

Antioxidant, antimicrobial activities and total flavonoid contents of *Cirsium bulgaricum* DC. leaf extracts

Ayca KARASAKAL, A. Şükrü DEMİRCİ , Nazan TOKATLI DEMİROK, Evren CABI

ABSTRACT

In this study, the antioxidant activities of methanol and acetone extracts of *Cirsium bulgaricum* DC. (Asteraceae) were evaluated by five antioxidant assays, including phosphomolybdate method, cupric ion reducing capacity (CUPRAC), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/persulfate) ABTS/Persulfate, N, N-dimethyl-p-phenylenediamine (DMPD) and a Ce(IV)-based reducing capacity (CERAC) methods. The 80% methanol and acetone extracts showed 0.005-6.16±0.03 mmol/g and 0.004-5.02±0.02 [(trolox (TR), catechin (CT), epicatechin (EC), rutin (RT), quercetin (QR), naringin (NG) in CUPRAC and ABTS/Persulfate methods, respectively]. According to DMPD methods, inhibition effect were showed 2.3%±0.02 for 80% methanol and 4.6%±0.07 for acetone. Ascorbic acid equivalent mmol (AA)/g extract in CERAC method was found to be 0.0834±0.09 for 80% methanol and 0.5622±0.05 for acetone. Ascorbic acid equivalent µg ascorbic acid (AA) /mL extract

were found 807.6±0.07 for 80% methanol and 292.4±0.01 for acetone in phosphomolybdate method. In the addition to the antioxidant activity of the extract, the total flavonoid content was measured. Total flavonoid content of 80% methanol and acetone extracts was found to be 105.5-645±0.04 µg/ml quercetin and rutin equivalent. For the determination of antibacterial activities *Escherichia coli* (E.coli) ATCC 25922, *Escherichia coli*(E.coli) O157:H7 ATCC 33150, *Salmonella* Enteritidis(S.Enteritidis) ATCC 13076, *Listeria monocytogenes*(L.monocytogenes) ATCC 7644, *Staphylococcus aureus*(S.aureus) ATCC 25923 bacteria were used as test bacteria. Acetone extracts showed antibacterial activity against all of the tested bacteria ranged between 17.00±1.72- 19.88±1.75 mm and 80% methanol extracts showed antibacterial activity against all of the tested bacteria ranged between 11.35±1.43-14.43±1.32 mm.

Keywords: Antioxidant Activity, Antimicrobial Activity, Total Flavonoid Contents, *Cirsium bulgaricum*

Ayca Karasakal
Department of Chemistry, Art and Science Faculty, Namik Kemal University, 59100 Tekirdag - TURKEY

A.Şükrü Demirci
Department of Food Engineering, Agriculture Faculty, Namik Kemal University, 59100 Tekirdag – TURKEY

Nazan Tokatlı Demirok
Department of Nutrition and Dietetics, School of Health, Namik Kemal University, 59100 Tekirdag – TURKEY

Evren Cabi
Department of Biology, Art and Science Faculty, Namik Kemal University, 59100 Tekirdag – TURKEY

Correspondence:

Ayca Karasakal
Tel: +902822502658
GSM: +90 5338117347
E-mail: aycakarasakal@yahoo.com

1. INTRODUCTION

Plants contain a wide variety of free radical scavenging molecules, such as flavonoids, anthocyanins, carotenoids, dietary glutathione, vitamins and endogenous metabolites and such natural products are rich in antioxidant activities (1-4). Herbs have been used in many domains including medicine, nutrition, flavoring, beverages, dyeing, repellents, fragrances, cosmetics, smoking, and other industrial purposes. Since the prehistoric era, herbs have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century (5,6). The preservative effect of many plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues (7). Recently, interest has increased considerably in finding naturally

occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (8). Flavonoids can be used directly to scavenge O_2^- and $\cdot OH$ by single electron transfer. The scavenging process can generally be followed by means of electron spin resonance (ESR) (9,10), but the expense of such instruments hinders their use by the average laboratory. The photochemical reduction of riboflavin was first used to determine the dismutation of O_2^- by superoxide dismutase (SOD) (11) and has been adapted for analysis of the dismutation of O_2^- by a model compound of superoxide dismutase and other natural compounds (12). Antioxidants are health beneficial compounds that fight reactive oxygen and nitrogen species and free radicals that may eventually give rise to various diseases. Since plant foods are rich in antioxidant vitamins, phenolic and hydroxycinnamic acids, flavonoids, carotenoids, and anthocyanins (13), consumption of vegetables and fruits in the diet is one of the most efficient ways of preventing these diseases. It is important to measure the antioxidant potency of food material and human plasma for food quality estimation and diagnosis and treatment of diseases, respectively. Recent literature states that a single "total antioxidant capacity (TAC)" index for food labelling may not be adequate because of the lack of standard quantitation methods (14), and thus a series of methods have to be employed to assess the antioxidant quality of food. The genus *Cirsium* Mill. (thistle; köygöçüren) belongs to subtribe *Carduinae* of the *Cardueae* tribe, family *Compositae* (*Asteraceae*). The members of the genus have been distributed mainly in Eurasia. There are about more than 250 species throughout the world, while in Turkey the genus is represented with 64 species and 76 taxa (15,16). Some species in the genus such as *Cirsium arvense* (L.) Scop. (Köygöçüren) and *C. vulgare* (Savi) Ten. (yaygın kangal) are considered as weeds in some parts of the world. The genus is also important for ethnobotanical aspect. Certain taxa are used for different purposes in Turkey (Table 1) *C. bulgaricum* DC. (pomak kangalı) is a biennial species and euxine element that grows in open mountain woods, roadsides, and meadow areas with the limited area in Kırklareli province in Turkey (15,16).

No previous literature study reported the antimicrobial activity of the extracts of *Cirsium bulgaricum* DC. examined in the present study. Therefore, this is the first study which demonstrates the antioxidant and antimicrobial activity of this plant. The aim of this research was to compare the efficiency of ABTS/Persulfate, DPMD, CUPRAC, CERAC and Phosphomolybdate assays to

Table 1. Some *Cirsium* taxa used for local people in Turkey.

Taxon	Vernacular name	Usage	Reference
<i>Cirsium arvense</i> (L.) Scop.	Köygöçüren	Stem, root/ Orexigenic, tonic, antihemorrhoidal, cough, bronchitis	(36)
<i>C. creticum</i> d'Urv. subsp. <i>creticum</i>	Eşek çalısı	Mushroom poisoning	(37,38)
<i>C. hypoleucum</i> DC.	Vişne kangalı	Diabetes	(39)
<i>C. rhizocephalum</i> <i>C.A. Mey. subsp.</i> <i>sinuatum</i> (Boiss.) PH. <i>Davis & Parris</i>	Medik	Boiled and drunk for dyspnea	(40)
<i>Cirsium vulgare</i> (Savi) Ten.	Yaygın Kangal	Areal parts are used for heating	(41)
		Stems pelled off and freshly eaten	(42)

estimate antioxidant activities. On the other hand, the objective of this work was to investigate the effects of solvents on the extraction of total flavonoid contents and antioxidant activities of *Cirsium bulgaricum* DC.

2. MATERIAL AND METHODS

2.1. Chemicals and Instruments

All chemicals, solvents, reagents and standards used in the experiments were purchased from Sigma Chemical Co. All chemicals were of analytical grade. All spectrophotometric measurements were made with a pair of matched Hellma quartz cuvettes using a Shimadzu-1601 UV-Vis spectrophotometer.

2.2. Preparation of plant extracts

Specimens of *C. bulgaricum* DC. were collected in July 2013 from Derekoy, Kırklareli and identified using the *Cirsium* key given in the Flora of Turkey (17,18) and other relevant literature (16). The specimens collected by the authors are deposited in the herbarium of Namık Kemal University (NAKU).

The leaves of *Cirsium bulgaricum* DC. were collected in July 2013 from Derekoy, Kırklareli and dried under shade to obtain dry sample. The dried samples were powdered in a Willy Mill to 60-mesh size and used for solvent extraction. For extract preparation, 1 g of dried leaf samples were added 40ml of 80% methanol and 100% acetone and extracts which is prepared with two different extraction techniques were kept in the dark at room temperature in stoppered flasks. The extracts were filtered through Whatman No. 1.

2.3. Preparation of solutions

For the CUPRAC test of TAC, the following solutions were prepared. CuCl_2 solution, 20 mM, was prepared by dissolving 0.8524 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ in water, and diluted to 250 mL. Ammonium acetate buffer at pH = 7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in ethanol and diluted to 250 mL. Neocuproine (Nc) solution, 7.5 mM, was prepared daily by dissolving 0.039 g Nc in absolute ethanol, and diluting to 25 mL with ethanol. For the ABTS test of TAC, the chromogenic radical reagent ABTS, at 7.0 mM concentration, was prepared by dissolving this compound in water and adding $\text{K}_2\text{S}_2\text{O}_8$ to this solution such that the final persulfate concentration in the mixture is 2.45 mM. The resulting ABTS radical cation solution was left to mature at room temperature in the dark for 12–16 h, and then used for ABTS/Persulfate assay. The reagent solution was diluted with EtOH at a volume ratio of 1:30 prior to use. For the N,N-Dimethyl-p-phenylenediamine (DMPD) method 10 mM Fe(III) stock solution was prepared: 0.0676 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was dissolved in 1.0 mL of 0.5 M H_2SO_4 and diluted to 25 mL with distilled water. To obtain 1.0×10^{-7} M Fe(III) working solution, this solution was appropriately diluted to 1.0×10^{-4} M intermediary stock solution and then diluted 10^3 times with 10 mM H_2SO_4 to avoid hydrolysis. The pH 5.7 buffer solution was prepared by mixing 45.25 mL of 2.0 M sodium acetate (NaOAc) with 4.75 mL of 2.0 M acetic acid (HOAc) solution. DMPD solution at 2.4×10^{-2} M concentration was prepared by dissolving 0.125 g of DMPD in water, followed by the addition of 0.25 mL of 0.5 M H_2SO_4 and final dilution to 25 mL with distilled water. The 3.0% H_2O_2 solution was prepared by diluting 30% commercial hydrogen peroxide with water.

The standard solutions at 1.0×10^{-3} M concentration of antioxidant compounds were all prepared in 80% MeOH, 100% acetone. All working solutions of antioxidant compounds were freshly prepared.

2.4. Determination of total flavonoid content

The total flavonoid contents were measured by a colorimetric assay (19,20). 100.0 μL aliquot of extracts in 80% methanol and acetone were added to a 10 mL volumetric flask containing 4 mL of distilled water. At zero time, 0.3 mL 5% sodium nitrite was added to the flask. After 5 min, 0.3 mL of 10% aluminium chloride was added. At 6 min, 2 mL of 1 M sodium hydroxide was added to the mixture. Immediately, the mixture was diluted to volume with the addition of 3.3 mL distilled water and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at

510 nm versus a blank containing all reagents except samples of extracts or fractions. Rutin and quercetin was used as standard for the calibration curve. Rutin and quercetin equivalents were calculated using standard graph of Rutin and quercetin.

2.5. Modified CUPRAC Assay

The CUPRAC method is based on the reduction of a cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the cuprous form (Cu(I)–Nc). 200 μL sample was taken from acetone extract and 80% methanol extract was diluted with methanol and acetone at a ratio of 1:10. 400 μL sample was taken from 1:10 diluted 80% methanol extract. To a test tube were added 0.5 mL each of Cu(II), 0.5 mL ethanol, 1 mL Nc and NH_4Ac buffer solutions. Sample or standard solution (x mL) and ethanol (1 – x) mL were added to the initial mixture so as to make the final volume: 4 mL. The tubes were stoppered, and after 1/2 h, the absorbance at 450 nm (A_{450}) was recorded against water blank (21). The Standard calibration curves of each antioxidant compound was constructed in this manner as absorbance vs. concentration, and the molar absorptivity of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned.

If a plant infusion (initial volume = V_{cup}) prepared from (m) grams of dried plant was diluted (r) times prior to analysis and a sample volume of V_s was taken for analysis from the diluted extract and colour development (after addition of reagents) was made in a final volume of (V_f) to yield an absorbance of (A_f) and antioxidant capacity of the plant was found using the equation:

$$\text{Capacity (in mmol/g)} = (A_f / \epsilon) (V_f / V_s) r (V_{\text{cup}} / m)$$

2.6. Determination of ABTS/persulfate assay

To 1 mL of the radical cation solution, 4 mL of ethanol were added, and the absorbance at 734 nm was read at the end of the six minute. The procedure was repeated for the unknown plant extract by adding 1 mL of the radical cation solution to x mL (x = 0.1 or 0.5 mL) of dilute plant extract (previously diluted with H_2O at a volume ratio of 1:20) and (4-x) mL of ethanol, and recording the absorbance (22). The absorbance difference (ΔA) was found by subtracting the extract absorbance from that of the reagent blank (pure radical solution). Dried plant was diluted (r) times prior to analysis and a sample volume of V_s was taken for analysis from the diluted extract and colour development (after addition of reagents) was made in a final volume of (V_f) to yield an absorbance of (A_f).

Antioxidant capacity of the plant was found using the equation:

$$\text{Capacity (in mmol/g)} = (\Delta A / \epsilon) (V_f / V_s) r (V_{\text{ABTS}} / \text{Persulfate} / m)$$

2.7. Determination of DMPD method

Extracts were diluted with its solution at a ratio of 1:10. 300 µL sample was taken from acetone extract and 300 µL sample was taken from 80 % methanol extract. To a test tube were added 0.5 mL of FeCl₃, 1 mL of acetate buffer solution (pH 5.7), 1 mL of H₂O₂ (3%), and x mL of sample solution, and the volume was completed to 9-x mL with distilled water. The mixture was shaken after each addition and then allowed to stand on a water bath at 25 °C for 5 min. After the addition of 4.8 × 10⁻³ M (1 mL) DMPD solution (DMPD solution at 2.4 × 10⁻² M described in the original method was diluted 5 times with distilled water to get a final absorbance of ~0.9–1.0 in the absence of scavenger sample solution), the mixture was kept on the water bath for an additional 20 min, and the absorbance in the absence or presence of sample was recorded against distilled water at 514 nm. The decrease in absorbance in the presence of sample linearly correlated with antioxidant concentration over a reasonable range (23). Percentage of inhibition for all of the dilutions was determined by the following equation:

$$\text{Inhibition \%} = (1 - A_f / A_0) 100 (\%)$$

where A_f is the absorbance of the non-scavenged radical and A₀ is the absorbance of the cation after 20 min of incubation with the antioxidant.

2.8. Determination of phosphomolybdate method

Extracts were diluted with its solution at a ratio of 1:10. 300 µL sample were taken from acetone and 80% methanol extract. Total antioxidant activity of the fractions was evaluated by the phosphomolybdate method using AA as a standard. The assay is based on the reduction of Mo (VI)-Mo(V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank using a spectrophotometer. Methanol (0.3 mL) in the place of extract is used as the blank. Ascorbic acid equivalents were calculated using standard graph of AA.(24).

The experiment was conducted in triplicates and values are expressed as equivalent of ascorbic acid in mg per g of extract.

2.9. Determination of CERAC method

Extracts were diluted with its solution at a ratio of 1:10. 200 µL sample were taken from acetone and 80% methanol extract. The method is based on the oxidation of antioxidants with cerium (IV) sulphate in dilute sulphuric acid at room temperature. The Ce(IV) reducing capacity of the sample is measured under carefully adjusted conditions of oxidant concentration and pH such that only antioxidants and not other organic compounds would be oxidized. The spectrophotometric determination of the remaining Ce (IV) was performed after completion of reaction with antioxidants. To a test tube were added 1 mL of 2 × 10⁻³ M Ce(IV), x mL of sample and (10-x) mL of water were added (total volume = 10 mL) and mixed well. Absorbance against a reagent blank was measured at 320 nm after 0.5 h.(25).

If a plant infusion (initial volume = V_E) prepared from m grams of dried plant was dilute (r) times prior to analysis and a sample volume of V_S was taken for analysis from the diluted extract and colour development (after addition of reagent) was made in a final volume of (V_F). The absorbance difference (ΔA) was found by subtracting the extract absorbance from that of the reagent blank and The molar absorptivity of ascorbic acid (ε) is 12950 L⁻¹cm⁻¹, then the ascorbic acid equivalent antioxidant capacity of the plant (in mmol AA per gram of plant, or simply mmol AA/g) was found using the equation:

$$\text{Capacity (mmol /g)} = [A_f / \epsilon] (V_f / V_s) r (V_E / m)$$

2.10. Bacterial Strains

80% methanol and acetone extracts of plant were individually tested against a panel of bacteria, including *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7, *Salmonella* Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 2592. All strains mentioned above were obtained as actively growing cultures from the American Type Culture Collection (ATCC). Stock cultures of *E. coli* ATCC 25922, *E. coli* O157:H7, *S. Enteritidis* ATCC 13076, *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 2592 were grown in Nutrient Broth (Acumedia Manufacturers, Inc., Maryland) at 37°C for 24 h and suspensions were adjusted to 0.5 McFarland standard turbidity (corresponding to 10⁷-10⁸ cfu/ml for bacteria, depending on genera).

2.11. Disc diffusion assay

Antimicrobial activity of methanol and acetone extracts of *Cirsium bulgaricum* DC. plant was investigated respectively. The agar disc diffusion method was employed

for the determination of antimicrobial activities of the extracts in question (29). Briefly, a suspension of the tested microorganism (0.1 mL of 10^8 cells per ml) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 20 μ L of the extracts and methanol (as negative control) and placed on the inoculated plates. After staying at 4°C (2h), all petri dishes were incubated at 37°C (24h), except *L. monocytogenes* that was incubated during 48 h. The diameters of the inhibition zones were measured in millimetres. Tests were carried out in triplicate. Values are presented as means \pm SD of three parallel measurements.

2.12. Determination of minimum inhibitory concentration (MIC)

Bacterial strains sensitive to the plant extracts in disc diffusion assay were studied for their minimal inhibition concentration (MIC) values using micro-well dilution assay method (26). MICs were defined as the lowest concentrations of the antimicrobial agents that inhibited visible growth of the microorganism. For the determination of antibacterial activities *E.coli* ATCC 25922, *E.coli* O157:H7, *S.Enteritidis* ATCC 13076, *L.monocytogenes* ATCC 7644, *S.aureus* ATCC 25923 bacteria were used as test bacteria.

The inocula of the microbial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts of plants dissolved in 10% DMSO were first diluted to the highest concentration (1000 μ g/mL) to be tested, and then serial twofold dilutions were made in a concentration range of 7.8 to 1000 μ g/mL in 10 mL sterile test tubes containing Nutrient broth.

The 96-well plates were prepared by dispensing 95 μ L of the cultures media and 5 μ L of the inoculum into each well. A 100 μ L aliquot from the stock solutions of extracts initially prepared at the concentration of 1000 μ g/mL was added into the first wells. Then, 100 μ L from their serial dilutions was transferred into seven consecutive wells. The last well containing 195 μ L of nutrient broth without compound and 5 μ L of the inoculum on each strip was used as the negative control. The final volume in each well was 200 μ L. contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated for 24 h at 37 °C.

Microbial growth was determined by the presence of a white pellet on the well bottom and confirmed by plating 5 μ L samples from clear wells on Nutrient Agar medium. The MIC value was defined as the lowest concentration of the extract required for inhibiting the growth of each

Table 2. Analysis results of antioxidants tested with CUPRAC and ABTS methods (n=3)

Antioxidant (mmol/g)	80% Methanol		Aceton	
	CUPRAC	ABTS/persulphate	CUPRAC	ABTS/persulphate
TR	0.18 \pm 0.02	0.14 \pm 0.03	0.02 \pm 0.09	0.01 \pm 0.02
CT	0.05 \pm 0.07	0.03 \pm 0.05	0.007 \pm 0.03	0.005 \pm 0.02
EC	0.04 \pm 0.09	0.05 \pm 0.08	0.006 \pm 0.06	0.007 \pm 0.07
RT	0.06 \pm 0.05	0.07 \pm 0.01	0.009 \pm 0.01	0.01 \pm 0.05
QR	0.04 \pm 0.05	0.03 \pm 0.04	0.005 \pm 0.08	0.004 \pm 0.04
NG	6.16 \pm 0.01	5.02 \pm 0.07	0.84 \pm 0.07	0.73 \pm 0.08

^a Values are expressed as mean \pm standard deviation(n=3)

^bTR: Trolox, CT: Catechin, EC: Epicatechin, RT: Rutin, QR: Quercetin, NG: Naringenin

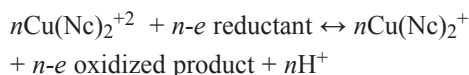
microorganism. All tests were repeated two times.

3. RESULTS AND DISCUSSION

In this study, it is aimed to evaluate the effect of solvent on the antioxidant behavior of phenolic compounds. The TAC measurements in different solvent media of other ET-based assays (i.e., ABTS/persulfate, DMPD, CERAC, Phosphomolybdate method) were compared to those of the CUPRAC assay. Methanol 80% and acetone 100% solvent media were chosen as variable solvent environments. The antioxidant compounds of different representative classes used in this work are trolox, catechin, epicatechin, rutin, quercetin naringenin and Vitamin C

3.1. CUPRAC and ABTS Persulfate method

The CUPRAC method of total antioxidant capacity (TAC) assay uses bis(2,9-dimethyl-1,10-phenanthroline: neocuproine)Cu(II) chelate cation as the chromogenic oxidant, which is reduced in the presence of antioxidants to the cuprous neocuproine chelate [Cu(I)-Nc] showing maximum light absorption at 450 nm. Colour development in the CUPRAC method is based on the following reaction: (26).



Antioxidants show some variation with solvent polarity. The various antioxidant compounds found with ET-based CUPRAC, ABTS/persulfate are shown in Table 2. In the CUPRAC assay results, The CUPRAC and ABTS/Persulfate values of TR, CAT, EC, RT, QR and NG were higher in MeOH 80% than in pure acetone (Table 2), probably due to facilitated e-transfer in ionizing solvents

Table 3. Inhibition effect of DMPD Method

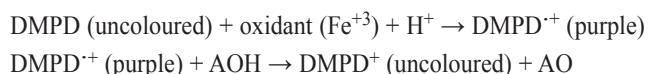
	Inhibition effect (%)
MeOH %80	2.3±0.02
Acetone	4.6±0.07

^a Values are expressed as mean ± standard deviation (n=3)

capable of anion (phenolate) solvation, because MeOH is the alcohol that best supports ionization. TAC_{ABTS}/Persulfate values were closer TAC_{CUPRAC} values.

3.2. DMPD method

The principle of the assay is that at an acidic pH and in the presence of a suitable oxidant solution DMPD can form a stable and colored radical cation (DMPD^{•+}). Antioxidant compounds which are able to transfer a hydrogen atom to DMPD^{•+} quench the color and produce a decoloration of the solution which is proportional to their amount.



the absorbance at 514 nm as percentage of the absorbance of the uninhibited radical cation solution (blank) according to the equation:

inhibition of A₅₁₄ (%) = $(1 - A_f / A_0) \times 100$, where: A₀ is the absorbance of uninhibited radical cation and A_f is the absorbance measured 20 min after the addition of antioxidant samples. The results of DMPD method is shown in Table 3. In the DMPD assay results, Inhibition effects were higher in acetone than in 80% methanol solution (23-27).

3.3. CERAC and Phosphomolybdate method

For CERAC method, the equation for the calibration line of ascorbic acid in the ascorbic acid concentration range 1.5×10^5 to 7.5×10^4 M was: $A_{320} = 1.295 \times 10^4 \text{ Casc.} - 0.9648$, ($R^2 = 0.99$) yielding a molar absorptivity of $1.295 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ for ascorbic acid. Antioxidants were quantified indirectly through their ability to reduce a fixed initial concentration of Ce(IV). For Phosphomolybdate method, the equation for the calibration line of ascorbic acid in the ascorbic acid concentration range 25 to 400 µg/ml was: $A_{695} = 3.8 \times 10^2 \text{ Casc.} - 0.0171$, ($R^2 = 0.99$) yielding a molar absorptivity of $3.8 \times 10^2 \text{ L mol}^{-1} \text{ cm}^{-1}$ for ascorbic acid. phosphomolybdate method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The phosphomolybdate

Table 4. CERAC and Phosphomolybdate analysis results

	Cerac Method (mmol AA/g)	Fosfomolibden Method (µg AA/ml)
MeOH %80	0.0834±0.09	807.6±0.07
Acetone	0.5622±0.05	292.4±0.01

^a Values are expressed as mean ± standard deviation (n=3)

method is a quantitative since the total antioxidant activity is expressed as AA (ascorbic acid) equivalent. In the CERAC assay results, AA were higher in acetone than in 80% methanol solution and In the Phosphomolybdate method assay results, AA were higher in methanol 80% than in acetone solution. Analysis results are shown in Table 4 (24-25).

3.4. Total flavonoid content

Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo-γ-pyrone structure. It is ubiquitous in fruits and vegetables. Total flavonoid contents can be determined in the sample extracts / fractions by reaction with sodium nitrite, followed by the development of coloured flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm. The total flavonoid content was expressed as rutin, quercetin equivalents (RE) in microgram per milliliter of extracts (28). Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenols. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. The flavonoid contents of *Cirsium bulgaricum* DC. were calculated using the standard plots of quercetin and rutin, respectively $y = 0.0011x - 0.018$, $R^2 = 0.997$ and $y = 0.0001x + 0.049$, $R^2 = 0.998$. Total flavonoid contents are shown in Table 5.

3.5. Statistical analysis

The data's are expressed as mean ± standard deviation (SD) from three parallel measurements. The Pearson correlation analysis was performed between antioxidant activity and total flavonoids. There were strong positive significant correlations between CUPRAC and contents of flavonoids, ABTS/Persulfate and contents of flavonoids. ($p < 0.01$). Pearson's correlation coefficient was calculated using Microsoft Excel 2010.

Table 5. Total flavonoid contents of *Cirsium bulgaricum* leaf extracts (n=3)

	QR ($\mu\text{g/mL}$)	RT($\mu\text{g/mL}$)
MeOH %80	119.5 \pm 0.03	645 \pm 0.004
Acetone	105.5 \pm 0.1	490 \pm 0.08

^a Values are expressed as mean \pm standard deviation(n=3)

3.6. Antimicrobial effect

Antibacterial activities of the methanol and acetone extracts of *Cirsium bulgaricum* DC. plant evaluated here were determined by the application of agar disc diffusion and MIC tests against a panel of pathogenic bacteria. The results are given in Table 6. As expected, the negative control treatment (absolute methanol) had no inhibitory effect on any of the test bacteria. As summarized in Table 6, methanol and acetone extracts of *C. bulgaricum* showed antibacterial activity against all pathogenic bacteria tested, particularly against gram-positive bacteria.

In general, the acetone extract possessed higher antibacterial activity than methanol extract of *C. bulgaricum* with diameter of inhibition zones ranging from 12.70-17.60 mm, while it was 11.60-14.43 mm for the methanol 80% extract. The results from the disc diffusion method, followed by measurement of minimum inhibitory concentration (MIC), indicated that *L.monocytogenes* ATCC 7644 was the most sensitive microorganisms tested, showing the highest inhibition zones (14.43 mm for methanol extract and 19.88 mm for acetone extracts) and the lowest MIC values (250 $\mu\text{g/mL}$ for both extracts). Other sensitive microorganism was *S.aureus* ATCC 25923 of which inhibition zone and MIC values are 13.55 (methanol), 18.99 (acetone) and 250 $\mu\text{g/mL}$ respectively. The methanol extract of the plant showed weak antibacterial activity against *S.Enteritidis* ATCC 13076 and *E.coli* O157:H7 in both disc diffusion and MIC tests.

The resistance of these Gram negative bacteria could be due to the complexity of their double layer cell membrane in comparison with the single membrane of Gram positive bacteria (31). In this regard, the extracts of the plant showed its best antibacterial activity in both disc diffusion and MIC tests on gram positive bacteria (*L.monocytogenes* ATCC 7644 and *S.aureus* ATCC 25923) tested. However, its inhibitory effect on Gram negative bacteria was also considerable. Overall the extracts of plant displayed moderate activity against the test bacteria.

Nazaruka was examined Antioxidant activity of *Cirsium* (Asteraceae): *C. arvense* (L.) Scop., *C. oleraceum* (L.) Scop., *C. palustre* (L.) Scop., *C. rivulare* (Jacq.).The plants were extracted with

Table 6. Antibacterial activity against pathogenic bacteria of the methanol and acetone extracts of *Cirsium bulgaricum* ^a (n=3)

Microorganisms	MeOH %80		Acetone		Negative Control
	Disc diffusion ^b	MIC ^c	Disc diffusion	MIC	Methanol
<i>E.coli</i> ATCC 25922	12.0 \pm 1.05	500	17.65 \pm 1.89	250	NA
<i>E.coli</i> O157:H7	11.6 \pm 1.00	500	17.00 \pm 1.72	500	NA
<i>S.enteritidis</i> ATCC 13076	11.35 \pm 1.43	500	17.60 \pm 1.46	500	NA
<i>L.monocytogenes</i> ATCC 7644	14.43 \pm 1.32	250	19.88 \pm 1.75	250	NA
<i>S.aureus</i> ATCC 25923	13.55 \pm 1.42	250	18.95 \pm 1.44	250	NA

^a Results are means of three different experiments (Mean \pm SD)

^b Diameter of inhibition zone including disc diameter of 6 mm.

^c MIC, minimum inhibitory concentration (as $\mu\text{g/mL}$)

NA: Not active

MeOH,CHCl₃, Et₂O, EtOAc and BuOH (32). Nazaruka et al. were investigated crude aqueous extracts from leaves of *Cirsium arvense*, *C. oleraceum*, *C. palustre*, *C. rivulare* and *C. vulgare*. Total phenolic content, analysed by using Folin-Ciocalteu's method, ranged between 54 and 96 mg g⁻¹, was expressed as milligrams of gallic acid per gram of dry extract. Phenolic acids were identified by HPLC method. Antimicrobial activity of those extracts was examined. The total antioxidant activity indicated by radical cation 2,2'-azini-di-(3-ethylbenzthiazoline sulphonate) ABTS^{•+}, expressed as total antioxidant status (TAS) ranged from 2.31 to 2.78 mM L⁻¹ (33). Yin et al. were investigated the antioxidant, antidiabetic activities and phenolic and flavonoid contents of methanol (MeOH) and water extracts from roots of *Cirsium japonicum in vitro* by the DPPH method, the aluminum chloride colorimetric method, Hydroxyl radical scavenging activity assay, reducing power assay, ferrous ions chelating activity assay, α -glucosidase inhibitory assay and Folin-Ciocalteu's method (34). In *Cirsium* species, presence of flavonoids (luteolin-7-glucoside, -7-glucuronide, cirsiliol, hispudilin, cirsitakaoside, cirsitakaogenin etc.), triterpenes, acyclic diterpenes (α -tocopherol, *trans*- phytol), polyolefins, fatty acids (linoleic, linolenic, palmitic, and stearic acids) and some widespread acetylenes have been reported (35).

4. CONCLUSION

This study was designed for a detailed investigation of the total flavonoid content profile, antioxidant activity and antimicrobial activity of *Cirsium bulgaricum* DC. leaves

growing in Turkey. Five different methods were applied to the test materials in antioxidant activity analysis. The results revealed that each of the methanol 80% and acetone 100% extract prepared from these leaves have antioxidant activity and antimicrobial activity. In conclusion, the results obtained from this work demonstrated that the antioxidant

behaviour of phenolic compounds show variations based on solvent type and polarity, reaction mechanism, solubility parameters as well as on an essential structural property, i.e., electron-transfer capability According to results , *Cirsium bulgaricum* DC. leaves have good antioxidant, antimicrobial activity and total flavonoid contents.

***Cirsium bulgaricum* yaprak ekstresinin antioksidan, antimikrobiyal etkinliği ve toplam flavonoid içeriği**

ÖZET

Bu çalışmada *Cirsium bulgaricum* DC. bitkisinin metanol ve aseton ekstraktları dört farklı antioksidan aktivite tayin yöntemi (CUPRAC, DMPD , CERAC ve Fosfomolibden yöntemi) ile incelendi. Cuprac ve ABTS/Persülfat metoduna göre 80% metanol ve aseton ekstraktları sırasıyla 0.005-6.16±0.03 mmol/g ve 0.004-5.02±0.02 aralıklarında mmol (TR, CT, EC, RT, QR, NG eşdeğeri olarak) /g bulundu. DMPD yöntemine göre 80% metanol ekstraktı için inhibisyon etkisi 2.3%±0.02 ve aseton için 4.6%±0.07 bulundu. CERAC yöntemine göre, 80% metanol ekstraktı için 0.0834±0.09 mmol AA/g, aseton ekstraktı için de 0.5622±0.05 mmol AA/g

bulundu. Fosfomolibden yöntemine göre, 80% metanol ekstraktı için 807.6±0.07 mmol AA/g , aseton ekstraktı için de 292.4±0.01 µg(AA)/ml bulundu. Total flavonoid içerikleri 80% metanol ve aseton ekstraktları için 105.5-645±0.04µg/ml aralığında quercetin ve rutin eşdeğeri olarak bulundu. Antibakterial etkinin belirlenmesinde test bakterileri olarak *Escherichia coli* (E.coli) ATCC 25922, *Escherichia coli*(E. coli) O157:H7, *Salmonella* Enteritidis(S.Enteritidis) ATCC 13076, *Listeria monocytogenes*(L.monocytogenes) ATCC 7644, *Staphylococcus aureus* (S.aureus) ATCC 25923 kullanıldı. Aseton ekstraktları için 17.00±1.72- 19.88±1.75 mm ve 80% metanol ekstraktları için 11.35±1.43-14.43±1.32 mm aralıklarında test edilen bakterilere karşı antimikrobiyal etki gözlemlendi.

Anahtar sözcükler: Antioksidan Etki, Antimikrobiyal Etki, Toplam Flavanoid İçeriği, *Cirsium bulgaricum*

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