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ANTIBACTERIAL POTENTIAL OF MACRO AND MICROALGAE EXTRACTS AGAINST PATHOGENS RELEVANT TO HUMAN HEALTH

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Abstract

The rising need to use natural resources for the production of innovative solutions is increased dramatically. Among these natural resources is algae which is one of the main sources of bioactive compounds. The potential of algae being antimicrobial depends on three factors including its type to be effective against pathogenic bacteria; the solvent used during the process of bioactive compounds extraction; and the concentration of each extract. The biochemical composition of three marine algae *Ulva* sp. (Chlorophyceae), *Sargassum* sp. and *Hormophysa* sp. (Phaeophyceae) Red Sea, Egypt in comparison to three fresh water blue green alga *Spirulina* sp., green alga *Chlorella* sp. and *Scenedesmus* sp. were screened for antibacterial activity against bacterial pathogens with relevance for aquaculture and human health in comparison to antibiotic susceptibility, in addition to the determination of minimum inhibitory concentration (MIC). The fresh water blue green algae *Spirulina* sp. that represent prokaryotes and the green algae *Scenedesmus* sp. that represent eukaryotes were selected to be identified molecularlyand the obtained accession number for *Spirulina* and *Scenedesmus* were MT897876 and MT900569, respectively. Moreover, Anti-inflammatory study by human red blood cell (HRBC) membrane stabilization method and the *In vitro* cytotoxicity assay were also determined. The highest ZIsagainst Gram positive bacteria (37 mm) was recorded for ethanol *Scenedesmus* sp. extract; followed by ethanol *Scenedesmus* sp. (28 mm). Whereas, for Gram-negative bacteria the highest ZIsvalue was found to be 29 mm with butanol *Scenedesmus* sp. extract; followed by chloroform *Ulva* sp. extract (28mm). The cytotoxicity of *Spirulina* sp. ethanol extract against HCT116 and MCF7 cell lines showed that, small amounts, 26.2 and 43.6 µg/ml were required to inhibit 50% (IC_{50}) of HCT116 and MCF7 cell lines, correspondingly. Whereas, higher concentrations of *Scenedesmus* sp. ethanol extract 58 and 87.7.6 µg/ml were required to inhibit 50% (IC_{50}) of HCT116 and MCF7 cell lines, respectively. This study revealed that algal bioactive compounds have potentials as antimicrobials and anticancer.

Key words: Antibacterial - fresh water algae - marine algae - cytotoxicity - anti-inflammatory

Introduction

Microorganisms have developed adaptation mechanisms against the action of antimicrobial drugs. The increasing resistance of microorganisms to antimicrobial drugs in use has attracted the attention of the scientific community (Höfling *et al.*, 2010). Tackling antibiotic resistance is a high priority for WHO. A global action plan on antimicrobial resistance, including antibiotic resistance was approved at the World Health Assembly in May 2015. The global action plan aims toensure prevention and treatment of infectious diseases with safe and effective medicines. Nowadays, the use of antibiotics has increased significantly due to heavy infections and the pathogenic bacteria becoming resistant to drugs are common due to indiscriminate use of antibiotic (Usharani *et al.,* 2015). Recently, new mechanisms of resistance have resulted in the simultaneous development of resistance to several antibiotic classes creating very dangerous multidrug resistant (MDR) bacterial strains, some also known as ‘‘superbugs”. The required number of new antimicrobial agents is higher than ever due to the rapid presence of new infections, emergence of multidrug resistance in common pathogens. To overcome the resistance of bacterial pathogens, continuous screening and development for new and safe antibacterial agents are necessary. Natural products from algae are diverse source of bioactive compounds due to the harsh environmental conditions in which these organisms survive (Bernhoft, 2010).

Algae are photosynthetic organisms that can be a source of natural compounds with interesting biological activity. Seaweeds represent a potential source of antimicrobial substances due to their diversity of secondary metabolites with antiviral, antibacterial and

antifungal activities (Bhoumick *et al.,* 2020). However, the search for similar antimicrobial activity in other algal species such as microalgae has gained important concern regarding the antibiotic resistant microorganisms (Jaki *et al.,* 1999). Compounds with cytostatic, antiviral, antihelminthic, antifungal and antibacterial activities have been detected in green, brown and red algae (Newman *et al.,* 2003). It contains protein, iodine, bromine, vitamins and substances of stimulatory and antibiotic nature. The phytochemicals produced by fresh and marine algae are extensively used in various industries such as food, confectionary, textile and pharmaceutical. Moreover, seaweeds are good sources of proteins, polysaccharides and fibers. Brown seaweeds are known to contain proteins forming 5 to 10% of dry weight. Green algae such as Ulva contain 18 to 26% proteins. Presence of these various compounds makes the seaweeds valuable in pharmaceutical industries, where they have been largely screened in drug development for antibacterial, antifungal antiviral and antitumor activities (Kolanjinathan and Stella 2009).

A promising strategy for the replacement of antibacterial and antifungal chemicals is to promote the natural biological control products obtained from microalgae. Microalgae is a rich source of novel bioactive compounds such as phytochemicals namely carotenoids, phenolic, amino acids, polyunsaturated fatty acids and sulphated polysaccharides. These compounds are providing excellent biological actions including, antioxidant, antimicrobial, antiviral, antitumor, anti-inflammatory and anti-allergy effects. However, some microalgae species such as *Chlorella* and *Spirulina* species have been used in several areas in nutraceutical, pharmaceutical and cosmetics (Chandrasekaran *et al.,* 2014).

One approach used to identify algae across all forms of life is DNA barcodes. DNA barcode is a tool that can be used to quickly identify species (Shen *et al*., 2013). DNA barcodes that are often used in plants generally are chloroplast DNA (cpDNA). The DNA sequence that has the opportunity to be used as a DNA barcode is the rbcL gene. The rbcL gene is about 1400 bp long so it provides many characters for phylogenetic studies (CBOL, 2009). The role of the *rbcL* gene that encodes the RuBisCO protein is thought to cause this gene sequence to have a low mutation level compared to other barcode genes in cpDNA so that the level of similarity between species is quite high (Asahina *et al*., 2010). Several gene loci, e.g. *rbcL*, ITS and *tufA*, have been recommend as the promising DNA barcodes for green algae (John *et al*., 2010).

This study aimed to estimate the biochemical composition and the antibacterial potential of three marine algae *Ulva* sp. (Chlorophyceae), *Sargassum* sp. and *Hormophysa* sp. (Phaeophyceae) Red Sea, Egypt in comparison to three fresh water blue green alga *Spirulina* sp., green alga *Chlorella* sp. and *Scenedesmus* sp. to evaluate the antibacterial activity of these algae against seven human pathogen bacteria.

Materials and Methods

Fresh water algae and culture conditions

The blue green alga *Spirulina* sp., green alga *Chlorella* sp. and *Scenedesmus* sp. were obtained from Hydrobiology Lab, Qanater Khayria, Qalubia, Egypt. Both *Chlorella* sp. and *Scenedesmus* sp. were cultivated on the BG-11 medium (Stanier *et al.,* 1971) while *Spirulina* sp. was cultivatedon Zarrouk's medium (Zarrouk, 1966). All cultures were kept under fluorescent light with 16 h light period at 25±2°C. The body mass of growth of all microalgae were collected by centrifuge (at 6000 rpm, 10 min). The supernatant of each microalga was discarded and then the wet biomass was frozen for 12h to detect pigments.

Marine algae collection

The marine seaweeds *Ulva* sp. (Chlorophyceae), *Sargassum* sp. and *Hormophysa* sp. (Phaeophyceae) were collected from the intertidal zone of the Gulf of Suez during summer. *Ulva* sp. from Ras El-adabiya, *Sargassum* sp. and *Hormophysa* sp. were collected from Ras Sedr. Ras El-adabiya is located on the western shore of Suez Bay (10 kilometers south of Suez city) and Ras Sedr at Lat. 29° 37' N; Long 32° 41' E.

The seaweeds were handpicked and washed thoroughly with sea water to remove all the unwanted impurities, epiphytes and adhering sand particles. Samples then washed thoroughly using tap water to remove salt on the surface of the sample and air dried (26°-30°C) with indirect light during 10 days followed by oven drying for 48 h at 50°C (Sivasankari *et al.,* 2006). Dried seaweeds were crushed and powdered with Mixer grinder. The powdered samples were then stored in refrigerator at 4°C.

Algal biochemical analysis

Quantitative analysis of biochemical in the fresh and marine algae were estimated. Pigments, Chlorophyll a & b and Carotenoids, were estimated by spectrophotometer according to APHA (1995). Total protein content was determined by Bradford (1976). Total carbohydrate content and total soluble lipid content was determined by method by Van Handel (1985). Phenol contents were determined according to Singleton *et al.,* (1999). The

composition of elements such as nitrogen, phosphate, potassium, calcium, magnesium, sodium, manganese, copper, zinc and iron were estimated using atomic absorption spectrophotometer Humpshires (1956), amino acid was detected as George (2019).

Preparation of algal crude extracts

Tengram of dried algal samples (fresh or marine algae) were placed in bottles separately with 100 mlusing different solvents (Methanol, Ethanol, Acetone, Chloroform, Butanol and Ether) for extraction. These bottles were covered with aluminum foils and were kept at room temperature in a shaker for two days, filtered and the solvent were evaporated and the dried extracts were stored at 4°C for antibacterial assay (Val *et al.,* 2001).

Antimicrobial activity

The antimicrobial activity of algal crude extracts were assayed against eight species of pathogenic bacteria, 3 Gram-positive bacteria *Bacillus megaterium* BMS4, *B. amyloliquefaciens* subsp. *plantarum* SA5 and *B. subtilis* subsp. *subtilis* BTN7A (with accession number, KC429572, KC438369 and KC438368 respectively) and five Gram negative bacteria *Salmonella enterica*, *Escherichia coli 0157:H7*, *Escherichia coli 8739, Pseudomonas aeruginosa 6538* and *Klebsiella pneumoniae MG461522*. Bacterial inoculum was prepared from the 24 h LB culture (Bertani,1952) of a loop full was inoculated in a tube containing 5 ml of LB broth, incubated at 37°C for 3-5 hours until it achieves the turbidity of OD at 620 = 0.1 (10^6CFU/ml) and then used for the determination of antibacterial activity.

Antibiotic susceptibility test

The antimicrobial susceptibility of the collected bacteria was assessed using the modified Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2012). Antibiotics used were Chloramphenicol (C, 30 mcg), Rifampin (RA, 5mcg), Streptomycin (S, 10 mcg), Neomycin (N, 30 mcg), Kanamycin (K, 30 mcg), Ampicillin (AMP, 10 mcg), Tetracycline (TE, 30 mcg) and Amikacin (AK, 30 mcg).

Agar plates were uniformly inoculated with the selected bacteria. The antibiotic disks were and incubated at 37°C for 18 h. Multiple antibiotic resistances (MAR) index was calculated for each tested bacteria by dividing the number of antibiotics to which the isolate is resistant by the total number of antibiotics tested (Olayinka *et al.,* 2009).

Antibacterial activity assay

Antibacterial activity of 6 algal crude extracts (100 mg/ml) were examined against all the tested bacterial species using well diffusion agar method (Stepanovi *et al.,* 2003). The plates were left for 10 to 20 min then 50 µl of each algal extract were dispensed into each well, separately. Dimethyl sulfoxide (DMSO) represented as a negative control. Inoculated plates were incubated at 37°C for 24h (Priadarshini *et al.,* 2013). The zonesofinhibition (ZIs) were measured.

Determination of minimum inhibitory concentration (MIC)

The determination of MIC was conducted using tube dilution method (Wiegand *et al.,* 2008). A 24 h culture of the tested bacterial species was diluted in 10 ml of L.B broth until it achieves the turbidity of OD620 = 0.1 (10^6 CFU/ml). Culture tubes containing nine different concentrations of each algal solvent extract (250, 125, 62.5, 31.25, 1.0, 0.75, 0.50, 0.25, 0.1 mg/ml in DMSO) were prepared independently. Each tube was inoculated with 100 µl of bacterial cell suspension and incubated at 37°C for 24 h. The growth of the inoculum in broth is indicated by turbidity of the broth and the lowest concentration of extract that inhibited the growth of the tested organism was determined as the minimum inhibitory concentration (MIC).

Molecular identification of *Scenedesmus* and *Spirulina*

Based on the best algal extract antibacterial activity, *Scenedesmus* sp.and *Spirulina* sp. were selected to be identified molecularly.

***rbcL* barcoding analysis:**

Amplification and sequence analysis of *rbcl* genome regions

a) PCR Reactions:

DNA barcoding analysis was performed with the plastidial *rbcL* region. For PCR amplification and sequencing of *rbcL*, the reaction mixture consisted of 1x buffer (Promega), 15mM$MgCl_2$, 0.2 mM dNTPs, 20pcoml of each primers, 1uL of Taq DNA polymerase (GoTaq, Promega), 40 ng DNA and ultra-pure water to a final volume of 50 uL.

Primer Code	Sequence	Product Size
rbcL-F	5'-ATGTCACCACAAA CAGAGACTAAAGC-3'	600bp
rbcL-R	5'-TCGCATGTAC CTGCAGTAGC-3'	

b) Thermo-cycling PCR program

PCR amplification was performed in a Perkin-Elmer/ GeneAmp® PCR System 9700 (PE Applied Biosystems)

programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 30 sec., an annealing step at 50ºC for 30 sec. and an elongation step at 72ºC for 30 sec. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

c) Detection of the PCR Products

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X Trace base (TBE) buffer at 95 volts. A 100bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000).

Purification of PCR Products

Amplified products for all PCR were purified using EZ-10 spin column PCR products purification PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes after that centrifuge, 750 ul of wash solution was added to the column and centrifuge at 10.000rpm for two minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and adds 50 ul of elution buffer, incubated at room temperature for 2 minutes and when store purified DNA at -20°C.

***rbc*L sequencing analysis**

The sequencing of the product PCR was carried through in an automatic sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using *rbcL* forward primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Microgen Company).

Computational analysis (BLASTn) *rbcL.*

The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST.

Phylogenetic analysis:

The alignment sequences of *Scenedesmus* and *Spirulina* samples were trimmed online using FaBox (Villesen, 2007) and then neighbor-joining (NJ) phylogenetic trees for each gene were constructed using the MEGA 7 program (Kumar *et al*., 2016) and 10000 bootstrap repeats, "Partial Deletion" for the Gaps/Missing Data Treatment parameter and the Jukes-Cantor nucleotide substitution model.

Anti-inflammatory study by human red blood cell (HRBC) membrane stabilization method

Membrane stabilizing activity of the samples was conducted and assessed by Regional Center for Mycology and biotechnology, Al-Azhar University, Cairo, Egypt using hypotonic solution-induced erythrocyte hemolysis The tested sample consisted of stock erythrocyte (RBCs) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (1000-7.81 µg/ml) or indomethacin. The control sample consisted of 0.5 ml of RBC mixed with hypotonic-buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g. In 96 well plates, the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis or membrane stabilization was calculated according to modified method described by Shinde *et al.*, (1999).

% Inhibition of hemolysis (membrane stabilization%) = 100 × {OD1-OD2/OD1}

Where: OD1 = Optical density of hypotonic-buffered saline solution alone

OD2 = Optical density of test sample in hypotonic solution.

The IC_{50} value was defined as the concentration of the sample to inhibit 50% RBCs hemolysis under the assay conditions. All determinations were carried out in triplicate manner and values are expressed as the mean ± SD. The IC_{50} value is defined as the concentration of inhibitor to inhibit 50% of its activity under the assayed conditions.

***In vitro* cytotoxicity assay**

The *in vitro* cytotoxicity assay was conducted and assessed by the Bioassay-Cell Culture Laboratory, Regional National Research Centre using the colorimetric method of Mosmann (1983). Two human cancer cell lines, colon cancer (HCT116) and breast cancer (MCF7) were subjected tothe most potent extracts against Gram -ve bacteria independently (*Spirulina* sp. and *Scenedesmus* sp.). Cells were suspended in RPMI 1640 medium in 96-well microtiter plastic plates at concentration of 103 cells/well and kept at 37°C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was

aspirated, fresh medium (without serum) was added and cells were incubated for 48 h, either alone (negative control) or with different concentrations of either extract or fraction to give a final concentration of (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, 150 and 200 mg/ml). The medium was aspirated 40 ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) of 2.5 mg/ml was added to each well and incubated for further four hours at 37°C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 ml of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37°C. A positive control composed of novantron standard (100 µg/ml) was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions.

The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm.

Results

Algal of biochemical analysis

Biochemical composition of algal species were estimated and presented in table 1. The maximum chlorophyll 'a' (12.9), chlorophyll 'b' (8.9) and carotenoid (4.41) were recorded in the *Ulva* sp. Total proteins content in the algae varied from 11.36 to 63.20.mg/g. The maximum protein content (63.20.mg/g) was recorded in the alga *Spirulina* sp. and the alga *Hormophysa* sp. recorded the minimum content (11.36mg/g). Total carbohydrates content of algae varied from 6.65 to 32.95 mg/g. The maximum carbohydrates content (32.95 mg/g) was recorded in the alga *Chlorella* sp. and the minimum (6.65 mg/g) was recorded in the alga *Sargassum* sp.

Total lipids content varied from 8.50 to 41.11 mg/g. The maximum lipids content (41.11 mg/g) was recorded in alga *Ulva* sp. and the alga *Spirulina* sp. recorded the minimum (8.50 mg/g). The total phenol and total amino acids content ranged between 9.91 to 153.33 mg/g and from 2.49 to 17.68%, respectively. The highest total phenol (153.33 mg/g) and total amino acids (17.68 %) was in *Sargassum* sp. and *Chlorella* sp., respectively.

Mineral composition The variation of mineral composition in the studied algae was presented in table 2. Among the 10 minerals analyzed the concentrations are N(1.9), K(7.19), Mg(4.9), Cu(0.0730) were higher in the blue green alga *Spirulina* sp., P(1.45), Mn(1.98), Fe(10.41), Zn(0.855) were high in the green alga *Chlorella* sp. and Na (407.5) was found to be the highest in the brown alga *Sargassum* sp. but the highest of Ca (9.2) was found in *Hormophysa* sp.

Antibiotic susceptibility

The antibiotics resistance or sensitivity were determined according to the interpretation of the vitro antibiotic susceptibility test as shown in table 3. Resistance of bacterial isolates to different tested antibiotics is shown in table 4. Among the 8 bacterial isolates: *Pseudomonas aeruginosa 6538, Klebsiella pneumoniae* and *E. coli 8739* were resistant to all the tested antibiotics, followed by *E. coli O157:H7, B. subtilis* subsp. *subtilis* BTN7A and *Bacillus megaterium* BMS4 which were resistant to 4 antibiotics. *B. amyloliquefaciens* subsp. *plantarum* SA5was resistant to 2 antibiotics only. The incidence of resistance to different tested antibiotics ranged between 37.5% (Neomycin and kanamycin) and 100% (Rifampin).

Antibacterial activity assay

In this study, the antimicrobial activities of six algal

Table 2: Mineral composition of algal species.

Elements / Algal species	%						(mg/L)			
	N	P	K	Ca	Mg	Na	Mn	Cu	Fe	Zn
Ulva sp.	1.24	0.053	0.43	7.8	2.64	370.5	0.638	0.0381	9.931	0.710
Hormophysa sp.	0.68	0.094	4.78	9.2	1.92	349.5	0.460	0.0090	6.847	0.611
Sargassum sp.	1.68	0.161	6.35	7.8	0.60	407.5	0.441	0.0118	6.911	0.637
Chlorella sp.	1.79	1.45	2.96	3.0	3.1	150.7	1.98	0.0181	10.41	0.855
Scenedesmus sp.	1.03	0.99	4.52	2.67	2.8	145.6	0.89	0.035	8.72	0.661
Spirulina sp.	1.90	1.06	7.19	4.2	4.9	165.0	1.66	0.0730	5.2	0.518

Table 1: Biochemical composition of algal species.

parameters / Algal Species		Pigments			Total proteins (mg/g)	Total carbohydrates (mg/g)	Total Lipids (mg/g)	Total phenols (mg/g)	Total amino acids (%)
		Chlorop-hyll (a)	Chlorop-hyll (b)	Carot-enoids					
Marine algae	*Ulva sp.*	12.9	8.9	4.4	14.42	9.24	41.11	102.58	6.11
	Hormophysa sp.	7.3	Nil	3.4	11.36	24.96	36.24	9.91	2.49
	Sargassum sp.	7.2	Nil	3.6	13.87	6.65	34.12	153.33	3.76
Fresh water algae	*Chlorella sp.*	7.3	3.4	3.9	22.28	32.95	17.28	45.94	17.68
	Scenedesmus sp.	6.6	2.7	3.2	18.02	21.02	16.54	36.96	13.6
	Spirulina sp.	4.3	Nil	2.9	63.20	18.91	8.50	59.10	13.76

Table 3: Interpretation of the *in vitro* antibiotic susceptibility test results.

Antibiotic agent	Disc Concen-tration (mcg)	Zone diameter inter-pretive standards (mm)		
		Sens-itive (S)	Moderately sensitive (MS)	Resis-tant (R)
Chloramphenicol	30	18>	13-17	12<
Rifampin	5	20>	17-19	16<
Streptomycin	10	15>	12-14	11<
Neomycin	30	17>	13-16	12<
Kanamycin	30	18>	14-17	13<
Ampicillin	10	20	----	19<
Tetracycline	30	19>	15-18	14<
Amikacin	30	24>	----	24<

extracts prepared by methanol, ethanol, acetone, chloroform, butanol, and ether were studied against some multidrug resistant bacteria. All algal extracts have different inhibition levels towards the tested microbial growth.

Inhibitory activity of algae had been evaluated against 8 bacterial isolates namely Gram-positive bacteria *Bacillus megaterium* BMS4, *B. amyloliquefaciens* subsp. *plantarum* SA5 and *B. subtilis* subsp. *subtilis* BTN7A (with accession number, KC429572, KC438369 and KC438368 respectively) and five Gram negative bacteria *Salmonella enterica*, *Escherichia coli 0157:H7*, *Escherichia coli 8739, Pseudomonas aeruginosa 6538* and *Klebsiella pneumoniae*. Algal crude extracts were active against the examined pathogens in different degrees table 5. In this regards, ZIs values ranged between 11-37mm for Gram-positive, 5-29 mm for Gram-negative bacteria. In this respect, the highest ZIsagainst Gram positive bacteria (37 mm) was recorded for ethanol *Scenedesmus* sp. extract; followed by ethanol *Scenedesmus* sp. (28 mm). Whereas, for

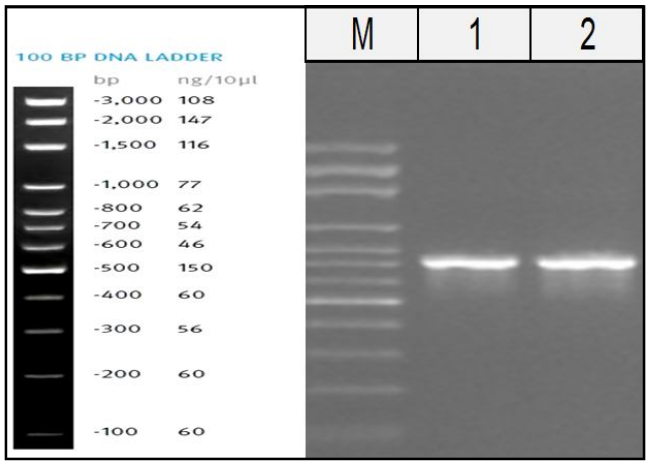

Fig. 1: An agarose gel (1 %) electrophoresis of PCR products showing the amplification of rbcL gene of *Spirulina* sp. and *Scenedesmus* sp.
Lane M, 100BP DNA marker; lane 1, *Spirulina* sp.; lane 2, *Scenedesmus* sp.

Gram-negative bacteria the highest ZIs value was found to be 29 mm with butanol *Scenedesmus* sp. extract; followed by chloroform *Ulva* sp. extract (28mm).

Results of the antibacterial activity as minimum inhibitory concentration (MIC) of the ethanol extracts of the two algae, *Scenedesmus* sp. and *Spirulina* sp. against *E. coli, Pseudomonas, B. megaterium, B. subtilis* and *B. amyloliquefaciens* were recorded in table 6. In general, It was found that the ethanol extract of *Scenedesmus* sp. alga showed the highest inhibitory effect against *Bacillus megaterium* BMS4 and *B. subtilis* subsp. *subtilis* BTN7A with MIC value 31.25 mg/ml followed by *Pseudomonas aeruginosa 6538, E. coli O157:H7 and B. amyloliquefaciens* subsp. *plantarum* SA5 with MIC values of 62.5 mg/ml. In contrast, *Spirulina* sp. extract was the least effective extract. Thus, the green alga *Scenedesmus* sp. can be used as a source for natural antibacterial agents.

The Amplification and Sequence analysis of *rbcL*

Table 4: Antibiotic susceptibility test for the tested bacteria.

Antibiotic disc (mcg) / Tested Bacteria	Inhibition zone diameter (mm)							
	C30	RA5	S10	N30	K30	AMP10	TE30	AK30
Bacillus megaterium BMS4	0R	0R	0R	18S	25S	0R	20S	25S
B. amyloliquefaciens subsp. *plantarum* SA5	33S	18R	20S	21S	25S	29S	17S	0R
B. subtilis subsp. *subtilis* BTN7A	35S	7R	7R	22S	26S	8R	0R	25S
Salmonella enterica	24S	10R	9R	22S	22S	20S	9R	21S
E.coli O157: H7	25S	8R	17R	20S	22S	8R	9R	24S
E. coli 8739	0R	0R	0R	0R	0R	0R	0R	0R
Pseudomonas aeruginosa 6538	0R	0R	7R	7R	0R	0R	0R	0R
Klebsiella pneumoniae MG461522	12R	9R	8R	9R	9R	8R	9R	11R
Resistance %	50	100	75	37.5	37.5	75	75	50

C: Chloramphenicol, RA: Rifampin, S: Streptomycin, N: Neomycin, K: Kanamycin,
AMP: Ampicillin, TE: Tetracycline andAK: Amikacin.

gene Fragment from *Spirulina* sp. and *Scenedesmus* sp. algae:

The results of *rbcL* gene amplification from algal samples was tested by electrophoresis presented in Fig. 1.

Table 5: Antibacterial activity of the investigated algal extracts.

Algal/ Solvent extracts	Diameter of inhibition zone (mm)							
	Gram +ve bacteria			Gram -ve bacteria				
	Bacillus megateriu mBMS4	***B. amyloli-quefaciens SA5***	***B. su-btilis BTN7A***	***Salm-onella enterica***	***E.coli O157 :H7***	***E. coli 8739***	***Pseudomonas aeruginosa 6538***	***Klebsiella pneumoniae MG461522***
	Ulva sp.							
Methanol	13	13	R	11	14	14	R	R
Ethanol	15	12	16	15	19	15	R	R
acetone	13	11	R	11	11	R	R	14
chloroform	R	R	21	28	R	R	R	17
butanol	R	R	18	22	14	R	R	15
ether	13	15	12	25	14	19	R	17
	Hormophysa sp.							
Methanol	R	R	R	R	R	R	R	R
Ethanol	R	R	R	R	R	R	R	R
acetone	R	R	R	R	R	R	R	R
chloroform	14	R	R	R	R	R	R	R
butanol	R	R	R	R	R	R	R	R
ether	R	R	R	R	R	R	R	R
	Sargassum sp.							
Methanol	R	15	R	R	16	R	7	11
Ethanol	R	11	15	R	R	R	R	11
acetone	R	15	R	R	R	R	R	R
chloroform	R	R	R	R	R	18	12	20
butanol	R	R	R	R	R	R	R	R
ether	R	R	R	R	R	R	R	18
	Chlorella sp.							
Methanol	14	13	20	R	17	R	18	5
Ethanol	14	12	20	R	17	R	11	18
acetone	11	14	20	11	16	R	12	16
chloroform	R	14	R	R	R	R	R	R
butanol	R	17	22	R	R	R	R	15
ether	R	R	R	R	R	R	R	19
	Scenedesmus sp.							
Methanol	R	14	19	12	10	R	16	R
Ethanol	37	18	28	14	16	R	21	14
acetone	19	15	23	R	12	R	15	R
chloroform	R	11	R	15	R	R	R	R
butanol	R	16	26	29	18	R	14	14
ether	R	R	27	R	R	R	25	R
	Spirulina sp.							
Methanol	R	15	13	R	16	R	15	14
Ethanol	R	19	13	R	R	15	17	12
acetone	R	15	12	R	R	15	13	13
chloroform	R	R	R	R	R	R	R	R
butanol	R	R	R	R	R	R	R	R
ether	R	R	R	R	R	R	R	R

R: Resistance

Table 6: Minimum inhibitory concentration (MIC) in mg/ml of ethanol extracts of *Scenedesmus* sp. and *Spirulina* sp. against some tested bacterial isolates.

Algal species / Bacteria species	MIC value (mg/m)	
	Scenede-smus sp.	*Spiru-lina* sp.
Pseudomonas aeruginosa 6538	62.5	250
E.coli O157: H7	62.5	250
*Bacillus megaterium*BMS4	31.25	-
B. subtilis subsp. *subtilis* BTN7A	31.25	125
B. amyloliquefaciens subsp. *plantarum* SA5	62.5	125

The amplification of the rbcL gene showed DNA bands with a size of ± 600 bp. The selected algae were identified by DNA barcoding analysis using *rbcL* region then comparing these sequences by the available sequences in Gene Bank using blast tool provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained results were submitted to Gene Bank.

Furthermore, BLASTn analysis for the selected algae showed that *Spirulina* sp. has 99% similarity with *Spirulinaplatensis*. For *Scenedesmus* sp., it has 99% homologous with *Scenedesmusobliquus*. The obtained accession number for *Spirulina* and *Scenedesmus* were MT897876 and MT900569, respectively.

Phylogenetic Analysis:

In order to identify phylogenetic analysis, selected algae were compared with data from the BLAST search results at NCBI. Neighbor Join (NJ) method was used. The rbcL gene can be used to clarify taxon positions in the identification of different species. Phylogenies trees show the relationship of species based on genetic similarities. The results showed that sample (1) *Spirulina* sp. is located in the same clade of *Arthrospira platensis* which indicate the high percent of homology 99%. Also, for sample (2) *Scenedesmus* sp. is in the same clade of *scenedesmus obliquus* with 98% similarity Fig. 2.

Anti-inflammatory activity (Membrane stabilization %)

The results of the HRBC assay of ethanol extract for *Spirulina* sp. and *Scenedesmus* sp. were given in

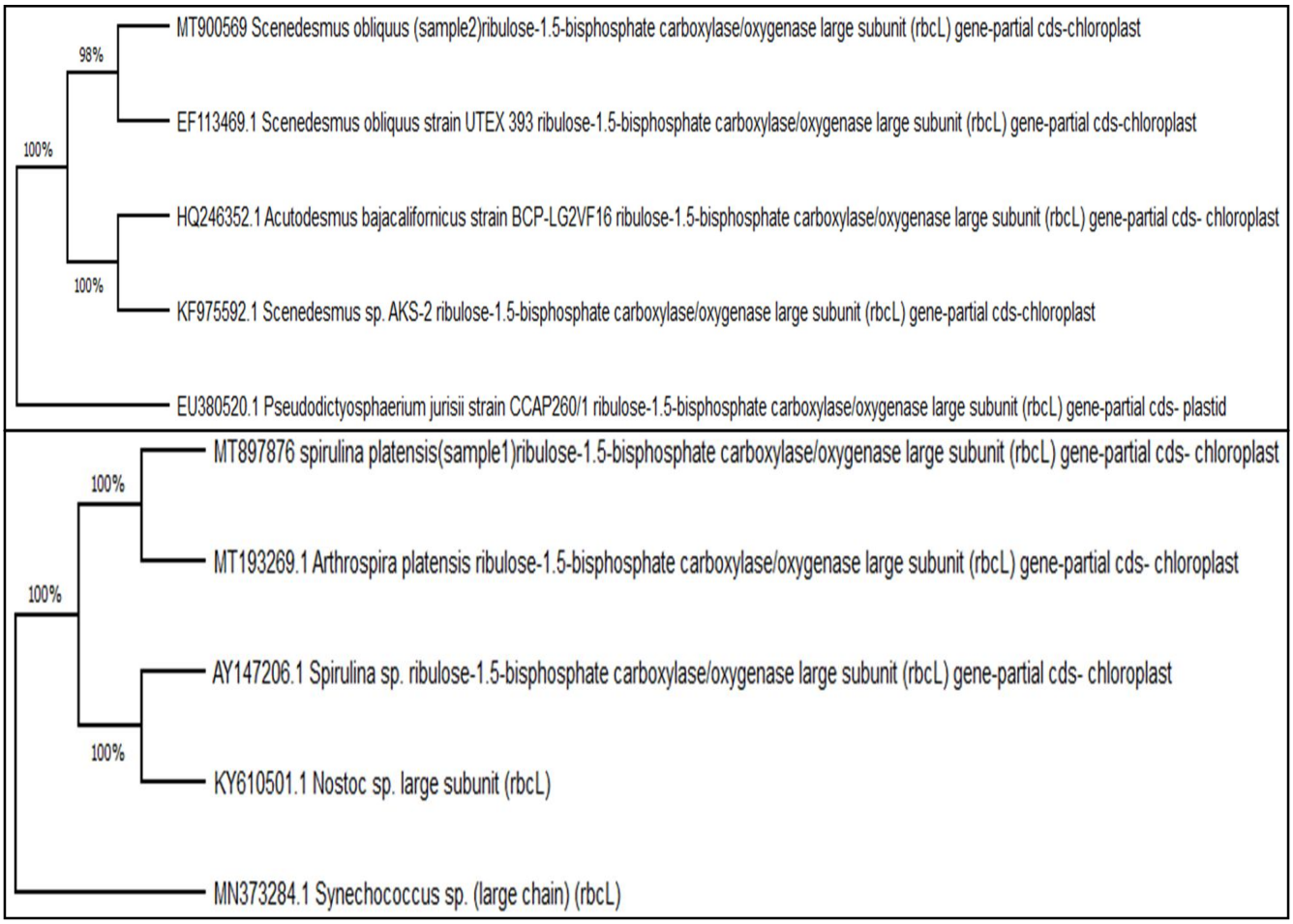

Fig. 2: Phylogram analysis depicting phylogenetic relationships of each morphological pattern of *Spirulina* sp. and *Scenedesmus* sp. samples used in this study based on *rbcL* sequences.

Sample conc. (µg/ ml)	(Membrane stabilization %)	S.D.
1000	76.32	1.5
500	71.44	0.63
250	69.35	2.1
125	62.47	0.58
62.5	54.32	2.1
31.25	43.24	1.6
15.63	22.57	0.92
7.81	12.76	0.94
0	0	0
IC50	50.31	

Fig. 3 & 4. It was depicted that maximum inhibition was 71.25±1.3% and 76.32 ±1.5% at a concentration of 1000 µg/ml for ethanol extract of *Spirulina* sp. and *Scenedesmus* sp. extracts, respectively.

Cytotoxicity of *Spirulina* sp. and *Scenedesmus* sp.

The cytotoxicity of *Spirulina* sp. ethanol extract against HCT116 and MCF7 cell lines are illustrated in Fig. 5, small amounts 26.2 and 43.6 µg/ml were required to inhibit 50% (IC_{50}) of HCT116 and MCF7 cell lines, respectively. Whereas, higher concentrations of

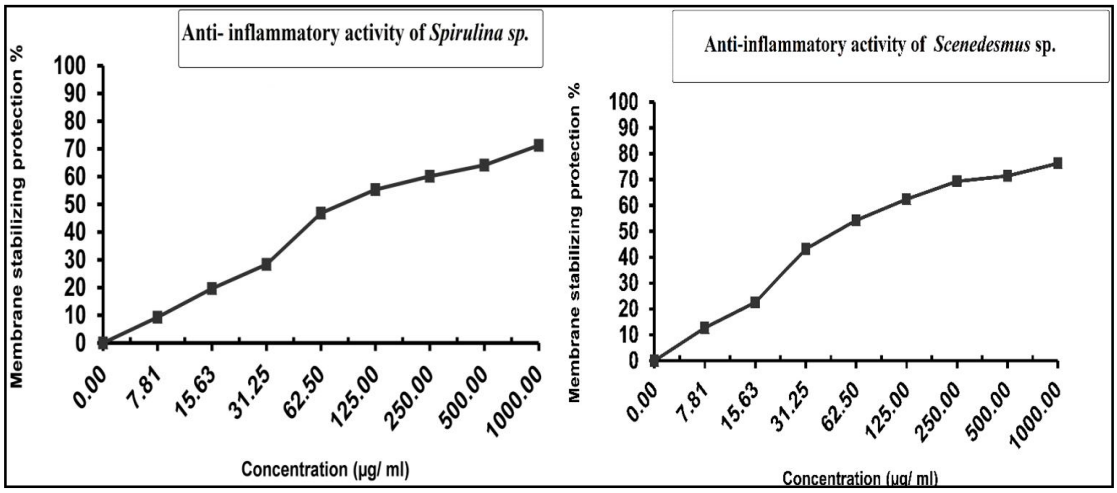

Fig. 3: Anti-inflammatory activity of *Scenedesmus* sp.

Sample conc. (µg/ ml)	(Membrane stabilization %)	S.D.
1000	71.25	1.3
500	64.13	1.6
250	60.13	2.1
125	55.32	0.58
62.5	46.85	2.1
31.25	28.32	2.1
15.63	19.63	0.58
7.81	9.31	0.63
0	0	0
IC50	58.7	

Scenedesmus sp. ethanol extract 58 and 87.7µg/ml were required to inhibit 50% (IC_{50}) of HCT116 and MCF7 cell lines, respectively.

Discussion

The ability of algal compounds (marine and/or fresh water) to constrain the growth of bacterial pathogens provides a potential alternative for antibiotics. This is due to the fact that pathogens are mostly resistant to antibiotics and research attention now has shifted to developing antimicrobial agents that are more effective

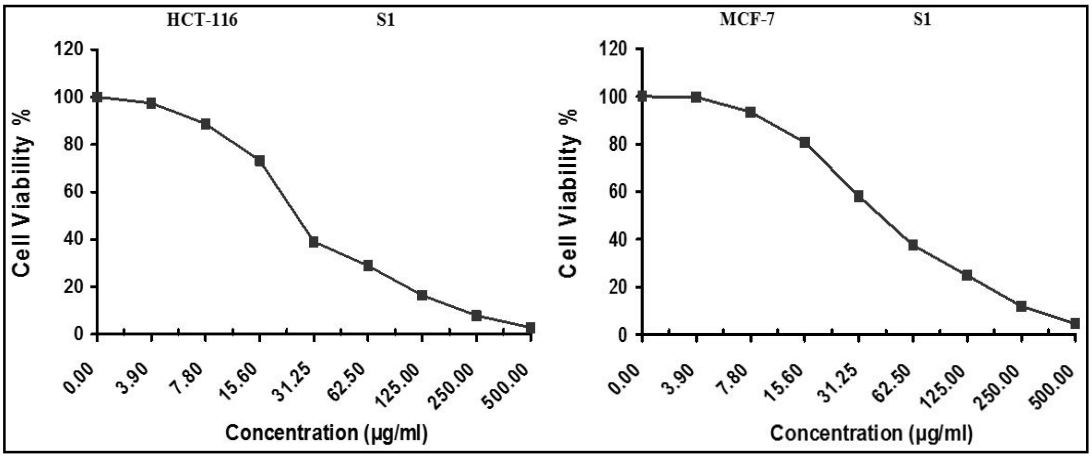

Fig. 4: Anti-inflammatory activity of *Spirulina* sp.

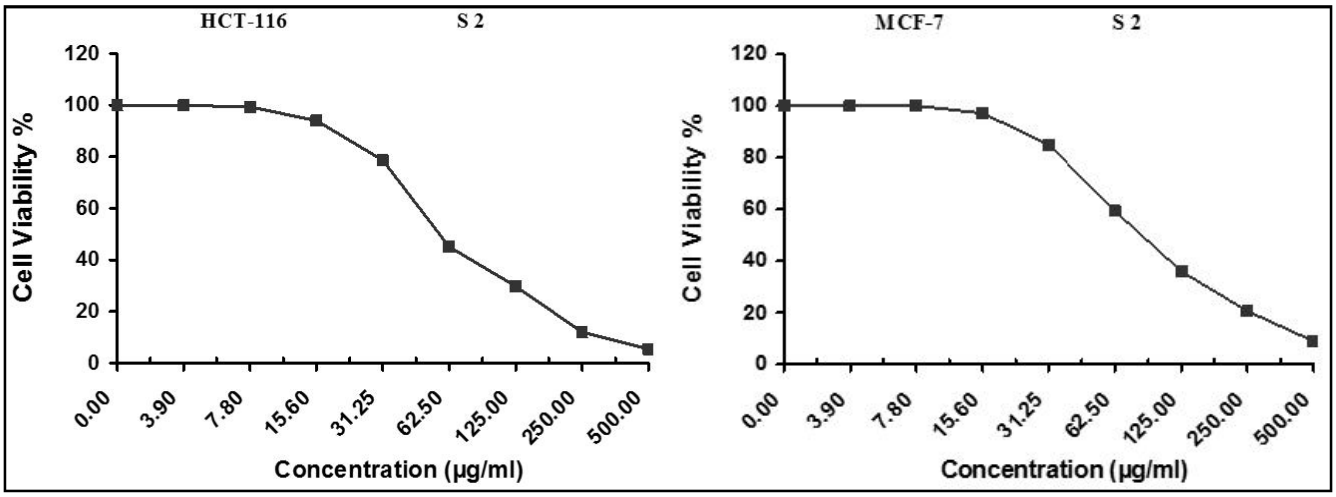

Fig. 5: Cytotoxicity assay of *Spirulina* sp. and *Scenedesmus* sp. ethanol extract on MCF7 and HCT116 cell lines.

and have minimal/no toxicity and less-side effects than antibiotics.

In this study, six algal extracts were prepared by methanol, ethanol, acetone, chloroform, butanol and ether from the blue green alga *Spirulina* sp., green algae *Chlorella* sp. and *Scenedesmus* sp. From the Hydrobiology Lab, Qanater Khayria, Qalubia, Egypt. The marine seaweeds *Ulva* sp. (Chlorophyceae), *Sargassum* sp. and *Hormophysa* sp. (Phaeophyceae) were collected from the intertidal zone of the Suez Gulf during the summer. The antimicrobial activity of algal crude extracts was assayed against eight species of pathogenic bacteria, 3 Gram-positive bacteria *Bacillus megaterium* BMS4, *B. amyloliquefaciens* subsp. *plantarum* SA5 and *B. subtilis* subsp. *subtilis* BTN7A (with accession number, KC429572, KC438369 and KC438368 respectively) and five Gram negative bacteria *Salmonella enterica*, *Escherichia coli 0157:H7*, *Escherichia coli 8739, Pseudomonas aeruginosa 6538* and *Klebsiella pneumoniae MG461522.*

Previous studies have shown that marine algae as well as microalgae are valuable sources of bioactive primary, secondary metabolites and compounds such as some vitamins, carotenoids and polyphenols which have antibacterial activity (El-Sheekh *et al.*, 2014). In another study, algal extracts had antibacterial and antifungal activities, especially *Bacillus subtilis* and *Candida albicans* which were the most sensitive species (Katircioglu *et al.,* 2006).

Chlorophyll from *Scenedesmus quadricauda* and *Chlorella vulgaris* showed significant antibacterial effects against gram positive *Bacillus subtilis*. Major carotenoids also showed antibacterial activity (Smith *et al.,* 2010). *Spirulina platensis* showed antibacterial effects against human pathogenic strains such as *Streptococcus* sp., *Escherichiacoli*, *Bacillus* sp. and *Pseudomonas* sp. These findings show the broad range of antibacterial activities displayed by algal pigments, which are dependent on the sources and habitats of the algae (Sabarinathan and Ganesan, 2008).

Many compounds in algae play a relevant role in preventing cardiovascular diseases or cancers (Wang *et al.*, 2018) and specifically phenolic compounds are involved in anti-inflammatory, anti-viral, or anti-ageing activities. Phenolic compounds, with a certain concentration, may affect growth and metabolism of bacteria and inhibit their growth (Bhoumick *et al.,* 2020). The phenolic contents in the *Sargassumdenticulatum* were supported by Matanjum *et al.,* (2009) who observed that the brown seaweeds contained a higher phenolic content than others seaweeds. In this study, it is also observed that the presence of phenolic compounds was the highest in the some tested algal samples.

Compared to water-based methods, organic solvents such as ethane, ethyl acetate and diethyl ether were the best solvents (water miscible and immiscible solvents) for the extraction of the active substances for antimicrobial activities. These solvents were effective against all microorganisms; however, ethanol extract indicated an antimicrobial activity against several microorganisms. This could be linked to the presence of bioactive metabolites which are soluble in ethanol but not in diethyl ether (Athperumalsamy *et al.,* 2010).

This may be caused by the toxins produced by its cells; for example, some blue green algae produce toxins which have potential pharmaceutical usages. The results of this study align with the study by Volk and Furkert (2006) who found that some microalgae had high biological activity against *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa*. This study also

confirmed the presence of metabolite like carbohydrates which might be the cause of antibacterial activity of these extracts against the types of pathogenic bacteria mentioned above.

In accordance with Najdenski *et al.,* (2013) who reported that *Scenedesmus obliquus* ethanolic extract had antibacterial activity against *S. aureus, B. cereus, E. coli, P. aeruginosa* and *S. typhi*, the current study showed that ethanol extract gave the highest results against tested bacteria. On the contrary, Beena and Krishnika (2011) confirmed that *Scenedesmus* sp. acetone, methanol, diethyl ether and hexane extracts had moderate antibacterial activity against *Pseudomonas* sp. Several studies reported the antimicrobial bacterial activity of *Scenedesmus obliquus* and *S. quandricanda* against different species of food borne pathogenic bacteria and mycotoxigenic fungi (Najdensk *et al.,* 2013). Also, *S. obliquus* extracts had anticancer activity against human breast MCF7, hepatic HePG2, colon HCT116 and human cervical adenocarcinoma HeLa cancer cell line (Abd El Baky *et al.,* 2014).

The results of this study are not compatible from what Abedin and Taha (2008) reported on acetone and diethyl ether extracts of *Spirulina platensis* having the highest antimicrobial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*. The antimicrobial activities of *S. platensis* could be related to different compounds belonging to a diverse range of chemical classes.

The results of the current study (*Scenedesmus* sp. and *Spirulina* sp.) revealed that Gram positive bacteria were more vulnerable than Gram negative bacteria. Taskin *et al.,* (2007) also reported that Gram positive bacteria were more effectively controlled than Gram negative bacterial by algal extract was due to the differences in their cell structure and their composition.

There are several factors that lead to the variation of antibacterial activity including the means of extraction, the solvents used and the season during which the samples were collected. Seenivasan *et al.,* (2010) explained that the variation observed in the potential antimicrobial components in seaweeds could be caused by external ecological factors such as herbivory, light, depth, salinity and nutrients of the environment. According to previous reports, anti-bacterial activity variation depends on algal species, the efficiency of the extraction method and the resistance of the pathogenic bacteria.

Microalgae such as *Chlorella* sp., *Spirulina* sp., *Dunaliella* sp. and *Scenedesmus* sp. are sources of valuable compounds which have potential benefits in the production of a wide spectrum of bioactive compounds (Catarina Guedes *et al.,* 2011). *Scenedesmus* sp. is a valuable source of new antimicrobial and anticancer compounds. *Spirulina* sp. is also known for its therapeutic properties and for being a source of bioactive compounds and therefore, it is considered one of the most commercially important microalga for the production of biomass (Vonshak and Tomaselli, 2000).

For the treatment of inflammatory diseases, the usage of synthetic drugs is very dominant nowadays. Specifically, the nonsteroidal anti-inflammatory drugs (NSAIDs) are prominently used for the treatment of inflammation and other related diseases. These drugs are carboxylic acid-based compounds reducing the enzyme activity and blocking the cyclooxy genase pathway. However, they have potential side effects such as pepticulcer, perforation and bleeding which is why medicinal research is working on finding alternatives with safe efficacy (Vonkeman and Van de Laar, 2010). *In vitro* anti-inflammatory effect of the extracts was analyzed by HRBC membrane stabilization method. Lysosomal enzymes released during the inflammation produce a variety of disorders. The stabilization of this lysosomal membrane is important in limiting the inflammatory response. The NSAIDs inhibit the release of lysosomal enzymes or stabilize the lysosomal membranes applying their healing property. The prevention of hypotonicity-induced HRBC membrane lyses can be followed as an *in vitro* measure of anti-inflammatory activity of natural products Since HRBC membrane is equivalent to lysosomal membrane components (Emamuzo *et al.,* 2010).

In this study, the percentage of membrane stabilization for ethanol extracts of *Spirulina* sp. and *Scenedesmus* sp. was done at 7.81, 15.63, 3.25, 62.5, 125, 250, 500 and 1000 µg/ml. Both extracts are found to be effective in inhibiting the heat produced by hemolysis of HRBC at the measured concentrations ranging from 7.81 to 100 µg/ml. It was found that the maximum inhibition was 71.25±1.3% and 76.32 ±1.5% at a concentration of 1000 µg/ml for ethanol extract of *Spirulina* sp. and *Scenedesmus* sp. extracts, respectively. The anti-inflammatory effects of the extracts are dose-dependent; increasing the concentration leads to the decrease of the membrane hemolysis which imply the increase of stabilization of HRBC membrane according to Emamuzo *et al.,* (2010). Even at a low concentration of 7.81 µg/ml, the percentage inhibition was in the range of 9.3±0.63-12.76±0.94% for *Spirulina* sp. and *Scenedesmus* sp. extracts correspondingly which showed that the two algal extracts have a significant anti-inflammatory activity in comparison to the typical drug Indomethacin.

In vitro anticancer activity of acetone extract of *Spirulina platensis* was evaluated by the MTT assay using MCF7 (human breast cancer) and HCT116 (human colon cancer) cell lines. MTT is considered one of the most convenient yet reliable methods due to their ease of use, accuracy, instant indication of toxicity, sensitivity and specificity. The assay is in vitro whole cell toxicity assay that employ colorimetric methods for determining viability cell based on mitochondrial dehydrogenase activity measurement. The reagent is bio reduced by dehydrogenase inside living cells to form a colored formazan dye (McCauley *et al.,* 2013). Ethanol extract of *Spirulina platensis* and *Scenedesmus* was dissolved in DMSO. DMSO is an amphipathic molecule, which makes it soluble in both aqueous and organic media. It is effectively used as a solvent for water-insoluble compounds in biological research (Wang *et al.,* 2012).

The cytotoxicity of *Spirulina* sp. ethanol extract against HCT116 and MCF7 cell lines are illustrated. Small amounts, 26.2 and 43.6 µg/ml were required to inhibit 50% (IC_{50}) of HCT116 and MCF7 cell lines, correspondingly. Whereas higher concentrations of *Scenedesmus* sp. ethanol extract 58 and 87.7.6 µg/ml were required to inhibit 50% (IC_{50}) of HCT116 and MCF7 cell lines, respectively. In contrast to Marrez *et al.,* (2019) argument that the highest anticancer effect of DEE was observed at IC_{50} against HCT116 cell line using just 24.6 mg ml^{-1} while, a higher concentration of 93.8 mg ml^{-1}, was required to exhibit its effect against MCF7. On other hand, Abd El Baky *et al.,* (2014) reported a high anticancer activity against human breast MCF7, hepatic HepG2 and colon HCT116 cancer cell lines at IC50 values 11.62, 14.5 and 15.22 mg ml^{-1} in *S. obliquus* oil which is extracted with chloroform: methanol (2:1 v/v).

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