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In vitro Anti-breast cancer and antifungal Bio-efficiency of some microalgal extracts

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ABSTRACT

Screening for sources of biologically active compounds capable of treating cancer is the main target of many researches nowadays. The anticancer as well as antifungal activities of the methanolic extracts of 4 microalgae (*Spirulina maxima, Chlorella salina*, *Nannochloropsis oceanica*, and *Nannochloropsis oculata*) were investigated. Among the microalgal extracts studied, the extract of *Spirulina maxima* exhibited promising anti-breast cancer activity causing 75.50±1.76% cytotoxicity at concentration of 200 µg/ ml, followed by *N. oceanica* extract (46.86±8.15% cytotoxicity) at the same concentration. Moreover, the same extract in addition to that of *Nannochloropsis oceanica* exerted anti-*Fusarium* activity. At the same time, both extracts showed weak antifungal activities against *Aspergillus niger* and *A. flavus*, while no activity was recorded against *Penicillum chrysogenum.*

INTRODUCTION

Cancer diseases are ranked as the second cause of mortalities after heart diseases worldwide. About 17 million new cases were reported in 2018, and this number is expected to be 27.5 million new cases each year by 2040, recently, it was reported that the mortalities related to cancer diseases reached 9.6 million in 2018 (World health

organization fact sheet, 2018). Breast cancer comes second in the list of the most common cancers occurring globally (about 2.1 million case in 2018 only), and ranked fifth among the most common causes of cancer death (627,000 deaths in 2018).

On the other hand, *Fusarim* species are causing many diseases and serious economic losses in livestock, humans, and agricultural crops. *Fusarium* causes a broad spectrum of human diseases such as fusariosis especially in immunocompromised patients (Antonissen *et al*., 2014). Moreover, *Fusarium* species can cause head blight, necrosis, wilting root rot, and damping-off among other diseases in crops such as cereals, cotton; rice, corn, beans, tomato, cucumber and others (Xia, 2019). Head blight was responsible for 2.6$ losses in only two years (from 1998 to 2000) in areas of North America (Bissonnette *et al*., 2018).

Microalgae are microorganisms typically found in freshwater and marine systems. More than 35,000 different species of these microorganisms have been identified (Ebenezer *et al*., 2012). Microalgae are characterized by their huge taxonomic diversity which attracts attention as a promising sources of variety of important compounds and products such as polyunsaturated fatty acids, proteins, enzymes, pigments stable isotope biochemicals, lipids, exopolysaccharides, and other biologically active metabolites (Gaignard *et al*., 2019). Such metabolites have interested applications in different fields as nutraceutical, cosmetic, pharmaceutical, and food/feed industries (Borowitzka, 2013). Novel drug discovery is one of the most attractive aspects of biotechnological applications of microalgae due to the vast possibilities although screening still relatively limited (Spolaore *et al*., 2006; El-Hack *et al*., 2019).

One of the important algal genus is *Nannochloropsis* which is characterized by spherical or slightly ovoid cells (Gwo *et al*., 2005). It has extremely important roles in the global mineral and carbon cycles (Fogg, 1995). Both *N. oculata* and *N. oceanica* are rich sources of polyunsaturated fatty acids, pigments, and proteins. Hence, they are commonly used for feeding purposes in aquaculture (Chua, and Schenk, 2017), and biofuel production (Hamidi *et al*., 2014).

Another important algal genus is *Spirulina* which is famous for its diverse biological activities, nutritional importance, and various applications in agricultural, food, pharmaceuticals, and perfumery fields (Hasanein *et al*., 2018). Moreover, *Spirulina* exhibited anticancer activities, and could inhibit carcinogenesis due to its anti-oxidant characteristics that protect tissues, and decrease toxicity of kidney, liver, and testes (Wu *et al*., 2016).

On the other hand, *Chlorella* represents an attractive alternative to currently well-established bacteria, yeast, and mammalian cell-based expression systems for production of many recombinant proteins such as enzymes, vaccines, monoclonal antibodies, and growth factors (Potvin *et al*., 2010). Since *Chlorella* has long been used as a health food and is approved to be safe to human, recombinant proteins derived from *Chlorella* may be readily acceptable by the public (Liu and Hu, 2013).

In this study, potential anti breast cancer activities of the methanol extracts originated from the algal species *Spirulina maxima, Chlorella salina, Nannochloropsis oceanica,* and *Nannochloropsis oculata* were investigated. Moreover, the antifungal activities of these extracts against some fungal isolates were evaluated. Finally, chemical analyses of promising extracts were performed in order to highlight compounds responsible for present activities.

MATERIALS AND METHODS

Microalgae cultivation

Four algal strains (*Spirulina maxima, Chlorella salina, Nannochloropsis oceanica* and *Nannochloropsis oculata*) were selected for this study from the culture collections maintained in the algal unit of the marine hatchery presented in the National Institute of Oceanography and fisheries, Alexandria, Egypt. All of these are unicellular of microscopic forms with the exception of *S. maxima* being filamentous. *N. oculata, N. oceanica,* and *C. salina* were grown in sterile filtered sterilized sea water was enriched with Walne's medium (Walne, 1970).

Mixing was done by sparing air from the bottom of the carboys through blower; lighting was supplied by four cool-white fluorescent tubes with an intensity of 5 000 lux at 20-25C^{o}. Cultivation of microalgal species in carboys were represented in Fig. 1, and at the end of exponential phase of growth all algal biomass were harvested through centrifuge.

On the other hand *S. maxima* was cultured in different medium composition, temperature and salinity. Modified Zarrouk medium (Zarrouk, 1966) at temperature 35±2.0C°, and salinity 5 g/L were used in scale up process from indoor to outdoor by the same method of culturing and harvesting techniques as described in (Hasanein *et al.*, 2018). For cultivation of *S. maxima,* brackish water medium enriched with modified Zarrouk medium. For Cost effectiveness, sodium chloride can be replaced by natural sea water and the whole medium salinity was adjusted to 5ppt.

Fig. (1). Cultivation of microalgal species in carboys

Extraction of algal metabolites

Different algal culture were extracted as described previously (Stranska-Zachariasova *et al*., 2016). Extraction using methanol was conducted at room temperature, and extracts were kept overnight prior to filtering. The resulting filtered extracts were concentrated at 50°C using a rotary evaporator till complete dryness. Obtained extracts were stored at 4ºC in a clean closed container until further use.

Effect of extracts on Human breast carcinoma tumor (MCF7) cell lines

Cell culture

MCF7 breast carcinoma human tumor cell line was cultured in 95% humidity, 5% CO_2 and 37°C. MCF7 was maintained in MEM media, supplemented with 10% fetal bovine serum and 1% antibiotic (Yang *et al*., 1996).

Cytotoxicity assay

Acid phosphatase assay was conducted to evaluate cytotoxicity as described previously (Yang *et al*., 1996). All samples were tested in triplicates, and 0.5% DMSO was used as negative control and 50 μM cisplatin was used as positive control. Algal extracts were tested at serial dilutions with final concentration of 200, 100, 50, and 25 μg/ml.

Percent cytotoxicity = [1-(D/S)] × 100, where D and S denote the optical density of drug and solvent treated wells, respectively.

Antifungal activity of microalgae extracts

The antifungal activities of *Spirulina maxima, Chlorella salina, Nannochloropsis oceanica, Nannochloropsis oculata* extracts were evaluated using the poison food technique (Wang *et al*., 2011; Bouson *et al.*, 2017). Briefly, PDA media were incorporated with microalgae extracts at concentration 10% (v/v). After solidification of medium surface, a fungal plug (5 mm in diameter) of the fungal indicator strains *Aspergillus niger, Aspergillus flavus, Fusarium solani, Fusarium oxysporum*, and *Penicillium chrysogenum* (5 days old) was placed at the center of each plate. Replicate plates were incubated at 30ºC. Control plate containing PDA medium was also inoculated with the fungal plug at the center of the plates. After an incubation period of 2 to 6 days, the radial growth of fungi (mm) in both treated and control plates was measured in perpendicular directions diametrically till the fungal growth in the control almost reach the wall of the petri dishes. The percentage of growth inhibition (I%) was calculated using the formula:

$$I\ (\%) = [(C-T)/C]\ X\ 100$$

The corrected inhibition (IC%) was then calculated as follow:

$$IC\ (\%) = [(C-T)/(C-C_0)]\ X\ 100$$

Where:

C is the diameter of the fungal radial growth in control petri dish.

T is the diameter of the fungal radial growth in treated petri dish.

C_0 is the diameter of the fungal agar plug placed in the center of petri dishes (5 mm).

GC-MS analysis of metabolites of selected microalgal extracts

Metabolites analysis was carried out as follows. Briefly 100 mg of selected finely powdered algae were individually extracted with 5 ml 100% methanol with sonication for 30 min with frequent shaking, followed by centrifugation at 12,000×g for 10 min to remove debris. 100 µl of the methanolic extract was aliquoted in a screw-cap vials and left to evaporate under a nitrogen gas stream until complete dryness. For derivatization, 150 µL of N-methyl-N-(trimethylsilyl)-tri fluoroacetamide (MSTFA) that was previously diluted 1:1% with an hydrous pyridine was added to the dried methanolic extract and incubated at 60 °C for 45 min prior to analysis using GC-MS (Farag *et al.*, 2018). The GC-MS analysis of the selected algal extracts was performed at Department of Medicinal and Aromatic Plants Research, National Research Center as described previously (Elkhateeb *et al.*, 2020), and with the following specifications, Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a Thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TR-5 MS column (30 m x 0.32 mm i.d., 0.25 µm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10 using the following temperature program: 60 °C for 1 min; rising at 4.0 °C/min to 240 °C and held for 1 min. The injector and detector were held at 210° C. Diluted samples (1:10 hexane, v/ v) of 1 µL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450.

RESULTS

The fight against lethal diseases spreading nowadays requires continuous screening for natural sources rich in biologically active compounds. Cancer diseases are ranked second as leading causes of mortalities globally. Hence, investigating for anticancer activities exerted by natural sources is of great interest. Microalgae are known for exhibiting promising biological activities, which made them interesting source of potential pharmaceuticals (Borowitzka, 2013; de Morais *et al.*, 2015).

The cytotoxicity of the four methanolic microalgal extracts were *in vitro* investigated against human breast cancer MCF7 cell line at different concentrations (25, 50, 100, 200 µg/ml). As shown in Fig. 2, the cytotoxicity exhibited by extracts was dose dependent. Extract of *N. oceanica* exhibited cytotoxicity ranging between 20.23±5.02%, and 46.86±8.92%; while *N. oculata* extract exerted cytotoxicity ranged between 8.63±1.06%, and 43.83±3.17%. On the other hand, *S. maxima* extract resulted in cytotoxicity ranging between 27.88±2.88%, and 75.50±1.76%; whereas *C. salina* extract recorded cytotoxicity ranging between 7.46±6.13%, and 42.73±4.99%. The strongest cytotoxicity (75.50±1.76%) was achieved by using *S. maxima* extract at concentration of 200 µg/ml. *N. oceanica* extract came second by achieving 46.86±8.92% for concentration 200 µg/ml, followed by *N. oculata* extract (43.83±3.17%.). On the contrary, *C. salina* has recorded the weakest anti-breast cancer activity (42.73±4.99%) among the tested extracts. The anticancer capability of *Spirulina* extracts have been previously reported in many studies (Schwartz *et al.*, 1988; Konícková *et al.*, 2014; Fayyad *et al.*, 2019). Also,

some studies have mentioned the anticancer activities of *Nannochloropsis* extracts (Sanjeewa *et al*., 2016; El-Hack *et al*., 2019).

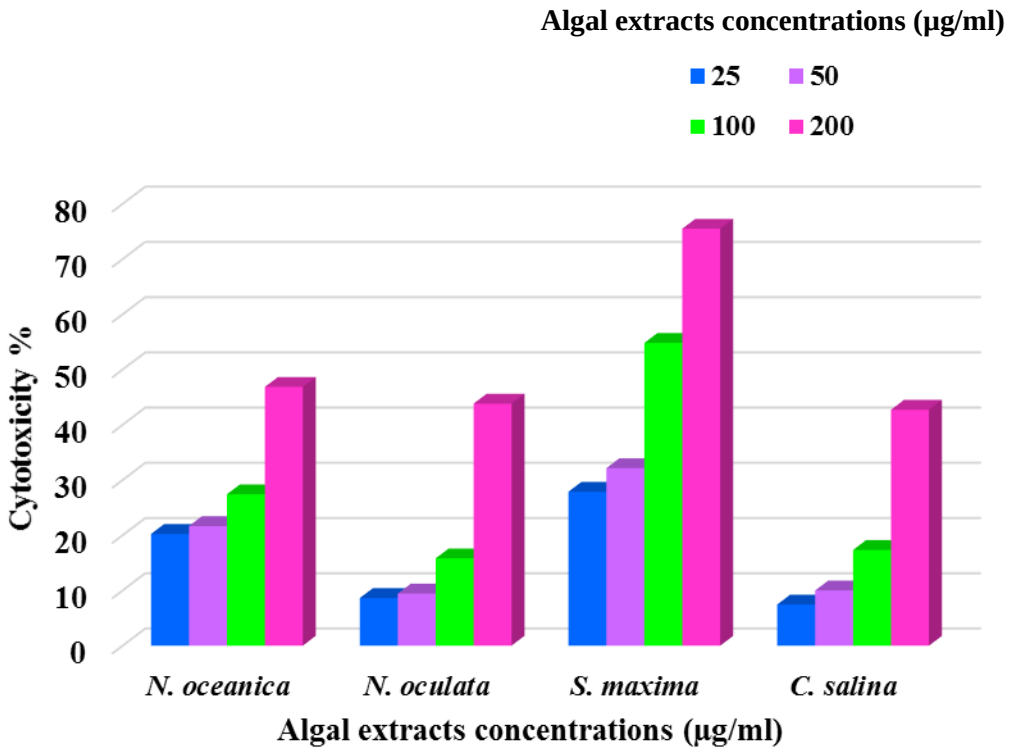

Fig. (2). Cytotoxicity % of algal extracts of *N. oceanica*, *N. oculata*, *S. maxima*, and *C. salina* against human breast cancer cell line. Values are presented as the means for three independent experiments.

Evaluation of antifungal activities of the methanolic microalgae extracts at concentration 10% (v/v) was conducted against the fungal strains *Aspergillus niger*, *A. flavus*, *Fusarium solani*, *F. oxysporum*, and *Penicillium chrysogenum*. Generally, extraction using organic solvents results in a higher efficiency in extracting compounds showing antimicrobial activities in comparison with water-based methods (Lima-Filho *et al.*, 2002). The antimicrobial activity of microalgae could be caused by their production of many acids, cyclic peptides, alkaloids and lipopolysaccharides (Katircioglu *et al*., 2006). As shown in Table 1, Microalgae exhibited varied antifungal activities. *N. oceanica* extract exerted promising anti-*Fusarium* activities with corrected inhibition of 76.25% against *F. solani*, and 75.62% against *F. oxysporum*. *S. maxima* extract recorded comparable results (75.0%, 76.25% against *F. solani*, and *F. oxysporum* respectively. Weak anti-*Aspergillus* activities have been noticed by using *N. oceanica* and *S. maxima* extracts. Recorded IC% were as low as 8.12% (*N. oceanica* extract against *A. flavus*), 8.75% (*N. oceanica* extract against *A. niger*), 9.62% (*S. maxima* extract against *A. flavus*), and 10.0% (*S. maxima* extract against *A. niger*). Antifungal capabilities of *Spirulina* species have been previously reported in many studies (Souza *et al*., 2011; Battah *et al*.,

2014). Similarly, *Nannochloropsis* species were reported for having antifungal activities (Scaglioni *et al*., 2019).
On the contrary, no activity was noticed by any tested extract against *Penicillium chrysogenum.* Furthermore, *N. oculata* and *C. salina* extracts showed no antifungal activities against all tested fungal indicator strains.

Table (1)**.** Antifungal activities of *m*icroalgae extracts against some fungal indicator strains

Fungal indicator strains	***S. maxima* extract**		***N. oceanica* extract**		***N. oculata* extract**		***C. salina* extract**	
	I%	**IC%**	**I%**	**IC%**	**I%**	**IC%**	**I%**	**IC%**
Aspergillus niger	9.41	10.0	8.23	8.75	0	0	0	0
Aspergillus falvus	9.06	9.62	7.64	8.12	0	0	0	0
Fusarium solani	70.59	75.00	71.76	76.25	0	0	0	0
Fusarium oxysporum	71.76	76.25	71.18	75.62	0	0	0	0
Penicillium chrysogenum	0	0	0	0	0	0	0	0

GC -MS analysis of the silylated microalgae extracts metabolites

GC-Ms analyses were performed for extracts of the microalgae exhibiting promising anti-breast cancer and antifungal activities namely *S. maxima and N. oceanica*. Metabolites profiles of their extracts were investigated in order to detect as much compounds is possible. As shown in Fig. 3 and Table 2, the GC-Ms analysis of *S. maxima* extract revealed the presence of 24 compounds such as amino acids, carboxylic and fatty acids along with glyceryl galactoside. The peak area% representing lactic acid was the major peak (39.14), followed by glycerol (11.38%), L-alanine (6.81%), trehalose (5.32%).

On the other hand, the GC-Ms analysis of *N. oceanica* extract (Fig. 4) revealed the presence of 24 compounds. Majority of these compounds were different from those detected in *S. maxima* extract. Only palmitic acid, stearic acid, lactic acid, and glycerol were detected in both extracts but represented by different peak areas indicating difference in their production ability between the two microalgae (Tab. 3).

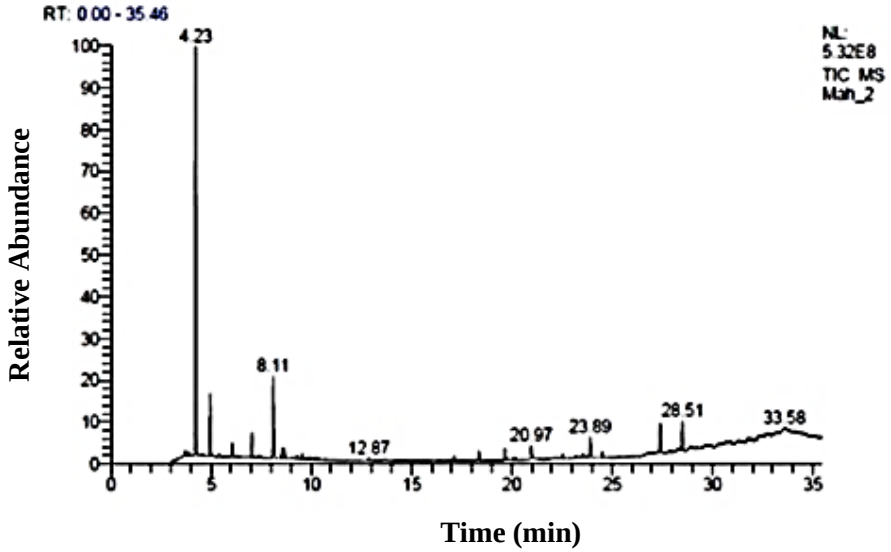

Fig. (3). GC-MS chromatogram of metabolites from the extract of *Spirulina maxima.* Major compounds peaks were illustrated.

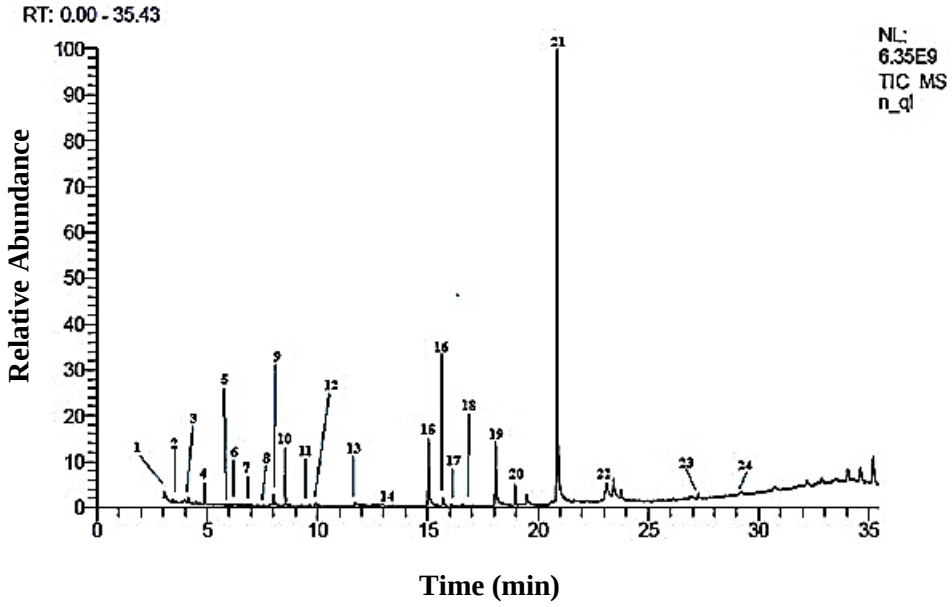

Fig. (4). GC-MS chromatogram of metabolites from the extract of *Nannochloropsis oceanica.* Major compounds peaks were illustrated.

Table (2). Identified compounds of *Spirulina maxima* extract using GC-MS analysis

No.	RT	Area %	Compound Name	Molecular Weight	Molecular Formula	SI
1	4.23	39.14	2-hydroxypropanoic acid (Lactic acid)	90	$C_3H_6O_3$	954
2	4.98	6.81	L-Alanine	89	$C_3H_7NO_2$	930
3	6.05	1.74	2- hydroxy Butanoic acid	104	$C_4H_8O_3$	900
4	7.04	3	L-Valine	117	$C_5H_{11}NO_2$	896
5	8.11	11.38	Glycerol	92	$C_3H_8O_3$	906
6	8.53	1.44	L-Isoleucine	131	$C_6H_{13}NO_2$	917
7	8.67	1.42	L-Proline	115	$C_5H_9NO_2$	860
8	9.55	1.22	pyrimidine-2,4-diol	112	$C_4H_4N_2O_2$	859
9	18.36	1.44	D-Glucose	180	$C_6H_{12}O_6$	866
10	19.66	1.8	α-D- Talopyranose	180	$C_6H_{12}O_6$	890
11	20.97	2.77	Hexadecanoic acid (Palmitic acid)	256	$C_{16}H_{32}O_2$	905
12	23.54	1.04	Octadecanoic acid (Stearic acid)	284	$C_{18}H_{36}O_2$	780
13	23.9	3.16	Glycerylgalactoside	254	$C_9H_{18}O_8$	860
14	24.52	0.86	Glycerylglycoside	254	$C_9H_{18}O_8$	801
15	27.38	4.74	sucrose	342	$C_{12}H_{22}O_{11}$	886
16	28.3	2.56	4-O-α-galactopyranosyl-D-mannopyranose	342	$C_{12}H_{22}O_{11}$	669
17	28.51	5.32	Trehalose	342	$C_{12}H_{22}O_{11}$	865
18	28.89	1.16	D-Turanose	342	$C_{12}H_{22}O_{11}$	637
19	30.67	0.91	14-α-H- PREGNA	288	$C_{21}H_{36}$	761
20	30.88	1.01	2,2-Dideutero Octadecanal	268	$C_{18}H_{34}D_2O$	760
21	31.76	1.69	Tetrapentacontane, 1,54-dibromo	914	$C_{54}H_{108}Br_2$	747
22	31.96	1.11	17-Pentatriacontene	490	$C_{35}H_{70}$	749
23	32.75	1.52	Dotriacontane	450	$C_{32}H_{66}$	766
24	33.55	1.62	9 (Z) Octadecenoic acid	282	$C_{18}H_{34}O_2$	749
Total identified % is 98.86 and SI ≥ 630						

Table (3). Identified compounds of *Nannochloropsis oceanica* extract using GC-MS analysis

No.	RT	Area %	Compound Name	Molecular Weight	Molecular Formula	SI
1	4.13	0.31	2-hydroxy Propanoic acid (D-Lactic Acid)	90	$C_3H_6O_3$	906
2	4.19	0.15	Indol-1-ylacetamide	174	$C_{10}H_{10}N_2O$	732
3	4.5	0.22	Hexanoic acid (Caproic Acid)	116	$C_6H_{12}O_2$	914
4	4.87	1.36	Alanine	89	$C_3H_7NO_2$	929
5	6.24	0.18	Heptanoic acid	130	$C_7H_{14}O_2$	881
6	6.93	0.07	L-norvaline	117	$C_5H_{11}NO_2$	875
7	7.89	0.12	Benzoic acid	122	$C_7H_6O_2$	891
8	7.99	0.77	Glycerol	92	$C_3H_8O_3$	902
9	8.07	0.39	Octanoic acid	144	$C_8H_{16}O_2$	902
10	8.54	4.28	Methylthiouracil	142	$C_5H_6N_2O_S$	825
11	9.67	0.12	Serine	105	$C_3H_7NO_3$	876
12	9.91	0.33	Nonanoic acid	158	$C_9H_{18}O_2$	876
13	11.7	0.63	Decanoic acid	172	$C_{10}H_{20}O_2$	834
14	12.96	0.4	1-(tert-butyl)-4-(hexa-1,3,5-triyn-1-yl)benzene	206	$C_{16}H_{14}$	923
15	15.02	6.97	Dodecanoic acid	200	$C_{12}H_{24}O_2$	924
16	16.59	0.25	N-Tridecanoic acid	214	$C_{13}H_{26}O_2$	639
17	16.89	0.08	D-(-)-tagatofuranose	180	$C_6H1_2O_6$	854
18	17.03	0.15	D-(-)-fructofuranose	180	$C_6H_{12}O_6$	864
19	18.07	7.78	Tetradecanoic acid	228	$C_{14}H_{28}O_2$	915
20	19.18	0.11	Octadecanenitrile	265	$C_{18}H_{35}N$	702
21	20.87	48.54	Hexadecanoic acid(Palmitic Acid)	256	$C_{16}H_{32}O_2$	896
22	23.43	2.51	Octadecanoic acid (Stearic Acid)	284	$C_{18}H_{36}O_2$	705
23	27.27	0.61	Sucrose	342	$C_{12}H_{22}O_{11}$	812
24	29.18-32.7	1.7	2,4,6-Tri[4,5-(methylenedioxy)phenyl]-s-triazine	441	$C_{24}H_{15}N_3O_6$	753
Total identified % is 78.03 and SI ≥ 700						

DISCUSSION

Compounds detected by GC-Ms analyses of metabolites of both extracts can explain the biological activities exerted by *S. maxima* and *N. oceanica* extracts. The anticancer activity exerted by *S. maxima* extract can be attributed to the presence of many compounds having anticancer activities such as the saturated fatty acid, stearic acid which has been previously reported to have anti-breast cancer properties in many *in vitro* and *in vivo* studies. Stearic acid inhibited proliferation of human breast cancer cell (**Hardy *et al.*, 1997, Evans *et al*., 2009a**), and breast tumorigenesis (**Habib *et al.*, 1987; Evans *et al.*, 2009b**). Moreover, stearic acid is capable of inducing breast cancer cells apoptosis (**Hardy et al., 2003**), and can arrest breast cancer cell-cycle (**Li *et al.*, 2011**). Amazingly, stearic acid was nominated in many epidemiological studies as a protecting agent that prevent and treat breast cancer (**Saadatian-Elahi *et a*l., 2004**). Other compounds known for their anticancer activity is palmitic acid (**Harada *et al*., 2002; Intisar *et al*., 2013**), and 9 (*Z*) Octadecenoic acid (**Yoo *et a*l., 2007**). On the other hand, presence of palmitic and stearic acid in *N. oceanica* extract is responsible for the anticancer activity. Furthermore, caproic acid detected in this extract has been known for its anticancer activities (**Narayanan *et al.*, 2015; Widiyarti *et al*., 2019**).

Investigating the antifungal activities of the four microalgae extracts resulted in promising antifungal properties of both *N. oceanica* and *S. maxima* extracts specially against the two tested *Fusarium* species. These promising results are of great value due to the economic loss and diseases caused by *Fusarium* species in humans, livestock, and agricultural crops. The antifungal activities of *S. maxima* and *N. oceanica* extracts can be explained by the presence of many compounds having antimicrobial activities such as palmitic, and stearic acids which are known to possess antibacterial and antifungal properties (**Agoramoorthy *et al.*, 2007**). Moreover presence of lactic acid affect microbial growth (**Machado *et al*., 2018**), 17-Pentatriacontene is known to exert antimicrobial activity (**Hassan and Shobier, 2018**), and octadecane which exhibits potent antimicrobial activity (**Ramasamy and Gopalakrishnan, 2013**). Nonanoic acid has antifungal activities, and known as a stable inhibitor of spore germination produced by fungi (**Breeuwer *et al*., 1997; Chadeganipour and Haims, 2001; Sahin *et al*., 2006**), and decanoic acid (capric acid) has antimicrobial properties (**Liu *et al*., 2008; Altieri *et al*., 2009; Pohl *et al*., 2011**).

CONCLUSION

Besides their known uses and economically important applications, this study has highlighted some of the biological activities of important microalgae as *S. maxima* and *N. oceanica*. Results in this study encourage further investigations for more potential applications of those promising microalgae. Continuous search for natural sources capable of fighting serious diseases such as cancer and exhibiting activity against phytopathogenic fungi such as *Fusarium* can contribute in discovering potent compounds able to make us win the battle against life threating enemies.

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