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Antioxidant activity of *Salvia jurisicii* Košanin ethanol extracts

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ABSTRACT: In this study, ethanol extracts (96%, 50%, 30% and 10%) of *Salvia jurisicii* Košanin whole plant and different parts (leaves and stems) were investigated for *in vitro* antioxidant 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) free radical-scavenging activity and total phenolic and flavonoid contents. The largest extract yields were obtained from the 50% and 30% leaf extracts (21.22% and 19.85%, respectively). The 50% and 96% ethanol extracts of whole plants showed the highest activities against DPPH radical (97.1 and 100.6 μg/ml, respectively) whereas leaf extracts were superior in the ABTS test (1.76 and 1.65 mg AAE/g, for 96% and 50%, respectively). Total phenolic and flavonoid contents were ranged in similar orders for the various extracts: 96%>50%>30%>10%. Ethanol extracts of leaves were the richest in phenols and flavonoids, followed by the whole plant and stems. A very strong linear correlation between ABTS activity and phenolic content of extracts was established (r=0.932). The results suggest that the herb *Salvia jurisicii*, particularly the leaves, could be considered as a potential source of natural antioxidants.

Key words: *Salvia jurisicii*, Lamiaceae, DPPH, ABTS, phenolics, flavonoids

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INTRODUCTION

The genus *Salvia* is one of the most widely-spread members of the Lamiaceae family, comprising several species with beneficial healing properties. Among diverse biological activities manifested by different constituents of the essential oil and/or extracts from the genus *Salvia*, an antioxidant effect was obtained in the study of Tosun *et al.* (2009). As the harmful effects of free radicals can be prevented by the intake of antioxidant substances, and synthetic antioxidants may induce some health disorders, many researcher groups have been searching for non-toxic antioxidants from natural sources, especially edible or medicinal plants.

Salvia jurisicii Košanin (Jurisic's cutleaf sage), an endemic species for the central part of the Republic of Macedonia, is a perennial herb inhabiting arid habitats (Hedge 1972). It is an attractive floricultural species due to a basal rosette of feathery foliage and dense flower spikes, but information on its morphological, anatomical, chemical characteristics, and biological effects of its essential oil or extracts is still lacking. The anatomical and micromorphological characteristics of this species were previously investigated by Alimpić *et al.* (2012). Janicsák *et al.* (2010) found a low antioxidant activity, in comparison with ascorbic acid, in aqueous-methanol extracts of *Salvia jurisicii*, one of eleven *Salvia* species studied.

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In the present study, the radical scavenging activities of ethanol extracts of *S. jurisicii* plant parts and concentrations of total phenolics and total flavonoids were determined. In this study, ethanol and water were chosen as extraction solvents because ethanol/water formulations are relatively safe for human consumption, compared with other organic solvents, such as acetone or methanol, frequently used by researchers. Further, ethanol extraction is widely used to obtain crude extracts of phytochemicals from plant materials in the herbal medicine industry for therapeutic applications (Spigno *et al.* 2007; Stagos *et al.* 2012).

The objective of this study was to determine the best ethanol to water ratio for preparing extracts from selected *S. jurisicii* plant parts to optimize their antioxidative activity and phenolic/flavonoid contents against *in vitro* free radicals.

MATERIAL AND METHODS

Chemicals. Methanol, ethanol and distilled water were purchased from Zorka Pharma, Šabac, Serbia. Gallic acid, quercetin, ascorbic acid, 2(3)-t-butyl-4-hydroxyanisole (BHA), 3,5-di-tert-butyl-4 hydroxytoluene (BHT) 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), potassium acetate ($C_2H_3KO_2$), potassium-persulfate ($K_2S_2O_8$), sodium carbonate anhydrous (Na_2CO_3), aluminium nitrate nonahydrate ($Al(NO_3)_3$ x $9H_2O$) and Folin-Ciocalteu phenol reagent were purchased from Sigma Chemicals Co., St Louis, MO, USA. All chemicals were of analytical grade purity.

Plant material. Aerial parts of *Salvia jurisicii* Košanin plants were collected at the end of the flowering period from natural populations in the locality Štip (Macedonia) in July 2011. Plant material was dried and kept in reduced light at room temperature for further processing. voucher specimen was deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade (BEOU; voucher No. 16674).

Preparation of plant extracts. Extracts were prepared of whole plants and leaves and stems, one 5 g sample of each. Plant material was ground in small pieces (2 - 6 mm) in a cylindrical crusher and individually extracted with 50 ml of ethanol (at concentrations of 10%, 30%, 50% and 96%) by maceration for 24h at room temperature (10% w/v). Subsequently, extracts were filtered through a filter paper (Whatman No.1) and evaporated to dryness under reduced pressure with a rotary evaporator (Buchi rotavapor R-114). The resultant crude extracts (Table 1) were stored in the fridge at +4° C for further experiments.

Evaluation of DPPH free radical-scavenging activity. For evaluation of antioxidant activity of extracts, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging method (Blois 1958) with slight modifications was used. This spectrophotometric assay uses stable DPPH radical as the reagent. Stock solutions of dry extracts were prepared in the appropriate solvent (10%, 30%, 50% and 96% ethanol) at a concentration of 1000 μg/ml. Stock extract solutions were diluted with a methanolic solution of DPPH (40 μg/ml) to adjust the final volume of reaction mixture (4 ml) of extract concentrations to 50, 75, 100, 125, 150 μg/ml. Methanol was used as a blank, while methanol with DPPH solution was used as a control. BHA, BHT and ascorbic acid were used as positive controls (standards). Each blank, sample and standard absorbances were measured in triplicate. Absorbance of the reaction mixture was measured after 30 min in the dark at room temperature at 517 nm using the JENWAY 6305UV/Vis spectrophotometer. The decrease of absorption of DPPH radical at 517 nm was calculated using the following equation:

$$\text{Inhibition of DPPH radical (\%)} = [(A_C - A_S)/A_C] * 100\%$$

where A_c is the absorbance of control (without test sample) and A_s is the absorbance of the test samples at different concentrations. IC_{50} values (μg/ml) (concentrations of the test samples and standard antioxidants providing 50% inhibition of DPPH radicals) were calculated from the DPPH absorption curve at 517 nm.

Table 1. The yields of aqueous ethanolic extracts of *Salvia jurisicii.*

Type of extract	% yield of ethanol extract (w/w)			
	96 % ethanol	50 % ethanol	30 % ethanol	10 % ethanol
Whole plant	11.18	19.03	17.38	15.06
Leaves	16.38	21.22	19.85	18.88
Stems	7.44	11.52	9.76	8.56

ABTS assay. The ABTS assay was performed according to the procedure of Miller *et al.* (1993) with some modifications. Fresh $ABTS^+$ solution was prepared 12-16 h before use by dissolving 35 µM of ABTS in 5 ml of 2.46 mM potassium-persulfate, then storing in the dark at room temperature. The $ABTS^+$ solution was dissolved in distilled water to obtain an absorbance for the working solution of 0.700 ± 0.020 at 734 nm. One hundred µl of test samples and/or standard solutions (1 mg/ml) were mixed with 4 ml of diluted $ABTS^+$ solution and incubated for 30 min at 30° C. Absorbance was recorded at 734 nm using a JENWAY 6305UV/Vis spectrophotometer. Distilled water was used as a blank. BHA and BHT dissolved in methanol at 0.1 mg/ml were used as standards. ABTS activity was calculated from an ascorbic acid calibration curve (0-2 mg/l) and expressed as ascorbic acid equivalents per gram of dry extract (mg AAE/g). All experimental measurements were carried out three times were presented as average ± standard deviation.

Determination of total phenolic content. The total phenolic content of *Salvia jurisicii* extracts was measured spectrophotometrically (Singleton & Rossi 1965). The reaction mixture was prepared by mixing 0.2 ml of ethanol extract at 1 mg/ml and 1 ml of 10% Folin–Ciocalteu reagent and after six min, 0.8 ml of 7.5% Na_2CO_3 was added. A blank was prepared to contain distilled water instead of extract. Absorbance was recorded at 740 nm after two h incubation at room temperature using a JENWAY 6305UV/Vis spectrophotometer. The same procedure was repeated with an aqueous solution of gallic acid to construct a calibration curve. Sample phenolic contents were calculated from the standard curve equation and expressed as gallic acid equivalents (mg GAE/g dry extract) averaged from three measurements.

Determination of flavonoid concentration. Flavonoid concentrations of samples were measured spectrophotometrically according to procedure of Park *et al.* (1997). The reaction mixture was prepared by mixing 1 ml of ethanol extract at 1 mg/ml, 4.1 ml of 80% ethanol, 0.1 ml of 10 % $Al(NO_3)_3$ x 9 H_2O, and 0.1 ml 1M CH_3COOK. A blank was prepared to contain 96% ethanol instead of extract. After 40 min incubation at room temperature, absorbance was measured at 415 nm using a JENWAY 6305UV/Vis spectrophotometer. The same procedure was repeated for a 96% ethanol solution of standard antioxidant quercetin to construct a calibration curve. Sample flavonoid concentrations (mg/ml) were calculated from the standard curve equation and expressed as quercetin equivalents (mg QE/g dry extract) averaged from three measurements.

Statistical analysis. All measurements were made in triplicate and data are expressed as average ± standard deviation. Analyses and construction of standard curves were performed using MS Office Excel, 2007.

RESULTS AND DISCUSSION

Yields of extracts. The yields of ethanol extracts obtained from 5 g of dried plant material varied according to the plant part and ethanol:water ratio used for extraction (Table 1). The highest yield was obtained for leaves, especially using 50% and 30% ethanol (21.22 and 19.85%, respectively). The lowest yield was obtained for the 96% ethanol extract of stems (7.44%). Veličković *et al.* (2003) obtained lower yields for *Salvia officinalis* dry ethanol extracts, but of a similar order: 3.5% (flowers) >3.1% (leaves) > 1.2% (stem). The yield of 96% ethanol extracts of fourteen Turkish *Salvia* species ranged from 1.37 to 5.94% (Orhan *et al.* 2013). In our study, the yields of whole plant extracts varied from 11.18 to 19.03%, according to the ethanol:water ratio.

DPPH and ABTS scavenging activity. DPPH and ABTS assays are the most widely used spectrophotometric assays for determination of antioxidant activity. IC_{50} values of ethanol extracts of *S. jurisicii* whole plants and different parts obtained using the DPPH method ranged from 95.81 to 246.39 µg/ml (Table 2). The most active extracts against the DPPH radical were whole plant extracts, especially 50% (95.81µg/ml) and 96 % (97.10 µg/ml), followed by stems and leaves. Leaf and stem extracts showed similar activities, particularly in 30% ethanol (131.39 and 131.13 µg/ml, respectively). Kamatou *et al.* (2010) found a wide range of IC_{50} values for methanol:chloroform (1:1) extracts of 16 South African *Salvia* species (from 1.6 to 74.5 µg/ml using DPPH). Tosun et al. (2009) found that IC_{50} values of methanol extracts of eight *Salvia* species from Turkey ranged from 18.3 to 88.2 µg/ml. In the current study, *Salvia jurisicii* whole plant extract was the most powerful against the DPPH radical. The 96% ethanol extracts showed the strongest activity against the ABTS radical, giving 1.76 (leaves), 1.51 (whole plant) and 1.35 (stems) mg AAE/g of dry extract (Table 3). Other aqueous ethanol extracts were ranged in a similar order, i.e. 50%>30%>10% and leaves>whole plant>stems. Some researchers preferred to express ABTS activity as IC_{50} value (Kamatou *et al.* 2010; Stagos *et al.* 2012) and more frequently as standard equivalents (Trolox, ascorbic acid, etc.). In this study, the ascorbic acid calibration curve was chosen for presentation of results. Re *et al.* (1999) reported that ascorbic acid showed an ABTS activity of 0.99 as Trolox equivalents (mM). To simplify comparison of results, the same concentration for all extracts was applied (1 mg/ml), although previous studies demonstrated a dose and/or time-dependent reducing ability (Re *et al.* 1999; Asadi *et al.* 2010). Asadi *et al.* (2010) showed that extracts of six Iranian *Salvia* species at a concentration of 100 µg/ml had activities ranging from about 15 to 55 mg of Trolox equivalents of plant extracts. Stagos *et al.* (2012) found that *Salvia* species were the most active against free radicals among three selected Lamiaceae genera (*Salvia, Sideritis,*

Table 2. DPPH activity of *Salvia jurisicii* aqueous ethanol extracts. Results are expressed as IC_{50} (μg/ml).

Salvia jurisicii extracts	96% ethanol	50% ethanol	30% ethanol	10% ethanol
Whole plant	97.10	95.81	112.27	176.48
Leaves	125.36	113.10	131.39	210.19
Stems	100.64	106.82	131.13	246.39
BHA	13.37			
BHT	17.94			
Ascorbic acid	5.12			

Table 3. ABTS activity of *Salvia jurisicii* aqueous ethanol extracts. Results are expressed as mg AAE/g of dry extract.

Salvia jurisicii extracts	96% ethanol	50% ethanol	30% ethanol	10% ethanol
Whole plant	1.51 ± 0.02	1.42 ± 0.04	1.29 ± 0.05	0.98 ± 0.01
Leaves	1.76 ± 0.01	1.65 ± 0.02	1.46 ± 0.03	1.23 ± 0.03
Stems	1.35 ± 0.02	1.18 ± 0.02	1.14 ± 0.02	0.77 ± 0.01
BHA (0.1 mg/ml)	2.82 ± 0.01			
BHT (0.1 mg/ml)	2.75 ± 0.02			

Table 4. Total phenolic content (TPC) and total flavonoid content (TFC) of aqueous ethanol extracts of different *Salvia jurisicii* parts expressed in terms of gallic acid equivalents (mg GAE/g dry extract) for total phenols and quercetin equivalents (mg QE/g dry extract) for flavonoids. Values are presented as mean ± standard deviation.

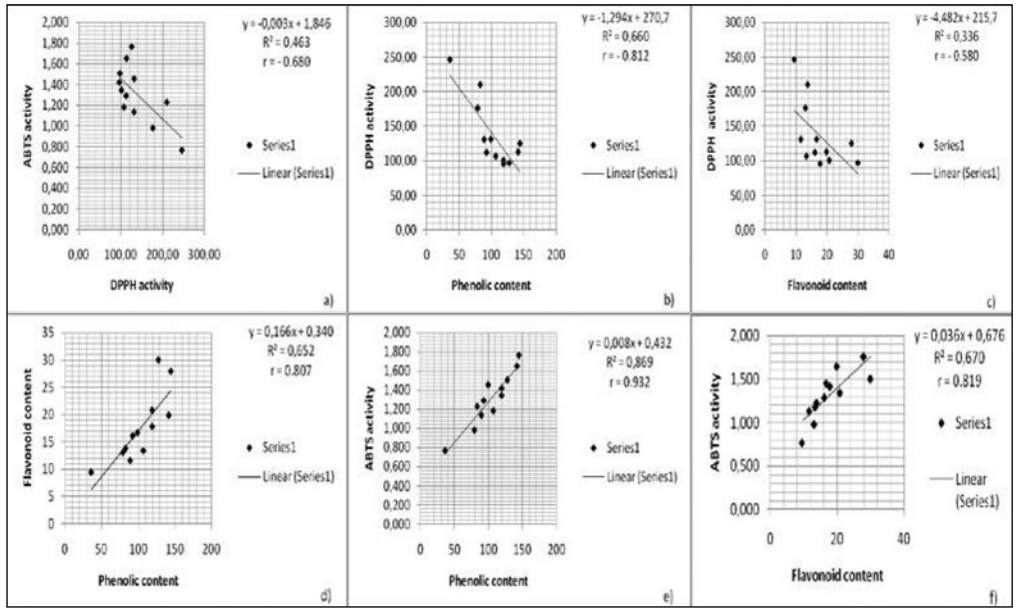
Salvia jurisicii extracts	96% ethanol		50% ethanol		30% ethanol		10% ethanol	
	TPC	TFC	TPC	TFC	TPC	TFC	TPC	TFC
Whole plant	127.71 ± 0.92	29.95 ± 0.55	119.07 ± 0.60	17.79 ± 0.23	92.60 ± 0.40	16.10 ± 0.19	79.10 ± 0.31	13.05 ± 0.35
Leaves	144.60 ± 1.18	27.87 ± 0.47	141.65 ± 1.90	19.85 ± 0.28	99.34 ± 0.67	16.67 ± 0.31	82.97 ± 1.00	13.77 ± 0.33
Stems	119.02 ± 0.71	20.77 ± 0.41	106.97 ± 1.10	13.38 ± 0.54	89.13 ± 1.50	11.59 ± 0.35	35.82 ± 0.80	9.44 ± 0.16

Mentha) with values of 21 μg/ml for DPPH and 25 μg/ml for the ABTS test. IC_{50} values of sixteen *Salvia* species encompassed a wide range (11.9-69.3 μg/ml) (Kamatou *et al.* 2010).

Total phenolic content and flavonoid concentration. Total phenolic contents of *S. jurisicii* ethanol extracts differed significantly depending on plant part and ethanol:water ratio used for extraction (Table 4). The richest in phenolics were 96 % ethanol extracts of leaves (144.60 mg GAE/g), whole plant (127.71 mg GAE/g) and stems (119.02 mg GAE/g), followed by 50%, 30% and 10% ethanol extracts, respectively.

The range of phenolic contents of ethanol extracts *S. jurisicii* in our research (35.82-144.60 mg GA/g) was much greater than phenol contents of methanol extracts of eight

Chart 1 a-f. Linear correlation coefficients between DPPH and ABTS scavenging activities and total phenolic and flavonoid contents.



Salvia species collected in Turkey, which varied from 50.3 to 101.2 mg GA/g (Tosun *et al.* 2009). Our results were very similar to the range (45 – 211 mg GAE/g) found for total phenol methanol/chloroform extracts of 16 South African *Salvia* species (Kamatou *et al.* 2010). Total phenol content of ethanol extracts for 14 Turkish *Salvia* species ranged from 57.10 to 218.09 mg GAE/g and 8.29-108.78 mg QE/g for flavonoids (Orhan *et al.* 2013).

Flavonoid concentrations ranged from 29.95 mg QE/g) in the 96% ethanol extract of leaves to 9.44 mg QE/g in the 10% ethanol extract of stems. Generally, the largest contents of flavonoids were measured in 96%, followed by 50%, 30% and 10% ethanol extracts. Plant parts differed in flavonoid contents, being richest in leaves and lowest in stems).

Correlation between antioxidant activity and total phenolic and flavonoid content. Correlation coefficients were calculated for relationships between ABTS, DPPH activity, total phenolic and total flavonoid contents and results are shown in Chart 1 a-f. The negative correlation shown in Chart 1a-c between DPPH activity and other parameters, is due to expressing DPPH activity via IC_{50} value (μg/ml). DPPH scavenging activity was more strongly correlated with total phenolic (r=-0.812) then flavonoid content (r=-0.580). ABTS activity was strongly and positively correlated with total phenolics (r=0.932, Chart 2e). The two antioxidative activity assays were weakly, but significantly correlated (r=-0.680, Chart 2a). Phenolic and flavonoid contents were moderately correlated (r=0.807, Chart 1d), significant at $p<0.01$. Similar results for other *Salvia* species were obtained by Asadi *et al.* (2010), Kamatou *et al.* (2010) and Stagos *et al.* (2012).

CONCULUSIONS

Ethanol extracts, especially 96% and 50%, obtained from whole plants and separate parts of *Salvia jurisicii* showed *in vitro* antioxidative activity against DPPH and ABTS free radical. Leaf extracts exhibited high contents of phenols and flavonoids. A strong linear correlation between ABTS scavenging activity and total phenolic content was established. The results of this study revealed the variability and importance of separate examination of phenol composition and antioxidative potential of plant parts and comparison to the whole plant providing a significant contribution to medicinal plant study and their pharmacological applications.

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REZIME

Antioksidativna aktivnost etanolnih ekstrakata vrste *Salvia jurisicii* Košanin

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U ovom radu je ispitivana *in vitro* DPPH i ABTS antioksidativna aktivnost kao i sadržaj ukupnih fenola i flavonoida etanolnih ekstrakata (96%, 50%, 30% i 10%) *Salvia jurisicii* Košanin dobijenih iz celih biljaka i pojedinih biljnih delova (listovi i stabla). Najveći prinosi su dobijeni kod 50% i 30% ekstrakta listova (21.22% i 19.85%). 50% i 96% etanolni ekstrakt celih biljaka su pokazali najbolju aktivnost u DPPH testu (97.10 i 100.64 μg/ml) za razliku od ABTS testa gde su najaktivniji bili 96% i 50% ekstrakti listova (1.763 i 1.651 mg AAE/g, respektivno). Etanolni ekstrakti listova su se pokazali najbogatijim po sadržaju flavonoida i fenola, a zatim cele biljke i stabla. Utvrđena je veoma jaka linarna korelacija između ABTS aktivnosti i sadržaja fenola ekstrakata (r=0.932). Dobijeni rezultati sugerišu da biljka *Salvia jurisicii*, a posebno njeni listovi, mogu biti značajan potencijalni izvor antioksidanasa prirodnog porekla.