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Phenolic Profile and Antioxidant Activity of Methanolic Extract of Carduus acicularis Bertol. (Asteraceae)

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Abstract. Phenolic acid and flavonoid profiles of *Carduus acicularis* were investigated for the first time. Eleven phenolic acids and eight flavonoids were identified and quantified in the inflorescences, by high performance liquid chromatography. The main phenolic compounds were found to be: sinapic acid (930.41 ± 21.72 μg/g dw), chlorogenic acid (582.66 ± 13.60 μg/g dw), rutin (545.65 ± 12.82 μg/g dw), apigenin (478.75 ± 11.38 μg/g dw), luteolin (288.46 ± 6.86 μg/g dw) and myricetin (276.32 ± 5.21 μg/g dw). The antioxidant activity of methanolic extract of inflorescences has been investigated, employing four different established testing systems: scavenging activity on 2,2-diphenil-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonate (ABTS) radical cation decolorization assay, ferric reducing antioxidant power (FRAP) and copper reduction antioxidant assays (FRAP). The highest antioxidant activity values were measured by the ABTS assay, among all performed methods.

Key words: *Carduus acicularis,* phenolic acids, flavonoids, HPLC analysis, antioxidant activity.

Introduction

Carduus acicularis Bertol. is one of the Bulgarian representatives of the genus *Carduus* from family *Asteraceae*. This species is an annual plant, 20-60 cm high, growing in grassy areas up to 800 m altitude. Its distribution in Bulgaria covers the Black Sea Coast, Mountain Strandzha, Valley of Struma River, Thracian Lowland and Tundzha Hilly Valley (DELIPAVLOV & CHESHMEDZHIEV, 2003). Some species of genus *Carduus* are medicinal plants with different applications mainly in folk medicine (PETKOV, 1982).

Previous phytochemical studies on the genus *Carduus* show the presence of different groups of substances - phenolics, terpenoids, sterols, volatiles, alkaloids and few other compounds (JORDON-THADEN & LOUDA, 2003). Phenolics are the most studied secondary metabolites, due to their role as antioxidants and positive influence on the human health (BECKMAN, 2000; GRAF *et al.*, 2005). That was the reason for the beginning of phytochemical studies on Bulgarian representatives of the genus *Carduus* for examining the presence of phenolic compounds (ZHELEV *et al.*, 2011; 2013; SLAVOV *et al.*, 2014). Moreover, the initial screening of several *Carduus* species, which grow in Bulgaria, for radical scavenging and antioxidant activity,

revealed that they could be evaluated as a rich source of antioxidants (ZHELEVA-DIMITROVA *et al.*, 2011).

The objective of the present study was to identify and quantify some of the widespread phenolic compounds (flavonoids and phenolic acids) by HPLC analysis, and to evaluate the related total antioxidant potential of methanolic extract from *Carduus acicularis*.

Material and Methods

Plant material

Inflorescences (flower heads) of *C. acicularis* were collected from a natural habitat (*Black Sea Coast* floristic region) in Bulgaria, during the 2014 vegetative season and then they were air-dried in darkness at room temperature. Species identification was carried out at the Department of Botany of the University of Plovdiv "Paisij Hilendareski", according to TUTIN *et al.* (1976) and DELIPAVLOV & CHESHMEDZHIEV (2003). Voucher specimen of this species was deposited in the Herbarium at the Agriculture University of Plovdiv, Bulgaria (Herbarium SOA - 059650).

Preparation of plant extracts

Dried plant material was grounded and 0.5 g of the accurately weighed sample was refluxed exhaustively three times with 70 % (v/v) methanol at 70ºC for 30 min. The extracts were combined and made up to 30 ml with methanol in a volumetric flask.

HPLC analysis

The HPLC analysis were performed by a Waters HPLC system, (Milford, MA, USA) equipped with binary pump (Waters 11525), a UV-VIS detector (Waters 2487) and Breeze 3.30 SPA software. Detailed conditions of HPLC analyses are reported previously (MARCHEV *et al.*, 2011). Concentration of each individual compound was calculated, basing on external standard method, and converted to µg compound per g dry weight (dw).

DPPH free-radical scavenging activity

This assay is based on the bleaching of purple colored methanol solution of DPPH. The DPPH radical scavenging activity was determined, following the method of BRAND–WILLIAMS *et al.* (1995). Freshly prepared $4x10^{-4}$ M methanolic solution of DPPH was mixed with the sample in a ratio of 2:0.5 (v/v). The reaction was performed at 37°C in a dark place. The light absorption was measured at 517 nm and the antiradical activity of samples was calculated and represented as function of the concentration of Trolox. The unit of Trolox equivalent antioxidant capacity (TEAC) was defined as the concentration of Trolox having equivalent antioxidant activity expressed as mMTE/g dry plant material.

ABTS radical cation decolorization assay

The total antioxidant activity of the sample was measured by improved ABTS radical cation decolorization assay, according to the method of RE *et al.* (1999). TEAC was defined as the concentration of Trolox having equivalent antioxidant activity expressed as mMTE/per gram dry weight (dw).

Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to BENZIE & STRAIN (1999). The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. 150 µl of plant extracts were allowed to react with 2850 µl of the FRAP reagent for 4 min at 37°C and the absorbance was recorded at 593 nm. The results were expressed as mM/TE g dw.

Copper reduction antioxidant assay (CUPRAC)

CUPRAC assay was performed according to the method of AK & GÜLÇIN (2008). 1 ml of $CuCl_2$ solution (1.0×10^{-2}M), 1 ml of neocuproine methanolic solution (7.5×10^{-3} M), and 1 ml NH_4Ac buffer solution (pH 7.0) were added to a test tube and then mixed; 0.1 ml of herbal extract (sample) followed by 1 ml of water were added (total volume = 4.1 ml), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of extracts was expressed as mM/TE g dw.

Statistical analysis

The presented results are average from two independent experiments carried out in triplicates. The results were expressed as

Mean ± SD and statistically analyzed using MS Excel 2003 software.

Results

Flavonoid and phenolic acid profiles

Six flavonoid aglycons and two glycosides were identified in *Carduus acicularis* (Table 1). Rutine was better represented glycoside (545.65 µg/g dw) compared with hyperoside 211.48 µg/g dw). Among all established aglycons, apigenin (478.75 µg/g dw), luteolin (288.46 µg/g dw) and myricetin (276.32 µg/g dw) showed the highest concentration.

Eleven phenolic acids were identified in the investigated methanolic extracts (Table 1). The highest concentration was found for sinapic acid (930.41 dw), followed by chlorogenic acid (582.66 µg/g). Ferulic and caffeic acids were comparatively well represented (Table 1). The presence of p-coumaric acid, 2-hydroxybenzoic acid, vanillic acid, 3,4-dihydroxybenzoic acid, syringic acid, cinnamic acid and gallic acid was also found.

Table 1. Content of flavonoids and phenolic acids in inflorescences of *Carduus acicularis* (µg/g dw).

Phenolic acids	**Mean ± SD**
Caffeic acid	112.97 ± 2.64
Cinnamic acid	29.66 ± 0.69
Chlorogenic acid	582.66 ± 13.60
p-Coumaric acid	84.77 ± 1.98
3,4-Dihydroxybenzoic acid	67.85 ± 1.58
Ferulic acid	188.45 ± 4.40
Gallic acid	19.08 ± 0.45
2-Hydroxybenzoic acid	82.59 ± 1.93
Sinapic acid	930.41 ± 21.72
Syringic acid	36.52 ± 0.85
Vanillic acid	66.37 ± 1.55
Flavonoid glycosides	**Mean ± SD**
Hyperoside	211.48 ± 5.03
Rutin	545.65 ± 12.82
Flavonoid aglycones	**Mean ± SD**
Apigenin	478.75 ± 11.38
Hesperedin	75.83 ± 1.81
Kaempferol	78.42 ± 1.86
Luteolin	288.46 ± .6.86
Myricetin	276.32 ± 5.21
Quercetin	6.08 ± 0.15

Antioxidant activity

For determination of antioxidant activity of prepared methanolic extract of inflorescences were conducted experiments with two stable radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, but the ferric reducing antioxidant power (FRAP) and copper reduction (CUPRAC) assays were also performed. The results were expressed as Trolox equivalent antioxidant capacity - TEAC (Table 2). TEAC values were in range of 10.69 mM/TE g dw (CUPRAC**)** to 32.28 mM/TE g dw (ABTS)**.** The highest antioxidant activity were measured by the ABTS assay, followed by FRAP assay.

Discussion

The results obtained suggested that phenolics (phenolic acids, flavonoid aglycones and glycosides) were important components of *C. acicularis.*

Table 2. In vitro antioxidant activity in inflorescences of *Carduus acicularis* (mM/TE g dw). *Legend:* TEAC - Trolox equivalent antioxidant capacity; DPPH: 2,2-diphenil-1-picrylhydrazyl; ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FRAP: Ferric reducing antioxidant power; CUPRAC: Copper reduction antioxidant assay; dw- Dry weight.

Method / **Sample**	TEAC $_{DPPH}$	TEAC $_{ABTS}$	TEAC $_{FRAP}$	TEAC $_{CUPRAC}$
methanolic extract	11.59±0.52	32.28±0.08	22.19±0.65	10.69±0.36

The established qualitative composition of flavonoids confirms previously data for the genus *Carduus* flavonoid aglycones and glycosides - apigenin, kaempferol, luteolin, quercetin, and rutin (BAIN & DESROCHERS, 1988; JORDON-THADEN & LOUDA, 2003; TERENTJEVA & KRASNOV, 2003; KOZYRA, 2013), as well as the myricetin and hyperoside, referred for the first time in the *C. thoermeri* from SLAVOV *et al.* (2014). Main flavonoid components in *C. acicularis* were rutin and apigenin, accompanied with luteolin, myricetin and hyperoside. The measured amounts of rutin and myricetin were higher (eight and three times, respectively) than those established for *C. thoermeri* (SLAVOV *et al.*, 2014). Many studies have suggested that flavonoids (especially abovementioned) exhibit biological activities, including antiallergenic, antiviral, antihypertensive, antiinflammatory and anticancerogenic properties (NIJVELDT *et al.*, 2001; ODONTUYA *et al.*, 2005; SANGUINE *et al.*, 2010; SHEN *et al.*, 2013). It is known that apigenin is protectant against cardiotoxic agents (BREINHOLT *et al.*, 1999).

Phenolic acids have been poorly studied in the genus *Carduus*. First data for their presence in *Carduus acanthoides* was pointed out by LIU *et al.* (2013) and SLAVOV *et al.* (2014) - in *Carduus thoermeri*. The present study showed that from eleven of the identified phenolic acids in *C. acicularis* sinapic and chlorogenic acids prevailed, accompanied by ferulic, caffeic, p-coumaric, 2-hydroxybenzoic acids.

Acid-phenols and their esters are known for their antioxidative properties. Caffeic, sinapic, ferulic and p-coumaric acids have antioxidative potency (CUVELIER *et al.*, 1992). Antioxidant $ONOO^-$scavenging ability of sinapic acid was indicated in the study of ZOU *et al.* (2002) and for anxiolytic-like effects in mice (YOON *et al.*, 2007). Other studies mention that ferulic acid and caffeic acid derivatives may have antitumor activity (LI *et al.*, 2012). Chlorogenic acid possesses pharmacological activities as antihypertensive effect (ZHAO *et al.*, 2011), anti-diabetic and anti-lipidemic effects (ONG *et al.*, 2013).

Good antioxidant capacity of the methanolic extract of C. *acicularis* were established in this study, especially towards $ABTS^{\bullet+}$and FRAP-assay. For comparison, ethanolic extract of the species were also found to have antioxidant activity (ZHELEVA-DIMITROVA *et al.*, 2011).

Conclusion

Phenolic acids and flavonoid profiles of *Carduus acicularis* were investigated for the first time. The present study reported eight flavonoids and eleven phenolic acids in the methanolic extract of flower heads. Exhibited antioxidant activity, as well as predominance of sinapic and chlorogenic acids, also rutin and apigenin from flavonoids, determine the species as a natural source of antioxidants.

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