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In vitro Assessment of Antimicrobial, Antioxidant and Anticancer Activities of some Marine Macroalgae

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THE PRESENT study evaluated ethanol, methanol, ethyl acetate, hexane, chloroform and acetone extracts of five green and red seaweed species from Abu-Qir bay, Alexandria, Egypt for their antimicrobial, antioxidant activities and cytotoxicity against four cell lines. Chloroform extracts of *Ulva lactuca* and *Ulva fasciata* exhibited the highest inhibition zones against the tested pathogenic bacteria (*Klebsiella pneumoniae* and *Proteus mirabilis*) and fungi (*Aspergillus flavus, Aspergillus fumingatus* and *Aspergillus niger*) as measured by disc diffusion method. The extracts of *U. lactuca and U. fasciata* showed the highest antioxidant activity (IC_{50} 6.32±0.29mg/ml and 6.61±0.27mg/ml, respectively), using DPPH (2, 2- diphenyl-1-picrylhydrazyl) scavenging method and total antioxidant capacity assay (2.13 and 1.51mg ascorbic acid equivalent/ gram dry weight, respectively). Cytotoxicity test using MTT assay showed that *U. lactuca* extract had strong activity against MCF-7 and Hela cell lines (IC_{50} 10.83±1.0, 12.43±1.3µg/ml, respectively), while *U. fasciata* had strong activity against PC3 and HepG2 cell lines (IC_{50} 12.99±1.2, 16.75±1.5µg/ml, respectively). As analyzed by Ultraviolet spectra, Fourier Transform Infrared Spectra and Gas Chromatography Mass Spectroscopy after purification by column chromatography, the most active antimicrobial fractions in *U. lactuca* and *U. fasciata* extracts include an aromatic compound that have different active group (-C=O, phenyl ring and –OH); its molecular weight of di-isooctyl phthalate equals 390.56g/mol and butylated hydroxytoluene equals 220.356g/mol, respectively. The studied seaweeds have distinct active metabolites being a promising source of antimicrobial and antiproliferative compounds that can be used effectively in pharmaceutical drug industry.

Keywords: Antimicrobial, Antioxidant, Cytotoxicity, Minimum inhibitory concentration (MIC), GC-MS.

Introduction

The increasing pathogenic microbial resistance of the antibiotics, as well as their side effects and much expense have led to search for new infection-fighting strategies to control microbial infections (Rajesh et al., 2015). Generally, the compounds produced naturally, if compared with the antibiotics, have better potential, less side effects and minimal toxicity (Thanigaivel et al., 2015). It is also worthwhile to test the marine antimicrobials for possible synergism with existing drugs (Cheung et al., 2014). Recently active compounds from marine seaweeds have always been of great interest to scientists working on different diseases (Hemraj et al., 2012). They include several structurally secondary metabolites including carotenoids, polyphenols, flavonoids and terpenoides. In addition, they contain other antioxidant pigments, vitamins (such as, ascorbic acid and β-carotene), polysaccharides and polyunsaturated fatty acids. All these compounds were exploited as functional ingredients (Kumar et al., 2008; Sachindra et al., 2010) with antimicrobial (Cox et al., 2010; Osman et al., 2010, Farasat et al., 2014; El Shafay et al., 2016; Mohamed& Saber, 2019; Metwally et al., 2020), antioxidant (Ismail et al., 2016; Di et al., 2017), antiviral (Gheda et al., 2016), laxatives, anticoagulants (De Zoysa et al., 2008), antiulcer and antitumor activities (Gheda et al., 2018).

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The antioxidant activity of seaweeds prevents many diseases, such as heart disease, cancer, hypertension and diabetes, providing the living organisms the resistance against damage, lipid peroxidation, protein damage and DNA breaking (Ismail et al., 2016; Ismail, 2017). The anticancer agent *in vitro* of seaweeds decreases tumor growth by apoptosis. This occurs by stimulation of cytotoxic cytokine on gene expression (Sznarkowska et al., 2017). The anticancer activity of the red alga *Jania rubens* against colon and breast cancer cell lines is due to their contents of polysaccharides (Gheda et al., 2018). Red marine alga *Gracilaria corticata* illustrated anticancer activity against human leukemic and breast cancer cell lines (Zandi et al., 2010; Namvar et al., 2014).

The study aims to evaluate the antimicrobial and antioxidant activities of seaweed extracts prepared from five green and red seaweed species collected from Abo-Qir bay, Alexandria, Egypt against pathogenic bacteria and fungi. The study extends to estimate the cytotoxicity of the seaweed extracts against four cell lines of hepatocellular (HepG2), mammary gland (MCF7), Epithelioid Carcinoma (Hela) and Human prostate cancer (PC3). Identification of the chemical composition of the potent seaweed extracts by Gas Chromatography Mass Spectrometer is also aimed.

Materials and Methods

Seaweed samples collection

Five seaweed samples from Abo-Qir bay area, Alexandria, Egypt were collected during summer of 2016. The samples were collected in plastic bags containing seawater to prevent evaporation. Seaweeds were cleaned from epiphytes and rock debris and given a quick freshwater rinse to remove surface salts. Some of the collected samples were preserved in 5% formalin in seawater for taxonomical identification. The formalin was changed periodically to keep the seaweeds vitality for years. The other portion of the harvested samples was air-dried in the shade at room temperature (30°C) on absorbent paper. The dried samples were grounded to fine powder in an electrical mill and stored at −20°C until use. The seaweed samples were identified according to Aleem (1993), Jha et al. (2009) and Guiry & Guiry (2016).

Preparation of algal extracts

The grinded seaweeds (1:15w/v) were soaked in six solvents (concentration of 80%) of acetone, chloroform, ethyl acetate, ethanol, hexane and methanol, and put into a rotary shaker at 150rpm and at room temperature (25-30°C) for 72hrs. The extracts were obtained after evaporation of solvents using rotary evaporator at 45ºC under reduced pressure. The obtained dry extracts were stored at -20ºC in air tight bottles (Osman et al., 2010).

Antimicrobial activity

Antimicrobial activity of seaweed extracts against different human pathogenic organisms was examined by the disk diffusion assay according to Clinical and Laboratory Standards Institute (CLSI, 2012). The tested pathogenic bacteria (*Klebsiella pneumoniae* and *Proteus mirabilis*) and fungi (*Aspergillus flavus*, *Aspergillus fumingatus* and *Aspergillus niger)* were obtained from Microbiology Laboratory, Faculty of Science, Tanta University. A suspension of 0.5ml of each tested pathogenic bacteria (1x 10^5CFU/ml) and fungi (0.1x 10^5CFU/ml) were spread on the surface of nutrient agar medium for bacteria and Capek's agar medium for fungi using sterilized cotton swabs. Sterilized filter paper disks were saturated with 20µl of seaweed extracts and transferred to the surface of each plate, separately. The plates were kept at 4°C for 2hrs to allow diffusion then incubated at 37°C for 24hrs for bacterial species and 28ºC for 3-4 days for fungal species. The inhibition zone was measured after incubation time in triplicate.

Determination of the minimum inhibitory concentration (MIC) using microtiter plates technique

The seaweed extracts exhibited highest antimicrobial activities were tested for minimum inhibitory concentration by microliter broth dilution method. Nutrient broth and Czapex media were used as diluents. A volume of 90µl of 10^5CFU/ml microbial culture for both bacteria and fungi were inoculated separately in each microliter plate well with 10µl of seaweed extract at various concentrations ranged from 6 to 15mg/ml. Tetracycline (15mg/ml) was used as positive control for bacteria and ketoconazole (15mg/ml) as positive control for fungi. After incubation at 37°C for 24hrs for bacteria and 25°C for 3-4 days for fungi, the growth turbidity were detected by measuring absorbance of microliter plates at 600nm using TRIAD multimode reader to

determine the minimum inhibitory concentration (MIC) values (triplicates). The MIC is defined as the lowest concentration of compound, which inhibited 90% of the growth when compared with that of the growth control (Maadane et al., 2017).

Antioxidant activity

DPPH radical scavenging activity assay

The antioxidant activity of seaweed extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging method according to Brand-Williams et al. (1995). To prepare stock solution 1gm of each seaweed was dissolved in 10ml chloroform, then evaporated to final concentration 10mg/ml. Different concentrations (0 to 10mg/ml) of seaweed extracts dissolved in chloroform were prepared, 100µl from each concentration was added to 3.9ml of DPPH solution (0.1mM) dissolved in 95% methanol. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30min, the absorbance were measured at 517nm. Blank consisted of 0.1ml of methanol and 3.9ml of DPPH solution was measured and ascorbic acid was used as standard antioxidant.

DPPH scavenging %= (Ao – As/Ao) X 100

where Ao is the absorbance of the blank, and As is the absorbance of sample at 517nm.

IC_{50} which denotes the amount (mg) of seaweeds in 1 ml solution required to reduce initial concentration of DPPH radicals by 50% was also calculated. The result evaluated by comparing the IC_{50} of the ascorbic acid 2.4± 0.20, lower IC_{50} value indicate higher antioxidant activity.

Total antioxidant capacity (TAC) assay

Total antioxidant activity of seaweed chloroform extracts was determined according to (Ahmed et al., 2012). Twenty five mg of an extract was dissolved in 10ml of chloroform to prepare test samples. Phosphomolybdate reagent was prepared by mixing 0.6M sulfuric acid (100ml), 4mm ammonium molybdate (100ml) and 28mm sodium phosphate (100ml) solution. In a test tube, 3ml of phosphomolybdate reagent was mixed with 300µl of the test sample in a capped vials. The test tube was heated at 95°C in a water bath for 90min. Then the content of the test tube was allowed to cool down, and the absorbance was measured at spectrophotometer at 695nm against a blank. Ascorbic acid was used as a standard.

Cytotoxicity assay

Hepatocellular carcinoma (HepG2), Mammary gland (MCF7), Epithelioid Carcinoma (Hela) and Human prostate cancer (PC3) human tumor cell lines were obtained from ATCC via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt.

The inhibitory effects of seaweed extracts were determined on cell growth using the MTT assay (Klajnert et al., 2006). Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100units/ml penicillin and 100µg/ml streptomycin at 37°C in a 5% CO_2 incubator. The cell lines were seeded in a 96-well plate at a density of 1.0x 10^4cells/well. At 37°C for 48hrs under 5% CO_2. After incubation the cells were treated with different concentration of seaweed extracts and incubated for 24hrs. After incubation period, 20µl of MTT solution at 5mg/ml was added and incubated for 4hrs. Dimethyl sulfoxide (DMSO) in volume of 100µl is added into each well to dissolve the purple formazan formed. The colorimetric assay is measured and recorded at absorbance of 570nm using a plate reader (EXL 800, USA).

The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated sample) X 100. Doxorubicin was used as a standard anticancer drug for comparison.

* IC_{50} (µg/ml): 1-10 (very strong), 11-20 (strong), 21-50 (moderate), 51-100 (weak) and above 100 (non-cytotoxic).
*DOX (µM): Doxorubicin as standard.

Fractionation, purification and characterization of the antimicrobial crude extract of selected seaweeds

Colum chromatography

Active crude extract of the promising seaweed (5g) was purified and fractioned by column chromatography on silica gel G EDWC; 60-200 mesh (Solomon & Santhi, 2008). A glass column (3cm x 20cm) was packed by silica gel (30-40g) in pure methanol eluted with gradients of mobile phase from 3:7% methanol: chloroform to 7:3% methanol: chloroform and the collected

fractions (21 fractions) were evaporated at 40°C under vacuum. The dried fractions were dissolved again in chloroform solvent and its UV absorption was measured using UV spectrophotometer (UV 2101/pc) at 200–400nm wavelengths. Appearance of different peaks at certain wavelengths may indicate the structure of the antimicrobial compound. Antimicrobial activity of each peak fraction was estimated using disk diffusion method against the tested bacterial and fungal isolates.

Determination of the chemical structure of antimicrobial compounds of selected seaweeds

UV spectra

The ultraviolet absorption of the most active antimicrobial fractions of the two best of selected seaweeds extract, which were collected from column chromatography, were measured using UV spectrophotometer (UV 2101/ pc) at 200-900nm range of wavelength

The infra-red spectra (IR)

Seaweed extract fraction was analyzed by infra-red spectrophotometer in Central laboratory of Tanta University using Perkin Elmer 1430 FT-IR. About 5mg of the sample in solid phase were mixed with 200mg KBr (FT-IR grade) and pressed in to a pellet. The pellet was immediately put in to the sample holder and FT-IR spectra were measured in the range of 400-4000cm^{-1} (Boeriu et al., 2004).

GC-MS analysis was used to determine the active components of the active antimicrobial seaweed extract in Central laboratory of Tanta University using Perkin Elmer, Clarus 580/560 S model system with (30.0mx 250µm) column. The GC conditions were carried out using helium as carrier gas (0.8ml/min) and the GC oven temperature was programmed from 60°C to 250°C at a rate 2°C/min. (Sparkman et al., 2011).

Statistical analysis

The results were expressed as mean (±) standard deviation. Data was statistically analyzed using analysis of variance (ANOVA test by SPSS version 6) software**.**

Results

Identification of the seaweeds

Seaweeds were identified according to morphological description as *Enteromorpha intestinalis, Ulva lactuca, Ulva fasciata* (Chlorophyta), *Jania rubens* and *Gelidium spinosum (Rhodophyta).*

Antimicrobial assay

Seaweed extracts were tested for their antimicrobial activities. The highest inhibition zones were detected with *U. lactuca* and *U. fasciata* chloroform extracts. Chloroform extract of *U. lactuca* was more effective on *K. pneumoniae* than *P. mirabilis* while chloroform and ethanol extracts of *U. fasciata* were more effective on *P. mirabilis* than *K. pneumoniae*. In case of the tested pathogenic fungi, it was found that both chloroform and methanol *U. lactuca* extracts were also more effective on *A. niger* than the other two pathogenic fungal species. On the other hand ethyl acetate extract showed lowest inhibition zone with *P. mirabilis* and *A. flavus* (Table 1).

The minimum inhibitory concentration (MIC)

The MIC was carried out on the most potent antimicrobial seaweeds. The MIC of active compound of the *U. lactuca* and *U. fasciata* was estimated using microtiter plate technique. The lowest concentration of the antimicrobial agent, which did not show any visible growth was considered as the MIC. The results were illustrated in (Table 2).

The lowest MIC concentrations of *U. lactuca* and *U. fasciata* were 9±0.3 and 7±1.01mg/ml against *A. flavus.* While the highest MIC concentrations were 12±0.1mg/ml for *P. mirabilis*.

Antioxidant assays

DPPH free radical scavenging assay

The antioxidant activity of the five chloroform extracts was evaluated by DPPH radical scavenging activity comparing the IC_{50} of the ascorbic acid, which was 2.4±0.20mg/ml. *U. lactuca* and *Ulva fasciata* extracts exhibited the highest antioxidant activities and the highest IC_{50} (6.32±0.29 and 6.61±0.27mg/ml), respectively (Table 3).

Total antioxidant capacity

The *U. Lactuca* and *U. fasciata* recorded the highest antioxidant activity (2.13 and 1.51mg ascorbic acid equivalent/gram dry weight, respectively), then *E.intestinalis*, *J. rubens* and finally *G. spinosum* (Fig. 1).

TABLE 1. Antimicrobial activity of different seaweed extracts using disk diffusion method.

Algae	Solvent	Diameter of inhibition zone (mm)				
		Bacteria		Fungi		
		K. pneumoniae	***P. mirabilis***	***A. flavus***	***A. fumingatus***	***A.niger***
U. lactuca	Ethanol	NA	NA	NA	NA	NA
	Methanol	18±0.58aA	NA	14±1.00aB	10±1.00aD	16±1.00aE
	Acetone	NA	NA	NA	NA	NA
	Ethylacetate	NA	6±0.58bA	9±1.00aB	NA	NA
	Chloroform	19±1.00aB	17±0.58bA	16±1.00cA	16±1.00aA	20±1.00eA
	Hexane	NA	NA	NA	NA	NA
U. fasciata	Ethanol	10±0.00aA	15±0.58bB	10±0.58cC	15±1.00cC	20±1.00aA
	Methanol	NA	15±0.58bB	9±0.58cC	14±1.00cD	NA
	Acetone	NA	NA	13±0.58aC	5±1.00bA	17±1.00aB
	Ethylacetate	NA	NA	NA	NA	NA
	Chloroform	16±0.58aC	18±1.00bC	15±1.00aC	20±0.58bC	24±1.00bE
	Hexane	NA	NA	NA	NA	NA
E. intestinalis	Ethanol	NA	NA	15±1.00aB	NA	11±1.00
	Methanol	NA	NA	NA	NA	NA
	Acetone	NA	NA	NA	NA	NA
	Ethylacetate	NA	2±1.00aA	12±1.00aA	NA	10±1.00aA
	Chloroform	11±1.00aA	15±1.00bA	14±1.00aA	12±1.00bC	18±1.00bC
	Hexane	NA	NA	NA	NA	NA
J. rubens	Ethanol	NA	NA	11±1.00aA	NA	10±1.00aA
	Methanol	8±0.58aA	7±0.58bB	NA	1±1.00aA	9±1.00aA
	Acetone	NA	NA	NA	NA	NA
	Ethylacetate	NA	NA	NA	NA	NA
	Chloroform	12±1.00aA	11±1.00bB	12±1.00aA	10±1.00bC	13±1.00bB
	Hexane	NA	NA	NA	NA	NA
G. spinosum	Ethanol	10±0.00aA	NA	15±1.00aA	NA	NA
	Methanol	NA	NA	NA	NA	NA
	Acetone	NA	NA	NA	NA	NA
	Ethylacetate	9±0.00aA	9±1.00bB	6±0.00cC	NA	NA
	Chloroform	12±1.00aA	10±1.35bB	13±1.00cC	7±1.00bB	10±1.00dA
	Hexane	NA	NA	NA	NA	NA
Amoxicillin		15±0.00aB	7±0.00bC	-	-	-
Ketoconazole		-	-	8±0.4cA	16±0.00dA	10±0.00aE

- Each value are the mean of three replicates ± standard deviation, NA: No activity recorded.
- Difference in manuscript letters in each row or column indicate significance at P ≤0.05 level.
- Amoxicillin 25µg/ml, ketoconazole 10µg/ml were used as standard positive control for bacteria and fungi, respectively.

TABLE 2. Minimum inhibitory concentration (MIC) of chloroform extract of *U. lactuca* and *U. fasciata* against the tested human pathogenic bacterial and fungal species.

Seaweed chloroform extracts	MIC (mg/ml)				
	K. pneumoniae	***P. mirabilis***	***A. flavus***	***A. fumingatus***	***A. niger***
U. lactuca	11±0.1	12±0.1	9±0.3	10±0.2	11±0.1
U. fasciata	10±0.2	12±0.1	7±1.01	9±0.3	11±0.1

Each value are the mean of three replicates ± standard deviation.

TABLE 3. Antioxidant activity of the tested seaweed extracts by DPPH assay.

Seaweeds	DPPH radical scavenger %						IC_{50} (mg/ml)
	Chloroform concentration (mg/ml)						
	0	2	4	6	8	10	
U. lactuca	0.00±0.00	21.23±1.53	40.15±1.32	45.95±1.75	59.08±1.20	68.54±0.85	6.32±0.29
U. fasciata	0.00±0.00	20.34±1.35	36.49±1.4	47.72±0.85	60.72±0.62	68.03±1.53	6.61±0.27
E. intestinalis	0.00±0.00	11.89±0.95	27.53±0.90	36.37±1.35	53.52±1.0	63.49±0.70	7.46±0.34
J. rubens	0.00±0.00	4.83±1.10	22.61±1.00	32.96±0.90	47.22±1.45	59.58±0.90	8.43±0.39
G. spinosum	0.00±0.00	6.09±0.57	21.35±0.80	31.57±0.65	45.2±1.35	56.55±1.20	8.78±0.38

- Each value are the mean of three replicates ± standard deviation.
- The stock solution of seaweeds extract 10mg/ml.

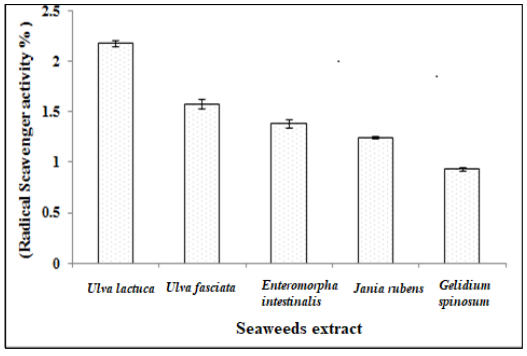

Fig. 1. Total antioxidant capacity (TAC) of tested seaweeds.

Cytotoxicity assay

Seaweeds extracts that showed highest antioxidant activity were selected for cytotoxic assay against four tested human tumor cell lines. *Ulva lactuca* extract showed very strong cytotoxic activity against MCF7 cell line (IC_{50} 10.83± 1.0µg/ml), strong cytotoxic activity against Hela (12.43±1.3µg/ml IC_{50}). *Ulva fasciata* showed strong cytotoxic activity against PC3 and HepG2 cell lines (IC_{50} 12.99± 1.2 and 16.75±1.5µg/ml), respectively. *Ulva lactuca* and *Ulva fasciata* extracts showed moderate activity against the 2 other cell *lines* (Table 4, Fig. 2).

Fractionation, purification and characterization of Ulva lactuca and Ulva fasciata extracts

Different fractions obtained by column chromatography were tested for antimicrobial activity. *U. lactuca* extract fraction No. 3 showed the highest antimicrobial activity against *K. pneumoniae* and *P. mirabilis* (16±0.02 and 18±0.05mm, respectively) and (18±0.00, 19±0.06 and 20±0.01mm) in case of *A. flavus*, *A. fumingatus* and *A. niger,* respectively. While fraction No.5 of *U. lactuca* extract showed the lowest antimicrobial activity (Table 5).

U. fasciata extract fraction No.4 has the highest antimicrobial activity against *K. pneumoniae and P. mirabilis* (15±0.11 and 16±0.02mm, respectively) and (17±0.1, 18±0.05 and 19±0.018mm) in case of *A. flavus, A. fumingatus and A. niger*, respectively. On the other hand, fraction No.5 showed the lowest antimicrobial activity (Table 6).

Determination of the chemical structure of U. lactuca and U. fasciata active antimicrobial compound

Ultra violet spectra analysis

The absorption peaks of the active two (Fr. 3, Fr. 4) fractions were of similar UV absorption at 245nm, suggesting an aromatic acid esters structure of *U. lactuca* and *U. fasciata* antimicrobial active agent (Fig. 3).

Fourier transform infra-red spectra (FTIR) spectra analysis

The infra-red absorption spectrum was used to identify the frequency of functional groups which found in the active compound of *U. lactuca* and *U. fasciata* chloroform extract.

TABLE 4. Cytotoxicity (IC_{50}) of the tested seaweed extracts on different cell lines.

Seaweed chloroform extracts	*In vitro* cytotoxicity IC_{50} (µg/ml)			
	HepG2	MCF7	PC3	Hela
Dox	4.50±0.2	4.17±0.2	8.87±0.6	5.57±0.4
U. lactuca	26.81±2.3	10.83±1.0	32.28±2.8	12.43±1.3
U. fasciata	16.75±1.5	29.71±2.5	12.99±1.2	35.18±3.1

Each value are the mean of three replicates ± standard deviation

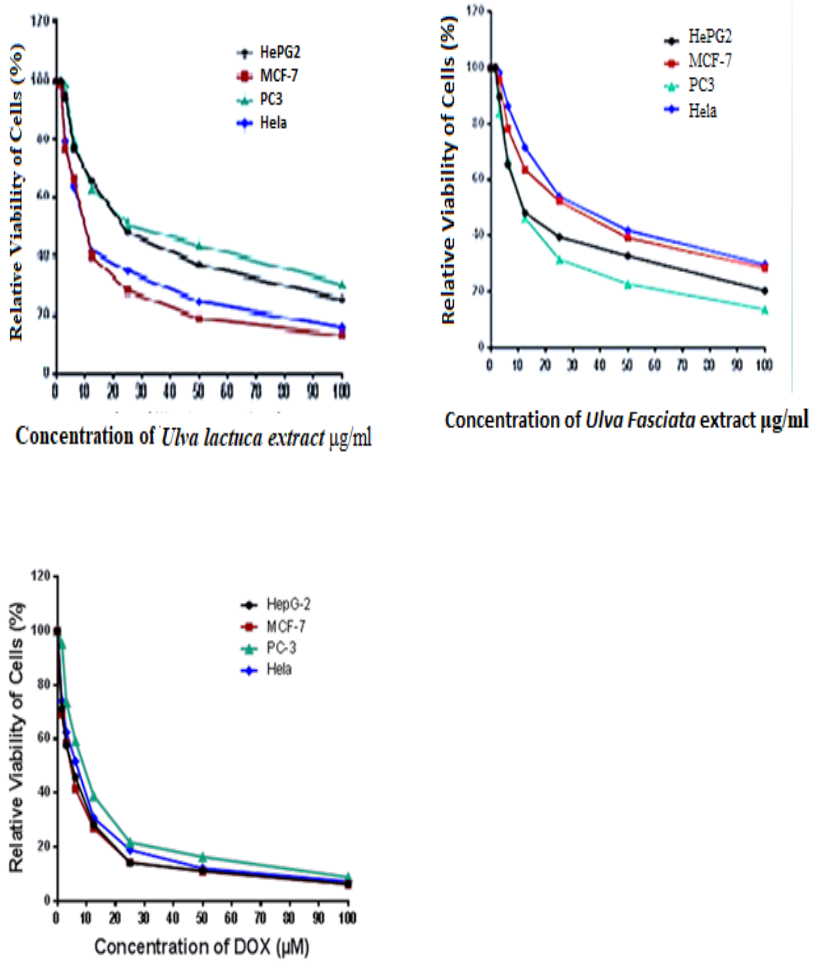

Fig. 2. Cytotoxicity assay of the selected seaweed extracts on different cell lines by MTT method.

TABLE 5. Antimicrobial activity of *U. lactuca* fractions obtained from the silica gel column chromatography against tested bacterial and fungal pathogens.

Bacterial and fungal species	Diameter of inhibition zone (mm)							
	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 8	Amoxicillin	Ketoconazole	Negative control
K. pneumoniae	12±0.1	16±0.02	8±0.01	6±0.1	13±0.5	14±0.12	-	-
P. mirabilis	11±0.7	18±0.05	9±0.00	7±0.01	14±0.3	17±0.00	-	-
A. flavus	14±0.3	18±0.00	10±0.05	8±1.7	15±0.02	-	13±0.30	-
A. fumingatus	11±0.7	19±0.06	12±0.01	11±0.1	14±0.6	-	15±0.58	-
A. niger	13±0.6	20±0.01	11±0.7	9±0.00	16±0.8	-	19±0.42	-

- Each value are the mean of three replicates ± standard deviation.
- Amoxicillin 25µg/ml, ketoconazole 10µg/ml were used as standard positive control for bacteria and fungi, respectively and solvent as negative control.

TABLE 6. Antimicrobial activity of *U. fasciata* fractions obtained from the silica gel column chromatography against tested bacterial and fungal pathogens.

Bacterial and fungal species	Diameter of inhibition zone (mm)							
	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Amoxicillin	Ketoconazole	Negative control
K. pneumoniae	9±0.1	15±0.11	10±0.1	8±0.00	12±0.02	14±0.12	-	-
P. mirabilis	11±0.2	16±0.02	12±0.01	9±0.01	13±0.01	17±0.00	-	-
A. flavus	12±0.11	17±0.1	8±1.7	14±0.4	15±0.3	-	13±0.30	-
A. fumingatus	10±0.1	18±0.05	9±0.01	13±0.2	14±0.00	-	15±0.58	-
A. niger	11±0.3	19±0.018	7±0.1	15±0.01	16±0.5	-	19±0.42	-

- Each value are the mean of three replicates ± standard deviation.
- Amoxicillin 25µg/ml, ketoconazole 10µg/ml were used as standard positive control for bacteria and fungi, respectively. and solvent as negative control.

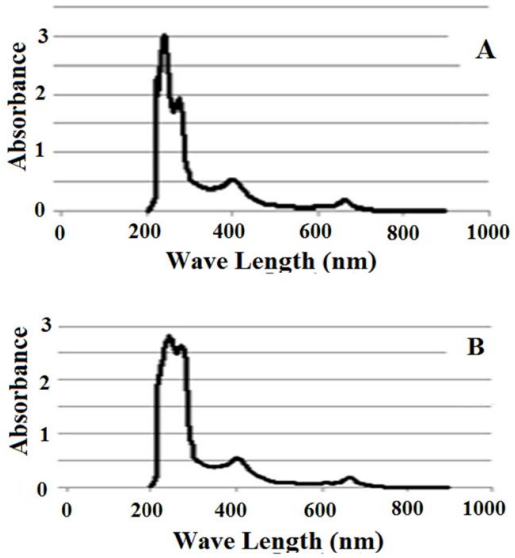

Fig. 3. The UV spectrophotometer active fraction of *Ulva lactuca* (A) and *Ulva fasciata* (B) crude extract resulted from column chromatography [A, fraction 3; B, fraction 4].

The highest stretching broad band (υ) of OH group was positioned at 3450 cm^{-1}. The υ of CH aliphatic (CH, CH2 and CH3) sharp band was at 2861.84cm^{-1}. A sharp band of C=O was positioned at 1730.8cm^{-1}.

A strong sharp (υ) of C=C and C-O were positioned at 1461.78cm^{-1} and 1277.6cm^{-1}, respectively. A band (υ) of ortho position, aromatic phenyl ring (CH, CH2 and CH3) was at 785.85cm^{-1} (Fig. 4 A, B).

Gas chromatography mass spectrometry

The GC-MS chromatogram of both *U. lactuca* and *U. fasciata* chloroform antimicrobial compounds indicated the presence of 3 peaks with different retention times and peak relative intensities. All peak areas of the chromatogram were relatively small except the parent peak at retention time of 36.357 and 13.038min which was corresponding to 100 and 27.38% relative intensity for *U. lactuca* and *U. fasciata, respectively.* It was identified according to its molecular mass to be an aromatic ester derivative compound Di-isooctyl phthalate (390.56g/mol) and butylated Hydroxytoulene (220.356g/mol) for *U. lactuca* and *U. fasciata*, respectively (Tables 7, 8 and Figs. 5 A, B).

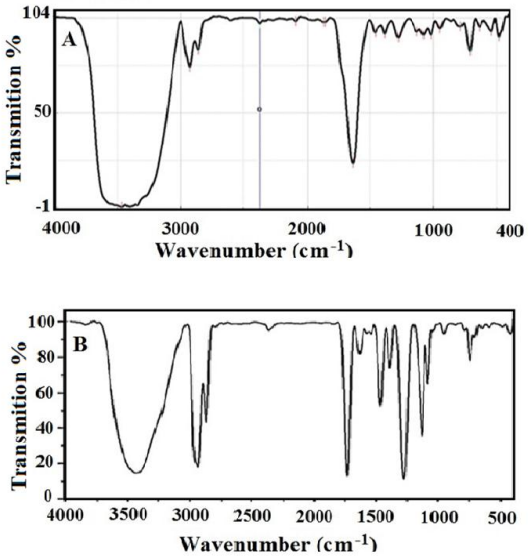

Fig. 4. FTIR spectra of antimicrobial compound active fraction of *Ulva lactuca* (A) and *Ulva fasciata* (B) [A, fraction 3; B, fraction 4].

TABLE 7. GC-MS analysis of *U. lactuca* antimicrobial compounds.

Retention Time (min)	Peak area%	Compound name	Molecular structure	Molecular weight	Norm %
5.775	0.479	2-Allyl-2-methyl-1,3-cyclopentanedione	$C_9H_{12}O_2$	152.1	0.80
17.314	5.151	Cyclododecanemethanol	$C_{13}H_{36}O$	198.3	8.62
36.357	59.725	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390.56	100.00

TABLE 8. GC-MS analysis of *U. fasciata* antimicrobial compounds.

Retention Time (min)	Peak Area%	Compound name	Molecular structure	Molecular weight	Norm %
13.038	16.523	Butylated Hydroxytoluene	$C_{16}H_{26}O_3$	220.356	27.38
19.48	1.120	Nonadecene	$C_{19}H_{38}$	266.5	1.86
29.2	1.062	Tetradecanol	$C_{14}H_{30}O$	214.3	1.76

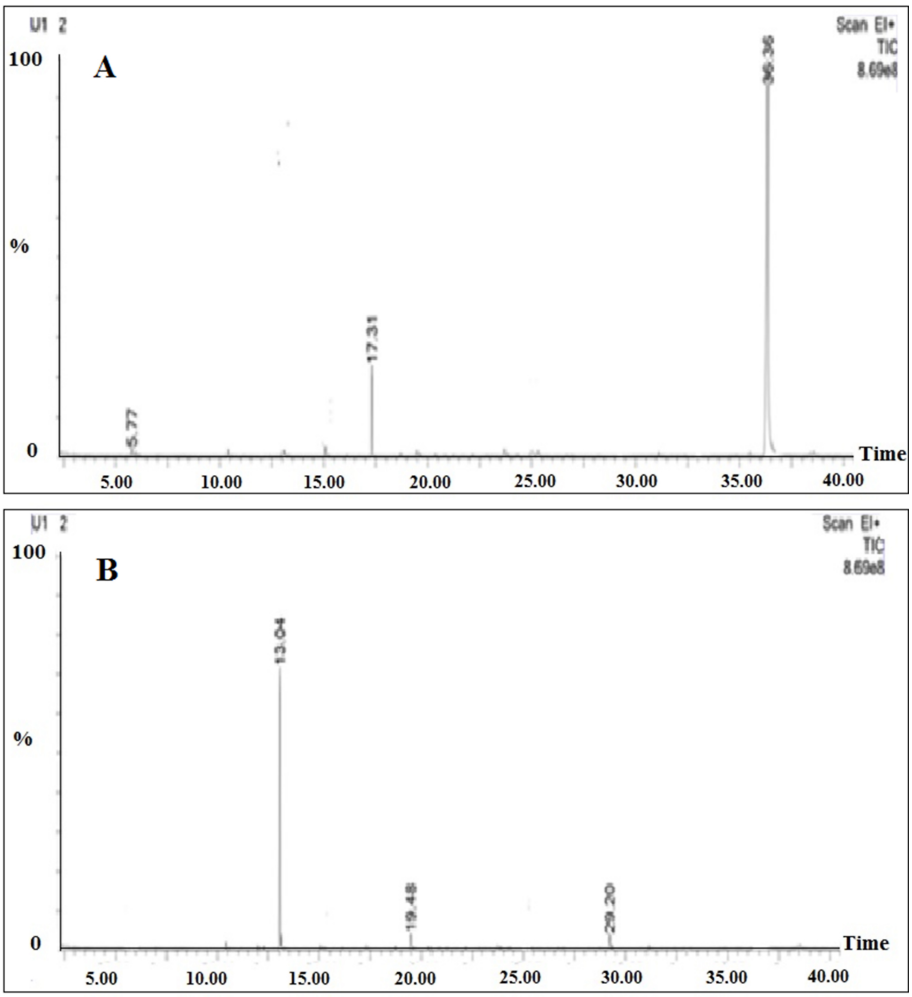

Fig.5. The GC-MS chromatogram of the antimicrobial compound active fraction of *Ulva lactuca* (A) and *Ulva fasciata* (B) [A, fraction 3; B, fraction 4].

Discussion

Seaweeds are rich in many antimicrobial compounds (Bhagavathy et al., 2011) and therefore nowadays become a cheap and safe source for these compounds in many medicinal and pharmacological applications (Adaikalaraj et al., 2012). However, the antimicrobial activity of

seaweeds may be influenced by some factors, such as habitat, season of algal collection, alga growth stages and experimental methods. Although a variety of solvents have been employed by many researchers to screen seaweeds for antimicrobial activity, it is still uncertain what kind of solvent is the most effective and suitable for extraction of seaweeds for antimicrobial applications.

The applied chloroform extracts of the studied seaweeds were significant if compared with the other applied extracts, having the strongest antimicrobial activity against both of the tested bacterial and fungal species using disk diffusion method. These results were consistent with those of Sastry & Rao (1994) and Val et al. (2001) as the chloroform extract is more effective than methanol in extracting antimicrobial compounds. However, it was found that the antimicrobial activity was different among the applied *U. lactuca* and *U. fasciata* chloroform extracts. The highest inhibition zones against *K. pneumonia* and *A. niger* were obtained using the *U. lactuca* extracts, while the highest zones against *P. mirabilis* and *A. niger* were obtained using the *U. fasciata* chloroform extracts.

In contrary to the significance effect of the chloroform extracts of the studied seaweeds, many researchers reported other extracts is more effective against microbes. Karthikaidevi et al. (2009) reported that the ethanolic extracts of green algae like *Codium adherens, Ulva reticulate and Halimeda macroloba* showed high antibacterial activity against *Staphylococcus sp., Propionibacterium acnes and Proteus mirabilis.* The ethanolic extract of the green seaweed *Caulerpa sertularioides* showed the maximum antibacterial activity against *Bacillus subtilis, E. coli and Proteus mirabilis* (Pushparaj et al., 2014). Also, the ethanolic extracts from seaweeds of *Sargassum binderi, Amphiroa sp. and Turbinaria conoides have* the strongest antibacterial activities *against the test organisms Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium acnes, and Proteus mirabilis and* antifungal *activity against Candida albicans* (Boonchum et al., 2011). The methanolic extract of *Enteromorpha flexuosa* (green seaweeds) exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria of *Staphylococcus aureus*, *Bacillus subtilis, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Salmonella typhimurium* (Senthilkumar et al., 2014). On the other hand, Osman et al. (2010) estimated that the most active seaweed extract against *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli, Salmonella typhi and Klebsiella pneumoniae* and the fungal species *Candida albicans* was the acetone extract of *Ulva fasciata* (Chlorophyceae).

The antimicrobial activity of the tested seaweeds may be attributed to the cytotoxic activity through damaging the plasma membrane of the microbial cell, leading to the leakage of intracellular K, change in the membrane permeability and disruption of the stressed cells (Ramsewak et al., 2001; Smit, 2004). Also, damaging the plasma membrane of the microbial cell may be due to the complete collapse of the microbial cell and the growth inhibition (Giamarellou, 2000). Fareed & Khairy (2008) explained that *U lactuca* possesses proteins or peptides responsible for the antibacterial activity, which function by binding to the microbial cell membrane and once embedded, forming pore like membrane defects that allow efflux of essential oils and nutrients from the bacteria. *Ulva lactuca* was reported to contain uniform distribution of antibiotics (Hornsey & Hide, 1974).

Antioxidant substances in seaweeds contribute to the endogenous defense mechanism against external stressful conditions (Ratnayake et al., 2013). The antioxidant activity of the studied five chloroform extracts, *U. lactuca* and *U. fasciata* extracts showed the highest ones. Antioxidant activity among the studied five seaweeds is arranged in decreasing order as of *U. lactuca*, *U. fasciata*, *E. intestinalis*, *J. rubens* and *G. spinosum*. Farasat et al. (2014) detected that *Ulva clathrate* exhibited high antioxidant activity with low IC_{50} followed by *U. intestinalis, U. linza* and *U. flexuosa*. The antioxidant activity in algae acts via several processes and compounds, such as lipophilic scavengers (carotenoids), enzymatic scavengers (catalase, superoxide dismutase and peroxidase), and polyphenols (Heim et al, 2002; Mittler, 2002). The antioxidant properties of these compounds mainly depend on the structure that signifies the geometric arrangement, number and positions of hydroxyl moieties, and the substitution of aromatic rings (Balasundram et al., 2006).

The studied seaweed extracts exhibited variable degrees of inhibitory activity toward

the four tested human tumor cell lines. Strong cytotoxic activity against MCF-7 and Hela cell lines was given by *U. lactuca* extract, while the strong cytotoxic activity against PC3 and HePG2 cell lines was given by *U. fasciata*. This was in accordance with Mashjoor et al. (2016) and Deviyani et al. (2018) who reported cytotoxic potential and activity of the brown seaweed *Turbinaria deccurrens* Bory and marine macro algal extracts against three cell lines including MCF7, HeLa and Vero. Also, seaweed extracts of *Palmaria palmate* were shown to be effective antioxidants, capable of inhibiting cancer cell proliferation. The alcoholic extract of the red alga *Acanthophora spicifera* has tumoricidal activity on Ehrlich's ascites carcinoma cells developed in mice (Vasanthi et al., 2004).

Several studies have attributed the anti-proliferative activity of seaweed extracts in cancer cell lines to their antioxidant activity. Water-soluble polysaccharides, such as laminarans, fucoidans, carragenans, and agar, are representative anticancer substances extracted from seaweeds (Gamal-Eldeen et al., 2009; Gheda et al., 2018). Enzymatic and polysaccharides extracts from brown seaweeds strongly showed antioxidant potential with dose-dependent radical scavenging activities (Heo et al., 2005) and suppressed the in vitro proliferation of selected cancer cell lines (Athukorala et al., 2006). In addition, phenolic compounds mostly found in seaweeds are reported to have several biological effects including antioxidant, antiapoptosis, anti-aging and anticarcinogenic properties and have important roles as antioxidants and chemoprotective agents (Namvar et al., 2014). Gawron & Kruk (1992) showed that phenolics reduce the amount of cellular protein and mitotic index, as well as colony formation during cell proliferation of cancer cells. Polyphenolic compounds inhibit cancer cells by xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens, while some flavonoids can also alter hormone production and inhibit aromatase to prevent the development of cancer cells (Zhao et al., 2007). The mechanism of anticancer activity of phenolics may also occur by disturbing cellular division during mitosis at the telophase stage.

The GC-MS chromatogram of *U. lactuca* chloroform antimicrobial compound indicated the presence of di-isooctyl phthalate as the major compound . In accordance with the obtained results, Sastry & Rao (1995) reported that dioctyl phthalate is a derived bioactive molecule from chloroform-methanol (2:1) extracts of *Saragassum wightii* with significant antimicrobial activities against *Staphylococcus aureus, Proteus vulgaris, E. coli, Salmonella typhi, Salmonella paratyphi, Salmonella typhiridium, Klebsiella pneumoniae, Shigella sonnie, Vibrio cholerae and Pseudomonas aeruginosa.* Phthalate esters found in aquatic organisms and can be potentially bio-accumulated due to their lipophilicity (Mackintosh et al., 2004). The biosynthesis of di-(2-ethylhexyl)-phthalate (DEHP) and di-n-butyl phthalate (DBP) have been reported by Chen (2004) in the red alga *Bangia atropurpurea*.

On the other hand, the GC-MS analysis of *U. fasciata* chloroform extract proves the presence of butylated hydroxy toluene phenol as a major component. It may be responsible the growth inhibition of the pathogenic bacteria and fungi due to its antimicrobial, antioxidant and anti-inflammatory activities (Mujeeb et al., 2014) and hence may be useful for curing diseases arising from oxidative deterioration (Kumar et al., 2008). Butylated Hydroxytoluene (BHT) is an organic chemical composed of 4-methylphenol modified with tert-butyl groups at positions 2 and 6. BHT is a lipophilic (fat-soluble) organic compound and inhibits autoxidation of unsaturated organic compounds. Therefore it is used in food (additive; E number E321), cosmetics, pharmaceuticals and industrial fluids to prevent oxidation and free radical formation.

Several studies are in accordance with the studied work. Phenolic compounds are commonly found in seaweeds and have been reported to have several biological activities including antimicrobial and antioxidant activity (Yuan et al., 2005). Srivastava et al. (2010) reported that the extracts of seaweeds contained phenolics, alkaloids and amino acids, which may be responsible for the antimicrobial activity. The bioactive secondary metabolites of concentrated extract of marine brown algae *Turbinaria conoides* were characterized by using gas chromatography and mass spectrometry (GC-MS) and demonstrated the presence of compounds with antimicrobial, antiphrastic, antitumor and anticancer properties (Kalaivani et al., 2016). Moreover, El- Shouny et al. (2017) concluded that *U. lactuca* green alga contain high content of phenolic compounds as analysed by GC/MS.

Conclusion

The study indicated that chloroform extracts of *U. lactuca, U. fasciata* showed antimicrobial activity and exhibited the highest antioxidant activity among other tested seaweeds. *U. lactuca* extract had strong cytotoxic activity against MCF-7, Hela cell lines. On the other hand, *U. fasciata* had strong cytotoxic activity against PC3 and HepG2 cell lines. The GC-MS chromatogram of chloroform extracts of *U. lactuca* and *U. fasciata* antimicrobial compounds indicated the presence of di-isooctyl phthalate and butylated hydroxy toluene phenol, respectively. These phenolic derivatives were the major constitutes that responsible for the antimicrobial against tested pathogens. Seaweeds are a promising candidate for further drug and pharmacological studies.

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التقييم المعملى للانشطة المضادة للميكروبات ومضادات الأكسدة ومضادات الأورام لبعض الطحالب البحرية

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في الدراسة الحالية تم تقييم مستخلصات الإيثانول والميثانول والإيثيل استات و الهكسان والكلوروفورم والأسيتون من أربع أنواع من الطحالب البحرية الخضراء والحمراء المجمعة من منطقة أبو قير بالإسكندرية؛ مصدر كمضادات للميكروبات ومضادات للإكسدة والسرطان ضد البعض أنواع من الخلايا. مستخلص الكلوروفورم الطحلبي الأولفا لاكتيوكا والأولفا فاسيتا أعطي أحسن تثبيط ضد أنواع البكتريا المختبرة (كلابسيلا بنوميا وبروتيس ميربيلس) وأيضا (فطر الأسبرجلس فلاقس والأسبرجلس فيمنجاتس والأسبرجلس نيجر) وذلك بطريقة disc diffusion. مستخلص الأولفا لاكتيوكا والألفا فاسيتا أعطت أحسن نشاط مضاد الأكسدة (IC_{50}) (6.32±0.29mg/ml) و (6.61±0.27 mg/ml) بالترتيب. بأستخدام طريقة DPPH وطريقة Total antioxidant capacity (2.13, 1.51 مليجرام اسكوربيك / جرام من الوزن الجاف بالترتيب).أختبار السمية بأستخدام طريقة MTT أوضح أن الاولفا لاكتيوكا لها نشاط قوي على خلايا السرطان الثدي MCF-7 وخلايا Hela cell lines (IC_{50} 10.83±1.0 µg/ml) و (12.43±1.3 µg/ml) بالترتيب بينما الأولفا فاسيتا أعطت نشاط قوي ضد خلايا، PC3، HePG2 (IC50 12.99±1.2µg/ml) (16.75±1.5µg/ml) بالترتيب. وبالتحليل بإستخدام الأشعة الفوق بنفسجية UV spectra والأشعة تحت الحمراء (FT/IR) وبتحليل كروماتوجرافيا الغاز(GC/MS) بعد التنقية Column chromatography عمود الفصل الكروماتوجرافي، أتضح أن معظم المواد المضادة للميكروبات النشطة في الأولفا لاكتيوكا والأولفا فاسيتا تشمل مركبات أروماتية لها مجموعات نشطة مثل C=O، حلقة فينيل ومجموعة OH)، والوزن الجزيئي لهذه المركبات Di -isooctyl phthalate هو 390.56 جرام / مول Butylated hydroxy toluene هو 220.356 جرام/مول. والأستنتاج من الدراسة أن الطحالب البحرية محل الدراسة تحتوى على العديد من المركبات النشطة التى تعتبر كمصدر مضاد للميكروبات ولها نشاط مضاد للأورام فمن الممكن أستخدامها في الصناعات الدوائية .