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HPLC ANALYSIS OF PHENOLIC ACIDS IN MOUNTAIN GERMANDER (*Teucrium montanum* L.) EXTRACTS

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The methanol, petroleum ether, chloroform, ethyl acetate, 1-butanol and water extracts were obtained by extraction of mountain germander (Teucrium montanum L.). The total phenolic content in extracts was measured by Folin-Ciocalteu method. The 1-butanol extract had the highest phenolic content (296.00 mg/g).

High performance liquid chromatography (HPLC) was employed to define qualitative and quantitative content of phenolic acids in mountain germander extracts. The largest number of phenolic acids were determined in ethyl acetate and 1-butanol extracts, while these acids were not present in petroleum ether extract. The highest content of phenolic acids (28.619 mg/g) had ethyl acetate extract and gentisic acid (14.432 mg/g) was its major component. Despite of a large number of phenolic acids in 1-butanol extract, their content was only 3.740 mg/g.

KEYWORDS: Mountain germander (*Teucrium montanum* L.); extraction; phenolic acids; HPLC

INTRODUCTION

Polyphenolic compounds, such as phenolic acids and flavonoids, are important constituents in many plants, and their identification and quantification can give vital information relating to antioxidant function, food quality, and potential health benefits. Phenolic acids are attractive as they are known to act as potentially protective factors against cancer and heart diseases (1-4). Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids (Fig. 1). The most common hydroxybenzoic acid derivatives are *p*-hydroxybenzoic, vanillic, gallic and protocatechuic acids which are mainly present in the form of glucosides in foods (5). The most common forms of hydroxycinnamic acid are *p*-coumaric,

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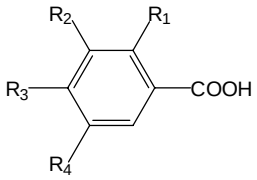
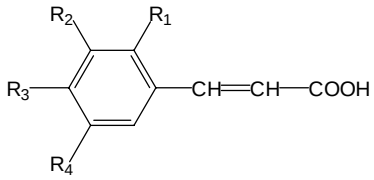
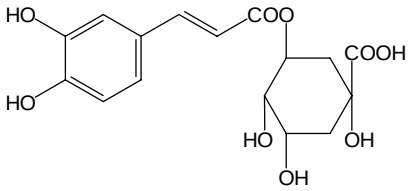
caffeic, and ferulic acids which frequently occur in foods as simple esters with quinic acid or glucose (5). Likely, the most familiar of these is chlorogenic acid.



$R_1=R_2=R_4=H$, $R_3=OH$	*p*-Hydroxybenzoic acid
$R_1=R_4=H$, $R_2=R_3=OH$	Protocatechuic acid
$R_1=R_4=H$, $R_2=OCH_3$, $R_3=OH$	Vanillic acid
$R_1=H$, $R_2=R_3=R_4=H$	Gallic acid
$R_1=R_4=OH$, $R_2=R_3=H$	Gentisic acid





Chlorogenic acid

$R_1=R_2=R_4=H$, $R_3=OH$	*p*-Coumaric acid
$R_1=R_4=H$, $R_2=R_3=OH$	Caffeic acid
$R_1=R_4=H$, $R_2=OCH_3$, $R_3=OH$	Ferulic acid

Fig. 1. Chemical structures of phenolic acids

Mountain germander (*Teucrium montanum* L.) is a grass crop which grows in zones of mountain scrub, low zones, preferably limestone, and blooms from May to end of summer (6). It has long been consumed both as an herbal medicine and as a nourishing food. In traditional medicine, it is widely used as diuretic, stomachic, analgesic and antispasmodic agent and also possesses antibacterial, antifungal, antiinflammatory and antioxidative activity.

This paper is concerned with high performance liquid chromatography (HPLC) qualitative and quantitative analysis of phenolic acids in mountain germander extracts obtained by successive extraction with solvents of different polarities.

EXPERIMENTAL

Methanol, ethyl acetate, petroleum ether, chloroform and 1-butanol were purchased from "Zorka" Šabac, Serbia and Montenegro. Standards of phenolic acids (gallic, protocatechuic, gentisic, vanillic, syringic, chlorogenic, caffeic, coumaric, ferulic and 3,5-dimethoxy-4-hydroxycinnamic acid), acetonitrile and acetic acid were supplied from Sigma Chemicals Co., USA. Folin-Ciocalteu reagent was from Fluka, USA.

Plant material mountain germander (*Teucrium montanum* L.) was collected in August 2002 from the region of Zlatibor. Plant material was dried in a drying cabinet with forced ventilation at 40°C for 2 days.

Preparation of extracts. Dried plant of mountain germander (20 g) was extracted with 70 % methanol (2 × 500 ml) at room temperature for 2 × 24 h. The 20% v/v of obtained extract was evaporated to dryness under reduced pressure and used further as methanol extract. The rest of the extract (80% v/v) was concentrated under reduced pressure. After removing methanol, the extract was successively treated with petroleum ether (2 × 20 ml), chloroform (2 × 20 ml), ethyl acetate (2 × 20 ml) and 1-butanol (2 × 20 ml). The petroleum ether, chloroform, ethyl acetate, 1-butanol and water extracts were evaporated to dryness under reduced pressure. The yields, average of triplicate analysis, of extracts were: methanol, m = 0.9588 ± 0.0398 g; petroleum ether, m = 0.1195 ± 0.0046 g; chloroform, m = 0.1554 ± 0.0052 g; ethyl acetate, m = 0.1065 ± 0.0053 g; 1-butanol, m = 1.1132 ± 0.0533 g and water, m = 2.2759 ± 0.1038 g.

Total phenolic content. Total phenolic compounds in extracts were determined spectrophotometrically using the Folin-Ciocalteu reagent. The results are expressed as chlorogenic acid equivalents per g dry weight (7). All measurements were performed at least in triplicate, and presented as mean ± SD.

High performance liquid chromatography. HPLC was performed on a liquid chromatograph HP1090 (Hewlett-Packard) with diode-array detector (DAD). Hypersil MOS column (200 × 2.1 mm) with a 5 μm particle size was used at the flow rate of 0.2 ml/min. The mobile phase was H_2O adjusted with acetic acid to pH 2.8 (solvent A) and acetonitrile (solvent B) and the HPLC separation was performed by the following linear gradient: 1-40% B in 50 min, 40-1% B in 10 min. The injected volume was 10 μl of 1% methanolic solution of extracts and standards. The spectra were acquired in the range 210-400 nm and chromatograms were plotted at 290/4 nm with reference wavelength 550/100 nm. All injections were performed in duplicate.

RESULTS AND DISCUSSION

The total phenolic content of different mountain germander extracts is shown in Table 1. 1-Butanol extract had the highest phenolic content (296.00 mg/g), while the phenolic compounds were not found in petroleum ether extract. In the methanol, chloroform, ethyl acetate and water extracts phenolic compounds were present in a lesser extent than 1-butanol extract.

Table 1. Total phenolic content in mountain germander extracts

Extract	Total phenolic content (mg/g)
Methanol	154.00 ± 6.7
Petroleum ether	0
Chloroform	0.0956 ± 0.003
Ethyl acetate	32.40 ± 1.56
1-Butanol	296.00 ± 12.72
Water	59.80 ± 2.54

Based on these results, HPLC analysis was employed to define qualitative and quantitative content of phenolic acids in mountain germander extracts.

HPLC chromatograms of mountain germander extracts are presented in Fig. 2. Hydroxyl derivatives of benzoic (gallic, protocatechuic, gentisic, vanillic and syringic acid) and cinnammic acid (chlorogenic, caffeic, *p*-coumaric, ferulic and 3,5-dimethoxy-4-hydroxycinnamic acid) were identified in the investigated extracts by comparing their retention times (RT) and on-line ultraviolet (UV) spectra with those of standards (Fig. 3, Table 2).

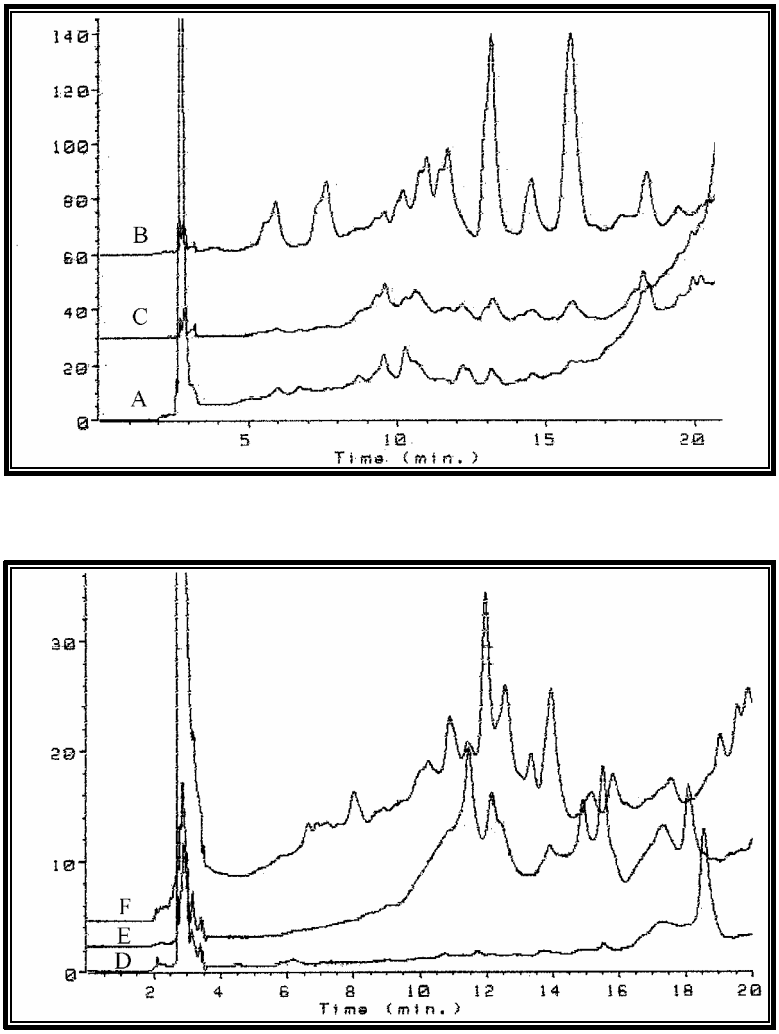


Fig. 2. HPLC chromatograms of mountain germander extracts: A – methanol extract, B – ethyl acetate extract, C - 1-butanol extract, D – petroleum ether extract, E – chloroform extract, F –water extract (see Table 2 for peak identification)

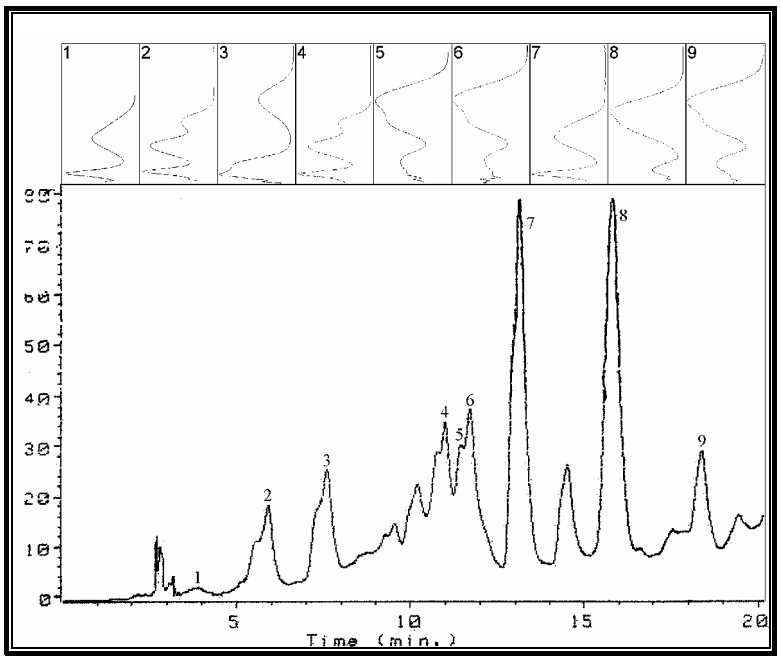


Fig. 3. HPLC chromatogram of ethyl acetate extract and UV spectra of identified phenolic acids
1 - Gallic acid, 2 - Protocatechuic acid, 3 - Gentisic acid, 4 - Vanillic acid, 5 - Caffeic acid, 6 - Chlorogenic acid, 7 - Syringic acid, 8 - Coumaric acid, 9 - Ferulic acid

The results of qualitative HPLC analysis showed that the greatest number of hydroxyl derivatives of benzoic and cinnammic acid are present in ethyl acetate and 1-butanol extracts (Table 2). These phenolic acids were not detected in petroleum ether extract, while chlorofom extract contained only caffeic and syringic acids.

Based on the results of quantitative HPLC analysis (Table 3), it can be concluded that the highest content of phenolic acids (28.619 mg/g) had ethyl acetate extract. The content of gentisic acid (14.432 mg/g) was shown to be the major component in ethyl acetate extract, while the other phenolic acids were present in a lesser extent. Despite of a large number of phenolic acids in 1-butanol extract, their content was only 3.740 mg/g. The content of phenolic acids in other investigated extracts was less than 0.5 mg/g.

In general, the total phenolic content in investigated extracts was higher than the content of phenolic acids identified by HPLC. The content of phenolic acids in ethyl acetate and chloroform extracts was 28.619 and 0.086 mg/g, respectively, and those values were somewhat lower than their total phenolic contents (32.40 and 0.0956 mg/g, respectively). However, the total phenolic content in methanol, 1-butanol and water extracts determined by the Folin-Ciocalteu method was significantly higher than the content of phenolic acids identified by HPLC. The obtained results can be explained by the fact that phenolic compounds are distributed in investigated extracts as a function of polarity of applied solvents (8). In our investigation, ethyl acetate appeared the best solvent for extraction of phenolic acids.

Table 2. Retention times (RT) and UV_{max} absorption of the phenolic acids in mountain germander extracts

Phenolic acid	Retention time (min)						UV max (nm)
	Methanol extract	Ethyl acetate extract	1-Butanol extract	Petroleum ether extract	Chloroform extract	Water extract	
Gallic	-	3.912	3.892	-	-	-	222, 270
Protocatechuic	5.969	5.908	5.961	-	-	-	212, 222, 258, 292
Gentisic	-	7.599	-	-	-	-	212, 222, 230, 324
Vanillic	-	10.994	9.570	-	-	12.568	222, 260, 290
Caffeic	10.576	11.445	10.282	-	13.907	13.957	214, 238, 296, 322
Chlorogenic	-	11.699	10.608	-	-	13.354	214, 218, 222, 240, 298, 324
Syringic	13.152	13.148	13.190	-	14.916	15.170	222, 274
Coumaric	15.872	15.827	15.891	-	-	19.052	216, 228, 294, 308
Ferulic	18.474	18.395	18.257	-	-	-	218, 236, 294, 322
3,5-Dimetoxy-4-hydroxycinnamic	-	-	20.224	-	-	-	214, 236, 322

Table 3. The content of phenolic acids in mountain germander extracts

Phenolic acids	Contents of phenolic acids in extract (mg/g)					
	Methanol extract	**Petroleum ether extract**	**Chloroform extract**	**Ethyl acetate extract**	**1-Butanol extract**	**Water extract**
Gallic	-	-	-	0.132	0.004	-
Protocatechuic	0.094	-	-	1.337	0.051	-
Gentisic	-	-	-	14.432	-	-
Vanillic	-	-	-	1.944	0.982	0.165
Caffeic	0.125	-	0.015	0.515	0.210	0.059
Chlorogenic	-	-	-	3.076	0.949	0.147
Siringic	0.148	-	0.071	4.588	0.536	0.052
Coumaric	0.018	-	-	1.794	0.174	0.0085
Ferullic	0.054	-	-	0.811	0.582	-
3,5-Dimetoxy-4-hydroxycinamic	-	-	-	-	0.252	-
Total	0.439	-	0.086	28.619	3.740	0.4315

CONCLUSION

1-Butanol extract had the highest phenolic content (296.00 mg/g), while petroleum ether extract did not contain phenolic compounds.

Emlpoying qualitative HPLC analysis, the largest number of hydroxyl derivatives of benzoic and cinnammic acid were determined in ethyl acetate and 1-butanol extracts, and these phenolic acids were not detected in petroleum ether extract.

The highest content of phenolic acids (28.619 mg/g) was determined in ethyl acetate extract using quantitative HPLC analysis. Gentisic acid (14.432 mg/g) was the major component in this extract.

Despite of a large number of phenolic acids in 1-butanol extract, their content was only 3.740 mg/g.

The content of phenolic acids in other investigated extracts was less than 0.5 mg/g.

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HPLC АНАЛИЗА ФЕНОЛНИХ КИСЕЛИНА У ЕКСТРАКТИМА ТРАВЕ-ИВЕ (*Teucrium montanum* L.)

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Екстракцијом траве-иве (*Teucrium montanum* L.) добијени су метанолни, петролетарски, хлороформски, етилацетатни, 1-бутанолни и водени екстракти. Садржај укупних фенолних једињења у добијеним екстрактима, одређен је спектрофотометријски, Folin-Ciocalteu методом. 1-Бутанолни екстракт је имао највећи садржај укупних фенолних једињења (296.00 mg/g), док у петролетарском екстракту она нису била присутна.

Квалитативни и квантитативни састав фенолних киселина у екстрактима траве-иве одређен је течном хроматогарфијом под високим притиском (HPLC). Етилацетатни и 1-бутанолни екстракти су садржавали највећи број хидрокси деривата бензоеве и циметне киселине. Ове киселине нису присутне у петролетарском екстракту, док је хлороформски екстракт садржи само кафену и сиринги чну киселину. Етилацетатни екстракт је имао највећи садржај фенолних киселина (28,619 mg/g), а гентисична киселина (14,432 mg/g) је била главни конституент. Упркос великом броју, садржај фенолних киселина у 1-бутанолном екстракту је износио свега 3,740 mg/g. Садржај фенолних киселина у метанолном, хлороформском и воденом екстракту био је мањи од 0,5 mg/g.

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