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Comparative Evaluation of Aqueous and Methanolic Extracts of *Spirulina platensis*: Antibacterial Efficacy, Cytotoxicity, and Gene Expression Profiling

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Running title: Antibacterial and Anticancer Properties of *Spirulina platensis* Extracts

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Abstract

Background: *Spirulina platensis* is a cyanobacterium with significant bioactive potential. This study evaluated the antibacterial and cytotoxic properties of the aqueous and methanolic extracts and explored associated changes in gene expression. The antibacterial activity of both extracts was tested against Gram-positive (*Bacillus cereus*, *Listeria monocytogenes*) and Gram-negative (*Klebsiella sp.*, *Salmonella sp.*, *Escherichia coli*) bacteria. The cytotoxic effect was determined in human hepatocellular carcinoma (HepG-2) and colorectal adenocarcinoma (Caco-2) cells using the MTT assay after 24 and 48 hours. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to evaluate the expression levels of RAF, LC3, and P53 genes. **Results:** At 500 mg/mL, the aqueous extract showed the most potent antibacterial activity, with the largest inhibition zone against *L. monocytogenes*, followed by *Klebsiella sp*. Cytotoxicity was time- and concentration-dependent. Cytotoxicity reached its highest level in the aqueous extract after 72 hours, with the 500 µg/mL concentration showing a 77.5% inhibitory effect on HepG-2 cells and the 250 µg/mL concentration showing a 70% inhibitory effect on Caco-2 cells. For the methanolic extract, the maximum cytotoxic effect was observed after 72 hours, with the 125 µg/mL concentration showing a 72% inhibitory effect on HepG-2 cells. Gene expression analysis revealed that both extracts markedly upregulated RAF, P53, and LC3 after 72-hour treatment. The aqueous extract induced the highest P53 expression, while the methanolic extract triggered the most remarkable LC3 upregulation.

Conclusion: Taken together, our findings highlight the differential bioactivity of *S. platensis* extracts. The aqueous extract demonstrated superior antibacterial and cytotoxic activity. Both extracts could modulate key

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genes involved in stress response, apoptosis (P53), and autophagy (LC3), suggesting potential mechanisms for their observed effects.

Keywords: *S. platensis*, aqueous extract, methanolic extract, HepG-2, Caco-2, Apoptosis

1. Introduction

Spirulina platensis (*Arthrospira*) is a free-floating filamentous spiral-shaped blue-green algae (cyanobacteria) that grows in alkaline water. Its structure is simple, but its composition is complex (1). *Spirulina* contains a diverse array of bioactive compounds and has emerged as a nutraceutical (2). Numerous studies support the assertion that *Spirulina* contains a high quantity of primary and secondary metabolites, which are beneficial for health maintenance. The species acts as a major source of protein, accounting for 55-70% of its dry weight. Apart from that, *Spirulina* is rich in lipids, vitamins (B, D, E, and ascorbic acid), and minerals, including major ions such as potassium, calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium, and zinc. The other components found in *Spirulina* are pigments such as chlorophyll a, xanthophyll, β-carotene, zeaxanthin, and phycobiliprotein complexes (mainly C-phycocyanin [CPC] and allophycocyanin [APC]) (3, 4). Due to its high nutritional value and therapeutic properties, Spirulina has become very popular as a dietary supplement (5). In addition, NASA asserts that the nutritional value of 1 kg of Spirulina is equal to that of 1000 kg of fruits and vegetables (6).

Several research articles highlighting the positive influence of *Spirulina* on various fronts have been published in recent years. The number of published articles showing the effect of *Spirulina* or its derivatives on human health indications is rising each year. Species like *Saccharina platensis* or *S. platensis* have demonstrated several therapeutic effects such as prevention of cancer, lowering blood cholesterol, reducing nephrotoxicity caused by drugs and toxic metals, and providing protection against radiation damage (1). In addition to this, high levels of *S. platensis* extracts have shown potential for being used as antibacterial agents. Articles (7) and (8) have revealed that Gram-positive bacteria are more sensitive to the effects of *S. platensis* extract than Gram-negative bacteria.

With rising incidence and mortality rates, cancer causes millions of deaths annually, making it a serious threat to world health (9). The World Health Organization (WHO) has, in fact, projected that there will be a substantial rise in cancer-related deaths and cases by 2030, with an estimated 13 million deaths and 21 million new cases per year (10).

Liver cancer is a significant health problem around the world. It ranks fifth amongst the most frequent cancers and fourth as the cause of death due to cancer globally. Hepatocellular carcinoma (HCC) is the most frequent type of liver cancer. Chemotherapy may work well, but it can also have negative effects on healthy tissues. Colorectal cancer is the third leading cause of death from cancer amongst adults. In 2022, there will likely be 52,580 fatalities and 106,180 new cases (14). Even with improved results from new treatments, the five-year relative survival rate remains low (15). Natural products have highly beneficial properties due to their wide chemical diversity, low toxicity, favorable safety profiles, and availability. *S. platensis*-derived peptides showed anti-proliferative properties on cancer cells with low cytotoxicity on healthy cells, indicating that they could be used as a natural anticancer drug in the pharmaceutical and nutraceutical industries (16).

Accordingly, a novel method for treating bacterial infections and/or cancerous cells has been developed utilizing *S. platensis* extracts. Thus, in the current study, aqueous and methanolic extracts of *S. platensis* were evaluated for antibacterial activity

against both Gram-negative (*Klebsiella, Salmonella, and Escherichia coli*) and Gram-positive (*Bacillus cereus* and *Listeria)* bacteria. The anticancer activities against human hepatocellular carcinoma (HepG-2) and colorectal adenocarcinoma (Caco-2) cell lines were also examined. Expression of certain genes participating in cell survival [Rapidly Accelerated Fibrosarcoma (RAF)], autophagy [Microtubule-associated protein 1A/1B-light chain 3 (LC3)], and apoptosis (P53) expressions) were measured. To the best of our knowledge, no previous study has compared aqueous and methanolic extracts in HepG-2 and Caco-2 cell lines and examined genetic alterations in genes involved in the survival (RAF), apoptotic (P53), and autophagy (LC3) pathways.

2. Materials and Methods

2.1. Preparation of *S. platensis* extracts:

The Blue-Green Alga *S. platensis* used in the present study was obtained from Biotech Egypt. Aqueous and methanolic extracts were prepared as previously described by (17) with some modifications. *S. platensis* was separated from the growth medium by using centrifugation at 7000 rpm for 2.30 minutes at 20°C. After that, the pellet was left in a Petri dish to dry in an oven at 37 °C for 3 days. The aqueous solution of *S. platensis* extract was prepared by dissolving 10 gm of dried *S. platensis* powder in 100 mL of distilled water.

In comparison, the methanolic extract was then prepared by dissolving 10 gm of dried *S. platensis* powder in 100 mL of 90% methanol. The two extracts (aqueous and methanolic) were left in a shaker at 30°C for 24 hr, then filtered using filter paper. The filtrates were incubated in the oven at 37°C for 24 hr to evaporate the solvents. The dried aqueous and methanolic extract powders were stored at -20°C. The stock solution of the aqueous extract of Spirulina platensis was prepared by dissolving 5 g of dried powder in 10 mL of distilled water. The stock solution of the methanolic extract was made by dissolving 5 g of dried powder of methanolic extract in 10 mL of 5% DMSO. Aqueous and methanolic extracts were prepared from 10 g of *S. platensis* in amounts of 2.52 g and 1.008 g, respectively.

2.2. Antimicrobial activity of *S. platensis* extracts:

Bacterial strains were obtained from the Egyptian Microbial Identification Center (EMIC), FBT, USC, Egypt. The Gram-positive bacterial isolates (Listeria monocytogenes and Bacillus cereus) were cultured on nutrient agar in Petri dishes, while the Gram-negative isolates (Klebsiella sp., Salmonella sp., and Escherichia coli) were cultured on appropriate media. *Klebsiella sp. was cultured in Petri dishes on nutrient agar medium; Salmonella sp. was cultured in Petri dishes on S.S. medium;* while *E. E. coli was cultured on TBX agar in a Petri dish*. All strains were incubated at 37ºC until separate colonies were obtained. Bacterial inoculum was prepared by adding a loopful of test bacterial strains into 20 mL of Nutrient broth, which was then incubated at 37°C overnight until moderate turbidity developed.

A layer from each bacterial suspension was spread on agar plates using the same medium used to grow the bacterial strains' colonies, with a sterile cotton swab, and the plates were kept dry. The antibacterial activity of *S. platensis* was assessed using the disc diffusion method (18). Six milliliter paper discs were produced and sterilized. Serial dilutions of 500, 400, 300, 200, and 100 mg/mL were prepared from aqueous and methanolic extract stock solutions for the antibacterial assay, and 20 µL of each *dilution* was added to the paper discs. In parallel, 20 µL of distilled water (as a control for aqueous extract) and 20 µL of DMSO (as a control for methanolic extract) were used.

2.3. Anticancer activity of *S. platensis* extracts:

The human colon carcinoma (Caco-2) and hepatocellular carcinoma (HepG-2) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). In 75 cm^2 tissue culture flasks, cells were grown in RPMI-1640 medium enriched with 10% heat-inactivated fetal bovine serum free of virus and mycoplasma, 10 mM

N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES), and antibiotics (100 µg/mL streptomycin and 100 units/mL penicillin). In a humidified incubator with 5% water and 95% air, cell cultures were kept at 37°C. Every 48 hours, the media was replaced. Trypsin/EDTA (1X) solution was used to detach confluent monolayer cells (≅90%) for subculturing. To examine the cytotoxic activity of *S. platensis* extracts, HepG-2 and Caco-2 cells were cultured in flat-bottom 96-well microtiter plates (200 µL/well) in complete RPMI-1640 medium at 37°C to allow cells to settle. The following day, monolayer cells were treated with serial concentrations (4000, 2000, 1000, 500, 250, 125, 62.5 µg/mL) prepared from aqueous and methanolic extract stock solutions for 24 hours, 48 hours, and 72 hours. Cells supplemented with complete culture media served as untreated negative controls.

S. platensis extracts cytotoxicity was assessed by the ability of living cells to reduce the yellow tetrazolium salt [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT] via mitochondrial dehydrogenase enzymes, creating crystals of the insoluble purple formazan that were dissolved in dimethylsulfoxide (DMSO), as mentioned earlier by (19). The medium was removed after incubation, and MTT was added. The purple formazan crystals were dissolved using DMSO. The absorbance was measured at 570 nm (SunriseTM, Tecan Group Ltd., Switzerland). The viability percentage was calculated by dividing the average absorbance value of the treated sample by the average absorbance value of the negative control group and multiplying the result by 100. All the chemicals were purchased from Sigma (Sigma Chemical Company, USA), while cell culture reagents were supplied by Biowest (Nuaillé, France), unless otherwise specified. The maximal toxicity effect of the concentrations of both extracts was used for the analysis of gene expression.

2.4. Analyzing the expression of apoptotic and antiapoptotic genes

2.4.1. RNA Isolation

Hep-G2 or Caco-2 cancer cells suspended in complete RPMI-1640 medium were added in triplicate to 6-well flat-bottom cell culture plates and treated with *S. platensis* extracts. Based on cytotoxicity results, the best concentration and duration (gene expression experiment) were selected. Following the manufacturer's guidelines, TRIzol reagent (Invitrogen, Thermo Fisher Scientific) was used to extract total RNA from treated cells. A NanoDrop^{2000} spectrophotometer (Thermo Fisher Scientific) was used to measure the quality and quantity of the purified RNA. For further analysis, RNA samples having A260/A280 ratios of 1.8–2.0 were utilized. Additionally, 1% agarose gel electrophoresis was used to confirm RNA integrity.

2.4.2. cDNA Preparation

The TOPscript RT Dry MIX (dT18/dN6 plus) kit (Enzynomics, Life Technologies (India) Pvt. Ltd.) was used to synthesize complementary DNA (cDNA) from total RNA, following the manufacturer's guidelines. RNase/DNase-free water was used to dissolve the RT dry Mix pellet. A total of 20 µL of nuclease-free water, 10 µL of RT Mix, and 1 µg of RNA make up the reaction mixture. The reverse transcription reaction was conducted using the following thermal profile: 25°C for 10 minutes, 42°C for 60 minutes, and 95°C for 5 minutes. The generated cDNA was stored at -20°C for subsequent PCR analysis.

2.4.3. Quantitative Real-Time PCR (qRT-PCR)

In a 20 µL reaction, the TOPrealTM qPCR 2X PreMIX (SYBR Green with low ROX) PCR Kit was used to perform qRT-PCR in compliance with the manufacturer's instructions. The primer sequences for RAF, LC3, and P53 detection are shown in Table 1. The following were the PCR cycles: 1 cycle for 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 34 seconds at 60°C, and 30 seconds at 72°C. The AriaMx Real-Time PCR System

(Agilent Technologies) was used to conduct the reaction. The experiments were done in triplicate for each sample. β-actin was the housekeeping gene used for normalization. The technique calculates fold differences in gene expression by comparing the cycle threshold (CT) values of target genes with those of the housekeeping gene (β-actin) and subsequently with controls. The $2^{\wedge(-\Delta\Delta CT)}$ method (24) was used to determine gene expression levels. Experiments were conducted in triplicate, and the results were expressed as fold change relative to untreated control cells.

2.5. Statistical Analysis of He

To ensure accuracy and reproducibility, each MTT assay was carried out in 96-well plates with six replicates per concentration and time point, while RT-PCR analyses were performed in triplicate. Data are presented as mean ± standard error (SE). Statistical evaluations were performed using IBM SPSS Statistics (version 21.0; SPSS, Inc., Chicago, IL, USA). Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for pairwise comparisons. A significance level of *p* < 0.05 was considered statistically significant.

3. Results

3.1 Antibacterial activity of *S. platensis* extracts:

Inhibition zones were measured to estimate the antibacterial activity of *S. platensis* aqueous and methanolic extracts against different bacterial strains, as reported in Table 2. The bacterial inhibition zone of *S. platensis* aqueous extract ranged from 8 mm to 33 mm in Gram-positive bacteria. The 500 mg/mL concentration showed the largest inhibition zones against all bacterial strains, with the largest (33 mm) against the Gram-positive *Listeria*. The bacterial inhibition zone of *S. platensis* methanolic extract ranged from 0 mm to 12 mm. As shown in the aqueous extract, 500 mg/mL showed the largest inhibition zones against all bacterial strains, with the largest (12 mm) against the Gram-positive *Listeria*.

Concerning the Gram-negative bacteria, the bacterial inhibition zone of *S. platensis* aqueous extract ranged from 9 mm to 25 mm. The 500 mg/mL concentration showed the largest inhibition zones against all bacterial strains, with the largest (25 mm) against *Klebsiella*. The bacterial inhibition zone of *S. platensis* methanolic extract ranged from 0 mm to 12 mm. The largest inhibitory zone against all bacterial strains was obtained at 500 mg/mL in the aqueous extract, with the largest (12 mm) against *E. coli*.

3.2. Effect of *S. platensis* extracts on the viability pG-2 cells:

The dose-response curves for *S. platensis* extracts in HepG-2 cells at different time points are shown in Figures 1 and 2. Aqueous extract of *S. platensis* demonstrates a degree of cytotoxicity in nearly all concentrations and all time intervals. After 24 hr, the compound has a significant inhibitory effect, reducing viability by 27-47% across all tested concentrations. The plateau at higher doses suggests that the highest effect for a 24hr exposure has been reached. Concentrations from 1000 µg/mL down to 500 µg/mL show the most potent effect, killing nearly 80% of the cells (viability ~22%). As the concentration increases from 62.5 µg/mL to 4000 µg/mL, viability generally increases (from 42.6% to 51.1%), indicating that cytotoxicity effect is inversely proportional to the dose. At 500 µg/mL, the greatest cytotoxic effect was seen after 72 hours (77.5% cytotoxicity). This is an extremely powerful molecule, as evidenced by the fact that the lowest dose (62.5 µg/mL) still kills more than half of the population after 72 hours (58%).

However, the treatment of HepG-2 cells with the methanolic extract is dose- and time-dependent. At 24 hr, most of the concentration augments cell division, leading to an elevation in cell viability. Extreme stimulation of viability is observed at high concentrations: 169.8% at 4000 µg/mL and 133.3% at 250 µg/mL. Most other concentrations are near or above 100% viability. This elevation is diminished

by time, with a reduction in viability (51.8%) by 48 hr reaching the maximum cytotoxic effect after 72 hr incubation (72% cytotoxicity) at a concentration of 125 µg/mL. The effect of *S. platensis* extract on the viability of HepG-2 cells was shown in Figure 3.

3.3. Effect of *S. platensis* extracts on the viability of Caco-2 cells:

The dose-response curves for *S. platensis* extracts on Caco-2 cells at different time intervals are illustrated in Figures 4 and 5. The effect of *the* aqueous extract of *S. platensis* on the viability of Caco-2 cells revealed that a high concentration (4000 µg/mL) increased cell viability rather than causing cytotoxicity. Viability values are clustered between ~71% and 94%. For instance, 500 µg/mL is the highest (94.6%), while 250 µg/mL is the lowest (71.2%). Very low cytotoxicity was observed after 24 hr and 48 hr of incubation. Concentrations between 125 µg/mL and 2000 µg/mL show substantial cytotoxicity (30.4%–70.0%) after 72 hours, with 250 µg/mL being the most potent (70.0% cytotoxicity) (Figure 6).

Alternatively, the methanolic extract showed transitions from being largely non-toxic at 24 hr to showing a potent, time-dependent, and complex biological effect by 72 hr. At 24hr, viability is high (>83%) at most concentrations, then a dramatic shift occurs after 48 hr, with viability dropping as concentration increases (from 52% to 86%). The highest concentration (4000 µg/mL) shows 196% viability, and the lowest (62.5 µg/mL) shows 128% viability. The middle concentrations (125 µg/mL, 500 µg/mL) show potent inhibition (43%, 56%). The highest level of cytotoxicity was observed at 125 µg/mL(57%) at 72 hr.

3.4. Effect of *S. platensis* extracts on apoptotic/antiapoptotic gene expression:

Compared with the HepG-2 untreated control cells (CM), incubating the cells for 72 hr with the aqueous extract (500µg/mL) caused a massive increase in RAF expression (~38.62), while the methanolic extract (125 µg/mL) also increased it substantially (~20.56). The aqueous extract moderately increased LC3 (~16.48-fold) vs. CM; the methanolic extract caused a huge increase (~41.31). Regarding P53, both extracts strongly increased P53 expression compared to the control (~48.3- and ~26.75-fold increases for the aqueous and methanolic extracts, respectively). Collectively, incubation of HepG-2 cells with *S. platensis* aqueous (500 µg/mL) and methanolic (125 µg/mL) extracts for 72 h induces RAF, LC3, and P53 production, with the maximum expression level of LC3 by the methanolic extract, while the maximum amount of P53 is produced in response to the aqueous extract.

Treating Caco-2 cells for 72 hr with aqueous extract (250 µg/mL) strongly downregulated RAF and LC3, while greatly upregulating P53 (~8.9-fold) compared to the Caco-2 untreated cells (CM). In contrast, methanolic extract (125 µg/mL) upregulated RAF (~1.76-fold) and LC3 (~4.4-fold) and downregulated P53 relative to the culture.

Table 1. Primer sequences used in gene expression detection

Primer	Sequence	References
Raf-1 sense	5’-TTTCCTGGATCATGTTCCCCT-3’	(20)
Raf-1 antisense	5’-ACTTTGGTGCTACAGTGCTCA-3’	
LC3 sense	5'-ATCATCGAGCGCTACAAGGGTGA-3'	(21)
LC3 anti-sense	5'-GGATGATCTTGACCAACTCGCTCAT-3'	
P53 sense	5’-TCAGATCCTAGCGTCGAGCCC-3’	(22)
P53 anti sense	5’-GGGTGTGGAATCAACCCACAG-3’	
β-actin sense	5’-GAGACCTTCAACAACCCAGCC-3’	(23)
β-actin antisense	5’-GGATCTTCATGAGGTAGTCAG-3’	

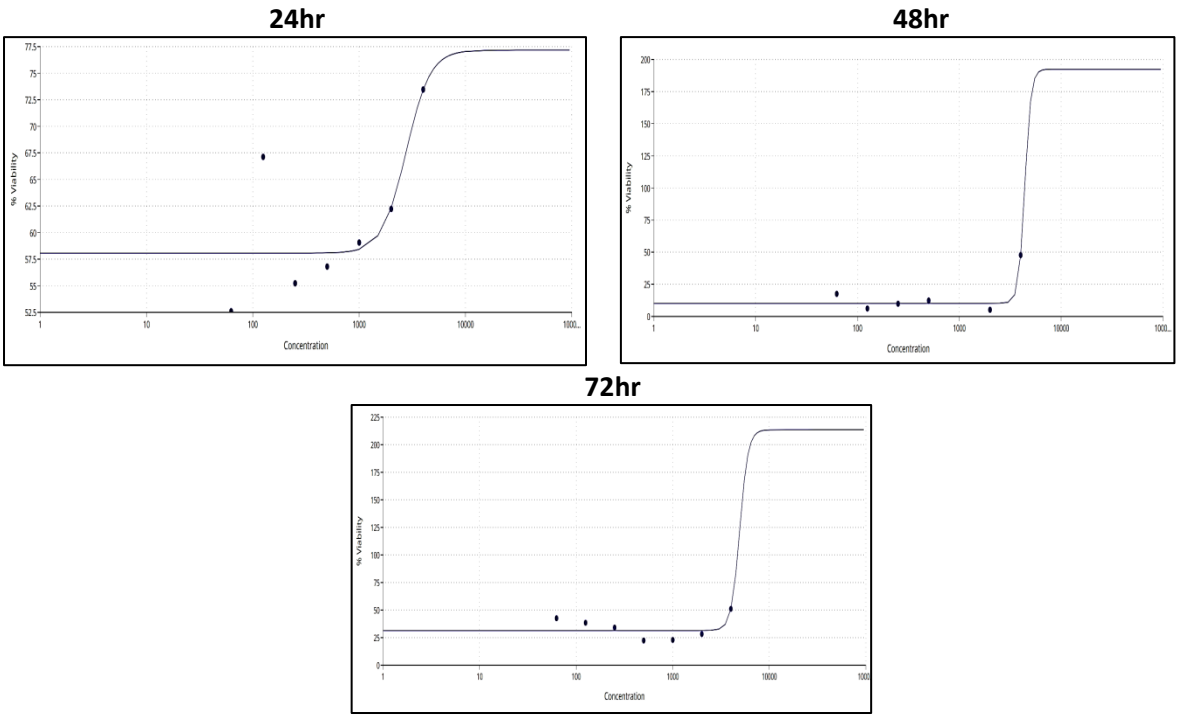

Figure 1. The dose-response curves for *Spirulina platensis* aqueous extracts on HepG-2 cells at different time intervals. (Each point represents the mean of 8 wells).

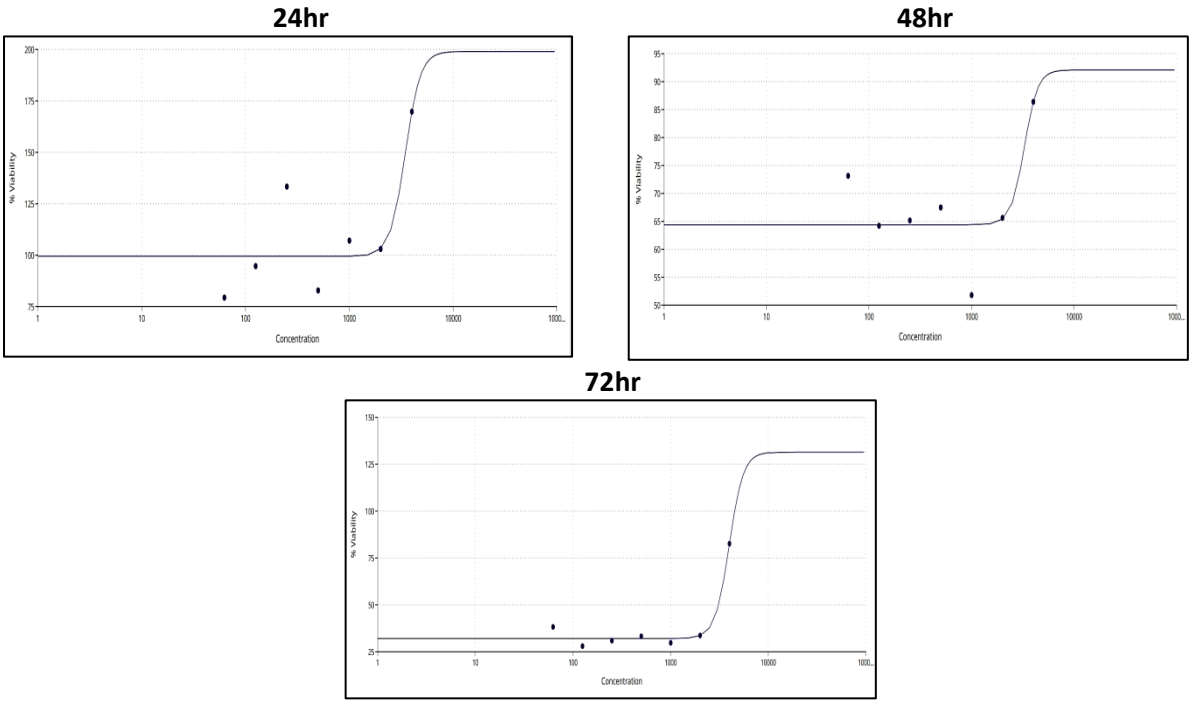

Figure 2. The dose-response curves for *Spirulina platensis* methanolic extracts on HepG-2 cells at different time intervals. (Each point represents the mean of 8 wells).

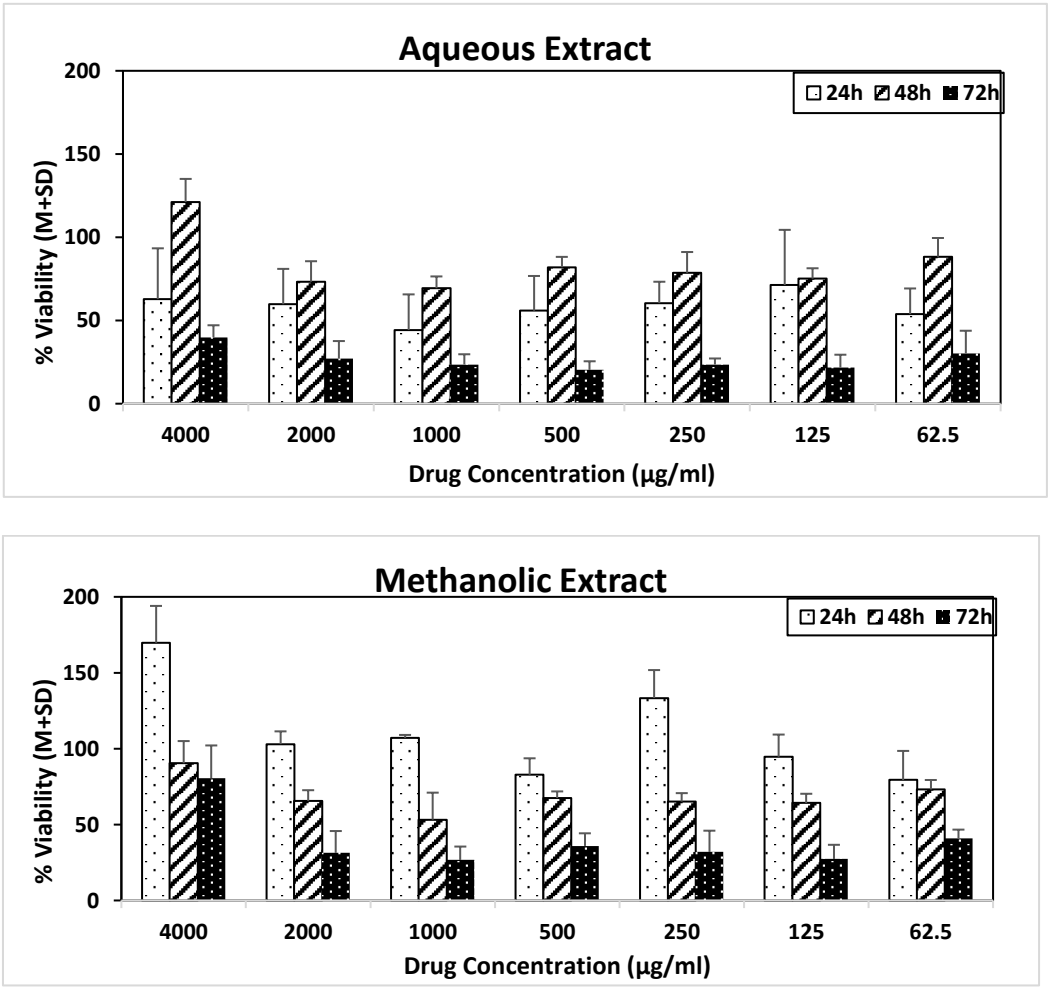

Figure (3). Effect of aqueous and methanolic extracts of *S. platensis* on the viability of HepG-2 cells. Cells were treated with several concentrations (62.5–4000 µg/mL) for different time intervals (2hr, 48hr and72 hr), and cell toxicity was measured by MTT assay.

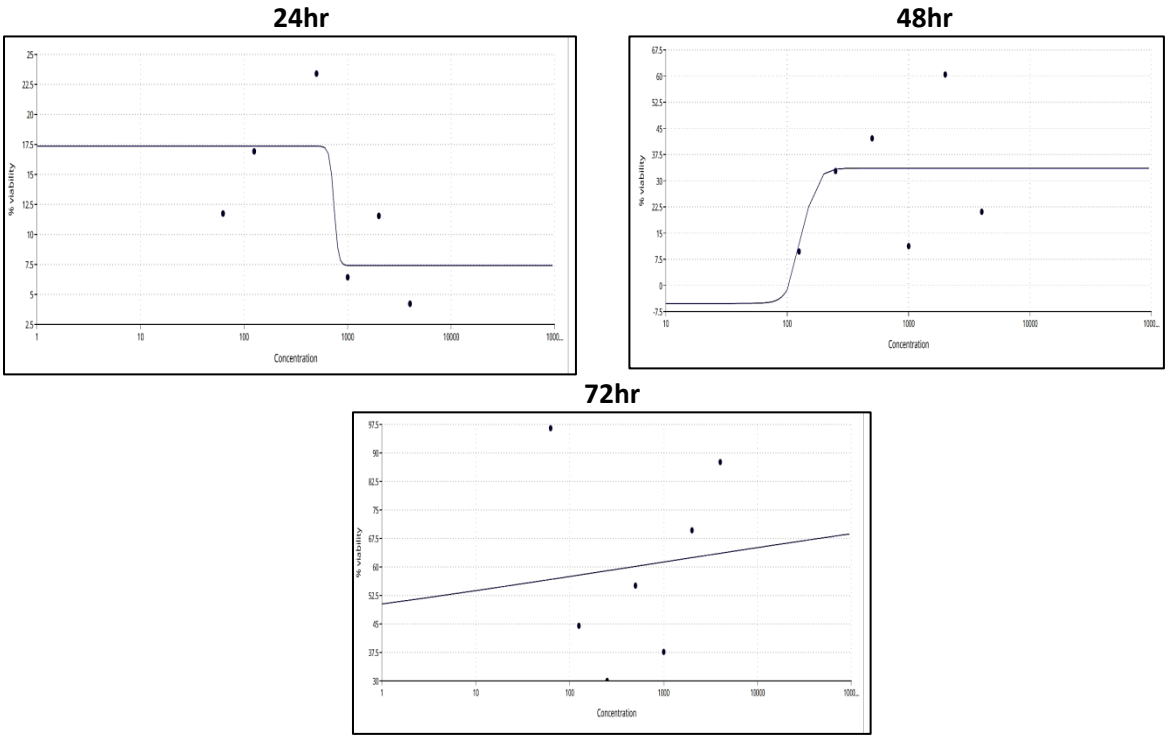

Figure 4. The dose-response curves for *Spirulina platensis* aqueous extracts on Caco-2-2 cells at different time intervals. (Each point represents the mean of 8 wells).

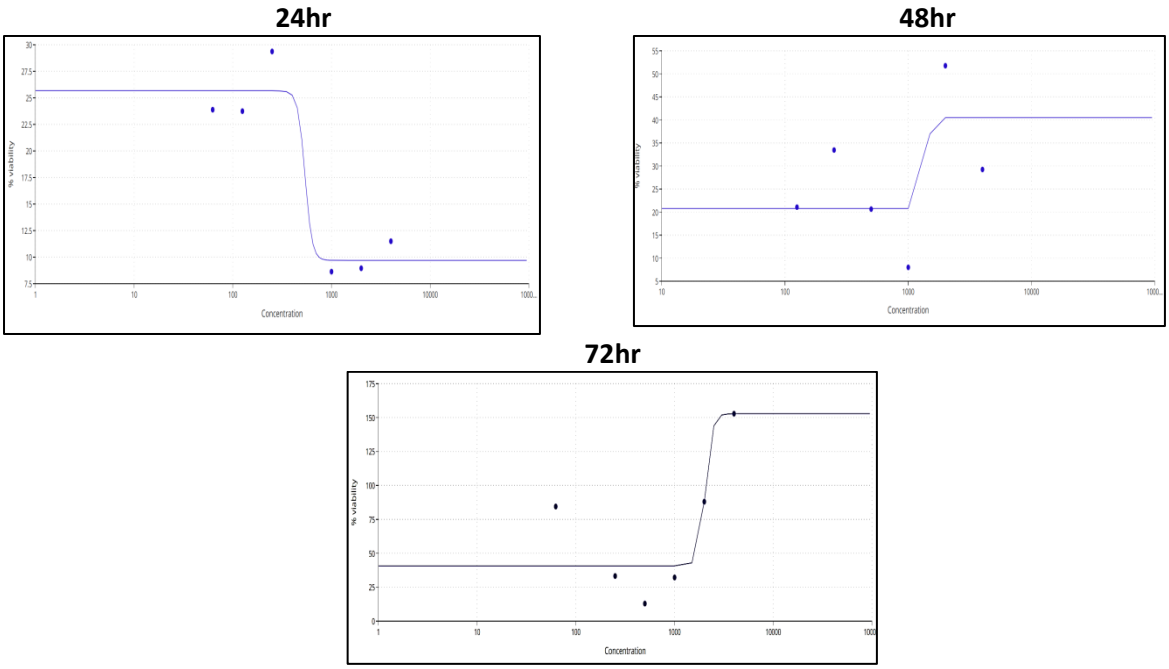

Figure 5. The dose-response curves for *Spirulina platensis* methanolic extracts on Caco-2 cells at different time intervals. (Each point represents the mean of 8 wells).

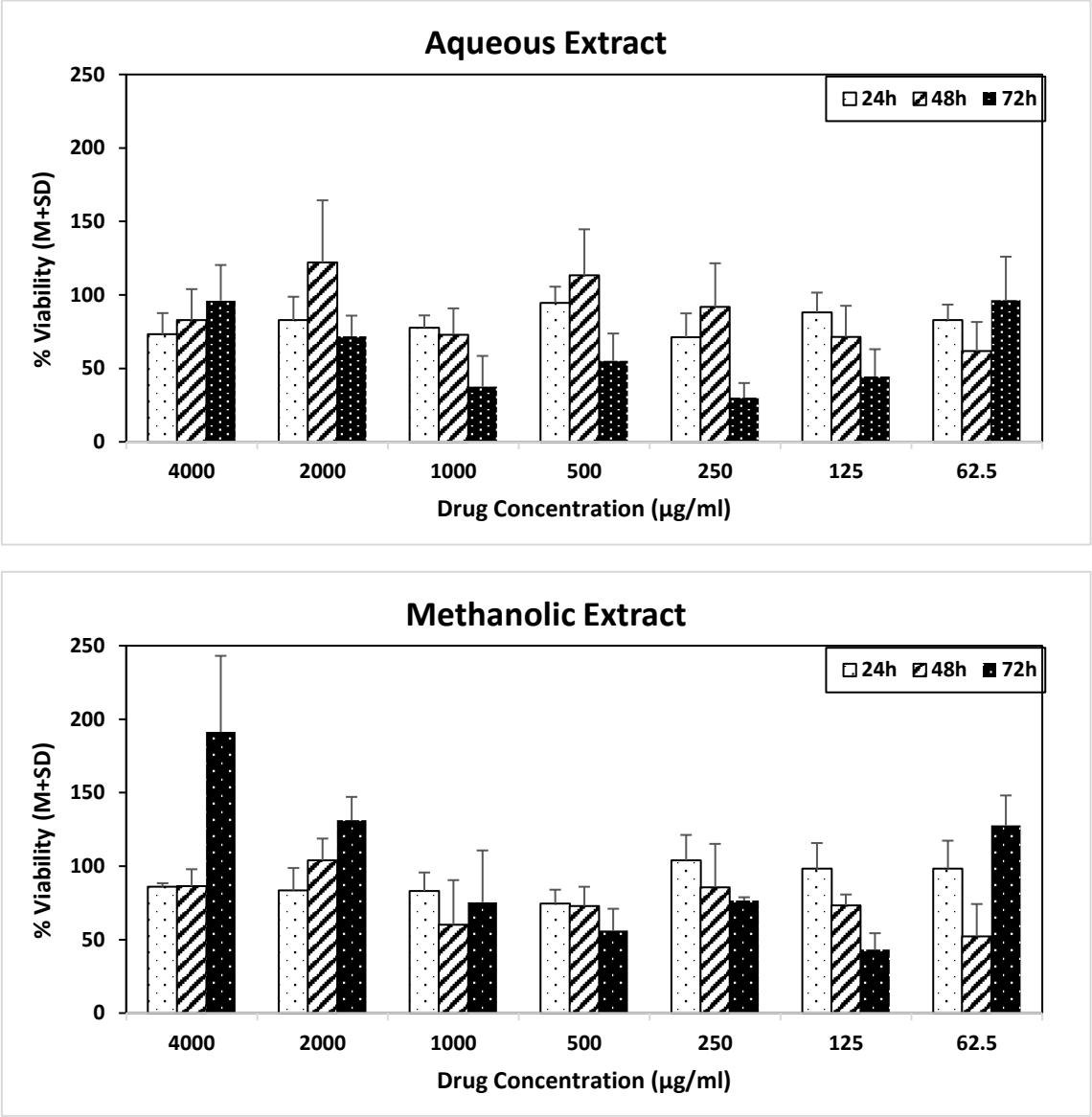

Figure (6). Impact of *Spirulina platensis* methanolic and aqueous extracts on Caco-2 cell viability. Cell toxicity was measured using the MTT assay after cells were treated with various doses (62.5–4000 µg/mL) for different durations (24, 48, and 72 hours).

Table (2): Antibacterial activity of *Spirulina* aqueous and methanolic extracts

Bacterial Strain		Extract concentration (mg/ml) 100	200	300	400	500
		Aqueous Extract				
		Inhibition zone (mm)				
Gram-positive	*Listeria monocytogenes*	22	25	27	29	**33**
	Bacillus cereus	8	9	10	11	**12**
Gram-negative	*Klebsiella sp.*	15	17	19	23	**25**
	Salmonella sp	9	10	11	11	**12**
	Escherichia coli	10	11	13	14	**15**
		Methanolic Extract				
		Inhibition zone (mm)				
Gram-positive	*Listeria monocytogenes*	8	9	9	10	**12**
	Bacillus cereus	0	5	7	8	**10**
Gram-negative	*Klebsiella*	8	8	8	9	**9**
	Salmonella	0	8	9	10	**11**
	Escherichia coli	9	10	11	11	**12**

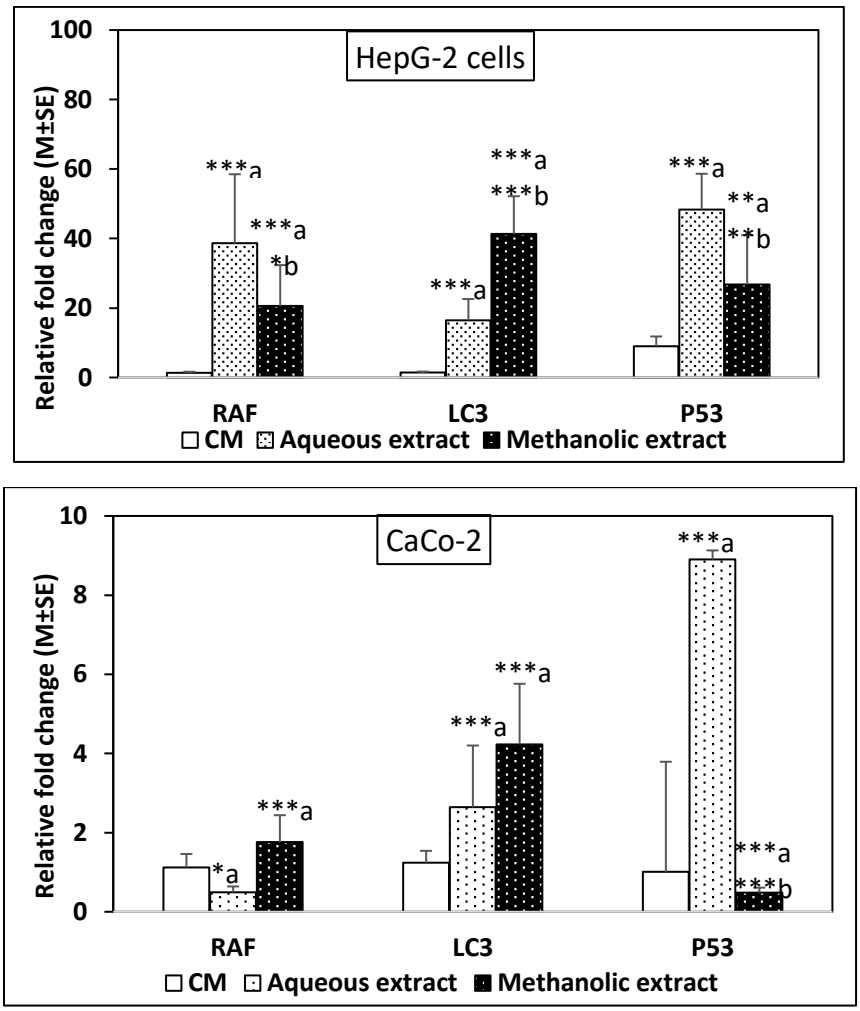

Figure 7. RT-qPCR study for RAF, LC3, and P53 gene expression levels following treatment of HepG-2 and Caco-2 cells with *Spirulina platensis* aqueous and methanolic extracts at the highest cytotoxic dose. Significant compared with CM (a) and aqueous extract (b). *($p<0.05$), **($p<0.01$), and ***($p<0.001$).

4. Discussion

The effects of *S. platensis* extracts (aqueous and methanolic) on various bacterial strains were investigated. For both extracts, the highest concentration (500 mg/mL) yielded the largest inhibition zones. This demonstrates a classic dose-dependent response and confirms that the observed inhibition zones are due to the extracted compounds and not an artifact. The higher activity of the aqueous extract suggests that the primary antibacterial agents in this *S. platensis* strain are water-soluble compounds. These likely include Phycobiliproteins such as Phycocyanin and Allophycocyanin (25,26), and Exopolysaccharides (27). Small peptides are known to have antibacterial properties, and the high protein content of certain algae is linked to their action. The significant antimicrobial activity of *S. platensis* extracts may be directly related to the abundance of proteins and small peptides in this cyanobacterium (28). A study by (29) using scanning electron microscopy has visually confirmed that distortion and damage to bacterial cells lead to leakage of cellular contents and cell death following treatment with *S. platensis* extract. They found that *S. platensis* extract has antibiofilm activity, reducing biofilm formation and making it harder for bacteria to survive.

The results of this investigation demonstrated that *S. platensis* extracts were more effective at inhibiting the growth of Gram-positive than Gram-negative bacteria. This impact might be caused by the intricate structure of Gram-negative bacteria's outer membrane or cell wall (7). (30) found that *S. platensis* exhibited stronger antibacterial efficacy against *Salmonella sp* and *E. coli*. An inhibition zone against Klebsiella sp. (25 mm) is an important result, as some studies report no inhibitory effect, while others demonstrate moderate activity. *Klebsiella pneumoniae* is a particularly hardy, highly antibiotic-resistant Gram-negative bacterium (31). The relatively weak activity of the methanolic extract indicates that lipophilic, methanol-soluble antimicrobials (e.g., certain fatty acids, tocopherols, or sterols) are less potent in this specific algal material. This finding aligns with studies reporting stronger antibacterial activity in aqueous or ethanol extracts than in chloroform or methanolic extracts (32). The ability of *S. platensis* extracts, especially the aqueous extract, to suppress these bacteria suggests that their active components can disrupt the bacterial outer membrane to reach internal targets. In contrast to our results, some studies found that *the* methanolic extract of *S. platensis* was the most effective antimicrobial agent compared with the aqueous extract (33).

In terms of cytotoxicity against HepG-2 cells, both extracts exhibited time-dependent effects, but with distinct patterns and cytotoxic profiles between the aqueous and methanolic extracts. The aqueous extract demonstrates immediate, potent, and consistent cytotoxicity across all time points and concentrations. In contrast, the methanolic extract exhibits a biphasic dose-response. This is consistent with hormesis—characterized by low-dose stimulation and high-dose inhibition —which has been documented previously (34,35). Evidence indicates that *S. platensis* may exhibit various beneficial health-promoting effects, including anticancer activities (36). (1) and (37) reported that *S. platensis* extract exhibited cytotoxic effects on HepG-2 cells. Over 120 chemical agents demonstrate hormetic responses in cancer cells, with maximum stimulatory responses typically not exceeding twice the control value, and stimulatory concentration ranges usually less than 100-fold. Our data suggest that the methanolic extract contains compounds that initially act as mitogens or growth-promoting agents (such as low-molecular-weight phenolic compounds) before their primary cytotoxic mechanisms dominate, as well as cytotoxic agents (such as lipids and fatty acids). Phenolic compounds at low concentrations can activate estrogen receptors and stimulate proliferation in cancer cells (38). The capacity of polyphenols to interact with and modify

hormone receptors may be the basis for their concentration-dependent biphasic effects (39). Moreover, Alkaloids in *S. platensis* extracts demonstrate potent anticancer activity, inducing an 80% reduction in cell viability (17).

The aqueous extract exhibits consistent, dose- and time-dependent cytotoxic activity against HepG-2 cells. This finding aligns with previous studies that have reported the potent anticancer properties of *S. platensis*, often attributed to water-soluble compounds, such as highly polar compounds (phycocyanin, polysaccharides, peptides) with direct cytotoxic action (40). It seems that C-phycocyanin's potential to influence mitochondrial pathways, encouraging necrosis and apoptosis, was what allowed it to have an anticancer effect in HepG-2 cells (41). The plateau effect at higher doses (e.g., between 500-4000 mg/mL) is characteristic of many cytotoxic compounds, indicating a saturation point where all target sites (e.g., receptors, enzymes) are engaged, and increasing concentration yields no additional effect (42). The capacity of phycocyanin to halt the cell cycle, prevent tumor cell proliferation, and induce autophagy and death in these cells has established its role in cancer (43).

Concerning Caco-2 cells, the aqueous extract exhibits erratic, non-dose-dependent, and time-varying effects and does not follow a classic dose-response curve. On the other hand, the effect of methanolic extract transitions from being largely non-toxic at 24 hr to a potent cytotoxic effect by 72 hours at 125 µg/mL. It has been reported that *S. platensis* extract exhibits cytotoxic effects on HCT116 and Caco-2 colon carcinoma cells (1,37). (44) discovered that giving animals with ulcerative colitis 300 mg/kg of S. *platensis* aqueous extract by gavage can reduce colonic mucosal damage and alter gut microbiota dysbiosis by reducing oxidative stress and inflammation.

Our data showed significant increases in RAF and p53 levels in HepG-2 and Caco-2 cancer cells treated with *S. platensis* aqueous extract. On the other hand, the methanolic extract produced a vast amount of LC3. The significant upregulation of RAF, LC3, and P53 points to complex, multi-mechanistic anticancer activity involving pro-apoptotic signalling, induction of autophagy, and potential modulation of cell proliferation pathways. This massive induction of P53 strongly suggests that *S. platensis* extracts induce significant genotoxic or oxidative stress in HepG-2 cells, activating the canonical p53 pathway. This aligns with the established literature, which shows that *S. platensis* bioactives, particularly C-Phycocyanin (C-PC), induce apoptosis in cancer cells by upregulating p53. C-PC can increase reactive oxygen species (ROS) in cancer cells, leading to DNA damage and p53 stabilization (45,46). A higher concentration of water-soluble proteins, including C-PC, a recognised p53 inducer, may be the cause of the higher fold change in the aqueous extract (43). Phycocyanin is generally recognised to downregulate the expression of antiapoptotic genes such as Bcl-2 and to increase the expression of proapoptotic genes such as p53 and Fas (47). Furthermore, the different phytochemical components of *S. platensis*, such as flavonoids, which have a range of effects on cancer cells, including inhibiting cell division and differentiation, lowering the activity of kinase enzymes, inducing cell cycle arrest, and triggering apoptosis, may be responsible for the cytotoxic and apoptotic actions of the extracts (48).

Through both intrinsic and extrinsic pathways, the methanolic extract induced DNA apoptosis (49). The marked increase in LC3, especially with the methanolic extract, indicates potent induction of autophagy. In conjunction with the massive upregulation of p53, this LC3 induction likely represents "autophagic cell death" or a stress response rather than pro-survival autophagy. p53 is a well-known positive regulator of autophagy. Numerous substances from S. platensis extracts, including phycocyanin, which has been shown to block the PI3K/Akt/mTOR signalling pathways and

induce autophagic cell death, have been reported to induce cytotoxic autophagy in cancer cells. Additionally, it stimulates NF-κB activation and nuclear translocation, which is crucial for maintaining the balance between phycocyanin-mediated autosis and apoptosis (50). Also, nanoformulated *S. platensis* methanolic extract was reported to induce the autophagy signal through activation of beclin-1 and LC3II, downregulation of p62 expression, and increased gene expression of paraoxonase-1 (PON-1) (51). The methanolic extract's superior effect may be due to its higher concentration of non-polar polyphenolic compounds, which have been shown to induce autophagy (52).

The induction of RAF (a serine/threonine kinase, part of the MAPK/ERK pathway—RAF/MEK/ERK) is the most complex result, and this is related to cell survival, proliferation, and differentiation. In certain contexts, particularly with sustained and strong activation, the MAPK pathway can paradoxically trigger cell cycle arrest and apoptosis (53,54). The activation of these 3 genes might be attributed to severe genotoxic stress previously observed during chemotherapy (55), which may have been induced by our *S. platensis* extracts. Massive DNA damage activates the p53 pathway (56). Many chemotherapeutic agents also induce oxidative damage, which is a potent inducer of autophagy (LC3 conversion) (57). Furthermore, DNA damage response pathways can crosstalk with and activate RAF/MEK/ERK signalling as part of a pro-survival or feedback loop, contributing to RAF elevation (58). In a neuronal cell line (59,60), persistent ERK activation contributes to glutamate-induced oxidative toxicity.

We face several limitations throughout the study. The proposed compound-activity relationships remain speculative, based on reports in the literature (25-29). This study's primary reliance on the MTT test for preliminary cytotoxicity screening is a limitation of our study. In order to differentiate metabolic suppression produced by mitochondria from actual cytotoxicity, We'll include viability tests (such as LDH release) in subsequent research, even though we only used MTT for the initial concentration-range trials. Moreover, gene expression data alone are not sufficient to confirm apoptosis or autophagic cell death; thus, the lack of protein-level confirmation (e.g., ELISA or Western blotting) for the studied genes is another limitation of this work,

In conclusion, this study confirms that *S. platensis* is a promising source of antimicrobial and anticancer agents. The aqueous extract produced the maximum antimicrobial activity and potent cytotoxic ability. *S. platensis* extracts, particularly the aqueous extract, demonstrated promising antibacterial and cytotoxic effects, while the expression of RAF, LC3, and P53 suggests possible involvement of stress-response, apoptosis, and autophagy pathways. Future research aimed at developing standardized *S. platensis* -based nutraceuticals or adjunctive therapies has to include quantitative profiling of total phenolics, flavonoids, phycobiliproteins, and polysaccharides to establish structure–activity relationships are warranted to establish structure–activity relationships.

5. List of Abbreviations

S. platensis: Spirulina platensis

HepG-2: Human hepatocellular carcinoma cell line

Caco-2: Human Colorectal adenocarcinoma cell line

qRT-PCR: quantitative reverse transcription polymerase chain reaction.

RAF: Rapidly Accelerated Fibrosarcoma

LC3: Microtubule-associated protein 1A/1B-light chain 3

HEPES: N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

DMSO: dimethylsulfoxide

6. Declarations

6.1. Ethics approval and consent to participate: Not applicable for this section

6.2. Consent for publication: All authors approved the final version of the manuscript

6.3. Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

6.4. Competing interests: The authors declare that they have no competing interests.

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