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Content of polyphenolic constituents and antioxidant activity of some *Stachys* taxa

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Abstract

Seven Croatian *Stachys* taxa (*S. alpina*, *S. officinalis*, *S. palustris*, *S. recta* subsp. *recta*, *S. recta* subsp. *subcrenata*, *S. salviifolia* and *S. sylvatica*) have been investigated in order to determine their content of biologically active compounds (polyphenols, tannins, phenolic acids and flavonoids) as well as their antioxidant activity. All taxa tested had a high content of total polyphenols, medium content of total phenolic acids, and a rather low content of tannins and flavonoids. The total phenolic acids content correlated significantly with total polyphenols content and the content of polyphenols unadsorbed on hide powder. A low correlation between plant phenolic acids and tannins was observed. Methanolic, ethanolic and dichloromethane extracts were investigated using DPPH, lipid peroxidase and xanthine oxidase assays. The extracts showed no inhibitory effects against lipid peroxidation and xanthine oxidase, but they had the ability to scavenge DPPH. The most effective in the DPPH assay were methanolic extracts of *S. recta* sub. *recta* and *S. palustris* whose radical scavenging activity was higher then that of the reference rutin. A low correlation between radical scavenging capacities of extracts with total flavonoids content was observed. The results indicate that investigated *Stachys* taxa exhibit potent antiradical activity and therefore could be a potential material for extracting free radical scavengers.

Keywords: *Stachys*; Polyphenols; Tannins; Phenolic acids; Flavonoids; DPPH; Lipid peroxidase; Xanthine oxidase

1. Introduction

Stachys L., is one of the largest genera of the family *Lamiaceae*. Plants of this genera have been used in folk medicine for centuries to treat genital tumors, sclerosis of the spleen, inflammatory diseases, cough and ulcers (Hartwell, 1982).

Recent investigations on the antioxidant activity of herbs that are used for treating cancer, show that polyphenols are the carriers of these properties (Agrawal, 1989; Chen & Ho, 1997; Kähkönen et al., 1999; Ferrari, 2000; Bors, Michel, & Stettmaier, 2001; Wei & Shiow, 2001; Ming et al., 2002; Yizhong, Qiong, Mei, & Harold, 2004). Phenolic compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because they are stable radical intermediates (Cuvelier, Ruchard, & Berset, 1992; Maillard, Soum, Boivia, & Berset, 1996). Probably the most important natural phenolics are flavonoids because of their broad spectrum of chemical and biological activities including radical scavenging properties (Agrawal, 1989; Kähkönen et al., 1999). Chen and Ho (1997) stated that phenolic acids show high scavenging DPPH potential. Previous investigations of *Stachys* taxa have shown the presence of flavonoids and phenolic acids (Bilušić Vundać, Maleš, Plazibat, Golja, & Cetina-Čižmek, 2005), indicating that these plants could possess antioxidant activity. The goal of this work was to determine the content of all polyphenols present and to find a correlation between them and antioxidant activity.

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2. Materials and methods

2.1. Chemicals and analytical instruments

All solvents used were of analytical grade and obtained from E. Merck (Darmstadt, Germany). Fisetin was purchased from C. Roth (Karlsruhe, Germany; purity >97%).

$FeCl_3 \times 4H_2O$, $FeCl_2 \times 4H_2O$ and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Fluka (Buchs, Switzerland). All other chemicals were purchased from Sigma (St. Louis, MO, USA). The spectrophotometric measurements were carried out using a Carry 300 Scan spectrophotometer (Varian, USA).

2.2. Plant material

The aerial parts of ten plants in full flower from the *Stachys* taxa investigated were collected from June until Semptember 2004. The plant material was air-dried at room temperature (20 ± 2 °C). Voucher specimens (collection numbers 807.1–807.7) were deposited at the Herbarium Croaticum (ZA), Department of Botany, Faculty of Science, University of Zagreb, Croatia. Detailed information about the plant material is presented in Table 1.

2.3. Extraction

Five grams of herb was ground into powder and extracted with MeOH, EtOH and CH_2Cl_2 (50 ml) by magnetic stirring (6000 rpm) for 12 h at room temperature. The extracts were filtered through filter paper (∅120 mm; Schleicher & Schuell GmbH, Germany), the solvents evaporated under reduced pressure and the residues freeze-dried. The extracts were sealed in glass bottles and stored at +4 °C until use.

2.4. Determination of total polyphenols, polyphenols unadsorbed on hide powder and tannins

The determination was performed by the spectrophotometric method with phosphorus tungstic acid according to the Yugoslav Pharmacopoeia (Ph. Yug. 5., Vols. 1–3, 2001). The results were calculated with regard to the dry matter.

All the extraction and dilution procedures were performed with light protection. Stock solution was prepared from 0.75 g of powderd sample mixed with 150 ml of water, treated in a water bath at 100 °C for 30 min, then cooled and water added up to 250 ml followed by filtration. Total polyphenols were determined from a 5 ml aliquot of the stock solution diluted to 25 ml. An aliquot of 5 ml of the formerly prepared solution was mixed with 1 ml phosphorus tungstic acid and 50 ml (150 g/l) of sodium carbonate solution. The adsorption was measured after 2 min at 715 nm (A_1) using water for compensation. Polyphenols unadsorbed on hide powder were determined from 10 ml of stock solution by addition of 0.1 g hide powder vigorously mixed at room temperature (20 ± 2 °C) for 60 min, and filtered. From this filtrate an aliquot of 5 ml was diluted to 25 ml with water, and the further procedure for the adsorption measurement was the same as the procedure described for the determination of total polyphenols (A_2). Standard pyrogallol solution was prepared from 50 mg pyrogallol in water diluted up to 100 ml. Five milliliters of thus prepared solution was mixed with 1 ml of phosphorus tungstic acid and 50 ml (150 g/l) of sodium carbonate solution. The adsorption at 715 nm (A_3) was measured 2 min after pyrogallol dissolution.

The tannin content was determined according to the following relationship: $\%\ \text{tannin} = 13.12 \times (A_1 - A_2)/(A_3 \times \text{m})$, where m is sample mass in grams.

2.5. Determination of total phenolic acid and flavonoid contents

The total phenolic acids content was determined according to the European Pharmacopoeia spectrophotometric method (Ph. Eu. 4., 2004). Stock solution was prepared from 0.2 g of the powdered drug mixed with 190 ml of ethanol (50 per cent v/v), heated in a water bath, under reflux for 30 min, then cooled and filtered. The filter was rinsed with 10 ml of ethanol, the filtrate and the rinsings combined and diluted to 200 ml with ethanol.

Phenolic acids were determined from 1 ml aliquot stock solution mixed with 2 ml hydrochloric acid (0.5 M), 2 ml of a solution prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water, and 2 ml of dilute sodium hydroxide solution. This solution was diluted to 10 ml with water and the adsorption was measured immediately at 505 nm by comparison with the compensation solution (1 ml of the stock solution, 2 ml of 0.5 M hydrochloric acid, 2 ml of dilute sodium hydroxyde solu-

Table 1
Sources of the plant material

Sample no.	Taxa	Locality	Coordinates	Collection no.
1	*Stachys alpina*	Medvednica	N45°53′00″; E15°56′30″	807.2
2	*Stachys officinalis*	Maksimir	N45°50′00″; E16°01′30″	807.3
3	*Stachys palustris*	Vrbani	N45°47′30″; E15°55′00″	807.7
4	*Stachys recta* subsp. *recta*	Velebit	N44°31′45″; E15°11′00″	807.5
5	*Stachys recta* subsp. *subcrenata*	Velebit	N44°32′00″; E15°08′50″	807.6
6	*Stachys salviifolia*	Vinjerac	N44°15′15″; E15°28′00″	807.4
7	*Stachys sylvatica*	Dolje	N45°53′00″; E15°56′30″	807.1

tion mixed together and diluted with water to 10 ml). The percentage of phenolic acids, expressed as rosmarinic acid, is calculated from the expression: $A \times 5/m$, where A is the absorbance of the test solution at 505 nm and m mass of the powdered drug, in grams. The rosmarinic acid was used as standard for calculating the calibration curve (0–100 mg/l).

The total flavonoid content was determined by the method of Christ and Müller (1960). Each powdered sample (0.5 g) was mixed with 20 ml of acetone, 2 ml of 25% hydrochloric acid and 1 ml of 0.5% hexametylentetramine solution and heated in a water bath under reflux for 30 min. After hydrolysis, the aglycones were measured spectrophotometrically at 425 nm by creating a complex with $AlCl_3$ in a methanol–ethyl acetate–acetic acid medium. The total flavonoid content was calculated using a standard curve with quercetin (0–50 mg/l) as the standard. The percentage of flavonoids, expressed as quercetin, is calculated from the expression: $A \times 0.772/b$, where A is the absorbance of the test solution at 425 nm and b the mass of the powdered drug, in grams.

2.6. Evaluation of antioxidant activity

2.6.1. DPPH radical scavenging assay

The radical scavenging activity of the extracts was measured by the slightly modified method of Hatano, Kagawa, Yasuhara, and Okuda (1988). One milliliter of a 0.2 mM DPPH methanol solution was added to 4 ml of various concentrations of the extracts in methanol. The mixture was shaken vigorously and left to stand at room temperature. After 30 min, the absorbance of the solution was measured at 517 nm and the antioxidant activity calculated using the following equation: scavenging capacity % = 100 − [(Ab of sample − Ab of blank) × 100/Ab of control]. Methanol (1 ml) plus plant extract solution (4 ml) were used as a blank, while DPPH solution plus methanol was used as a negative control. The positive control was DPPH solution plus 1 mM rutin. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration. The tests were carried out in triplicate.

2.6.2. Assay of non-enzymatic lipid peroxidation in liposomes

The ability of plant extracts to inhibit peroxidation of membrane lipids was determined by the method of Houghton, Zarka, De La Heras, and Hoult (1995). Liposomes were prepared from bovine brain phospholipids and peroxidation induced by addition of Fe^{3+} ascorbate. Triplicate reactions contained 10 μl of the inhibitor or its vehicle, 0.5 ml of a 5 mg/ml suspension of Type VII Folch bovine brain extract sonicated at 0–4 °C until opalescent, 0.3 ml phosphate-buffered saline (PH 7.4), 0.1 ml 1.0 mM $FeCl_3$ and peroxidation was initiated by adding 0.1 ml 1 mM ascorbic acid. All reagents were prepared freshly. After 60 min at 37 °C, the extent of peroxidation was essessed using the TBA test: 1.0 ml of thiobarbituric acid (1% w/v in 0.05 M NaOH), 1.0 ml 2.8% w/v trichloroacetic acid and 0.1 ml 2% w/v butylated hydroxytoluene were added and the tubes heated at 80 °C for 20 min. After cooling and centrifugation for 5 min at 3200 rpm, the absorbance was read at 532 nm. The inhibition of lipid peroxidation was calculated in percentages by comparing control tubes without any extract added. Fisetin was used as positive control. Results are means of experiments conducted in triplicate.

2.6.3. Xanthine oxidase assay

The inhibition of xanthine oxidase was measured by the method of Cos et al. (1998). The plant extracts were dissolved in a small amount of 0.2% MeOH or 0.2% EtOH and diluted with buffer (PH 7.5) to 1 mM. Test solutions were prepared by adding xanthine (final concentration 50 μM), hydroxylamine (final concentration 0.2 mM), EDTA (final concentration 0.1 mM), and plant extracts in various concentrations. The reaction was started by adding 0.2 ml of xanthine oxidase (6.25 mU/ml) in a phosphate buffer solution (pH 7.5, 200 mM). The mixture (1 ml) was incubated for 30 min at 37 °C and the reaction was stopped by adding 0.1 ml of HCl (0.58 M). The absorbance of the mixture was measured at 290 nm. The uric acid production was calculated from the differential absorbance with a blank solution in which the xanthin oxidase was replaced by buffer solution. A test mixture containing no plant extracts was prepared to measure the total uric acid pro-

Table 2
Content of polyphenolic compounds in *Stachys* taxa

Plant material	Polyphenols content (%)[a]				
	Total	Unadsorbed on hide powder	Tannins	Phenolic acids	Flavonoids
Stachys alpina	5.74 ± 0.17	4.72 ± 0.14	1.02 ± 0.01	2.01 ± 0.04	0.30 ± 0.01
Stachys officinalis	6.75 ± 0.20	5.39 ± 0.16	1.36 ± 0.02	2.67 ± 0.08	0.15 ± 0.00
Stachys palustris	10.8 ± 0.32	8.77 ± 0.26	2.03 ± 0.02	6.37 ± 0.22	0.28 ± 0.01
Stachys recta subsp. *recta*	13.8 ± 0.41	12.8 ± 0.38	1.01 ± 0.03	5.76 ± 0.46	0.17 ± 0.01
Stachys recta subsp. *subcrenata*	6.75 ± 0.20	5.06 ± 0.15	1.69 ± 0.02	1.65 ± 0.14	0.18 ± 0.01
Stachys salviifolia	6.75 ± 0.20	5.41 ± 0.16	1.35 ± 0.02	2.59 ± 0.11	0.13 ± 0.01
Stachys sylvatica	3.37 ± 0.10	2.73 ± 0.08	0.64 ± 0.00	2.41 ± 0.07	0.29 ± 0.01

[a] Results are means ± SD of three different experiments.

Table 3
Free radical-scavenging capacities of *Stachys* extracts measured by the DPPH assay[a]

Plant material	Extract	IC_{50} (μg dry ext/ml ± SD)
Stachys alpina	MeOH	5.20 ± 0.10
	EtOH	46.6 ± 0.1
	CH_2Cl_2	112 ± 0.5
Stachys officinalis	MeOH	10.6 ± 0.2
	EtOH	13.8 ± 0.1
	CH_2Cl_2	387 ± 0.3
Stachys palustris	MeOH	2.67 ± 0.03
	EtOH	5.42 ± 0.29
	CH_2Cl_2	168 ± 0.5
Stachys recta subsp. *recta*	MeOH	1.96 ± 0.86
	EtOH	6.37 ± 0.68
	CH_2Cl_2	212 ± 0.5
Stachys recta subsp. *subcrenata*	MeOH	5.97 ± 0.92
	EtOH	23.0 ± 0.6
	CH_2Cl_2	142 ± 0.9
Stachys salviifolia	MeOH	10.7 ± 0.23
	EtOH	9.87 ± 0.08
	CH_2Cl_2	680 ± 0.4
Stachys sylvatica	MeOH	16.1 ± 0.4
	EtOH	21.9 ± 0.1
	CH_2Cl_2	172 ± 0.3

[a] Results are means of four different experiments.

duction. Allopurinol was used for comparison. Tests were carried out in triplicate.

2.6.4. Statistical analysis

Values shown in Tables 2 and 3 were the mean of at least three determinations ± SD. Correlation coefficients (R) to determine the relationship between two variables were calculated using the MS Excel software (CORREL statistical function).

3. Results and discussion

Polyphenols are an important group of pharmacologically active compounds. They are considered to be the most active antioxidant derivatives in plants (Bors et al., 2001). The results of the polyphenol content are presented in Table 2.

The content of total polyphenols ranged from 13.8 (*S. recta* subsp. *recta*) to 3.37% (*S. sylvatica*). The content of polyphenols unadsorbed on hide powder was between 12.8 (*S. recta* subsp. *recta*) and 2.73% (*S. sylvatica*), and the content of tannins varied between 2.03 (*S. palustris*) and 0.64% (*S. sylvatica*). *S. recta* subsp. *recta*, which was the richest in total polyphenols and polyphenols unadsorbed on hide powder, had a low content of tannins (1.01%). The content of phenolic acids was between 6.37 (*S. palustris*) and 1.65% (*S. recta* subsp. *subcrenata*), and showed almost no correlation ($R = 0.087$) with the flavonoid content, which ranged from 0.30 (*S. alpina*) to 0.13% (*S. salviifolia*). In addition, the content of total phenolic acids correlated significantly with the total polyphenol content ($R = 0.86$) and the content of polyphenols unadsorbed on hide powder ($R = 0.84$). A low correlation was observed between plant phenolic acids and tannins ($R = 0.33$). The flavonoid content showed a negative correlation to total polyphenols ($R = -0.26$), polyphenols unadsorbed on hide powder ($R = -0.24$) and tannins ($R = -0.17$).

Stachys species were extracted with methanol, ethanol and dichlormethane. The antioxidative capacities of the extracts were assessed by employing three tests: DPPH radical scavenging assay, assay of non-enzymatic lipid peroxidation in liposomes and xanthine oxidase assay. The results are shown in the Table 3. Since the reaction followed a concentration-dependent pattern, only concentrations of active extracts providing 50% inhibition (IC_{50}) were included in the table. Polar extracts exhibited stronger activity than non-polar extracts. Methanolic extracts were the most effective DPPH radical scavengers. IC_{50} values of *S. recta* subsp. *recta* (1.96 μg/ml) and *S. palustris* (2.67 μg/ml) methanolic extracts were superior to that of the reference rutin (3.01 μg/ml). IC_{50} values of other methanolic extracts were also noteworthy and ranged from 5.20 to 16.1 μg/ml. The ethanolic extracts were slightly less effective radical scavengers compared to methanolic extracts, with IC_{50} ranging from 5.42 (*S. palustris*) to 46.6 μg/ml (*S. alpina*). The only exception was *S. salviifolia*: the ethanolic extract ($IC_{50} = 9.87$ μg/ml) was slightly more effective than the methanolic extract ($IC_{50} = 10.7$ μg/ml). Dichlormethane extracts exerted the weakest antioxidant activity, with their IC_{50} values ranging from 112 (*S. alpina*) to 680 μg/ml (*S. salviifolia*). The antioxidant components of the investigated extracts are most likely to be polar, since the antioxidant activity depended on the polarity of the solvent used.

A low correlation was found between the DPPH results and the flavonoid contents (EtOH extract $R = 0.15$; MeOH extract $R = 0.01$; CH_2Cl_2 extract $R = 0.17$). Other authors have also found a low correlation between DPPH and flavonoids (Miliauskas, Venskutonis, & Van Beek, 2004; Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). It is known that only flavonoids of a certain molecular structure, particularly those with a certain hydroxyl position, will determine the antioxidant properties.

The DPPH results of all investigated extracts showed negative correlations with total phenolic acids, polyphenols and tannins content and content of polyphenols unadsorbed on hide powder. It has been shown in other studies (Meda et al., 2005) that the phenolic content does not necessarily correspond to the antioxidant activity. The antioxidant activity is the result of the combined activity of a wide range of compounds, including phenolics, peptides, organic acids and other components (Choi et al., 2002; Gallardo, Jiménez, & García-Conesa, 2006).

Although the DPPH radical-scavenging abilities of the dichlormethane extracts were weak, it was evident that the methanolic and ethanolic extracts did show significant proton-donating ability and could serve as free radical

inhibitors or scavengers, acting possibly as primary antioxidants.

The investigated *Stachys* extracts neither exhibit any peroxidation inhibiting activity in the liposome peroxidation system nor on xanthin oxidase under the described experimental conditions. No encouraging results, obtained in lipid peroxidase and xanthin oxidase assay are probably due to the fact that not only the level of antioxidants, but also a synergy occuring between them and other plant constituents might influence the antioxidant ability of plant extracts (Capecka, Mareczek, & Leja, 2005).

4. Conclusion

All investigated *Stachys* taxa contained polyphenols, tannins, phenolic acids and flavonoids. Although the tested extracts were not active as lipid peroxidation and xanthin oxidase inhibitors, methanolic and ethanolic extracts seem to be relevant as DPPH scavengers, suggesting that they possess high radical scavenging activity. Methanol extracts of *S. recta* subsp. *recta* and *S. palustris* were the strongest radical scavengers in DPPH assay among the plant extracts tested. Their antiradical activity was higher then that of the reference rutin, which is very effective in terms of antioxidant activity.

All the plants of *Stachys* taxa seem to be promising as natural sources to develop free radical scavengers. Further investigations are needed to isolate and identificate radical scavenging components of the studied extracts.

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