RESEARCH ARTICLE

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Phenolic acid profiles of endemic species *Verbascum anisophyllum* and *Verbascum davidoffii*

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ABSTRACT
The profiles of methanol extractable and methanol insoluble bound phenolic acids of two species: *Verbascum anisophyllum* Murb (Balkan endemic) and Verbascum davidoffii Murb. (Bulgarian endemic) were determined. Free radical scavenging activity and total phenolic content of studied extracts and fractions were evaluated by DPPH antioxidant method and Folin–Ciocalteu reagent, respectively. Phenolic acid profiles were analyzed by GC/MS. Sixteen phenolic acids and their derivatives were detected. Ferulic acid was the major individual phenolic acid presented in all extracts and fractions. Hydroxycinnamic, vanillic and p-hydroxybenzoic acids were also abundant in the studied phenolic acid profiles. The presence of gentisic, syringic, isoferulic, dihydroferulic, eudesmic, 3,5-di-tert-butyl-4-hydroxybenzoic acids were reported for the first time to *Verbascum* species. The greatest variety of phenolic acids was found in the fractions containing methanol insoluble bound hydrolysable phenolic acids. The highest free radical scavenging activity and total phenolic content were established for methanol extractable alkaline hydrolysable fractions. Phenolic acid profiles and free radical scavenging activity of both species were similar although *V. davidoffii* contained few more components. The present study is the first report on the phenolic acid profiles and free radical scavenging activity of *V. anisophyllum* and *V. davidoffii* as well as the first detailed study of the phenolic acid profiles of the *Verbacum* species.

Key words: alkaline and acid hydrolysis, DPPH, GC/MS, mullein

Introduction

With the development of modern analytical chemical methods, it has been possible to study the content of biologically active substances in rare, endemic plant species using minimal amounts of plant material. As part of an extensive phytochemical and genetic study of rare Bulgarian species, the present study characterizes the content of phenolic acids in two endemic species of the *Verbascum* genus. *Verbascum davidoffii* Murb. (*Scrophulariaceae*) is a biennial herbaceous plant, Bulgarian endemic (Petrova, 2006), protected by the National Biodiversity Act (2002), included in the Red List of vascular plants in Bulgaria (Assyov & Denchev, 2009) and in the Red Data Book of the Republic of Bulgaria (Assyov & Denchev, 2015) classified as "Critically Endangered" according to the IUCN criteria. All known localities are within Pirin National Park. *Verbascum anisophyllum* Murb. is a Balkan endemic plant. The species is critically endangered according to the IUCN criteria. It is included in the Red Data Book of Bulgaria and protected by the Biodiversity Act (2002). In Bulgaria, the species exist under three populations: one in Konyavska Planina Mt. (Znepole floristic region) and the other near Vukovo village (floristic region Valley of River Struma).

Verbascum species are valuable medicinal plants used mainly in the treatment of respiratory diseases (Nikolov, 2007). Phenolic constituents of *Verbascum* species are considered to be responsible for the antioxidant activity of the herb (Grigore et al., 2013). Phenolic acids are compounds that possess one carboxylic acid to the phenolic ring. Structurally, they are two main types: hydroxycinnamic and hydroxybenzoic. Phenolic acids are found in plants mainly in the bound form as esters, glycosides and other structures. Therefore, various hydrolysis procedures have been developed to characterize the phenolic acid content in plants (Robbins 2003; Kim et al., 2006; Dvořáková et al., 2008).

The aim of the present study was to determine the profiles of methanol extractable free, methanol extractable alkaline hydrolysable, methanol insoluble bound alkaline and acid hydrolysable phenolic acids of two *Verbascum* species: *V. anisophyllum* Murb and *V. davidoffii* Murb. Free radical scavenging activity and total phenolic content of studied extracts and fractions were estimated.

Materials and Methods

Plant material

Leaf samples from 5 individuals of *V. anisophyllum* and *V. davidoffii* each were randomly collected at flowering stage in July 2015. The population of *V. anisophyllum* is from Vukovo village (N 42.20414; E 22.97331), at 800 m alt. The population of *V. davidoffii* is situated in Pirin Mt., over Banderitsa hut (N 41.76827: E 23.42633) at 1800 m alt.

Extraction procedure

A sample of 100 mg –of dried plant material was placed in 2 mL Eppendorf tubes and extracted with 1 mL of MeOH for 24 h at room temperature. Aliquots of 200 µL from the methanol extract was placed in a glass vial and evaporated to examine for methanol extractable free phenolic acids. Aliquot of 800 µL was transferred in other Eppendorf tubes and was added of 500 µL H_2O and 500 µL of $CHCl_3$, vortexing for 2 min, and the mixture was centrifuged. The chloroform fraction was removed. The rest of the aqueous fraction was hydrolyzed with 0.5 mL of 1N NaOH for 18 h at 60°C. After acidification to pH 1-2 with conc. HCl, the phenolic compounds were extracted with EtOAc (2x500 µL) which was dried with anhydrous Na_2SO_4 and evaporated to obtain methanol extractable alkaline hydrolysable phenolic acids. Plant material remaining after methanol extraction was hydrolyzed subsequently first by 2 M NaOH, 4 h, at room temperature followed by acid hydrolysis by 6 M HCl 18 h at 60°C resulting in two fractions methanol insoluble bound alkaline and acid hydrolysable phenolic acids, respectively.

The obtained extractions and fractions were silylated with 50 µL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 µL of pyridine for 2 h at 50°C.

GC–MS analysis

The GC–MS spectra were recorded on a Thermo Scientific Focus GC coupled with Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. ADB-5MS column (30 m x 0.25 mm x 0.25 µm) was used. The temperature program was: 100-180 °C at 15 °C x min-1, 180-300 20 at 5 °C x min-1 and 10 min hold at 300 °C. The injector temperature was 250 °C. The flow rate of carrier gas (Helium) was 0.8 mL x min-1. The split ratio 1:10 1 µL of the solution was injected. The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant-specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Free radical scavenging activity

The effect of methanolic extracts on DPPH radicals was estimated according to Stanojević et al. (2009). The results were calculated by Software Prizm 3.00. All experiments were carried out in triplicate.

Total phenolic content

Total phenolic content of the methanolic extracts was determined by employing the method given in the literature involving Folin–Ciocalteu reagent and gallic acid as standard (Giorgi et al., 2009; Nićiforović et al., 2010). The content of total phenols was presented as mean ±standard deviation of tree independent analyses (n=3).

Results

The profiles of methanol extractable free, methanol extractable alkaline hydrolysable, methanol insoluble bound alkaline and acid hydrolysable phenolic acids were determined. Sixteen phenolic acids and their derivatives were detected (Table 1 and Table 2). Ferulic acid was the major individual phenolic acid presented in all extracts and fractions. p-Hydroxycinnamic, vanillic and p-hydroxybenzoic acids were also abundant in the studied phenolic acid profiles. Methanol extractable free phenolic acids were established in trace amounts. Only 4(p)-hydroxycinnamic acid was found of V. anisophyllum extract. V. davidoffii extract contains 4(p)-hydroxybenzoic acid additionally. The largest variety of phenolic acids was established in the fractions containing methanol insoluble bound alkaline and acid hydrolysable phenolic acids. Protocatechuic, siringic, 3,4,5-trimethoxybenzoic, dihydroferulic acids were determined only in these fractions. Phenolic acid profiles of both species were similar although V. davidoffii contained few more components: trans-cinnamic, eudesmic, isoferulic and dihydroferulic acids.

Free radical scavenging activity and total phenolic content of methanolic extract and fractions of both species were determined. The results shown in Fig. 1 and Fig. 2 indicate that methanol extractable alkaline hydrolysable fractions showed the highest activity and total phenolic content. Antiradical activity determined as IC_{50} showed values under 50 µg/mL that is indicator for significant activity. Methanolic extract and other fractions exhibited moderate activity with IC_{50} values about 200 µg/mL. Antiradical activity correlates positively with the content of total phenols in all tested

Table 1. *Phenolic acid profiles of methanolic extract and fractions of Verbascum anisophyllum.*

Phenolic acid		Methanol extractable phenolic acids		Methanol insoluble bound phenolic acids	
	RI	F	A	A	B
4-Hydroxybenzoic acid	1637		3.9±1.1	10.4±1.4	6.6±1.4
cis-4-Hydroxycinnamic acid (cis-p-Coumaric acid)	1684			0.6±0.2	
4-Hydroxy-3-methoxybenzoic acid (*Vanillic acid*)	1753		17.4±2.5	17.9±2.6	18.0±1.6
4'-Hydroxy-3'-methoxyacetophenone	1778				6.8±1.6
2,5-Dihydroxybenzoic acid (Gentisic acid)	1783			0.03±0.0	2.5±0.9
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	1811				0.7±0.4
cis- 4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)	1863		3.3±0.9	1.1±0.5	0.7±0.4
3,5-Dimethoxy-4-hydroxybenzoic acid **(**Syringic acid)	1888			0.7±0.2	0.6±0.3
trans-4-Hydroxycinnamic acid **(**trans-p-Coumaric Acid**)**	1934	100	17.5±2.4	11.3±0.8	9.9±1.8
3,5-Di-tert-butyl-4-hydroxybenzoic acid	2034			0.7±0.3	
trans-- 4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)	2063		66.2±3.8	58.3±2.2	46.7±3.4
trans -3,4-Dihydroxycinnamic acid (Caffeic acid)	2142				0.01±0.0

Legend: F - Free phenolic acids; A - Alkaline hydrolysables phenolic acids; B - Acid hydrolysables phenolic acids; *-Data are expressed as percentage of the total peak area of phenolic acids [%] All values are mean ± SD of the three replicates

Table 2. *Phenolic acid profiles of methanolic extract and fractions of Verbascum davidoffi.*

Phenolic acids		Methanol extractable phenolic acids		Methanol insoluble bound phenolic acids	
	RI	F	A	A	B
trans-Cinnamic acid	1376		0.6±0.2	2.3±0.9	1.2±0.3
4-Hydroxybenzoic acid	1637	30.50	0.7±0.3	2.9±0.3	3.0±0.7
cis-4-Hydroxycinnamic acid (cis-p-Coumaric Acid)	1684		10.4±1.9	2.3±0.5	
3,4,5-trimethoxybenzoic acid (Eudesmic acid)	1736			1.4±0.3	0.6±0.2
4-Hydroxy-3-methoxybenzoic acid (*Vanillic acid*)	1753		4.9±0.9	20.9±2.2	30.7±2.2
3-Hydroxy-4-methoxycinnamic acid (isoferulic acid)	1767		2.6±0.8		0.1±0.05
4'-Hydroxy-3'-methoxyacetophenone	1778				0.9±0.2
2,5-Dihydroxybenzoic acid (Gentisic acid)	1783		2.8±1.2	0.04±0.02	1.4±0.9
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	1811			0.5±0.2	1.4±0.6
Benzenepropanoic acid, 4-hydroxy-3-methoxy (dihydroferulic acid)	1845			0.7±0.3	
cis- 4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)	1863		1.6±0.5	1.5±0.3	
3,5-Dimethoxy-4-hydroxybenzoic acid **(**Syringic acid)	1888			1.4±0.5	0.9±0.3
trans-4-Hydroxycinnamic acid **(**trans-p-Coumaric Acid**)**	1934	69.49	39.5±1.5	26.3±1.5	29.2±1.6
3,5-Di-tert-butyl-4-hydroxybenzoic acid	2034		3.7±0.9	0.6±0.1	1.7±0.4
trans- 4-Hydroxy-3-methoxycinnamic acid (Ferulic acid)	2063		36.3±2.3	37.5±2.8	25.2±1.6
trans -3,4-Dihydroxycinnamic acid (Caffeic acid)	2142		0.29±0.1	2.4±0.7	5.0±0.9

Legend: F - Free phenolic acids; A - Alkaline hydrolysables phenolic acids; B - Acid hydrolysables phenolic acids; *-Data are expressed as percentage of the total peak area [%] All values are mean ± SD of the three replicates

samples. Extracts and fractions of both species displayed similar activity.

Discussion

Phenolic acid profiles of *Verbascum anisophyllum* and *V. davidoffii* were analysed. Sixtheen phenolic acids and their derivatives were identified from which the main were ferulic, p-hydroxycinnamic, vanillic and p-hydroxybenzoic acids. This result is consistent with data reported previously for other species of the genus *Verbascum* (Tatli et al., 2004; Armatu et al., 2011; Boğa et al., 2016). The presence of gentisic, syringic, 3,5-Di-tert-butyl-4-hydroxybenzoic, 3-hydroxy-4-methoxycinnamic (isoferulic acid), benzenepropanoic, 4-hydroxy-3-methoxy (dihydroferulic acid) 3,4,5-trimethoxybenzoic (eudesmic acid) acids were reported for the first time for *Verbascum* species for the best of our knowledge.

Expected the received results confirmed the presence of hydrolysable phenolic acids mainly. Thus, the profiles of phenolic acids are largely determined by the hydrolysis method used. Hydrolysis is mainly performed with a base (NaOH), acid (HCl) or enzymes (pectinases, cellulases, and amylases), but the variety of methods is due to the conditions under which they are carried out (Kim et al., 2006; Stalikas 2007; Dvořáková et al., 2008; Khoddami et al., 2013).

It has been observed that under acidic hydrolysis at high temperature (95°C) cinnamic acid derivatives have been degraded (Kim et al., 2013). In the present study acid

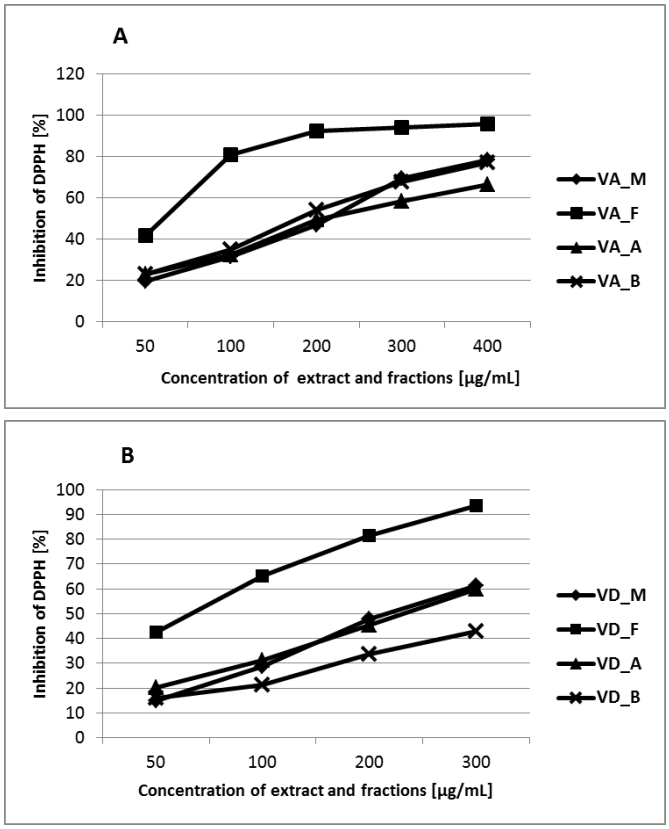

Figure 1. *Free radical scavenging activity of methanolic extracts and hydrolysable fractions of V. anisophyllum (A) and V. davidoffii (B). Legend: VA - V. anysophyllum; VD - V. davidoffii; M - methanolic extracts; F - fraction with methanol extractable alkaline hydrolyzable phenolic acids; A - fraction with methanol insoluble alkaline hydrolyzable phenolic acids; B - fraction with methanol insoluble acid hydrolyzable phenolic acids.*

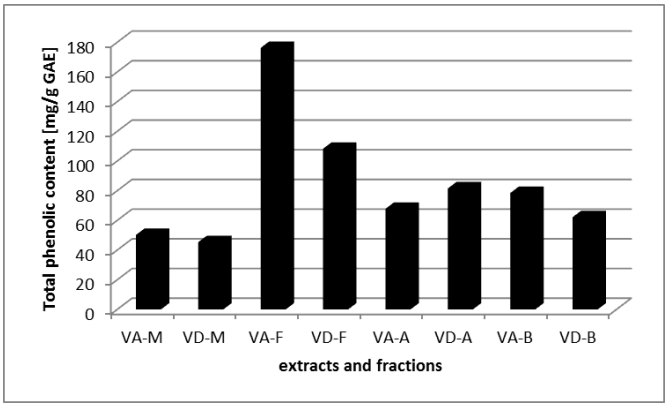

Figure 2. *Total phenolic content of extracts and fractions of V. anisophyllum and V. davidoffii. Legend: VA - V. anysophyllum; VD - V. davidoffii; M - methanolic extracts; F - fraction with methanol extractable alkaline hydrolyzable phenolic acids; A - fraction with methanol insoluble alkaline hydrolyzable phenolic acids; B - fraction with methanol insoluble acid hydrolyzable phenolic acids.*

hydrolysis was carried out at lower temperatures (18°C) but for longer period (18 h), which may have prevented the degradation of these derivatives and as a result the variety of phenolic acids and their amounts in acid-hydrolyzed fraction was found to be similar to alkaline hydrolyzed fraction, even richer. Methanolic extracts and hydrolyzed fractions of both species were evaluated for antiradical activity and total phenolic content. The received results for methanolic extracts of both species are similar to that reported in the literature for other *Verbascum* species (Ozcan et al., 2011). The highest free radical activity and total phenolic content were established for methanol extractable alkaline hydrolysable fractions. These fractions were not the richest in phenolic acids, but the presence of other phenolic compounds such as flavonoid aglycones may explain the high antioxidant action (Shakeri and Farokh 2015). The present study is the first report on the phenolic acid profiles and free radical scavenging activity of Verbascum anisophyllum and *V. davidoffii*. For the best of our knowledge, this is the first detailed study of the phenolic acid profiles of the Verbacum species.

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RESEARCH ARTICLE

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