Acta Pharm. *55* (2005) 207–214 Short communication

In vitro antioxidant activity of some *Teucrium* species (*Lamiaceae*)

TATJANA KADIFKOVA PANOVSKA[1]*
SVETLANA KULEVANOVA[2]
MARINA STEFOVA[3]

[1]*Institute of Toxicology, Faculty of Pharmacy, 1000 Skopje, R. Macedonia*

[2]*Institute of Pharmacognosy, Faculty of Pharmacy, 1000 Skopje, R. Macedonia*

[3]*Institute of Chemistry, Faculty of Science, P.O. Box 162, 1001 Skopje R. Macedonia*

Received September 17, 2004
Accepted April 22, 2005

The chemical composition and antioxidant activity of different extracts (diethyl ether, ethyl acetate and *n*-butanol) obtained from *Teucrium* species (*T. chamaedrys*, *T. montanum*, *T. polium*) were investigated in this work. Phytochemical screening of the plant extracts proved the presence of flavonoids luteolin, apigenin and/or diosmetin. The chemical composition of extracts was evaluated by HPLC and spectrophotometry. Antioxidant activities of the extracts were evaluated using three complementary *in vitro* assays: inhibition of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, inhibition of hydroxyl radicals and protection of β-carotene-linoleic acid model system. In the first two assays, strong inhibitory activity was shown by *T. montanum* and *T. chamaedrys* extracts. In the β-carotene-linoleic acid model system, extracts from *T. polium* showed remarkable activity. These findings demonstrated that *Teucrium* species possess free radical and hydroxyl radical scavenging activity as well as antioxidant activity *in vitro*.

Keywords: Teucrium, flavonoids, antioxidant activity, reactive oxygen species, thiobarbituric acid-reactive substances

Since ancient times, many official herbs have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infections and preservation of foods from the toxic effects of oxidants. The preservative effect of many plant species and herbs suggests the presence of antioxidative and antimicrobial constituents (1). Many medicinal plants contain large amounts of antioxidants other than vitamin C, vitamin E, and carotenoids (2). Many herb species, especially those belonging to the *Lamiaceae* family, such as sage, oregano, and thyme, show strong antioxidant activity (1). A number of phenolic compounds with strong antioxidant activity have been identified in these plant extracts (3). The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists

* Correspondence, e-mail: taka@baba.ff.ukim.edu.mk

and food manufacturers as consumers move towards functional foods with specific health effects (4). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (2). The antioxidative effect is mainly due to phenolic components, such as flavonoids (5), phenolic acids, and phenolic diterpenes (6). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (7). Many of these phytochemicals possess significant antioxidant capacity that may be associated with lower incidence and lower mortality rates of cancer in several human populations (2).

The purpose of this study was to evaluate some *Teucrium* species as new potential sources of natural antioxidants. The antioxidant activities were determined by *in vitro* assays: inhibition of DPPH (1,1-diphenyl-2-picrylhydrazyl) and hydroxyl radicals and protection of β-carotene-linoleic acid model system.

EXPERIMENTAL

Plant material. – Aerial parts of *Teucrium chamaedrys* L., *Teucrium polium* L. and *Teucrium montanum* L. were collected in the early stages of vegetation from the Lazaropole district and Belasica Mountain, Macedonia, during the summer of 2001. Voucher specimens were deposited at the Herbarium of the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia.

Reagent and authentic samples. – The reagents used were of highest purity (> 99.95%) and were purchased from Sigma Chemical Co. (Germany). Authentic samples of rosmarinic acid, caffeic acid, myricetin, eriodyctiol, luteolin, naringenin, apigenin, kaempferol, chrysoeriol and diosmetin were the products of Extrasynthese (France).

Preparation of plant extracts. – Dried powdered plant material (10 g) was extracted by continous mixing in 100 mL 70% ethanol, 24 h at room temperature. After filtration, ethanol was evaporated until only water remained. Water phase was subsequently extracted with diethyl ether, ethyl acetate or *n*-butanol. These extracts were dried over anhydrous sodium sulfate, filtered and concentrated under vacuum up to a concentration of 1 g per 1 mL of extract. They were further diluted with diethyl ether (E), ethyl acetate (EA) or *n*-butanol (B), to obtain 0.01 g mL^{-1} solutions used in the experiments.

Identification and determination of flavonoids. – The flavone aglycones in the extracts were identified by the HPLC method already reported (8), using Varian HPLC system (Bulgaria) equipped with a ternary pump Model 9012 and UV-diode-array detector Model 9065. A reverse phase column C18 (250 × 4.6 mm, 5 μm particles) was used. The mobile phase consisted of three solvents: H_2O (A), CH_3CN (B) and CH_3OH (C) and the elution program for screening the extracts was the following: 0–2 min 80% A, 20% B; 5–25 min 75% A, 25% B; 30–40 min 70% A, 25% B, 5% C; 40–50 min 40% A, 25% B, 35% C. The flow rate was 1.5 mL min^{-1} and the temperature was set to 25 °C. The elution was monitored at 348 nm. Identification was made according to the retention times and UV spectra of the components compared to those of the authentic samples of flavonoids and phenolic acids.

Total flavonoid content in dried aerial parts of the plants was determined by the known spectrometric method (9). A Perkin-Elmer UV-Vis spectrometer Lambda 16 (Germany) was used. 10 mL of the extract prepared by the standard procedure (a) transferred into a 25 mL volumetric flask, 1 mL 2% $AlCl_3$, was added and the solution was filled to volume with methanol-acetic acid. After 30 min, the absorbance was measured at 390 nm against the same solution without $AlCl_3$ being blank. Luteolin was used to construct the calibration curve in the concentration range 1.0–10.0 μg mL^{-1}.

Free radical scavenging activity in 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) assay. – The antioxidant activity using the DPPH assay was assessed by the method of Tagashira and Ohtake (10). A test sample solution (200 μL) was added to 4 mL of 100 mmol L^{-1} ethanolic DPPH. After vortexing, the mixture was incubated for 10 minutes at room temperature and the absorbance at 517 nm was measured. The difference in absorbance between a test sample and a control (ethanol) was considered as activity. The activity was shown as IC_{50} value (50% of inhibitory concentration in mg mL^{-1}). Luteolin, quercetin and silymarin (Extrasynthese) (100 μg mL^{-1} in ethanol) were used as reference substances. All values are shown as the mean of three measurements.

Evaluation of the hydroxyl radical scavenging activity. – Hydroxyl radical scavenging activity was carried out by measuring the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Attack of the hydroxyl radical on deoxyribose led to TBARS (thiobarbituric acid-reactive substances) formation (11). The formed TBARS were measured by the method given by Ohkawa *et al*. (12). The extracts were added to the reaction mixture containing 2.8 mmol L^{-1} deoxyribose, 100 μmol L^{-1} $FeCl_3$, 104 μmol L^{-1} EDTA, 100 μmol L^{-1} ascorbic acid, 1 mmol L^{-1} H_2O_2 and 230 mmol L^{-1} phosphate buffer (pH 7.4), making a final volume of 1.0 mL. One milliliter of thiobarbituric acid TBA (1%) and 1.0 mL trichloroacetic acid (TCA 2.8%) were added to the test tube and incubated at 100 °C for 20 min. After cooling, absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Reactions were carried out in triplicate. In the series of control experiments, reference substances: luteolin, quercetin and silymarin (100 μg mL^{-1} in phosphate buffer pH 7.4) were used instead of the extract solution. The reaction mixture was incubated at 37 °C for 1 h.

Evaluation of antioxidant activity. – The antioxidant activity of the extracts was evaluated using a β-carotene/linoleate model system (13). A solution of β-carotene was prepared by dissolving 2.0 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipetted into a round-bottom flask. After chloroform was rotary evaporated at 40 °C under vacuum, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier and 50 mL of distilled water were added to the flask under vigorous shaking. Aliquots (5 mL) of this emulsion were transferred into a series of tubes containing 2 mg of each extract or 2 mg of BHA (butylated hydroxyanisole) for comparison. An aliquot (5 mL) of emulsion without any further addition was used as control. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 10-min intervals by keeping the sample in a water bath at 50 °C until the visual color of β-carotene in the control sample disappeared (about 120 min).

RESULTS AND DISCUSSION

Flavone aglycones were identified by HPLC and the total content of flavonoides in dried aerial parts of the plants were determined spectrophotometrically. The *Teucrium* species studied have highly similar flavonoid composition. HPLC analysis revealed that luteolin was present in each of the diethyl ether extracts. Diosmetin was found in *T. polium* and *T. montanum*, while apigenin was identified only in *T. polium* (Fig. 1).

The amount of total flavonoids, determined by spectrophotometry, varied in different *Teucrium* species and ranged from 0.15 to 0.20% (Table I). The concentration of total flavonoids in the tested *Teucrium* species was lower than in the other *Lamiaceae* plants such as *Thymus vulgaris* (14), *Mentha piperita*, *Melissa officinalis* and *Rosmarinus officinalis* (15).

Free radical scavenging activity (FRSA) of *Teucrium* extracts was evaluated by comparing it with the activities of substances such as luteolin, quercetin and silymarin, which possess some antioxidant potential (reference substances). Table II shows the FRSA values obtained for plant extracts together with reference substances in the DPPH assay. Various extracts showed different activity in this assay. Ethyl acetate extracts derived from *T. polium* and *T. chamaedrys* and ether extract from *T. montanum* showed the highest inhibitory activity with IC_{50} of 10, 11 and 10 mg mL^{-1}, respectively. When compared to the reference substances, the *Teucrium* extracts were found to be less efficient in radical scavenging. Luteolin, quercetin and silymarin interacted intensively with DPPH (IC_{50}:

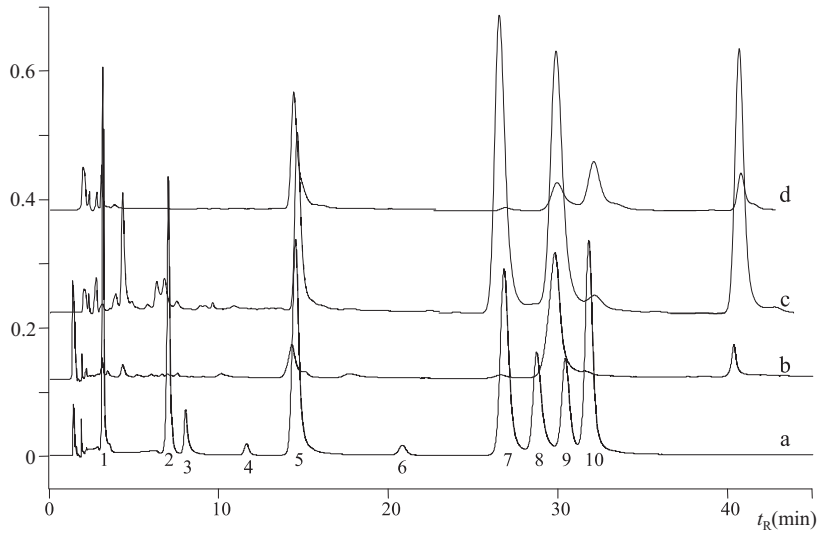

Fig. 1. HPLC chromatograms (348 nm) of mixture of: a) standard substances (1 – rosmarinic acid, t_r = 3.18; 2 – caffeic acid, t_r = 6.99; 3 – myricetin, t_r = 8.01; 4 – eriodyctiol, t_r = 11.64; 5 – luteolin, t_r = 14.51; 6 – naringenin, t_r = 20.79; 7 – apigenin, t_r = 26.78; 8 – kaempferol, t_r = 28.71; 9 – chrysoeriol, t_r = 30.39; 10 – diosmetin, t_r = 31.78) and diethyl ether extracts of b) *Teucrium chamaedrys*, c) *Teucrium polium* and d) *Teucrium montanum*.

Table I. Concentration of total flavonoids in air-dried aerial parts of Teucrium *species determined by spectrophotometry*

Plant material	Total flavonoids (%)	RSD (%)
Teucrium chamaedrys	0.18	3.3
Teucrium polium	0.20	2.4
Teucrium montanum	0.15	2.9

[a] $n = 5$

0.08, 0.06 and 1.96 mg mL^{-1}, respectively). The scavenging effects of the examined extracts could be due to the flavonoids, but could also be a result of the activity of other secondary biomolecules present in the extracts.

The effect of *Teucrium* extracts on inhibition of hydroxyl radical production was assessed by the iron(II)–dependent deoxyribose damage assay. The Fenton reaction generates hydroxyl radicals ($OH^{\bullet}$) that degrade deoxyribose using Fe^{2+} salts as an important catalytic component (11). Oxygen radicals may attack the sugar, which leads to sugar fragmentation. Addition of transition metal ions such as iron at low concentrations to deoxyribose causes degradation of the sugar into malondialdehyde and other related compounds which form a chromogen with thiobarbituric acid (TBA). Table II presents the results of the effects of the examined *Teucrium* extracts as well as control solutions on $OH^{\bullet}$ radical production. They show that all extracts of *T. montanum*, *T. chamaedrys* and *T. polium* and control solutions inhibited the production of $OH^{\bullet}$ radicals. All extracts of

Table II. Free radical scavenging activity of the Teucrium *extracts*[a]

Sample	DPPH assay (IC_{50}, mg mL^{-1})	$I_{OH^{\bullet}}$ (%)[b]
Teucrium montanum		
diethyl ether extract	10	45
ethyl acetate extract	20	45
n-butanol extract	30	46
Teucrium chamaedrys		
diethyl ether extract	16	43
ethyl acetate extract	11	46
n-butanol extract	30	41
Teucrium polium		
diethyl ether extract	55	42
ethyl acetate extract	10	43
n-butanol extract	70	44
Luteolin	0.09	48
Quercetin	0.06	42
Silymarin	1.96	43

[a] $n = 3$
[b] $OH^{\bullet}$ radicals production.

Teucrium species exhibited a significant inhibitory effect, ranging from 41 to 46%, which was comparable to the reference solutions of quercetin (42%), silymarin (43%) and luteolin (48%).

It has been recently shown that quercetin and its glycosides exert inhibitory activity against lipid peroxidation (16). Since luteolin and its derivates, along with quercetin and rutin, belong to the same group of compounds – flavonoids, the following antioxidative mechanism can be proposed: conjugation of the double bound in position 2,3 with C_4--carbonyl group, and also the existence of a free OH group on C_5 and C_7, enable the formation of chelate complexes with d-elements (Fe^{2+}, Cu^{2+}, Zn^{2+}). The formation of a complex with Fe^{2+} prevents the production of $OH^{\bullet}$ radicals (Fenton's reaction), which was used to evaluate the inhibitory effects.

The antioxidant activity of each extract of the *Teucrium* species examined, BHA and control is presented in Table III. According to the preventive activity against bleaching of β-carotene, *T. polium* is the most promising species. Its *n*-butanol extract showed the highest antioxidant activity comparable to that of BHA (Table III). The extracts from *T. montanum* showed good antioxidative effects during the first 15 minutes, whereas the extracts of *T. chamaedrys* showed lower activity. Although *Teucrium* extracts exhibited relatively strong antioxidant activity, all of them showed lower antioxidant activity than BHA in the β-carotene/linoleic acid model system.

Chemical analysis showed that the highest total flavonoid levels were detected in the *T. chamaedrys* and *T. polium* species, while the lowest in the *T. montanum* (Table I). This indicates that the concentration of flavonoids is not the only factor related to the antioxidant activity. The possible synergism of flavonoids with other components present in the extracts may be responsible for this observation.

Table III. Effect of diethyl ether (E), ethyl acetate (EA) and n*-butanol (B) extracts of* T. montanum, T. polium *and* T. chamaedrys, *BHA and the control on oxidation of the β-carotene/linoleic acid model system*

	A_{470} (% of initial value)[a]								
	Time (min)								
Sample	0	15	30	45	60	75	90	105	120
Teucrium chamaedrys (E)	100	86	83	71	63	52	49	41	36
Teucrium chamaedrys (EA)	100	88	84	74	66	56	56	45	41
Teucrium chamaedrys (B)	100	89	85	73	63	54	53	46	42
Teucrium montanum (E)	100	89	84	73	65	55	51	43	39
Teucrium montanum (EA)	100	90	85	74	65	55	51	43	39
Teucrium montanum(B)	100	91	85	75	66	57	54	45	40
Teucrium polium (E)	100	87	84	73	65	55	52	44	40
Teucrium polium (EA)	100	92	87	76	68	59	56	48	42
Teucrium polium (B)	100	93	87	77	68	59	56	49	43
BHA	100	95	92	81	74	68	64	59	56
Control	100	65	60	48	30	22	18	14	10

[a] $n = 3$

CONCLUSIONS

Based on the results described, it may be concluded that luteolin, diosmetin and/or apigenin are present in *Teucrium chamaedrys*, *T. montanum* and *T. polium*, respectively. The major flavonoid in *T. montanum* is luteolin followed by diosmetin; the identification of major components of *T. chamaedrys* and *T. polium* still remains to be done. Diethyl ether, ethyl acetate and *n*-butanol extracts from *Teucrium* species possess significant free radical scavenging, hydroxyl radical scavenging and antioxidant activity *in vitro*, which offer the possibility of using these extracts as natural antioxidants.

REFERENCES

1. K. Hirasa and M. Takemasa, *Spice Science and Technology*, Marcel Dekker, New York 1998.
2. Y. S. Velioglu, G. Mazza, L. Gao and B. D. Oomah, Antioxidant activity and total phenolics in selected fruits, vegetables and grain products, *J. Agric. Food Chem.* 46 (1998) 4113–4117.
3. N. Nakatani, *Antioxidants from Spices and Herbs*, in *Natural Antioxidants: Chemistry, Health Effects, and Applications* (Ed. F. Shahidi), AOCS Press, Champaign, 1997, pp. 64–75.
4. J. Loliger, *The Use of Antioxidants in Food*, in *Free Radicals and Food Additives* (Eds. O. I. Aruoma and B. Halliwell), Taylor and Francis, London 1991 pp. 129–150.
5. P. G. Pietta, *Flavonoids in Medicinal Plants*, in *Flavonoids in Health and Diseases* (Eds. C. A. Rice-Evans and L. Packer), Marcel Dekker, New York, 1998, pp. 61–110.
6. F. Shahidi, P. K. Janitha and P. D. Wanasundara, Phenolic antioxidants, *Crit. Rev. Food Sci. Nutr.* **31** (1992) 67–103.
7. T. Osawa, *Novel Natural Antioxidants for Utilization in Food and Biological Systems*, in *Postharvest Biochemistry of Plant Food-materials in the Tropics* (Eds. I. Uritani, V. V. Garcia and E. M. Mendoza), Japan Scientific Societies Press, Tokyo, 1994, pp. 241–251.
8. S. Kulevanova, M. Stefova, T. Kadifkova Panovska and T. Stafilov, HPLC identification and determination of myricetin, quercetin, kaempferol and total flavonoids in herbal drugs, *Maced. Pharm. Bull.* **48** (2002) 25–30.
9. S. Kulevanova, M. Stefova, T. Kadifkova Panovska, J. Tonic and T. Stafilov, Determination of flavones in species of *Thymus* L. (*Lamiaceae*) from Macedonian flora, *Maced. Pharm. Bull.* **47** (2001) 9–14.
10. M. Tagashira and Y. Ohtake, A new antioxidative 1,3-benzodioxole from *Melissa officinalis*, *Planta Med.* **64** (1998) 555–558.
11. B. Halliwell and J. Gutteridge, Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. The role of superoxide and hydroxyl radicals, *FEBS Lett.* **128** (1981) 347–352.
12. H. Ohkawa, N. Ohishi and K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal. Biochem.* **95** (1979) 351–358.
13. U. Wanasundara, R. Amarowicz and F. Shahidi, Isolation and identification of an antioxidative component in Canola meal, *J. Agric. Food Chem.* **42** (1994) 1285–1290.
14. M. P. Kahkonen, A. I. Hopia, H. J. Vuorela, J. P. Rauha, K. Pihlaja, T. S. Kujala and M. Heinonen, Antioxidant activity of plant extracts containing phenolic compounds, *J. Agric. Food Chem.* **47** (1999) 3954–3962.
15. W. Zheng and S. Y. Wang, Antioxidant activity and phenolic compounds in selected herbs, *J. Agric. Food Chem.* **49** (2001) 5165–5170.
16. N. C. Cook and S. Samman, Flavonoids – chemistry, metabolism, cardioprotective effects, and dietary sources, *J. Nutr. Biochem.* **7** (1996) 66–76.

S A Ž E T A K

In vitro antioksidativno djelovanje nekih *Teucrium* vrsta (*Lamiaceae*)

TATJANA KADIFKOVA PANOVSKA, SVETLANA KULEVANOVA i MARINA STEFOVA

U radu je opisan kemijski sastav i antioksidativno djelovanje različitih ekstrakata (dietil-eter, etil-acetat, *n*-butanol) iz *Teucrium* vrsta (*T. chamaedrys*, *T. montanum*, *T. polium*). Fitokemijska istraživanja ekstrakata HPLC metodom i spketrofotometrijom pokazala su prisutnost flavonoida luteolina, apigenina i/ili diosmetina. Antioksidativni učinci ekstrakata praćeni su *in vitro* pomoću tri komplementarne metode koje se temelje na inhibiciji DPPH (1,1-difenil-2-pikrilhidrazil) radikala, inhibiciji hidroksil radikala ili na zaštiti sustava β-karoten-linoleinska kiselina. U prva dva modela dokazan je snažni inhibitorni učinak ekstrakata vrsta *T. montanum*, *T. chamaedrys*, dok su u trećem modelu ekstrakti iz *T. polium* pokazala značajni učinak. Rezultati ukazuju da *Teucrium* vrste imaju sposobnost vezanja slobodnih i hidroksil radikala i antioksidativni učinak *in vitro*.

Ključne riječi: Teucrium, flavonoidi, antioksidativni učinak, reaktivne vrste kisika, reaktivni derivati tiobarbiturne kiseline

Institute of Toxicology, Faculty of Pharmacy, Skopje, Macedonia

Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia

Institute of Chemistry, Faculty of Science, Skopje, Macedonia