# Antioxidant Activity of Some *Carduus* Species Growing in Bulgaria

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## ABSTRACT

**Background:** Species of genus *Carduus* are traditionally used in Bulgarian folk medicine as diuretic, cardiotonic and antihemorroidal remedies. *C. candicans* ssp. *globifer* and *C. kerneri* ssp. *austro-orientalis* are Balkan endemic, whereas *C. acanthoides*, *C. nutans*, *C. thoermeri* C. are invasive alien weeds in the Americas, Australia and New Zealand, and causes major economic losses. The aim of the present study was to screen some *Carduus* spesies growing in Bulgaria for radical scavenging and inhibitition of lipid peroxidation in order to discover new natural sources of antioxidants for further investigation. **Methods:** Antioxidant activity of *Carduus* species were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) free radicals; ferric reducing antioxidant power (FRAP) assay and inhibition of lipid peroxidation in linoleic acid system by the ferric thiocyanate method (FTC). Butylated hydroxytoluene (BHT) and ascorbic acid were used as positive controls. In addition, the quantification of total water soluble polyphenols and flavanoids were determinate by Folin-Chiocalteu reagent and $AlCl_3$, respectively. **Results:** The highest concentrations of total water soluble polyphenols and flavonoids were found in *C. thoermeri* (2.06 ± 0.03 g /100 g dw and 3.31 ± 0.12 g /100 g dw, respectively), followed by *C. nutans* (1.88 ± 0.01 g/100 g dw; 2.60 ± 0.09 g /100 g dw, respectively) and *C. candicans* ssp. *globifer* (1.85 ± 0.04 g/100 g dw). **Conclusions:** All tested extracts demonstrate significant antioxidant activity moreover *C. thoermeri*, *C. nutans* and *C. candicans* ssp. *globifer* were found to be the most potent and can be a good new source of natural antioxidants.

**Keywords:** Radical scavenging, Antioxidant activity, *Carduus*, Asteraceae

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## INTRODUCTION

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources[1]. In order to discover new antioxidant substances we evaluated the radical scavenging activity and inhibition of lipid peroxidation of extracts from six *Carduus* species: *Carduus acanthoides* L., *C. acicularis* Bertol., *C. candicans* Waldst.& Kit. ssp. *globifer* (Vel.) Kazmi, *C. nutans* L, *C. kerneri* Simonkai ssp. *austro-orientalis and C. thoermeri* Weinm[2,3].

These species are members of the family Asteraceae, tribe Cynareae and are commonly known as thistles[4]. Two of them *C. candicans* ssp. *globifer* and *C. kerneri* ssp. *austro-orientalis* are Balkan endemic. In previous phytochemical studies the presence of flavonoids, lignans, pentacyclic triterpenoids, isoquinoline alkaloids, phenolic acids, sesquiterpene lactones, phenylpropanoids, and saturated fatty acids has been established[4-9]. No reports on the antilipoxygenase and antioxidant activity of studied species are presently available, moreover this is the first report on quantification of phenolic compounds and flavonoids in these plants. Radical scavenging and antioxidant activity of ethanol extracts from *Carduus* species were examined using DPPH[10] and ABTS[11] free radicals; FRAP assay[12] and inhibition of lipid peroxidation in linoleic acid system by FTC method[13]. Butylated hydroxytoluene (BHT) and ascorbic acid were used as positive controls. In addition, the quantification of total water solubles and flavanoids were determinate using Folin-Chiocalteu reagent and $AlCl_3$, respectively[14].

## MATERIALS AND METHODS

### Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, ferrous chloride, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), butylated hydroxytoluene (BHT), potassium persulphate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™) were from Sigma-Aldrich USA. All the other chemicals used including the solvents, were of analytical grade. All solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO).

### Plant material

*Carduus* anthodia used for this study were collected during the flowering season from wild habitats. The voucher specimen of all studied species was confirmed and deposited in herbarium at the Agriculture University of Plovdiv, Bulgaria (Herbarium SOA) (Table 1). The collected plant matherial was air-dried in darkness at room temperature. The EtOH extracts (10 mg dw/ml) were subjected to determined antioxidant activities.

### Quantification of total water soluble polyphenols

The determination of total water soluble polyphenols in the anthodia was performed according to the European Pharmacopoeia[14] involving Folin-Chiocalteu reagent and pyrogallol as standard. The analyses were carried out at 760 nm. The measurements were carried out using a Specord UV-VIS spectrophotometer (Germany). All determinations were performed in triplicate (n = 3).

### Quantification of flavonoids

The content of the flavonoids in the anthodia was spectrophotometrically determined at 430 nm by creating a complex with $AlCl_3$ according to the European Pharmacopoeia[14] with some modifications. The content of flavonoids was calculated as quercetin. The measurements were carried out using a Specord UV-VIS spectrophotometer (Germany). All determinations were performed in triplicate (n = 3).

### Determination of antioxidant activity

*DPPH radical-scavenging activity:* Scavenging activity of ethanol extracts against DPPH radical was assessed according to the method of Blois with some modifications[10]. Briefly, 2 ml of each extract was mixed with 2 ml of DPPH methanol solution (0.01 mg/ml). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid (10 mg/ml in methanol) and BHT (10 mg/ml in methanol) were used as references. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity,

$$(\%) = 1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}} \times 100$$

where $Abs_{sample}$ is the absorbance of DPPH radical solution mixed with sample; $Abs_{blank}$ is the absorbance of plant extract (2 ml) with methanol (2 ml); $Abs_{control}$

**Table 1.** Collection locality, altitude and voucher specimen of the studied Carduus species

| Species | Collection locality, altitude [m] | Voucher specimen in Herbarium SOA-Plovdiv |
|---|---|---|
| *Carduus acanthoides* L. | v.Targovishte, Northeast Bulgaria, 218 m | 059 652 |
| *Carduus acicularis* Bertol. (*C. argentatus*) | Tzarevo, Black Sea Coast, 30 m | 059 650 |
| *Carduus candicans* W.et K. *ssp. globifer* (Vel.) Kazmi **Balkan endemic** | Kotel, Stara planina Mountains, 542 m | 059 653 |
| *Carduus kerneri* Simonkai ssp. *austro-orientalis* Franco (*C. scardicus*) **Balkan endemic** | Beglika, Rhodope Mountains, 1550 m | 059 651 |
| *Carduus nutans* L. | v.Hrabrino, Rhodope Mountains, 340 m | 059 659 |
| *Carduus thoermeri* **Weinm**. *(C. leiophyllus)* | v.Ticha, Stara planina Mountains, 331 m | 059 657 |

is the absorbance of DPPH radical in methanol. All determinations were performed in triplicate (n=3).

*ABTS radical scavenging assay:* For ABTS assay, the procedure followed the method of Arnao *et al*., with some modifications[11]. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 2ml ABTS solution with 30 ml methanol to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. A fresh ABTS solution was prepared for each assay. One ml of extract was allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min. The ABTS scavenging capacity of the compound was compared with that of BHT and ascorbic acid and percentage inhibition calculated as

ABTS radical scavenging activity,

$$(\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ is the absorbance of ABTS radical in methanol; $Abs_{sample}$ is the absorbance of an ABTS radical solution mixed with sample. All determinations were performed in triplicate (n=3).

*Total antioxidant activity (FRAP):* The FRAP assay was done according to Benzie and Strain, 1996 with some modifications[12]. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \times 3H_2O$ and 16ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3 \times 6H_2O$ solution and then warmed at 37 °C before using. 0.2 ml of each extract was allowed to react with 1.4 ml of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. The standard curve was linear between 30 and 500 mM Trolox. Results are expressed in mM Trolox equivalent TE. Ascorbic acid and BHT was used as references. All determinations were performed in triplicate (n=3).

*Determination of antioxidant activity in linoleic acid system by the FTC method:* The antioxidant activity of studied compounds against lipid peroxidation was measured through ammonium thiocyanate assay, as described by Takao *et al*., with some modifications[13]. The reaction solution, containing 0.2 ml of 0.1 mM compound in MeOH, 0.2 ml of linoleic acid emulsions (25 mg/ml in 99% ethanol) and 0.4 ml of 50 mM phosphate buffer (pH 7.4), was incubated in the dark at 40 °C. A 0.1 ml aliquot of the reaction solution was then added to 3 ml of 70 % (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without compound) reached maximum value. BHT was used as positive control. All determinations were performed in triplicate (n=3).

## RESULTS

### Total water soluble polyphenols and flavonoids content

The amount of total water soluble polyphenols was expressed as pyrogallol equivalent (PE) g/100 g dry weight (dw), the flavonoid content was expressed as g quercetin equivalent (QE) g/100 g dw (Figure 1). The amounts of polyphenols ranged from 1.36 ± 0.02 mg/100 g dw in *C. kerneri* ssp. *austro-orientalis* to 2.06 ± 0.03 g/100 g dw in *C. thoermeri*. The highest concentration of flavonoids was also found in *C. thoermeri* (3.31 ± 0.12g/100g dw) although, the lowest amount was established in *C. acicularis* (1.86 ± 0.02 g/100g dw). *C. nutans* and *C. candicans* ssp. *globifer* took second place after *C. thoermeri* on total water soluble polyphenols and flavonoids content (Figure 1). *C. kerneri* ssp. *austro-orientalis* has the lowest content of total water soluble polyphenols and moderate quantity of flavonoids (2.12 g/100 g dw ± 0.09).

### DPPH, ABTS radical-scavenging and total antioxidant activity

The radical scavenging and total antioxidant activity of *Carduus* extracts (10 mg dw/ml) were compared with those of BHT and ascorbic acid at the same concentration and expressed as % of inhibition against DPPH, ABTS and mM TE/g dw, respectively (Table 2). *C. thoermeri* demonstrated the highest DPPH and FRAP activity (97.2 % ± 0.1; 116.5 mM TE/g dw ± 3.1, respectively) and significantly quenched ABTS (94.0 % ± 0.2). These results well correlate with the high concentration of polyphenols and flavonoids in the species. *C. candicans* ssp. *globifer* and *C. nutans* significantly quenched DPPH (95.6 % ± 0.4; 95.2 % ± 0.9) and ABTS (94.7 % ± 1.0; 94.1 % ± 0.2), and demonstrated a moderate total antioxidant activity (98.5

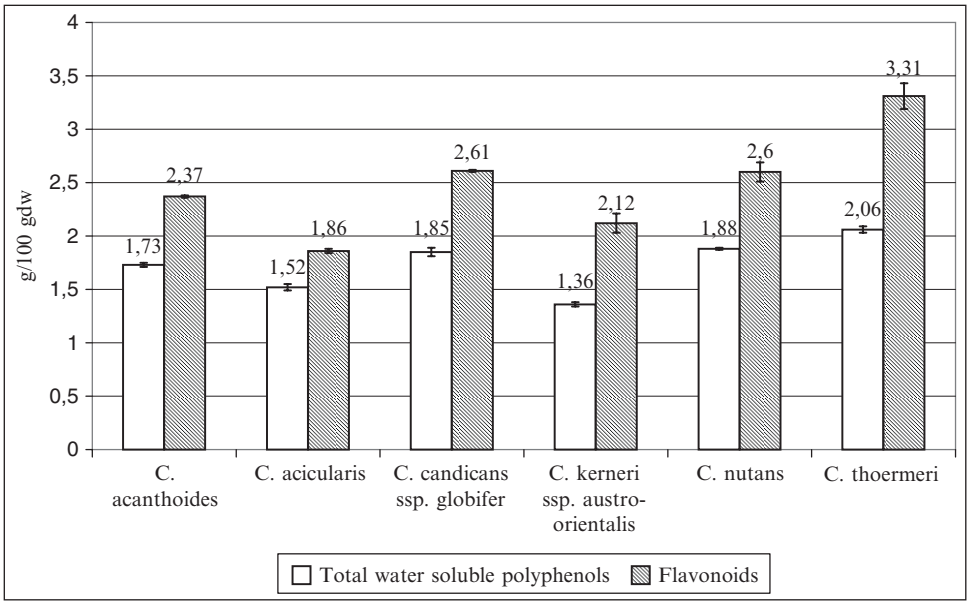


**Figure 1.** Contents (g/100 g dw) of total water soluble polyphenols and flavonoids in the studied Carduus species.

**Table 2.** DPPH, ABTS-radical scavenging and FRAP-activities of the studied Carduus species

| Sample | DPPH % | ABTS % | FRAP mM TE/g dw |
|---|---|---|---|
| *C. acanthoides* | 95.4 ± 0.3 | 97.0 ± 0.2 | 103.1 ± 3.2 |
| *C. acicularis* | 93.8 ± 0.4 | 89.3 ± 0.2 | 86.4 ± 5.5 |
| *C. candicans* ssp. *globifer* | 95.6 ± 0.4 | 94.7 ± 1.0 | 98.5 ± 5.5 |
| *C. kerneri* ssp. *austro-orientalis* | 91.4 ± 0.2 | 91.5 ± 0.2 | 114.0 ± 4.2 |
| *C. nutans* | 95.2 ± 0.9 | 94.1 ± 0.2 | 106.5 ± 4.5 |
| *C. thoermeri* | 97.2 ± 0.1 | 94.0 ± 0.2 | 116.5 ± 3.1 |
| BHT | 97.9 ± 0.2 | > 100 | 79.0 ± 2.0 |
| Ascorbic acid | 98.2 ± 0.2 | > 100 | 148.5 ± 4.0 |

Results are represented as means ± standard deviation, n = 3



mM TE/g dw ± 5.5; 106.5 mM TE/g dw ± 4.5) compared to ascorbic acid (148.5 mM TE/g dw ± 4.0). The strongest ABTS activity was showed by *C. acanthoides* (97.0 % ± 0.2). The scavenging ability of *C. kerneri* ssp. *austro-orientalis* has low values (91.4 % ± 0.2 for DPPH and 91.5 % ± 0.2 for ABTS), although the species demonstrated moderate total antioxidant activity probably due to the presence of flavonoids. Comparatively to other species the lowest antioxidant activity was presented by *C. acicularis*.

### *Antioxidant activity in linoleic acid system*

The antioxidant activity of *Carduus* extracts was determined by peroxidation of linoleic acid using the ferric thiocyanate method (FTC) - Table 3. As shown in Table 3 the absorption of the control at 500 nm increased to a maximal value of 3.11 ± 0.09 after 96 h. Significant diminution of the absorption was demonstrated by all *Carduus* extracts.

## DISCUSSION

In the present study the radical scavenging and inhibition of lipid peroxidation of anthodia from *C. acanthoides, C. acicularis, C. candicans ssp. globifer, C. nutans, C. kerneri* ssp. *austro-orientalis* and *C. thoermeri* were investigated for the first time. In addition the quantification of the total water soluble polyphenols and flavonoids was carried out. Although flavonoids are related to polyphenol compounds, lower than flavonoids levels of polyphenols depend on

**Table 3.** Antioxidant activity of studied Carduus species in linoleic acid system

| Sample | Absorption at 500 nm | | | | |
|---|---|---|---|---|---|
| | 1-st day | 2-nd day | 3-rd day | 4-th day | 5-th day |
| Control | 0.71 ± 0.01 | 1.05 ± 0.07 | 1.78 ± 0.01 | 2.78 ± 0.22 | 3.11 ± 0.09 |
| *C. acanthoides* | 0.62 ± 0.01 | 0.56 ± 0.01 | 0.48 ± 0.03 | 0.47 ± 0.02 | 0.45 ± 0.03 |
| *C. acicularis* | 0.63 ± 0.01 | 0.58 ± 0.02 | 0.57 ± 0.01 | 0.55 ± 0.03 | 0.54 ± 0.02 |
| *C. candicans* ssp. *globifer* | 0.60 ± 0.02 | 0.55 ± 0.01 | 0.36 ± 0.09 | 0.34 ± 0.04 | 0.36 ± 0.01 |
| *C. kerneri* ssp. *austro-orientalis* | 0.66 ± 0.01 | 0.59 ± 0.01 | 0.50 ± 0.02 | 0.34 ± 0.03 | 0.30 ± 0.01 |
| *C. nutans* | 0.68 ± 0.03 | 0.59 ± 0.01 | 0.50 ± 0.02 | 0.49 ± 0.01 | 0.48 ± 0.02 |
| *C. thoermeri* | 0.53 ± 0.03 | 0.53 ± 0.03 | 0.46 ± 0.03 | 0.42 ± 0.01 | 0.41 ± 0.01 |
| BHT | 0.65 ± 0.02 | 0.63 ± 0.03 | 0.57 ± 0.01 | 0.56 ± 0.02 | 0.55 ± 0.01 |
| Ascorbic acid | 0.65 ± 0.01 | 0.25 ± 0.04 | 0.16 ± 0.01 | 0.13 ± 0.01 | 0.12 ± 0.01 |

Results are represented as means ± standard deviation, n = 3

the different methods of extraction used. The polyphenols determination was carried out with water extraction in which most flavonoids are not soluble. In contrast, 90 % ethanol was used for quantification of the flavonoids. All tested species demonstrated high levels of flavonoids, especially *C. thoermeri, C. nutans* and *C. candicans ssp. globifer.*

DPPH and ABTS assays are based on the ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. ABTS or DPPH radical scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of components. The scavenging effect of species and standards on the DPPH radical decreased in the order Ascorbic acid > BHT ≈ *C. thoermeri* > *C. acicularis* > *C. candicans* ssp. *globifer* ≈ *C. acanthoides* ≈ *C. nutans* > *C. kerneri* ssp. *austro-orientalis*. The ABTS activity of the samples is as follow: Ascorbic acid = BHT > *C. acanthoides* > *C. candicans* ssp. *globifer* ≈ *C. nutans* ≈ *C. thoermeri* > *C. kerneri* > *C. acicularis.*

In FRAP assay reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue color) at low pH can be monitored by measuring the change in absorption at 593 nm. The reaction is non specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture. The FRAP activity of the tested samples decreased in the order Ascorbic acid > *C. thoermeri* > *C. kerneri* ssp. *austro-orientalis* > *C. nutans* > *C. acanthoides* > *C. candicans* ssp. *globifer* > *C. acicularis* > BHT. All *Carduus* species demonstrated higher FRAP activity than BHT.

During linoleic acid peroxidation, peroxides were formed and these compounds oxidized $Fe^{2+}$ to $Fe^{3+}$. The $Fe^{3+}$ ion formed a complex with $SCN^-$, which had a maximum absorbance at 500 nm. Thus, a high absorbance value was an indication of high peroxide formation during the emulsion incubation. However, the antioxidant activity of *Carduus* species was slightly less effective than that of ascorbic acid, all of them inhibited lipid peroxidation stronger than BHT. *C. kerneri*, C. *candicans* ssp. *globifer* and *C. thoermeri* showed the strongest effect at this method. Furthermore, all plant species demonstrated at these concentrations tendency to decrease the initial quantity of peroxides.

Previous studies on the chemical composition of the genus *Carduus* discuss only *C. acanthoides* and *C. nutans*, moreover *C. candicans* ssp. *globifer* and *C. kerneri* are Balkan endemic and this is the first investigation on these species.

## CONCLUSION

All tested *Carduus* species exhibited radical scavenging, antioxidant activity and strongly inhibited lipid peroxidation compared to the control. *C. thoermeri, C. nutans,* and *C. candicans* ssp. *globifer* contain significant amount of polyphenols and flavonoids. Ethanolic extracts from these species were found to be the most potent. The results obtained demonstrated that these plants are possible new

powerful natural sources of antioxidants and could be useful in therapy of free radical pathologies. Furthermore the investigation completes the knowledge about pharmacological activity and chemical composition of genus *Carduus*. Further isolation and identification of the active compounds from these plant species is required for a better understanding of the antioxidant mechanisms involved and for the possible application as a food supplement or in the pharmaceutical industry.

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