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Short communication

HPLC and UV-spectrophotometry analysis of flavonoids in spray-dried and freez-dried extracts of *Teucrium polium* L. (Lamiaceae)

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Abstract

The aim of the study was identification and determination of the content of flavonoids in dry extracts of *Teucrium polium*, collected from Republic of Macedonia. Two different drying procedures were used, freeze and spray draying. In freeze-dried (FDE) and spray-dried (SDE) extracts of aerial parts of *T. polium* five flavone aglycones (luteolin, apigenin, cirsiliol, cirsimaritin and cirsilineol) were identified by HPLC method, on the base of retention times and UV spectral data of the components of the extracts in comparison to the authentic samples of flavonoids. Additionally, seven glycosides of apigenin and luteolin were tentatively identified. No significant differences in the composition nor in the content of identified flavonoids were found between both extracts. The content of total flavonoids determined by UV-spectrophotometry with $AlCl_3$ was 72.8 ± 0.62 mg Lut/g and 73.34 ± 0.53 mg Lut/g in FDE and SDE extract of *T. polium*, respectively.

Key words: *Teucrium polium*, freeze-dried extract, spray-dried extract, flavonoids, HPLC analysis, total flavonoids

Introduction

Teucrium polium L. (Lamiaceae) is widely distributed wild-growing plant in Republic of Macedonia (RM) (Micevski, 1998) and abounds in southwestern Asia, Europe, and North Africa (Mashreghi and Niknia, 2012). This flowering perenial small shrub can grow up to 30 cm and has a callous white exterior. Infusion of the leaves and flowers is consumed as a appetite stimulating beverage. Some biological and therapeutic effects have been reported for the plant such as anti-oxidant (Kadifkova Panovska et al., 2005; Ardestani et al., 2008; Esmaeili et al., 2009), anti-inflammatory (Tariq et al., 1989; Capasso et al., 1983), anti-nociceptive (Baluchnejadmojarad et al., 2005; Abdollahi et al., 2003), anti-pyretic (Autore et al., 1984), antimicrobial (Autore et al., 1984; Zerroug et al., 2011), hypolipidemic (Rasekh et al., 2001, Esmaeili and Yazdanparast, 2004; Stefkov et al., 2011), hepatoprotective (Panovska et al., 2007), cytotoxic and apoptotic effects (Rajabalian et al., 2008). It is also found that protects against ethanol-induced gastric mucosal damage (Mashreghi and Niknia, 2012).

Even so, there is a presence of different classes of secondary metabolites, such as diterpenes (Malakov and Papanov, 1983; Marquez and Valverde, 1979), essential oil (Cozzani et al., 2005; Moghtader, 2009; Kabouche et al., 2007), phenylethanoid glycosides (Oganesyan et al.,1991), *etc*, probably the most important group of compounds in *T. polium* are the flavonoids. Mainly, flavone glycosides with

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highly methylated aglycons are identified until now (Verykokidou-Vitsaropoulou and Vajias, 1986; Rizk et al., 1986; Kawashty et al., 1999; Harborne et al., 1986; Sharififar et al., 2009). Recently was published that freeze-dried extract of *T. polium* that contain flavonoids posses insulinotropic and antihyperglycemic effects (Stefkov et al., 2011). It is known that freeze-draying or lyophilisation or cryodesication is dehydratation process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-draying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase. Spray-drying is a method of producing a dry powder from a liquid or slurry by rapidly drying with hot gas. This is the preferred method of drying of many thermally-sensitive materials such as food and pharmaceuticals. Today, freeze-dried and spray-dried extracts are the preferable forms of dry extracts in production of herbal medicinal products. Spray-dried extracts are suitable for production of cold water soluble tea and herbal extracts, hot water soluble tea, industrial use for the food and beverage industries, dry sugar mixes, "Ready to Drink" tea beverages, various food products, and tea powder blends like "2 in 1" or "3 in 1" milk mixes. Freeze dried extracts are mainly hot water soluble, suitable for consumer packs of high value products.

Freeze- and spray-drying are different process and works on different ways. The aim of the present study is analysis of flavonoids in dry extracts of *T. polium*, obtained by two methods of draying, as a part of the first step of eventual standardization of these herbal preparations for further use in production of medicinal products.

Material and methods

Plant material

The over-ground parts of the flowering plant *Teucrium polim* (20-25 cm from the top) were collected during the summer in 2007 near v. Koleshino, south-east part of R. Macedonia. 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, RM.

Reagents and authentic samples

Reagents of HPLC purity were purchased from Sigma Chemical Co. (Germany). Authentic substances: apigenin and luteolin were products of Extrasynthese (France) and cirsimaritin, cirsilineol and cirsiliol 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 plant extracts

A quantity of 5 g of plant material was submitted to hydroalcoholic extraction, in 500 ml ethanol 70% (*V*/*V*). The extractions were made with constant mixing on magnetic stirrer for 24 h. To obtain a solid substance, the alcoholic extracts were concentrated in a rotary evaporator to only water residue remained. The concentrated solution was divided in two equal portions.

First portion was spray-dried under following conditions: inlet temperature 110 °C, outlet temperature 69 °C, pump activity 30%, flow rate of extract 8 ml min^{-1}, and aspiration 100%. Atomization pressure was 1.5 bars. This led to obtain spray-dried extract (SDE) of *T. polium*. The second portion of concentrated extract was freeze-dried on -36 °C under 12 Pa pressure, 12 h, after previous freezing at -80 °C, 30 minutes. This led to obtain freeze-dried extract (FDE) of *T. polium*.

HPLC analysis

Flavonoid aglycones and glycosides in dried extracts were identified 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), and the elution program for extracts screening was the following: 0–5 min 85% A; 10–20 min 80% A; 25–30 min 75% A; 40–45 min 65% A; 50-55 min 55 A; and 65-70 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 (Stefkov, 2006).

Part of the dried extracts were dissolved in appropriate amount of methanol for HPLC analysis. The elution was monitored in the whole UV range and the chromatograms for flavones 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 comparing the peak area of flavones in the HPLC chromatograms at 348 nm.

UV-spectrophotometry of total flavonoids

The content of total flavonoid was determined using modified UV-spectrophotometric method for determination of flavonoids by Ph.Eur.7. 10 mg of dry SDE or FDE extracts were hydrolyzed in 50 ml acetone with 2 ml conc. HCl in a presence of 0.5 ml 10% solution of urotropine, 1 h. After cooling the mixture was transferred to 50 ml volumetric flask and filled up with acetone. 20 ml of this were transferred into separating funnel and after adding 20 ml water extracted with ethyl acetate, 3 x 15 ml. Ethyl acetate

Table 1. Identified flavonoids in spray-dried (SDE) and freez-dried extract (FDE) of *Teucrium polium*

Flavone	Structure	SDE (t_R)	FDE (t_R)	St (t_R)
Apigenin-glycoside	-	5.80	5.81	-
Luteolin-glycoside	-	14.30	14.35	-
Luteolin-glycoside	-	15.41	15.47	-
Apigenin-glycoside	-	16.53	16.57	-
Apigenin-glycoside	-	17.74	17.79	-
Apigenin-glycoside	-	18.80	18.84	-
Apigenin-glycoside	-	20.67	20.70	-
Luteolin	3',4',5,7-tetrahydroxyflavone	31.35	31.39	31.35
Apigenin	4',5,7-trihydroxyflavone	41.20	41.23	41.21
Cirsiliol	3',4',5-trihydroxy-6,7-dimethoxyflavone	43.24	43.28	43.25
Cirsimaritin	4',5-Dihydroxy-6,7-dimethoxyflavone	52.20	52.22	52.23
Cirsilineol	4',5-dihydroxy-3',6,7-trimethoxyflavone	53.70	53.74	53.72

St - mixture of standard substances of flavonoids

fractions were collected together, washed with water, 3 x 25 ml, dried with anhydrous Na_2SO_4, filtrated into another 50 ml volumetric flask and filled up with ethyl acetate. 10 ml of this was used for developing complex with 1 ml $AlCl_3$ in 25 ml volumetric flask, filled up with acetic methanol. The absorbance was measured after 30 min at 390 nm, against the same prepared solution without $AlCl_3$ used as a blank. The content of total flavonoids was expressed in term of luteolin equivalent (mg Lut / g extract). All meausures were repeated three times.

Results and discussion

Spray-dried extract of *T. polium* (SDE) was dry, pale green, amorphous powder with characteristic smell and extremely bitter taste, with 3.73% moisture. Freez-dried extract (FDE) was dry, dark green powder with characteristic smell, extremely bitter taste and 3.27% moisture.

The HPLC analysis of dissolved dry extract in methanol (1 mg ml^{-1}) and comparison of the retention times and UV spectral data of selected picks with the appropriate data obtained for standard substances showed presence of 5 different flavones in both dried extracts of *T. polium* and seven glycosides of apigenin and luteolin (Table 1). The free flavone aglycones could be easily identified as luteolin, apigenin, cirsiliol, cirsimaritin and cirsilineol. Identification of the flavone-glycosides was made tentatively as apigenin- and luteolin-glycosides, identified in both, SDE and FDE, extracts of *T. polium,* recognized by comparing the UV-spectral data with literature and previously published data (Andersen and Markham, 2006). The obtained HPLC chromatograms are presented in Fig. 1.

The obtained results showed no differences in the composition of flavonoids (Table 1, Fig. 1.), in both dry extracts of *T. polium* identical free flavones and flavone glycosides were identified. Semi–quantitative analysis of peak area of the components in the extracts have shown almost identical abundance of identified flavonoids (Table 2).

Table 2. Semi-quantitative HPLC analysis of flavonoids in spray-dried (SDE) and freez-dried extract (FDE) of *Teucrium polium*

Flavone	SDE	FDE
Apigenin-glycoside	+	+
Luteolin-glycoside	+	+
Luteolin-glycoside	+	+
Apigenin-glycoside	+	+
Apigenin-glycoside	+	+
Apigenin-glycoside	+	+
Apigenin-glycoside	+	+
Luteolin	+	+
Apigenin	+++	+++
Cirsiliol	+++	+++
Cirsimaritin	+	+
Cirsilineol	tr	tr

(+) – present; (+++) – dominate; tr – trace amounts

The content of total flavonoids

Determination of the content of flavonoids can be made by different methods and most often HPLC or UV-spectrophotometry with $AlCl_3$ (complex-making reagent) are used. In case when appropriate authentic substances of flavonoids were not commercially available such it was the case with *T. polium* flavone glycosides, UV-spectrophotometry was chosen as more appropriate. The content of flavonoids was expressed in terms of luteolin equivalent (the standard curve equation of luteolin was: $y = 0.0132 + 0.0044$; $R^2 = 0.9993$), mg Lut/g extract. The obtained results showed no significant differences in the content of to-

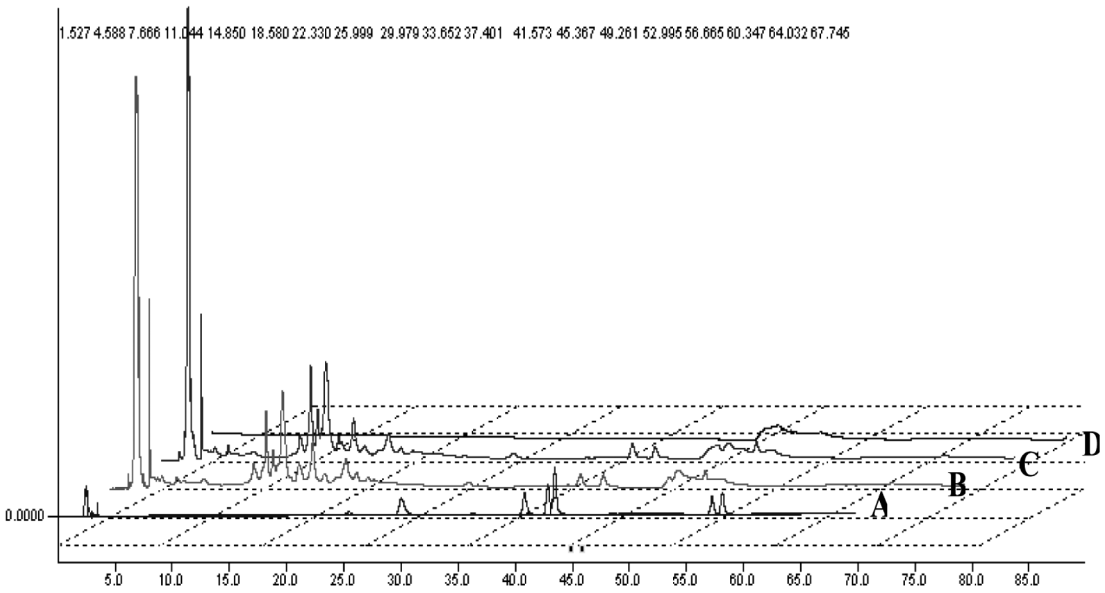

Fig. 1. HPLC chromatograms of mixture of standard substances (A), SDE extract (B), FDE extract (C) and blank (D).

tal flavonoids as 72.8 ± 0.62 mg Lut/g and 73.34 ± 0.53 mg Lut/g was found in FDE and SDE extract of *T. polium*, respectively. These are the first results on content of total flavonoids in dry extract of *T. polium*. Previously, it was reported that dried aerial parts of *T. polium* from RM contain 0.2% of total flavonoids, determined by UV-spectrophotometry (Kadifkova Panovska et al., 2005).

Conclusion

In freeze-dried (FDE) and spray-dried (SDE) extracts of aerial parts of *T. polium* five flavone aglycones (luteolin, apigenin, cirsiliol, cirsimaritin and cirsilineol) were identified by HPLC method, on the base of retention times and UV spectral data of the components of the extracts in comparison to the authentic samples of flavonoids. Additionaly seven glycosides of apigenin and luteolin were tentatively identified. No differences in the composition or in the semi-quantitative abundance of identified flavonoids were found between both extracts, in spite of differences in draying procedure. The content of total flavonoids determined by UV-spectrophotometry with $AlCl_3$ was 72.8 ± 0.62 mg Lut/g and 73.34 ± 0.53 mg Lut/g in FDE and SDE extract of *T. polium*, respectively.

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Резиме

HPLC and UV-спектрофотометриска анализа на флавоноиди во суви екстракти од *Teucrium polium* L. (Lamiaceae), добиени со спреј сушење и со лиофилизација

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Клучни зборови: *Teucrium polium*, лиофилизиран екстракт, спреј-сушен екстракт, флавоноиди, HPLC анализа, вкупни флавоноиди

Цел на испитувањето е идентификација и определување на содржина на флавоноиди во суви екстракти од *Teucrium polium*, собран од Република Македонија. Користени се две различни постапки за сушење, постапка со лиофилизација и постапка на спреј-сушење. Во лиофилизираните (FDE) и во спреј-сушените (SDE) екстракти од надземните делови на *T. polium,* со HPLC анализа и врз база на ретенционите вемиња и UV спектралната анализа, идентификувани се пет флавонски агликони (лутеолин, апиге-

нин, цирсилиол, цирсимаритин и цирсилинеол), во споредба со автентични примероци од флавоноиди. Дополнително, тентативно се идентификувани седум различни хетерозиди на апигенин и лутеолин. Во двата екстракти не се забележани разлики ниту во составот ниту во содржината на идентификуваните флавоноиди. Содржината на вкупните флавоноиди е определена со UV-спектрофотометрија со $AlCl_3$ и изнесува 72.8 ± 0.62 mg Lut/g и 73.34 ± 0.53 mg Lut/g во FDE и SDE екстракт од *T. polium*, соодветно.