Antioxidant Activity of Four Endemic *Stachys* Taxa

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Methanol extracts of aerial flowering parts of four endemic *Stachys* taxa: *S. anisochila* Vis. et Pančić, *S. beckeana* Dörfler & Hayek, *S. plumosa* Griseb. and *S. alpina* L. ssp. *dinarica* Murb. were investigated on their antioxidant activity. The extracts were studied for total antioxidant activity (TAA), along with 1,1-diphenyl 2-picryl hydrazyl (DPPH) and OH radical scavenging activity, and lipid peroxidation (LP). High correlations between total phenolics content, TAA and scavenging DPPH radical indicate that polyphenols are the main antioxidants. All *Stachys* extracts, with the exception of *S. plumosa*, exhibited high anti-DPPH activity (IC_{50}<50 μg/ml). In concentration range from 6.25 to 50 μg/ml, all extracts scavenged OH radical above 40%, with maximal inhibitions for *S. anisochila*, *S. alpina* ssp. *dinarica* and *S. beckeana* extracts of 50.22%, 50.94% and 64.97%, respectively. Only *S. plumosa* extract achieved maximal activity of 60.67% at 100 μg/ml. As for LP, IC_{50} values for *S. beckeana* and *S. alpina* ssp. *dinarica* extracts were 25.07 and 49.00 μg/ml, respectively, while *S. anisochila* and *S. plumosa* extracts did not reach 50% of LP inhibition.

Key words *Stachys*; polyphenol; antioxidant activity; 1,1-diphenyl 2-picryl hydrazyl (DPPH); hydroxyl radical; lipid peroxidation

Free radicals are constantly formed in the human body, but defense mechanisms such as enzymes (glutathione peroxidase, catalase, superoxid dismutase), glutathion, ferritin and others, neutralise them and maintain the balance.[1)] Large production of some free radicals in the organism, particularly reactive oxygen species (ROS) and their high activity leads to oxidative stress, condition in which endogenous antioxidant mechanisms are insufficient for scavenging ROS. Oxidative stress is considered to be substantial, if not crucial, in initiating and developing of many conditions and diseases of modern time: inflammation, autoimmune diseases, cataract, cancer, Parkinson's disease, artherosclerosis and ageing.[2)]

Having all that in mind, it is reasonable to believe that exogenous antioxidants could play important role in preventing oxidative damage in cells and tissues, by means of scavenging ROS. Vitamin C, tocopherols, carotenoids, some synthetic substances (BHA, BHT) have been evidenced as antioxidants and are widely used in many areas of human life. Since synthetic antioxidants have shown some toxic effects, investigations of antioxidants are now focused on naturally occurring substances, especially plant polyphenols. This large group of second plant metabolites includes: flavonoids, phenolic acids, tannins and others. In spite of large structure diversity they all share the same chemical pattern—one or more phenolic groups, for which they react as hydrogen donors and in that way neutralize ROS. Many studies have proven antioxidant and free radical scavenging activity of various polyphenols.[3)] It was comparable with classic antioxidants, and in some cases this activity was much higher. Polyphenols also possess many biological effects and these are generally attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals.[4,5)]

Stachys species are traditionally used in different conditions: headache, neuralgia, nervous conditions, as tonic at dyspepsia and for treating wounds and skin inflammation,[6)] as astringent and antidiarrheal.[7)] In pharmacological studies *Stachys* species showed variety of effects: anti-inflammatory,[8,9)] antibacterial,[10,11)] anti-nephritic[12—14)] and anxiolitic.[15)] Methanol extract of *S. spruneri* showed antioxidant activity similar to α-tocopherol,[16)] while ethanol extract of *S. sylvatica* had low anti-ABTS activity.[17)] Phytochemical studies of *Stachys* species revealed the presence of several secondary plant metabolites: different polyphenols (flavonoids, tannins, phenolic acids, phenylethanoid glycosides), iridoids, terpenoids and sterols.[8,9,18)]

This work deals with antioxidant activity of four Balkan endemic *Stachys* taxa: *S. anisochila* Vis. et Pančić, *S. beckeana* Dörfler & Hayek, *S. plumosa* Griseb. and *S. alpina* L. ssp. *dinarica* Murb. All of them are perennial, herbaceous plants. *S. beckeana* is endemic Illyrian (Dinaric) species, which inhabits Dinaric Mountains of Balkan Peninsula (N Albania, Montenegro, Herzegovina, S Bosnia). *S. anisochila* is also Illyrian endemic (W Serbia, Bosnia, Herzegovina and Albania; data for Bulgarian flora are doubtful and still unchecked). In contrast to *S. beckeana* it inhabits lower mountain areas and gorges in the north. Both species belong to very polymorphic *S. recta* L. complex. *S. plumosa* is endemic Moesian-Scardo-Pindhic species and grows on dry pastures and mountain rocks in central and southeastern parts of Balkan Peninsula (SE Serbia, Macedonia, W Bulgaria and N & C Greece).[19)] *S. alpina* belongs to the polymorphic *S. germanica* L. complex. *S. alpina* ssp. *dinarica* Murb. is endemic for Balkan Peninsula (Croatia, Bosnia and Herzegovina, Montenegro, Serbia and SW Bulgaria). In contrast to the typical subspecies, it mostly grows at higher altitudes, on mountain pastures and meadows.[19—21)]

MATERIALS AND METHODS

Chemicals 1,1-Diphenyl 2-picryl hydrazyl (DPPH), Folin-Ciocalteu reagent and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.); thiobarbituric acid (TBA) from Merck (Darmstadt, Germany); trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and L-ascorbic acid from Lachema

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(Neratovice, Czech Republic); all other reagents used were of analytical grade.

Plant Material and Extraction Aerial flowering parts of plants were collected from natural populations as follows: *S. anisochila* in gorge of Beli Rzav (W Serbia) in June 2003; *S. beckeana* from Mt. Durmitor (Montenegro) in August 2003; *S. plumosa* in Jelašnička klisura gorge (SE Serbia) in June 2002; *S. alpina* ssp. *dinarica* on Mt. Jahorina (Bosnia and Herzegovina) in July 2004. Voucher specimens are deposited in Herbarium collection of Natural History Museum in Belgrade (BEO—ko620041/4, ko820033/6, ko320025/6 and ko720049/83, respectively). Plant material was air dried at room temperature and finely grounded. Each sample was bimacerated with chloroform (3 and 2 d; plant material : solvent ratio=1 : 7). The marc was further extracted in the same manner with methanol. Solvent was evaporated under reduced pressure and obtained dry methanol extracts were used for all investigations.

Thin-Layer Chromatography Fifty microliters of each extract dissolved in methanol (1%, w/v) was applied on silica gel plates (Merck) and developed in system ethyl acetate–acetic acid–formic acid–water (100 : 11 : 11 : 27, v/v/v/v). Flavonoids were detected by spraying with 1% 2-aminoethyldiphenyl borate solution in methanol followed by 5% poly-ethylene glycol 4000 in absolute ethanol at 365 nm.[22] DPPH test performed directly on TLC plates (0.2% DPPH in methanol used as spray reagent) revealed contributions to the antioxidant activity of different compounds separately.[23,24]

Determination of Total Phenolics Total phenolics were determined using Folin-Ciocalteu reagent as previously described,[25] with slight modifications. 100 μl of the extract (1 mg/ml) dissolved in methanol was mixed with 750 μl of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min; 750 μl of Na_2CO_3 (60 g/l) solution was added to the mixture. After 90 min the absorbance was measured at 725 nm. Results were expressed as catechin equivalents.

FRAP Assay Total antioxidant activity (TAA) was investigated using Ferric Reducing Antioxidant Power (FRAP) assay, in which reductants in the sample reduce Fe^{3+}-TPTZ complex, present in stoichiometric excess, to the blue colored ferrous form, with an increase in absorbance at 593 nm.[26,27] FRAP reagent was freshly prepared by mixing 25 ml acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ solution (10 mM TPTZ in in 40 mmol/l HCl) and 2.5 ml $FeCl_3$ (20 mM) water solution. 150 μl of each sample (1 mg/ml) dissolved in methanol was added in 4.5 ml of FRAP reagent, stirred and after 5 min absorbance was measured at 593 nm, using FRAP working solution as blank. Calibration curve of ferrous sulfate (100—1000 μmol/l) was used, and results were expressed in μmol Fe^{2+}/mg dry weight extract. The relative activity of the samples was compared to L-ascorbic acid.

DPPH Radical Assay All extracts were dissolved in methanol. An aliquot of this solution was mixed with 1 ml of 0.5 mM DPPH in methanol, and final volume adjusted up to 5 ml. Final concentrations of the extracts were in a range from 5—200 μg/ml. Mixtures were virgously shaked and left 30 min in dark. Absorbance was measured at 517 nm using methanol as blank. 1 ml of 0.5 mM DPPH dilluted in 4 ml of methanol was used as control.[24] Inhibition of DPPH radical was calculated using the equation: $I(\%)=100\times(A_0-A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the test compound), and A_s is the absorbance of the tested sample. The IC_{50} value represented the concentration of the extract that caused 50% inhibition.

2-Deoxyribose Assay The 2-deoxyribose method was used for determining the scavenging effect on hydoxyl radical. Investigated extracts were applied in different concentrations (6.25—200 μg/ml). Each reaction mixture contained the following reagents, in the final concentrations stated: $FeCl_3$ (100 μM), EDTA (100 μM), H_2O_2 (2.2 mM), 2-deoxyribose (2.5 μM), L-ascorbic acid (100 μM). Phosphate buffer (pH=7.4, I=0.1) was added up to a final volume of 4 ml. The mixtures were incubated 1 h at 37 °C, then 1 ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH and 1 ml of 2.8% (w/v) trichloroacetic acid (TCA) were added in each mixture and heated 15 min at 100 °C. After cooling on ice absorbance was measured at 532 nm.[28] Inhibition of 2-deoxyribose degradation in percents was calculated in the following way: $I(\%)=100\times(A_0-A_s)/A_0$, where all symbols have the same meaning as in DPPH radical assay.

TBA Test Lipid peroxidation (LP) was measured using preparation of liposomes containing 0.03 g lecithin/ml. Liposomes were prepared from the commercial preparation "Lipotech 10", which contains 10% of lecithin, diluting it with distilled water in ultrasonic bath for 30 min. Reaction mixture contained 20 μl $FeSO_4$ (0.075 M), 50 μl of liposome suspension, 10 μl of extract dissolved in MeOH in different concentrations (0.5—10%, w/v), 20 μl of L-ascorbic acid (0.1 M) and phosphate buffer (pH=7.4, I=0.1) up to a final volume of 4 ml. Samples were incubated 1 h at 37 °C. Then, 0.2 ml of EDTA (0.1 M) and 1.5 ml of TBA reagent (3 g TBA, 120 g TCA and 10.4 ml $HClO_4$ in 800 ml of distilled water) were added in each sample and heated for 15 min at 100 °C. After cooling on ice, samples were centrifugated for 10 min (3000 rpm) and absorbance of supernatant was measured at 532 nm.[29] Inhibition of LP was calculated the same way as described in DPPH radical assay.

Statistical Analysis One way analysis of variance was used to compare the data, and the values were considered statistically significant at $p<0.05$.

RESULTS

Total phenolics content and FRAP values for investigated extracts have shown in Table 1. *S. anisochila* and *S. beckeana* extracts had the highest polyphenols content (192.16 and 189.67 μg catechin equivalent/mg), as well as the highest TAA (1.87 and 1.83 μmol Fe^{2+}/mg, respectively). *S. plumosa* extract had the least polyphenols (65.63 μg catechin equiva-

Table 1. Total Phenolics Content and FRAP Values for *Stachys* Extracts

Sample	Total phenolics[a]	FRAP value[b]
S. anisochila	192.16	1.87
S. beckeana	189.67	1.83
S. plumosa	65.63	0.46
S. alpina ssp. *dinarica*	141.53	1.36
L-Ascorbic acid		7.41

a) Expressed as μg catechin equivalent/mg dry weigh extract. *b*) In units μmol Fe^{2+}/mg dry weigh extract.

lent/mg) and had minimum of TAA (0.46 μmol Fe^{2+}/mg). Correlation between polyphenols content and antioxidant potential of the extracts was found to be high (r=0.999) (Fig. 1). All extracts had TAA lower than L-ascorbic acid used as standard (7.41 μmol Fe^{2+}/mg).

In DPPH assay all extracts showed concentration dependent activity. Concentrations at which etxracts decrease DPPH radical by 50% (IC_{50} values) were 17.90 μg/ml, 20.90 μg/ml, 26.14 μg/ml and 101.61 μg/ml for *S. anisochila*, *S. beckeana*, *S. alpina* ssp. *dinarica* and *S. plumosa* extracts, respectively (Table 2). IC_{50} value for L-ascorbic acid, used as standard, was 4.09 μg/ml. Results of DPPH radical assay were also correlated with total phenolics and the results obtained in the FRAP assay (Fig. 2).

Investigated extracts scavenged OH radical in concentration dependent manner (Table 3). In concentration range from 6.25 to 50 μg/ml, all extracts inhibited 2-deoxyribose degradation above 40%, with maximal inhibitions for *S. anisochila*, *S. alpina* ssp. *dinarica* and *S. beckeana* extracts of 50.22%, 50.94% and 64.97%, respectively. Only *S. plumosa* extract achieved maximal activity of 60.67% at 100 μg/ml. In higher concentrations for all extracts lower activity was observed. IC_{50} of quercetin, used as reference compound, was 3.1 μg/ml.

Inhibition of lipid peroxidation (LP) was also concentration dependent (Table 4). In applied concentration range (from 12.5 to 250.0 μg/ml), IC_{50} values for *S. beckeana* and *S. alpina* ssp. *dinarica* extracts were 25.07 and 49.00 μg/ml, respectively, while *S. anisochila* and *S. plumosa* extracts did not reach 50% of LP inhibition. Quercetin, used as a standard, reached 50% of LP inhibition at 0.75 μg/ml.

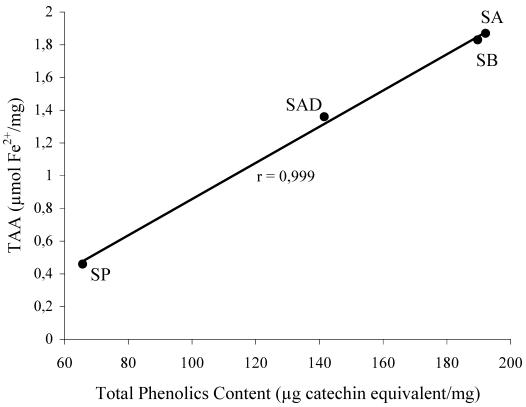

Fig. 1. Values of Total Phenolics Content and Total Antioxidant Activity (FRAP Values) of *Stachys* Extracts

SP: *S. plumosa*; SAD: *S. alpina* ssp. *dinarica*; SB: *S. beckeana*; SA: *S. anisochila*. Results show strong correlation (r=0.999) between the two parameters.

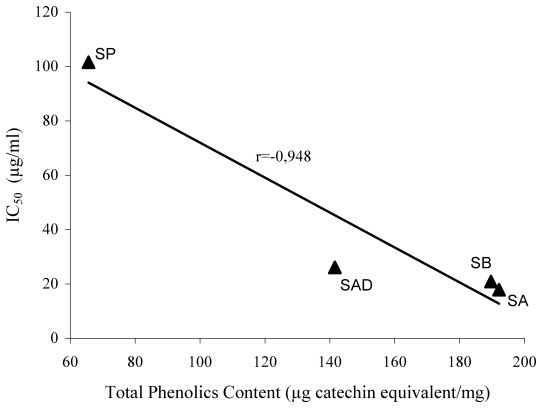

Fig. 2. Values of Total Phenolics Content and DPPH Scavenging (IC_{50}) of *Stachys* Extracts

SP: *S. plumosa*; SAD: *S. alpina* ssp. *dinarica*; SB: *S. beckeana*; SA: *S. anisochila*. Results show good correlation (r=−0.948) between the two parameters.

Table 2. Scavenging Effect (% Inhibition) of *Stachys* Extracts Obtained in DPPH Radical Assay

Concentration of the extracts (μg/ml)	*S. anisochila*	*S. beckeana*	*S. plumosa*	*S. alpina* ssp. *dinarica*
5	19.71±1.67	12.88±1.12	6.22±0.02	6.82±1.33
10	31.97±0.95	22.76±0.08	7.80±0.63	18.18±0.49
20	54.56±1.63	47.87±0.74	13.24±0.02	35.57±0.35
50	95.33±0.16	91.66±1.57	25.73±0.35	91.60±0.07
100	96.24±0	95.45±0.08	49.30±0.18	94.47±0.42
200	96.13±0.04	95.16±0.17	87.45±1.06	—
IC_{50}	17.90	20.90	101.61	26.14

Values are expressed as mean of three replications±S.D.

Table 3. Scavenging Effect (% Inhibition) of *Stachys* Extracts Obtained in 2-Deoxyribose Assay

Concentration of the extracts (μg/ml)	*S. anisochila*	*S. beckeana*	*S. plumosa*	*S. alpina* ssp. *dinarica*
6.25	43.50±2.55	62.64±0.35	46.87±0.11	50.23±1.57
12.5	48.60±1.72	64.97±0.01	52.97±1.36	50.94±0.63
25	50.22±1.50	60.99±2.02	54.25±2.13	49.63±2.21
50	47.60±3.90	58.02±1.16	58.43±1.20	45.77±3.24
100	37.70±3.76	48.59±0.12	60.67±1.25	35.53±1.64
200	19.70±3.26	39.17±1.99	49.44±1.48	22.41±1.52

Values are expressed as mean of three replications±S.D.

Table 4. Inhibitory Effect (% Inhibition) of *Stachys* Extracts on Lipid Peroxidation (LP) in Liposomes

Concentration of the extracts (μg/ml)	*S. anisochila*	*S. beckeana*	*S. plumosa*	*S. alpina* ssp. *dinarica*
12.5	21.68±3.01	41.35±1.74	30.49±2.12	4.64±0.82
25.0	17.05±1.22	48.73±1.94	42.46±0.29	18.72±2.21
62.5	46.02±3.77	50.70±0.70	48.73±0.68	60.49±4.49
125.0	37.13±1.13	53.63±1.39	36.60±0.75	69.24±2.26
250.0	41.47±3.27	58.81±0.03	14.71±2.98	43.35±4.25
IC_{50}	—	25.07	—	49.00

Values are expressed as mean of three replications±S.D.

DISCUSSION

In previous investigations of *Stachys* species, presence of various polyphenol compounds was reported. In methanol and ethanol extracts of aerial parts of investigated four species were identified 7-*O*-glycosides of: apigenin and luteolin (*S. plumosa*),[31] isoscutelarein (*S. beckeana*, *S. anisochila*, *S. plumosa*, *S. alpina*),[31—34] hypolaetin (*S. beckeana*, *S. anisochila*, *S. alpina*),[31—33] and chrysoeriol (*S. plumosa*).[31] In *S. plumosa* phenylethanoid glycosides acteoside, martinoside and forsithoside B were found.[34] Caffeic, sinapic, protocatechuic, chlorogenic and rosmarinic acids were identified in *S. alpina* extract.[35]

Some of these compounds were assesed on their antioxidant activity earlier. Acteoside was found to be strong antioxidant, possessing activity comparable to the synthetic antioxidant BHT and clearly superior to natural α-tocopherol.[36] Martinoside[36] and forsithoside B[37] were also strong DPPH scavengers. Phenolic acids exhibited strong DPPH scavenging activity in the following order: rosmarinic>caffeic>chlorogenic>α-tocopherol>ferulic acid>BHT.[38] Isoscutellarein 7-*O*-glucoside, along with rosmarinic acid, caused high activity of *Rosmarinus officinalis* leaf water extract against linoleic acid peroxidation.[39] 8-*O*-Glucuronides of isocutellarein and hypolaetin possess higher anti-DPPH activity than kempferol.[40] Luteolin was also found to be an excellent inhibitor of Fe^{3+}-catalyzed liposome peroxidation, superior in comparison to TBHQ.[41]

All investigated *Stachys* extracts exhibited substantial antioxidant activity. High correlations between total phenolics content, TAA and scavenging DPPH radical (Figs. 1, 2, respectively) indicate that polyphenols present in these extracts are the main antioxidants. This is in agreement with previous findings[37,42,43] and results of TLC analysis obtained in this work. Namely, TLC chromatograms of all four extracts have shown presence of several polyphenol compounds, identified as flavonoids and phenolic acids. With DPPH reagent few yellow spots appeared immediately after the TLC plate had been sprayed, while others revealed during 30 min period and later. Comparison of obtained chromatograms showed that main spots, identified as flavonoids, are in fact the most potent scavengers of DPPH.

Though inhibition of DPPH radical was obtained in higher concentrations comparing to L-ascorbic acid, all investigated extracts, with the exception of *S. plumosa*, exhibited high anti-DPPH activity ($IC_{50}<50\ \mu$g/ml).[30]

As for inhibition of OH radical and LP, generally all extracts exhibited substantial scavenging activity, though weaker than quercetin used as standard. These effects were more pronounced at lower concentrations of the extracts applied, giving inverted U-shaped concentration–response curve. This could be explained with pro-oxidative effects, phenomena observed for plant extract and polyphenols earlier.[5,45] In addition, differences among extracts could be also explained by differences in content and type of polyphenols present in investigated extracts, considering complex mechanisms of OH and hydroperoxyl radicals generation.

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