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In vitro assessment of the cytotoxic effects of secondary metabolites from *Spirulina platensis* on hepatocellular carcinoma

Mahboobeh Akbarizare, Hamideh Ofoghi*, Mahnaz Hadizadeh and Nasrin Moazami

Abstract

Background: *Spirulina platensis*, an edible cyanobacterium, is considered as a valuable and natural resource of novel anticancer agents. This study aimed to investigate the anticancer potential of major bioactive metabolites from *Spirulina platensis* on hepatocellular carcinoma cells. The total phenolic and alkaloid content of *S. platensis* were determined using spectrophotometric procedures and thin-layer chromatography. Cellular viability of HepG2 cancer cells and normal fibroblasts was evaluated using MTT assay after 24 h treatment with 0.02–2 mg/ml of alkaloids, phenolic compounds, aqueous, and methanol extracts from *Spirulina platensis*.

Results: Total phenolic and total alkaloid compounds were 150.5 ± 1.18 mg gallic acid equivalents/mg extract and 11.4 ± 0.05 mg atropine equivalents/mg extract, respectively. All tested extracts and compounds demonstrated the inhibitory effect on the viability of HepG2 cells in a dose-dependent manner without cytotoxicity on normal cells. The most potent anticancer activity was induced by alkaloids (2 ± 0.001 mg/ml) with 80% reduction in cell viability and an IC_{50} of 0.53 ± 0.08 mg/ml. IC_{50} values of the aqueous extract, the methanolic extract, and phenolic compounds were 1.7 ± 0.14, 1.28 ± 0.22, and 0.86 ± 0.14 mg/ml, respectively.

Conclusions: This is the first report to demonstrate anticancer effects of alkaloids and phenolic compounds of *Spirulina platensis* in relation to liver cancer.

Keywords: Hepatocellular carcinoma, Cyanobacterium, *Spirulina platensis*, Alkaloid, Phenolic compounds, Anticancer

Background

Hepatocellular carcinoma (HCC) that is considered as the most frequent type of liver cancer is the sixth most common neoplasm in the world, and its incidence continues to rise annually [1]. It is a type of tumor with a poor prognosis and highly resistant to chemotherapeutic agents. Therapeutic options for the treatment of patients with early stage of HCC include surgical resection and transplantation which can improve the 5-year survival rate by 25% [2, 3]. Unfortunately, in most cases, HCC is diagnosed at an advanced stage. Sorafenib, an inhibitor kinase for systemic chemotherapy, is the only approved treatment that increases survival in patients with advanced stage HCC. However, sorafenib is very expensive and has some significant side effects including hemorrhagic complications and thromboembolic and cardiac ischemic events [4]. Therefore, there is a need for searching new efficient agents for the treatment of HCC with low toxicity and side effects. Bioactive compounds from natural sources have received increasing attention due to their broad spectrum of therapeutic properties with minimal side effects [5]. Marine microorganisms have been reported as new sources of a huge number of bioactive compounds with interesting pharmaceutical activities [6]. Among the marine microorganisms, cyanobacteria as an exceptional rich source of bioactive compounds are an interesting target for future researches [7]; these Gram-negative photosynthetic prokaryotes produce bioactive secondary metabolites as quorum sensing inhibitory compounds and also as a chemical defense against invading pathogens. Recently, the role of some of these secondary metabolites in killing cancer cells or as antimicrobial, anti-inflammatory, and antiviral agents has been also proved [8, 9].

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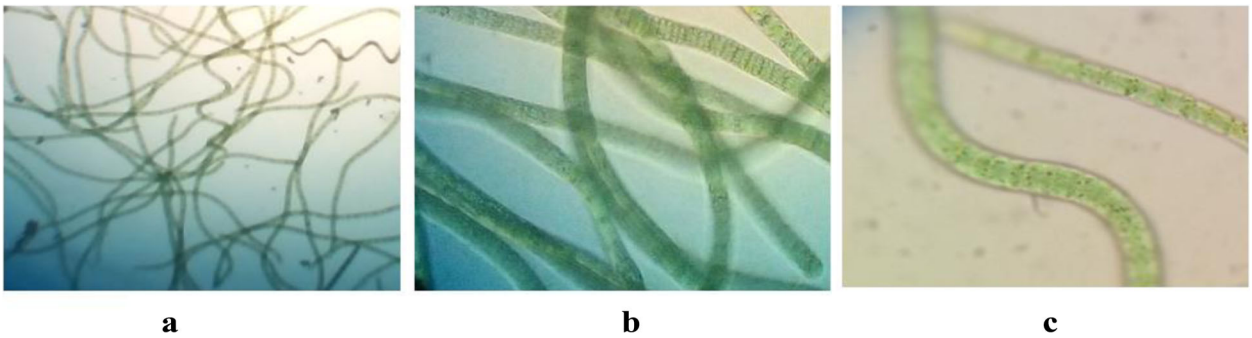

Fig. 1 Light microscopic observation of *Spirulina platensis*. **a** Magnification × 10. **b** Magnification × 40. **c** Magnification × 100

Spirulina platensis (*S. platensis*), a well-known cyanobacterium with a long history of safe human food consumption, is a multicellular filamentous, spiral-shaped (Fig. 1), and photosynthetic microorganism that can easily grow in fresh water, marshes, and seawater [10]. It contains diverse nutrients such as protein, polyunsaturated fatty acids, vitamins (A, E, and B_{12}), minerals, and various pigments. In addition to nutritional value, its potential therapeutic has been also reported [11].

Several studies illustrated that *Spirulina* extracts can stimulate the immune system, improve glucose and lipid metabolism, and prevent or inhibit different types of cancer [12–15]. *Spirulina* has also been claimed to prevent chronic diffusion of liver disease [16]. However, there is little information about the effect of secondary metabolites isolated from this cyanobacterium on human tumor cells, and to data, no studies of alkaloids and phenolic compounds extracted from *S. platensis* were reported on human liver cancer. Although structures of these secondary bioactive metabolites and their pharmacological activities have been determined in plants, there are few studies in cyanobacteria such as *Spirulina* [17, 18]. Therefore, the aim of this study was to determine the total alkaloid and phenol content and cytotoxic effects of the aqueous and methanolic extracts, and the bioactive compounds which are isolated from *S. platensis* on the growth of human liver cancer cell line HepG2 as a model of the hepatocellular carcinoma.

Methods

Materials

Each constituent of Zarrouk's medium was obtained from Merck. Folin-Ciocalteu reagent, MTT (3-4,5-dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide), gallic acid, bromocresol green, and all other chemicals and organic solvents were purchased from Sigma Chemical. Co. Fetal bovine serum (FBS) and other cultural materials were obtained from Invitrogen Corporation (USA). All other chemicals and reagents used in this study were of analytical grade.

Microorganism and growth conditions

The cyanobacterium *S. platensis* utilized in this work was obtained from Dr. M. Amin Hejazi (Agricultural Biotechnology Research Institute of Iran) and was grown in Zarrouk's medium [19], under the condition of pH 10 and 30 ± 2 °C under the illumination of 4 klx light intensity for 15 days. The harvested biomass was dried in a freeze dryer and stored at 4 °C.

Extract preparation

To obtain the aqueous extract, the biomass of *S. platensis* was ground and dried at 40 °C. One gram of dried powder was mixed with 10 ml distilled water and stirred for 60 min. The extract was then centrifuged at 7000 rpm for 20 min. Then the supernatant was separated and dried.

To obtain the methanolic extract, 10 g of dried powder from *S. platensis* was extracted with 90% methanol three times. After filtration, the extract was evaporated at 40 °C to dryness.

Finally, both the extracts (aqueous and methanol) were dissolved separately in DMSO and diluted with culture medium for the cytotoxicity tests.

Isolation of alkaloids

Ten grams of ground *S. platensis* biomass was extracted with methanol. The methanolic extract then was filtrated, and the solvent was evaporated at 40 °C. The crude extract was then extracted with 5% aqueous acetic acid and filtrated. The filtrate was extracted with CH_2CL_2, and the aqueous phase was then basified to pH 10 with 10% Na_2CO_3. The crude alkaloid mixture was then separated from other materials by extraction with CH_2CL_2 and the organic solvent evaporation.

Isolation of phenolic compounds

To isolate phenolic compounds from *S. platensis*, 0.1 g of the biomass was dissolved in 10 ml of distilled water and incubated in shaking water bath at 80 °C for 10 min. Next, the extract was cooled to room temperature and

centrifuge (6000 rpm, 5 min). The resulting supernatant was then separated and dried. Different concentration of the isolated phenolic compounds was prepared with sterile distilled water and used for testing the cytotoxicity activity.

Determination of total alkaloid content

Total alkaloid content was determined using back titration method. One gram of the extract was dissolved in chloroform (25 ml); 25 ml of H_2SO_4 (0.02 N) was then added, and the solution was warmed for the removal of chloroform. Thereafter, the solution was cooled, and excess acid was back titrated with NaOH (0.02 N) and methyl red as an indicator. Each milliliter of sulfuric acid was considered to be 5.8 mg of alkaloids [20].

Determination of total phenolic content

Total phenolic content of the *S. platensis* extracts was determined by the Folin-Ciocalteu method using the gallic acid as a standard (10–100 μg/ml). Briefly, 1 ml of the extract was diluted 1:10 with distilled water and mixture was incubated with 1 ml of the Folin-Ciocalteu phenol reagent for 5 min at room temperature. Then, 10 ml of 7% Na_2Co_3 solution was added to the mixture and adjusted with distilled water to a final volume of 25 ml. The absorbance of the reaction was determined at 720 nm. The total phenol content was calculated from the gallic standard curve [21].

Thin-layer chromatography

The presence of alkaloids and phenolic compounds of *S. platensis* was qualitatively performed by thin-layer chromatography (TLC). Methanolic extracts of *S. platensis* were spotted on pre-coated silica gel 60 F264 plates. Solvent systems used for the separation of alkaloids and phenols were a mixture of methanol:demineralized water: ethyl acetate (16.5:13.5:100) and chloroform:methanol (27: 0.3). After separation of bioactive compounds, Dragendorff and Folin-Ciocalteu reagents were used respectively to identify the alkaloids and phenolic compounds.

Cell culture

Liver cancer cell line HepG2 and normal human fibroblast cells were obtained from the National center for sciences, Pasteur Institute, Tehran, Iran. The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 V/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 incubator. After 90% confluency, cells were treated with 0.25% sterile trypsin and seeded at a concentration of 1.0×10^4 cells/well into 96-well microplates for 24 h prior to the addition of test compounds.

In vitro evaluation of cellular viability

Liver cancer HepG2 and fibroblast normal cells were plated at a density of 1.0×10^4 cells/well into a flat bottom 96-well plate and incubated at 37 °C in a humidified 5% CO_2 incubator. On the second day, cells were exposed to various concentrations (0.02–2 mg/ml) of the aqueous and methanol extracts or the bioactive compounds (phenolic compounds and alkaloids) extracted from *S. platensis*. The well-containing cells treated with DMSO (0.5%) instead of a test compound were utilized as controls. After 24 h of test compound treatment, 10 μl of the MTT solution (0.5 mg/ml) was added to the wells and plates were incubated for 4 h at 37 °C. The supernatant was then removed and the violet formazan crystals were dissolved with 100 μl DMSO, and adsorption at 570 nm was measured using a microplate ELISA Redder (Biotek). The IC_{50} values were calculated from the dose-response inhibition curve.

Evaluation of morphological changes in cells

The morphology and viability of HepG2 cancer cells and normal fibroblast cells were observed in an inverted microscope (CETi) with magnification × 40 after 24 h incubation of cells with 0.02–2 mg/ml of the phenolic compounds, alkaloids, and methanolic extracts from *S. platensis.*

Results

Total phenol and alkaloid content

The content of total phenolic compounds in *S. platensis* extract using the Folin-Ciocalteu reagent was estimated as milligram of gallic acid equivalents per gram of dry weight (mg GA/g DW) of *S. platensis*. The amount of total phenolic compounds extracted from *S. platensis* was found 150.5 ± 1.18 mg GA/g DW. The equation obtained from the standard curve was $y = 0.00017x + 0.0008$.

The alkaloid content was determined in the *S. platensis* extract and expressed in terms of atropine equivalents as milligram of atropine per gram of dry *S. platensis* weight (mg GA/g DW).

The standard curve equation was $y = 0.0009x + 0.0761$. According to the standard curve equation, the alkaloid content of *S. platensis* was found to be 11.4 ± 0.05 mg AT/g DW.

Thin-layer chromatography

The methanol extracts of *S. platensis* were evaluated for the presence of phenolic compounds and alkaloids using the TLC technique. As shown in Fig. 2, a total of four different bands were recorded for alkaloids with Rf values of 0.645, 0.375, 0.16, 0.91, and 0.75 (Fig. 2).

The TLC profile of total phenolic compounds showed more bands (7) than total alkaloids with Rf values ranging from 0.07 to 0.65 (Fig. 3).

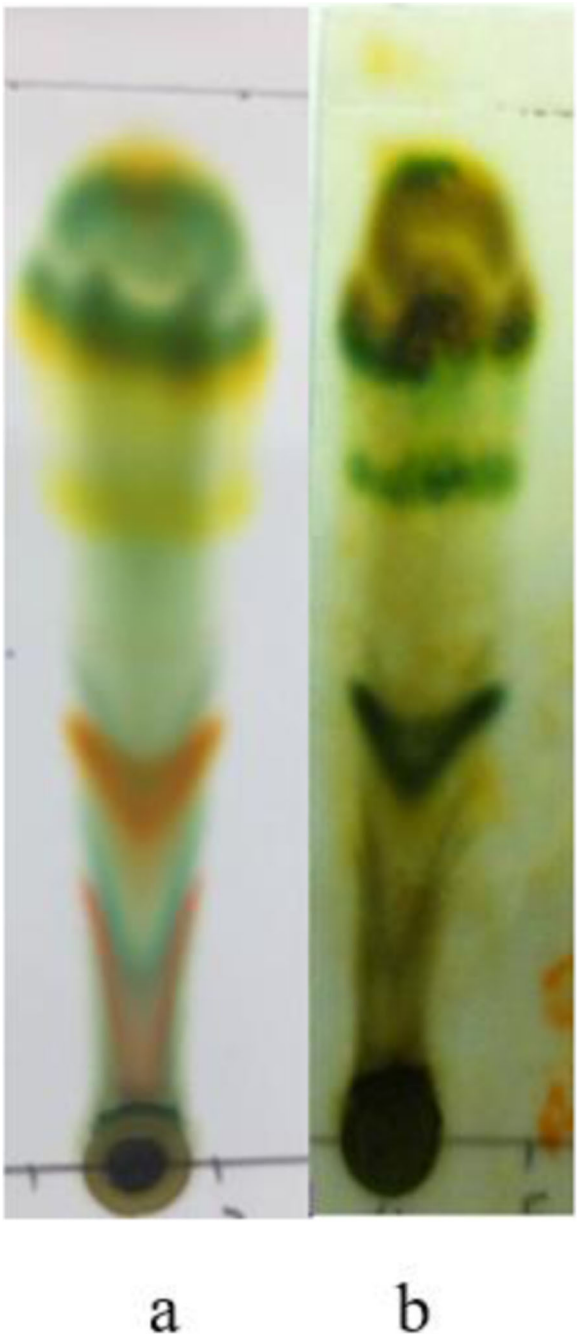

Fig. 2 The profile of alkaloid compounds of *S. platensis* **a** with visible light treatment, before sprayed with Dragendorff's reagent **b** and after sprayed with Dragendorff's reagent

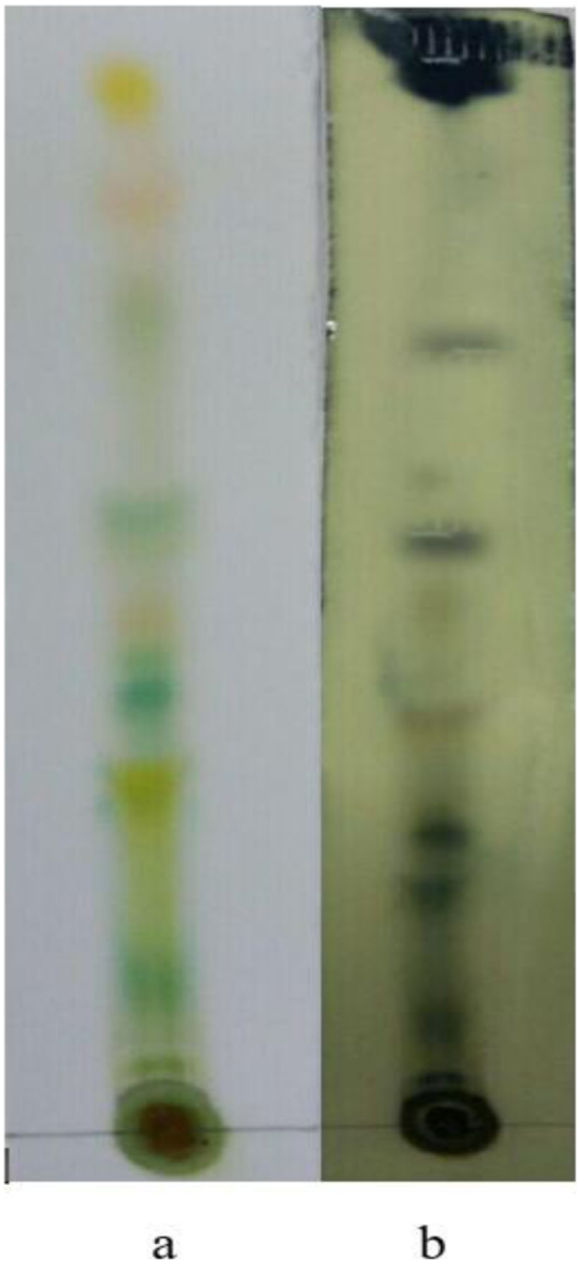

Fig. 3 Detection of phenolic compounds in the aqueous extract from *S. platensis* with **a** light treatment visible and **b** visible, after sprayed with Folin-Ciocalteu reagents

Evaluation of cellular viability of *S. platensis* extracts and some of its bioactive compounds on HepG2 cells

The aqueous and methanolic extracts, phenolic compounds, and alkaloids of *S. platensis* were individually assessed for their effects on cell viability of HepG2 cells. All the tested compounds have shown a cytotoxic effect on liver cancer HepG2 cells in a concentration-dependent manner (Fig. 4).

The result also showed that the bioactive compounds extracted from *S. platensis* were more active than crude extracts against HepG2 cells (~ 3-fold). The most cytotoxic effect against HepG2 cells was observed with total alkaloids with IC_{50} value of 0.56 mg/ml. The IC_{50} values after treatment with aqueous and methanolic extracts and phenolic compounds were 1.9, 1.3, and 0.77 mg/ml respectively (Table 1).

To evaluate the specificity of the tested extracts and compounds to HepG2 cancer cells, the activity of

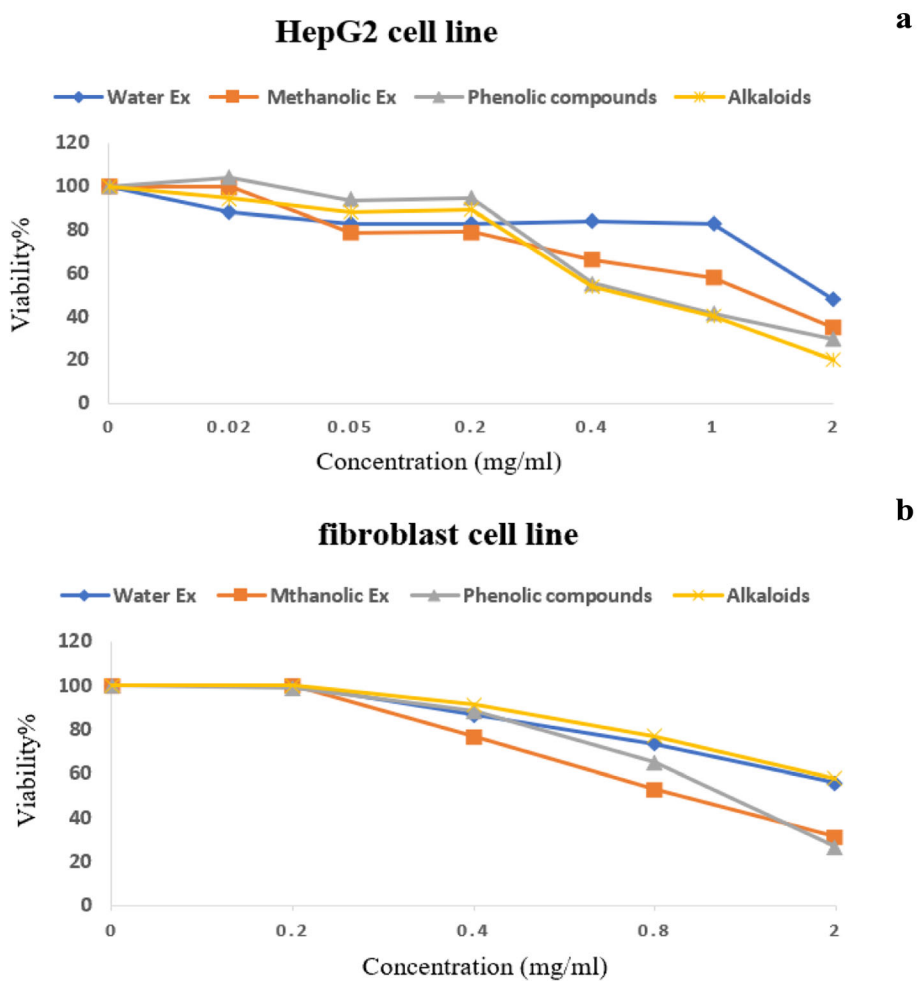

Fig. 4 Evaluation of the cellular viability with different concentrations of phenolic compounds, alkaloids, and aqueous and methanolic extracts of *S. platensis* on HepG2 cells (**a**) and fibroblast cells (**b**) after 24 h incubation. Values are mean ± SD ($n = 3$)

these compounds on the viability of normal cells was also examined by cytotoxicity assay using human fibroblast cells. The cells were treated with the same concentration of compounds that were used for HepG2 cancer cells for 24 h. As shown in Fig. 4, all the tested compounds did not affect the viability of normal fibroblast cells with $IC_{50} > 1$ mg/ml and were more specific to cancer HepG2 cells.

Table 1 IC_{50} values (mg/ml) obtained for the phenolic compounds, alkaloids, aqueous, and methanolic extracts of *S. platensis* on HepG2 and fibroblast cells

Bioactive compounds	$HepG_2$	Fibroblast
Aqueous extract	1.70 ± 0.14	2.34 ± 0.06
Methanolic extract	1.28 ± 0.22	2.43 ± 0.04
Total phenolic compounds	0.86 ± 0.14	1.07 ± 0.07
Total alkaloids	0.53 ± 0.08	1.46 ± 0.05

Microscopic examination of morphological changes in HepG2 cells

After treatment, HepG2 cancer cells and normal fibroblast cells with the phenolic compounds, alkaloids, and aqueous and methanolic extracts from *S. platensis*, the morphology of HepG2 cells was abnormal compared with untreated cell control, but no significant morphological changes were observed in the normal fibroblast cell (data not shown). All the samples induced severe morphological changes in treated HepG2 cells such as rounding, shrinkage, and floating cells in the medium. It was clear that phenolic compounds and alkaloids had the most cytotoxic effect on HepG2 cells (Fig. 5).

Discussion

Cyanobacteria are one of the natural sources that demonstrate potential in anti-carcinogenesis. Essentially, the adverse and beneficial effects of extracts from cyanobacteria are due to their phytochemical content. Secondary

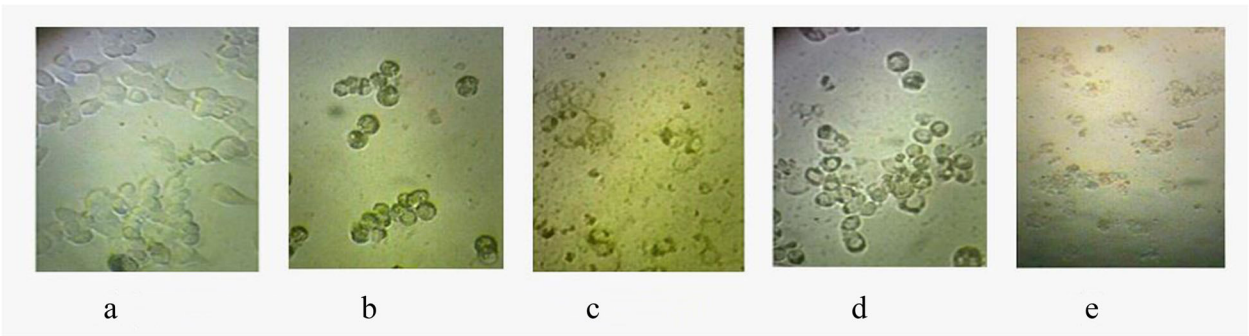

Fig. 5 Morphological changes of HepG2 cell line after 24 h treatment with various extracts and bioactive compounds obtained from *S. platensis* **a** control, **b** 1.8 mg/ml aqueous extract, **c** 1.3 mg/ml methanolic extract, **d** 0.7 mg/ml phenolic compounds, and **e** 0.5 mg/ml alkaloids

metabolites produced by these microorganisms are excellent antioxidant and chemotherapeutic agents which are easily available, safe, and affordable [22].

Among cyanobacteria species, *S. platensis* is one of the most well-known and popular cyanobacteria due to its valuable constituents and many pharmaceutical activities such as antimicrobial, antioxidant, anti-inflammatory, antiaging, and anticancer activities [23].

The results of this study showed that the aqueous and methanolic extract from *S. platensis* can inhibit the proliferation of liver cancer cells (HepG2). However, two major secondary metabolites isolated from this cyanobacterium (phenolic and alkaloid compounds) exhibited greater anticancer activity that approximately was 3-fold and 2.5-fold more cytotoxic, respectively, than the *S. platensis* extracts. This could be because *Spirulina* extract is a complex mixture of substances and the part of bioactive compounds may be relatively low. The anticancer capacity of *S. platensis* extracts and some of its bioactive compounds was previously reported for different cell lines including Kasumi-1, K562 [24], pancreatic cancer cells [25], HCT116 colon carcinoma cells [15], Mcf-7 breast cancer cells [26, 27], and HepG2 cells [27]. However, to the best of our knowledge, our study represents the first report on the anticancer activity of alkaloids and phenolic compounds extracted from *S. platensis* against hepatocellular carcinoma in vitro. Although the mechanism of anticancer activity of these compounds remains to be determined, our studies also indicated that *S. platensis* extracts and its bioactive compounds in the concentration range used (0.02–2 mg/ml) only specifically inhibited the growth of liver cancer cells but not significantly affect the proliferation and morphology of normal human fibroblasts. The water and methanolic extracts from *Spirulina* exhibited dose-dependent cytotoxicity against HepG2 cells with IC_{50} of 1.7 ± 0.14 and 1.28 ± 0.22 mg/ml, respectively. Similarly, Wu et al. [28] reported that the water extracts of *Spirulina* and *Chlorella* have potent antiproliferative effects on HSC and HepG2 cells. However, the IC_{50} values were lower than those reported here. Another study [27] revealed that incubation of breast cancer Mcf-7 and liver cancer $HepG_2$ cells with ethanol and chloroform extracts from *S. platensis* and *Chlorella vulgaris* (100 μg/ml for 24 h) induced some cell inhibition growth, but did not reach 50% inhibition (IC_{50}). The highest concentration of the extracts was 100 μg/ml, while we examined the effect of higher concentration of water and methanolic extracts from *S. platensis* and achieved 50% inhibition, of HepG2 cell growth after 24 h treatment. Currently, Hernandez et al. [24] have reported the cytotoxic effects of *S. platensis* extracts on chronic leukemia K562 and leukemia Kasumi-1 cell line. The IC_{50} values of *Spirulina* water extract were found 15.77 mg/ml and 9.44 mg/ml, respectively, which were higher than those we obtained for HepG2 cells. These differences could be due to constituent variance among *Spirulina* species, changes in the type and amount of biologically active substances under different growth conditions, and/or type of cells tested. Therefore, in this study, the amounts of two major bioactive metabolites including alkaloids and phenolic compounds isolated from *S. platensis* were measured. Our results showed that *S. platensis* under the growth conditions listed in the "Methods" section contains 1.14% and 15.05% of alkaloids and phenolic compounds, respectively. There are various reports on the content of *Spirulina*-containing bioactive compound. For example, a study by Agustini et al. [29] indicated the absence of alkaloids in *Spirulina*, while Ali et al. [30] reported higher phenolic compounds (21.88 ± 1.67 mg $GAEg^{-1}$ dry WT) and total alkaloids (3.02 ± 0.06%) than those reported in the present study for *S. platensis*. Moreover, although in this study, higher total amount and diversity of phenolic compounds isolated from *Spirulina* were confirmed by the TLC technique compared with its alkaloids, the cytotoxicity studies using these compounds revealed that alkaloids had stronger anticancer activity than the phenolic compounds against hepatoblastoma HepG2 cell. Although, according to

the literature [31–33], both alkaloids and phenolic compounds isolated from natural sources have shown potent anticarcinogenic activities in vitro and in vivo, based on the results obtained in the present study, we think that molecules with stronger anticancer effects may exist among the alkaloids extracted from *S. platensis* compared with its phenolic compounds. Therefore, further investigation on alkaloids of *S. platensis* seems vital. Some of the alkaloids such as camptothecin and vinblastine have already been successfully developed into chemotherapeutic drugs [34]. These secondary metabolites function as therapeutics by modulating key signaling pathways involved in apoptosis, cell cycle, proliferation, and metastasis, as well as inhibiting the enzyme topoisomerases, which disrupts DNA synthesis and DNA repair [35–37]. In summary, this study reports for the first time that alkaloids and phenolic compounds extracted from *S. platensis* cause a dose-dependent inhibition of HepG2 cell growth without significant cytotoxicity in normal human fibroblasts.

Therefore, further studies are needed to isolate, identify, and purify the bioactive molecules present in these compounds. Moreover, research on mechanisms of action will be necessary to better understand the anticancer activity of these bioactive metabolites against hepatocellular carcinoma.

Conclusion

It can be concluded from the results obtained in this study that alkaloids and phenolic compounds extracted from *S. platensis* have the potential to further develop as new natural anticancer agents effective against hepatocellular carcinoma, but more confirmatory studies and clinical trials are necessary before the introduction of them for the treatment of HCC.

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Authors' contributions

MA performed experiments and prepared the manuscript; HO designed and directed the study; MH co-supervised the study and NM co-advised the study. All authors read and approved the final manuscript.

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Availability of data and materials

Data and materials were available for study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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