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ANTICANCER ACTIVITY OF *SPIRULINA PLATENSIS* METHANOLIC EXTRACTS AGAINST L20B AND MCF7 HUMAN CANCER CELL LINES

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Abstract

Spirulina platensis was isolated and identified microscopically and genetically through phycocyanin (cpcBA) genes detection and safeguarding the safety of isolated *Spirulina platensis* through detection of microcystin producing gene *(mcyE)* gene via PCR technique. To assess the cytotoxic effects of *Spirulina platensis* hot methanolic extracts on L20B and MCF7 human cancer cell lines, Various concentrations of *Spirulina platensis* extracts (mg/mL) obtained with 70% methanol solvents were used to treat cell lines after 24 h. and 48 h. exposure time, MTT assay was achieved For cytotoxic effect studies, Chemical analyses and finally GC mass analysis for crude extracts were done to identify the most active chemical compounds, hot methanolic *Spirulina* extract exhibited notable cytotoxicity against two tested cancer cell lines, the highest percentage (32.5%), (71.5%) of growth inhibition was observed with the treatment using 25 mg/mL, 12.5 mg/ml against L20B and MCF7 respectively. These percentage were increased after 48 hr. application to (35.5%) against L20B, and (78%) against, phytochemical analysis showed that the active chemical compounds from extracts were contains alkaloids, phenols, Terpenes, Steroids, Flavones, Resins, Saponines, proteins, amino acids and tannins. Finally the result of GC mass analysis for extracts proved the existence of many biologically active compounds including 11 anticancer compounds.

Key words: *Spirulina platensis*, methanolic extracts, anticancer agent, bioactive compounds.

Introduction

Cancer is one of the most severe diseases that threaten the health of human all over the world, one of the main treatments commonly used to treat cancer by killing or inhibiting the growth of cancer cells is chemotherapy. Besides that, this group of drugs are associated with toxicity and very unpleasant also may be life threatening. There is a growing interest in marine biological resources, especially microalgae and seaweeds as sources of bioactive materials (Monteiro *et al*., 2014). There has been a lot of devotion to natural substances obtained from marine algae to discover their therapeutic and medicinal properties for instance anticancer, antioxidant and antibacterial effects (Tannoury *et al*., 2017). Numerous screening studies have been accomplished over the past years to discover new antibiotic or cytotoxic metabolic compounds of microalgae particularly cyanobacteria and green algae (Fayyad & Dwaish, 2016 a). Because algal types were used for cancer treatment, many crude extract and compounds derived from different algae have been estimated for their antitumor activities (Mohamed *et al*., 2012) (mcf7) *Spirulina*, a filamentous cyanobacterial genus, researchers deals with botany classify it as a micro alga belonging to class cyanophyceae, its structure is simple but a composition is complex. *Spirulina* and its constituents have been shown to have positive advantages across arange of human health indications from overcoming malnutrition to using as antioxidant. One of its species *Spirulina platensis* or its extract revealed therapeutic properties, such as preventing cancer ability (Abu Zaid *et al*., 2015).

Among large number of *Spirulina* species, three species of, including *Spirulina platensis (Arthrospira platensis), Spirulina maxima (Arthrospira maxima)* and *Spirulina fusiformis (Arthrospira fusiformis)* are most widely investigated as those *Spirulina* species that edible with high nutritional and potential therapeuticvalues (Deng & Chow, 2010). *Spirulina platensis* or itsextract show pharmaceutical properties, such as the ability tofight cancers, reduce the level of blood cholesterol, decrease nephrotoxicity of drugs and toxic metals and protect against the harmful radiation effects (Kumar *et al*., 2011) (water extracts) *Spirulina platensis* is cultivated under controlled culture conditions still, certain other harmful cyanobacteria grow along with it, and contaminating it. These cyanobacteria produce metabolic substances (cyanotoxins) that including microcystins (MCs), MCs principally cause changes in functioning and morphology of the hepatocytes thus inhibiting the activity of phosphatase protein both *in vivo* and *in vitro*. Controlling the growth of harmful cyanobacterial species is struggled, but the contamination still occurs (Manali *et al.,* 2018). Many challenges in curing cancer for patients, including decreasing treatment-related

adverse events, managing triple-negative breast cancerdespite poor outcomes and the lack of a therapeutic target and balancing treatment toxicity with quality of life in patients with metastatic cancer who have already received inclusive therapy. (Yezhelyev *et al*., 2006).

To overcome these difficulties, researchers have suggested the use of *Spirulina platensis* methanolic crude extract against human breast cancer cell line (MCF7) and against the human cancer cell line L20B. after safeguarding the safety of isolated *Spirulina platensis* through detection of microcystin producing gene via PCR technique.

Materials and Methods

Collection, Isolation and Purification of Spirulina platensis

The samples were collected from water canal of Baghdad university campus. This station located in AL-gadrieyah in Baghdad. This station located on longitude(44° 24' 4.9026"E) and latitude (33° 21' 56.2026"N) and are isolated by streak plate method (Stein, 1973). Zarrouck nutrient solution solidified by 2% agar-agar and adjusted pH to about 10 then autoclaved, after sterilization with 45-50 ºC was poured in petri-dishes and left to solidify. Then the surface of each plate was inoculated with 1 ml of sampled water, the inoculum distributed with a sterile spreader or streaking using a sterile loop. The inoculated plates were kept in a cooled illuminated incubator with about 200 µE/m²/s light intensity and 26± 2 ºC for 10-12 days. Aggregated colonies were observed on the surface of plates. Part from these colonies was stroke on other plates. Each subculture was examined intervally, this method was repeated till a uni algal culture or cultures have been gained (Stein, 1973). A small part of uni algal culture (which was microscopically confirmed as uni algal culture) was transferred into Zarrouck nutrient solution within a 250 ml sterile flask and incubated for 2-3 weeks according to method of (Jawad, 1982) to get appropriate growth. In order to sustain the viability of the uni algal growth, these cultures should be renewed every two weeks by sub culturing into another Zarrouck nutrient solution obtained pellets have been used for extraction.

Table 1 : Ingredients of selective medium (Zarrouck medium) (12)(Zarrouk, 1966).

ingredients	$NaHCO_3$	NaCl	$MgSO_4$, $6H_2O$	$FeSO_4$, $6H_2O$	K_2SO_4	$CaCl_2$, $2H_2O$	$NaNO_3$	K_2HPO	EDTA	Distilled water
Amount (g/L)	16.8	1.0	0.2	0.01	1.0	0.04	2.5	0.5	0.08	1000 ml

Morphological and molecular identification of isolates

Obtained algal isolates were identified according to its morphological features with help of classical algal classification reference (Desikachary, 1959). Also molecular identification of isolated Spirulina was done by using primers PCβF: GGCTGCTTGTTTACGCGACA, PCαR: CCAGTACCACCAGCAACTAA. Via PCR technique .This set of primers produced a 650 bp. gen fragment from phycocyanin operon. these primers are used to confirm the presence of cyanobacterial genome (Nguyen *et al*., 2014), Also chlorophyte Chlorella sp. Was used as control negative for phycocyanin producing gene. For PCR analysis DNA was extracted and PCR reaction was programmed according to (Fayyad & Dwaish, 2016 b).

Molecular detection of microcystin producing genes

Molecular detection for the ability of isolated *Spirulina to produce cyanotoxin* (*microcystin and nodularin*) by amplification of aminotransferase (AMT) domain which is located on the module *mcy* E of the microcystin synthetas gene cluster because of its essential function in the synthesis of all microcystin and nodularin. by using (HEPF/ HEPR) primer which amplify a 472bp. HEPF: TTTGGGGTTAACTTTTTTGGGCATAGTC, HEPR:AATTCTTGAGGCTGTAAATCGGGTTT. PCR product from the AMT domain of all tested hepatotoxic species (Jungblut & Neilan, 2006). By using PCR analysis. *Microcystis aeruginosa* was used as control positive for *mcy*E gene. DNA was extracted and PCR reaction was programmed according to (Fayyad & Dwaish, 2016 b).

Preparation of Algal Extracts

S. platensis isolate were cultivated inbioreactors with Zarrouck Spirulina nutrient medium in order toobtain a high concentration of vegetative cells. After a period of 2 weeks in aerated bioreactors. To harvest biomass, cells were centrifuged and used for extraction after removal of excess water content. Fresh algal biomass grinded and 1 g of fresh biomass was used for every 10 mL of solvents: 70% methanol, and then extracted by using Soxhlet. After 24 h, the solution was

centrifuged for 15 min at 10000 r/min, and then collected liquid phase was used for further process. The solvent was evaporated using a rotary evaporator at 50 C. After measuring the weight of dry extracts, stock solution of 100 mg /ml prepared by dissolving 2 gm of dry weight in 20 ml dimethyl sulfoxide solution(DMSO). The extracts used for evaluation were sterilized by filtration with 0.20 mm membrane and kept at −80 C in the dark till used for further analysis.

In vitro Anticancer Activity

The anticancer efficacy of methanolic extract from *Spirulina platensis* against L20B and MCF7 cell line was evaluated. The colorimetric cell viability MTT assay was used as described by (Chih *et al*., 2004) & (Freshney, 2012) At first, 100 µL/well of RD cells (106 cell/ mL) were cultured in 96-well tissue culture plate. Different concentrations of Spirulina extract test solution were prepared to evaluate cytotoxic effect against two examined cell line (50, 25, 12.5 mg/ mL) in water. Then, 100 µL of various concentrations was added to each well and incubated at 37 ºC for 24h, 28h. After the incubation, 10µL of MTT solution (5 mg/ mL) was added to each well and incubated at 37 ºC for 4 h. Finally, 50 µL of DMSO (dimethyl sulfoxide) was added to each well and incubated for 10 min. L20B and MCF7 cells were cultured in complete medium without algal extract solution as a control. The absorbance was measured for each well at 620 nm using an ELISA reader. Only viable cells able to take the stain while the dead cells were not. The live cells, percentage of viability and inhibition ratio were calculated according to the formula

$$GI\% = \frac{(OD\,of\,control\,wells - OD\,of\,test\,wells)}{OD\,of\,control\,wells} \times 100.$$

Evaluation Some of the Active Compounds in the Algal Extracts

The presence of active compounds in the studied algae was determined by adopting standard protocols (Trease&Evans, 1989), (Harborne, 1998)

Gas Chromatography-Mass Spectrometry

For GC-MS analysis, a high-temperature column (Inert cap 1MS; 30 m × 0.25 mm id ×0.25µm film thickness) was purchased from Agilent Technologies (SHIMADZU-Japan),by employing a high-temperature column. Derivatization of each sample was eliminated. The injector and detector temperatures were set at 280°C while the initial column temperature was set at100°C. A 5 µL sample volume was injected into the column and ran using split (1:10)mode After 1 min, the oven temperature was raised to 225°C at a ramp rate of12.5°C/min (hold time 4 min). The oven temperature was then raised to 300°C at a ramp rate of 7.5°C/min (hold time 5 min).The helium carrier gas was programmed to maintain a constant flow rate of 17.5mL/min and the mass spectra were acquired and processed using both Agilent GC-Mass. Solution (SHIMADZU—Japan) and postrun software. The compounds were identified bycomparison of their mass with NIST library search and authentic standards.

Results and Discussion

Morphological and molecular identification of isolates

The main morphological feature of *Spirulina platensis* is non-heterocystous multicellular cylindrical trichome arranged in an open helix shape with evident cross-walls (Fig.1).

Fig. 1: Microscopic morphology of isolated *Spirulina* (40X)

In the current study, the phycocyanin operon gene fragment containing the IGS (*cpcBA*-IGC) from *Spirulina platensis* isolate was amplified. A distinct amplicon patterns was produced from DNA extracts with a size about 650 bp. While there was no amplification for chlorophyte *Chlorella* sp. when analyzed in gel electrophoresis (Fig. 2), confirming the presence of cyanobacterial DNA from *Spirulina* isolate.

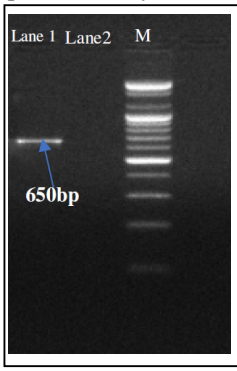

Fig. 2: Gel electrophoresis in Agarose (1.5%), 5 V/cm for 2 hr., stained with ethidum bromide and visualized under a UV transilluminator (A): **amplified cpcBA-IGC (650bp.)** Lane 1: isolated *Spirulina platensis*, Lane 2: negative control *Chlorella sp*., Lane M:100 bp DNA ladder

Microcystin producing gene detection in term to MCs producing gene detection, the results revealed The absence of amplification of mcye gene in *Spirulina*. while microcystin producing gene is present for *Microcystis aeruginosa* this certified the safety of isolated *Spirulina platensis*-based using as anticancer drug (Fig. 3)

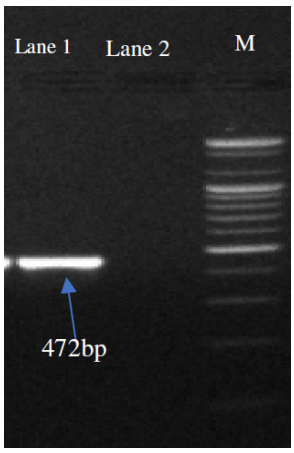

Fig. 3 : Gel electrophoresis in Agarose (1.5%), 5 V/cm for 2 hr., stained with ethidum bromide and visualized under a UV transilluminator : amplified mycE(472bp), Lane 1:positive control *Microcystis aeruginosa* , Lane 2: isolated *Spirulina platensis,* Lane M:100 bp DNA ladder

Cell line growth and cytotoxicity assay

To evaluate the potential as anticancer drugs, the hot methanolic extracts of blu-green alga *Spirulina platensis,* was investigated by using in vitro cytotoxicity against human breast cancer cell line (MCF7) and against the human cancer cell line L20B.

The cytotoxic activity of methanol extracts of *Spirulina* against both tested human cell line exhibited a dose and time-dependent inhibitory effect. The methanol extracts of *Spirulina*., showed potential inhibitory effect as compared to the cell control. on the growth of L20B and MCF7 the results are represented in tables 2 & 3Three different concentrations (50, 25, 12.5 mg/mL) of methanol extract were applied and growth inhibition percentage was calculated after 24 and 48 hr. after application. The highest percentage (32.5%), (71.5%) of growth inhibition was observed with the treatment using 25 mg/mL, 12.5 mg/ml against L20B andMCF7 respectively. of methanol extract. These percentage were increased after 48 hr. application to (35.5%) against L20B and (78%) against MCF7, While the lowest growth inhibition percentage was calculated as (20%) and (35.1%) with the treatment using 50 mg/mL against L20B andMCF7 respectively, also these percentages were increased after 48 hr. application to (21.4%) against L20B and (68%) against MCF7 (Fig. 4, 5).

Table 2: Cytotoxic effect of various concentrations of *Spirulina platensis* methanolic extract on growth of L20B cell lines after 24 and 48 hr. incubation time.

	After 24 hr.		**After 48 hr.**	
Concentration	OD. Mean+-sd	GI%	OD. Mean+-sd	GI%
A	0.0794+-0.004	20	0.142+-0.007	21.4
B	0.067+-0.007	32.5	0.117+-0.0007	35.5
C	0.23+-0.165	31.5	0.102+-0.008	43.8
Control	0.0994+-0.07		0.181+-0.03	

A:50 mg/ml,B:25 mg/ml, C:12.5 mg/ml, OD: optical density, GI%: growth inhibition percentage

Table 3: Cytotoxic effect of different concentrations of *Spirulina platensis* methanolic extract on growth of MCF7 cell lines after 24 and 48 hrs incubation time.

	After 24 hr.		**After 48 hr.**	
Concentration	Mean+-sd	GI%	Mean+-sd	GI%
A	0.0645+-0.002	35.1	0.432+-0.03	68
B	0.0965+-0.062	71.2	0.344+-0.04	74
C	0.0955+-0.009	71.5	0.285+-0.04	78
Control	1.354+-0.19		0.336+-0.06	

A:50 mg/ml,B:25 mg/ml, C:12.5 mg/ml, OD: optical density,GI%: growth inhibition percentage

The results of the present study recommend that the methanol extract of *Spirulina platensis*, possibly will used as anti-cancer drug in the near future. These results agreed with (Mofeed *et al*., 2018), which reported that Human breast adenocarcinoma cell line growth was inhibited by using crude extract of *Spirulina platensis* also Several studies indicated anti-tumor effect of *Spirulina platensis* crud extract against several human cell line, as (Abd El Sadek *et al*., 2017) who used methanolic extract of *spirulina* as potentially anticancer agent to treat Ehrlich Ascites Carcinoma (EAC). (Abu-zaid *et al*., 2015) reported that cancer chemotherapeutic drugs observing Many side effects include hairloss, diarrhea, mouth sores nausea, vomiting, loss of appetite

and fatigue. thus, new anticancer drug should beinvestigated from various resources. A great number of antitumor compounds are natural products or their derivatives, mainly manufactured from blue-green algae. Further studies are suggested to identify and purify the specific anti-cancer compounds in the pointed extracts for the development of cancer therapy. The identification of specific metabolites from seaweeds is also recommended for the discovery of potential anti-proliferative or anticancer compounds. Due to a diverse chemical ecology, the marine organisms, especially marine flora have a great promise for production of powerful, cheaper, and safer antitumor drugs, which bring in an extensive investigation.

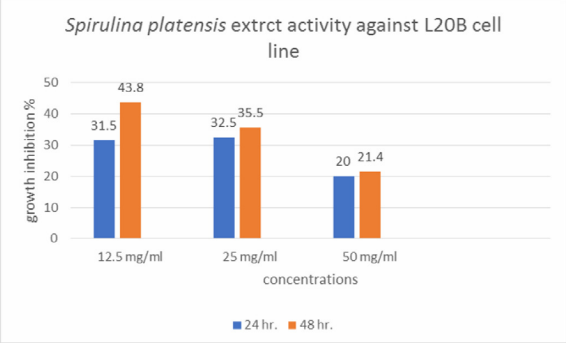

Fig. 4 : Effect of various concentrations of *Spirulina platensis* methanolic extract on cancer cell line (L20B) during different exposure times.

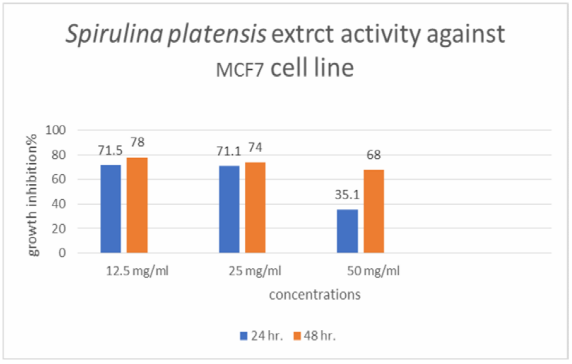

Fig. 5 : Effect of various concentrations of *Spirulina platensis* methanolic extract on cancer cell line (MCF7) during different exposure times

Evaluation of Phytoactive Compounds:

The primary detection (Presence or absence) for the active components shown in Table (4) for hot methanolic algal extract, the results showed that the crude methanolic extract contains many active chemical compounds such as Saponines, phenols, tannins, glycosides, alkaloids, Flavonoids, polysaccharides, proteins, Resins and amino acids the mean of pH extracts was (5.5-6)

Table 4 : Presence or absence of active compounds in *Spirulina platensis* Hot Methanol Extracts

Chemicals Compound	Phenols	Tannins	alkaloids	glycosides	Flavonoids	Polysaccharides	Proteins& amino acids	Resins	pH
Presence	+	+	+	+	+	+	+	+	5.5-6

+:presence

This results supports the findings of many researchers (ALI & Doumandji, 2017) who identify same compounds in *Spirulina platensis*. Among the known phytochemicals, flavonoids are one of the most popular compounds with a variety of biological activities at nontoxic concentrations. Flavonoids have been widely discussed as promising anticancer agents. This group of compounds are also registered to produce a various effects against tumor cells such as cell growth inhibition, apoptosis induction and inhibition of kinase enzymes (Weng *et al*., 2007) Flavonoids have many effects on cancer cells including inactivation of carcinogen, antiproliferation, arrest of cell cycle. (Chahar *et al.,* 2011). Terpenoids has numerous therapeutic properties including anticancer, antiallergenic, antiparasitic, anti-inflammatory and immuno-modulatory activities (Das, 2015). Similarly, the other compounds detected in the extracts, saponins, and alkaloids are also reported to have anticancer effect by various authours. Inhibition of growth and induction of apoptosis effects of saponins in tumor xenograft and human colon cancer cells have been reported (Chanu *et al*., 2018) also resins and polyphenols showed high antioxidant and anticancer power activity (Rahman, 2018).

Evaluations of Gas Chromatography-mass Spectrometry for Algal Extracts

GC-MS analysis of the hot methanolic extract *Spirulina platensis* showed Thirty six compounds (Fig. 6), most of these compounds possessing different biological activities, chemical compounds that may observed anticancer and antioxidant activities are listed in table (5) which together accounted for 66.88% of the total mass, the cytotoxicity exhibited by Spirulina extract to cancer cell lines might be due to the presence of N-Methyl-N-methoxyacetamide with beak area 14.35%, n-Hexadecanoic acid% 10.6, Octanoic acid, 2-ethylhexyl ester 14.09%, 8-hexadecyn-1-o 21.87% that were reported previously as constituents of the extract

because they are occupying the larger area. So crude extracts of Spirulina can be used as a source to develop anticancer drugs., our finding agreed with (Mofeed *et al*., 2018) and (Diana & Parthipan, 2015) who reported themost similarly compound where isolated from blue-green algae.

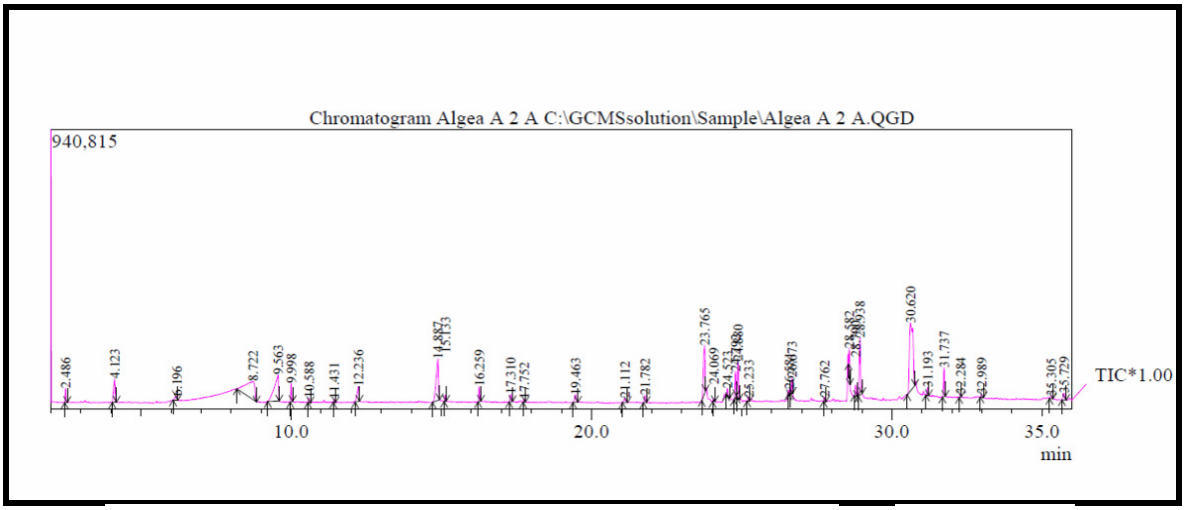

Fig. 6 : The chromatogram of GC-Mass spectrophotometery showed the **70%** methanolicextract of *Spirulina platensis*

Table 5 : Major Phyto-components and its biological activities obtained through the GC/MS Study of *Spirulina platensis* have been listed

no.	Name of compound	RT	Area%	Biological activity	References
1	N-Methyl-N-methoxyacetamide	8.722	14.35	antitumor, antimicrobial, inhibitor of anthrax lethal factor, antiinflammatory, trypanocidal, antidiabetic, andantimalarial agents	Ismail *et al*., 2015
2	n-Hexadecanoic acid	12.236	(10.6)	Anti-inflammatory, Antioxidant, nematicide,pesticide,antiandrogenic flavor, hemolytic, 5-Alpha reductase inhibitor	Aparna *et al*. (2012)
3	Hexadecanoic acid, ethyl ester	15.133	(1.23)	5 Alpha reductase inhibitor Antiandrogenic Antioxidant FlavourNematicide Pesticide Antioxidant Hypercholestrolemic	Diana and Parthipan, 2015
4	:Octanoic acid, 2-ethylhexyl ester	16.259	14.09	Cancer preventive Anti-inflammatory Perfumery Flavor InsectifugeHypocholesterolemicAntiandrogenic	Diana and Parthipan, 2015
5	Tridecanoic acid ethyl ester	17.752	0.28	Anticancer NematicideHypercholestrolemic Lubricant Cosmetic Antioxidant	Vasudevarao and Sravanthi, 2017
6	Tetradecanoic acid	17.310	(1.44)	Anticancer,Nematicide,Cosmetic Antioxidant	Khairy & El-Kassas,2 010
7	Phytol 2-Hexadecen-1-ol	24.069	0.24	Anticancer,Antimicrobial Anti-inflammatoryDiuretic,preventive and therapeutic results against arthritis	Ogunlesi *et al*., 2009
8	tetracosane	28.790	(1.38)	Antioxidant,pesticides	Kalegar *et al*.,2012
9	8-hexadecyn-1-o	30.620	21.87	anticancer,antioxidant,antiinflammatory,antidiuretic ,antimicrobial	Dwaish *et al*., 2018
10	Decanohydrazid	32.989	0.34	Anticancer,anti-inflammatory anticonvulsant antiviral .	Popiołek, 2017
11	2H-1-Benzopyran-6-sulfonamide	35.729	1.06	Anticancer,Antibacterial,antiviral	Ghorab *et al*., 2017

Conclusions

It can be concluded from this study that *S. platensis* biomass showed considerable content of bioactive Compounds explaining the high anticancer and antioxidant capacity, in addition *S. platensis* water extracts showed antiproliferative properties against breast cancer adenocarcinoma cell line (MCF-7) and mice intestine carcinoma cell line (L20B) suggesting that new promising anticancer natural products from blue-green algae are possible. However, further studies are needed to display *S. platensis* anticancer properties towards other kinds of cell lines and to fully discover the mechanisms by which its extracts cause cell death; this will be the subject of interest in our future researches.

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