances were measured again (A_{120}) at the same wavelength. The percentage of lipoperoxidation inhibition was calculated using the equation (28):

$$\% \text{ inhibition} = [(A_{120}/A_0)]\times 100$$

IC_{50} of the extract was calculated from a curve which represented the dependence of the concentration and % of inhibition. Ascorbic acid and α-tocopherol served as positive controls.

Statistical analysis

The results present mean values of three measurements ± standard deviations. The results were analyzed by one-way analysis of variance (ANOVA). Differences among means were compared with Duncan test at $p < 0.05$ considered as significant. Correlations among components and antioxidant activities of the extracts were analyzed following the Pearson's correlation coefficient method ($p < 0.05$). The software package SPSS 17 (SPSS, Inc., Chicago, IL) was used for statistical analyses.

Results

The results of the extraction yield and the content of rosmarinic and caffeic acid are shown in Table 1. The best extraction yield was noted for the

Table 1. Yields and rosmarinic (RA) and caffeic acid (CA) contents in *Salvia aethiopis* L. extracts

	yield	RA	CA
extract	%	μg/mg	μg/mg
E	3.60	140.24±3.21[a]	1.33±0.03[a]
E80	8.45	231.09±4.11[b]	3.02±0.11[b]
E60	14.05	72.53±1.05[c]	4.39±0.80[c]
M	8.05	222.18±3.01[d]	1.16±0.05[a]
M80	9.20	223.26±2.24[d]	1.99±0.03[d]
EA	3.10	5.89±0.33[e]	1.00±0.01[a]

The results present mean values of three measure-ments ± standard deviations.
Values in the columns with different lower-case letters are significantly different at $p<0.05$.

Table 2. Antioxidant potentials of *Salvia aethiopis* L. extracts estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and *β*-carotene/linoleic acid (BC) systems

	DDPH	BC
extract	μg/ml	μg/ml
E	32.29±1.83[a]	79.54±1.98[a]
E80	24.34±0.56[b]	78.16±0.99[a]
E60	47.83±3.58[c]	37.82±1.45[b]
M	26.58±0.91[a,b]	58.86±4.26[c]
M80	23.79±0.42[b]	41.66±1.64[b]
EA	145.64±7.79[d]	108.29±7.96[d]
positive controls		
ascorbic acid	4.74±0.34[e]	22.95±1.52[g]
α-tocopherol	10.40±1.73[f]	0.15±0.00[h]

The results present mean values of three measurements ± standard deviations.
Values in the columns with different lower-case letters are significantly different at $p<0.05$.

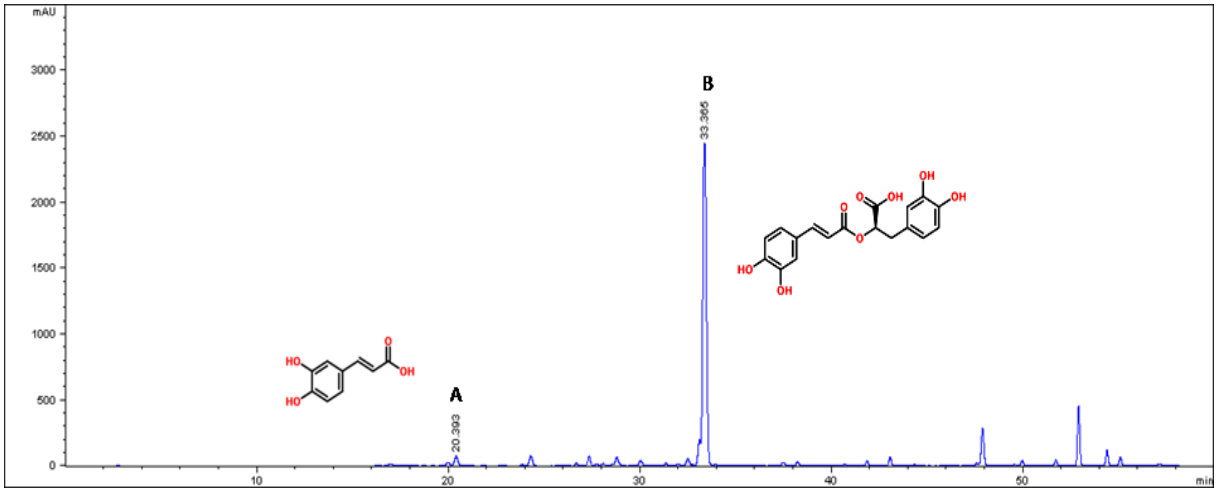

Figure 1. HPLC-chromatogram of the extract E80 of *Salvia aethiopis* L. at 330 nm. Peaks: A – caffeic acid, B – rosmarinic acid

extract E60 made up with the most polar solvent. This extract also contained the highest amount of caffeic acid, while extract E80 contained the greatest quantity of rosmarinic acid (Figure 1). Ethyl acetate was the least efficient in the process of extraction, as well as in the iso-lation of the phenolic acids.

The values of the concentrations of the extracts that inhibited the 50% of free radicals are shown in Table 2. As it is obvious, the extract M80 was the most efficient in DPPH system, immediately followed by E80 and M, while E60 expressed the best antilipoperoxidant activity in β-carotene/linoleic acid method. EA extract was the weakest in both antioxidant methods, as expected. Ascorbic acid and α-tocopherol, positive controls, were uneqaled in free radical reduction.

Discussion

The polarity of a solvent is the main factor for extraction yield and amount of extracted compounds. However, the extraction technique including temperature, time, pH and chemical structure of the components in the plant material are highly contributable (29). In this study, considering the same extraction technique used for all extracts, the main factors for different yields and quantity of the phenolic acids are their chemical structure, the type of used solvents and change of their polarity. The smallest yield was noted in EA extract due to the lowest solvent's polarity. Concentrated and absolute alcohols gave lower yields compared to their aqueous-alcoholic forms. This implies that adding water, by increasing the polarity, provided better extraction yields and that the plant material contained mostly polar compounds.

The literature data show that S. *aethiopis* extracts contain rosmarinic, caffeic, p-coumaric, oleanolic, ursolic and chlorogenic acid. Apigenin, apigenin-7-O-glucoside, luteolin, luteolin-7-3',4'-trimethylether and salvigenin were identified as the representatives of flavonoids (30-32). Veličković et al. (2002) (22) identified volatile compounds of S. *aethiopis* extracts, whereby 1,8-cineol and α-thujone were most present. It is interesting that there are specific compounds, diterepenes, salvipisone and aethiopinone found in the roots, as well as, sesterterpene lactones, salviaethiopisolide and 13-epi- salviaethiopisolide found in aerial parts of the herb (33-35). Chemical compositions of S. *aethiopis* essential oils are well studied whereby α-copaen, β-caryophyllene and germacrene D are the most dominant compounds (36).

This study confirmed the presence of rosmarinic and caffeic acid in S. *aethiopis* in our locality, the surroundings of Niš, Serbia. The content of rosmarinic acid in the extracts ranged from 5.89 μg/mg to 231.09 μg/mg. Ethyl acetate as non-polar solvent extracted the lowest quantity of rosmarinic acid, as expected, and on the other side, E80 was the richest in its amount. Absolute and 80% methanols were highly effective solvents for its isolation, as well. It is interesting that the extract E60, prepared with the most polar solvent, contained a low amount of rosmarinic acid compared to other polar solvents. This suggests that rosmarinic acid is less isolated from plant material when highly polar solvent is used too. Compared to the previous researches the contents of rosmarinic acid in our extracts are definitively the highest. Namely, the ethanolic and methanolic leaves extract of Hungarian S. *aethiopis* contained 1.8 μg/mg and 2.8 μg/mg, respectively (37, 38). Furthermore, methanolic extract of S. aethiopis from Romanian flora contained 1.1189 μg/mg (32). The scope of caffeic acid content in our extracts was 1–4.39 μg/mg. The lowest quantity was determined again in EA extract, but it does not significantly differ from the extract E and M. It is obvious that the addition of water to concentrate ethanol and absolute methanol increased the extraction of caffeic acid, so that its quantity rose in the following order M<E<M80< E80<E60. In the aforementioned extracts of other researchers, the caffeic acid contents were also significantly lower and the values ranged from 0.07-0.1185 μg/mg (32, 37, 38).

The assessment of antioxidant activity was carried out by two complementary methods. DPPH method assessed the ability of the extracts to capture free radicals while β-carotene bleaching model was used to examine their potency to inhibit lipid peroxidation (39). EA extract was the weakest in both model systems, as expected. Extract M80 was the most superior in DPPH system with IC_{50} of 23.79±0.42 μg/ml. On the one hand, E60 was least active in antiradical activity among the extracts prepared with polar solvents, but on the other hand, it had the strongest antilipo-peroxidant activity which is significant from the standpoint of its rosmarinic and caffeic acid content. Statistically significant is the connection between rosmarinic acid and DPPH antioxidant activity ($r = -0.853$, $p < 0.05$). This correlation indicates that rosmarinic acid is highly responsible for antiradical activity, in DPPH system, what other researches established as well (1). In contrast, caffeic acid is partially, but statistically significantly, accountable for antilipoperoxidant activity ($r = -0.423$, $p < 0.05$). Antiradical activity of S. *aethiopis* extracts investigated by other researchers varied. Firuzi et al. (2013) (21) determined poor activity of the S. aethiopis extract with IC_{50} of 237.37 μg/ml, while Tepe et al. (2006) (40) even found the total absence of antiradical activity. Best efficacy was performed by the methanolic extract from Turkey which inhibited 68.72% of free radicals with the concentration of 25 μg/ml (24). Great antilipoperoxidant activity was observed in the extract reported by Tosun et al. (2009), whereby 2 g/l of the methanolic extract inhibited 70.40% of linoleic-free radicals (41).

Rosmarinic and caffeic acid have been widely investigated lately in terms of its antioxidant activities. Rosmarinic acid is able to increase the

oxidative and physical stability of liposomes (42). In addition, it was found that rosmarinic acid protects a phospholipid membrane against oxidative damage by the reduction of radical propagation (43). The production of reactive oxygen species and release of interleukin (IL)-6 are significantly decreased in the presence of rosmarinic acid, and at the same time it can prevent UVB damage of human keratinocytes (44). The antioxidant effect of rosmarinic acid is considered to be involved in the prevention of DNA damage induced by doxorubicin. It is important that it reduces the frequency of micronuclei and does not express genotoxic effects (45). Osakabe et al. (2002) (46) suggests that rosmarinic acid could act as a liver protector due to the scavenging and reducing activities of superoxide or peroxynitirite in mice hepatocytes. Due to the ability to scavenge peroxynitrites, rosmarinic acid may protect against cognitive dysfunction in mice induced by amyloid beta protein which is in direct connection with Alzhemier disease (47). In addition to its neuroprotective effect, it is shown that rosmarinic acid significantly suppresses H_2O_2-induced reactive oxygen species (ROS) formation in human dopaminergic cell line, SH-SY5Y, and rats astrocytes (48, 49). Its antioxidant activity might be responsible for anti-angiogenic potential due to the reduction of ROS-associated expression of vascular endothelial growth factor and release of IL-8 *in vitro* (16). The treatment with rosmarinic acid diminishes pancreatic β-cell dysfunction and glucolipotoxicity-mediated oxidative stress in high-fat diet streptozotocin (STZ)-induced type 2 diabetes in albino rats possibly thanks to its antioxidant effect (50). It is also responsible for the alleviation of the ROS production in human gingival fibroblasts pretreated with lipopolysaccharide (51). Caffeic acid is also an effective antioxidant agent in biochemical reactions. It significantly de-creases the effects of pro-oxidants such as iron, sodium nitroprusside and quinolinic acid which causes increase in the malondialdehyde contents of the brain (52, 53). Caffeic acid inhibits the oxidative stress induced by H_2O_2 and suppresses the IL-8 secretion and its mRNA expression in human intestinal epithelial Caco-2 cells (54). Khan et al. (2012) (55) concluded that caffeic acid attenuated 12-O-tetradecanoyl-phorbol-13-acetate-induced lipid peroxidation in mouse skin along with the expression of nuclear factor kappa B and cyclooxygenase-2 and tumor necrosis factor-α release suggesting its possible anticarcinogenic activity. It has a protective role in ethanol metabolism-induced oxidative damage in SK-Hep-1 cells by blocking ROS production and enhancing antioxidant potentials (56). Caffeic acid may be beneficial in the attenuation of iron nitrilotriacetate- and nickel-induced oxidative damage in the rat kidney and liver, respectively (57, 58). Nardini et al. (1998) demonstrated that this phenolic acid is able to reduce glutathione depletion and lipid peroxidation in U937 human monocytic cells exposed to t-butyl hydroperoxide (59).

Conclusion

Considering the reported results, S. *aethiopis* extracts are the important sources of, primarily, rosmarinic acid along with caffeic acid. The presence of these phenolics in the diet may play an important role in the modulation of oxidative reactions. An extract with particularly high level of rosmarinic acid is E80, while extract E60 contains the largest amount of caffeic acid. All extracts, except ethyl acetate, were superior in antioxidant activities expressions in both models. Therefore, S. *aethiopis* extracts, made up with polar solutions, could be of great importance for usage in the conditions where oxidative reactions have signi-ficant destabilizing and pathological role. Further investigations are recommended for *in vivo* anti-oxidant testing of the extracts, and for the identifi-cation and quantification of other present com-pounds as well.

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SADRŽAJ RUZMARINSKE I KAFENE KISELINE I ANTIOKSIDATIVNI POTENCIJAL EKSTRAKTA BILJNE VRSTE *SALVIA AETHIOPIS* L.

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Aromatične biljne vrste predstavljaju izvor farmakološki aktivnih jedinjenja sa visokim antioksidativni delovanjem. Među njima su *Salvia* L. vrste, žalfije, od davnina poznate širom sveta kao začini, arome, lekovita sredstva, prirodni konzervansi i antioksidansi. Podaci iz literature pokazuju da *Salvia aethiopis* L. ispoljava različite biološke efekte. Cilj ovog istraživanja bio je utvrditi količinu ruzmarinske i kafene kiseline, često zastupljene u žalfijama, u ekstraktima biljne vrste *S. aethiopis,* pripremljeni sa različitim rastvaračima i proceniti njihove antioksidativne efekte. Nadzemni delovi S. *aethiopis* su prikupljeni u periodu cvetanja u okolini Niša, Ploče, Srbija. Biljni materijal je osušen na vazduhu, pulverizovan i ekstrahovan pomoću apsolutnog i 80% metanola, 96%, 80% i 60% etanola i etilacetata (M, M80, E, E80, E60 i EA, redom) u ultrazvučnom kupatilu. Fenolne kiseline su kvantifikovane tečnom hromatografijom visokih performansi, dok je antioksidativni efekat procenjen pomoću dve *in vitro* komplementarne metode: 2,2-difenil-1-pikrilhidrazil (DPPH) i β-karoten/linolne kiseline (BC). Ekstrakt E80 sadržao je najveću količinu ruzmarinske kiseline (231,09±4,11 μg/mg), a E60 je bio najbogatiji u količini kafene kiseline (4,39±0,80 μg/mg). M80 je bio najefikasniji u DPPH testu, dok je E60 izrazio najbolju antilipoperoksidnu aktivnost u BC modelu. Prisustvo značajnih količina ruzmarinske kiseline, uz kafenu kiselinu, i odlična antioksidativna aktivnost, mogu značajno doprineti potencijalnoj upotrebi ekstrakata u različitim patološkim stanjima, posebno u modulaciji oksidativnog stresa. *Acta Medica Medianae 2017;56(3):121-128.*

Ključne reči: *Salvia aethiopis L., ekstrakti, ruzmarinska kiselina, kafena kiselina, antioksidativna aktivnost*