EVALUATION OF ANTIOXIDANT ACTIVITY OF *in vitro* PROPAGATED MEDICINAL *Ceratophyllum demersum* L. EXTRACTS

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ABSTRACT

Ceratophyllum demersum L. is a medical plant of the family Ceratophyllaceae that has been traditionally used for the treatment of diseases such as ulcer, diarrhoea, wounds and fever since ancient times. This study presents antioxidant activities of water and methanol extracts of *in vitro* propagated *C. demersum*. Shoot tip explants of *C. demersum* for *in vitro* plant regeneration were cultured on liquid Murashige and Skoog (MS) medium containing 0.25–1.25 mg/L 6-Benzylaminopurine (BAP) and 0.10 mg/L Thidiazuron (TDZ) combinations for eight weeks. Maximum number of plantlets per explant (110.67) was obtained on MS medium supplemented with 0.75 mg/L BAP + 0.10 mg/L TDZ. In order to determine antioxidant activities of *C. demersum*, antioxidant contents (*β*-carotene, flavonoid, lycopene and total phenols), metal chelating and reducing power capacities of methanol and water extracts obtained from *C. demersum* were investigated. It was detected that water extract was more effective in all activities. Total phenols (76.55 μg/mg) in water extract were measured as the highest antioxidant component. Based on the water extracts data, as IC_{50} value for chelating on ferrous ions was 9.24 mg/mL, EC_{50} value for reducing power activity was 8.23 mg/mL. It was also determined that the metal chelating and reducing power activities of the both extracts are increased depending on the concentration. Therefore, all these data suggest that both tested extracts of *in vitro* propagated *C. demersum* would exert beneficial effects by virtue of their antioxidant properties and may be utilized as a promising source of therapeutics.

Key words: shoot regeneration, antioxidant component, metal chelating, reducing power

INTRODUCTION

Chemical processes that occur in humans, especially oxidation, cause formation of free radicals. Free radicals that are highly reactive can easily react with different molecules and thus damage cells [Vikram et al. 2010]. The cells exposed to oxidative stress can cause lipid peroxidation, protein oxidation, DNA mutations and fractures, cytotoxic effects and impaired signalling [Mena et al. 2009].

Antioxidants are the agents that slow the autoxidation of oils. They prevent free radical-induced cell damage reacting with free radicals and decrease the risk of cell abnormalities and tumor formation. As cell destruction is reduced, there is an increased chance of the life that is healthier and has a minimum of aging effects [Devasagayam et al. 2004]. An ideal antioxidant can chelate redox metals and regenerate them associating with other antioxidants in the antioxidant network. Antioxidant component presented in appropriate physiological levels in tissues and body fluids has positive effects on gene expression and functions both in liquid media and membranes [Carocho and Ferreira 2013].

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The use of synthetic antioxidants in foods began with the understanding that butylated hydroxyanisole (BHA) and gallic acid esters inhibited oxidation in the 1940s. Possible carcinogenic effects of synthetic antioxidants cause a growing response. Thus, many countries do not allow synthetic antioxidants such as BHA to be used in food and there is a general desire for the use of natural antioxidants in place of synthetic antioxidants [Nikoo and Benjakul 2015]. Both epidemiological and clinical trials have found evidences that phenolic antioxidants in cereals, fruits and vegetables reduce the severity of degenerative and chronic diseases. Natural antioxidants found in many foodstuffs can be used as stabilizers in composite foods or can be added to other foods by extracting [Augustyniak et al. 2010].

Natural antioxidants naturally occur in all parts of plants. These include carotenoids, vitamins, phenols, flavonoids, glutathione and endogenous metabolites. Plant-derived antioxidants function as singlet and triplet oxygen exterminators, free radical scavengers, peroxide scavengers, enzyme inhibitors and synergists [Abourashed 2013]. Many researchers and food scientists have been working on natural antioxidants that can replace synthetic antioxidants in recent years. For this purpose, it is directed to vegetal resources that have wide distribution on the earth. The aim is to add natural antioxidants obtained from these sources to the food instead of synthetic antioxidants during the processing of the foods [Pokorný 2007].

Ceratophyllum demersum L. commonly known as hornwort or coontail belongs to the Ceratophyllaceae family. *C. demersum* is a cosmopolitan submerged macrophyte and commonly found in ponds, ditches, lakes, and quiet streams [Keskinkan et al. 2004; Arber 2010]. This species can inhabit shallow as well as deep littoral habitats. It is a loosely attached to the bottom or free-floating vascular macrophyte, which builds dense stands in nutrient-rich waters [Pełechaty et al. 2014].

C. demersum has been traditionally used in the treatment of diarrhoea, fever, dysentery, ulcer, wounds, burning sensation, haemorrhoids or piles, intrinsic haemorrhages, epistaxis, hyperdipsia and haematemasis due to its active compounds and its therapeutic properties [Taranhalli et al. 2011]. It contains more than 50 components including essential oils such as 2-Methylpropanoic acid 3-hydroxy-2,4,4-trimethylpentyl ester, 2-methylpropanoic acid 2,2-di-methyl-1-(2-hydroxy-1-methylethyl) propyl ester, 1,2-benzenedicarboxylic acid bis (2-methylpropyl) ester, β-ionone-5,6-epoxide, toluene, hexanal, dihydroactinidiolide, pentadecanal and β-asarone [Qiming et al. 2006]. It was also reported that the aqueous, chloroform, ethanol and methanol extracts of *C. demersum* have antimicrobial effect against isolated strains of bacteria and fungi [Fareed et al. 2008]. There are also various studies on isolation of different secondary metabolites of *C. demersum* such as tricin, esculetin and palmitic acid [Bankova et al. 1995; Xiao-Li et al. 2007].

In recent years, there has been a growing interest in finding plants with antioxidant activity for food and medicinal applications. It has been emphasized that more research is required on antioxidant activity of the medicinal plants such as *C. demersum*. In the present study, we focused on the analyses of antioxidant components, metal chelating and reducing power activities of different extracts of *in vitro* grown *C. demersum*.

MATERIALS AND METHODS

***In vitro* plant regeneration.** *C. demersum* plants were obtained from Department of Biology, Karamanoğlu Mehmetbey University, Karaman, Turkey and sterilized accordingly to Dogan [2013]. Shoot tip explants were isolated under sterile conditions and cultured in liquid [Murashige and Skoog 1962] medium (MS) containing 0.25, 0.50, 0.75, 1.00 and 1.25 mg/L 6-Benzylaminopurine (BAP) and 0.10 mg/L Thidiazuron (TDZ) combinations in Magenta GA^7 vessels for eight weeks. Also, the explants were cultured on hormone-free MS medium (MSO) as control experiment. pH was adjusted to 5.6–5.8 using either 1 N NaOH or 1N HCl prior to autoclaving at 121°C and 118 kPa for 20 min. All cultures were placed in culture room at 24°C and 16 h light photoperiod (1500 lux) using white Light Emitting Diodes (LED) lights. The data were recorded for shoot regeneration after eight weeks of culture.

Extraction of bioactive ingredients. Entire samples of *C. demersum* were used for extraction. Fine dried powdered samples were prepared using liquid nitrogen, mortar and pestle from 10 g of whole samples. The extracts were obtained in 250 ml of different solvent systems such as methanol and water using Soxhlet extraction apparatus for two days. Thereafter, solvents were evaporated using rotary evaporator under vacuum to dryness and extract with 2, 4, 6, 8 and 10 mg/mL concentration were prepared with 5% dimethyl sulfoxide (DMSO). Extraction of methanol and water yielded 22.14 and 25.46% (w/w) of substances, respectively. The yields were based on dry materials of plant sample.

Determination of antioxidant potentials. Possible antioxidant potentials of methanol and water extracts of *C. demersum* were evaluated by measuring their total flavanoids, *β*-carotene and lycopene amount and phenolic contents as well as reducing power and metal chelating activity. The methods used to evaluate these indicators of antioxidative capacity are given in the following parts.

Determination of total phenolic contents. Concentration of total phenolic compounds in different extracts of *C. demersum* was determined spectrophotometrically according to method described previously by Taga et al. [1984] with slight modifications. In this method, gallic acid with various concentrations (0.01–1.0 mM) was used as standard phenolic compound. This method was adapted to microtiter plate measurements in triplicate. 20 µl of standards or different extracts (10 mg/mL) were mixed with same amount of Folin and Ciocalteu's phenol reagent (2N) and kept at dark for 3 min. Afterwards, 20 µl of 35% sodium carbonate (w/v) and 140 µl dH_2O were added to start incubation period for 10 min. After that, the absorbance was measured at 725 nm and the results were calculated from gallic acid calibration curve and expressed as mg of gallic acid equivalents/mg of extracts.

Determination of total flavonoid contents. Total flavonoids of methanol and water extracts obtained from *C. demersum* were determined using protocols reported by Pal et al. (2010) with slight modifications. 50 µl of extracts (10 mg/mL) were mixed with 215 µl of ethyl alcohol (80% v/v), 5 µl of aluminum nitrate (10% w/v) and 5 µl potassium acetate (1 M) in microtiter plates and incubated for 40 min at room temperature. After reading at 415 nm, total flavonoid contents were calculated according to following equation:

$$\text{Total flavonoid contents (µg/mg extract)} = (A_{415} + 0.01089) / 0.002108$$

Determination of *β*-carotene and lycopene contents. Methanol and water extracts obtained from *C. demersum* were re-extracted with 10 ml of acetone : hexane (4 : 6) mixture and filtered through Whatman No. 4 filter paper in order to determine *β*-carotene and lycopene contents. After filtration, absorbance of the filtrates was measured at 453, 505 and 663 nm. *β*-carotene and lycopene contents were determined according to following equations [Pal et al. 2010].

$$\beta\text{-carotene content (mg/100 mg)} = 0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}$$

$$\text{Lycopene content (mg/100 mg)} = -0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$$

Determination of metal chelating activity. Chelating abilities of the extracts from *C. demersum* were determined using ethylenediaminetetraacetic acid (EDTA) as a standard chelating agent [Oyetayo et al. 2009]. Different concentrations (50 µl) of extracts (2, 4, 6, 8, 10 mg/mL) and standard (0.1–5 mM) were added to microplate wells and mixed with 10 µl ferrozine (5 mM), 5 µl iron (II) chloride (2 mM) and 185 µl absolute methanol. After incubation for 10 min at room temperature, absorbances were read at 562 nm and median inhibitory concentration (IC_{50}) values were calculated.

Determination of reducing power. Reducing powers of different extracts of *C. demersum* were determined according to the method of Karamac et al. [2000] with an adaptation to microplate measurement. Gallic acid (0.01–0.1 mM) was used as standard antioxidant. In the method, various concentrations of 50 µl *C. demersum* extracts (2, 4, 6, 8,

10 mg/mL) were mixed with 75 µl phosphate buffer (0.2 M pH: 6.6) and 75 µl potassium ferricyanide (1% w/v) in a total volume of 200 µl and incubated at 50°C for 20 min. After adding 75 µl trichloroacetic acid (10% w/v), samples were centrifuged for 10 min at 1000 g. Supernatant (75 µl) were transferred to another microtiter plate and mixed with 75 µl distilled water and 15 µl iron (III) chloride (0.1% w/v). After reading the absorbance at 700 nm, median effective concentration (EC_{50}) values at which the absorbance was 0.5 for reducing power was calculated.

Statistical analyses. All the assays were carried out at least in triplicate measurements. Antioxidant activities of the plants and differences among the values about the plant production were compared with analysis of variance (ANOVA) by Duncan's multiple range test for comparing groups. EC_{50} and IC_{50} values were calculated with Probit regression analysis and associated 95% confidence limits for each treatment. Relations among the variables were tested by bivariate correlation analysis. These calculations were carried out using Statistical Package for Social Sciences (SPSS®, version 21.0).

RESULTS AND DISCUSSION

Throughout the history of mankind, many diseases have been tried to be treated using plants. Especially after the 1990s, new usage areas of medicinal and aromatic plants increase the use of these plants day by day [Lange 2006]. Naturally occurring substances in many plant species are used in various treatments and show positive results [Emsen et al. 2016, 2017]. This study presents antioxidant activities of water and methanol extracts of *C. demersum* propagated via tissue culture techniques.

The shoot regeneration frequencies in the shoot explants ranged from 83.33% to 100.00%, and they were found statistically insignificant ($p < 0.01$). The highest shoot regeneration frequency (100%) was determined in explants cultured on MS medium containing 0.50 and 0.75 mg/L BAP + 0.10 mg L TDZ, whereas the lowest shoot regeneration frequency (83.33%) was recorded in explants cultured on hormone-free MS (MSO) medium and MS medium containing 1.25 mg/L BAP + 0.10 mg/L TDZ. Contrarily, Gnanaraj et al. [2011] reported increased
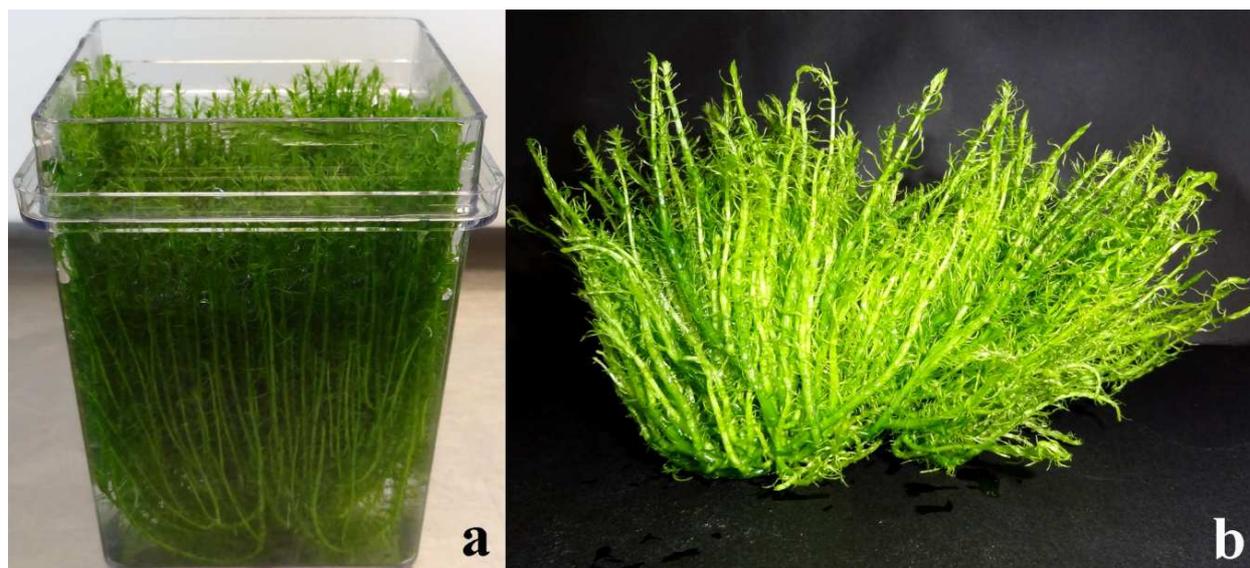

Fig. 1. *In vitro* plant regeneration from shoot tip explant of *C. demersum* cultured on liquid MS medium containing 0.75 mg/L BAP and 0.10 mg/L TDZ, after eight weeks of culture

Table 1. Effect of different combinations of BAP and TDZ on multiple shoot regeneration from shoot tip explants of *C. demersum* after eight weeks of culture

Plant growth regulators (mg/L)		Shoot regeneration frequency (%)	Mean number of shoots per explant	Shoot length (cm)
BAP	TDZ			
0	0	83.33 a*	1.57 d*	5.15 a*
0.25	0.10	94.44 a	73.00 c	4.76 b
0.50	0.10	100.00 a	86.33 bc	4.29 c
0.75	0.10	100.00 a	110.67 a	3.85 d
1.00	0.10	94.44 a	97.00 ab	3.70 d
1.25	0.10	83.33 a	75.67 c	3.66 d

*Values followed by different small letters in the same column differ significantly at $p < 0.01$

shoot regeneration frequency with increased BA concentration in *Alternanthera sessilis*.

The mean number of regenerated plantlets per explant ranged from 1.57 to 110.67 and was statistically significant at $p < 0.01$ level (tab. 1). The maximum number of plantlets per explant was achieved on the MS medium containing 0.75 mg/L BAP + 0.10 mg/L TDZ (110.67), followed by MS medium supplemented with 1.00 mg/L BAP + 0.10 mg/L TDZ (97.00). The minimum number of plantlets per explant (1.57) was obtained on MSO medium (the control group), followed by MS medium containing 0.25 mg/L BAP + 0.10 mg/L TDZ (73.00). The results showed that higher and lower BAP ratios have adversely affected on the number of plantlets per explants. The present findings are in agreement with those observed in other plant species such as *Aloe barbadensis* Mill. [Baksha et al. 2005], *Musa* spp. [Muhammad et al. 2007], *Chlorophytum borivilianum* Sant. & Fernandez [Ashraf et al. 2014] and *Aloe vera* (L.) [Gupta et al. 2014]. In contrast, Jo et al. [2008] reported that mean number of shoots per explant in *Alocasia amazonica* increased with increase in BA concentrations and Sharma et al. [2007] also reported negative effects of increased BA concentration on shoots per explant of *B.monnieri*. The results reported in this paper confirm that some plant species have enough levels of endogenous hormones and do not require any extra amount of exogenous growth regulators for their regeneration [Hussey 1982].

It was observed that in the presence of BAP affected shoot length significantly ($p < 0.01$) (tab. 1). The highest shoot length was recorded as 5.15 cm on MSO, followed by MS medium supplemented with 0.25 mg/L BAP + 0.10 mg/L TDZ (4.76 cm) (tab. 1). The shoot lengths have gradually decreased with the increase of the BAP ratio and therefore, the shortest shoot lengths were recorded on MS medium containing 1.25 mg/L BAP + 0.10 mg/L TDZ (3.66 cm). The results are in line with Eng et al. [2014], cultured shoot tip explants of *Citrus hystrix* on MS medium containing various BAP concentrations (0–13.33 µM) and reported a decrease in shoot length depending on the increase in BAP concentration. Öztürk et al. [2004] also observed pronounced inhibition or suppression in elongation and growth of regenerated shoots of *Ludwigia repens* on media containing BAP. In contrast, Ashraf et al. [2014] reported the shortest shoot length (3.67 cm) in *C. borivilianum* cultured on MS medium containing the highest BAP concentration (26.6 µM).

Antioxidant components. There are many researches on the sources and use of natural antioxidants. It was proved that many plant species had more antioxidant capacity than synthetic antioxidants [Augustyniak et al. 2010]. Plants that have a broader bioactivity profile are natural antioxidants that can be used as an alternative due to their unique flavour, aroma, antimicrobial and antioxidant properties [Soumia et al. 2014]. The plants gain importance in

many different areas such as medicine, food industry, perfumery and cosmetics because of antioxidant compounds in their structure. Especially plants containing terpenic compounds, flavonoids, phenolic acids show significant antioxidant activities [McKay et al. 2015]. Studies conducted in previous years showed that the antioxidant effects of plants were different from each other since their chemical components were diverse. It was found that the thymol and carvacrol phenolic components in thyme structure gave it antioxidant properties [Ozkan et al. 2007]. Similarly, another study on the antioxidant effect of active ingredients was carried out on sage tea and it was determined that carnosol, carnosic acid and rosmanol were phenolic compounds that had effective antioxidant capacity [Lu and Yeap Foo 2001].

In this study, antioxidant contents (β-carotene, flavonoid, lycopene and total phenols) of methanol and water extracts obtained from *C. demersum* determined with the results shown in Table 2. Among these extracts, the highest component levels were detected in water. Considering total phenolic contents, water extract of *C. demersum* had the highest quantity (76.55 µg/mg). The antioxidant effect of phenolic compounds is due to their properties such as free radical scavenging, compounding with metal ions and inhibiting the formation of single oxygen [Carvalho Costa et al. 2015]. As for flavonoid content, amounts of both extracts were close. β-carotene contents in the extracts were one of the high antioxidant component values. The difference among lycopene contents in methanol and water extracts was considerable level (1.92 and 6.28 µg/mg, respectively). There are studies that different extracts of the same plant show antioxidant activity at different ratios. Gulcin et al. [2004] reported that chloroform extract of *Salvia sclarea* L. had higher antioxidant activity than acetone extract and the total antioxidant activities of both extracts were higher than that of α-tocopherol.

Chelating abilities of metal ions. Antioxidants are systems that destroy the effects of free radicals. There are many enzymatic or nonenzymatic endogenous antioxidant defence mechanisms to prevent the formation of reactive oxygen species and their damage [Valko et al. 2007]. Protective antioxidants that delay or completely stop free radical formation are metal chelators. They bind iron and copper ions that are responsible for the production of reactive species [Hofer et al. 2014]. The protective antioxidant effects of many plant extracts are detected through the potential of chelating Fe^{2+} ions.

Table 2. Antioxidant contents of different extracts

Extract	Content (µg/mg)*			
	β-Carotene	Flavonoid	Lycopene	Total phenols
Methanol	10.35 ±1.25	2.63 ±0.34	1.92 ±0.38	64.73 ±1.16
Water	18.25 ±1.27	4.26 ±0.33	6.28 ±0.62	76.55 ±1.11

* Each value is expressed as mean ± standard deviation ($n = 3$)

Table 3. Correlation between different variables for antioxidant activities

	Concentration	Chelating activity	Reducing power
Concentration	1		
Chelating activity	0.91*	1	
Reducing power	0.67*	0.84*	1

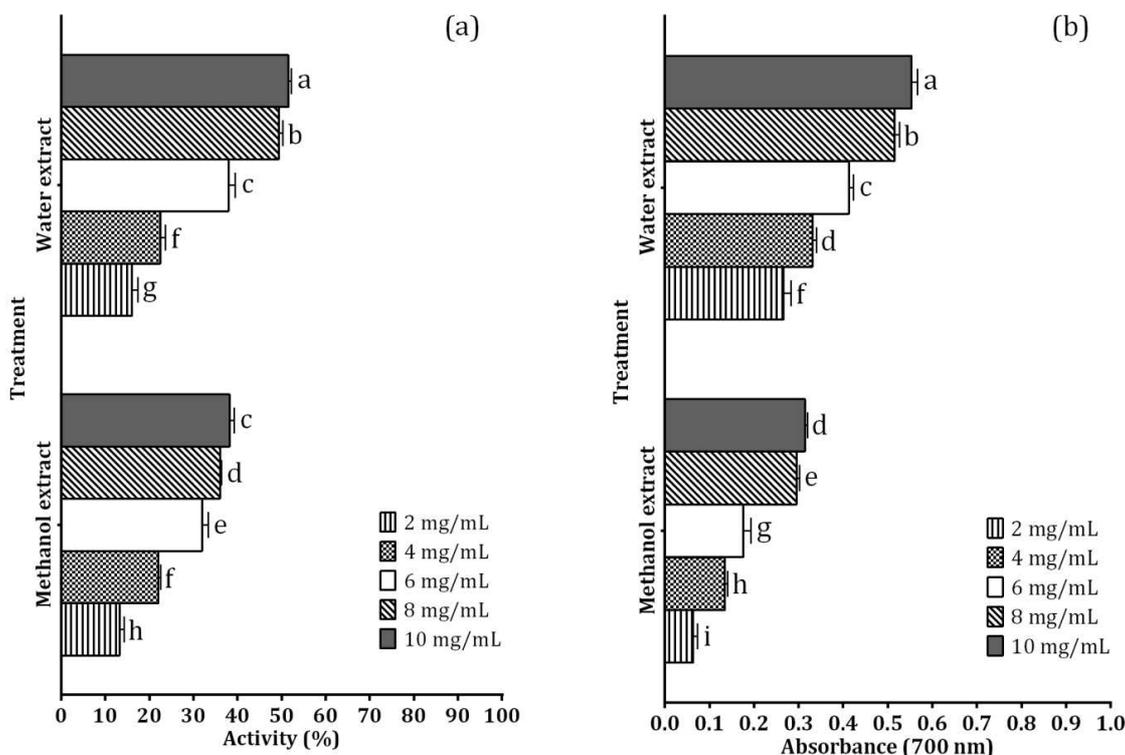* Correlation is significant at the 0.01 level

Fig. 2. Antioxidant activities of extracts in different concentrations from *C. demersum* (a) Chelating abilities on ferrous ions (b) Reducing powers. Each value is expressed as mean ± standard deviation ($n = 3$). Values followed by different small letters differ significantly at $p < 0.05$

Table 4. IC_{50} values (mg/mL) of different extracts and standard for chelating on ferrous ions

Treatment	IC_{50} (limits)	Slope ± Standard error (limits)
Methanol extract	16.31 (12.85–23.70) c*	1.21 ±0.15 (0.92–1.51)
Water extract	9.24 (8.23–10.72) b	1.63 ±0.15 (1.34–1.92)
EDTA	0.16 (0.13–0.18) a	1.25 ±0.07 (1.12–1.38)

* Values followed by different small letters in the same column differ significantly at $p < 0.05$

The present study has been focused on metal chelating activities of methanol and water extracts from *C. demersum*. The results revealed that metal chelating activity increased in a concentration-dependent manner for the both extracts. This relation was also revealed through bivariate correlation analysis. There was very high level of positive correlation between concentration and chelating activity [Pearson correlation coefficient (Pcc) = 0.91] (tab. 3). Water extract

at concentration of 10 mg/mL displayed the highest metal chelating activity (51.56%). There were also statistically ($p < 0.05$) significant differences between all values measured on the extracts (fig. 2a). In addition, as shown in Table 4, for chelating on ferrous ions, highly potent inhibition was found by water extract from *C. demersum* (IC_{50} = 9.24 mg/mL), when compared with standard (EDTA) inhibitor (IC_{50} = 0.16 mg/mL). It was determined that IC_{50}

values calculated for both extracts were statistically (p < 0.05) different from each other. Many researchers also demonstrated metal chelating activities of different plant species by determining their chemical content [Liu et al. 2014]. Heimler et al. [2007] pronounced that high chelating capacity in *Eruca sativa* might result from antioxidant compound glucosinolates.

Determination of reducing power. Reduction of Fe^{3+} to Fe^{2+} is an important indicator of potential antioxidant activity [Meir et al. 1995]. The Fe^{3+} reducing capacities of the compounds indicate their electron donating abilities. Because of these properties, some compounds may play a role in terminating the free radical chain by converting reactive free radical species into more stable non-radical ones [Sudha et al. 2016]. In some studies it also reported that there was a strong correlation between the Fe^{3+} reducing capacity and the inhibition of lipid peroxidation [Hinneburg et al. 2006].

Table 5. EC_{50} values (mg/mL) of different extracts and standard for reducing power

Treatment	EC_{50}
Methanol extract	15.16 c*
Water extract	8.23 b
Gallic acid	0.48 a

* Values followed by different small letters in the same column differ significantly at p < 0.05

In this study, it was determined that the reducing power activities of the methanol and water extracts obtained from *C. demersum* are increased depending on the concentration. It was showed high positive correlation at 0.01 level between concentration and reducing power (Pcc = 0.84) (tab. 3). The treatment with the highest reducing power was the solution of 10 mg/mL of water extract with an absorbance value of 0.55. Furthermore, many studies performed with different plant species such as *Eruca sativa* [Sarwar Alam et al. 2007], *Rumex vesicarius* [Beddou et al. 2015], *Artemisia annua* [Wan et al. 2016] and *Momordica charantia* [Svobodova et al. 2017] in previous years revealed that reducing power activity increased in concentration-dependent manner.

When the statistical analyses were examined, it was determined that reducing power activities of concentrations of methanol extract at 10 mg/mL and the water extract at 4 mg/mL were not different at the p > 0.05 level. When the activities of each extract were examined within themselves, it was found that the values were statistically different (p < 0.05) (fig. 2b).

Based on the reducing power activities, effective concentrations of extracts were determined by calculating EC_{50} values. Moreover, these values revealed more effective extract. It was determined that the lowest EC_{50} value was 0.48 mg/mL and it belonged to standard (gallic acid). It was found that the water extract having a very low EC_{50} (8.23 mg/mL) value in comparison with the methanol extract (EC_{50} = 15.16 mg/mL) has more effective reducing power level. It was also reflected in study results that the EC_{50} values of gallic acid and extracts were statistically different (p < 0.05) (tab. 5).

CONCLUSIONS

Considering important contents and low side effects of the plants, they are indispensable livings in the treatment processes. Therefore, numerous studies have been carried out on the use of medicinal plants for the treatment of diseases. The plants used in these studies are usually collected from nature and this situation may lead to degradation of ecological balance and decrease of biodiversity. In the present study, we used *C. demersum* plant produced by tissue culture technique, which provided us with significant advantages like multiple and rapid production of disease-free plants without adhering to external conditions, as well as protection of biodiversity. Afterwards, antioxidant activities of *C. demersum* were examined and the subject was tried to be illuminated in terms of usability instead of synthetic antioxidants. Today, the antioxidants that can replace synthetic antioxidants for the future are rapidly exploited. It is envisaged that plants with high antioxidant activity such as *C. demersum* will be replicated in desired amounts and their antioxidant effects in food systems will be investigated to ensure continuity of industrial application through the present study. At the same time, the results obtained suggest that *C. demersum*,

which has a high antioxidative capacity, is a plant that will be able to utilized in the combined treatment methods of oxidative stress-induced diseases.

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