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Cytotoxic effect of *Spirulina platensis* extracts on human acute leukemia Kasumi-1 and chronic myelogenous leukemia K-562 cell lines

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ABSTRACT

Objective: To evaluate the cytotoxic effects of *Spirulina platensis* extracts on acute leukemia Kasumi-1 and chronic leukemia K-562 cancer cell lines.
Methods: Various concentrations of *Spirulina platensis* extracts (0.25–50.00 mg/mL) obtained with different solvents were used to treat cell lines for 72 h. For cytotoxic effect studies, cell viability test with trypan blue solution, MTT assay and microscopic cyto-morphological assessment were done.
Results: *Spirulina* extract obtained with 70% ethanol showed significant cytotoxicity in K562 and Kasumi-1 cell lines. With trypan blue solution, IC_{50} values were found to be 4.64 mg/mL for K-562 and 3.68 mg/mL for Kusumi-1 cell lines. *Spirulina* aqueous extract also showed cytotoxicity with trypan blue method, at a slightly higher dose; where IC_{50} values were 12.68 mg/mL for K-562 and 2.13 mg/mL for Kusumi-1 cell lines. The IC_{50} values were found 0.40 mg/mL for K-562 and 0.31 mg/mL for Kusumi-1 cell lines for the 70% ethanol extract according to the MTT assay. *Spirulina* extract obtained with water also showed cytotoxicity but the dose was a little higher where IC_{50} values were 15.77 mg/mL for K-562 and 9.44 mg/mL for Kusumi-1 cell lines. The effect of cytotoxicity with ethanol extract is quite comparable with that observed for cyclophosphamide, which is a chemical used as anticancer agent.
Conclusions: The cytotoxicity exhibited by *Spirulina* extract to cancer cell lines might be due to the presence of phytopigments (carotenoids, chlorophyll, phycocyanin) as well as polysaccharides that were reported previously as constituents of the extract. So crude extracts of *Spirulina* can be used as a source to develop anticancer drugs.

1. Introduction

Chemotherapy is one of the main treatments commonly used to cure cancer. Besides that, a group of drugs are used to kill or inhibit the growth of cancer cells. These drugs are associated with toxicity, which is very unpleasant and may be life threatening. There is a growing interest in marine bioresources, particularly seaweeds and microalgae as sources of bioactive substances [1]. *Spirulina platensis* (*S. platensis*) draw attention because of its nutritional and various medicinal properties [2]. According to previous reports, both polysaccharides and phycobiliproteins from certain algal species are capable of inhibiting the growth of tumor cells [3–8]. Phycocyanin extracted from *S. platensis* has inhibitory effect on the growth of cancer cells. C-Phycocyanin (C-PC) is one of the major biliproteins in *S. platensis*, and functions as a light-harvesting protein in cyanobacteria [2,4]. C-PC is stable and soluble in aqueous solution and content is high in cyanobacteria (up to 15% of proteins in *S. platensis*). Despite of the fact that few human studies have been done so far about the benefits of *Spirulina* to health, the evidence for its potential therapeutic application is still not well interpreted in the areas of immunomodulation, anticancer, antiviral, and cholesterol-reduction effects. To further confirm such findings and to explore the effectiveness of the crude extracts of *S. platensis*, we studied the cytotoxic effects of *Spirulina* extracts obtained with different solvents in the growth of human leukemia cell lines like Kusumi-1 and K-562.

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2. Materials and methods

2.1. Preparation of algal extracts

S. platensis used in our study is a strain identified by scientists of UTEX Culture Collection of Algae, 205 W. 24th St, Biological Labs 218, the University of Texas at Austin (A6700), Austin, TX 78712, USA. They have their own depository and this strain we used was collected from them containing *S. platensis* LB2340. *S. platensis* LB2340 were cultivated in bioreactors with Utex *Spirulina* nutrient medium in order to obtain a high concentration of vegetative cells. The culture conditions were at 25 °C under a cycle of light: dark (16:8 h) with 2.5 KLux light intensity for a period of 2 weeks in aerated bioreactors. To harvest biomass, cells were centrifuged and used for extraction after removal of excess water content. Criteria for the extraction phase were constant for each sample, which is cultures of 15–18 days and optical density near 2 at 560 nm were used, which did not show any contamination microscopically.

Fresh algal biomass grinded with liquid nitrogen and 1 g of fresh biomass was used for every 10 mL of solvents: absolute ethanol, 70% ethanol, absolute acetone and water. After 2 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, they were dissolved in cell culture mediums for different cell lines with required concentration. The extracts used for evaluation were sterilized by filtration with 0.20 μm membrane and kept at −80 °C in the dark.

2.2. Cell culture

Cell cultures of Kasumi-1 (CRL-2724) and K-562 (CCL-243) lines were obtained from the American Type Culture Collection (ATCC, USA) for use. Cell line chronic K-562 leukemia was grown in Iscove's modified Dulbecco's medium supplemented with fetal bovine serum 10% and the cell line acute leukemia Kasumi-1 was grown in RPMI-1640 supplemented with fetal bovine serum 20%. Both lines were maintained in humidity 95%, 37 °C and 5% CO_2.

2.3. Cell viability assay

This assay was evaluated by performing triplicate 96-well plates which were inoculated at 100 μL per well of cell suspensions of leukemia cell line Kasumi-1 and K-562 in concentrations of 1×10^5 cells/mL in which dilutions were made inoculating extracts previously prepared with culture medium keeping a final volume of 200 μL and final required concentrations depending on extraction solvent. They were then incubated at 37 °C with 5% CO_2 for a period of 72 h. Then 200 μL of each cell suspension was removed and mixed with an equal volume of 0.4% trypan blue solution (Sigma–Aldrich, St. Louis, MI, USA). After incubation at room temperature for 5 min, the number of unstained (viable) and stained (non-viable) cells were counted using a hemocytometer (Warren Strober 1997).

Cell viability (V) was calculated using the following equation:

$$V = (Cv/Ct) \times 100$$

where Cv is viable cell number and Ct is total cell number.

2.4. MTT cytotoxicity assay

MTT assay method is based on the fact that metabolically active cells can reduce the MTT by the mitochondrial enzyme succinate dehydrogenase to form insoluble purple formazan crystals that are solubilized subsequently, and thus one can measure the metabolic activity of cells by spectrophotometry. This experiment was done by using Cell Titer 96® Non-Radioactive Cell Proliferation Assay from Promega. The cytotoxic activity of *S. platensis* extract was assessed in K-562 and Kasumi-1 leukemia cell lines. The cells were plated in 96-well plates (1×10^5 cells per well) in triplicate and incubated overnight at 37 °C. After 24 h, the *Spirulina* extracts were added from a stock diluted to concentrations ranging from 1.06 to 50.00 mg/mL. A volume of 50 μL of each concentration of *Spirulina* extract was added in triplicate to selected wells. This procedure was carried out in the presence of a blank for each dilution which consisted of 50 mL medium supplemented with 1% fetal bovine serum dilutions and 50 mL of extracts; a negative control consisted of 50 mL of the cell suspension without any treatment spiked with 50 mL of their respective culture medium supplemented with 1% FBS, and a positive control consisted of an anticancer drug (cyclophosphamide) at concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.313 mg/mL. The cells were then incubated for 24 h. Following incubation, 15 μL of the MTT labeling reagent was added to each well and incubated in a humidified atmosphere at 37 °C for 4 h. Following incubation, 100 μL of the solubilizing reagent, sodium dodecyl sulfate (10%) was added to each well and mixed gently for 1 h at room temperature. The absorbance of each well was measured at 570 nm using a spectrophotometer and percent of viability was calculated. The mean extract concentration that was cytotoxic to 50% of the cells (IC_{50}) was calculated.

2.5. Cytomorphological assessment of cell death processes

It was carried out by staining with propidium iodide treated cells from IC_{50} concentration, and pictures were taken with a fluorescence microscope at 24, 48 and 72 h to identify if there was damage to the treated cells to identify necrotic cells in possible apoptosis process or fragmentation of the nucleus in presence of a negative control (untreated cells).

2.6. Statistical analysis

The results of each series of experiments (performed in triplicates) are expressed as the mean values ± SD. Statistical significance of the data was determined using the independent *t*-test; a value of $P \leq 0.05$ was accepted as statistically significant. ANOVA was used to perform statistical analyses. Multifactor ANOVA (two factors) was performed for the results obtained for trypan blue method in the cells lines K-562 and Kasumi-1 treated whit different concentrations of extracts using the STATGRAPICS Centurion program.

3. Results

3.1. Extraction

S. platensis extracts were adjusted to pH 7.2 ± 0.2 and then filtered and stored at −80 °C. The characteristics of extracts were variable in color and end pH, which are summarized in Table 1.

Table 1
Characteristics of different extracts of *Spirulina*.

Extract type	pH	Color
Water extract	7.026	Blue green
Absolute ethanol	7.114	Yellow
Ethanol 70%	7.263	Yellow
Acetone	7.222	Orange yellow

But only water and 70% ethanol extracts were interpreted in this paper showing effective results in cell viability test.

3.2. Determination of the cytotoxic effect of the extracts by trypan blue method

With trypan blue method we found different dose response on cell viability with different cell lines with the type of extract when exposed for 72 h. The 70% ethanol extract was found to be more effective (Tables 2 and 3) on both the cell lines in comparison to absolute ethanol extract and water extract of *Spirulina*. The effects of 70% alcoholic extracts on the viability of the human leukemia cell line K-562 and Kasumi-1 cells are shown in Tables 2 and 3. The IC_{50} value of Kasumi-1 and K-562 cell lines treated with 70% alcoholic extracts was determined as 4.64 mg/mL and 3.68 mg/mL (Table 4) respectively which is very much comparable with the result of a commercial anticancer drug cyclophosphamide. In case of *Spirulina* water extract, the cell viability of cancer cell lines also decreased with increase of the dose of the extract (Table 5), but the IC_{50} values were slightly higher at 2.13 mg/mL and 12.68 mg/mL with Kasumi-1 and K-562 cell lines respectively (Table 4). Absolute ethanol extract of *Spirulina* also has considerable effect on cell viability but less effective than 70% ethanolic extract. Statistical analysis also demonstrated that 70% ethanoic extract had significant effect.

3.3. Determination of the cytotoxic effect of the extracts by MTT method

We examined the cell viability of cancer cell lines with crude *Spirulina* aqueous extracts and the alcoholic extract. With MTT method we found different dose response on cell viability with different cell lines with the type of extract when exposed for 72 h. 70% ethanolic extract was found to be more effective (Table 6) on both the cell lines in comparison to water extract of *Spirulina*. The effects of 70% alcoholic extracts on the viability percentage of the human leukemia cell line K-562 and Kasumi-1 cell are shown in Table 6. The IC_{50} values of Kasumi-1 and K-562 cell lines treated with 70% alcoholic extracts were found to be 0.31 and 0.40 mg/mL, respectively (Table 4), which is very much comparable with the result of a commercial anticancer drug cyclophosphamide. In case of cyclophosphamide, IC_{50} value of Kasumi-1 and K-562 cell lines were found at 4.38 and 3.13 mg/mL, respectively (Table 4 and Figure 1) which has a higher IC_{50} value than 70% alcoholic *Spirulina* extract (Table 6). In case of *Spirulina* water extract, the cell viability of cancer cell lines also decreased with increase of dose of the extract (Table 5), but the IC_{50} values were slightly higher at 9.44 and 15.77 mg/mL with Kasumi-1 and K-562 cell lines, respectively (Table 4). Absolute ethanol extract of *Spirulina* also had considerable effect on cell viability, but found less effective than 70% ethanolic extract.

Table 2
Viability percentage of the Kasumi-1 cell line (trypan blue method) in different types of *Spirulina* extracts (%).

Concentration (mg/mL)	Ethanol 70%	Absolute ethanol	Water
50	0.00 ± 0.00	25.77 ± 9.74	0.59 ± 1.04
25	1.18 ± 2.05	61.35 ± 9.08	2.99 ± 1.04
12.5	5.92 ± 1.02	74.85 ± 8.30	7.78 ± 1.04
6.25	21.89 ± 2.71	93.25 ± 16.70	17.36 ± 4.15
3.12	56.21 ± 2.71	85.28 ± 9.26	24.55 ± 9.89
1.56	68.47 ± 5.49	98.77 ± 12.25	64.67 ± 7.19
Control	100.00 ± 4.10	100.00 ± 12.89	100.00 ± 4.15

Table 3
Viability percentage of the K-562 cell line (trypan blue) in different types of *Spirulina* extracts (%).

Concentration (mg/mL)	Ethanol 70%	Absolute ethanol	Water
50	0.32 ± 0.56	25.77 ± 9.74	0.00 ± 0.00
25	0.65 ± 0.56	61.35 ± 9.08	23.17 ± 5.59
12.5	10.06 ± 5.36	74.85 ± 8.30	50.41 ± 10.37
6.25	43.18 ± 9.76	93.25 ± 16.70	74.39 ± 15.85
3.12	56.49 ± 6.08	85.27 ± 9.26	65.85 ± 8.54
1.56	74.02 ± 5.92	98.77 ± 12.25	91.87 ± 27.50
Control	100.00 ± 3.69	100.00 ± 12.89	100.00 ± 9.21

Table 4
IC_{50} value of Kasumi-1 and K-562 cell lines treated with different type of extracts and cyclophosphamide.

Cell lines	Treatment	IC_{50} value by MTT (mg/mL)	IC_{50} value by trypan blue (mg/mL)
Kasumi-1	*Spirulina* ethanol extract	0.31	3.68
	Spirulina water extract	15.77	2.13
	Cyclophosphamide	4.38	NA
K-562	*Spirulina* ethanol extract	0.40	4.64
	Spirulina water extract	9.44	12.68
	Cyclophosphamide	3.13	NA

NA: Not applied.

Table 5
Viability percentage of the cell lines (MTT method) treated with water extract of *Spirulina* (%).

Concentration (mg/mL)	K-562	Kusumi-1
26.64	14.17 ± 4.65	1.29 ± 0.56
13.32	58.06 ± 3.43	26.86 ± 3.42
6.66	106.74 ± 2.11	66.55 ± 1.45
3.33	103.03 ± 5.82	85.54 ± 0.67
1.66	96.57 ± 3.90	90.18 ± 1.45
0.83	91.59 ± 1.90	84.03 ± 2.45
Control	100.00 ± 4.07	100.00 ± 3.08

Table 6
Viability percentage of the cell lines (MTT method) treated with ethanolic (70%) extract of *Spirulina* (%).

Concentration (mg/mL)	K-562	Kusumi-1
8.04	14.05 ± 4.86	0.43 ± 2.20
4.02	16.18 ± 5.75	18.01 ± 2.61
2.01	26.52 ± 1.21	23.84 ± 1.22
1.01	34.74 ± 0.91	30.63 ± 0.49
0.5	40.31 ± 1.83	38.51 ± 1.80
0.25	64.72 ± 2.29	53.29 ± 1.49
Control	100.00 ± 3.58	100.00 ± 3.08

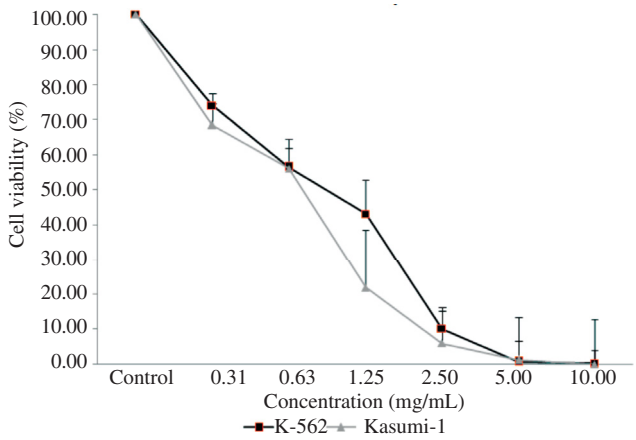

Figure 1. Cell viability (%) with 70% ethanol *Spirulina* extract (trypan blue method).

3.4. Assessment of cell death processes by cyto-morphological studies

In Kasumi-1 cell line, cells treated with *Spirulina* water extract, 70% ethanol extract, or cyclophosphamide, showed cells of varying morphology after 24, 48 and 72 h of treatment. Some were smaller and some were symmetrical segmentation of the nucleus and some with apoptotic bodies (Figures 2 and 3). But in untreated cells, nuclei were large, with their own characteristic cell line morphology and no abnormalities were found. After 72 h of treatment the apoptotic effect was more prominent.

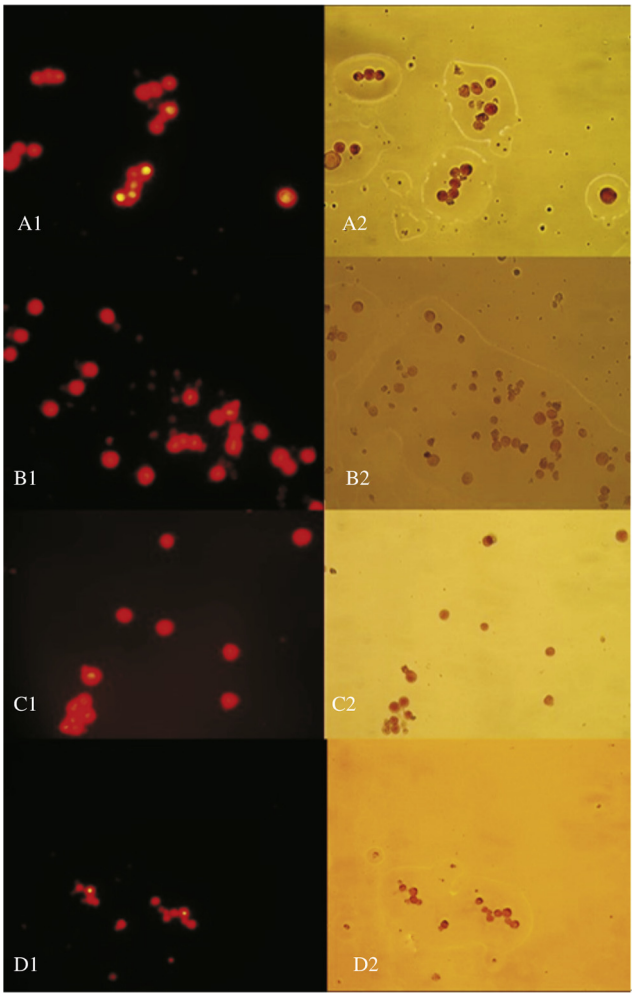

Figure 2. Cells treated for 24 h with *Spirulina* extract in line Kusumi-1. A: Untreated; B: Water extract; C: 70% Ethanol extract; D: Cyclophosphamide (40×). In Figure A1, B1, C1, D1 cells with propidium iodide in fluorescence microscopy and Figure A2, B2, C2, D2 in bright field.

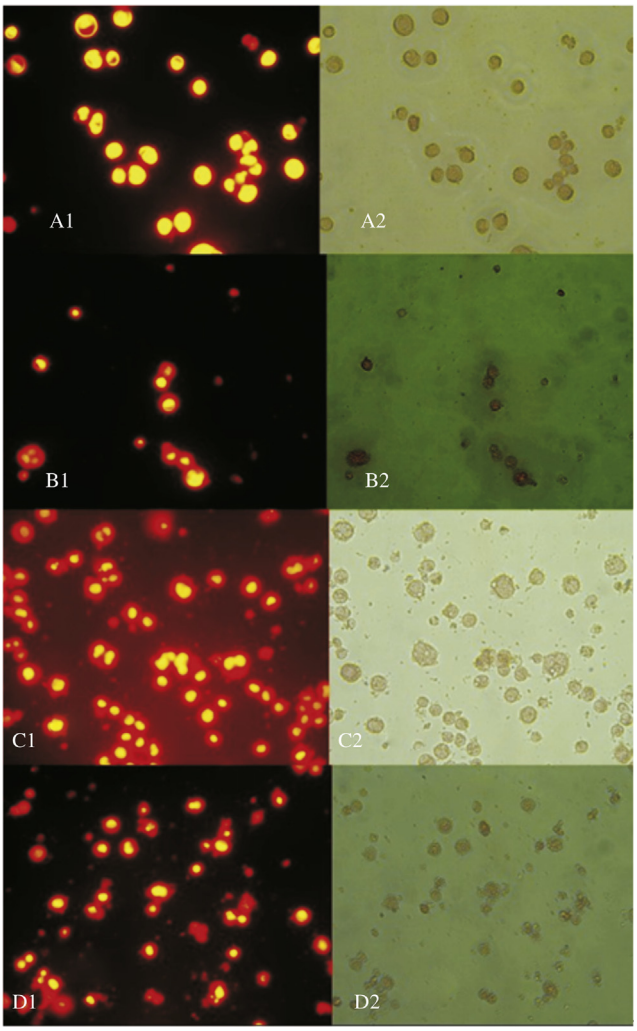

Figure 3. Cells treated 24 h with *Spirulina* extract in line K-562. A: Untreated; B: Water extract; C: 70% Ethanol extract; D: Cyclophosphamide (40×). In Figure A1, B1, C1, D1 cells with propidium iodide in fluorescence microscopy and Figure A2, B2, C2, D2 in bright field.

In K-562 cells treated with *Spirulina* water extract, 70% ethanol extract, or cyclophosphamide, it also showed cells of varying morphology, some were smaller and some were with segmentation of the nucleus and some with apoptotic bodies (Figures 2 and 3). In comparison to Kusumi-1 cell line, K-562 cells showed more apoptotic bodies and abnormal cell morphology. But in untreated cells, nuclei were large, with their own characteristic cell line morphology and normal cells were found (Figures 2 and 3). Here photographs were taken only for the treatment of the IC_{50} dose in both the cell lines. It was clear from the image about the cytotoxic effect of the *Spirulina* extracts at its IC_{50} dose showing apoptotic bodies.

Due to the values for $P \leq 0.05$ with a 95% of confidence level, it was concluded that extracts at different concentrations showed to have a significant effect on cell viability.

4. Discussion

Recent studies have demonstrated *S. platensis* crude extract has antioxidant, antimutagenic, antiviral, and anticancer, anti-allergic, immune enhancing, hepato-protective, blood vessel

relaxing, blood lipid lowering effects and immune functions by promoting immune competent cell proliferation or differentiation [9–12]. Therefore, in this study, we examined the cell viability of cancer cell lines with crude *Spirulina* aqueous extracts and the alcoholic extract.

Spirulina, used in daily diets of natives in Africa and America, has been found to be a rich natural source of proteins, carotenoids and other micronutrients. *Spirulina* contains various phytopigments (carotenoids, chlorophyll, phycocyanin) [13]. So it can elevate the activity of all the antioxidant related enzymes *viz*., superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase significantly. Apart from that, *Spirulina* contains phenolic acids, tocopherols and β-carotene that are known to exhibit antioxidant properties [14].

Phycocyanin reported as a stable and soluble compound in aqueous solution, present in cyanobacteria up to 15% of proteins (*S. platensis*), and non-toxic and for this it has been used in many research applications [2]. Phycocyanin reported as a very important pigment treating cancer in animals and stimulating the immune system [15]. The mechanism behind the anti-tumor effects of phycocyanin may be due to the interference of DNA synthesis in tumor cells [16,17] or by improving the immune functions [18]. It was recently reported that phycocyanin bound to the mitogen receptor on the tumor cell membrane and the surface expression of CD59 stimulated activating the cellular apoptotic signal transduction [19]. *Spirulina* has been evaluated by analyzing in blood cells of *Spirulina* consumers showing that in humans it acts directly on myeloid lineages and either directly or indirectly on NK cells [9]. Feeding of *Spirulina* phycocyanin extract leads to correcting effect within 4 weeks when Wistar rats exposed to X-rays. HeLa cells treated with highly purified C-PC showed a significant decrease in the number of survived cells [19]. Liu *et al.* [6] studied K-562 cells lines and found IC_{50} value of the phycocyanin was 72.5 mg/L for growth inhibition. It was still not well understood the molecular mechanism of growth inhibition and they suggested growth inhibition of K-562 cells by pathways other than apoptosis and a change in expression pattern of the c-myc protein may be involved in this case. But Subhashini *et al.* [12] reported that highly purified C-PC induces apoptosis in K-562 cells by cytochrome *c* release from mitochondria into the cytosol, Poly (ADP-ribose) polymerase cleavage and down regulation of Bcl-2.

Again water soluble polysaccharide from *S. platensis* was investigated and found that the presence of the polysaccharide increased both the repair activity of radiation damaged DNA excision and the unscheduled DNA synthesis [20]. Zhang *et al.* [21] reported polysaccharide extract of *S. platensis* has chemoprotective and radioprotective capability.

β-Carotene is another prominent bioactive compound present in *Spirulina*. Saleh *et al.* [22] reported the β-carotene content ranged from 171.1 to 231.7 (μg/g dry weight) in the *S. platensis* strains. Usually cancerous cell lines are not able to receive growth controlling chemical signals from other cells. β-Carotene opens the membrane communication channels of cancerous and pre-cancerous cells, allowing the body to signal the cancerous line to stop dividing. Thus, foods rich in carotenoids or β-carotene are able to prevent cancers [23]. Particularly, β-carotene and vitamin C have multiple biological activities such as anticarcinogenic [24], antimutagenic [25], antioxidant, anti-inflammatory, antiproliferative [26], and antiatherogenic properties [27]. In addition, these compounds have been used as chemo-preventive agents against cancer diseases in various organs such as the lung, stomach, colon, breast, and prostate [28]. People with diet rich in β-carotene found lower incidence of various cancers [29]. Researchers found that β-carotene exerts a protective effect on cervical cancer [28]. β-Carotene also helps to prevent skin cancers [30].

β-Carotene also helps to prevent skin cancers [31]. Hundreds of plant products are available as nutritional supplements, many of which have not been scientifically evaluated. Here the goal was to find out the effective dose of *S. platensis* crude extracts with different solvents on human leukemia cell lines like K-562 and Kusumi-1. In this study, the different concentrations of water extract were (0.83–26.64 mg/mL) chosen and all shown to inhibit the growth of both cell lines K-562 and Kusumi-1. As the concentration of crude extract increased, this inhibition was more apparent. It may be due to the water soluble fractions of *Spirulina* like phycocyanin and polysaccharide which has anticancer properties reported earlier. In this study, instead of pure fractions of phycocyanin and polysaccharide, crude water extracts were used for its simplicity for extraction and less expensive to see whether it has the same inhibitory effects on K-562 and Kusumi-1 cell lines. The IC_{50} values were found 15.77 mg/mL and 9.44 mg/mL which is practically not very high dose to apply for cell lines.

There are some reports on *S. platensis* ethanol extracts on different cell lines such as breast cancer (MCF7), normal cell (WRL68) and human liver cancer cell line (HepG2). The inhibition growth of cell lines varies according to solvent type, concentration of the solvent and treatment periods. In our study 70% ethanol extract also applied to K-562 and Kusumi-1 cell lines with different solvent concentrations (0.25–8.04 mg/mL) and seemed to have performed better inhibition (IC_{50}) with low effective concentration at 72 h. IC_{50} value found 0.40 mg/mL and 0.31 mg/mL for K-562 and Kusumi-1 cell lines which is very less concentration as comparison to chemical anticancer agent cyclophosphamide that showed IC_{50} value of 3.13 mg/mL and 4.38 mg/mL for the cell lines. So here crude 70% ethanol extract of *S. platensis* showed very significant effect on leukemia cell line growth inhibition. It was more effective than absolute ethanol extract.

Again the observation of morphological characteristics remains a key parameter for determining apoptosis, and its recognition in a transmission electron microscope is considered as the standard method for confirming the apoptotic state in a cell [31]. However, it can only detect late stages of apoptosis, besides being a highly qualitative and subjective to observation and detection method [31,32]. In our experiment, after 24–72 h of treatment, the cells have shown a smaller size than control cells, and some can be seen in a symmetrical fragmentation of the nucleus and with numerous apoptotic bodies. It implies the growth inhibitory effects of *Spirulina* extract on leukemia cell lines.

Currently, increased cost of health care has become a driving force in the shift towards interest in less expensive alternative medicine. So this work suggests *Spirulina* crude extract obtained with water and 70% ethanol can be used effectively for K-562 and Kusumi-1 cell line growth control. Still further confirmatory studies have to be done to identify bioactive compounds present in *Spirulina* extract.

Conflict of interest statement

We declare that we have no conflict of interest.

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