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Anti-angiogenesis and apoptogenic potential of the brown marine alga, *Chnoospora minima*

Shabana Parveen and Varalakshmi K. Nadumane^{*}

Abstract

Background: Algae being one of the dominant organisms in nature can provide best opportunity for the discovery of new anti-cancer drugs. The aim of the present study was to investigate the anti-cancer and anti-angiogenic potential of the brown marine alga *Chnoospora minima*.

Result: The methanol extract of *C. minima* and its bioactive fraction (CF4) have highly significant cytotoxic effects to HepG2, HeLa and MCF-7 cancer cell lines. The fraction's ability to induce apoptosis in the cancer cells was evidenced by increased caspase activity (caspase-3, 7 and 10), DNA fragmentation pattern and upregulated expressions of Bax and p53 genes. The bioactive fraction was not toxic to human peripheral lymphocytes. HPLC, ESI-MS and GC-MS analysis of CF4 fraction indicated the presence of the compound hexadecanoic acid which might be responsible for the observed anti-cancer activity of *C. minima*. The methanol extract of *C. minima* exhibited anti-angiogenic effects on chick embryos.

Conclusion: It can be concluded that fraction, CF4, from *C. minima* is a promising source of an anti-cancer lead molecule.

Keywords: Angiogenesis, Apoptosis, Bax, Caspase, *Chnoospora minima*, p53 expression

Background

Cancer is a major challenge for humans throughout the world, being the second leading cause of death [1]. Cancer affects all age groups, genders, organs and tissues. Despite the advancements in the technology to diagnose and treat this disease, the mortality rate is very high among the affected people. The current treatment modalities of chemotherapy, surgery and radiotherapy are not efficient enough due to their significant side effects and unequal responses. Search for novel and efficient compounds from natural sources thus becomes very important in cancer management. Many molecules have been isolated from natural sources like plants/microorganisms which are being used as anti-cancer agents.

The marine environment with its enormous undiscovered compounds is an attractive source for bioactive compounds. Already, more than 20,000 compounds have been isolated from marine invertebrates. Marine algae comprise one of the fascinating groups of organisms that provide us a large window of opportunity to find novel compounds, owing to their diverse subspecies and variety of unknown, unimaginable molecules which can be used in research against cancer. Many marine algae have been reported to possess anti-tumour activities [2–4]. To find an alternative therapy for cancer, the present research work is focussed to evaluate the brown marine alga, named *Chnoospora minima*, which comes under the phaeophyceae group of marine algae.

* Correspondence: kn.varalakshmi@jainuniversity.ac.in

Department of Biotechnology, School of Sciences, Jain (Deemed-to-be-University), #18/3, 9th Main, 3rd Block, Jayanagar, Bangalore, Karnataka 560011, India

Methods

Collection of algal sample and preparation of extracts

Marine algal sample was collected from Gulf of Mannar, south east coast of India (Long N 9 16.313 Lat E 79 00.073) Rameshwaram in the state of Tamilnadu. The sample was identified and authenticated as *Chnoospora minima*. Samples were washed with tap water, rinsed with distilled water for 3–4 times and allowed to dry under the shade. Dried samples were powdered in an electric mixer. Ten grammes of the sample was extracted with methanol using a soxhlet apparatus. Extracts were filtered using Whatman filter paper and concentrated in a rotary evaporator under reduced pressure.

Purification of methanol extract by thin-layer chromatography (TLC)

The methanol extract of *C. minima* (2 g) was partially purified by preparative TLC using commercially available Silica gel-coated chromatography sheets (50 × 20 cm size, Merck), following standard methods [5].

HPLC analysis of fraction CF4

The partially purified fraction CF4 was purified by running the sample in a semi-preparative HPLC (LC-20 AD plus Detector, Shimadzu, Japan) connected to a system with LabSolutions software. The pumps (pump A and pump B) were used to pass the pressurized liquid solvent (water to methanol) along with the sample. Pump A was always maintained as water (default) and pump B was set as methanol. The fractions from the sample were separated based on the gradient applied in the system as shown in Table 1.

When the TLC-purified sample (2 µL) was injected into the analytical column (C18, 5 µM: dimension 4.6 × 250 mm), at a flow rate of 0.5 mL/min, the sample was separated into different fractions at different retention times. To collect the separated fractions from the sample mixtures, preparatory column was used. Hundred microlitres of injection volume were used in the preparatory column (5 µM, C18: 10 × 50 mm dimensions) with a flow rate of 1.2 mL/min and total run was for 25 min.

Table 1 Separation of samples based on the gradient applied in the HPLC programme

Time (min)	Module	Command	Value (%)
0.01	Pumps	Pump B conc	10
2.00	Pumps	Pump B conc	10
6.00	Pumps	Pump B conc	90
10.00	Pumps	Pump B conc	90
13.00	Pumps	Pump B conc	10
20.00	Pumps	Pump B conc	10
25.00	Controller	Stop	

ESI-MS analysis

The major fractions separated through HPLC were subjected to QTOF Mass spectrometer ESI-QUAD-TOF (Bruker Impact HD, USA) at Indian Institute of Science (IISc, Bengaluru). Spectra were acquired at a maximum rate of 50 Hz.

GC-MS analysis

The bioactive fraction of *C. minima* (CF4) was subjected to GC-MS analysis at Central Silk Technological Research Institute, Bangalore. The system is equipped with HP-5MS capillary column of 30 mm × 0.25 mm and 0.25 mm of 1 mm thickness with 70 eV of ionization energy. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. One microlitre of the sample was injected into the GC-MS system, the injector port and detector temperature maintained at 280 °C. The initial temperature was 50 °C for 3 min, then increased to 280 °C and held for 2 min. The resulting GC-MS peaks of unknown compounds were analysed and were compared with the database present in the MS library and other anti-cancer compound databases.

Cell lines and culture conditions

The breast cancer cell line MCF-7, cervical cancer cell line HeLa and liver cancer cell line HepG2 were procured from National Centre for Cell Sciences (NCCS), Pune. They were grown in minimal essential medium (MEM, HiMedia, India) containing 10% foetal bovine serum, 1000 U/mL penicillin, 100 µg/mL streptomycin and pH 7.4 in 25 cm² culture flasks. The flasks were incubated at 37 °C in a CO₂ Incubator (Thermofisher, USA).

MTT Assay

Exponentially growing cells were plated in 96-well plates at a density of 3×10^3 cells/mL in 100 µL of culture medium and were allowed to adhere for 16 h before treatment. Increasing concentrations of samples prepared in DMSO were added. Cytotoxicity was checked using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as per the standard protocol [6]. Each experiment was performed in triplicates.

Viability by trypan blue staining

Trypan blue staining solution was prepared at 0.4% concentration in phosphate buffer saline (PBS). The cells, 48 h after treatment with sample, were harvested by trypsinization and resuspended in 1 mL of PBS. Equal volumes of the dye and the cell suspension were thoroughly mixed in a sterile vial. The stained cells were observed under a microscope and counted using a haemocytometer [7].

Cytotoxicity assessment by LDH assay

The cytotoxicity was analysed by using Cytoscan™ LDH Assay method (G-Biosciences, India, kit #786-210) [8]. In this method the cytosolic enzyme lactate dehydrogenase (LDH) was measured in the cancer cells after treatment with the sample. The enzymatic reaction results in the conversion of iodinitrotetrazolium (INT) to red-coloured formazan. The reduction of INT was measured quantitatively at 490 nm over a defined time period. The percentage cytotoxicity was calculated using the formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{O.D}_{490} \text{ of sample} - \text{O.D}_{490} \text{ of blank}}{\text{O.D}_{490} \text{ of positive control}} \times 100$$

Caspase-3, 7 and 10 activity assay

The activity of caspases (caspase-3, 7 and 10) was assessed in the cancer cells after sample treatment using the Caspase Colorimetric Assay Kit (G Biosciences, India, kit # 786-205A). The assay was performed following the manufacturer's instructions.

DNA fragmentation analysis

Extract treated or untreated cells were harvested by trypsinization and centrifugation. For DNA fragmentation analysis, cellular DNA was extracted using the Blood and Cell Culture Mini DNA kit as per the manufacturer's instructions (Bangalore Genei, India). The DNA was visualized by ethidium bromide staining after agarose gel electrophoresis [9].

Flow cytometry for cell cycle analysis

Propidium iodide staining

The cells were treated with the sample for 48 h. The adherent cells were harvested by trypsinization and washed with serum-containing media. To fix the cells, 0.3 mL of cell suspension was taken in a vial and 0.7 mL of chilled 100% ethanol was added and overnight stored at 4 °C. The cells were centrifuged at 2500 rpm and washed twice with PBS prior to staining. Appropriate quantity of RNase (final concentration of 0.2–0.5 mg/mL) was added and incubated at 37 °C for 20 min. To the cell suspension, PI stock stain solution (to get a final concentration of 40 µg/mL) was added and incubated in the dark for 10 min. The cells were scored by FACScan flow cytometer (488 nm) and the data were analysed using MACS Quant analyser [10].

Bax and p53 expression studies by quantitative real-time PCR (qRT-PCR)

The cells (2×10^6 cells/mL) were treated with 50 µg/mL of the bioactive fraction (CF4) for 48 h. The cells were harvested by trypsinization followed by centrifugation at 1000 rpm for 10 min. Total RNA was extracted from the cell pellet according to the instruction given in the kit manual. After RNA isolation, the concentration and

purity of RNA were assessed using a spectrophotometer (Sartorius, Germany). The purity of RNA was confirmed with an absorbance ratio at 260 and 280 nm (A260/A280) between 1.8 and 2.2. For reverse transcription, cDNA was first synthesized from RNA using cDNA synthesizer provided in the kit. The cDNA was confirmed by performing PCR for actin gene (housekeeping gene) and it was analysed by 2% agarose gel electrophoresis. The sample was run in applied Biosystem one-step real-time PCR using SYBR Green Chemistry kit (Sensifast SYBR HiRoxkit, Bioline, USA) according to the procedure given in the instruction manual. The qRT-PCR conditions were started with an initial denaturation at 95 °C for 10 min and continued with 40 cycles of 15 s at 95 °C, annealing at 60 °C for 30 s and extension at 72 °C for 30 s using the specific primers for p53 and Bax genes (Table 2). β -actin was used as the housekeeping gene.

The results were analyzed by calculating the relative quantification (RQ) values both in calibrator and treated sample using qbase plus software 13. To calculate the expression of a target gene (TG) relative to the EC, the comparative Ct ($\Delta\Delta\text{Ct}$) method 148 (Step one Software v2.2.2) was used as per the following equation:

$$\Delta\Delta\text{Ct} = (\text{Ct target gene}) - (\text{Ct EC}) - (\text{Ct target gene}) - (\text{Ct EC}) \text{ test sample calibrator.}$$

The $\Delta\Delta\text{Ct}$ values will be converted to a linear form using the formula: $E^{-\Delta\Delta\text{Ct}}$

$$\Delta\text{Ct} = \text{Average Ct of test sample} - \text{Average Ct of calibrator.}$$

The ΔCt values will be converted to a linear form using the formula: $E^{-\Delta\text{Ct}}$, where E = amplification efficiency.

Assay for anti-angiogenesis on chick embryos

Inhibition of blood vessel formation was analysed by the modified chick chorioallantoic membrane (CAM) assay [11]. Instead of using filter paper or silicone ring on the CAM layer, the sample was directly injected to 8-day-old chick embryos and its effect on the blood capillaries was checked after 72 h. Fertilized chicken eggs were collected from Veterinary College, Hebbal, Bangalore, and were maintained in CO₂ incubator at 55–60% humidity. Fertilized eggs (8th–12th day) were used for the study. The eggs were wiped with 70% ethanol, a mark was made below the air space of the eggs and a hole was made on the mark using an egg puncher under sterile

Table 2 Forward and reverse primer sequences used for the study

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
p53	AGAGTCTATAGGCCACCCC	GCTCGACGCTAGGATCTGAC
Bax	TTTGCTTCAGGGTTTCATCC	CAGTTGAAGTTGCCGTGACA
β -actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG

conditions. The needle of the syringe was carefully placed at 45° angle into the allantoic cavity. A hundred microlitres of different concentrations of the samples (0.5, 1.0 and 2.0 mg/mL) was directly injected to the eggs. A hundred microlitres of DMSO in phosphate buffered saline (1:9) was used as the negative control and eggs without any injections were used as normal controls. After injection, the hole was sealed with parafilm wax to avoid contamination from external sources. The eggs were returned to the incubator for 72 h. The anti-angiogenesis activity was examined both macroscopically and microscopically after 72 h of treatment. The number of eggs used for the study was six and all the experiments were carried out thrice.

Macroscopic observation

The egg shell was opened and the effect was visualized under the dissection microscope. The quantification and inhibitory effect of extracts on the blood vessels were calculated manually by counting the number of blood

vessels in control as well as in the treated group, using the following formula [12].

Inhibition of blood vessel (%) = $\frac{\text{No. of blood vessel in control} - \text{No. of blood vessel in treated}}{\text{No. of blood vessel in control}} \times 100$

Histopathological studies

Slides were prepared from different layers of control and treated chick embryos for histopathology and were stained with haematoxylin and eosin stain. The stained slides were examined under a binocular microscope, (Olympus, Germany 5X 541) and were photographed. Slides were scored for (1) structure of blood vessel mainly highlighting endothelial cells, (2) number and frequency of blood vessels and (3) membrane integrity.

Statistical analysis

The experiments were carried out in triplicates and the results were expressed as mean \pm standard error. Data from the experiments were analysed using one-way analysis of variance (ANOVA) with the help of GraphPad

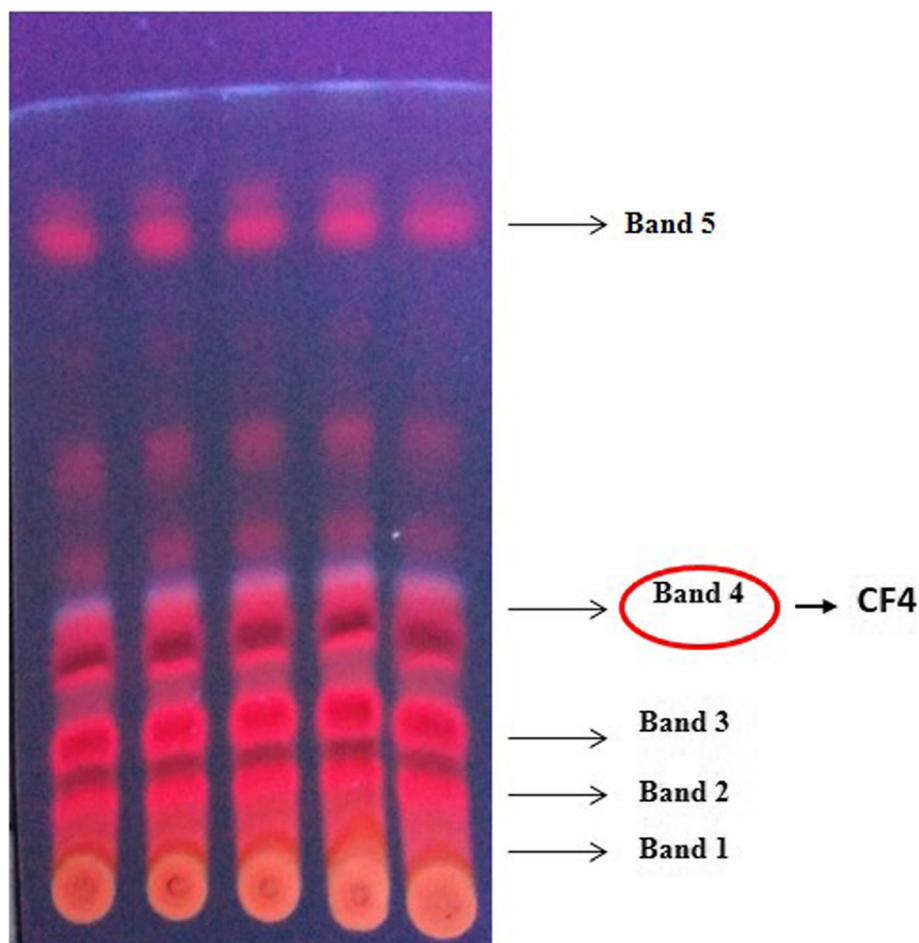


Fig. 1 TLC separated fractions of *C. minima* as observed under UV transilluminator

Prism 6.0 software. Using Duncan's multiple range test (DMRT), the sample means were compared. Differences were considered significant at $p < 0.05$.

Results

Purification of *C. minima* extract by TLC

The partial purification of the methanol extract of *C. minima* by TLC using the solvent combination of dichloromethane and chloroform (3:7) resulted in five different bands (Fig. 1)

HPLC purification

Out of the five fractions separated by TLC, the bioactive fraction CF4 was purified by HPLC method and the resulting chromatogram showed maximum peak at retention times (RT) 0.4–0.9 min and 6.5–6.6 min (Fig. 2a).

ESI-MS analysis

When the HPLC eluted fractions were further analysed by mass spectrometry, maximum spectral intensity was observed for the eluent with an m/z value of 274.3 (Fig. 2b).

GC-MS Analysis

As per GC-MS results, the compounds present in the bioactive fraction are hexadecanoic acid, undecane, ethanol 2-

phenoxy,7,7,9,9,11,11-hexamethyl-3,6,8,10,12,15-hexaoxa-7,9,11-trisilaheptadecane, cyclotetrasiloxane, octamethyl-, diethyl phthalate, hexasiloxane, tetradecamethyl-, 16-octadecenoic acid, methyl ester and bis (2-ethylhexyl) phthalate. The maximum peak was obtained at 18.1 retention time and the compound laid on that peak was hexadecanoic acid (Fig. 3a, b).

Cytotoxicity of *C. minima* to different cancer cell lines

When the methanol extract from *C. minima* was screened for cytotoxicity by MTT assay on MCF-7, HeLa and HepG2 cancer cell lines at two different concentrations (50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$), it was found that the extract was effective against all the cell lines and maximum effect was on MCF-7 cell line with a viability of 63% at 100 $\mu\text{g/mL}$ (Fig. 4a). The screening of all 5 TLC separated fractions of *C. minima* extract indicated the 4th fraction (CF4) as having highest cytotoxicity to MCF-7 cells with 48% viability and was identified as the bioactive fraction (Fig. 4b). When cancer cells were treated with 50 $\mu\text{g/mL}$ of CF4, the percentage viability of the treated MCF-7 cells was 48%, HeLa was 54% and HepG2 was 55% (Fig. 4c). The IC_{50} concentration of CF4 fraction was 37 $\mu\text{g/mL}$ in the case of MCF-7 cells, 50 $\mu\text{g/mL}$ on HeLa cells and 45 $\mu\text{g/mL}$ on HepG2 cells

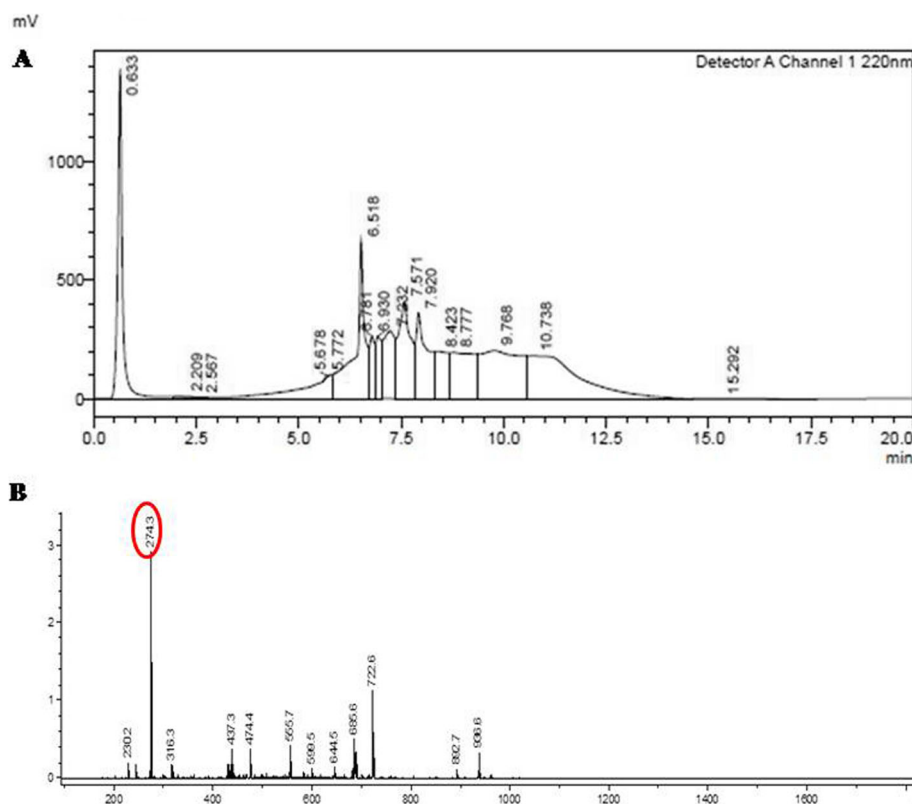


Fig. 2 Analysis of CF4 fraction by HPLC. **a** The HPLC chromatogram of the fraction CF4. **b** Mass spectrum of the HPLC purified eluent at RT 6.5–6.6 min

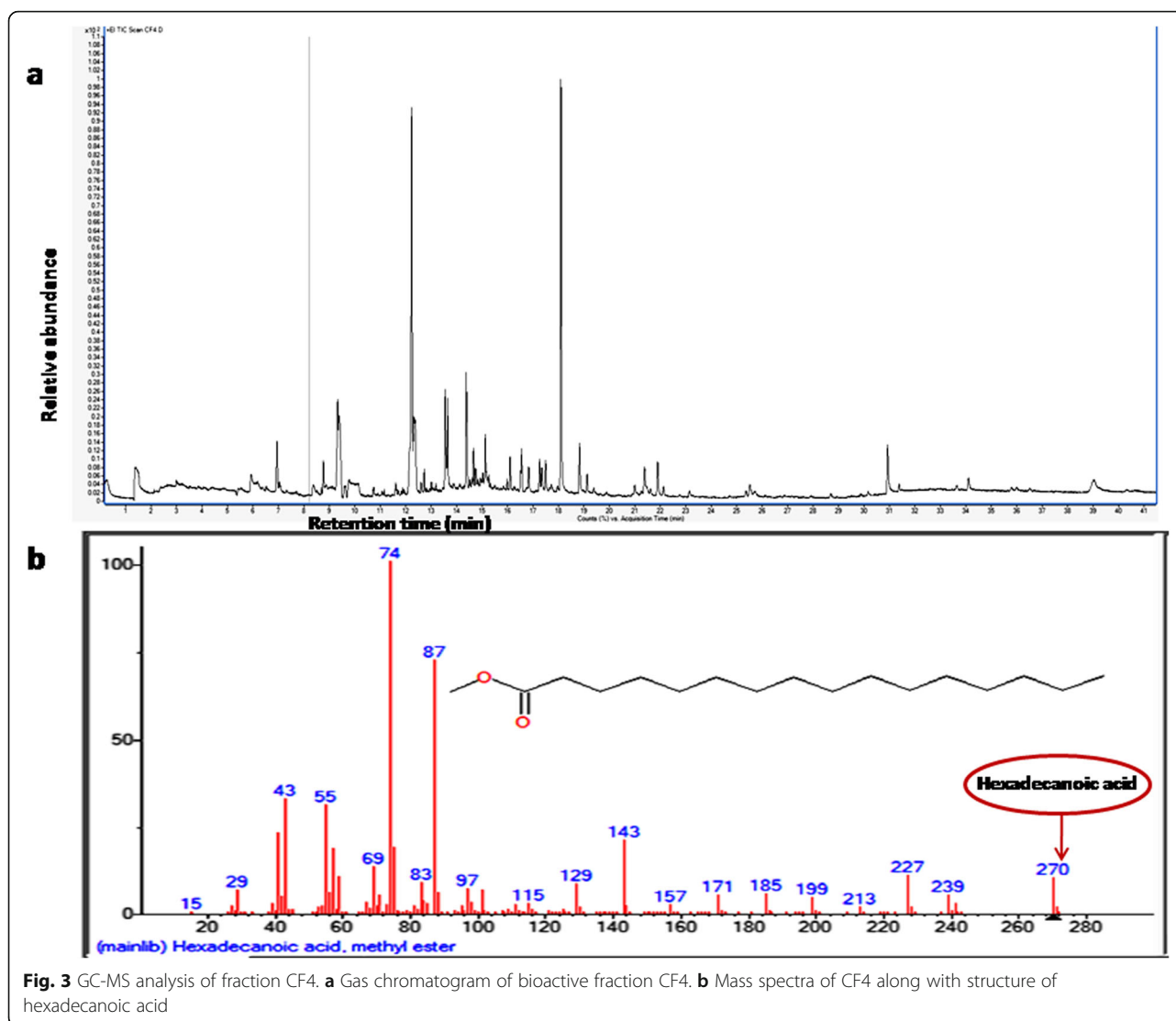


Fig. 3 GC-MS analysis of fraction CF4. **a** Gas chromatogram of bioactive fraction CF4. **b** Mass spectra of CF4 along with structure of hexadecanoic acid

for 72 h of treatment. The effect was found to be dose- and time-dependent, thus indicating the anti-proliferative property of CF4. When fraction CF4 was tested on normal human peripheral lymphocytes, the percentage viability of lymphocytes was found to be ~ 100 at all treatment periods, indicating the safety of CF4 to normal cells.

Cell concentration and cell viability

The cancer cells treated by CF4 were found to be affected at a concentration 50 µg/mL with a cell concentration of 7.2×10^6 cells/mL, 3.15×10^6 cells/mL and 5.8×10^6 cells/mL for HeLa, MCF-7 and HepG2 cells, respectively (Fig. 5a). These cell concentrations were significantly lesser than that of the control cells. The percentage viability of CF4-treated normal lymphocytes was almost close to 100.

LDH cytotoxicity

The maximum release of LDH was observed for CF4 treatment on MCF-7 cells with a cytotoxicity of 42.75% (Fig. 5b), followed by 31.63% in HeLa cells and 11.24% in HepG2 cells. The effect of CF4 was cell line-specific. No cytotoxicity was caused by CF4 to normal human lymphocytes.

Caspase-3, 7 and 10 activities

When caspase enzyme activity was analysed in the treated cancer cells, we found a gradual increase of activity in MCF-7, HeLa and HepG2 cells (Fig. 6). The activity was 49.59% higher in MCF-7, 40% higher in HeLa and 7.3% higher in HepG2 cells as compared to the control cells.

DNA fragmentation

When the DNA extracted from the treated and untreated cancer cells were run in agarose gel, it was found

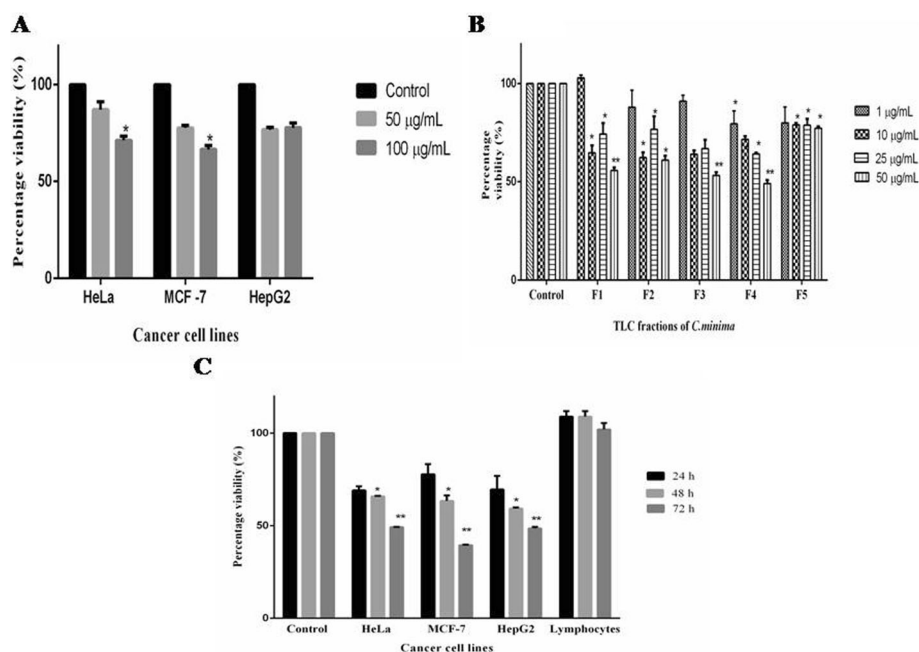


Fig. 4 Effect of *C. minima* extract and CF4 fraction on human cancer cell lines. **a** Percentage viability of HeLa, MCF-7 and HepG2 cells treated with *C. minima*. **b** Effect of TLC fractions of *C. minima* on MCF-7 cells for 48 h. **c** Effect of CF4 on HeLa, MCF-7, Hep G2 and lymphocyte cells for 24, 48 and 72 h. The values are expressed as mean \pm SE. An asterisk denotes significance at $p < 0.05$ and two asterisks denotes significance at $p < 0.01$

that the DNA was appearing as a smear due to fragmentation in the treated cancer cells (MCF-7, HepG2 and HeLa) as compared to the control groups (Fig. 7).

Cell cycle of CF4-treated cancer cells

When the cells were analysed through flow cytometry after 48 h of treatment with CF4, we found that (Fig. 8) in MCF-7 cells, a significant proportion (35.7%) of cells were in sub G0/G1 phase, indicating apoptosis. In the case of HeLa cells, G2/M phase arrest was seen with 85.9% of cells in this phase as compared to the control group where only 19.3% cells were in G2/M phase. In the case of HepG2 cells, a higher proportion of apoptotic cells (43.0%) were found in sub G0/G1 phase. S phase cells increased from 13.5 to 17.3% along with a decrease of G2/M phase cells from 13.5 to 9.7%.

Relative expression of Bax and p53 genes

When the expression pattern of Bax and p53 in MCF-7 cells treated with the sample were checked by qRT-PCR, we found 3.2-fold higher expression of Bax gene than that of the untreated control cells (Fig. 9a). The expression of p53 was also found to be upregulated (2.7-folds higher) than that of the untreated control cells (Fig. 9b).

Anti-angiogenesis effect

Macroscopic observation

When different concentrations (0.5, 1.0 and 2.0 mg/mL) of *C. minima* extract were injected to 8-day-old chick embryos, anti-angiogenic property was evidenced after 72 h of treatment. At 2 mg/mL concentration of treatment, we found significant inhibition of blood capillaries, while moderate inhibition of blood vessels was seen at 0.5 and 1 mg/mL concentrations (Fig. 10a, b).

Histopathology observation of chick embryos

The anti-angiogenic potential of *C. minima* was observed by studying the histological preparations of the blood vessel formed in the chick embryos. From Fig. 11, it can be seen that the control embryos had thick-walled endothelial cells and the number of blood vessels was higher. In the case of treated embryos, significant reduction of large and small blood vessels along with the presence of thin endothelial cells was seen. The anti-angiogenic effect of *C. minima* was found to be dose-dependent.

Discussion

One way to control the proliferation of cancer growth is by inducing cell death in the cancer cells. Hence, drugs should be designed in order to induce cell death either by apoptosis or necrosis. Marine algae comprise one of the important sources of natural bioactive compounds

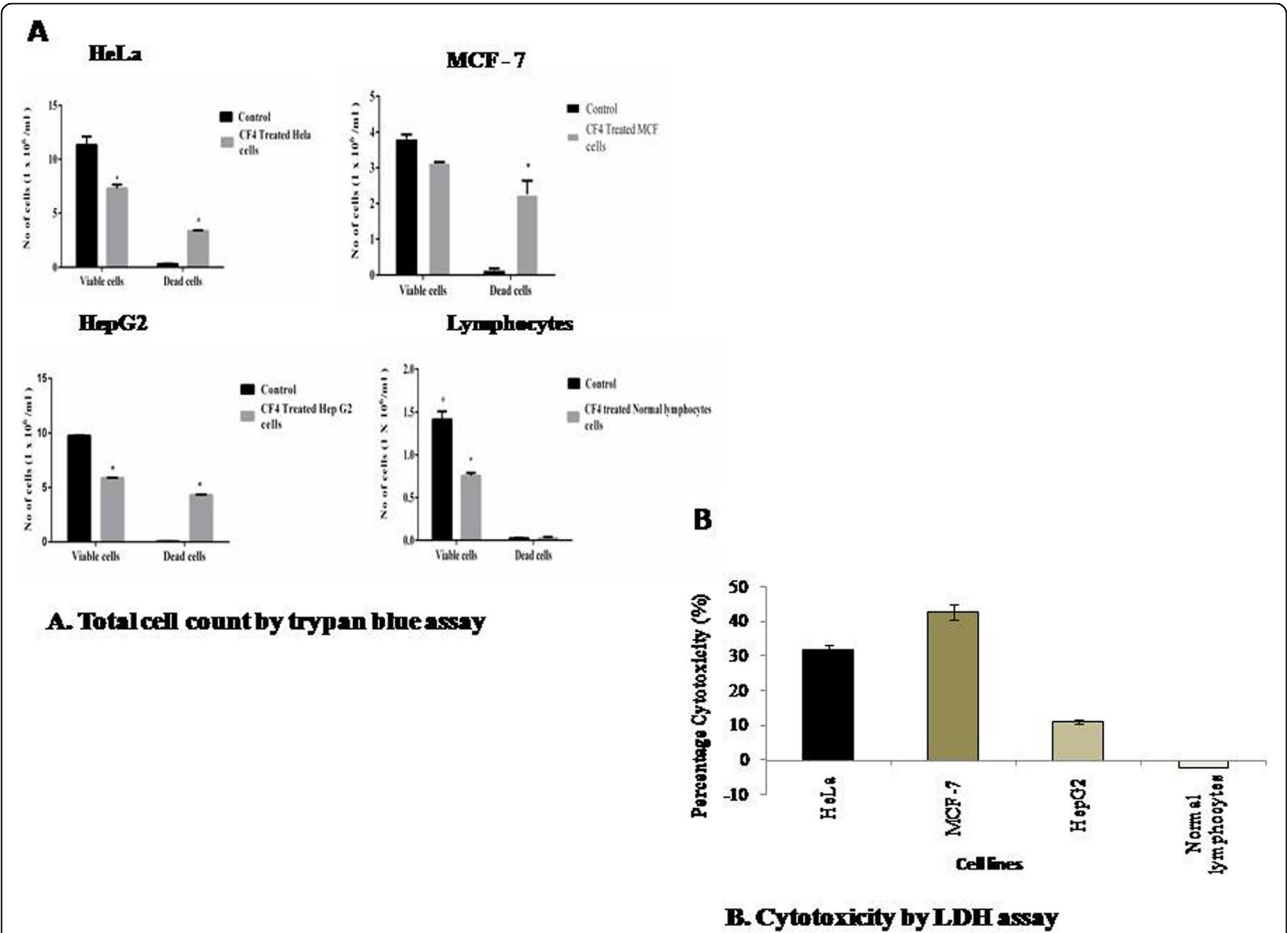


Fig. 5 In vitro cytotoxicity assays. **a** Cell count of HeLa, MCF-7, HepG2 and lymphocyte cells as per trypan blue assay. **b** Effect of CF4 fraction on LDH activity of different cell lines. The values are expressed as mean \pm SE. An asterisk denotes significance at $p < 0.05$ and two asterisks denotes significance at $p < 0.01$

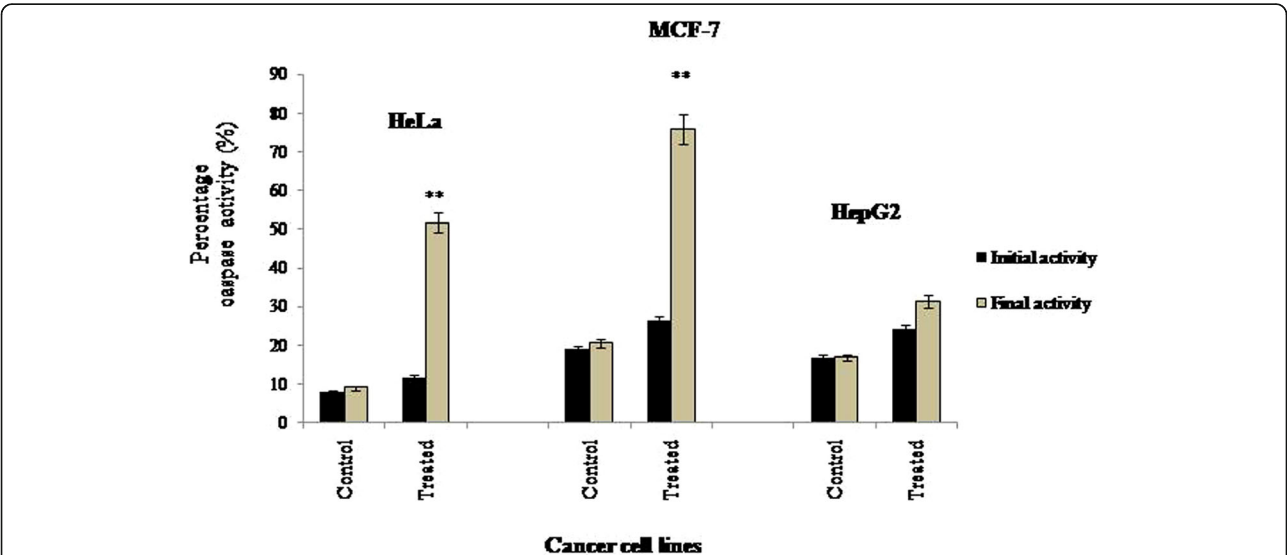
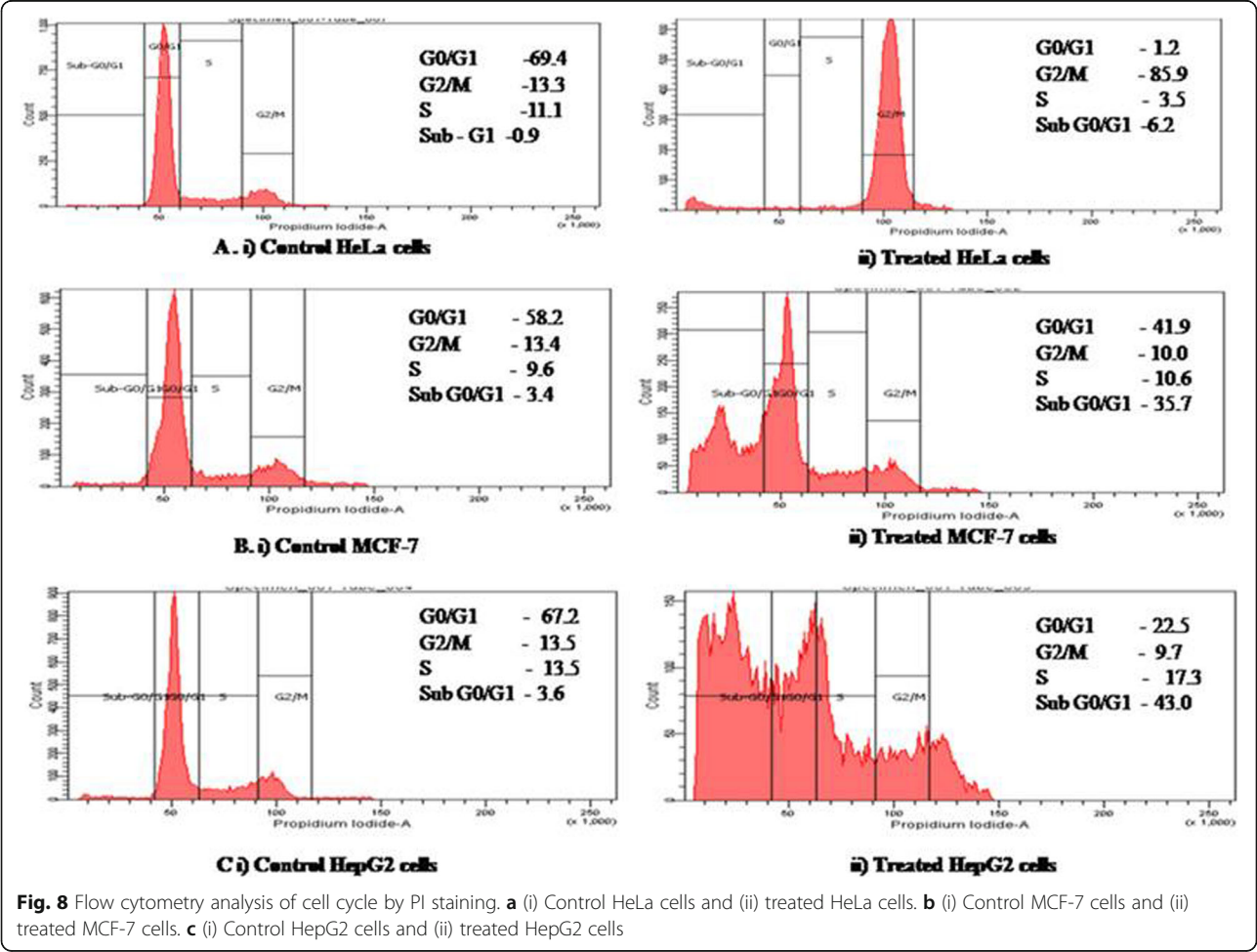
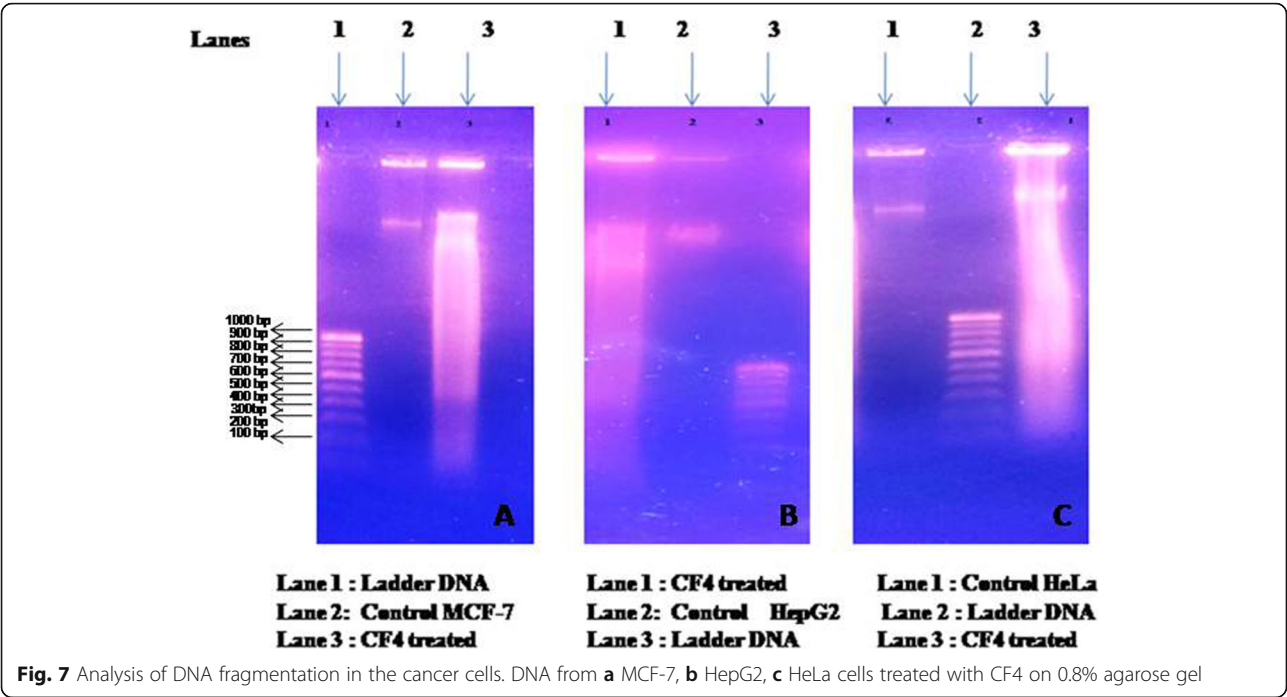


Fig. 6 Caspase activity of the cancer cells. Caspase-3, 7 and 10 activity of CF4-treated HeLa, MCF-7 and HepG2 cells. The values are expressed as mean \pm SE. An asterisk denotes significance at $p < 0.05$ and two asterisks denotes significance at $p < 0.01$



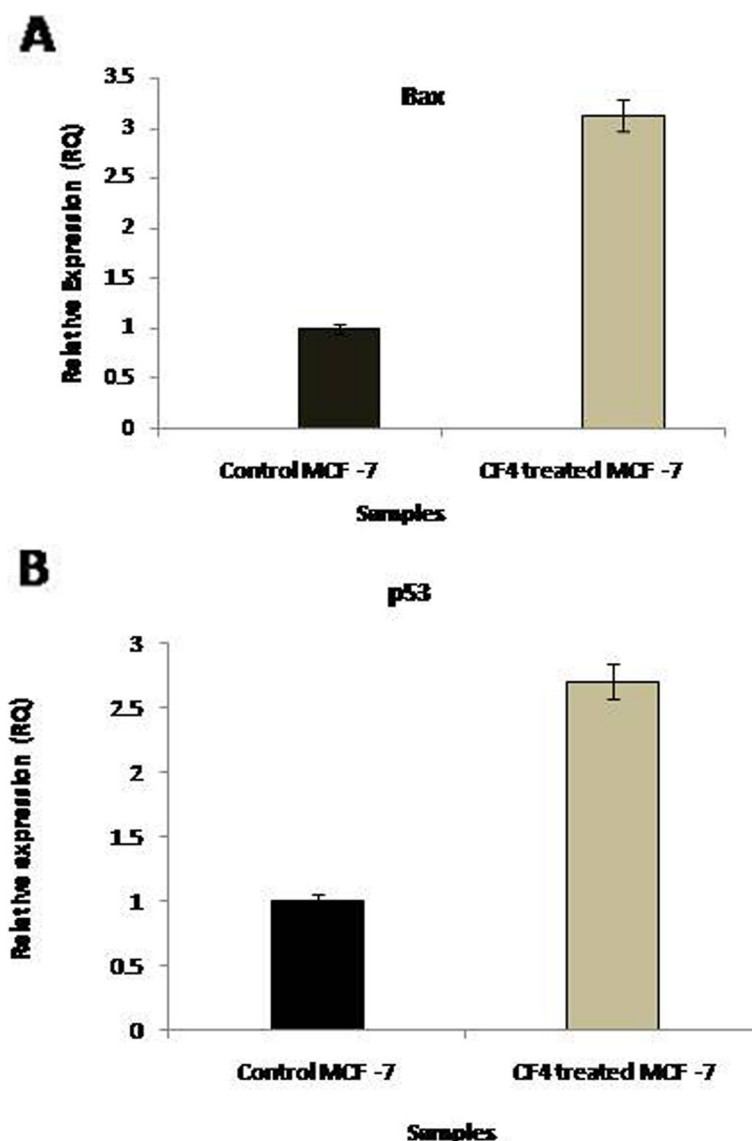


Fig. 9 Analysis of gene expression by qRT-PCR. **a** Relative expression of Bax gene in treated and untreated cells. **b** Relative expression of p53 gene in treated and untreated cells

[13, 14]. In the current study, the brown marine alga, *Chnoospora minima*, demonstrated significant anti-proliferative activity against the tested cancer cell lines. In the mitochondrial activity assay, the bioactive fraction CF4 demonstrated an effective response against the proliferation of cells. The IC_{50} concentration of this fraction was 37 $\mu\text{g/mL}$ on MCF-7, followed by 50 $\mu\text{g/mL}$ on HeLa and 45 $\mu\text{g/mL}$ on HepG2 cells for 72 h. In another study [15], the seaweed *Chnoospora implexa* was reported as to inhibit the growth of MCF-7 cell lines. The LC_{50} value (lethal concentration) of *C. implexa*, *U. lactuca* and *C. hornemanni* seaweeds were 125 $\mu\text{g/mL}$. In one more study, the fractions of brown algae such as *Sargassum swartzii*, *Cystoseira myrica* and *Colpomenia*

sinuosa had anti-proliferative effects against cancer cell lines. Here, the IC_{50} values were greater than that of methotrexate, the positive control [16]. From these reports, we came to the conclusion that *C. minima* has effective cytotoxicity to the cancer cells at a lower concentration (50 $\mu\text{g/mL}$). TLC purified fraction showed higher activities than the whole extracts. This could be due to the presence of lesser quantities of bioactive components in whole extracts and these compounds becoming concentrated during TLC fractionation [17]. The mechanism of action of the bioactive fraction CF4 in inhibiting cancer cell growth appears to be through the induction of apoptosis as well as by preventing angiogenesis.

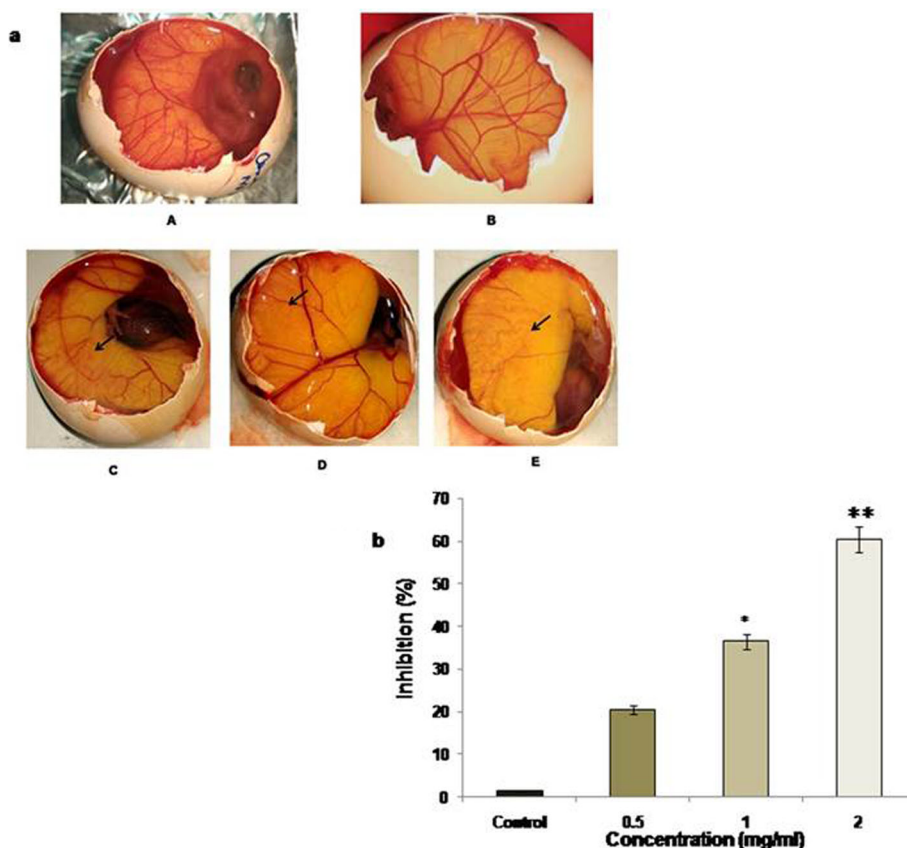


Fig. 10 Anti-angiogenesis effect of *C. minima* on chick embryos. **a** (A) Control chick embryo, (B) DMSO treated control, (C) 0.5 mg/mL treated, (D) 1.0 mg/mL treated, (E) 2.0 mg/mL treated. Arrow indicates the inhibition of blood vessel branch points **b** Percentage Inhibition of blood vessels in 8-day-old chick embryos treated with *C. minima*. The values are expressed as mean \pm SE. An asterisk denotes significance at $p < 0.05$ and two asterisks denotes significance at $p < 0.01$

Caspases are a family of protease enzymes involved in playing crucial roles such as initiation and execution of programmed cell death. Accordingly, we found an increase in the activity of caspase-3, 7 and 10 in *C. minima* treated cancer cells giving an evidence for the ongoing apoptotic process in these cells. Correlating well with this event, DNA fragmentation was also observed in the treated HeLa, MCF-7 and HepG2 cells indicating that CF4 is causing the cancer cell death through caspase activation and inducing apoptosis. The initiation of DNA fragmentation can be related to the activation of caspase-3 activity [18].

The growth of tumour and its metastasis are mainly dependent on angiogenesis as suggested by Folkman [19] that the inhibition of angiogenesis could be a valuable approach for cancer therapy. The use of animal models for the study of angiogenesis is an important tool for screening new agents that might inhibit or activate angiogenesis. The chick embryo assay is a widely used method for in vivo study to analyse the anti-angiogenesis activity. Through our study on chick embryos, it was found that *C. minima* extract efficiently

inhibited the formation of new blood capillaries from the pre-existing blood vessels [20]. The formation of capillary plexus is accompanied by decrease in CAM thickness and reduction in mesenchymal cells [21]. Namvar et al. [22] reported that the marine brown alga, *Sargassum muticum*, has anti-angiogenic effect. Ganesan et al. [23] reported the anti-angiogenesis effect of the siphonaxanthin from the green seaweed. In our study, *C. minima* extract suppressed angiogenesis by inhibiting the migration of mesodermal blood vessels to the basement membrane leading to the decrease in the capillary plexus and thereby reducing the thickness of CAM layer.

A strong evidence for apoptosis being induced by the bioactive compound from *C. minima* was provided by the results of qRT-PCR analysis of Bax and p53 genes. Generally, cancer cells proliferate due to the higher expression of the anti-apoptotic genes and downregulating the pro-apoptotic genes and thereby evading apoptosis. In this study, the expression of pro-apoptotic Bax gene was upregulated in the treated cells indicating that the cells are undergoing apoptosis due to Bax overexpression. In the same cells, the tumour suppressor gene, p53, was

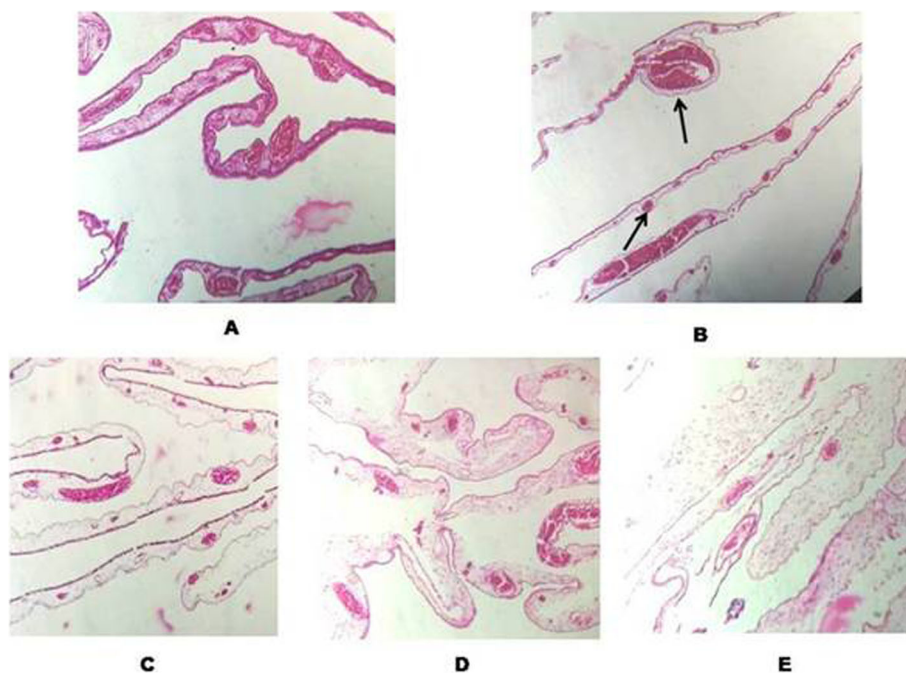


Fig. 11 Histopathological examination of chick embryos at $\times 40$ magnification. **a** Control CAM. **b** DMSO treated control. **c** 0.5 mg/mL treated. **d** 1 mg/mL treated. **e** 2 mg/mL treated. Black arrow indicates the presence of large and small blood vessels

also found to be overexpressed. This p53 gene plays a vital role in inducing cell death after DNA damage and genomic instability, as it is a nuclear transcription activator of the Bax gene [24] which interacts with Bcl-2 to enhance outer mitochondrial membrane permeabilization. The increased expression of p53 might be responsible for the increase in the Bax to Bcl-2 ratio, resulting in the release of cytochrome c, the activation of caspases and ultimately apoptosis [25, 26]. This was strongly supported by the bioactive fraction CF4 induced apoptosis in the MCF-7 cells where a 2.7-fold increase in the mRNA level of p53 in the treated group was observed when compared to the control cells.

Due to the promising anticancer potential demonstrated by the TLC-purified fraction CF4, it was further purified by HPLC and the major fraction was further characterized through ESI-MS analysis. The maximum spectral intensity was observed at 274.3 m/z ratio. The molecular ion peak obtained from MS data was found to be closely related to the compound hexadecanoic acid (m/z ratio 270) separated out from the GC-MS results. The molecular ion peaks for methylpalmitate was at 270 m/z ratio [27]. Characterization of CF4 through GC-MS studies also indicated hexadecanoic acid as a major component in CF4. All these results collectively suggest that the major compound present in *C. minima* as an analogue of the fatty acid hexadecanoic acid. Hexadecanoic acid is a saturated fatty acid, synonyms of palmitic acid and is known to have anti-microbial, anti-oxidant

activities and known to reduce the risk of coronary artery diseases [28]. In the present study, we found that the bioactive compound in *C. minima* has anti-proliferative activity against the cancer cell lines at 50 $\mu\text{g/mL}$ concentration. Relevant to this study [29], there is a report that palmitic acid from the marine red alga *Amphiroa zonata* as a cytotoxic substance at 50 $\mu\text{g/mL}$ concentration to human leukemic cells. N-hexadecanoic acid extracted from the leaves of *Kigelia pinnata* demonstrated anti-cancer activity [30]. Palmitic acid was reported as to be present in higher amounts in some marine red algae [31–38]. The previous studies on *C. minima* reported its anti-inflammatory and anti-oxidant activities. According to a previous report [39], polysaccharides from *C. minima* (SLBS11P) had high anti-oxidant activities. To the best of our knowledge, the anti-cancer potential of *C. minima* is being reported for the first time through the outcomes of the current study.

Conclusion

In summary, it can be concluded that the bioactive fraction from the brown macroalga, *C. minima*, is efficient in suppressing the growth of the cancer cells and at the same time is non-toxic to normal human peripheral lymphocytes. It also demonstrated anti-angiogenic activity on chick embryo model, providing the alternative source for cancer therapy. Presence of hexadecanoic acid along with other bioactive compounds appears as the main reason for the anti-cancer property of *C. minima*.

To the best of our knowledge, this study on *C. minima* is the first published report about its anti-proliferative activity. Further in vivo studies need to be carried out in order to find the efficiency of hexadecanoic and its analogues as anti-cancer compounds.

Abbreviations

Hela: Cervical cancer cell line; MCF-7: Breast cancer cell line; HepG2: Liver cancer cell line; TLC: Thin-layer chromatography; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye; HPLC: High-performance liquid chromatography; GC-MS: Gas chromatography-mass spectrometry; ESI-MS: Electro-spray ionization mass spectrometry; qRT-PCR: Quantitative real-time PCR; MARS: Marine Algal Research Station; CAM: Chick chorioallantoic membrane; PI: Propidium iodide

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Authors' contributions

VKN and SP: designed the study of the work. SP: a research scholar who carried out the sample collection, analysis and interpretation of data and writing of the manuscript. VKN: guiding the student to design the work, analysis and interpretation of data and editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author (VKN), upon reasonable request. A voucher specimen of this marine alga has been deposited in the Jain University herbarium with the number- JUBTMA01

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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References

- Aggarwal BB, Danda D, Gupta S, Gehlot P (2009) Models for prevention and treatment of cancer: problems vs. promises. *Biochem Pharmacol* 78(9):1083–1094. <https://doi.org/10.1016/j.bcp.2009.05.027>
- Zhuang H, Itoh T, Mizuno, Ito H (1999) Antitumor active fucoidan from the brown seaweed, *Umitoranoo* (*Sargassum thunbergii*). *Biosci Biotechnol Biochem* 59(4):563–567. <https://doi.org/10.1271/bbb.59.563>
- Coombe DR, Parish CR, Ramshaw IA, Snowden JM (1987) Analysis of the inhibition of tumour metastasis by sulphated polysaccharides. *Int J Cancer* 39 (1):82–88. <https://doi.org/10.1002/ijc.2910390115>
- Vischer P, Buddecke E (1991) Different action of heparin and fucoidan on arterial smooth muscle cell proliferation and thrombospondin and fibronectin metabolism. *Eur J Cell Biol* 56(2):407–414
- Kirchner JG, Miller JM, Keller GJ (1951) Separation and identification of some terpenes by new chromatographic technique. *Anal Chem* 23 (3):420–425. <https://doi.org/10.1021/ac60051a008>
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65(1–2): 55–63. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4)
- Strober W (2001) Trypan blue exclusion test of cell viability. *Current protocols in immunology*, John Wiley & Sons 21(1):77. <https://doi.org/10.1002/0471142735.ima03bs21>
- Weyermann J, Lochmann D, Zimmer A (2005) A practical note on the use of cytotoxicity assays. *Int J Pharm* 288(2):369–376. <https://doi.org/10.1016/j.ijpharm.2004.09.018>
- Shidoji Y, Ogawa H (2004) Natural occurrence of cancer-preventive geranylgeranoic acid in medicinal herbs. *J Lipid Res* 45:1092–1103. <https://doi.org/10.1194/jlr.M300502-JLR200>
- Pazarowski P, and Darzynkiewicz Z (2004) Analysis of cell cycle by flow cytometry. *Methods Mol Bio* 281: 301–311. <https://doi.org/10.1385/1-59259-811-0:301>
- Ribatti DL, Nico B, Bertossi M (1987) Effects of exogenous heparin on the vasculogenesis of the chorioallantoic membrane. *Acta Anat* 130:257–263
- Hardeep S, Tuli S, Sandhu, Anil KS, Puneet G (2014) Anti angiogenic activity of the extracted fermentation broth of an entomopathogenic fungus *Cordyceps militaris*. *Int J Pharm Sci* 6(7):1–6. <https://doi.org/10.1186/2045-824X-4-8>
- Riley RD, Ridley G, Williams K, Altman DG, Hayden J (2007) Prognosis research: toward evidence-based results and a Cochrane methods group. *J Clin Epidemiol* 60 (8): 863–865. <https://doi.org/10.1016/j.jclinepi.2007.02.004>
- Riley RD, Lambert PC, Abo-Zaid G (2010) Meta-analysis of individual participant data: rationale, conduct, and reporting. *BMJ* 340:c221. <https://doi.org/10.1136/bmj.c221>
- Shyamala V, Anagha K TN (2013) In vitro antioxidant and antiproliferative activities of macro algae against MCF-7 cell line. *J Pharm Biomed Sci* 32: 1413–1424
- Khanavi M, Nabavi M, Sadatin, Ardekani SM, Sohrabipour J, Mohammed S, Nabavi, Ghael P, Ostad N (2010) Cytotoxic activity of some marine brown algae against cancer cell lines. *Biol Res* 43(1):31–37. <https://doi.org/10.1007/s12010-0000100005>
- Babayi H, Kolo I, Okogun JI, Ijah UJJ (2004) The antimicrobial activities of methanolic extracts of *Eucalyptus camaldulensis* and *Terminalia catappa* against some pathogenic microorganisms. *Biochem* 16 (2):106–111. <https://doi.org/10.4314/biokem.v16i2.32578>
- Walters J, Pop C, Scott FL (2009) A constitutively active and uninhibitable caspase3 zymogen efficiently induces apoptosis. *Biochem J* 424(3):335–345. <https://doi.org/10.1042/BJ20090825>
- Folkman J (1971) Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285:1182–1186. <https://doi.org/10.1056/NEJM197111182852108>
- García-Caballero M, Cañedo L, Fernández-Medarde A, Medina MÁ, Quesada AR (2014) The marine fungal metabolite, AD0157, inhibits angiogenesis by targeting the Akt signalling pathway. *Mar Drugs* 12(1):279–299. <https://doi.org/10.3390/md12010279>
- Melkonian G, Munoz N, Chung J, Tong C, Marr R, Talbot P (2002) Capillary plexus development in the day five to day six chick chorio-allantoic membrane is inhibited by cytochalasin D and suramin. *J Exp Zool* 292:241–254. <https://doi.org/10.1002/jez.10014>
- Namvar F, Mohamad R, Baharara J, Balanejad SZ, Fargahi F, Rahman HS (2013) Anti oxidant, anti proliferative and anti-angiogenesis effect of poly phenol-rich seaweed (*Sargassum muticum*). *Biomed Res Int* 2013: 604787. <https://doi.org/10.1155/2013/604787>
- Ganesan P, Matsubara K, Ohkubo T et al., (2010) Anti-angiogenic effect of siphonaxanthin from green alga, *Codium fragile*. *Phytomedicine* 17(14): 1140–1144. <https://doi.org/10.1016/j.phymed.2010.05.005>
- Miyashita T, and Reed JC (1995) Tumor Suppressor p53 Is a Direct transcriptional activator of the human Bax gene. *Cell* 80:293–299. [https://doi.org/10.1016/0092-8674\(95\)90412-3](https://doi.org/10.1016/0092-8674(95)90412-3)
- Rassouli FB, Matin MM, Iranshahi M, Bahrami AR, Behravan J, Mollazadeh S, and Neshati V (2011) Investigating the enhancement of cisplatin cytotoxicity by combination with mogoltacin on 5637 cells. *Toxicol In Vitro* 25:469–474. <https://doi.org/10.1016/j.tiv.2010.11.015>

26. Luo G et al (2008) Thesphingolipid long-chain base-Pkh1/2-Ypk1/2 signaling pathway regulates eisosome assembly and turnover. *J Biol Chem* 283(16): 10433–10444. <https://doi.org/10.1074/jbc.M709972200>.
27. Basumatary S, Deka DC (2012) Identification of fattyacid methyl esters in biodisel from *Pithecellobiummonodelphum* seed oil. *Der Chemica Sinica* 3(6):1384–1393
28. Bodoprost J, Rosemeyer H (2007) Analysis of phenacyl ester derivatives of fatty acids from human skin surface sebum by reversed-phase HPLC: chromatographic mobility as a function of physico-chemical properties. *Int J Mol Sci* 8(11):1111–1124. <https://doi.org/10.3390/i8111111>.
29. Harada H, Yamashita U, Kurihara H, Fukushi E, Kawabata J, Kamei Y (2002) Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. *Anticancer Res* 22(5):2587–2590
30. Ravi L, Krishnan K (2017) Cytotoxic potential of N-hexadeconic acid extracted from *Kigelia pinnata* leaves. *Asian J Cell Biol* 12(1):20–27. <https://doi.org/10.3923/ajcb.2017.20.27>.
31. Baghel RS, Kumari P, Reddy CRK, Jha B (2014) Growth, pigments, and biochemical composition of marine red alga *Gracilaria crassa*. *J Appl Phycol* 26(5):2143–2150. <https://doi.org/10.1007/s10811-014-0250-5>.
32. Guaratini T, Lopes NP, Marinho-soriano E, Colepicolo O, Pinto E (2012) Antioxidant activity and chemical composition of the nonpolar fraction of *Gracilaria domingensis* (Kützinger) Sonder ex Dickie and *Gracilaria birdiae* (Plastino & Oliveira). *Rev Bras Farmacogn* 22(4):724–729. <https://doi.org/10.1590/S0102-695X2012005000063>
33. Hornicar VB, Parfene G, Tyagi AK, Gottardi D, Dinica R, Guerzoni ME, Bahrim G (2014) Extraction and characterization of volatile compounds and fatty acids from red and green macroalgae from the Romanian Black Sea in order to obtain valuable bioadditives and biopreservatives. *J Appl Phycol* 26:551–559. <https://doi.org/10.1007/s10811-013-0053-0>
34. Kang JY, Benlirio IMP, Lee IJ, Choi JY, Joo J, Choi YS, Hwang DS, Hong NYK (2014) Viability, fatty acid composition, and structure of the coralline alga *Corallina pilulifera*. *Bot Sci* 92 (1):103–109. <https://doi.org/10.17129/botsci.24>
35. Kendel M, Wielgoz-collin G, Bertrand S, Roussakis C, Bourgounon N, Bedoux G (2015) Lipid composition, fatty acids and sterols in the seaweeds *Ulva armoricana*, and *Solieria chordalis* from Brittany (France): an analysis from nutritional, chemotaxonomic, and antiproliferative activity perspectives. *Mar Drugs* 13(9):5606–5628. <https://doi.org/10.3390/md13095606>.
36. Rodrigues D, Freitas AC, Pereira L, Rochasantos TAP, Vasconcelos MW, Roriz M, Rodríguez-alcala LM, Gomes AMP, Duarte AC (2015) Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West coast of Portugal. *Food Chem* 183: 197–207. <https://doi.org/10.1016/j.foodchem.2015.03.057>.
37. Santos SAO, Vilela C, Freire CSR, Abreu MH, Rocha SM, Silvestre AJD (2015) Chlorophyta and Rhodophyta macroalgae: A source of health promoting phytochemicals. *Food Chem* 183:122–128. <https://doi.org/10.1016/j.foodchem.2015.03.006>.
38. Shimid M, Guiheneuf F, Stengel DB (2014) Fatty acid contents and profiles of 16 macroalgae collected from the Irish Coast at two seasons. *J Appl Phycol* 26(1): 451–463. <https://doi.org/10.1007/s10811-013-0132-2>.
39. Fernando IPS, Sanjeewa KKA, Samarakoon KW, Lee WW, Kim HS, Kang N, Ranasinghe P, Lee HS, Jeon YJ (2017) A Fucoidan fraction purified from *Chnoospora minima*; a potential inhibitor of LPS-induced inflammatory responses. *Int J Biol Macromol* 104: 1185–1193. <https://doi.org/10.1016/j.ijbiomac.2017.07.031>.

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