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ORIGINAL ARTICLE

Gracilaria foliifera (Forssk.) Børgesen Ethanolic Extract Triggers Apoptosis via Activation of p53 Expression in HepG2 Cells

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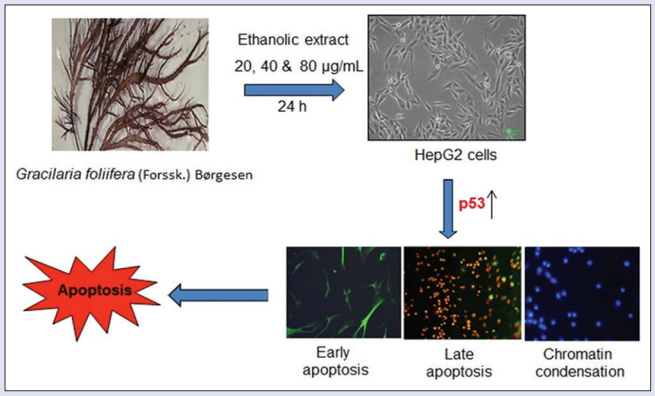
ABSTRACT

Background: Hepatocellular carcinoma is one of the most common types of malignancy and causes significant morbidity and mortality worldwide. *Gracilaria foliifera* (Forssk.) Børgesen, a brown marine alga, is shown to have growth inhibitory potential against various cancer cell lines other than human hepatoma HepG2 cells. **Objective:** To investigate the cytotoxic potentials of *G. foliifera* in HepG2 cells. **Materials and Methods:** HepG2 cells were fed with culture medium supplemented with different concentrations of ethanolic extract of *G. foliifera* (20, 40, and 80 µg/mL). After 24 h of treatment, the cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Induction of early apoptosis was investigated by annexin V-fluorescein isothiocyanate immunofluorescence. Induction of late apoptosis and necroptosis was investigated by annexin V and propidium iodide (PI) staining. Nuclear chromatin condensation was evaluated by Hoechst staining. p53 protein expression was analyzed using Western blotting. **Results:** *G. foliifera* treatment in HepG2 cells caused a significant cytotoxic effect. Phosphatidylserine translocation confirms the induction of early apoptosis. Analysis of late apoptosis using annexin V/PI staining showed that the percentage of apoptotic cells was increased in a concentration-dependent manner. Hoechst nuclear staining further confirms the nuclear chromatin condensation. *G. foliifera* treatment also induced the tumor suppressor p53 protein expressions. **Conclusion:** The present study demonstrated that *G. foliifera* induced apoptosis in HepG2 cells through activation of p53.

Key words: Brown marine alga, chromatin condensation, cytotoxicity, *Gracilaria foliifera*, hepatocellular carcinoma

SUMMARY

- *Gracilaria foliifera* induces cytotoxicity in human hepatoma HepG2 cells
- *G. foliifera* causes apoptosis-related morphological changes in HepG2 cells
- *G. foliifera* upregulates tumor suppressor p53 protein expression in HepG2 cells.



Abbreviations used: HCC: Hepatocellular carcinoma; DMEM: Dulbecco's minimum essential medium; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCCS: The National Center for Cell Science; FBS: Fetal bovine serum; PI: Propidium iodide; PS: Phosphatidylserine.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is an aggressive cancer and the third most common cause of cancer-related death worldwide.[1,2] Approximately 70%–90% of patients with HCC is said to have an established background of chronic liver disease and cirrhosis,[3] with major risk factors for developing cirrhosis including chronic infection with hepatitis B and C virus, chronic alcohol consumption, intake of aflatoxin-contaminated food, diabetes and obesity, and nonalcoholic steatohepatitis.[4] Patients with HCC are often asymptomatic, and late diagnosis consequently makes the disease untreatable. The treatment modalities for HCC are surgery, ablation, and liver transplantation.[5-7] Many treatments and management modalities exist with differing advantages and disadvantages. One of the current treatments employed for patients with advanced HCC is sorafenib, a MAP kinase pathway inhibitor. Sorafenib is used to improve the survival of HCC patients; however, it has several adverse effects including hyperbilirubinemia, hand and foot skin reactions, and fatigue.[8,9] Despite the significant advancement in HCC management, its incidence continues to rise.[10] Hence, there is an urgent need for the development of therapeutic modalities for the management of HCC that should have minimal or no side effects. In this study, we investigated the cytotoxic and antiproliferative potential of the ethanolic extract obtained from *Gracilaria foliifera* (Forssk.) Børgesen (*Gracilariaceae*), a brown marine alga, against human hepatoma HepG2 cells.

Several bioactive compounds from marine organisms have been experimentally tested and came out with the promising results.[11]

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Studies on the bioactivities of marine algae have revealed numerous health benefits, including antioxidative, anti-inflammatory, antimicrobial, and anticancer effects.[12,13] Studies have shown that seaweed can be a source of new anticancer drugs.[14,15] Accordingly, the cytotoxic effect of *G. foliifera* has been reported previously against different types of breast cancer cell lines.[16] However, the cytotoxic potential of *G. foliifera* has not been evaluated against human hepatoma HepG2 cells. Hence, the present study investigated the above lacunae.

MATERIALS AND METHODS

Reagents

Dulbecco's minimum essential medium (DMEM)-low glucose, penicillin, streptomycin, trypsin-EDTA, 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetal bovine serum (FBS) were obtained from GIBCO BRL (Gaithersburg, MD, USA). All other chemicals were of analytical grade.

Collection and processing of seaweeds

The brown seaweeds *G. foliifera* (Forssk.) Børgesen were collected from the Mandapam coast of Tamil Nadu, India. They were identified in the Central Salt and Marine Chemicals Research Institute, located at Mandapam Camp, Ramanathapuram, India. The seaweeds were powdered and 10 g of the powder was extracted with ethanol using a Soxhlet apparatus. The crude extracts were concentrated under reduced pressure. Ethanolic extracts of *G. foliifera* were used in the present study.

Cell cultures and treatment

The HepG2 human HCC cell line was procured from NCCS with the passage number of 17. The cells were maintained in DMEM with low glucose supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. The cells were grown in 25 cm^2 culture flasks, and after a few passages, the cells were seeded for experiments. The experiments were done at 70%–80% confluence. Upon reaching confluence, the cells were detached using 0.25% trypsin-EDTA solution.

HepG2 cells were plated at 10,000 cells/cm^2. After 24 h, the cells were fed with fresh expansion culture medium supplemented with different final concentrations of *G. foliifera* extract (20, 40, and 80 μg/mL) or the corresponding volumes of the vehicle. After 24 h of treatment, the cells were collected by trypsin application. The total cell number was determined by counting each sample in triplicate under the inverted microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The cytotoxic evaluation of *G. foliifera* was done using the MTT assay.[17] The cells were plated in a 96-well plate at a concentration of 1×10^4 cells/well. After 24 h, the medium was replaced with 100 μL of medium containing *G. foliifera* at different concentrations and incubated for 24 h. At the end of the treatment period, media from control and *G. foliifera*-treated cells were discarded, and 50 μL of MTT (0.5 mg/mL of PBS) was added to each well. The cells were then incubated for 4 h at 37°C in CO_2 incubator. MTT was then discarded, and the colored crystals of produced formazan were dissolved in 150 μL of DMSO. The purple-blue formazan formed was measured using an ELISA reader (BIORAD) at 570 nm. The optical density of control and each sample was compared, and graphs were plotted.

Annexin V/propidium iodide staining and phosphatidylserine translocation analysis

HepG2 cells were plated at a density of 1×10^4 in 48-well plates. They were allowed to grow at 37°C in a humidified CO_2 incubator until 70%–80% confluent. Then, they were treated with *G. foliifera* for 24 h. After 24 h, the culture medium was aspirated and the cells were gently rinsed twice with PBS at room temperature. In annexin V/propidium iodide (PI) staining, they were collected after treatments from control and experimental groups and then resuspended in PBS and incubated with annexin V reagent in HEPES buffer containing PI and viewed immediately under a Nikon inverted fluorescence microscope (Ti series, Tokyo, Japan).

In some experiments, to investigate the phosphatidylserine (PS) translocation, the cells were incubated with fluorescein isothiocyanate-conjugated annexin V and viewed immediately under the fluorescent microscope.

Analysis of nuclear chromatin condensation by Hoechst staining

To investigate the apoptosis-related chromatin changes in nucleus, Hoechst staining was performed according to the manufacturer's instructions. Briefly, at the end of 24 h of *G. foliifera* treatment, the cells were collected and then 10 μL of Hoechst 33342 stain was added to 1 mL of the cell suspension and incubated for 37°C for 5–15 min and viewed immediately under the Nikon inverted fluorescence microscope (Ti series, Tokyo, Japan).

Western blot analysis

HepG2 cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate sodium and 0.1% protease, and phosphatase inhibitor cocktails (Sigma-Aldrich, India). After extraction, protein concentration was estimated by the Bradford method (Bio-Rad Laboratories) with bovine serum albumin (BSA) as standard. Total protein extracts were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond™-ECL, Amersham, CA, USA). The membranes were blocked with 5% BSA, probed overnight at 4°C with p53 primary antibodies (monoclonal) and 2 h at room temperature with corresponding secondary antibodies. Immunoreactive bands were detected by enhanced chemiluminescence with protein A-horseradish peroxidase and the SuperSignal chemiluminescent system (Pierce, Rockford, IL, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. (San Diego, CA, USA). The data were expressed as mean ± standard error of the mean and analyzed by one-way ANOVA following Dunnett's test to determine the significant differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of *Gracilaria foliifera* on the proliferation of HepG2 cells

We evaluated the cytotoxic effect of *G. foliifera* in HepG2 cells. The morphology of HepG2 cells treated with *G. foliifera* is depicted in Figure 1a. Initially, we selected broad concentration ranges (10, 20, 40, 80, and 100 μg/mL) to find out the effective concentrations in HepG2 cells.

The *G. foliifera* treatment for 24 h significantly ($P < 0.001$) inhibits the proliferation of HepG2 cells [Figure 1b].

Analysis of apoptosis by annexin V/propidium iodide staining

Annexin V/PI staining was also used to identify apoptotic cells by fluorescence microscopy. Annexin V is a protein that is conjugated to a green fluorescent dye to detect apoptosis, while PI is a red fluorescent dye that stains DNA of both necrotic and late apoptotic cells with damaged membranes. In the present study, *G. foliifera*-treated cells were positive to annexin V and PI, indicating the presence of early and late apoptotic cells. The control cells were viable and hence negative to annexin V and PI stains [Figure 2].

Effect of *Gracilaria foliifera* on the plasma membrane translocation of phosphatidylserine

To find out the exact mechanism behind the *G. foliifera* induced a decrease in proliferation, we evaluated the plasma membrane translocation of PS

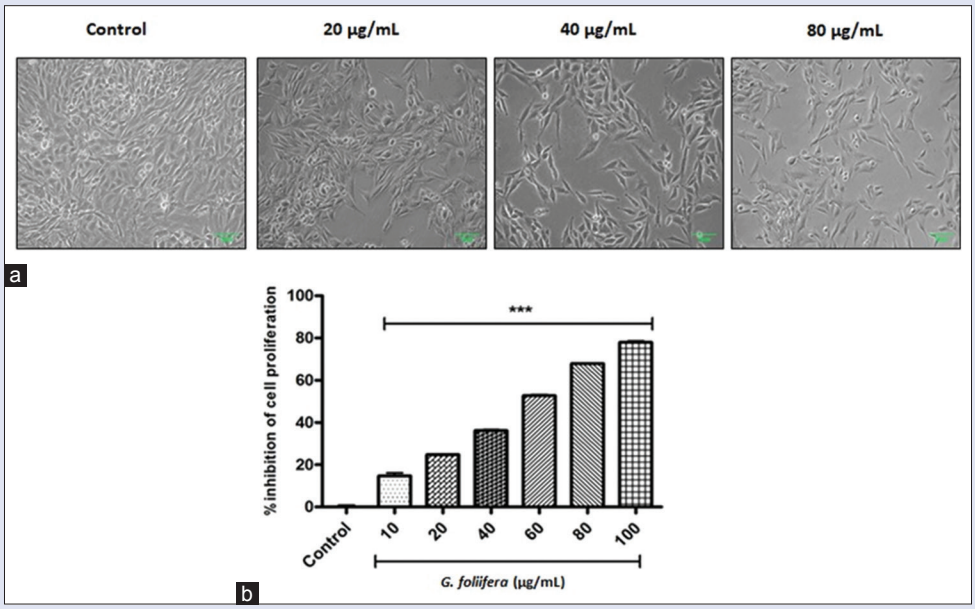

Figure 1: Effect of *Gracilaria foliifera on* HepG2 cell (a) morphology (×10) and (b) cell proliferation. Values are expressed as mean ± standard error of the mean ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. ***$P < 0.001$ was considered statistically significant

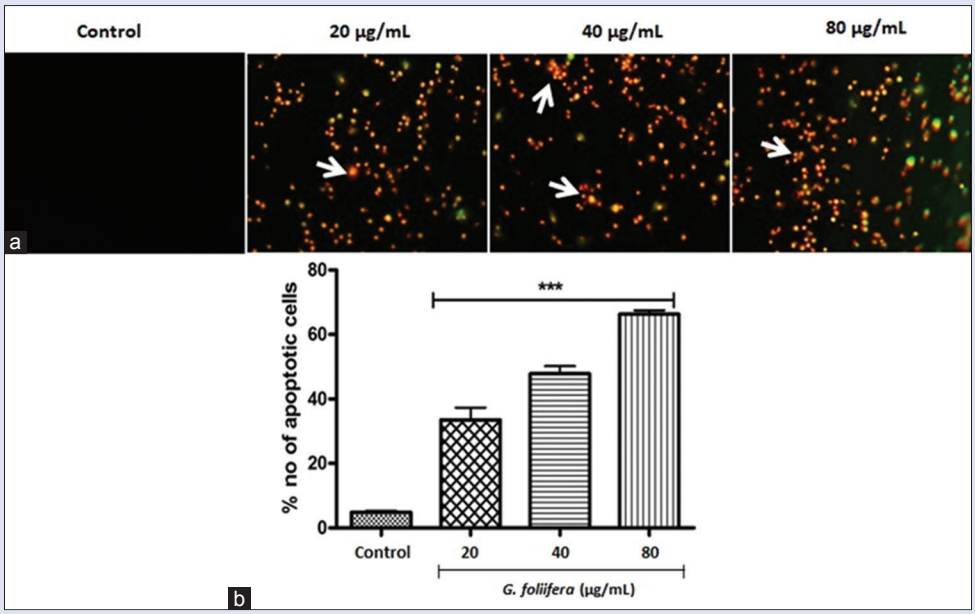

Figure 2: Annexin V and propidium iodide staining. (a) Representative images of HepG2 control and *Gracilaria foliifera*-treated cells. (b) Percentage of number of apoptotic cells stained with annexin V and propidium iodide. Values are expressed as mean ± standard error of the mean ($n = 3$). Apoptotic cells were individually calculated as percentage of apoptotic cells relative to the total number of cells in each random field. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. ***$P < 0.001$ was considered statistically significant

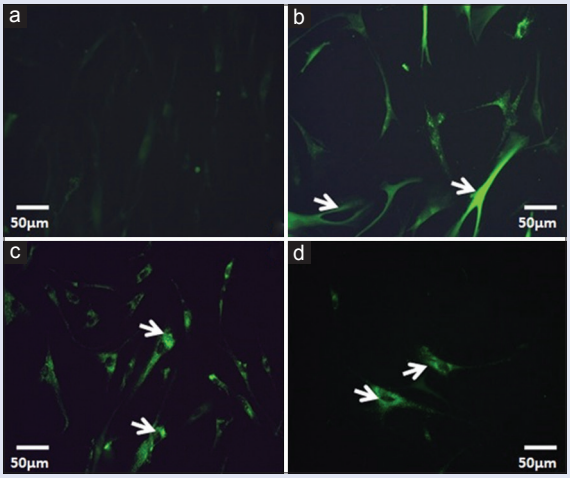

Figure 3: Effect of *Gracilaria foliifera* on phosphatidylserine staining (annexin V fluorescein isothiocyanate). (a) Control and (b-d) 20, 40, and 80 μg/mL of *Gracilaria foliifera* treatments, respectively

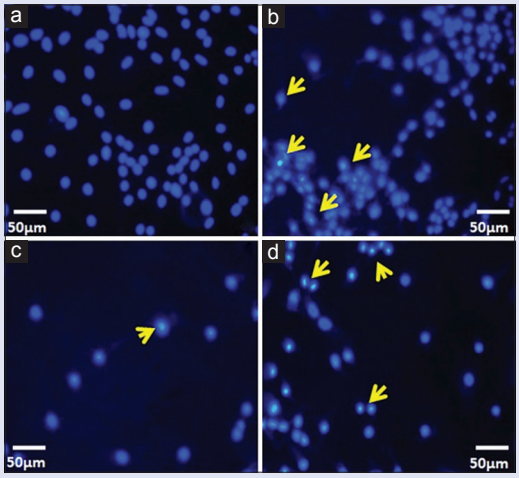

Figure 4: Effect of *Gracilaria foliifera* on nuclear chromatin condensation in HepG2 cells by Hoechst staining. (a) Control and (b-d) 20, 40, and 80 μg/mL *Gracilaria foliifera* treatments, respectively. Arrows show bright chromatin condensed nucleus observed only in the experimental groups

by fluorescein-labeled annexin V staining. PS translocation is one of the earliest features of cells undergoing apoptosis. In this study, *G. foliifera* treatment (20, 40, and 80 μg/mL) caused translocation of PS from the inner side of the plasma membrane to the surface. The intensity of annexin V immunofluorescence is more positive in the experimental groups when compared to control [Figure 3].

Apoptotic nuclear chromatin analysis by Hoechst staining

Apoptosis-induced chromatin changes were investigated by Hoechst staining using fluorescence microscopy. After treatment with different concentrations of *G. foliifera* for 24 h, HepG2 cells began to exhibit apoptotic characteristics, such as cell shrinkage, nuclear condensation, and fragmentation. The condensed chromatin in apoptotic cells appears more brightly in experimental groups than the chromatin in normal cells [Figure 4].

Gracilaria foliifera effect on the p53 protein expression

We further investigated the changes in the tumor suppressor p53 protein expression. *G. foliifera* treatment caused a significant upregulation of p53 expression at protein level than that of control. The high expression of p53 was observed with the maximum *G. foliifera* concentration used in this study, i.e., 80 μg/mL. β-actin is used for normalization of p53 protein expression [Figure 5a and b].

DISCUSSION

Natural products comprise a large portion of current-day pharmaceutical agents, especially in cancer therapy.[18] It is reported that over and above 60% of the anticancer drugs were derived in one way or another from natural sources.[19] Various bioactive compounds and biomaterials derived from marine algae are important ingredients in many products, such as cosmetics and drugs for treating cancer and other diseases.[20] In this study, *G. foliifera* caused a significant growth inhibitory effect in HepG2 cells. The cytotoxic effect of *G. foliifera* has been reported previously against human breast cancer cell lines.[16] Other brown algae species of *Gracilaria* were found to inhibit the proliferation of various *in vitro* cancer cell lines,[21,22] and our current results are in line with these results.

The annexin V/PI staining is a commonly used method for qualitatively evaluating the apoptotic cells. Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS, and binds to exposed apoptotic cell surface PS. In addition, the characteristics of late apoptosis include some loss of membrane integrity and PI to pass through the membranes, intercalate into nucleic acids, and display red fluorescence.[23,24] In view of the above reports, the PI-stained cells in the experimental groups indicate that HepG2 cells are in the late apoptotic stage. It was reported that annexin V/PI staining is not suitable for the evaluation of early apoptotic cells,[24] and hence, we examined the early apoptotic cells through plasma membrane translocation of PS. Translocation of membrane PS from the inner side of the plasma membrane to the surface is reported as one of the earlier events of apoptosis.[25] *G. foliifera* treatments induced significant translocation of PS to the periphery of HepG2 cell membrane, indicating strong evidence of early apoptosis. Further, the nuclear chromatin condensation, that is characteristic of apoptotic cell death, was also investigated by Hoechst staining. This staining is usually performed to investigate the nuclear damage, especially chromatin condensation upon apoptosis induction or DNA damage in cancer cells.[26] As confirmation of apoptosis, *G. foliifera* treatment resulted in the condensation of nuclear chromatin in apoptotic cells that appears more brightly when compared to untreated cells, and these results further correlate with the above findings.

The p53 tumor suppressor protein regulates cell cycle progression and cell survival in response to cellular stress.[27] This protein is also responsible for the regulation of cellular senescence and cell cycle.[28] DNA damage or oncogenic stress induces p53 protein levels, allowing elimination of incipient tumor cells by apoptosis. When DNA damages are induced, the cell cycle is arrested and p53 is activated for DNA repair.[29] If DNA repair is not successful, then p53 causes apoptosis by induction of Bax.[30] It is also noteworthy to mention that p53 participates directly in the intrinsic apoptosis pathway by interacting with the multidomain members of the Bcl-2 family to induce mitochondrial outer membrane permeabilization.[31] In this study, *G. foliifera* treatment induced upregulation of p53 protein expression, which could probably be due to the apoptosis induction in HepG2 cells.

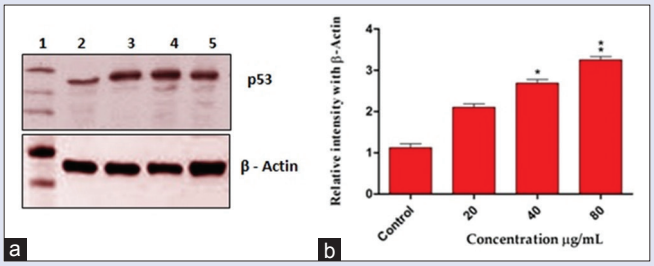

Figure 5: p53 protein expression. (a) p53 protein expression pattern and (b) quantification of protein expression. 1 – protein ladder, 2 – control, 3, 4, and 5 – 20, 40, and 80 µg/mL of *Gracilaria foliifera* treatments, respectively. Values are expressed as mean ± standard error of the mean ($n = 3$). *$P < 0.05$; **$P < 0.01$ versus control

CONCLUSION

The overall finding of the present study suggests that *G. foliifera* treatment in HepG2 cells caused (i) the externalization of PS at early stages after the induction of apoptosis, (ii) late apoptosis and necroptosis, (iii) nuclear fragmentation and chromatin condensation, and (iv) activation of p53 protein expression. These findings undoubtedly confirm the induction of apoptosis in HepG2 cells upon *G. foliifera* treatment. *G. foliifera* is likely to be valuable for the management of HCC.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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