

Research Article

Enteromorpha compressa Exhibits Potent Antioxidant Activity

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The green macroalgae, *Enteromorpha compressa* (Linnaeus) Nees, *Ulva lactuca*, and *E. linza*, were seasonally collected from Abu Qir bay at Alexandria (Mediterranean Sea). This work aimed to investigate the seasonal environmental conditions, controlling the green algal growth, predominance, or disappearance and determining antioxidant activity. The freshly collected selected alga (*E. compressa*) was subjected to pigment analysis (chlorophyll and carotenoids) essential oil and antioxidant enzyme determination (ascorbate oxidase and catalase). The air-dried ground alga was extracted with ethanol (crude extract) then sequentially fractionated by organic solvents of increasing polarity (petroleum ether, chloroform, ethyl acetate, and water). Antioxidant activity of all extracts was assayed using different methods (total antioxidant, DPPH [2, 2 diphenyl-1-picrylhydrazyl], ABTS [2, 2 azino-bis ethylbenzthiazoline-6-sulfonic acid], and reducing power, and β -carotene linoleic acid bleaching methods). The results indicated that the antioxidant activity was concentration and time dependent. Ethyl acetate fraction demonstrated higher antioxidant activity against DPPH method (82.80%) compared to the synthetic standard butylated hydroxyl toluene (BHT, 88.5%). However, the crude ethanolic extract, pet ether, chloroform fractions recorded lower to moderate antioxidant activities (49.0, 66.0, and 78.0%, resp.). Using chromatographic and spectroscopic analyses, an active compound was separated and identified from the promising ethyl acetate fraction.

1. Introduction

For many generations, marine algae have been extensively used in coastal regions in the Far East as food for humans and animals and as organic fertilizers. The high protein, lipid, and vitamin content of marine algae have encouraged their cultivation and use as a food source in many parts of the world [1]. These algae also contain various secondary products such as flavonoids, terpenoids, and alkaloids, some with potent antioxidant, antimicrobial, antineoplastic, and antiviral action [2]. Periodic exposure to high solar flux, elevated temperature, and dehydration during low tidal periods generates acute physiological stress such as increased levels of reactive oxygen species (ROS) which are modulated by the increased production of various compounds with antioxidant activity [3]. Species of *Ulva* and *Enteromorpha* attached to rocky substrata in the intertidal zone located in the Mediterranean Sea at Abu Qir bay exemplify those marine algae exposed to severe environmental stress [4].

This study was designed to identify the mechanism by which *E. compressa* combats this stress at the intracellular level and possibly associate these defense mechanisms with antioxidant activity.

2. Materials and Methods

2.1. Chemicals. All chemicals were of analytical grade and obtained either from Sigma-Aldrich or Merck Chemical Co. (Darmstadt, Germany).

2.2. Location and Algal Collection. *Enteromorpha compressa* (Linnaeus) Nees was harvested seasonally (15th day of January, April, July, and October) from rocky substrate in Abu Qir bay at Alexandria during 2009-2010 (morning time at 7–10 am). Adhered sand was removed from the algae by washing with seawater and the material was transported to the laboratory of Phycology at Cairo University. A small amount of algal sample was preserved in the Herbarium

and in 4% formalin for subsequent formal identification by Dr. S. Shanab (Prof. of Phycology). Some of the fresh alga (2.0 kg) was stored at -30°C for determination of antioxidant enzymes, essential oil content, and pigment analysis.

The remaining material was thoroughly washed sequentially with tap water and distilled water then allowed to air dry at $25 \pm^{\circ}\text{C}$ away from direct light. The dried material was ground (using electric mortar, moulinex) to a fine powder and transferred to labeled brown bottles until required.

2.3. Water Analysis

2.3.1. Physical Analysis. Sea water was seasonally (15th day of January, April, July, and October) sampled from the site of algal collection, and the pH and water temperature were measured *in situ* as well as the light intensity on the water surface (by an *in situ* pH meter, thermometer (Ordinary thermometer graduated from 0 to 100°C), and luxmeter, resp.).

2.3.2. Chemical Analysis. Sea water samples were seasonally picked up (15th day of January, April, July, and October) at the same time and place as the physical measurements were made and analyzed according to APHA [5].

2.3.3. Pigments Analysis. Chlorophyll and carotenoids contents were determined by the method of Holden [6]. Pigment levels in the filtered extract were determined by the absorbance at 663, 645, and 450 nm in a 1 cm quartz cell against a blank.

2.4. Antioxidant Enzyme Determination. Ascorbate oxidase and catalase activity were determined by the method of Nakano and Asada [7]. Ascorbate oxidase activity was measured at 265 nm and expressed as the number of enzyme units $\times 10^3/\text{mg}$ algal protein. Catalase activity was determined at 240 nm and expressed as $\mu\text{mole H}_2\text{O}_2/\text{min}/\text{mg}$ algal protein.

2.5. Protein Determination. Protein content in different algae was determined spectrophotometrically at 650 nm, using the Folin-Ciocalteu reagent [8].

2.6. Extract Preparation. A known mass (100 g) of each ground sample of both *Ulva* and *Enteromorpha* species was extracted three times with 70% ethanol for twenty-four hours in a dark environment at room temperature. The extracts were separately filtered through filter paper (Whatman number 102, 18 cm), and the solvent was removed using rotary evaporator with the water bath set at 45°C . Each residue was weighed, transferred to a brown bottle, and stored at 4°C until required. The crude ethanolic extract was sequentially extracted with petroleum ether, chloroform, ethyl acetate, and distilled water according to Rossenthaler [9]. The removal of the aqueous solvent was achieved by using a freeze drier.

2.7. Total Phenolic Contents. The phenolic content of each extract was determined by the method of Taga et al. [10]. An aliquot of each extract was dissolved in a known amount

of appropriate solvent and its absorption was measured at 720 nm. The phenolic contents were expressed as gallic acid equivalents *per gram* (GAE/g).

2.7.1. Essential Oil Extraction. 100 g of each dried extract was distilled in a Clevenger-type apparatus [11] and the resultant oil samples were dried and stored in the dark at 4°C . The amount of oil obtained per sample was calculated as: oil (5 V/W) = observed volume of oil (mL/mass of sample (g) $\times 100$).

2.7.2. GC/MS Analysis of Essential Oil. Essential oil was analyzed by the method of Adams [12] using a Thermoquest-Finnigan Trace GC-MS system with a DB-5 (5% phenyl) methylpolysiloxane column (60 m/0.25 mm id, film thickness 0.25 μm). The injection temperature was 220°C and the oven temperature was raised from 40°C (3 min hold) to 250°C at a rate of $5^{\circ}\text{C}/\text{min}$, and then held at 250°C for 2 min; the transfer line temperature was 250°C . 1 μL of sample was injected into helium adjusted to a flow rate of 1.0 mL/min. The mass spectrometer scan was between 40 and 500 m/z with an ionising voltage of 70 eV; identification of essential oil components was performed using the standard mass library of the National Institute of Standards and Technology (NIST version 2.0).

2.8. Assay of Antioxidant Activities. Antioxidant activity of different extracts was determined using the following methods:

2.8.1. DPPH Radical Assay. The scavenging effect of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured by the method of Chou et al. [13]. Various concentrations of algal extract (100 and 200 $\mu\text{g}/\text{mL}$) were separately incubated with 0.1 mL of a 1 mM DPPH methanol solution. The absorbance of each solution after 30 and 60 min incubation was measured at 517 nm against a blank of butylated hydroxyl toluene (BHT). The DPPH radical scavenging activity was expressed as the inhibition percentage as calculated by: absorbance of control-sample absorbance/control absorbance $\times 100$.

2.8.2. Reducing Power. The reducing power was determined by the method of Chou et al. [13]. Various concentrations of algal extract (100 and 200 $\mu\text{g}/\text{mL}$) were mixed with 0.25 mL of sodium phosphate buffer (200 mM, pH 6.6) and 0.25 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min; then 0.25 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (0.5 mL) was mixed with 0.4 mL of deionized water and 0.1 mL of 0.1% ferric chloride solution and allow standing for 10 min. The absorbance was measured at 700 nm. A higher absorbance indicated a higher reducing power, and standard BHT was used for comparison.

2.8.3. Total Antioxidant Activity. This was determined by the method of Prieto et al. [14] where the absorbance is

TABLE 1: Physical and chemical parameters of sea water collected at different seasons (2009-2010).

Parameters	Units	Seasons			
		Spring	Summer	Autumn	Winter
Physical parameter					
Temperature	°C	20.00	28.00	25.00	18.00
Light intensity	k lux	132.00	840.00	255.00	112.00
EC	mmose/cm	58.00	61.00	60.00	45.00
EC	µg/mL	46400.00	48800.00	4800.00	36000.00
PH		7.50	7.30	7.35	7.25
Chemical parameter (mg/L)					
Dissolved anions					
Carbonate		0.00	0.00	0.00	0.00
Nitrate		22.32	28.42	1.96	10.33
Phosphorus		0.00	0.044	0.53	0.029
Bicarbonate		2.62	1.62	1.70	1.90
Chloride		705.20	624.0	636.0	358.46
Sulphate		62.18	238.38	232.30	269.64
Dissolved cations					
Calcium		30.00	21.60	25.40	115.50
Zinc		0.159	0.006	0.00	0.004
Ammonium		6.48	2.94	3.92	1.72
Copper		0.00	0.008	0.002	0.016
Magnesium		107.00	141.48	117.16	31.75
Sodium		630.00	685.26	712.62	464.60
Potassium		3.00	15.66	14.82	18.15
Residual sodium carbonate		0.00	0.00	0.00	0.00
Adsorbed sodium %		76.09	75.89	84.43	54.15

TABLE 2: Antioxidant activity of crude ethanolic extract of three green macroalgal species against DPPH radical scavenging assay.

Algal species	Antioxidant activity %	
	30 min	60 min
<i>Enteromorpha compressa</i>	36.5 ± 0.5	55.7 ± 4.3
<i>Enteromorpha linza</i>	48.8 ± 1.5	48.4 ± 1.7
<i>Ulva Lactuca</i>	49.5 ± 2.4	50.4 ± 1.6
BHT (Synthetic standard)	89.2 ± 4.7	88 ± 3.6
LSD	0.0326	0.0326

Each value is presented as mean of triplet treatments; LSD: Least significant difference at $P \leq 0.01$ according to Duncan's multiple range test.

measured at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid.

2.8.4. β -Carotene-Linoleic Acid Bleaching Method. The antioxidant activity was determined by the method of Koleva et al. [15]; the absorbance was measured at 450 nm and the percentage of antioxidant activity was determined as the % inhibition of the β -carotene bleaching.

2.8.5. ABTS Radical Cation Scavenging Assay. This assay was based on the ability of different substances to scavenge 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid (ABTS+)

radical cation in comparison to a standard (BHT, 100 µg/mL). The radical cation was prepared by mixing a 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4–16 hrs until the reaction was completed and the absorbance was stable. The ABTS.+ solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photometric assay was conducted on 0.9 mL of ABTS.+ solution and 0.1 mL of tested samples (100 and 200 µg/mL) and mixed for 45 s; measurements were taken at 734 nm after 1 min. The antioxidative activity of the tested samples was calculated by determined the decrease in absorbance at different concentrations by using the following equation: $E = ((Ac-At)/Ac) \times 100$, where at and Ac are the respective absorbance of tested samples and ABTS.+ [16].

2.9. Purification and Characterization of the Active Antioxidant Ethyl Acetate Fraction. Thin layer chromatography (precoated TLC F254) was used to separate the various using the eluent mixtures: petroleum ether:ethyl acetate (9.5:0.5 v/v) petroleum ether:ethyl acetate (9:1 v/v); petroleum ether:ethyl acetate (7:3 v/v) and petroleum ether:ethyl acetate (8.5:1.5 v/v). The final solvent combination was found to be the most promising, and R_f values were calculated for the separated compounds, and its locations was indentified by UV Lamp. The resultant spots were eluted

TABLE 3: Antioxidant activity (%) of different extracts from *Enteromorpha compressa* using DPPH method after 30 min at 100 and 200 $\mu\text{g/mL}$.

Extracts	Seasons								LSD
	Spring		Summer		Autumn		Winter		
	Conc. ($\mu\text{g/mL}$)								
	100	200	100	200	100	200	100	200	
Crude (ethanolic extract)	46 \pm 1.6	49 \pm 2.6	47 \pm 3.6	50 \pm 1.0	50.53 \pm 3.2	55.44 \pm 0.74	41.5 \pm 1.5	44.5 \pm 1.6	0.0325
Pet. ether	63 \pm 1.3	66 \pm 1.9	50.7 \pm 3.90	53.7 \pm 3.2	46.02 \pm 1.8	55.3 \pm 3.65	49.1 \pm 0.7	52.1 \pm 1.0	0.0325
Chloroform	75.5 \pm 6.0	78 \pm 4.6	44 \pm 4.0	47 \pm 1.6	60.6 \pm 4.6	66.8 \pm 4.9	40.2 \pm 0.6	43.24 \pm 1.0	0.0325
Ethyl acetate	79.5 \pm 5.5	82.8 \pm 7.8	44.3 \pm 2.45	47.3 \pm 1.0	32.6 \pm 0.9	46 \pm 1.25	49.1 \pm 2.7	52.1 \pm 5.0	0.0325
Water	90 \pm 2.0	93 \pm 7.7	45.5 \pm 3.61	48.5 \pm 1.7	48.8 \pm 4.6	50.13 \pm 0.99	40.7 \pm 1.0	43.7 \pm 1.7	0.0325
BHT	84 \pm 2.0	88.5 \pm 2.6	84 \pm 1.85	88.5 \pm 3.6	85 \pm 4.9	88.5 \pm 4.6	84 \pm 2.6	88.5 \pm 1.9	0.0325
LSD	0.0262	0.0262	0.0262	0.0262	0.0576	0.0668	0.0262	0.0262	

Each value is presented as mean of triplet treatments; LSD: least significantly different at $P \leq 0.01$ according to Duncan's multiple range test.

TABLE 4: Antioxidant activity (%) of different extracts from *Enteromorpha compressa* using DPPH method after 60 min at 100–200 $\mu\text{g/mL}$.

Extracts	Seasons								LSD
	Spring		Summer		Autumn		Winter		
	Conc. ($\mu\text{g/mL}$)								
	100	200	100	200	100	200	100	200	
Crude (Ethanolic extract)	46 \pm 4.5	55.7 \pm 6.9	47 \pm 7.0	50.7 \pm 4.7	50.66 \pm 2.6	55.03 \pm 2.7	41.5 \pm 2.6	44.7 \pm 1.6	0.0325
Pet. ether	63 \pm 2.0	69 \pm 4.3	50.7 \pm 3.2	54.3 \pm 6.3	45.88 \pm 1.4	54.24 \pm 1.3	49.1 \pm 1.5	52 \pm 3.6	0.0325
Chloroform	75.5 \pm 2.6	82 \pm 4.5	44 \pm 4.5	48 \pm 2.5	59.3 \pm 1.0	65.12 \pm 6.2	40.2 \pm 3.6	44.4 \pm 2.7	0.0325
Ethyl acetate	79.5 \pm 1.6	95.7 \pm 1.6	44.3 \pm 5.3	48.5 \pm 8.6	34.6 \pm 3.2	45.75 \pm 0.9	49.1 \pm 2.5	52.7 \pm 1.0	0.0325
Water	90 \pm 4.6	95.4 \pm 4.8	45.5 \pm 4.6	50 \pm 2.5	49.6 \pm 1.8	50.66 \pm 2.5	40.7 \pm 1.3	44 \pm 4.6	0.0861
BHT	83 \pm 4.6	88 \pm 4.8	83 \pm 6.8	88 \pm 3.2	85.4 \pm 2.9	88.85 \pm 7.0	83 \pm 1.5	88 \pm 2.6	0.0325
LSD	0.0262	0.0262	0.0262	0.0262	0.0642	0.05132	0.0262	0.0262	

Each value is presented as mean of triplet treatments; LSD: Least significant difference at $P \leq 0.01$ according to Duncan's multiple range test.

with the chosen eluent, the mixture was filtered prior to solvent evaporation. The residue was used for the bioassays.

2.10. Determination of the Chemical Structure of the Antioxidant Compound

2.10.1. Mass Spectra (MS). To detect the chemical structure and M.wt of compound from each fraction, the mass spectrometer scanned over the range of 40–500 nm/z set at an ionizing voltage of 70 eV; values were compared with those from the standard mass library of the National Institute of Standard and Technology (NIST Version 2.0).

2.10.2. The Infrared Spectra (IR). The molecular structures of the separated antioxidants were partially identified by means of their functional groups using a Perkin Elmer 1430 infrared spectrophotometer and scanning between 400–4000 nm.

2.10.3. Proton Nuclear Magnetic Resonance (^1H NMR) Spectra. Samples were dissolved in dimethyl sulfoxide (DMSO)

and their hydrogen atoms were identified using a Varian Gemini 200 MHz.

2.11. Statistic Analysis. Data were subjected to an analysis of variance, and the means were compared using the least significant difference (LSD) test at the 0.05 and 0.01 levels were determined according to the method of Snedecor and Cochran [17].

3. Results and Discussion

The presence of three green macroalgal species all over the year was encouraging to study the genera *Enteromorpha* and *Ulva* which are mostly growing on the rocky substrata of the intertidal zone of Abu Qir bay at Alexandria.

Genus *Enteromorpha* was represented by the two species *E. compressa* and *E. linza* while genus *Ulva* was represented by the species *U. lactuca* only.

E. compressa was found growing abundantly in spring season, and its growth was found to decrease gradually

TABLE 5: Antioxidant activity (%) of algal crude extract, spring-collected ethyl acetate (EA) extract and standard BHT by different antioxidant methods at 100 and 200 $\mu\text{g}/\text{mL}$.

Extract	DPPH		ABTS		Total antioxidant		Reducing power		β -carotene bleaching	
	100	200	100	200	100	200	100	200	100	200
Crude (ethanolic extract)	46 \pm 1.6	55.7 \pm 1.9	30.3 \pm 0.90	34.5 \pm 0.7	2.7 \pm 0.3	9.0 \pm 0.2	5.15 \pm 1.0	6.18 \pm 1.0	65 \pm 1.0	67 \pm 1.88
EA (spring)	79.5 \pm 4.6	95.7 \pm 7.9	16.5 \pm 0.6	20.5 \pm 1.2	10.7 \pm 0.9	17.50 \pm 0.1	6.5 \pm 0.3	7.47 \pm 0.5	18 \pm 1.2	20 \pm 2.6
BHT	83 \pm 2.8	88 \pm 6.3	83.6 \pm 4.9	84.6 \pm 5.9	32.20 \pm 1.6	37.1 \pm 4.6	15.03 \pm 2.3	18.12 \pm 2.1	66 \pm 3.7	68 \pm 1.8
LSD	0.338	0.338	0.041	0.041	4.130	4.130	0.024	0.024	0.413	0.413

Each value is presented as mean of triplet treatments; LSD: Least significant difference at $P \leq 0.01$ according to Duncan's multiple range test.

TABLE 6: Pigments (chl a, b, total chlorophylls, and carotenoids) and phenolic compounds (of spring collected *E. compressa*) in ethyl acetate (EA) extracts expressed as mg/g and GAE.

Extract	Pigments (mg/g)				Gallic acid equivalent (GAE)	
	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids	Phenolic compounds	
Crude (Ethanolic extract)	0.51 \pm 0.01	0.967 \pm 0.2	1.47 \pm 0.3	0.067 \pm 0.02	0.057 \pm 0.01	
EA (Spring)	4.05 \pm 0.7	3.15 \pm 0.6	7.2 \pm 0.5	0.127 \pm 0.01	0.064 \pm 0.0	
LSD	0.0701	0.007	0.0737	0.0177	0.005	

Each value is presented as mean of triplet treatments; LSD: Least significant difference at $P \leq 0.01$ according to Duncan's multiple range test.

in summer, autumn seasons and was greatly reduced in winter.

E. linza was moderately growing during spring, summer and autumn while it completely disappeared in winter.

Ulva lactuca species was only recorded in reduced quantities during spring and summer seasons and was mostly attached to rocks and stones of the high tide region near the shore.

The optimum physical and chemical water analyses including moderate temperature (20°C), light intensity (132 klux), hydrogen ion conc, pH 7.5, dissolved important anions and cations (Table 1) induced an enhancement to growth rates of all the three green macroalgae especially in spring season, while a gradual retardation was observed in all species in other seasons and complete absence of *Ulva lactuca* in autumn and winter and *E. Linza* in winter. Growth of *E. compressa* was highly encouraged by spring environmental conditions and slightly affected by summer and autumn conditions, while in winter its growth was severely reduced (Figure 1)

The abundant presence and high rate of growth of *Enteromorpha compressa* and its preliminary high antioxidant activity (55.7%, Table 2) encourage our scientific interest to investigate seasonally the antioxidant efficiency of its different extracts, fractionate the promising one, and identify the pronounced antioxidant active compound(s) of this fraction by chromatographic and spectroscopic methods.

We thought that the tolerance of this alga to different environmental conditions and its persistence all the year must be due to its internal defense mechanism(s) and special metabolism with which this alga not only tolerate the conditions of the habitat, but also grow and dominate its inhabiting area (the intertidal zone).

The defense mechanism of an alga against the stressed environmental conditions especially of high temperature,

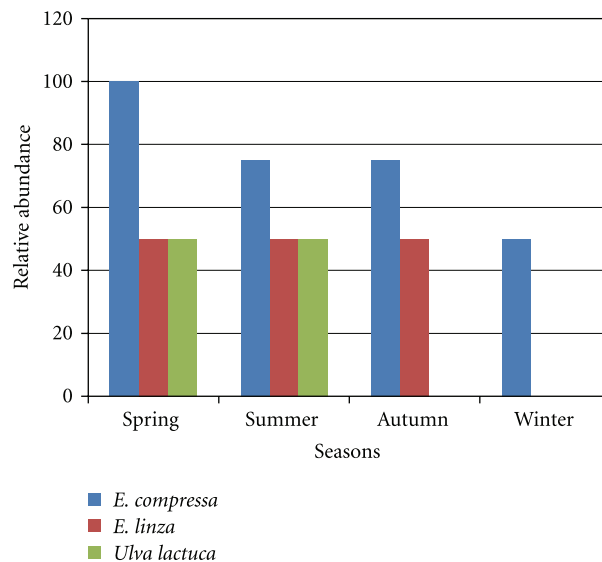


FIGURE 1: % abundance of green macroalgal species collected at different seasons from Abu Qir bay.

high light intensity, and pollutants was expressed by algal production of defensive substances and enzymes belonging to the antioxidant system.

Under stressed conditions, different free radicals are produced in biological system, and if they were not scavenged properly in time, they will attack the biomolecules and the biomembranes causing serious and adverse alternations and modifications as recorded by Plaza et al. [3].

Thus, the stressed conditions induced remarkable antioxidant and defense mechanism(s) in the alga including the production of antioxidant enzymes and low-molecular-weight substances to scavenge or even inactivate the reactive

TABLE 7: Antioxidant activity (%) of essential oil of *E. compressa* collected during spring compared with BHT as standard.

Extract	DPPH %	Total antioxidant	Reducing power	ABTS %	β -Carotene %
Essential oil	41.0 \pm 2.4	40 \pm 2.4	2.07 \pm 0.2	12.7 \pm 1.6	14 \pm 1.0
BHT	89.5 \pm 5.6	37.1 \pm 1.7	18.12 \pm 0.8	83.6 \pm 4.6	68 \pm 2.9
LSD	0.0702	0.074	0.1403	0.0702	0.07

Each value is presented as mean of triplet treatments; LSD: Least significant difference at $P \leq 0.01$ according to Duncan's multiple range test.

TABLE 8: Essential oil constituents of *E. compressa*.

Peak number	RT	Name of compound	Area %
1	15.36	2-Cyclohexene-1-one	0.46
2	15.62	Benzaldehyde, 4-methoxy	0.2
3	16.17	Benzene, 1-methoxy-4-(1-propenyl)	0.44
4	22.79	Heptadecene	0.24
5	28.58	Octadecane, 1-chloro-	0.21
6	29.64	Butyl hexadecanoate	2.42
7	29.82	Docosane	0.69
8	29.89	Hexadecane, 1-chloro-	0.32
9	30.98	Tricosane	0.97
10	31.96	Octadecanoic acid, butyl ester	2.07
11	32.10	Tetracosane	1.62
12	33.10	Phthalic acid, diisooctyl ester	0.21
13	33.18	Hexatriacontane	1.38
14	33.85	1,2-Benzenedicarboxylic acid	82.23
15	34.24	Hexacosane	1.52
16	35.22	Heptacosane	1.31
17	36.18	Eicosane	1.38
18	37.25	Nonacosane	0.99
19	38.43	Eicosane	0.86
20	39.78	Hexatriacontane	0.48
Total			100%

TABLE 9: Ascorbate oxidase and catalase content of *Enteromorpha compressa* collected in different seasons.

Seasons	Ascorbate oxidase ($\text{Eu}\chi 10^3/\text{mg protein}$)	Catalase ($\mu\text{mole H}_2\text{O}_2/\text{min}/\text{mg protein}$)
Spring	28.15 \pm 2.4	5.85 \pm 0.6
Summer	41.8 \pm 3.6	6.95 \pm 0.2
Autumn	35.86 \pm 1.4	6.35 \pm 1.0
Winter	30.05 \pm 0.9	5.80 \pm 0.6
LSD	10.754	1.237

Each value is presented as mean of triplet treatments; LSD: least significantly different at $P \leq 0.01$ according to Duncan's multiple range test.

oxygen species (ROS) or free radicals normally produced internally in the alga growing under adverse stressed conditions. Seasonal algal collection and extraction using organic solvents of different polarities, (petroleum ether, chloroform, ethyl acetate, ethanol, and water) were performed.

Antioxidant efficiency of each extract was carried out by DPPH method. This method was used as a principle

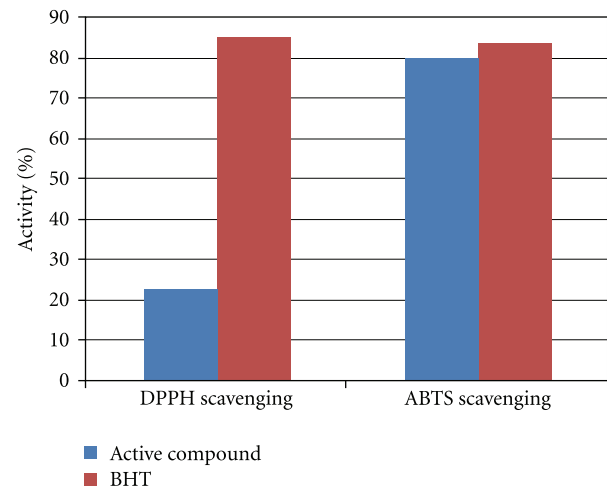


FIGURE 2: Antioxidant activity of the separated pure compound from ethyl acetate extract (of spring collected alga) by DPPH and ABTS methods at 100 $\mu\text{g}/\text{mL}$.

antioxidant as fast test (H-donor method) and the other four methods are used to know and understand the mechanism of antioxidant activity (electron donor as in case of ABTS (e-donor), total antioxidant, reducing power, and β -carotene bleaching).

The obtained results using the antioxidant bioassays of all seasonal extracts (by different solvents) showed that the antioxidant activity was concentration and time dependant. Tables 3 and 4 recorded the activity (using DPPH method) which increased by doubling both the extract concentration (100 and 200 $\mu\text{g}/\text{mL}$) and time (30 and 60 min).

The antioxidant activity of the spring-collected algal ethyl acetate extract was 79.5% at 100 $\mu\text{g}/\text{mL}$ (after 30 and 60 min) and increased to 95.7% on doubling the extract concentration to 200 $\mu\text{g}/\text{mL}$. Water extract (9.5 g/100 g of crude ethanolic extract) gave comparable results to that of ethyl acetate extract (40 g/100 g of crude ethanolic extract) at the same conditions. Summer, autumn, and winter extracts of different solvents recorded much lower antioxidant activities (by DPPH method) even at high concentration (ranged between 32.6–66.8%).

Table 5 recorded the antioxidant activity of crude ethanol extract using two concentrations (100 and 200 $\mu\text{g}/\text{mL}$) as well as the spring collected ethyl acetate extract by different antioxidant assays compared with the standard BHT.

The obtained data clearly showed that the DPPH method recorded the highest antioxidant activity (95.7%) at higher ethyl acetate extract concentration, (200 $\mu\text{g}/\text{mL}$) which exceeds both of the standard BHT (88.0%) and the crude

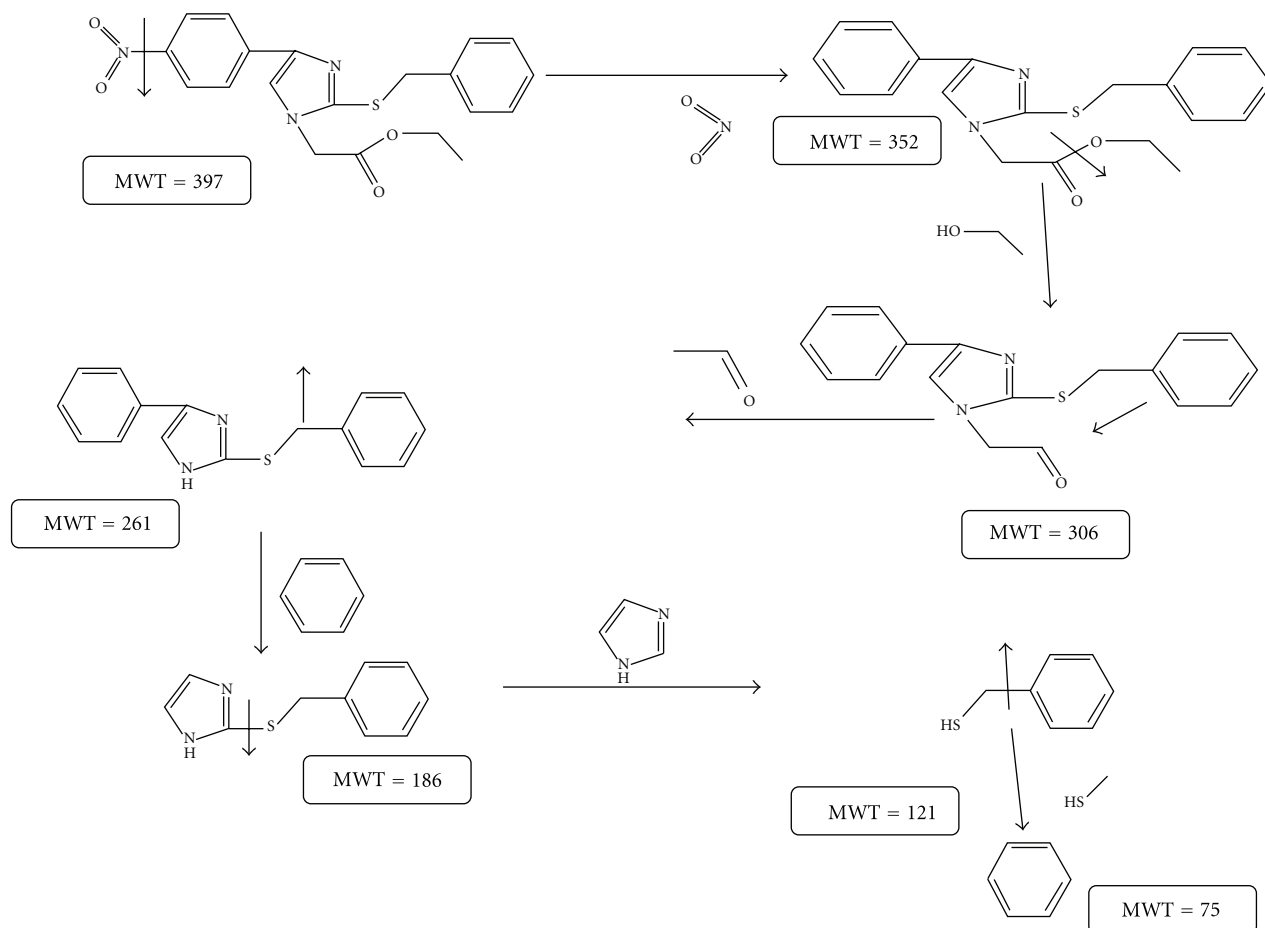


FIGURE 3: MS fragmentation pattern of active ingredient separated from ethyl acetate fraction of *E. compressa*.

extract (55.7%). All the other antioxidant methods (ABTS, total antioxidant, reducing power, and β -carotene/linoleic acid) recorded much lower activities than those of both the crude extract and the standard BHT, as illustrated in Table 5.

So it was clear now that the spring collected alga, the ethyl acetate extract, and the DPPH method were the highly pronounced results. Algal ethyl acetate extract may contain various compounds including pigments (chl a, b, carotenoids), alkaloids, and flavonoids (not determined here) phenolic compounds, as well as essential oil which can participate in the obtained great antioxidant activity.

Table 6 recorded the pigments and phenolic content in the crude extracts and the ethyl acetate extract (of the spring collected alga).

Chlorophyll a, b, total chlorophyll, and carotenoid contents in the ethyl acetate extract were highly pronounced comparing with those in the crude extract (4.05, 3.15, 7.2, and 0.127 mg/g chlorophyll a, b, total chlorophyll and carotenoids) compared with 0.51, 0.967, 1.47, and 0.067 mg/g of the same pigments in the crude extracts.

Phenolic content of the ethyl acetate extract was slightly greater than that in the crude extract (0.064 and 0.057 GA, resp.).

Table 7 recorded the antioxidant activity of the essential oils in the spring collected alga by the different (five) antioxidant methods used. The obtained data clearly demonstrated that the DPPH method reported the highest activity (41%) compared with those of the other methods and about 50% of that of standard BHT (89.5%). The antioxidant activity of essential oil may be due to the high concentration of 1, 2-benzenedicarboxylic acid in the crude oil (82.32%) as shown in Table 8. This compound is rich with unsaturated bonds and bind with two electronegativity groups (carboxylic acid) which has great ability for scavenging the free radical [18].

All these data (Tables 5, 6, and 7) confirmed that ethyl acetate extract of the spring-collected alga has greater content of pigments, phenolic compounds, and essential oils to which attributed the highest antioxidant activity by DPPH method. This means that synergistic effects may occur between these constituents leading to the pronounced antioxidant activity of the ethyl acetate extract (containing the antioxidant active components). On the contrary the crude extract recorded not only lower pigment contents but also lower content of phenolic compounds together with other undetermined compounds which manifested reduced antioxidant activity (antagonism may occur between the components of the crude extract).

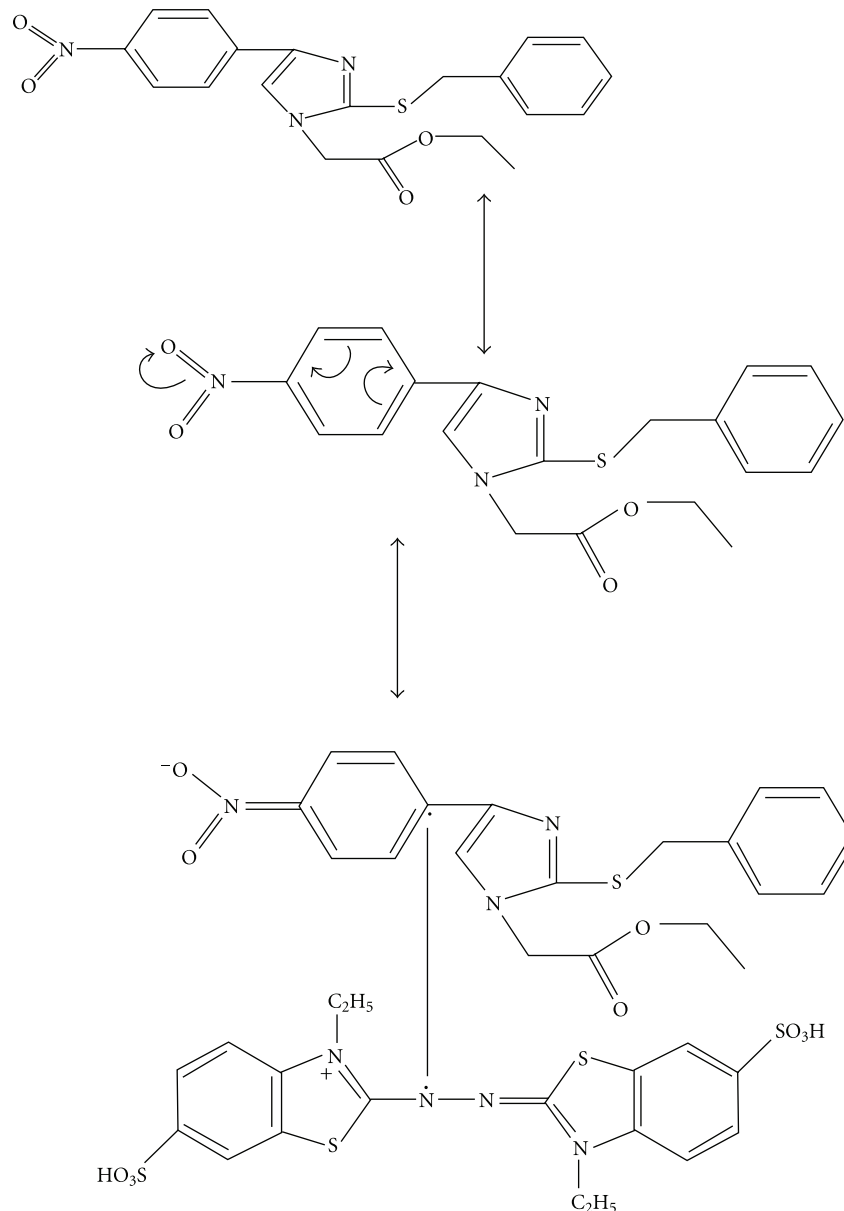


FIGURE 4: First suggested mechanism for the antioxidant activity of active ingredient separated from *E. compressa* against ABTS radical.

These results were confirmed by those obtained by Matsukawa et al. [19] who screened the antioxidant activity (by DPPH method) of 17 seaweed species (11 brown, 5 red, and 1 green algae (*E. linza*)). The ethanolic extracts of these seaweeds were highly inhibitory than the aqueous ones.

Our results also went parallel with Lim et al. [20] who reported that dichloromethane fraction from methanol extract exhibited the strongest antioxidant activity (in red blood cell hemolysis and lipid peroxidation assays). Further fractionation by column chromatography, TLC, UV, and IR showed that the separated four subfractions contain phenolic compounds and manifested potent antioxidant activities.

Dichloromethane extract of three seaweed species (one brown and 2 red) studied by Shanab [21] exhibited higher antioxidant activity (free radical scavenging by DPPH and

antilipid peroxidation by Fe^{++} /ascorbate assay) of 86.16 and 83.44%, respectively, comparing with that of the standard silymarin (92 and 96.5%) which was attributed to the contents of carotenoids and phenolics.

Most of the reported antioxidant activity of different seaweed species (green, brown, and red) using extracts of different polarities and concentrations. The activity may be determined by different assays (DPPH, ABTS, β -carotene/linoleic acid bleaching method, reducing power, and free radical scavenging methods). The recorded activity either high or low was shown to correlate with the total phenolic content in the algal extract(s) as reported by many investigators (Sachindra et al. [22], Sivakumar and Bajagopal [23], Demirel et al. [24], and Sadati et al. [25]). On the other hand, few studies as Heo and Cha [26] reported that

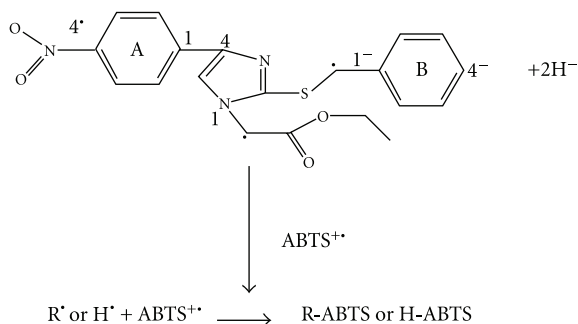


FIGURE 5: Second suggested mechanism for the antioxidant activity of active ingredient separated from *E. compressa* against ABTS radical.

the antioxidant activity (determined by different methods), using large number of algal species (10 green and 25 brown seaweed species), not always correlate with the total phenolic content in each algal extract. These results may indicate the possible participation of other active substances which exhibit antioxidant activity as pigments (chlorophyll, carotenoids), essential oils, and low molecular weight polysaccharides. Chlorophyll may connect to the negatively charged peroxy radical, act as potent synergist of vitamin E [24, 25], and enhance the antioxidant activity of α -tocopherol [27].

Carotenoids (carotene and xanthophylls) are known to quench the excited sensitized molecule and singlet oxygen [28] and may act as antioxidant under conditions, where singlet oxygen is not formed [29].

Crude extract and ethyl acetate extracts may contain both parts of the constituents (pigments and phenolic compounds) which differ certainly in proportion according to the polarity of solvents and compounds; in addition to other components which may act synergistically or antagonistically leading to either increase or decrease of the antioxidant activity [30].

So to determine the active antioxidant compound(s) in the ethyl acetate extract, other than the pigments and phenolic compound, fractionation of the extract was performed using TLC F_{254} and n-hexane/ethyl acetate as mobile phase (7:3, v/v).

The separated spot seen under the UV lamp was scratched, eluted in the same mobile phase, and filtered, and solvents were evaporated and weighted.

Identification of the separated compound was performed using mass spectrum then complete identification was carried out by infrared spectrum and proton NMR spectrum. The antioxidant activity of this pure compound was carried out by both DPPH and ABTS methods as recorded in (Figure 2) and was found to be 22.7 and 80.4%, respectively. The pure compound was identified as ethyl [2-(benzylsulfanyl)-4-(4-nitrophenyl)-1H-imidazol-1-yl] acetate.

Chromatographic and spectroscopic analysis of active compound separated from ethyl acetate fraction suggested that, Ethyl[2-(benzylsulfanyl)-4-(4-nitrophenyl)-1H-imidazol-1-yl] acetate (Figure 3) was present with molecular weight 397 Dalton and molecular formula $C_{20}H_{19}N_3O_4S$.

This compound was shown to exert potent antioxidant activity of 80.4% and 22.7% against both ABTS and DPPH radicals compared to those of the synthetic antioxidant standard BHT (85.6 and 85.5%, resp.).

This active compound was identified using different spectroscopic analysis methods as an alkaloid (Figure 3) and this is the first record for the separation of this compound from *E. compressa* and was shown to exert potent antioxidant activity (Figure 2).

The mass spectrum of separated active ingredient indicates the presence of the following fragment ions: 397, 352, 306, 261, 186, 121, and 75 Dalton as shown in Figure 3.

These results were confirmed by the complete identification of active compound by IR and 1H -NMR. The IR spectrum of active compound showed absorption at 3437 (NH group). Region between 1500 and 1600 (C=C of aromatic ring), 2731 (COO-), 2930, 2866 (-CH), 1460 (Aliphatic CH_2 scissor for the methylene group).

The 1H -NMR data indicated that, the compound under study had the following types of protons; A multiplex signal at δ 7.2 ppm which is characteristic for aromatic protons (Aromatic B ring; H-3⁻, H-4⁻, and H-5⁻) and A multiplex signal at δ 7.4 ppm is characteristic for aromatic protons (Aromatic B ring; H-2⁻ and H-6⁻). Moreover, A multiplex signal at δ 8.06 ppm which is characteristic for aromatic protons (Aromatic A ring; H-2⁻, H-3⁻, H-5⁻ and H-6⁻) the singlet signal at δ 2.5 ppm was characteristic of the protons of methylene group (- CH_2 -) and the singlet signal at δ 1.9 ppm was characteristic of protons of methyl group (- CH_3).

3.1. Suggested Mechanism. The antioxidant activity of the promising active compound which was separated from *E. compressa* against ABTS radical may be due to one of the following reasons.

3.1.1. First Reason. The resonance phenomena of double bonds and lone pair atoms (N, S, O) in the chemical structure of the active compound. This structure may lead to radical formation in more than one site for example: benzene ring (A), this ring is near from highly negativity group (nitro group) this condition helps the benzene ring to convert it to radical form and formation of new covalent bond with ABTS radical as shown in Figure 4.

3.1.2. Second Reason. The presence of different electronegativity groups in the structure may lead to less stability of different atoms (e.g., methylene group) because these groups can attach electron from methylene group and convert it to radical form. So the activity of the active compound may be due to the reaction between methylene group radicals or hydrogen proton with ABTS radical as shown in (Figure 5).

In addition to the determination of antioxidant compounds in the extract (as pigments, phenolic compounds, and essential oils). Activity of the antioxidant enzymes as ascorbic acid oxidase and catalase was determined in the extracts of the seasonally collected *E. compressa* and recorded in Table 9. The results indicated that the concentration of defense enzymes during different seasons was ordered as the following: summer > autumn > winter = spring this

ordered antioxidant enzymes may due to the environmental stress especially the abiotic stress (temperature, light etc.) on algal cell. So, the algae protect themselves by secretion of different antioxidant compounds and enzymes. These results are in agreement with the results obtained by Plaza et al. [3] who reported that, the absence of structural damage in the algae leads to the consideration that these organisms are able to generate the necessary compounds to protect themselves against oxidation. In this respect, algae can be considered as an important source of antioxidant compounds that could be suitable also for protecting our bodies against the reactive oxygen species formed by our metabolism or induced by external factors (as pollution, stress, UV radiation, etc.).

4. Conclusions

The green macroalga *E. compressa* is the only green species predominated all over the year in the intertidal zone of Abu Qir bay in Alexandria (Egypt) the crude ethanolic extract of the spring collected alga recorded high antioxidant activity by DPPH method, and the ethyl acetate fraction of the crude ethanolic extract showed the highest antioxidant activity (%) when compared with the other fractions. Using chromatographic and spectroscopic analyses, an active compound was separated from the promising ethyl acetate fraction.

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