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New Information about Polyphenols of Wild (Amygdalus webbii) and Cultivated (Amygdalus communis) Almonds from Southwestern Bulgaria

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Abstract. Plant material (flowers and leaves) of wild *Amygdalus webbii* Spach. and cultivated *A. communis* L. individuals growing in the same area were examined for their polyphenolic composition with aim to receive data about the adaptive potential and relationship between them. Eight flavonoid glycosides and two phenolic acids were detected by thin layer chromatography (TLC). They were identified by co- chromatography with authentic markers as quercetin-3-rutinoside (rutin), isorhamnetin-3-rutinoside, kaempferol-3-rutinoside, quercetin-3-galactoside (hyperoside), quercetin-3-glucoside, kaempferol-3-glucoside, quercetin-3-rhamnoside, kaempferol-3-rhamnoside, chlorogenic and cafeic acids. It is a flavonoid composition of the samples of flowers, the leaf samples showed a simpler qualitative flavonoid composition. Rutin, hyperoside and quercetin-3-glucoside were detected as main components of the leaf samples. The comparative TLC analysis of polyphenolic profiles of all studied samples showed that same polyphenolic compounds were present in each almond individual with small variations in relative levels. Total phenolic and flavonoid content of the studied samples were evaluated by spectrophotometric method. Generally the samples of *A. webbii* displayed higher amount of phenolics and flavonoids than that of *A. communis* but the differences were statistically significant only for phenolics in leaf samples. The received results suggest that adaptive capacity definite by phenolic compounds achieved in the early stage of species formation of *A. webbii* is retained and is effective in the cultivated *A. communis* (Bulgarian origin). To the best of our knowledge this is first report for flavonoid composition of flowers of *Amygdalus*.

Key words: flavonoids, phenolic acids, TLC.

Introduction

Phenolic compounds represent the most abundant and the most widely present class of plant natural products. The development of the ability for synthesis of phenolic compounds is a key evolutionary moment in the successful adaptation of amphibious plants to land. So phenolic compounds have been selected throughout the course of evolution in different plants to help them to adapt to variable biotic and abiotic environment (BOUDET, 2007; CHEYNIER *et al.*, 2013).

Almond is an ancient nut crop of southwest Asia, but its wild ancestor has not been properly identified although *Amygdalus fenzliana* (Fritsch) Lipsky has been indicated as the most plausible almond progenitor (LADIZINSKY, 1999). Almond nuts have been studied extensively for their biological properties and nutritional value including phenolic content (MANDALARI *et*

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al., 2010; YILDIRIM *et al.*, 2010; ESFAHLAN *et al.,* 2012; ESFAHLAN & JAMEI, 2012). However, limited information is available concerning phenolics of the leaves of *Amygdalus* sp (MISIRLI *et al.,* 2001; BABAEI *et al.,* 2008). Furthermore to the best of our knowledge there are no data about flavonoid composition of flowers of *Amygdalus*.

In Bulgaria two wild and two cultivated species of *Amygdalus* L. have been spread respectively *A. nana* L., *A. webbii* Spach and *A. communis* L., *A. triloba* (Lindl.)Ricker (VALEV, 1973). Morphologically *A. webii* is distinguished from *A. communis* only by spinose shoots. The present study aimed to obtain some primary data about the adaptive potential and relationship on the basis of polyphenolic composition between the wild *A. webbii* and cultivated *A. communis* individuals growing in the same areas in relatively similar ecological conditions. For this purpose comparative analysis of extracts of flowers and leaves of five individuals of *A. webbii* and *A. communis* was carried out in relation to their polyphenolic composition.

Material and Methods

Plant material. Plant material (flowers and leaves) of five individuals of *Amygdalus webbii* and *A. communis* were collected in the spring of 2013 year. Detail information about origin of the materials is presented at Table 1.

Table 1. Origin of the experimental material.

Taxon	Country	Region	Soil	Altitude	Exposure
Amygdalus webbii	Bulgaria	Western Border Mountain – Maleshevska Mountain, locality Ljubina skala	dry calcareous	about 400 m s.a.l.	E
Amygdalus communis	Bulgaria	Struma valley, hills upper the village of General Todorov, locality "Pripechene"	dry calcareous	about 200 m s.a.l.	NE

Preparation of the extracts. Dry, ground plant material (1g) was extracted with 80% methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subject to subsequent analysis.

Thin layer chromatographic analysis of flavonoid glycosides and phenolic acids. The methanol extracts were examined for flavonoid glycosides and phenolic acids by TLC analysis. Two TLC sorbents and several mobile phases were used. Ethyl acetate:formic acid:acetic acid:MeCOEt: water (50:2:3:30:10) and ethyl acetate:formic acid:acetic acid:water (100:11:11:27) were used as mobile phase for the development of the methanol extracts on silica gel plates Kiselgel 60 F_{254}. Acetic acid–water (15:85, v/v) was used for cellulose plates DC-Alufolien Cellulose 5552 (10 x 20 cm, 0.1 mm layer). Chromatograms were viewed under UV light before and after spraying with 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol. The identification of the compounds was achieved by co-chromatography with authentic markers obtained from Prof. Eckhard Wollenweber.

Determination of total phenolic content. Total phenolic content of the methanol extracts was determined by Folin–Ciocalteu reagent and gallic acid as standard (GIORGI *et al.*, 2009, NIĆIFOROVIĆ *et al.*, 2010). Plant extracts were diluted to the concentration of 1 µg mL^{-1}, and aliquots of 0.5 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of Na_2CO_3 (6%). After 1 h of staying at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract).

Determination of total flavonoid content. An aluminum chloride colorimetric method was used for flavonoid determination, using rutin as a reference compound (MILIAUSKASA *et al.* 2004). One mL of plant extract in methanol (10 g L^{-1}) was mixed with 1 mL aluminium trichloride in ethanol (20 g L^{-1}) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at room temperature. Blank samples were prepared from1 mL plant extract and 1 drop acetic acid, and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts in rutin equivalents (RE).

Statistical analysis. Statistical analysis was carried out using excel. Results were presented as a mean value ± standard deviation (SD). Significant levels were defined at $p<0.05$ as analyzed by t-test.

Results and Discussion

Plant material (flowers and leaves) of five individuals of *Amygdalus webbii* from wild population and cultivated *A. communis* growing in the same area were examined for their polyphenolic composition. Eight flavonoid glycosides were detected by thin layer chromatography (TLC) and comparison with known compounds. They were identified as kaempferol-3-rutinoside (**1**), kaempferol-3-glucoside (**2**), kaempferol-3-rhamnoside (**3**), quercetin-3-rutinoside – rutin (**4**), quercetin-3-galactoside (hyperoside) (**5**), quercetin-3-glucoside (**6**), quercetin-3-rhamnoside (**7**), isorhamnetin-3-rutinoside (**8**). The leaf samples showed a simpler qualitative flavonoid composition. Rutin, hyperoside and quercetin-3-glucoside were detected as main components as well as quercetin-3-rhamnoside and kaempferol-3-glucoside in trace of the leaf samples (Table 2). Additionally flower samples contain kaempferol-3-rutinoside, kaempferol-3-rhamnoside and isorhamnetin-3-rutinoside. Chlorogenic and caffeic acids were also detected in the extracts of studied samples. The content of chlorogenic acid was found to be significantly higher in the extracts of wild species.

The comparative TLC analysis of polyphenolic profiles of all studied samples showed that same polyphenolic compounds were present in each almond individual with small variations in relative levels. Rutin, hyperoside, quercetin-3-glucoside and quercetin-3-rhamnoside found to be dominant compounds (Table 2).

Total phenolic and flavonoid content of the studied samples were evaluated by spectroscopic method. The extracts of the flowers have a higher content of flavonoids in comparison with the samples of leaves (Table 3). Generally the samples of *A. webbii* displayed higher amount of flavonoids than that of *A. communis* but the differences were not statistically significant. Concerning the phenolic compounds the leaf samples of *A. webbii* displayed twice as much phenolics than that of *A. communis*.

The received data displayed that there are no quality differences in the polyphenolic composition between *A. webbii* and *A. communis*. These results showed that besides morphological proximity both species have similar composition of phenolic compounds. Taking into account that phenolics play a major physiological role, especially in resistance to various stress factors and diseases (TREUTTER, 2007; BOUDET, 2007; CHEYNIER *et al.*, 2013) these results suggest that adaptive capacity of the studied species to the changing environment definite by contained in them phenolic compounds is similar. Identical polyphenol composition of the two species supposed that they have common origin. According to DIMITROVSKI & RISTEVSKI (1973), wild almond *Amygdalus webbii* is dwarf rootstock for cultivated almond. The present results are in confirmation of this hypothesis. It is not excluded *A. webbii* to be ancestral species lying in the base line of the long selection of the cultural species.

Table 2. Flavonoid glycosides and phenolic acids detected in the flower and leaf samples of examined *Amygdalus* species

Studied samples	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A. webbii										
flowers	X	X	XX	XX	X	X	X	X	XX	XX
leaves		tr	X	X	X			tr		
A. webbii										
flowers	tr	tr	X	X	X	tr	X	tr	XX	X
leaves		tr	X	X	X			tr		
A. communis										
flowers	X	tr	X	X	X	tr	X	X	X	X
leaves		tr	X	X	X			tr		
A. communis										
flowers	tr	X	X	XX	XX	X	X	tr	tr	X
leaves		tr	X	X	X			tr		
A. communis (out of culture)										
flowers	tr	X	X	XX	XX	X	X	tr	tr	X
leaves		tr	X	X	X			tr		

Legend: kaempferol-3-*O*-rutinoside (1), kaempferol-3-*O*-glucoside (2) kaempferol-3-*O*-rhamnoside (3) quercetin-3-*O*-rutinoside (rutin) (4) quercetin-3-*O*-galactoside (hyperoside) (5) quercetin-3-*O*–glucoside (isoquercetin) (6) quercetin-3-*O*–rhamnoside (quercetrin) (7) Isorhamnetin-3-*O*-rutinoside (8); chlorogenic acid (9) caffeic acid (10); tr- trace.

Table 3. Total phenol and flavonoid content of the flower and leaf samples of examined *Amygdalus* species

Studied samples	**Total phenolics* mg GAE/g extract**		**Total flavonoids* mg RE/g extract**	
	flower	**folia**	**flower**	**folia**
A. webbii	41,72±3,9057 a	64,79±1,0905 a	2,322±0,0177 a	1,95±0,4274 a
A. webbii	39,61±3,1015 a	58,01±2,2683 a	2,405±0,1233 a	1,79±0,1944 a
A. communis	29,50±1,9974 b	30,04±0,1484 b	2,066±0,1732 a	1,596±0,0250 a
A. communis	36,24±3,0033 a	36,89±0,8550 b	2,056±0,1246 a	1,409±0,3950 a
A. communis (out of culture)	35,01±3,1816 a	35,67±2,0451 b	2,008±0,0951 a	1,542±0,2960 a

Legend: * values represent mean ±SD; Values with the same letter are not significantly different, $p>0.05$; GAE-gallic acid equivalents; RE – rutin equivalents.

Conclusion

The present study provides data about flavonoid composition of flowers of Amygdalus species for the first time. The received results showed that there is no divergence of *A. webbii* and *A. communis* in respect to their polyphenolic composition. Adaptive and resistant capacity achieved in the early stage of species formation of *A. webbii* is retained and is effective in the *A. communis* (Bulgarian origin).

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