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ORIGINAL ARTICLE

Phenolic Profile and *In vitro* Antioxidant Activity of Endemic Bulgarian *Carduus* Species

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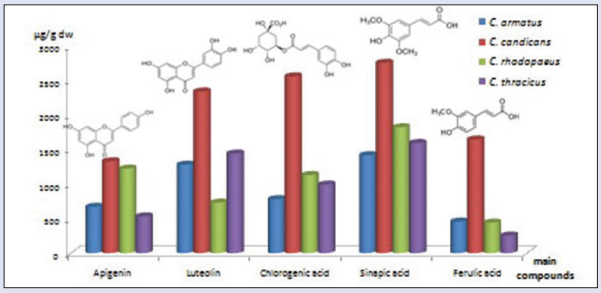
ABSTRACT

Background: Plant species from genus *Carduus* are widely distributed in the world and represented in Bulgaria by 14 species. Previous investigations on this genus demonstrated a strong antioxidant potential of extract from some Bulgarian *Carduus* species. **Objective:** The present study investigates the phenolic profile and the antioxidant potential of different extracts obtained from four endemic *Compositae* herbs, growing wild in Bulgaria: *Carduus armatus* Boiss and Heldr., *Carduus candicans* Waldst. et Kit ssp. *globifer* (Velen.) Kazmi., *Carduus rhodopaeus* Velen. and *Carduus thracicus* (Velen.) Hayek. **Materials and Methods:** Antioxidant capacity of the obtained extracts was estimated with 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), and ferric reducing antioxidant power and copper reduction antioxidant assays. Phenolic profile was estimated by high performance liquid chromatography. **Results:** Eleven phenolic acids and eight flavonoids were quantified in the inflorescences. Sinapic (2760.72 ± 15.68 μg/g dry weight [dw]), chlorogenic (2564.50 ± 19.73 μg/g dw) and ferulic acids (1648.71 ± 19.57 μg/g dw), as well as luteolin (2345.45 ± 18.61 μg/g dw) and apigenin (1332.75 ± 12.05 μg/g dw) were found to be the predominant compounds. The above contents are the highest values found in *C. candicans* ssp. *globifer*. The highest established antioxidant activity (AOA) was in favor of the ethanolic extracts, and the extract of *C. rhodopaeus* affirmed with the highest AOA among the investigated plant species. **Conclusion:** All identified phenolic compounds were reported for the 1st time in the studied endemic *Carduus* species, as well as their antioxidant capacities. The present study revealed that these plant species could be used as sources of antioxidants with potential medicinal properties.

Key words: *Carduus species*, high-performance liquid chromatography, *in vitro* antioxidant activity, phenolic profile

SUMMARY

- Phenolic acids and flavonoid profiles of four endemic compositae herbs, growing wild in Bulgaria: *Carduus armatus*, *Carduus candicans* ssp. *globifer*, *Carduus rhodopaeus* and *Carduus thracicus*, were quantified for the first time by high performance liquid chromatography (HPLC). Eleven phenolic acids and eight flavonoids were determined in the inflorescences. Sinapic, chlorogenic and ferulic acids, as well as luteolin and apigenin were found to be the predominant compounds. The highest values of established phenolic compounds were found in *C. candicans* ssp. *globifer*. The studied plant extracts of *Carduus* species possessed antioxidant activity in favor of *C. rhodopaeus* and results confirmed 70 % ethanol as more appropriate solvent. The present study revealed that these plant species could be used as sources of antioxidants with potential medicinal properties.



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INTRODUCTION

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens.[1] In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor, and oxidative stability. Polyphenols and other food phenolics are currently subjects of increasing scientific interest because of their possible beneficial effects on human health. Toward the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long-term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis, and neurodegenerative diseases.[2,3] Plant species from genus *Carduus* belongs to family *Asteraceae*. It is widely distributed in Europe and represented in Bulgaria by 14 species.[4,5] Some species of this plant genus are well-known herbs and have been used in phytotherapy as diuretic, cardiotonic, liver tonic, and antihemoroidal remedy.[6]

The flavonoids are more exhaustive studied phenolic compounds (present as different aglycons and glycosides attached) in different *Carduus* species,[7-10] but the profile of phenolic acids is less studied for this plant genus. First data for the presence of phenolic acids in genus *Carduus* was showed by Liu *et al.*,[11] for *Carduus acanthoides* and Slavov *et al.*,[12] for *Carduus thoermeri*.

As a result of the conducted phytochemical screening of Bulgarian *Carduus* L. species in terms of main phenolic compounds content, flavonoids, and total phenols were revealed as dominant constituents, followed by phenolic acids and anthocyanins.[13] Furthermore, the

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Table 1: Collection locality, floristic region, altitude, and voucher specimen number of the studied *Carduus species*

Species	Collection locality, floristic region in Bulgaria	Altitude (m)	Voucher specimen, number
Carduus armatus Boiss and Heldr. (syn. *Carduus tmoleus* ssp. *armatus* Boiss and Heldr.), *Asteraceae* Balkan endemic plant	"Bulgarka" Nature park, mountain range Stara planina (central)	1415	059,781
Carduus candicans Waldst. and Kit. ssp. *globifer* (Velen.) Kazmi *Asteraceae* Balkan endemic plant	Starosel, Thracian Lowland	349	059,723
Carduus rhodopaeus Velen. (syn. *Carduus adpressus* C.A. Mayer), *Asteraceae* Bulgarian endemic plant	Rozhen, Rhodope mountains (central)	1431	059,776
Carduus thracicus (Velen.) Hayek *Asteraceae* Balkan endemic plant	Zlatograd, Rhodope mountains (central)	498	059,778

dw: Dry weight

screening of *Carduus* species in terms of antioxidant activity (AOA) revealed that the species *C. thoermeri* and *Carduus candicans* ssp. *globifer* are more potent and could be evaluated as sources of antioxidants.[14,15]

The aim of the present investigation was to establish the phenolic profile (flavonoids and phenolic acids), to examine the total phenolic content, and to evaluate the related total antioxidant potential in four endemic *Carduus* species, which showed good quantitative values according our previous screening studies. Thus, this research will enrich the scanty available information on these plant species and outlines the promising potential practical application.

MATERIALS AND METHODS

Plant material

The plant materials (inflorescences) were collected from natural habitats from Bulgaria, during the 2011–2013 vegetative seasons [Table 1]. The collected plant materials were air-dried in darkness at room temperature. Species identification was carried out at the Department of Botany of University of Plovdiv "Paisij Hilendareski," according to Tutin *et al.*,[4] and Delipavlov and Cheshmedzhiev.[5] Voucher specimens of the different species were deposited in the herbarium at the Agriculture University of Plovdiv, Bulgaria (Herbarium SOA).

Preparation of plant extracts

Dried plant material of all four *Carduus* species was grounded and 0.5 g of the accurately weighed sample was refluxed exhaustively triple with 70% (v/v) methanol at 70°C (raw material to solvent 1:20) for 30 min. The extracts were combined and made up to 30 ml with methanol in a volumetric flask.

High-performance liquid chromatography analysis

The high performance liquid chromatography (HPLC) analysis of phenolic acids and flavonoids were performed by using Waters 1525 Binary Pump HPLC systems (Waters, Milford, MA, USA), equipped with Waters 2484 dual Absorbance Detector (Waters, Milford, MA, USA) and Supelco Discovery HS C18 column (5 µm, 25 cm × 4.6 mm), operated under control of Breeze 3.30 software. Detailed conditions of HPLC analyses are reported previously.[16] Concentration of each individual compound was calculated based on the external standard method and was converted to microgram compound per gram dry weight (dw).

Total polyphenol content determination

The total polyphenol content (TPC) was analyzed using the Folin–Ciocalteu method[17] with some modifications.[18] Each sample (1 ml) was mixed with 5 ml of Folin–Ciocalteu phenol reagent and 4 ml of 7.5% Na_2CO_3. The mixture was then vortexed well and left for 5 min at 50°C. After incubation, the absorbance was measured at 765 nm. The TPC in the extracts was expressed as milligram gallic acid equivalent (GAE) per gram dw.

2,2-diphenyl-1-picrylhydrazyl free-radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined following the described method[19] which was slightly modified.[18] Freshly prepared 4×10^{-4} M methanolic solution of DPPH was mixed with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox - Trolox equivalent antioxidant capacity (TEAC) and was defined as the concentration of Trolox having equivalent AOA expressed as µM/Trolox equivalent (TE) gram dw.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay

The scavenging activity of the extract against radical cation 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was estimated according to Re *et al.*,[20] Briefly, ABTS was dissolved to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Afterward, the $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. After the addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution to 10 ml of samples, the absorbance reading was taken at 30°C after 6 min. The results were expressed as TEAC value (µM/TE gram dw).

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was carried out according to the procedure of Benzie and Strain[21] with some changes.[18] The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. One-hundred and fifty microliters 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 µM/TE gram dw.

Copper reduction antioxidant assay

Copper reduction antioxidant (CUPRAC) assay was performed according to the method of Ak and Gülçin.[22] To a test tube were added 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), and mixed with 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 a standard and total antioxidant capacity of extracts was expressed as µM/TE gram dw.

Statistical analysis

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

RESULTS AND DISCUSSION

Flavonoid and phenolic acid profile

Flavonoids as a main class of phenolic compounds demonstrate a wide range of biochemical and pharmacological effects.[23,24]

In the present study, the methanol extract obtained from flower heads of the species was subjected to HPLC analysis for the presence of flavonoids. The analysis showed eight flavonoids and flavonoid glycosides in studied *Carduus* species. Among all established flavonoid, aglycones luteolin and apigenin were found to be with the highest content [Table 2].

Luteolin was the predominant compound in *C. candicans* (2345.45 ± 18.61 μg/g dw), followed by *Carduus thracicus* (1444.79 ± 8.00 μg/g dw) and *Carduus armatus* (1282.24 ± 13.58 μg/g dw) extracts, respectively. Luteolin expresses antihypertensive,[25] anti-inflammatory,[26] and anticancer activity.[27] Apigenin was evaluated in similar concentrations in *C. candicans* and *Carduus rhodopaeus* extracts (1332.75 ± 12.05 and 1225.65 ± 11.78 μg/g dw, respectively), as well as in *C. armatus* and *C. thracicus* extracts (671.10 ± 7.10 and 531.82 ± 5.34 μg/g dw, respectively). As antioxidant apigenin significantly induced glutathione transferase it is a protectant against cardiotoxic agents.[28]

The glycosides hyperoside and rutin were detected to be present in all four species, as the highest concentration of rutin was established in *C. candicans* extract - 1006.58 ± 35.61 μg/g dw and of hyperoside in *C. rhodopaeus* extract - 643.85 ± 6.18 μg/g dw [Table 2].

In brief, all identified flavonoids were reported for the 1st time in the investigated *Carduus* species. However, in comparison with the study established by Slavov *et al.*,[12] for the profile of *C. thoermeri,* the concentrations of luteolin and myricetin were higher in the present study of endemic *Carduus* species (especially in *C. candicans* - 4–5 time higher, respectively).

Eleven phenolic acids were identified in the investigated extracts, except *C. candicans* extract, where they were 10 [Table 3]. Sinapic, ferulic, and chlorogenic acids were the main phenolic acids identified in the investigated extracts. The presence of caffeic acid, p-coumaric acid, 2-hydroxybenzoic acid, vanillic acid, syringic acid, gallic acid, cinnamic acid, and 3,4-dihydroxybenzoic acid was also established.

In *C. candicans* extract, sinapic, chlorogenic, and ferulic acids were with the highest concentrations (2760.72 ± 15.68, 2564.50 ± 19.73 and 1648.71 ± 19.57 μg/g dw, respectively), followed by *C. rhodopaeus, C. thracicus*, and *C. armatus* [Table 3]. However, relatively high concentrations of syringic acid (477.93 ± 7.29 μg/g dw) and vanillic acid (427.76 ± 9.80 μg/g dw) in *C. candicans,* as well of p-coumaric acid (246.56 ± 2.37 μg/g dw) in *C. rhodopaeus* were established. Comparatively, Slavov *et al.*,[12] reported sinapic, ferulic, and chlorogenic acids as predominant acids in *C. thoermeri,* as well.

Ferulic and sinapic acids as natural phenolic compounds are known for free-radical scavenger and reactive toward free-radicals.[29,30] Other studies suggest that ferulic acid may have antitumor activity.[31,32] Chlorogenic acid possess other pharmacological activities such as antihypertensive effect,[33] improved lipid and glucose metabolism,[34] protection of dopaminergic neurons in neuroinflammatory conditions,[35] and also antitumor activity.[31] Briefly, in the present investigation, the above-mentioned phenolic acids were established and quantified for the 1st time for the studied species.

Table 2: Content of flavonoids (aglycons and glycosides) in inflorescences of *Carduus* species, (μg/g dw)

	C. armatus	*C. candicans*	*C. rhodopaeus*	*C. thracicus*
Flavonoid aglycons				
Apigenin	671.10±7.10	1332.75±12.05	1225.65±11.78	531.82±5.34
Hesperidin	117.62±1.25	162.11±9.86	126.62±1.22	133.89±3.86
Kaempferol	65.19±0.69	99.62±6.75	48.69±0.47	78.37±2.26
Luteolin	1282.24±13.58	2345.45±18.61	731.94±7.03	1444.79±8.00
Myricetin	184.56±1.96	341.00±6.31	111.10±1.07	272.67±7.86
Quercetin	4.49±0.01	33.50±0.81	11.80±0.11	8.45±0.24
Flavonoid glycosides				
Hyperoside	280.93±2.97	521.05±4.94	643.85±6.18	188.44±5.44
Rutin	129.81±1.37	1006.58±35.61	53.94±0.52	89.83±2.59

dw: Dry weight; *C. armatus: Carduus armatus; C. candicans: Carduus candicans; C. rhodopaeus: Carduus rhodopaeus; C. thracicus: Carduus thracicus*

Free-radical scavenging activity

Nowadays, the current focus of researchers is toward natural antioxidants, especially polyphenolics with plant origin. More than one extraction system is recommendable for the detailed assessment of the antioxidant properties of plants; therefore, we investigated two types of solvent extraction.

Since polyphenols contribute significantly to the overall antioxidant capacity, it was reasonable for us to determine their total amount in the

Table 3: Content of phenolic acids in inflorescences of *Carduus* species, (μg/g dw)

Phenolic acids	*Carduus armatus*	*Carduus candicans*	*Carduus rhodopaeus*	*Carduus thracicus*
Gallic acid	19.50±0.21	34.03±1.52	28.01±0.27	48.64±1.40
3,4-dihydroxybenzoic acid	63.57±0.67	49.99±2.51	49.67±0.48	44.26±1.28
2-hydroxybenzoic acid	76.79±0.82	104.99±9.36	131.68±1.30	95.04±2.75
Chlorogenic acid	782.52±8.28	2564.50±19.73	1131.63±10.86	994.38±8.68
Vanillic acid	118.85±1.26	427.76±9.80	76.67±0.74	161.04±4.65
Caffeic acid	50.74±0.54	0.00±0.00	80.24±0.77	49.57±1.44
Syringic acid	58.12±0.62	477.93±7.29	60.93±0.59	46.11±1.33
p-coumaric acid	90.97±0.96	138.85±8.77	246.56±2.37	91.85±2.65
Sinapic acid	1421.47±15.05	2760.72±15.68	1825.15±17.54	1597.29±16.07
Ferulic acid	457.72±4.85	1648.71±19.57	442.59±4.25	254.50±7.34
Cinnamic acid	18.21±0.19	113.80±7.97	22.90±0.22	14.38±0.41

dw: Dry weight

Table 4: Total phenol content (mg GAE/g dw) and *in vitro* antioxidant activity (μM/TE g dw) in inflorescences of *Carduus species*

Sample/ analyses	*Carduus armatus*		*Carduus candicans*		*Carduus rhodopaeus*		*Carduus thracicus*	
	Water	70% ethanol	Water	70% ethanol	Water	70% ethanol	Water	70% ethanol
TPC	6.35±0.02	10.29±0.11	6.80±0.04	10.26±0.09	6.59±0.18	10.69±0.04	5.68±0.07	8.34±0.08
$TEAC_{DPPH}^{\bullet}$	38.37±0.21	59.83±0.75	43.73±0.38	56.66±0.49	42.54±0.06	64.06±0.30	29.61±0.17	42.88±0.30
$TEAC_{ABTS}^{\bullet+}$	63.72±0.44	79.92±0.63	71.74±1.50	89.36±3.01	71.25±0.01	93.86±0.03	56.20±0.21	73.66±1.17
FRAP	71.74±1.36	110.30±3.59	78.39±1.57	113.43±1.57	83.6±0.75	119.65±1.05	55.96±0.93	92.03±0.22
CUPRAC	95.15±1.86	145.82±2.50	108.05±1.06	172.05±2.19	119.44±1.40	200.08±2.01	85.66±2.15	140.08±1.08

TPC: Total phenolic content; TEAC: Trolox equivalent antioxidant activity; 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; GAE: Gallic acid equivalent; dw: Dry weight

investigated extracts of *C. armatus, C. candicans*, *C. rhodopaeus*, and *C. thracicus*. The established total phenolic compounds concentrations by Folin–Ciocalteu`s method varied widely and are shown in Table 4. By comparing the used solvents, better results were established by 70% ethanol extracts. However, the best result among the investigated samples was found for the *C. rhodopaeus* extracts 10.69 ± 0.04 mg GAE/g dw. It has to be noted that the other investigated species (excl. *C. thracicus*) showed quite similar results in prevalence of the 70% ethanol as the extraction solvent.

The observed differences could be explained by the different polarity of the polyphenol compounds present in the investigated plant species. These findings are in agreement with the previously reported by Kratchanova *et al*.,[36] significant influence of the extraction solvent over the efficiency of the polyphenol content in the extract.

It is known that phenolics have various functions in plants. The role of polyphenols in preventing many chronic diseases including cancer, cardiovascular diseases, and diabetes has been well-documented.[37] Therefore, the presence of those compounds in the studied extract suggests their important role in the plant and might contribute toward its probable AOA.

In order to investigate the AOA, experiments with two stable radicals $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were conducted. In addition, the FRAP and copper reduction (CUPRAC) assays were also performed. The results were expressed as TEAC-values [Table 4]. $TEAC_{DPPH}^{\bullet}$ values were in range of 29.61 ± 0.17–64.06 ± 0.30 μM/TE g dw and the $TEAC_{ABTS}^{\bullet+}$ values were in range from 56.20 ± 0.21 to 93.86 ± 0.03 μM/TE g dw, respectively. Higher TEAC value indicates that a sample has a stronger antioxidant potential. Therefore, based on the results it can be conducted in agreement with the total polyphenolic content that the best effectiveness was achieved by extracting *C. rhodopaeus* with 70% ethanol.

In addition, the same tendency was observed for the FRA*P* values of the investigated extracts of *Carduus* species as the results were in range from 55.96 ± 0.93 to 119.65 ± 1.05 μM/TE g dw, respectively. Using the CUPRAC assay the highest values were found to be 200.08 ± 2.01 μM/TE g dw [Table 4] by the *C. rhodopaeus* 70% ethanol extract, corresponding to the results mentioned already in this research. Thus, the ethanol extracts of *C. rhodopaeus* definitely affirmed with the highest AOA among the investigated plant species in relation to the estimated polyphenolic content.

The results of all conducted antioxidant capacity assays showed that the investigated extracts possessed AOA, which for the 70% ethanol extracts was higher than the capacity of the water ones. This confirmed the results obtained from the TPC assay. Therefore, it can be concluded that the 70% ethanol is more efficient as a solvent in order to obtain extracts with higher content of biologically active substances in terms of AOA. This is in agreement with the study reported by Sultana *et al*.,[38] for better results achieved with aqueous organic solvent.

A slight difference among the results of the applied assays was observed. Interestingly, the highest antioxidant values were measured by the CUPRAC assay. This could be explained by the unique mechanism and the unequal sensitivity of each method applied. The authors, therefore, strongly suggested that, when analyzing the AOA of samples, it is better to use at least two methods due to the differences between the test systems.[39]

CONCLUSIONS

In the present study, the phenolic acids and flavonoid profiles of *C. armatus, C. candicans* ssp. *globifer*, *Carduus rhodopaeus*, and *C. thracicus* from Bulgaria were investigated and quantified for the 1st time. The main compounds established were sinapic, chlorogenic, and ferulic acids, and the flavonoids luteolin, apigenin, and rutin as well using HPLC-methods. Among all investigated species, *C. candicans* ssp. *globifer* was with the highest concentration of identified phenolic compounds. The studied *Carduus* species extracts revealed AOA with in favor of Bulgarian endemic *C. rhodopaeus*. The detailed investigation confirmed 70% ethanol as a more appropriate solvent according to all conducted assays.

The present study revealed that the investigated *Carduus* species could be used as sources of bioactive compounds with potential medicinal properties, in particular, the AOA.

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Conflicts of interest

There are no conflicts of interest.

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