Antimicrobial and Anticancer Activity of Some Microalgae species

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Abstract:

Algae are known to produce a wide variety of bioactive secondary metabolites and several compounds have been derived from them with potential for use in the development of new pharmaceutical agents. In this study *Chlorella vulgaris*, *Nannochloropsis oculata* and *Amphora coffeaeformis* were examined for their antimicrobial activity. Methanol extracts of the selected algae were assayed for antimicrobial activity against *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli* as Gram negative bacteria and *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus mutans* as Gram positive bacteria. The used fungi were *Aspregillus niger*, *Penicillium expansum* and *Syncephalastrum racemosum* and one examined yeast was *Candida albicans*. Results indicated that *Nannochloropsis oculata* methanol crude extract showed the highest activity against bacteria while *Chlorella vulgaris* methanol crude extract showing the highest activity against fungi and yeast. Moreover, the crude extracts obtained were assayed for cytotoxicity against human Hepatocellular cancer cell line. The active compound was identified by the GC-mass.

Key words: *Chlorella vulgaris*, *Nannochloropsis oculata*, *Amphora coffeaeformis*, Antibacterial, Human Hepatocellular cancer cell line

Introduction

Algae are very large and diverse group of simple, typically autotrophic organisms that can carried out photosynthesis since they have the ability to capture energy from sunlight (**Sharma *et al*., 2012**). As the resistance of bacteria and fungi to antibiotics has necessitated the development of new alternatives (**Ireland *et al*., 1988; Smith *et al*., 1994**), researchers begun to study algae as a

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new alternative, they found that algae have wide range spectrum of biological activities such as antimicrobial (**Bouhlal *et al.*, 2011**), antiviral (**Kim and Karadeniz, 2011),** antifungal (**De Felício *et al.*, 2010**) and antioxidant activities (**Devi *et al.*, 2011**). They produce a variety of active metabolites in their surroundings as a weapon to protect themselves against other settling organisms (**Bhadury and Wright, 2004**).

Both marine and fresh water algae produce wide varieties of chemically active secondary metabolites which act as a chemical defense against predators and herbivores under environmental stress and competition for space (**Isassi *et al.*, 2000**).These bioactive metabolites are synthesized by certain species of marine and fresh water microalgae. These molecules have antibacterial, antialgal and antifungal properties which are effective in the remediation of various diseases, bio-fouling and are employed in other aspects such as in therapeutics (**Syed *et al.*, 2015**).

The important compounds identified as antimicrobial are fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing hetero cyclic compounds, carbohydrates and phenols (**Kannan *et al.*, 2010**). Polyunsaturated fatty acids, sulfated polysaccharides, phyco sterols, heat-induced proteins, phenolic compounds, and pigments including carotenoids are also have positive effects on the health of man and animals (**Pulz and Gross, 2004**).

Cancer is a significant challenge of the 21st century. Globally, one of the most prominent and deadly diseases, cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells (**Ferlay *et al.*, 2012**).The malignant tumor are cause of death in humans and have high impact in industrial countries (**Fukahori *et al.*, 2008; Smyrniotopoulos *et al.*, 2010**). In the last years, the developments of new drugs have increased, and nature became a good resource for the discovery of anticancer compounds. More than 60% of anticancer drugs have made from natural materials, such as plants, microbes, and marine organisms (**Newman *et al.*, 1981**), natural compounds may act more specifically and have fewer or less severe adverse side effects than synthetics (**Paterson and Anderson, 2005**). The present investigation aimed to evaluate important bioactive compounds in *Chlorella vulgaris*, *Nannochloropsis oculata* and *Amphora coffeaeformis* and assessing the antimicrobial and anticancer activities of these algal extracts.

Materials and Methods

Chlorella vulgaris, *Nannochloropsis oculata and Amphora coffeaeformis* were obtained from Algal Biotechnology Unit, National Research Centre, Egypt. All of these organisms were subjected to mass production in different photobioreactor types with a final capacity of 1200-1500 liter. Harvesting was done when culture reached about 1.0 $g.l^{-1}$ (fresh weight) as mentioned by **Khan *et al.*** (**2015**).

Preparation of algal extract

The extraction of algal samples was done using methanol. 50g of algal powder were extracted in 250 ml solvent for 24 hrs. Then the soaked samples were filtered and concentrated under reduced pressure using rotary evaporator. Each dried residue was re-dissolved in the corresponding solvent and preserved at 5 ^{O}C until use.

Bacterial strains

Isolates of *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli* as Gram negative bacteria and *Staphylococcus aureus, Bacillus subtilis* and *Streptococcus mutans* as Gram positive bacteria were tested. The used fungi are *Aspregillus niger*, *Penicillium expansum* and *Syncephalastrum racemosum* and yeast (*Candida albicans*). These isolates were obtained from Algal Biotechnology Unit, National Research Centre, Egypt and The Regional Center for Mycology and Biotechnology, Al Azhar University.

Antitumor activity assay

Antitumor activity assay of extracts was determined by Human hepatocellular carcinoma (HepG2),Cell line were obtained from VACSERA Tissue Culture Unit. The cells were grown on RPMI-1640 medium contains 10% inactivated fetal calfserum and 50μg/ml gentamycin. The cells were preserved at 37^{O}C in a humidified with 5% CO_2 and were subcultures two to three times a week according to (**Mosmann, 1983; Gomha *et al.*, 2015**).The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of

intact cells, was estimated from graphic plots of the dose response curve for each conc. Using Graphpad Prism software (San Diego, CA. USA)

Separation and purification of the active compounds of the tested alga extract:

The crude extracts were applied on TLC plates and (silica column chromatography according to the method described by (**El-Sheikh *et al.*, 2009 and 2013**).

The active fractions were analyzed by G.C. mass spectroscopy was performed (Agilent Technologies 7890B GC Systems combined with 5977A Mass Selective Detector). Capillary column was used (HP-5MS Capillary; 30.0 m × 0.25 mm ID × 0.25 µm film) and the carrier gas was helium at a rate of flow of 1.8 ml/min with 1 µl injection. The sample was analyzed with the column held initially for 3 min at 40 °C after injection, then the temperature was increased to 300°C with a 15°C/min heating ramp, with a 4.0 min hold. Injection was carried out in splitless mode at 300 ^{O}C. MS scan range was (*m*/*z*): 50–550 atomic mass units (AMU) under electron impact (EI) ionization (70 eV). Silylation agent: BSA. N,O-Bis(trimethylsilyl) acetamide.

The reaction is carried out by adding 100 µL of BSA + amount of the sample after extraction and heating in water bath at 70^{O}C for two hours and after that inject into GC/MS under the above conditions. The constituents were determined by mass fragmentations with The NIST mass spectral search program for the NIST/EPA/NIH mass spectral library Version 2.2 (Jun 2014).

Results

Antimicrobial activity of the algal extracts

The results in Figures (1and 2) demonstrated the antimicrobial activities of the three studied microalgae. The methanolic extracts of algae were examined against *Pseudomonas aeruginosa*, *Proteus vulgaris* and *E.coli* as Gram negative bacteria and *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus mutans* as Gram positive bacteria. The used fungi are *Aspergillus niger*, *Penicillium expansum* and *Syncephalastrum racemosum* and yeasts (*Candida albicans*).

Data obtained in Figure (1) showed different reactions against bacteria with different solvent extracts. The methanol extract of C. *vulgaris*, *A. coffeaeformis*

and *Nannochloropsis oculata* indicated moderate antibacterial activity against *Bacillus subtilis* (7, 8 and 6 mm respectively) while *Staphylococcus aureus* was affected by C. *vulgaris* and *N. oculata* with high and moderate activities (10 and 7 mm respectively).

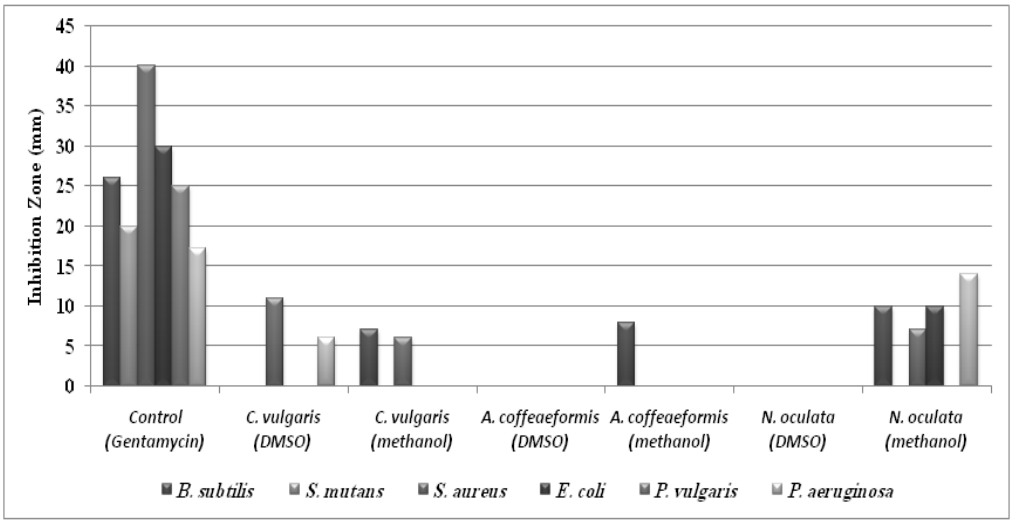


Figure (1): Antibacterial activity of the three algal extracts against pathogenic bacteria (growth of inhibition expressed as mm diameter)

Also, result cleared that *Nannochloropsis oculata* showed the highest activity against *Pseudomonas aeruginosa* with inhibition zone (14mm) followed by *Chlorella vulgaris* (6 mm). On the other hand, *A. coffeaeformis* showed no effect on *P. aeruginosa*.

Meanwhile, the results of DMSO extract showed that only *Chlorella vulgaris* (in which the crude extract re-dissolved in Dimethyl sulfoxide) showed high antibacterial activity against *Staphylococcus aureus* with inhibition zone (11mm) while no other effects were observed for DMSO extracts.

Result in Figure (2) revealed that methanolic extract of *Chlorella vulgaris* have the highest antifungal activity against *Aspergillus niger* and *Candida albicans* with inhibition zone (8 and 14mm) respectively, followed by *Nannochloropsis oculata* (7 and 10mm inhibition zone) respectively.

On the other hand, *Amphora coffeaeformis* showed 6mm inhibition zone against *C. albicans* but no antifungal activity against other organisms.

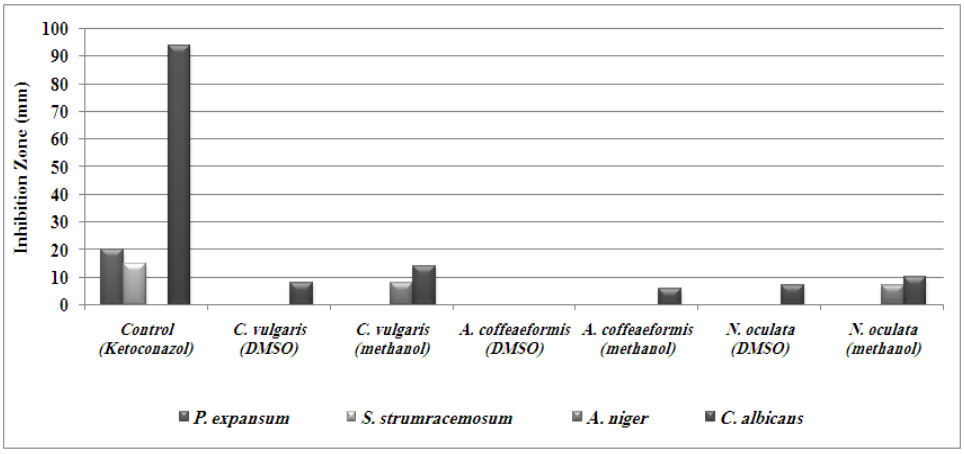


Figure (2): Antifungal activity of the three algal extracts against pathogenic fungi (growth of inhibition expressed as mm diameter)

Evaluation of cytotoxicity activity for algal extracts

Cytotoxicity assay of the methanol extract of *C. vulgaris Amphora coffeaeformis*, and *Nannochloropsis oculata* was determined in terms of IC_{50} value based on the percentage of free radical scavenging activity.

The selected algae were examined for their anticancer activity against hepatocellular carcinoma cell line all the algae show remarkable anticancer activity (Table 1).

Anticancer activity of the methanol extract of the three algae were determined in terms of IC_{50} value based on the percentage of free radical scavenging activity of *C. vulgaris*, *A. coffeaeformis* and *N. oculata* showed 50% inhibition (IC_{50}) of HepG2 at (97.6, 114 and 50.1 µg/ml) respectively.

Table (1): Evaluation of cytotoxicity for the three studied alga against HepG-2 cell line.

Sample conc. (µg/ml)	Viability %		
	Amphora coffeaeformis	***Chlorella vulgaris***	***Nannochloropsis oculata***
500	**17.94**	**14.21**	**6.43**
250	**33.26**	**31.59**	**20.75**
125	**46.87**	**42.76**	**31.21**
62.5	**65.42**	**59.28**	**43.96**
31.25	**81.96**	**70.34**	**59.18**
15.6	**90.68**	**86.20**	**72.34**
7.8	**98.41**	**94.75**	**89.65**
3.9	**100**	**98.23**	**94.82**
0	**100**	**100**	**100**

The antibacterial investigantion of the methanolic extract showed that *Chlorella vulgaris* had the highest antibacterial activity against *S. aureus*, further study of it´s most active compounds was conducted.

From Table (2) it was clear that fractions No.(8,15 and 18) exhibited the highest antibacterial activity.The mass spectrum of the fractions 8, 15 and 18was analyzed to give the mass fragmentation of its constituents.

The proposed configuration satisfies and complies with the analytical identification in characteristics shown by GC/MS can be deduced that the compound has the following characteristics for fractions No.(8,15 and 18): First: Palmitic acid derivatives. The molecular weight is 328.605, and its molecular formula is $C_{19}H_{40}O_2Si$. Finally, the nomenculature of the compound is identical to (rank) of GC mass figures, trimetylsilyl palmitate chemical structure and mass spectrum (Figures 3 and 4). Second: Myristic acid derivatives. The molecular weight is 300.552, and its molecular formula is $C_{17}H_{36}O_2Si$. Finally, the nomenclature of the compound is identical to (rank) of GC mass figures, trimetylsilyl mynstate chemical structure and mass spectrum (Figures 5 and 6). Third: Butanedioic acid derivatives. The molecular weight is 262.45, and its molecular formula is $C_{10}H_{22}O_4Si_2$. Finally, the nomenclature of the compound is

identical to (rank) of GC mass figures, butanedioic acid chemical structure and mass spectrum (Figures 7 and 8).

Table (2): The antibacterial activity of isolated fractions from *Chlorella vulgaris* against *Staphylococcus aureus*.

Fraction	Activity	Fraction	Activity	Fraction	Activity	Fraction	Activity	Fraction	Activity
1	-	16	+	31	-	46	-	61	+
2	-	17	-	32	-	47	-	62	-
3	+	18	+++	33	-	48	-	63	-
4	+	19	++	34	-	49	-	64	-
5	+	20	+	35	-	50	-	65	-
6	-	21	-	36	-	51	-	66	-
7	+	22	-	37	-	52	-	67	-
8	+++	23	-	38	-	53	-	68	-
9	++	24	-	39	-	54	-	69	-
10	++	25	-	40	-	55	+		
11	+	26	-	41	-	56	+		
12	-	27	-	42	-	57	++		
13	+	28	-	43	-	58	++		
14	++	29	-	44	-	59	++		
15	+++	30	-	45	-	60	+		

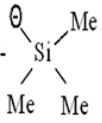
Ms m/z (%): 328.605(M^+), 313(M^+- CH_3), 145(M^+ - CH_3-$(CH)_{10}$, 117(M^+-CH_3- $(CH_2)_{14}$ – CH_2), 73(M^+- )

Figure (3): Chemical structure of Palmitic acid

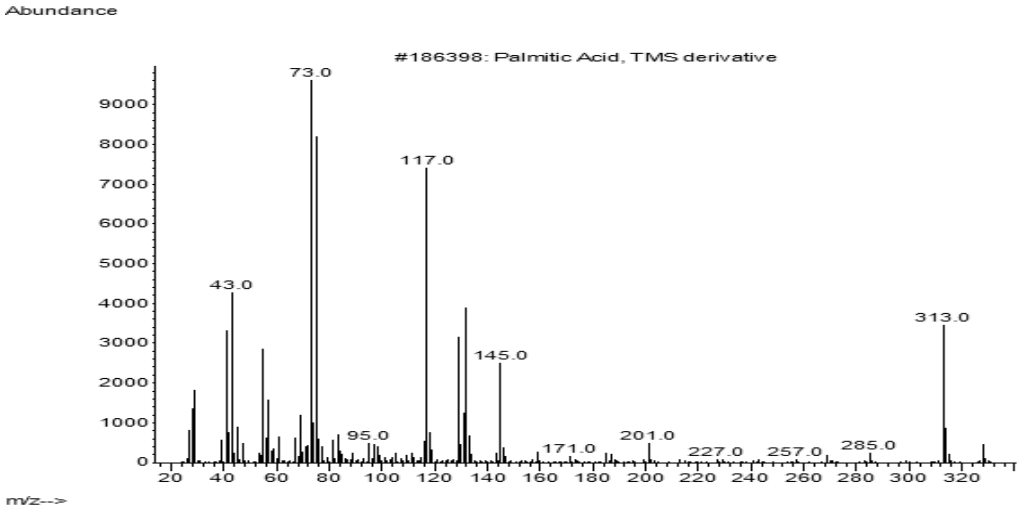


Figure (4): The mass spectrum of Palmitic acid obtained from *Chlorella vulgaris*

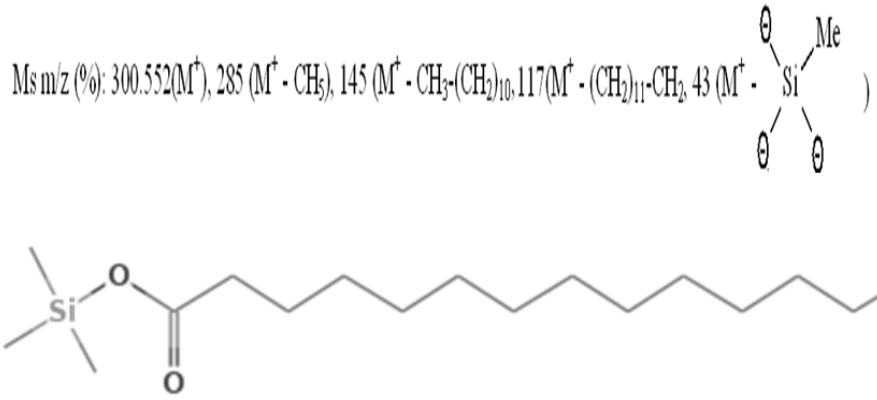


Figure (5): Chemical structure of Myristic acid

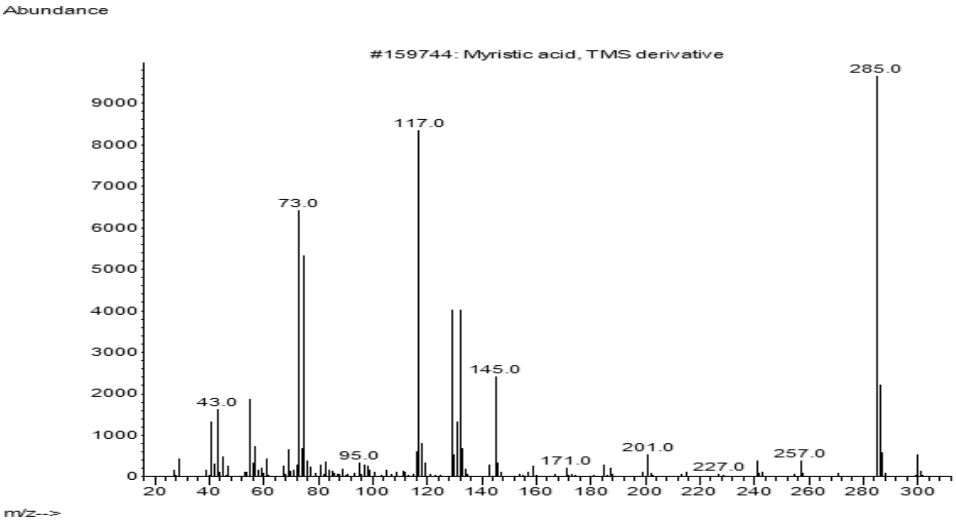
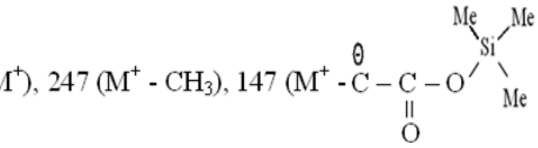
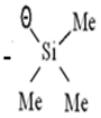


Figure (6): The mass spectrum of Myristic acid obtained from *Chlorella vulgaris*

Ms m/z (%): 262.45(M^+), 247 (M^+ - CH_3), 147 (M^+ -  , 73 (M^+ - )

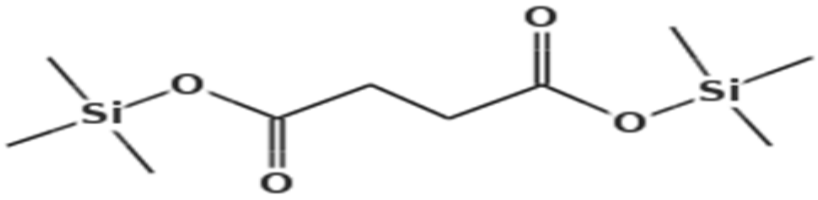


Figure (7): Chemical structure of Butanedioic acid

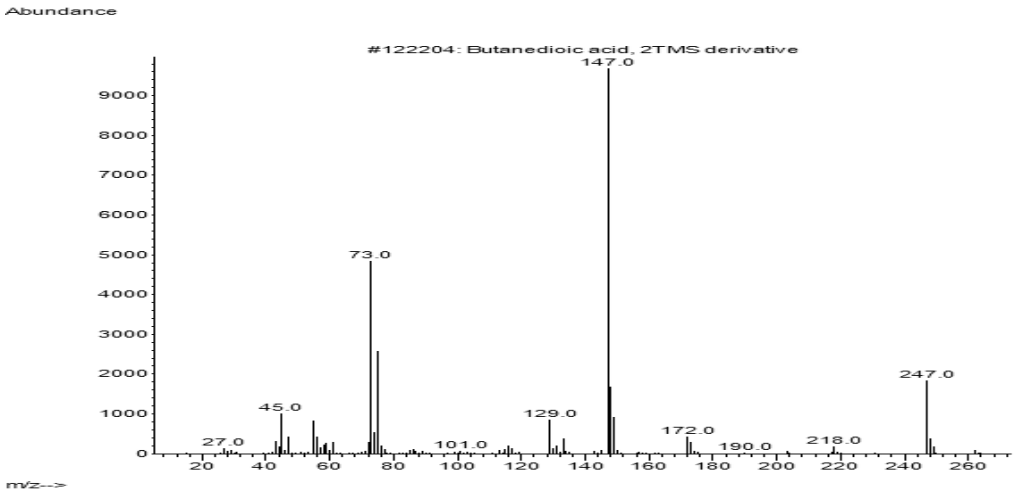


Figure (8): The mass spectrum of Butanedioic acid obtained from *Chlorella vulgaris*

Discussion

The present study was achieved aiming at the screening of antimicrobial activities of three algae belonging to Chlorophyta (*Chlorella vulgaris*), Bacillariophyta (*Amphora coffeaeformis)* and Chrysophyta (*Nannochloropsis oculata*) against some Gram positive, Gram negative bacteria and some fungi. Also this study includes screening of anticancer activities of the mentioned algae. The main reason for this is that bacteria developed a resistance mechanism to fight against most of the synthetic family of antibiotics. Also synthetic drugs have many side effects. The resistant of microbes is due to indiscriminate utilization of commercial antimicrobial. Many scientists investigation been conducted for medicines supported by modern antimicrobial substances from several medicinal plants and algae (**Alagesaboopathi and Kalaiselvi, 2012**). **Kulik** (**1995**) reported that cyanobacteria and eukaryotic algae occur in fresh water, marine and terrestrial soil habitats produce various biologically active compounds. These include antibiotics which in laboratory tests inhibited bacteria and fungi that cause diseases of humans and plants.

In the present study, the methanol was chosen for extraction method. *Chlorella vulgaris* showing the highest antifungal activity against *Aspergillus niger*, *Candida albicans* in agreement with **Ghasemi *et al*.** (**2004**) who found that *C. vulgaris* have antifungal activity against (*A. niger* and *C. albicans*), but **Salem *et al*.** (**2014**) showed that the *Chlorella* methanol extract have no antifungal activity against the four fungal strains tested, this contrast may be due to differences in environmental factors. Also, *C. vulgaris* crude extract showed antibacterial activity against *S. aureus*, *B. subtilis* and *P. aeruginosa*.These results were compatible with the study of **Salem *et al*.** (**2014**) on the antibacterial activity *of C. vulgaris extract against B. subtilis*, *S. aureus, and K. pneumonia* with inhibition zones (17.5, 17 and 14.5 mm) respectively. *Nannochloropsis oculata* showing the highest activity against *B.subtilis*, *E.coli* and *P. aeruginosa*. Also, *Nannochloropsis oculata* showing antifungal activity against *A.niger* and *C. Albicans*. *Amphora coffeaeformis* showing antibacterial activity against *B. subtilis* and antifungal activity against *C. albicans*. All of the tested algae have anticancer activity against human Hepatocellular cancer cell line.anticancer activity of selected algae was also observed by (**Hasegawa *et al*., 2002; Lauritano *et al*., 2016; Sanjeewa *et al*., 2016**).

Anticancer activity of the methanol extracts of the algae was determined in terms of IC_{50} value, high anticancer activity was observed at the concentration of 500 µg/ml. *Nannochloropsis oculata* has the lowest IC_{50} value, so it need less concentration of *Nannochloropsis oculata* extract to achieve the desired effect.

In the present investigation, the test organism, *Chlorella vulgaris* contain several compounds that were inhibiting the growth of pathogenic bacterium. Myristic acid, palmitc acid and butanedioic acid have good antimicrobial activity. Myristic acid, palmitc acid are fatty acids, the bactericidal and antifungal properties of fatty acids are well known (**Kabara *et al*., 1972; (McGaw *et al*., 2002; Seidel and Taylor, 2004**) reported that palmitic and myristic acids are known to have potential antibacterial and antifungal agents. Butanedioic acid is a dicarboxylic acid. **Mokbel and Hashinaga** (**2005**) tested the effect of butanedioic acid against some Gram negative and positive bacteria and found that butanedioic acid has antibacterial activity against *S. aureus, B. subtilis*, *B. cereus, S. enteritidis* and *E. coli***.**

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نشاط مضادات الميكروبات ومضاد للسرطان لبعض أنواع الطحالب الدقيقة

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تم في هذا البحث اختبار 3 طحالب معزولين من بيئات مختلفة للتعرف علي فاعليتهم ضد البكتيريا والفطريات، كما تم اختبار فاعليتهم كمثبط للخلايا السرطانية في الكبد، وقد اوضحت النتائج ان كل من الطحالب قيد الدراسة له فاعلية بدرجة ما ضد علي الاقل نوع من البكتيريا ونوع من الفطريات، كما وجد ان جميع الطحالب لها تاثير مثبط ضد الخلايا السرطانية. تم اجراء تجزئة بواسطة عمود الكروماتوجرافي لطحلب كلوريلا فولجارز للتعرف علي المواد الفعالة ضد البكتيريا، وتم الحصول علي 3 اجزاء كان لها تأثير ضد البكتيريا وتم تحليلهم بواسطة الجي سي ماس لمعرفة مكوناتهم.