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Original scientific paper

Seasonal variation of flavonoids in *Teucrium polium* L. (Lamiaceae)

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Abstract

The aim of the present study was identification of flavone aglycones and determination of the content of each and the content of total flavonoids as well as investigation of the eventual seasonal variations of flavonoids in *Teucrium polium* L. (Lamiaceae). The plant samples were collected at six different locations in Republic of Macedonia, during summer in 1999, 2000 and 2003. For determination of seasonal variations, the samples were collected in v. Koleshino, in 2004, each month during the whole season. Six flavone aglycones (luteolin, apigenin, diosmetin, cirsiliol, cirsimaritin and cirsilineol) were identified in the hydrolyzed extracts of the over ground part of *Teucrium polium* by HPLC method. The most abundant flavone was luteolin, followed by apigenin and cirsimaritin. Great seasonal variations were found in the content of each and in the content of total amount of flavonoids. The most abundant flavone during the whole season was luteolin with the highest content in May. The content of total flavonids was the highest in the period from May to July, which could be recommended as the most convenience period in the season for collecting of the plant material from *Teucrium polium*.

Key words: *Teucrium polium*, flavonoids, HPLC analysis, seasonal variation

Introduction

Teucrium polium L. (Lamiaceae) is a sub-shrub plant native to the Mediterranean region and the Middle East. In Republic of Macedonia it is widely distributed and traditionally used by native inhabitants, as herbal hypoglycemic tea. The decoctum of *T. polium* is used as an appetizer especially in children and also as a spice. Some biological and therapeutic effects have been reported for the plant such as antioxidant (Esmaeili et al., 2009; Ardestani et al., 2008), antiinflammatory (Tariq et al., 1989; Capasso et al., 1983), antinociceptive (Baluchnejadmojarad et al., 2005; Abdollahi et al., 2003), antipyretic (Aggelis et al., 1998; Autore et al., 1984), anti-microbial (Autore et al., 1984), hypolipidemic (Rasekh et al., 2001), hepatoprotective (Panovska et al., 2007), cytotoxic and apoptotic effects (Rajabalian et al., 2008). The plant poses complex chemical composition with presence of new clerodane type diterpenes (Malakov and Papanov, 1983; Marquez and Valverde, 1979), essential oil with dominating sesquiterpene alcohols and pinenes (Cozzani et al., 2005; Moghtader, 2009; Kabouche et al., 2007), phenylethanoid glycosides such as verbascoside and poliumoside (Oganesyan et al.,1991), flavone glycosides with highly methylated aglycons (Verykokidou-Vitsaropoulou and Vajias, 1986; Rizk et al., 1986; Kawashty et al., 1999; Harborne et al., 1986; Sharififar et al., 2009), etc.

Flavonoids are representing the most important group of active components of *Teucrium* species, and many of the activities of these plants are attributed to the flavonoid

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class of secondary metabolites. It is also well known that the composition and the content of the flavonoids in plant material could be variable depending of the season, location and environmental condition of plant growth as well as the influence of other different factors (Liu et al., 1994; De Castro, et al., 2006; Bagdonaite et al., 2009). Seasonal variations of flavonoids were also studied in different plant species (Luengas-Caicedo et al., 2007; Akabori, 1978; Ioku et al., 2005; Xu et al., 2009).

Until now, there is no published data about the seasonal variation in the composition and the content of the flavonoids in *Teucrium polium*. Taking into account all of these, the aim of the present study were identification, quantification and determination of the seasonal variations of the flavonoids in *Teucrium polium* from Macedonian origin.

Material and methods

Plant material

The over-ground parts of the plant of 6 different populations of *T. polium* were collected during the summer of 1999, 2000, 2003 and 2004 (Table 1). The plant material was air dried, packed in paper bags and kept in a dark and cool place until analysis. Plant identity was verified and voucher specimens were deposited at the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, R. Macedonia.

Reagents and authentic samples

Reagents of HPLC purity were purchased from Sigma Chemical Co. (Germany). Authentic substances apigenin, luteolin, chryseriol, diosmetin, acacetin, genkwanin, naringenin and eryiodictiol were the products of Extrasynthese (France). Cirsimaritin, cirsilineol, and 5,4'-OH and 6,7,8,3'-OCH_3 flavones were kindly donated by Dr. B. Voirin from the Laboratoire de Phytochimie, U. E. R. des Sciences de la Nature, Université Claude Bernard Lyon, France.

Preparation of hydrolyzed extracts

Grounded plant material (1 g) was extracted in an Erlenmeyer flask with reflux in a water bath with mixture of 25 ml acetone, 1 ml of concentrated HCl and 0.5 ml of 1% solution of urotropine. The extraction was performed twice, first for 40 min at 60 °C and then for 20 minutes more on the same temperature. The extracts were cooled, filtered and transferred to a 50 ml volumetric flask and filled up with acetone. 10 ml were transferred to a separating funnel. Water (25 ml) was added and extraction with ethyl acetate was repeated 3 times with 10 ml portion. The ethyl acetate fractions were collected, washed three times with 25 ml of water, then dried with anhydrous Na_2SO_4, filtered, and evaporated to dryness under low pressure. The residue was dissolved in 1 ml methanol and the solution was used for analyses of flavonoid aglycones by HPLC.

HPLC analysis

Flavonoid aglycones in the hydrolyzed extracts were analyzed by the HPLC method, using a Varian HPLC system equipped with a ternary pump Model 9012 and UV diode-array detector Model 9065. A reverse phase column C18 (250 x 4.6 mm, 5 μm particles) was used. The column was stabilized in thermostat on 35 °C with heater of column (CH-30) and temperature controller (TC-45). The mobile phase consisted of H_2O with pH adjusted to 3 with H_3PO_4 (A) and CH_3CN (B). The elution program for extracts screening was the following: 0–5 min 70% A; 10–20 min 65% A; 25–30 min 55% A; 40–48 min 35% A. The flow rate was 1 ml min^{-1}, the temperature was set to 35 °C and the injection volume was 20 μl.

The elution was monitored in the whole UV range and the chromatograms for flavone screening were best seen at 348 nm, which is in the region where flavones exhibit an absorption maximum. Identification was made according to the retention times and UV spectra of the components compared to those of authentic samples of flavonoids. Semi-quantification of flavones was performed on the basis of the peak areas of flavones in the HPLC chromatograms at 348 nm.

Table 1. The locations of collection of samples of *T. polium*

Species	Voucher specimen	Location	Mount and year of collection	Collection for determination of seasonal variation
Teucrium polium	T_p	v. Koleshino	July - 2003	Each month, from January to December, in 2004.
Teucrium polium	T_7	v. Janche	July - 1999	
Teucrium polium	T_9	v. Rashtak	July - 2000	
Teucrium polium	T_{11}	v. Gari	July - 2000	
Teucrium polium	T_{12}	Alshar	July - 2000	
Teucrium polium	T_{15}	Arkutino	July - 2000	

Results and discussion

HPLC analysis of flavonoids

The identification of flavone aglycons in the extracts of *T. polium,* was done by comparing the retention times and UV spectral data of the extract components with those of authentic flavonoid substances. Two mixtures of authentic substances of flavonoids were used, labeled as St_1 and St_2. The composition of the mixtures and relating retention times of flavonoids are presented in Table 2. The HPLC chromatograms of both mixtures of standards are presented on Fig.1.

Six flavone aglycons, luteolin, apigenin, cirsiliol, diosmetin, cirsimaritin and cirsilineol (Table 3) were identified in the hydrolyzed extracts of *T. polium*. As the authentic samples for the component cirsiliol was not available, identification was made using previously published data by Stefova et al. (2007) and other literature data (Verykokidou-Vitsaropoulou and Vajias, 1986; Rizk et al., 1986).

Table 2. Retention times (t_R) of the components in two mixture of authenthic samples of flavonoids (St_1 and St_2)

Mixture	Structure	t_R/min
St_1		
Apigenin	5,7,4'-OH flavone	15,59
Diosmetin	5,7,3'-OH, 4'-OCH3 flavone	17,65
Cirsimaritin	5,4'-OH 6,7-OCH3- flavone	26,77
Cirsilineol	5,4'-OH 6,7,3'-OCH3- flavone	28,43
Genkwanin	5, 4'-OH 7-OCH3 flavone	32,98
St_2		
Eryodictiol	5,7,3',4'-OH flavanone	9,15
Luteolin	5,7,3',4'-OH flavone	10,79
Naringenin	5,7,4'-OH flavanone	13,82
Apigenin	5,7,4'-OH flavone	15,73
Chryseriol	5,7,4'-OH, 3'-OCH_3 flavone	17,31
Diosmetin	5,7,3'-OH, 4'-OCH_3 flavone	17,77
Acacetin	5,7 -OH 4'-OCH_3 flavone	32,09
Genkwanin	5, 4'-OH 7-OCH_3 flavone	32,98

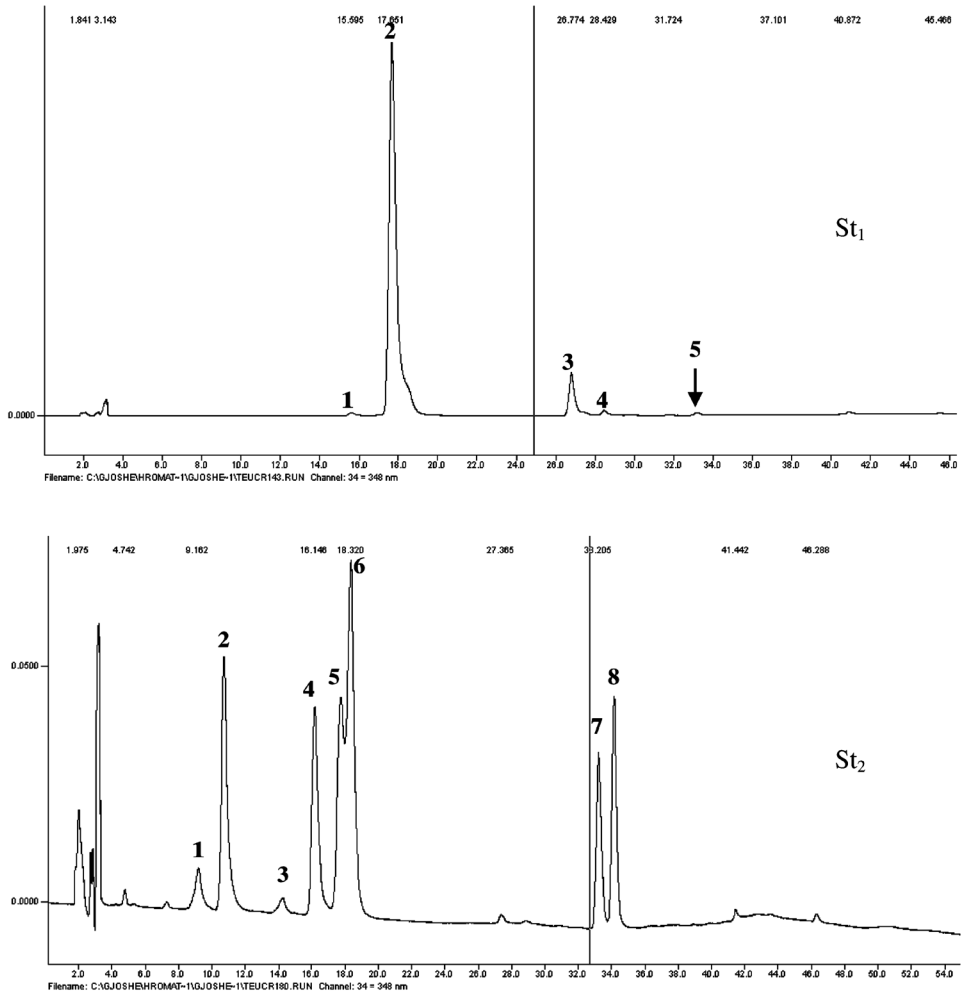

Fig. 1. *HPLC* chromatograms (348 nm) of two mixtures of standard substances of flavones. St_1: 1-apigenin, 2 – diosmetin, 3- cirsimaritin, 4 – cirsilineol, 5 – genkwanin; St_2: 1 – eryodictiol, 2 – luteolin, 3 – naringenin, 4 – apigenin, 5 – chrysoeriol, 6 – diosmetin, 7 – acacetin, 8 - genkwanin

The HPLC chromatogram of hydrolyzed extract of *T. polium* is presented at Fig. 2. The results of semi-quantitative analysis of the content of each flavones and the amount of total flavonoids are presented in Table 3.

Comparing to literature data, flavones identified in extracts of Macedonian *T. polium* are well known components of this species. Previously, in *T. polium* cirsimaritin, cirsiliol, cirsilineol, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, salvigenin, apigenin 5-galloyl-glycoside, apigenin-7--glycoside, vicenin-2 and luteolin-7-glycoside were reported (Esmaeili and Yazdanparast, 2004; Esmaeili at al., 2009; Harborne et al., 1986; Verykokidou-Vitsaropoulou and Vajias, 1986; Rizk et al., 1986, Kwahtsy et al., 1997; Panovska et al., 2007).

Seasonal variation of flavonoids

For determination of the eventually presented seasonal variation, the composition and the content of flavonoids were determined by HPLC in the samples of *T. polium* collected from the same location (v. Koleshino, south-eastern part of Republic of Macedonia), each month (except June) during 2004. The same HPLC method mentioned above was used and the hydrolyzed extracts were prepared on a same way, as it was made in a purpose of identification and quantification of the flavonoids.

In all investigated samples of *T. polium* that were collected for purpose of determination of seasonal variations, the same flavone aglycons were identified (Table 4), but in variable ratio during the season. For expression of the relative ratio of flavones, the peak area of cirsilineol measured in October was the lowest, and this area was expressed as one. The all other peak areas of all determined flavones were expressed in appropriately larger values presented in Table 4. The content of total flavonoids is presented as a sum of these numerical values for each month separately. The seasonal variation in the content of six flavones aglycones in *T. polium* is presented at Fig. 4, while the relative abundance of the flavonoids is presented on Fig. 5.

Table 3. Semi-quantitative determination of flavones aglycons in hydrolyzed extracts of *T. polium*

Sample	Luteolin	Apigenin	Cirsiliol	Diosmetin	Cirsimaritin	Cirsilineol
Tp	++	++	++	+	++	tr
T_7	++	+	+	tr	+	tr
T_9	++	++	+	-	+	tr
T_{11}	++	++	+	+	+	tr
T_{12}	++	+	+	tr	+	tr
T_{15}	++	+	+	+	+	tr

(++) - Dominantly present; (+) - present; tr – presented in traces; (-) – not detected

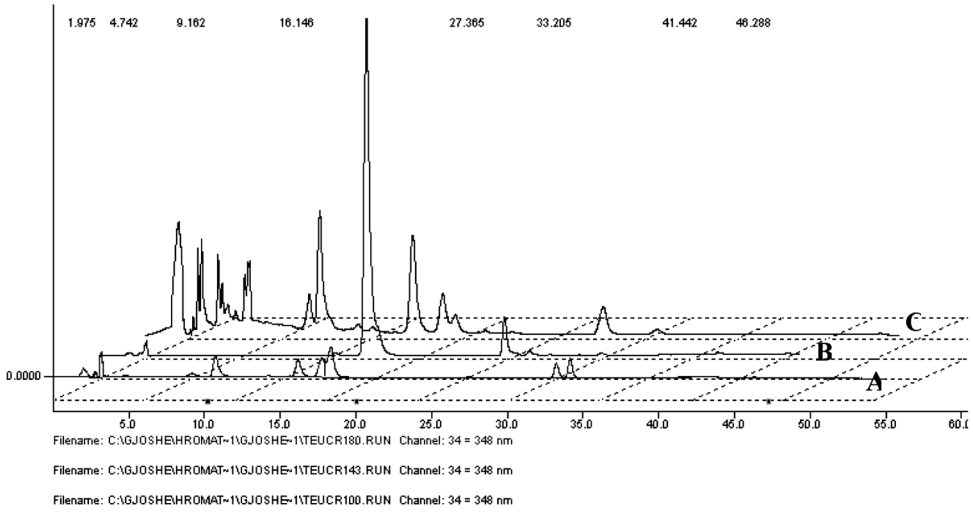

Fig. 2. *HPLC* chromatogram of hydrolyzed extract of *T. polium* – C, and mixtures of standard substances: St_1 – B and St_2 – A.

Table 4. Total amount and relative abundance of flavone aglycones during the one season of *Teucrium polium,* with normalized peak areas values of flavonoids accounted on peak area of cirsilineol, expresed as value one.

	Mounts										
	III	IV	V	VII	VIII	IX	X	XI	XII	I	II
		Spring			Summer			Autumn		Winter	
Luteolin	64	95	107	90	60	39	28	41	102	82	59
Apigenin	2	5	30	56	21	25	14	28	6	4	3
Cirsiliol	17	47	38	38	22	11	6	22	43	25	19
Diosmetin	5	19	17	8	5	3	4	6	13	15	7
Cirsimaritin	12	23	28	42	17	18	5	21	27	21	15
Cirsilineol	5	9	6	8	7	3	1	11	9	8	8
Total:	105	198	226	242	143	99	58	129	200	155	111

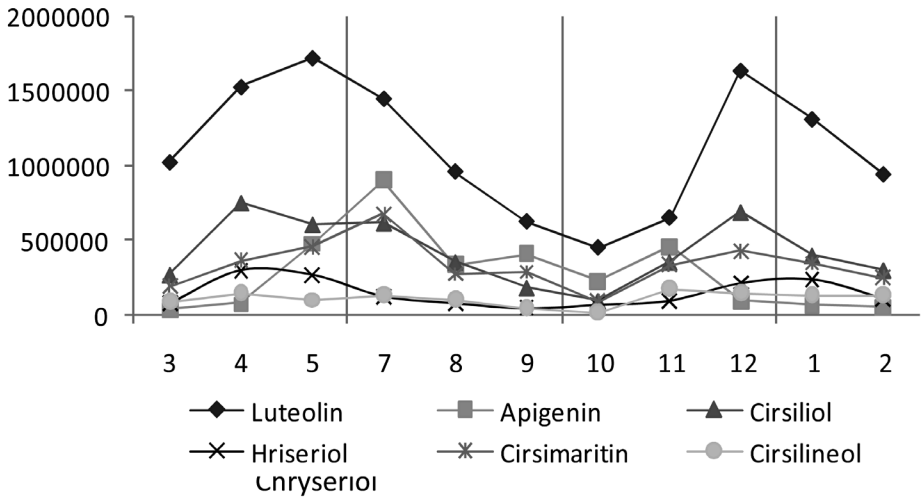

Fig. 3. Seasonal variations in the content of six flavone aglycones in *Teucrium polium* (1-12 number of mounths)

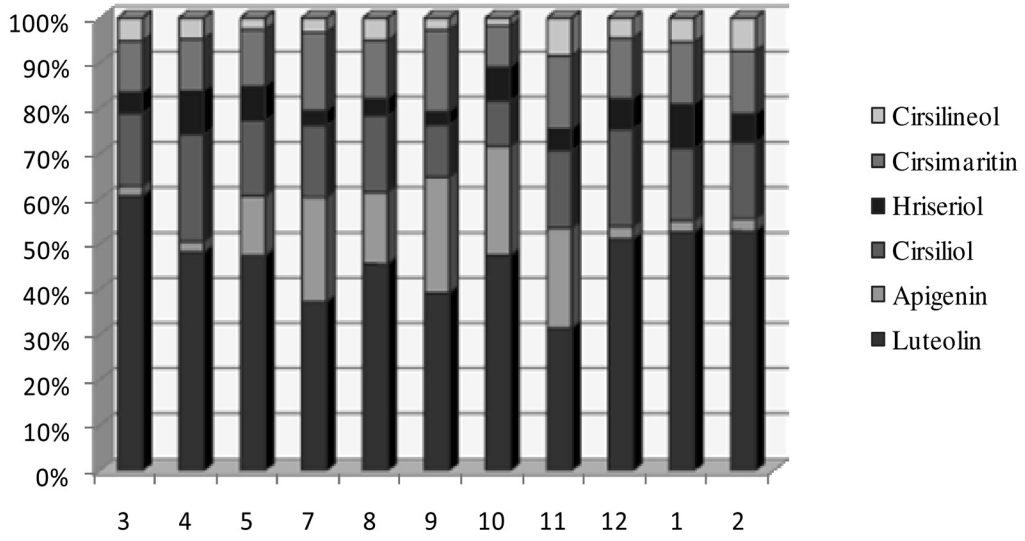

Fig. 4. Relative ratio of the flavonoids in *Teucrium polium* during one season (1-12 numbers of months)

From the presented results (Table 4 and Fig. 3 and Fig. 4), it could be noticed that there are no qualitative variations in the flavone aglycons in *T. polium* during one season, as all six flavone aglycones were identified in each month. On the other side, the content of the each flavone aglycone and the content of the total flavonoids vary a lot during the whole season. For instance, cirsilineol ranged from 1 in October to 9 in April and in December, cirsimaritin from 5 in October to 42 in July, while luteolin from 28 in October rise to 107 in May. The variations of each flavonoid were different and the highest amount of lutolin was found in May, for apigenin in July, for cirsiliol and diosmetin in April, for cirsimaritin in July and for cirsiliol in November. The content of total flavonoids was the highest in the period from May to July, when in the composition of flavonoids, luteolin was dominated aglycone, followed by apigenin and than by cirsiliol and cirsimaritin. From the Fig. 5, it could be seen that luteolin was the dominated aglycon during the whole season. According to these results, the over ground parts of *T. polium* collected in the period from May to July will have the highest quantity of total flavonoids. This period correspond to flowering phase of *T. polium*. The period from August to November is period of fruiting and is not convenience for collecting of the plant material, as the content of total flavonoids declines.

Conclusion

Six flavone aglycones (luteolin, apigenin, diosmetin, cirsiliol, cirsimaritin and cirsilineol) were identified by HPLC method in the hydrolyzed extracts of the over ground part of *Teucrium polium* from R. Macedonia. The most abundant flavone was luteolin, followed by apigenin and cirsimaritin. Great seasonal variations were found in the content of each flavonoid and in the total flavonoids amount. The content of all flavonoids was the highest in the period from May to July, which could be recommended as the most convenient period for collecting of plant material from *T. polium,* rich in flavonoids. Opposite to this, the period of maturation was characterized with the lowest total amount of the flavonoids and represents the period in the year when the collection of plant material should be highly avoided.

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Резиме

Сезонски варијации на флавоноиди во *Teucrium polium* L. (Lamiaceae)

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Клучни зборови: *Teucrium polium*, флавоноиди, HPLC анализа, сезонски варијации

Целта на трудот е идентификација на флавонски агликони и определување на содржина на поединечни и на вкупни флавоноиди, како и испитување на можни сезонски варијации на флавоноиди во *Teucrium polium* L. (Lamiaceae). Примероците од растението се собирани од шест различни локалитети во Република Македонија, во текот на летото во 1999, во 2000 и во 2003 година. За утврдување на сезонските варијации примероците се собирани во с. Колешино, во 2004 година, секој месец во текот на целата година. Во хидролизираните екстракти од надземните делови од *Teucrium polium*, со HPLC метод се идентификувани шест флавонски агликони (лутеолин, апигенин, диосметин, цирсилиол, цирсимаритин и цирсилинеол), меѓу кои доминираат флавонот лутеолин, а потоа апигенин и цирсимаритин. Во содржината на поединечните, како и во содржината на вкупните флавоноиди најдени се големи сезонски варијации. Доминантен флавон во текот на целата сезона е лутеолин, најмногу застапен во текот на месец мај. Содржината на вкупните флавоноиди е најголема во периодот од мај до јули и овој период може да се препорача како најсоодветен период во вегетационият развој на растението за собирање на растителниот материјал од *Teucrium polium*.