

Identification of Antioxidant Activity by Different Methods of a Freshwater Alga (*Microspora* sp.) Collected From a High Mountain Lake

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ABSTRACT

Algae contain a large amount of biological active compounds functioning as antioxidant, antimicrobial and anti-tumour. In the present research, antioxidant activity of freshwater alga *Microspora* sp. (Chlorophyta) extract prepared in different solvents (methanol, acetonitrile, ethanol, acetone and ethyl acetate) was determined with three different methods; 2,2-diphenyl-1-picrylhydrazyl (DPPH•), Iron (III) Reduction / Antioxidant Power (FRAP) Assay and Copper (II) Reductive Antioxidant Activity (CUPRAC). The alga samples collected from the high mountain lake (2733 m above sea level) localized in Gümüşhane province of Turkey. The algal extracts prepared in different solvents showed antioxidant activity. The values of DPPH• varied from 8.97% (in acetonitrile extract) to 31.15% (in methanol extract). As in the DPPH• method, the lowest and highest values in the FRAP method were measured based on μM TEAC (Trolox Equivalent Antioxidant Capacity) in methanol and acetonitrile extracts as 60.303 μM and 359.394 μM respectively. While the lowest value of CUPRAC test was determined in acetonitrile extract (0.041 μM TEAC) as in the other tests, highest value was measured in ethanol extract (0.101 μM TEAC). The values of highest antioxidant activity in both DPPH• and FRAP methods was measured in the methanol solvent whilst was determined in the ethanol solvent for CUPRAC.

Keywords:

Algae; *Microspora* sp.; Antioxidant; DPPH•; FRAP; CUPRAC

INTRODUCTION

Algae having both macroscopic and microscopic forms and growing in all environments including the extreme conditions are photosynthetic organisms that contribute greatly to primer production of aquatic ecosystems. There are many records about they have been used as food and drug since centuries [1-3]. Particularly, their use in the food and pharmaceutical industry depends on presence of large number of various bioactive compounds. The variety of these bioactive compounds is related to the stress conditions in the extreme environments such as high altitude regions [4].

In order to survive, algae are resistant to stress factors such as high salinity, high and low temperature, low nutrients, hot alkaline and acidic environments,

and non-thermal acidic environments, high and low light conditions. Thus, they well adapt to extreme environmental conditions with synthesizing many natural products also called as secondary metabolites [5-7]. The natural products of algae include compounds such as polyphenols, flavonoids, amino acids, lignins, terpenoids, tocopherols, fatty acid, phenolic acids and have antioxidant, antimicrobial, antifungal and antiviral activity [8, 9]. Generally, researchers have focussed terrestrial plants (flower, fruits, seed, leaf, stem, bark and root) in determining antioxidant substances. However, the source of natural antioxidants is not only terrestrial plants, but also algae have been reported to be rich in natural antioxidant compounds [9-11]. Algae having species richness are potential sources of natural bioactive compounds for new demand in medicine and food industry

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[4, 12]. Among these natural compounds, phenolics composed of one or more aromatic rings containing hydroxyl groups are considered to be one of the most important classes of natural antioxidants [13]. Especially, marine macroalgae have a large number of these compounds. Red algae (Rhodophyta), brown algae (Phaeophyta) and green algae (Chlorophyta) contains some phenolic compounds such as phlorotannins, bromophenols, terpenoids, mycosporine-like amino acids, meroditerpenoids, coumarins vanillic acid. The phlorotannins are the most studied phenolic compounds in marine algae and are used in preparation of nutraceutical, cosmeceutical, and pharmaceutical products [13, 14].

Genus *Microspora* Thuret is classified in the Chlorophyta division that distributed in both freshwater and marine environments. It has H-shaped cell wall, cells slightly swollen, cylindrical, and quadrate, arranged uniseriate. The filaments are unbranched and occurring free or basically attached. Chloroplast is parietal or coarse net or net, not present pyrenoid. Aplanospores or akinetes are present [15, 16].

Although Turkey has a rich diversity of freshwater algae [17, 18], there are few studies to determine their chemical composition and biological activity [19-24]. Although, there are many systematic, taxonomic and ecological studies on the genus *Microspora*, the number of studies based on its chemical composition, antioxidant and antimicrobial activity is limited in the literature [25-27]. The purpose of this study was to determine the efficiency of antioxidant activity of an alga *Microspora* sp. in different solvents with commonly used three antioxidant activity determination methods and to provides availability of freshwater algae for research in the food and pharmaceutical industry.

MATERIALS AND METHODS

Samples Collection

The samples of *Microspora* sp. were collected from the Acembol Lakes (Lake AC-3) of the Artabel Lakes Natural Park located in the Gümüşhane province in August 2016. The lake was 2733 m above sea level and located at 40° 25' 03" N – 39° 03' 28" E. Samples of *Microspora* sp., which occurred blooms on the surface of the lake, are collected from the 0 - 0.5 m below surface. The samples were washed several times with distilled water for removal epiphytic organisms and other substance in the laboratory. Then, algae samples were dried at room temperature for 2 weeks and blandered into powder [24]. However some algal specimen was stored in 4% formaldehyde solution. Algae identified according to related books [15, 16].

Preparation of Algal Extracts

Five different extracts (acetone, acetonitrile, ethanol, ethyl acetate and methanol) were prepared from powde-

red *Microspora* sp. samples at room temperature. In the preparation of each extract 20 g of the sample (powder) was dissolved in 50 mL of solvent. Extractions were mixed by a magnetic stirrer for 2 hours. Then the extracts were filtered consecutively with filter paper and syringe filter (Minisart, NY 0.45 µm). Clear solutions obtained at the end of the process were stored at room temperature and in a cool, dark place until the test.

2,2-diphenyl-1-picrylhydrazyl (DPPH•) Scavenging Activity

DPPH• method developed by Cuendet et al. [28] was used for determining antioxidant activity extracts prepared with different solvents of *Microspora* sp. Firstly, 100 µM DPPH• radical solution was prepared and the solution was dissolved in the magnetic stirrer for 30 minutes. Each of algal extract was pre-tested and diluted 1/5 and analyzed in triplicate. However, each concentration of samples were run for a samples and reagent blank after 50 minutes, the absorbance values of the tubes mixed by the DPPH• reagent were read at 517 nm at spectrophotometer and the % inhibition (DPPH• purification) values were calculated as follows

$$\% \text{ Inhibition (radical cleaning power)} = [(ADPPH - A_{\text{Sample}}) / ADPPH] \times 100$$

ADPPH : Absorbance value of the DPPH solution

A_{Sample} : Absorbance value of the sample extract

Iron (III) Reduction / Antioxidant Power (FRAP) Assay

Absorbance in this method occurs at 595 nm and forms $K_3(Fe)CN_6$ color complex [29]. As a standard, the antioxidant Trolox was used at different concentrations (1000-500-250-125-62.5 µM). Different extracts of *Microspora* sp. were run from stock solutions (20 g + 50 mL solvent). Three replications were prepared for all standard and extracts. Firstly, 50 µl samples and standard solutions were transferred. Then 50 µl of sample solvents (methanol and distilled water) were transferred to the reagent blind tubes. After transferring 50 µL of the sample solutions to the sample blind tubes, 1.5 mL of FRAP solution (60% methanol pure water) was added. Thus, 1.5 mL newly prepared FRAP reagent was added to the other tubes at 20 second intervals. The tubes were mixed and after 20 minutes the absorbance values were read at 595 nm spectrophotometer device. The results were calculated as TEAC in comparison with the standard antioxidant Trolox.

Copper (II) Reductive Antioxidant Activity (CUPRAC)

Copper metal is used in CUPRAC and this method is based on the reduction of antioxidant substance Cu (II) to

Cu (I) [30]. In this method, Cu (II) chloride solution, neocuproin solution with 96% ethanol, ammonium acetate buffer (pH = 7) and analysis solutions were added, respectively. Then, 4.1 mL of the final solution was completed and it was kept at room temperature during 30 minutes. At the end of this procedure, absorbance values were measured at 450 nm and antioxidant capacities of the samples were calculated as Trolox equivalent [31].

RESULTS AND DISCUSSION

There are some synthetic antioxidants used in the food industry commercially available. However, some studies indicate that synthetic antioxidants have toxicological and carcinogenic effects on cell of experimental animals [32]. Due to these unfavorable effects, use of synthetic antioxidants was restricted in food industry. Therefore, natural antioxidants are increasingly interested by producers and consumers [10, 32]. Natural antioxidants were commonly obtained from terrestrial plants. But it is not limited to them. Also, aquatic organisms, especially algae have organic and inorganic substances such as polysaccharides, lipids, proteins, carotenoids, phenolic compounds, vitamins and minerals. The content play important role in exhibiting high antioxidant activity of algae [9, 33].

In the present study, *Microspora* extracts were prepared in the five different solvents. Antioxidant activities of each of the algal extracts in these solvents were determined with three different method DPPH•, FRAP and CUPRAC and antioxidant activities results for each methods are compared each other (Table 1).

Table 1. Antioxidant activity (DPPH•, FRAP and CUPRAC) of *Microspora* sp. extracts in different solvents

	% DPPH•	FRAP (μ MTEAC)	CUPRAC (μ MTEAC)
Methanol	31.15 \pm 2.139	359.394 \pm 53.431	0.085 \pm 0.002
Ethanol	26.01 \pm 0.571	345.455 \pm 23.689	0.101 \pm 0.002
Acetonitrile	8.97 \pm 0.713	60.303 \pm 7.348	0.041 \pm 0.003
Acetone	13.00 \pm 0.142	294.545 \pm 36.056	0.098 \pm 0.009
Ethyl acetate	22.58 \pm 0.571	315.455 \pm 22.212	0.079 \pm 0.002

Values are means \pm standard deviations of three (n=3) measurements

DPPH• which is the most commonly used scavenging activity detection method showed the efficacy of highest and lowest in methanol extract (scavenging activity 31.15%) and acetonitrile extract (scavenging activity 8.97%) respectively (Fig. 1). In the less number of antioxidant activity studies carried on *Microspora*, For example, Laungsuwon and Chulalaksananukul [25] determined % DPPH• radical scavenging activity of *Microspora floccosa* in different solvent extracts which consist of water (6.6% \pm 1.4), ethyl acetate (8.9% \pm 1.6), methanol (9.3% \pm 2.2) and hexane (19.7% \pm 1.4). Order of % DPPH• values of solvents of *Microspora floccosa*

is methanol > ethyl acetate compatible with our results partially. However there are differences between % values of DPPH• in both studies. However, Suanmali et al. [27] indicated that environmental factors are effective in changing the antioxidant activity values and reported that values of the % DPPH• varied from 23 to 49.6 in methanol extracts of *Microspora* collected from five different areas. However there are differences between % values of DPPH• in both studies. In addition to this, Suanmali et al. [27] indicated that environmental factors are effective in changing the antioxidant activity values and reported that values of the % DPPH• varied from 23 to 49.6 in methanol extracts of *Microspora* collected from five different areas. In some studies on other filamentous algae in the Chlororophyta, for example Laungsuwon and Chulalaksananukul [25] also measured values of % DPPH• for *Cladophora glomerata*. (Linnaeus) Kützing and the values were determined in different solvent such as methanol (16.7 \pm 2.6), water (18.4 \pm 2.5), hexane (37.4 \pm 2.3), ethyl acetate (49.8 \pm 2.7). In contrary to present study, the lowest value of % DPPH• was detected the in methanol extract of *Cladophora glomerata*. Each species of algae include antioxidant compounds in different qualities and quantities, and the solvents used in extraction is also an important factor for determining the antioxidant capacity because of polarity differences of compounds in algae [34].

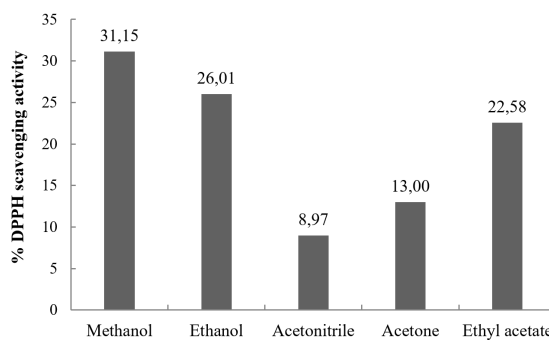


Figure 1. % DPPH scavenging activity

In the FRAP method belonging to the electron-transfer reaction group; Fe (III) salt, Fe (III) (TPTZ)₂Cl₃ (TPTZ = 2,4,6-tripyridyl s-triazine) is used as oxidant [29]. At low pH, the Fe (III) -TPTZ complex is reduced to Fe (II) form. This complex is dark blue in color and the absorption maximum is 595 nm [35]. Due to its simplicity, speed and low cost, the method is useful for the identification of hydrophilic and lipophilic antioxidants. In determination of Iron (III) reduction/antioxidant power (FRAP) Trolox standard calibration graphic with different concentrations was designed (Fig. 2). According to the calibration graphic, μ M TEAC (Trolox Equivalent Antioxidant Capacity) values of algae extracts prepared with different solvents were determined. μ M TEAC values of the algae extracts were ranged from 359.54 μ M (for methanol solvent extracts) to 60.303 μ M, (acetonitrile solvent extracts) (Fig. 3). Therefore, accor-

ding to the FRAP antioxidant assay method, the highest and the lowest antioxidant activity were observed in methanol extract and the acetonitrile extract respectively. The order of the antioxidant values of the solvent extracts measured in the FRAP and DPPH methods is similar (Methanol > Ethanol > Ethyl acetate > Acetone > Acetonitrile).

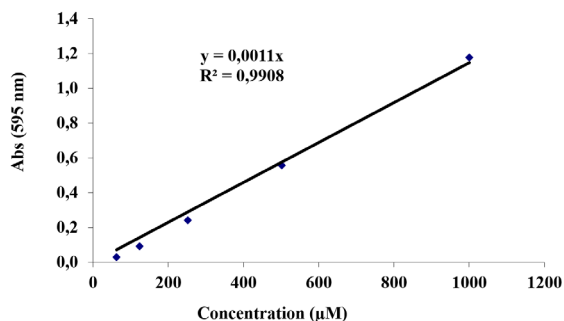


Figure 2. Calibration curve of Trolox equivalent by FRAP Method

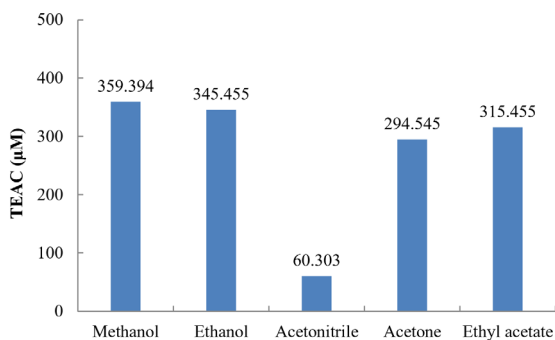


Figure 3. Comparison of FRAP values ($\mu\text{M TEAC}$) of different solvent extracts

CUPRAC is a method based on the reduction of Cu (II) to Cu (I) with present antioxidants in a sample. Simple sugars and citric acid, which cause interference in the FRAP method, are not oxidized by the CUPRAC reagent [31]. It is fast enough to oxidize thiol-type antioxidants. In the method, Trolox standard calibration graphic was designed with six different concentrations of the standart (Fig. 4). $\mu\text{M TEAC}$ values of each algae extract prepared with different solvents were calculated bases on values of the calibration graphic. However, values ($\mu\text{M TEAC}$) of CUPRAC antioxidant activity test of the algal extract were measured in each solvents that are ethanol (0.101 μM), acetone (0.098 μM), methanol (0.085), ethylacetate (0.079 μM), and acetonitrile (0.041 μM) (Fig. 5). Methanol solvent extracts showed the highest antioxidant activity in DPPH• and FRAP methods, while its activity value in CUPRAC method was significant. Safar et al. [10] pointed out methanol is the most preferred solvent in terms of antioxidative power compared to others.

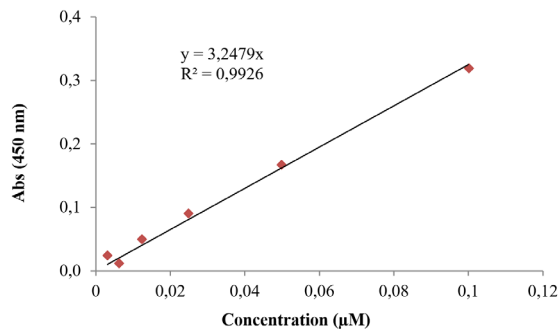


Figure 4. Calibration curve of Trolox equivalent by CUPRAC Method

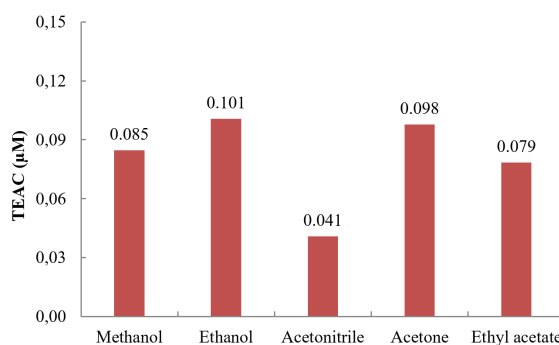


Figure 5. Comparison of CUPRAC values ($\mu\text{M TEAC}$) of different solvent extracts

CONCLUSION

It has been shown that *Microspora* sp. has antioxidant activity in three antioxidant activity assay methods. However, each of the five different solvents extracts have antioxidant activity In DPPH• and FRAP antioxidant assay methods showed the highest and lowest values of antioxidant activity in methanol extract and the acetonitrile extract respectively. While the lowest value of CUPRAC test is determined in acetonitrile extract as in the other tests, highest value is measured in ethanol extract as different from the others. Further studies should be undertaken on the identification of others biological activities of the alga *Microspora* sp and other freshwater algae in order to determine their potential use in the food and drug industry.

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