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BIOCHEMICAL COMPOSITION AND BIOACTIVITY SCREENING OF VARIOUS EXTRACTS FROM *DUNALIELLA SALINA*, A GREEN MICROALGA

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ABSTRACT

The current study examines the antimicrobial and antioxidant properties of different extracts of the microalga *Dunaliella salina* Teodoresco (Dunaliellaceae), their fatty acid composition and the antimicrobial activity of the oil. Antimicrobial and antioxidant activities were evaluated by obtaining extracts of *D. salina* in n-hexane, dichloromethane, ethanol, and methanol. To evaluate the antimicrobial activity, the extracts, and fatty acids from *D. salina* were assessed by the disc diffusion and microdilution techniques against pathogenic microorganisms including fish and clinical/food-borne. The MBC or MFC values of the extracts and fatty acids ranged from 0.63 to 10.00 mg/ml. The antioxidant activity was studied by phosphomolybdenum and DPPH assays and β-carotene/linoleic acid tests. In addition, the total phenolic and flavonoid contents were evaluated and the fatty acid composition was determined using gas chromatography. Palmitic, alpha-linolenic, and oleic acids were discovered to be the major components of the fatty acids. These findings have demonstrated that the extracts and oil from *D. salina* could be used as natural antimicrobials and antioxidants in the food/feed and pharmaceutical industry and as a biodiesel because of its high unsaturated fatty acid content.

Keywords: Fatty acid composition, antimicrobial, antioxidant, Lake Tuz

INTRODUCTION

Algae constitute a major group of living organisms that are an important source of a variety of useful products with widespread applications in the biodiesel, food, and pharmaceutical industries, as well as in biotechnology. Algae have developed a chemical defense system with the secondary metabolites that are synthesized by the means of different metabolic enzymes to survive in a competitive environment (Puglisi et al., 2004; Barros et al., 2005). In addition to their role in protecting the organism against stress conditions such as temperature, light, salinity, and drought, most components have some useful biological activities and are essential in human nutrition (Mayer and Hamann, 2004).

The salinity of Lake Tuz in Turkey (Salt) ranges from 30 % to 45 % (Saygı and Demirkalp, 2002). This high salinity gener-

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ates extreme stress in several living organisms, and limits life in Lake Tuz. *Dunaliella salina* Teodoresco (Dunaliellaceae), a green alga species, is one of the rare organisms can naturally thrive in Lake Tuz. *D. salina* is one of the richest sources of β-carotene, a secondary metabolite and a lipid-soluble orange pigment, used in food and feed as a coloring agent. *D. salina* normally contains 5–10 mg of β-carotene per gram dry weight (Del Campo et al., 2007). However, the ratio increases up to 14 % of dry weight in stress conditions such as high salinity, high light intensity, food scarcity, and extreme temperature (Jin and Melis, 2003). β-carotene exhibits several important biological activities, including preventing coronary heart disease and malignant tumors, increasing cell division of lymphocytes, expanding the immunological response, and control of growth (Wald et al., 1988; Stryker et al., 1990; Knekt et al., 1990; Challem, 1997; Buiatti, 1997; Chidambara et al., 2005; Raja et al., 2007). In addition, the antioxidant properties of β-carotene help eliminate free radicals (Burton and Ingold, 1984). Thus, β-carotene, which has applications in several different industrial sectors because of its numerous properties, is of great economic importance.

β-carotene accumulates within the lipid globules in the spaces between thylakoids located in chloroplast (Ben-Amotz et al., 1982). Thus, it has been proposed that the increase in fatty acid content under stress conditions can be partially attributed to the increase in β-carotene (Mendoza et al., 1999). Previous studies examining the fatty acid profile of *D. salina* identified high expression of palmitic (C16: 0), alpha-linolenic (C18: 3), and oleic acid (C18: 1) (Herrero et al., 2006; Lamers et al., 2010). Of these fatty acids, alpha-linolenic (C18: 3) essential fatty acid and ω3 fatty acids are the most well-known. ω3 fatty acids are of great importance in growth and development, brain development, and prevention of cardiovascular diseases. Marine organisms are an important source of these fatty acids (Meyer et al., 2003).

Microalgae have been used for therapeutic applications for several years, and their extract and/or extracellular products present antimicrobial activity (Kellan and Walker, 1989; Ozemir et al., 2004; Herrero et al., 2006). A study by Herrero et al. (2006) discovered the antibacterial and antifungal activities of various *D. salina* extracts on several microorganisms, which are important in the food industry; in addition, it highlighted the fatty acid profile of these extracts. In our study, we examined the antimicrobial activity of various extracts and fatty acids of *D. salina* on a total of 19 fish as well as clinical and food-borne pathogens, and emphasized the potential use of these extracts in the food/feed and pharmaceutical industries.

Studies conducted with the macro- and microalgae have shown that most algae have antioxidant activity (Jiménez-Escrig et al., 2001; Li et al., 2007), which stems from the expression of compounds such as polyphenols, ascorbic acid, and carotenoids in the algae (Cornish and Garbary, 2010).

The present study aimed to determine the oil content of *D. salina* naturally present in Lake Tuz, and to investigate the antimicrobial effect of the oil, as well as the antioxidant and antimicrobial effects of various organic *D. salina* extracts.

MATERIALS AND METHODS

Sample collection

Samples were collected on July 14, 2012 during the period when *D. salina* bloom along with a net plankton of 8 µ. The collected samples were examined under the binocular microscope to ensure that they consisted of pure *D. salina*. These samples were desalinated by maintaining them in distilled water followed by several rounds of dialysis to generate desalinated pure *D. salina*. Subsequently, the samples were completely dried in the oven at 35 °C and then used to obtain lipids and extracts.

Lipid extraction

The lipids from *D. salina* were homogenized three times with 10 ml of chloro-

form:methanol (2:1, v/v) ultraturrax. Subsequently, the solvent was completely evaporated till it reached a constant weight, and the total lipid content was determined. The lipid fatty acid methyl esters (FAME) were prepared by saponification with 0.5 N methanolic NaOH and esterification with BF_3-methanol complex (20 % solution in methanol), in accordance with IUPAC (1979). Then, 1 µl of the prepared FAME was analyzed using the HP 6890N gas chromatograph (Agilent Technologies, USA), and using the HP-88 (100-m length, 0.25-mm id, and 0.2-mM film thickness) capillary column. The identification of fatty acids was conducted by comparing retention times of the peaks derived from Altech and Accu standards. Analyses were conducted following three experiments, and the mean values were provided with SD.

The organic solvent portion of the fatty acid was completely evaporated under nitrogen (N_2) till it reached a constant weight, and then used to determine the antimicrobial activity of fatty acids. Subsequently, following dissolution in dimethylsulphoxide (DMSO), it was sterilized by using a 0.45 µm Millipore filter, and stored at +4 °C for use in future studies.

Preparation of extracts

Extracts were prepared using the in-line extraction method, by modifying the method of Sánchez-Saavedra et al. (2010). The dried *D. salina*, which was first dissolved in n-hexane (H), was vortexed and then sonicated on ice in an ultra-sonic bath (Bandelin Sonorex RK 100H), and was then incubated on a shaker for 2 h in the dark. After centrifugation, the pellet and supernatant were transferred to different tubes. Then, the solvent in the supernatant was evaporated in a drying oven at 50 °C, while the remaining solid extract was dissolved with DMSO. The pellet in the other tube was similarly processed using various solvents: dichloromethane (DCM), methanol (M), and ethanol (E). The solid extract obtained following DCM extraction was dissolved in DMSO, while the solid extracts obtained following methanol and ethanol extraction were dissolved with their respective solvents. The extracts were sterilized using a 0.45-µm Millipore filter and stored at +4 °C before used in the analysis of their antimicrobial and antioxidant activities.

Determination of antimicrobial activity

Microbial strains

For the determination of antimicrobial activity, the fish pathogenic strains, *Yersinia ruckeri, Lactococcus garvieae, Vibrio anguillarum* (M1 and A4 strains, obtained from different companies), and *Vibrio alginolyticus* were used. In addition, for the determination of antimicrobial activity on clinical and food-borne pathogenic microorganisms, *Yersinia enterocolitica* NCTC 11175, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Micrococcus luteus* NRRL B-4375, *Bacillus cereus* RSKK 863, *Escherichia coli* O157:H7, *Escherichia coli* ATCC 11229, *Escherichia coli* ATCC 35218, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Shigella sonnei* Mu:57, *Bacillus subtilis* RSKK 244, *Salmonella enteritidis* RSKK 171, and *Candida albicans* ATCC 10231 strains were used. Nutrient agar (NA) and Tryptic Soy Agar (TSA) were used for the growth of bacteria, and YPD medium was used for the growth of yeast. Fish pathogenic bacteria were incubated at 25 °C and other bacterial cultures were incubated at 37 °C for 24 h. Yeast cultures were incubated at 30 °C for 48 h.

Determination of antimicrobial effect

The antimicrobial activity was determined by the disc diffusion technique (Murray et al., 1995), in duplicate using filter paper discs (6 mm in diameter). The culture suspensions, adjusted by comparing with 0.5 McFarland, were spread on plates. The paper discs, impregnated with 10 µl of the extract (500 µg extract/disc) and 20 µl of fatty acids (250 µg fatty acid/disc), were placed on the inoculated plates. The plates were stored in a

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refrigerator (2 h) to enable prediffusion of the extracts into the agar, followed by incubation for 24 h and 48 h for bacterial and yeast strains, respectively. Amikacin (AK, 30 µg/disc), ampicillin (Amp, 10 µg/disc), gentamicin (CN, 10 µg/disc), and fluconazole (FCA, 25 µg/disc) were used as positive controls. Paper discs loaded with solvents were used as negative controls. The inhibition zones (mm) were used to determine the antimicrobial activity.

Determination of Minimal Bactericidal (MBC) or Fungicidal Concentration (MFC)

The MBC and MFC values of the extracts were determined with two-fold dilutions (Chandrasekaran and Venkatesalu, 2004). MBC and MFC values were determined for the microorganisms that presented an inhibition zone by the disc diffusion method. The extracts were collected in tubes, with the highest concentration of 40.00 mg/ml, followed by serial dilutions in tubes until the lowest concentration of 0.16 mg/ml was achieved. The final volume of 100 µl in each tube was attained by using the respective medium, before adding 2.5 µl of the microorganism (0.5 McFarland) to each tube. Tubes containing 2.5 µl of inoculum and growth medium were prepared as a positive control, and tubes containing 2.5 µl of extract and 100 µl of growth medium were prepared as a negative control. After thoroughly mixing the contents of the tube, it was incubated for 24 h at the appropriate incubation temperatures. Subsequently, 5 µl of the sample was withdrawn from each tube and spot inoculated on the petri containing the solid medium. The concentration that inhibited growth in its medium was recorded as MBC or MFC.

Antioxidant activity

Total phenolic and flavonoid content

Total phenolic content was determined by using the Folin–Ciocalteu reaction, in accordance with the method of Slinkard and Singleton (1977). The method was applied by measuring absorbance at 765 nm after adding 1 ml Folin–Ciocalteu reagent and 2 ml of 7.5 % Na_2CO_3 to 0.2 ml of extract at 2 mg/ml concentration, stirring in 7 ml of deionized water, and incubating at room temperature for 2 h. The results provided the gallic acid equivalent, which is the standard phenolic compound (GAE mg/g extract).

Total flavonoid content was determined by the method of Arvouet-Grand et al. (1994). In brief, 1 ml of a 2 % methanolic $AlCl_3$ solution was mixed with 1 ml of 2 mg/ml extract, and its absorbance was determined at 415 nm. Quercetin was used as the standard and the results were expressed as µg/g quercetin equivalent (QE).

Total antioxidant capacity

Total antioxidant capacity was evaluated by a method that uses phosphomolybdenum (Prieto et al., 1999). In brief, the mixture of 0.3 ml extract (2 mg/ml) with 3 ml of reagent solution (6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was incubated at 95 °C for 90 min. The absorbance of the mixture was then measured against blank at 695 nm. Antioxidant capacity of the extracts was expressed as ascorbic acid (mg AE/g extract) equivalent.

Free radical scavenging activity (DPPH, 2,2-diphenyl-1-picrylhydrazyl)

The method was applied with a partial modification of the method of Kirby and Schmidt (1997). In brief, 0.5 ml of the sample extract, with concentrations ranging from 0.2 to 1 mg/ml was mixed with 3 ml of a 6.10^{-5} M DPPH solution, and the mixture was incubated for 30 min at room temperature in the dark. The absorbance was subsequently measured at 517 nm by using the following equation and the % inhibition (I %) was determined;

$$I(\%) = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

(A_0 = absorbance of the control,

A_1 = sample/absorbance of standard)

BHT was used as the standard antioxidant. Results were expressed by calculating IC_{50} values. A low value of IC_{50} indicates a higher inhibitory activity.

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β-Carotene/linoleic acid bleaching assay

The method was applied with a partial modification of the method of Sokmen et al. (2004). In brief, 0.5 mg β-carotene was dissolved in 1 ml of chloroform, and then 25 µl of linoleic acid and 200 mg of Tween 40 were added. The chloroform was completely evaporated, distilled water was added, and quickly shaken and saturated with oxygen. To 2.5 ml of the reaction mixture, 350 µl of the extract (2 mg/ml) was added, and the reaction mixture was incubated at 50 °C for 2 h. The same procedure was used for the standard antioxidants BHA and BHT, which were used as a positive control, and for the blank. By measuring the absorbance of the mixture at 490 nm, inhibition was calculated according to the equation;

$$R = \ln (a/b)/t \quad (2)$$

(ln = natural log,
a = absorbance at time 0,
b = absorbance at time t (120 min)].

The antioxidant activity (AA) was calculated by the following equation as % inhibition:

$$AA = [(R_{Control} - R_{Sample})/R_{Control}] \times 100 \quad (3)$$

RESULTS

The fatty acid composition of lipids from *D. salina* is provided in Table 1. The total oil content of *D. salina* was found to be 24.85 %. Fifteen prominent fatty acids were identified in *D. salina*, including saturated fatty acids (SFA), monounsaturated acids (MUFA), and polyunsaturated acids (PUFA). SFA and PUFA were the dominant fatty acid groups, while the levels of MUFA were relatively low. The primary fatty acids identified in the samples were palmitic (C16:0), linolenic (C18:3 ω3), and oleic (C18:1 ω9) acids.

The antimicrobial activities of n-hexane, DCM, methanol, and ethanol extracts and fatty acids of *D. salina* against five fish and 14 clinical and food-borne pathogenic microorganisms were determined.

Table 1: Fatty acid composition and oil content of *Dunaliella salina*

Fatty Acids	*Dunaliella salina*
C 12:0	0.37±0.05*
C 14:0	1.60±0.01
C 15:0	0.09±0.01
C 16:0	45.41±0.35
C 17:0	0.19±0.02
C 18:0	1.65±0.43
C 20:0	0.11±0.01
ΣSFA[a]	**49.40±0.09**
C 14:1 ω5	0.07±0.01
C 15:1 ω5	0.29±0.01
C 16:1 ω7	0.25±0.06
C 17:1 ω7	0.49±0.06
C 18:1 ω9	11.69±0.06
C 18:1 ω11	6.78±0.06
ΣMUFA[a]	**19.55±0.01**
MUFA-18:1	**7.87±0.06**
C 18:2 ω6	9.87±0.01
C 18:3 ω3	21.19±0.10
ΣPUFA[a]	**31.06±0.09**
ΣUFA[a]	**50.61±0.09**
EFA[a]	**31.06±0.09**
Σω3	**21.19±0.10**
Σω6	**9.87±0.01**
ω3/ω6	**2.15±0.01**
ω6/ω3	**0.47±0.01**
PUFA/SFA	**0.63±0.01**
Oil Content	**24.85**

[a] SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, UFA: Unsaturated fatty acids, EFA: Essential fatty acids.
* Values expressed are means ± S.D. of three parallel measurements

Due to the increasing use of antimicrobial drugs in aquaculture, bacteria are developing resistance, and these bacteria can then pass on to humans consuming these fish (WHO, 2006). Therefore, the use of natural antimicrobial agents as an alternative to synthetic antimicrobials in aquaculture is very important and public's awareness and interest in these issues is gradually increasing. The first phase of our study was intended to reveal whether various extracts and fatty acids of *D. salina* have antimicrobial activity on certain fish pathogens, and its potential

utilization as an alternative to synthetic antimicrobials, used to prevent various diseases or as an inhibitor. According to the results of the disc diffusion technique conducted against the pathogenic microorganisms from fish, the highest inhibitory effect was identified with the ethanol extract: 18.21 mm against *L. garviae* (Table 2). In general, the methanol and ethanol extracts demonstrated a better inhibition zone than n-hexane and DCM against fish pathogenic microorganisms other than *V. alginolyticus*. The MBC values of extracts against fish pathogens were identified as 0.63–10.00 mg/ml. In parallel with the results of the disc diffusion method, the MBC values of methanol and ethanol extracts were observed to be lower than the other extracts. The lowest MBC value of the ethanol extract was identified in *V. anguillarum* (strain A4) and *V. alginolyticus*.

Based on the results of the disc diffusion method, the antibacterial activity of fatty acids obtained from *D. salina* on fish pathogenic microorganisms resulted in a 9.99-12.67 mm zone of inhibition (Table 2). However, the MBC values of the extracts were identified as 2.50 mg/ml for all bacteria. Compared with ampicillin (10 µg/disc, standard antibiotic), the fatty acids (250 µg/disc) showed higher inhibitory activity against *V. anguillarum* (strain A4 and M1), as demonstrated by the disc diffusion method.

The inhibitory activity of different extracts of *D. salina* on clinical and food-borne pathogens was also researched in this study. According to the results of the disc diffusion method, the extract of ethanol, and then the extract of methanol showed the highest inhibitory effect for all microorganisms, with respect to other extracts (Table 3), although the methanol extract was not effective against *B. subtilis* RSKK 244 and *S. enteritidis* RSKK 171. However, Sánchez-Saavedra et al. (2010) reported that the hexane extract of *D. tertiolecta* showed better antimicrobial activity than DCM and methanol extracts. While all three extracts in their study could inhibit *S. aureus* and *B. subtilis*, the extracts were ineffective against *M. luteus*, *E. coli*, *P. aeruginosa*. However, in our study, each of the three extracts showed antimicrobial activity against these three bacteria.

According to the results of the disc diffusion method, the ethanol extract (500 mg/disc) showed higher inhibitory activity against *C. albicans* (ATCC 10231) than FCA (25 µg/disc), which is the standard antifungal antibiotic. MBC and MFC values of the extracts were determined as 0.32 to 10.00 mg/ml. When the antimicrobial activity of the fatty acids on clinical and food-borne pathogens was studied, a maximum zone of inhibition of 11.82 mm was observed and a MBC of 0.63 mg/ml was estimated against *Y. enterolitica* NCTC 11175. The fatty acids demonstrated effective antimicrobial activity against all microorganisms to various degrees, except *M. luteus* NRRL B-4375. MBC and MFC values of the fatty acids varied between 0.63–10.00 mg/ml (Table 3).

Antioxidant activity of four different (hexane, dichlormethane, methanol, and ethanol) extracts obtained from *D. salina* was determined by using total antioxidant capacity, free radical scavenging activity and β-carotene/linoleic acid bleaching assay. In addition, the total phenolic and flavonoid contents of the extracts were researched (Table 4).

The total phenolic content of *D. salina* was determined in hexane and DCM extracts. No phenolic content was observed in the remaining extracts (methanol and ethanol). In hexane (53.27 mg GAE/g) and DCM (56.45 mg GAE/g) extracts, a low phenolic content was found. None of the *D. salina* extracts expressed any flavonoids (Table 4).

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Table 2: Antimicrobial activity of *Dunaliella salina* against fish pathogens

Test microorganisms	MBC[a] (mg/ml)					Inhibition zone diameter[b] (mm)					Antibiotics Inhibition zone diameter[b] (mm)			
	M-E	*E-E*	*H -E*	*DCM-E*	*FA[d]*	*M-E*	*E-E*	*H–E*	*DCM-E*	*FA*	*Amp*	*CN*	*AK*	*FCA*
L. garviae	*2.50*	*2.50*	*10.00*	*5.00*	*2.50*	*14.26±0.17*	*18.21±0.09*	*9.65±0.74*	*10.10±0.06*	*10.63±0.46*	*33.1±0.12*	*15.1±0.10*	*10.3±0.08*	-
Y. ruckeri	*1.25*	*1.25*	*10.00*	*10.00*	*2.50*	*14.40±0.22*	*15.64±0.13*	*9.62±0.04*	*9.26±0.05*	*9.99±0.07*	*32.3±0.15*	*18.8±0.05*	*18.6±0.12*	-
V. anguillarum M1	*2.50*	*1.25*	*5.00*	*5.00*	*2.50*	*15.51±0.16*	*15.44±0.25*	*11.56±1.74*	*13.22±0.94*	*10.30±0.51*	*9.02±0.04*	*12.3±0.09*	*9.46±0.12*	-
V. anguillarum A4	*1.25*	*0.63*	*5.00*	*2.50*	*2.50*	*17.64±0.21*	*15.26±0.28*	*12.09±0.49*	*12.55±1.76*	*10.51±1.20*	*9.40±0.11*	*15.1±0.15*	*12.0±0.13*	-
V. alginolyticus	-	*0.63*	*5.00*	*5.00*	*2.50*	-[c]	*7.39±0.15*	*16.92±0.53*	*11.02±0.60*	*12.67±0.68*	*13.5±0.09*	*15.0±0.07*	*15.0±0.03*	-

a: Minimal Bactericidal Concentration (MBC); b: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of two separate experiments; c: Indicates no antimicrobial activity; d: Fatty acids

Table 3: Antimicrobial activity of *Dunaliella salina* against clinical and food-borne pathogens

Test microorganisms	MBC[a]/MFC[b] (mg/ml)					Inhibition zone diameter[c] (mm)					Antibiotics Inhibition zone diameter[c] (mm)			
	M-E	E-E	H-E	DCM-E	FA[d]	M-E	E-E	H –E	DCM-E	FA	Amp	CN	AK	FCA
B. cereus RSKK 863	5.00	0.63	1.25	0.63	2.50	16.53±0.13	15.06±0.09	10.11±0.11	11.23±0.16	10.48±0.03	37.68±0.03	18.02±0.11	18.72±0.07	-
E. coli O157:H7	2.50	2.50	10.00	5.00	5.00	16.36±0.06	18.82±0.04	10.02±0.15	9.71±0.05	11.92±0.04	25.92±0.15	18.37±0.17	22.58±0.09	-
S. sonnei Mu:57	2.50	2.50	10.00	5.00	1.25	15.27±0.12	17.90±0.21	9.32±0.13	9.58±0.08	10.42±0.24	38.43±0.16	19.49±0.05	27.07±0.04	-
M. luteus NRRL B-4375	2.50	1.25	5.00	5.00	-	11.76±0.17	16.52±0.14	11.29±0.06	11.14±0.07	-	34.65±0.12	13.48±0.22	19.55±0.14	-
Y. enterocolitica NCTC 11175	1.25	1.25	5.00	1.25	0.63	16.36±0.21	22.91±0.19	10.57±0.12	11.41±0.08	11.82±0.14	11.58±0.09	16.17±0.11	21.19±0.07	-
E. coli ATCC 11229	2.50	0.63	10.00	2.50	5.00	13.62±0.12	15.39±0.14	10.78±0.06	11.61±0.16	11.02±0.57	27.99±0.14	14.98±0.12	19.81±0.13	-
P. aeruginosa ATCC 27853	2.50	1.25	10.00	2.50	2.50	15.04±0.23	17.06±0.09	10.90±0.21	11.17±0.12	10.94±0.04	-	15.89±0.05	19.71±0.08	-
S. aureus ATCC 25923	1.25	1.25	10.00	5.00	5.00	14.10±0.08	16.42±0.11	10.56±0.15	10.57±0.14	10.81±0.08	34.82±0.06	15.52±0.14	19.46±0.16	-
E. coli ATCC 35218	2.50	2.50	10.00	5.00	10.00	13.06±0.08	16.14±0.12	7.82±0.23	10.98±0.16	12.32±1.02	25.78±0.19	12.17±0.21	20.03±0.09	-
S. enteritidis ATCC 13076	2.50	1.25	10.00	5.00	5.00	14.58±0.11	15.94±0.21	8.59±0.09	8.11±0.03	11.47±0.01	29.49±0.15	16.38±0.17	17.27±0.11	-
L. monocytogenes ATCC 7644	2.50	2.50	10.00	5.00	5.00	15.56±0.22	19.73±0.18	10.06±0.07	9.37±0.14	10.87±0.05	25.13±0.06	20.63±0.16	20.52±0.21	-
B. subtilis RSKK 244	-	0.63	2.50	0.32	1.25	-[d]	14.31±0.14	8.92±0.07	10.45±0.12	10.16±0.20	37.57±0.08	15.35±0.04	16.86±0.07	-
S. enteritidis RSKK 171	-	2.50	10.00	2.50	5.00	-	15.28±0.12	9.29±0.09	12.11±0.12	11.80±0.73	31.27±0.11	12.05±0.14	15.67±0.09	-
C. albicans ATCC 10231	2.50	1.25	10.00	5.00	5.00	14.71±0.27	18.99±0.03	9.22±0.09	9.05±0.24	11.67±0.43	-	-	-	17.09±0.07

a: Minimal Bactericidal Concentration (MBC); b: Minimal Fungicidal Concentration (MFC); c: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of two separate experiments; [d]: Indicates no antimicrobial activity; [d]: Fatty acids

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Table 4: Total phenolic, flavonoid contents, antioxidant capacities, free radical scavenging activity and inhibition activity against on linoleic acid oxidation of extracts from *D. salina* and standard antioxidants

	TPC[a] (mg GAE/g)	TFC[b] (µg QE/g)	TAC[c] (mg AE/g)	IC_{50} (mg/ml^{-1})[d]	Inhibition (%)[e]
Hexane	53.27±7.71[f]	nd	151.85±2.18	3.07±0.48	25.79±3.77
DCM	56.45±4.50	nd	454.92±16.32	1.92±0.28	36.83±0.67
Methanol	nd	nd	nd	0.45±0.01	63.99±0.75
Ethanol	nd	nd	1.08±0.26	3.46±0.75	68.88±0.14
BHA	-	-	-	nd	89.57±0.22
BHT	-	-	-	0.05±0.01	93.84±0.38

[a] TPC: Total phenolic content (mg GAE/g extract); [b] TFC: Total flavonoid content (µg QE/g extract); [c] TAC: Total antioxidant capacity (mg AE/g extract); [d] Results of DPPH assay; [e] Results of β-caroten/linoleic acid bleaching assay; [f] Values are reported as means ± S.D. of three parallel measurements; –: not tested; nd: not detected. BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

The DPPH method is widely used to determine free radical scavenging activity. This activity is evaluated by determining the IC_{50} (the amount of extract required to scavenge 50 % of DPPH radicals) values for each extract being evaluated (Table 4). A low IC_{50} value indicates a high level of activity. Among the *D. salina* extracts, the methanol extract has the highest DPPH removal activity, with the value of IC_{50} = 0.45 mg/ml. The IC_{50} values of DCM, hexane, and ethanol extracts are 1.92, 3.07, and 3.46 mg/ml, respectively. BHT showed higher activity than other extracts, with the value of IC_{50} = 0.05 mg/ml.

Oxidation of linoleic acid, using the β-carotene/linoleic acid bleaching test, produces peroxyl free radicals depending on the cleavage of the hydrogen atoms from deallic methyl (Laguerre et al., 2007). The resulting free radicals cause the oxidation of β-carotene, which is an unsaturated molecule, and its orange color becomes lighter. The β-carotene oxidation inhibition values of different extracts of *D. salina* range between 25.79 %-68.88 % (Table 4). In this test, the inhibitory rates of BHA and BHT were 89.57 % and 93.8 %, respectively. The methanol and ethanol extracts were particularly noteworthy with their high inhibition values. *D. salina* extracts have a high inhibitory effect because of the increased β-carotene expression.

DISCUSSION

Several studies have reported the total oil content of *D. salina* as well as the fatty acid content (Herrero et al., 2006; Lamers et al., 2010; Diaz-Palma et al., 2012). *D. salina* has been reported to have 7.9 % of oil content (Diaz-Palma et al., 2012). The study by Lamers et al. (2010) identified fatty acids as the major component of *D. salina* (C16:0, C16:4, C18:1, C18:2, and C18:3), and indicated that these fatty acids constitute approximately 95 % of the total fatty acid content. The study by Herrero et al. (2006) identified palmitic, alpha-linolenic, and oleic acids as the essential fatty acid components. Our present study discovered that the fatty acid profile of the *D. salina* samples from Lake Tuz is primarily composed of palmitic, linolenic, and oleic acids, which complements the previous reports. The ω3/ω6 fatty acid isomer ratio is high, particularly in fishes, and it is a useful indicator for comparing the nutritional value of the fats (Piggot and Tucker, 1990). Studies conducted on the fatty acid composition of fishes have identified that the ratio varies between 1.03-2.80 (Guler et al., 2007; Donmez, 2009; Cakmak et al., 2012). In the present study, the ω3/ω6 ratio is 2.15. Accordingly, the nutritional value of the fat obtained from *D. salina* is higher than that of some fishes.

Herrero et al. (2006) reported that ethanol and hexane extracts of *D. salina* showed

antimicrobial activity against *E. coli*, *S. aureus,* and *C. albicans*, and that this antimicrobial activity might result from fatty acids such as palmitic, *α*-linolenic, and oleic acids, which are a major component of the extracts. Krishnika et al. (2011) identified the antimicrobial activity of different extracts of eight microalgae, and reported that *Dunaliella* sp. showed a high degree of inhibitory effect on *Shigella boydi*, *Salmonella paratphi* A, and *Pseudomonas fluorecens*. However, the antimicrobial activity of DCM, hexane, ethanol, and methanol extracts on *E. coli* was not observed. These results contradicted those of our study; we demonstrated that the aforementioned extracts of *D. salina* showed inhibitory effects against different strains of *E. coli*. The contradictions could be explained by the different strains used in the determination of the antimicrobial activity, the different methods used in the preparation of extracts or the concentration of the extracts used. In addition, the presence of different types of *Dunaliella* and the difference in location might affect the antimicrobial activity.

The antimicrobial activity of fatty acids has been reported by many studies over several years (Galbraith et al., 1971; Kabara and Vrable, 1977; Desbois et al., 2008, 2009; Abdelillah et al., 2013). Due to this important feature, fatty acids can have potential applications in various fields such as agriculture, medicine, cosmetics, food preservation, and in particular as an alternative to the forbidden antibiotics (Desbois and Smith, 2010). It is reported that the antimicrobial activity of fatty acids depends on both the chain length and the degree of unsaturation (Kabara and Vrable, 1977). Fatty acids with chain length less than 8 carbon atoms are defined as short-chain and those higher than 16 carbon atoms are defined as long chain (Desbois and Smith, 2010). In *D. salina*, only medium and long chain fatty acids were identified. Several studies have reported that medium and long chain fatty acids have antimicrobial activity (Galbraith et al., 1971; Bergsson et al., 1999). Also, in this study, the unsaturated fatty acid content of *D. salina* was found to be higher than the saturated content. It is reported that unsaturated fatty acids, because they contain double bonds, have higher antimicrobial activity in comparison with the saturated fatty acids (Ouattara et al., 1997).

Microalgae need to adapt their metabolism to their environment in order to survive in different habitats, and this characteristic equips them to resist environmental stress conditions by producing various secondary metabolites. Due to these features, microalgae hold an important place in biotechnology, healthcare, food industry, and aquaculture (Andersen, 1996). The stress evoked by the high salinity in Lake Tuz has sharpened the production of secondary metabolites in *D. salina* and, consequently, may have increased antimicrobial activity. As a result of this study, the antimicrobial potential of extracts and fatty acids obtained from *D. salina* against some fish and human pathogens was revealed; the isolation and characterization of the components responsible for the antimicrobial activity will be the basis for future studies.

Phenolic compounds are obtained from plant secondary metabolites, and they are major components that determine the antioxidant capacity of plants. Polyphenols have several beneficial effects, including anticarcinogenic activity (Carroll et al., 1999), and reducing the risk of coronary heart diseases (Williams and Eliot, 1997). There is very limited information on the phenolic content of microalgae. Li et al. (2007) examined the phenolic content of hexane, ethyl acetate, and water extracts from 23 microalgae, and demonstrated that hexane extracts have 2.12-39.87 mg GAE/g, ethyl acetate extracts have 0.01-9.80 mg GAE/g, and water extracts have 1.09-10.68 mg GAE/g. Another study examining the total polyphenol content of some marine algae identified the expression of phloroglucinol equivalent (PGE) between 5.3-41.4 g PGE/kg (dry matter) (Jiménez-Escrig et al., 2001). According to the phosphomolybdenum assay, the DCM extract showed the highest activity at 454.92 mg

AE/g, followed by hexane (151.85 mg AE/g) and ethanol (1.08 mg AE/g) extracts. Similarly, in our previous study, *Anabaenopsis* sp. Woloszyńska (Cyanophyceae). DCM extract demonstrated (34.15 mg AE/g) the highest total antioxidant capacity; the methanol extract (13.77 mg AE/g) had relatively low activity, while water and ethanol extracts did not show any activity (Ozusaglam et al., 2013).

Hu et al. (2008) compared the free radical scavenging effect of algal extracts of *D. salina* with some commercially carotenoids (all-trans forms of lutein, zeaxanthin, α-carotene, and β-carotene), which is also in the composition of *D. salina*, and determined that the algal extract showed higher activity than each of the listed carotenoids. Accordingly, while the EC_{50} value of the algal extract was 8.36 mg/ml, the carotenoids demonstrated a much greater activity in the range of 22.82–24.54 mg/ml (Hu et al., 2008). In our study, different extracts of *D. salina* showed higher activity than demonstrated in the study by Hu et al. (2008).

In conclusion, our study shows that *D. salina* has high oil content. Analysis of the composition of this oil content concluded that more than 50 % of the total fatty acids are mono- and poly-unsaturated, and it is expected to have beneficial health effects. With a high content of ω3/ω6, *D. salina* may have a positive effect on the risk of coronary disease. The present study also demonstrated that this oil has antimicrobial activity. Further, the various extracts obtained from *D. salina* have antimicrobial and antioxidant activity. Thus, we can conclude that due to its beneficial fatty acid composition, antimicrobial and antioxidant properties, *D. salina* is a safe food additive, a good feed source for fish and other aquatic organisms, and it can be utilized in the field of pharmacology due to its biological activities. And also it can be used as a new sources of essential fatty acids because of its high their content.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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