

Novel anticancerous compounds from *Sargassum wightii*: *In silico* and *in vitro* approaches to test the antiproliferative efficacy

S. M. Fazeela Mahaboob Begum¹, S. Priya^{1,2}, Raji Sundararajan³, S. Hemalatha¹

¹Department of Life Sciences, B.S. Abdur Rahman University, Chennai, Tamil Nadu, India, ²Department of women society, Xinovem, Golden Jubilee Biotech Park, Chennai, India, ³Department of Electrical & Computer Engineering Technology, Purdue University, West Lafayette, IN 47907, USA

Correspondence: S. Hemalatha, School of Life Sciences, B.S. Abdur Rahman University, Chennai, Tamil Nadu, India. E-mail: hemalatha.sls@bsauniv.ac.in

ABSTRACT

Non-small cell lung cancer (NSCLC) contributes to 80% of lung cancer death. The poor survival rate is contributed by the uncontrolled proliferation, evasion of apoptosis, ubiquitous expression of cell survival genes, and resistance to anticancer therapies. This prompts the search for novel and potent drugs for the effective treatment and management of NSCLC. Marine seaweeds are rich in novel bioactives widely employed in pharma, medical, cosmetic, and food industries. For the current study, the ethyl acetate extract of *Sargassum wightii* is utilized to test antiproliferative efficacy against the NSCLC cell line A549. From ethyl acetate extract, two compounds, namely, n-hexadecanoic acid and l-(+)-ascorbic acid 2,6 dihexadecanoate were identified by mass spectrometry analysis. These compounds interacted with the cell survival protein PI3K which is upregulated in most of human cancers. The *in silico* results demonstrated that the algal compounds interacted with the target PI3K with a C score of 5. The *in vitro* antiproliferative activity of the ethyl acetate extract was analyzed by MTT assay. The apoptotic hallmarks including fragmentation of nuclei and DNA were observed in treated cells. The real-time polymerase chain reaction analysis of gene encoding PI3K showed the downregulation of the gene. The current results suggest that the compounds of *S. wightii* have antiproliferative activity and can control lung cancer through induction of apoptosis.

Keywords: Non-small cell lung cancer, *Sargassum wightii*, antiproliferative property, n-hexadecanoic acid, l-(+)-ascorbic acid 2, 6 dihexadecanoate

Introduction

Lung cancer is the major cause of cancer mortality worldwide accounting for 1.5 million deaths in 2012.^[1] It constitutes 13% of the newly diagnosed cancer cases in 2015.^[2] Among the major two types of lung cancer, the non-small cell lung cancer (NSCLC) contributes to 80% of lung cancer deaths which urges the need for novel therapies in the effective treatment and management of NSCLC.^[3]

Apoptosis or programmed cell death maintains the balance between cell proliferation and cell death. Uncontrolled proliferation results in oncogenesis. In NSCLC cell lines, the deletion or inactivation of the tumor suppressor genes directly contributes to the uncontrolled proliferation and prolonged survival of cancer cells.^[4] Hence, drugs that can inhibit uncontrolled proliferation and induce apoptosis may be effective in the management and treatment of cancer.

A number of FDA-approved anticancer drugs are derived from the sea including cytarabine, eribulin mesylate, and trabectedin. This has triggered the pharmaceutical industries to focus on marine natural products, and many marine bioactives have entered into the pre-clinical and clinical trials.^[5] Marine seaweeds are a rich source in novel bioactives which are widely employed in pharma, medical, cosmetic, and food industries. The marine brown algae of genus *Sargassum* is reported to possess antithrombotic, antiplatelet, antiviral, and anticancer properties.^[6-8] In the current study, the marine brown algae *Sargassum wightii* was extracted, and the phytoconstituents were analyzed for the antiproliferative efficacy against the NSCLC cell line

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A549. The outcomes of the study showed that the algal bioactives inhibited cancer cell proliferation through the induction of apoptosis in A549 cells. The results thus suggested that the algal bioactives can be utilized for the development of novel anticancer agents in the treatment of NSCLC.

Materials and Methods

Collection and identification of marine algae

The marine brown algae *S. wightii* was collected from the Mandapam coast of Tamil Nadu, India. The algae were identified and authenticated by Dr. Saravanan, Scientist, CMFRI, Mandapam, Tamil Nadu, India. The algae were washed in water to remove debris, shade dried, powdered, and were used for further studies.

Extraction and characterization of the algal extract

The dried algal powder was subjected to sequential extraction.^[9] The phytoconstituents analysis was carried out in the extracts for detection of alkaloids, flavonoids, saponins, tannins, glycosides, carbohydrates, proteins, fats, and oils.^[10] The total polyphenol content was estimated.^[11] Gallic acid was used as a standard. The experiment was done in triplicates and the data were recorded as mean \pm standard deviation (SD) based on the phytochemical analysis, the ethyl acetate extract (SEA) was subjected to Fourier-transform infrared (FTIR) analysis in JASCO spectrometer, and the spectra in the range 400–4000 cm^{-1} were recorded.^[12] The SEA (5 mg) was filtered in 0.45 μm filter and subjected to high-performance liquid chromatography (HPLC) analysis in Shimadzu HPLC 9A fitted with LC 20AD binary gradient pump, SPD-M20A Diode array detector, and RF-fluorescence detector. The separation was carried out in Enable C18 G column of 250 \times 4.6 mm fitted with a C-18 guard column using acetonitrile and water as the mobile phase in the ratio of 70:30. The flow rate was maintained as 1 ml/min. The injection volume was 20 μl . The total running time was 25 min. The retention time (RT) of the compounds in all extract was recorded.^[13] The advanced mass spectroscopy (Shimadzu GC-MS QP2010 ultra) with direct injection port was used for the mass spectrometry analysis of SEA. The sample was directly sent to mass spectra using the direct injection port (200°C), and the emerging fragment ions were collected. The ion source was maintained at 200°C and interface at 250°C. The detector voltage was maintained at 0.7kV throughout the analysis. The probable structure based on the ion fragmentation pattern was derived from the National Institute of Standards and Technologies 14 library search.

Molecular docking analysis

The structures of the compound n-hexadecanoic acid (PubchemCID:985) and l-(+)- ascorbic acid 2,6 dihexadecanoate (PubchemCID:54686917) identified in SEA by MS analysis were obtained from PubChem database (www.ncbi.nlm.nih.gov/pubchem), and the X-ray crystal structures of the target receptor (PI3K) of humans were retrieved from protein data bank (<http://www.rcsb.org/pdb>). The compounds for docking were prepared as per the ligand preparation program, and Dock suite program of the

SYBYL- \times 1.3 was utilized. The prepared ligands were docked with the target protein PI3k based on the induced fit docking protocol. The interaction of the algal compounds with the target was visualized in Pymol, a python-based visualization tool (www.Pymol.org).

In vitro cell viability assay

The human adenocarcinoma cell line (A549) procured from NCCS, Pune, India, was used to test the antiproliferative efficacy of SEA. The cells were cultured in Dulbecco's modified Eagle's Medium (HiMedia) supplemented with 10% fetal bovine serum (HiMedia) and 1% antibiotic cocktail (Himedia). The cells were seeded in 96-well plate (1.5×10^5 cells) and treated with different concentrations of SEA (1, 500, 1000, 1500, and 2000 $\mu\text{g}/\text{ml}$) for 24 h to analyze the cell viability and determine the inhibitory concentration (IC_{50}) by MTT assay.^[14] A549 cells treated with 1500 $\mu\text{g}/\text{ml}$ of SEA for 24 h were analyzed for the hallmarks of apoptosis by staining with DAPI and acridine orange and ethidium bromide (1:1) mixture under fluorescent microscope (Zeiss Axio). DNA was isolated from the SEA-treated and control cells by Trizol method (Takara) and separated by electrophoresis (1.5% Agarose) at 75V for 40 min (BioRad) and observed in ChemiDoc (Eppendorf). The control was represented by ethyl acetate-treated cells, and the experiment was performed in triplicate.

Analysis of PI3K by real-time polymerase chain reaction (PCR)

Total RNA was isolated from control and SEA-treated cells by trizol method, followed by cDNA synthesis using the high capacity cDNA reverse transcription kit (Applied Biosystem) as per manufacturer's instruction. The cDNA synthesized was used for real-time PCR analysis of cell survival gene (PI3K) using SYBR Green PCR master mix (Bio-Rad). The analysis was done in triplicate, the fold change in gene expression was calculated based on $2^{-\Delta\Delta\text{ct}}$, and the expression was normalized with the housekeeping gene β actin. The primers used are PIK3CA (F) TGCAAAGAATCAGAACAATGCC: PI3KCA (R) CACGGAGGCATTCTAAAGTCA. β Actin (F) TAGAAGCCTCTTCATGGACAAC: β Actin (R) GTATCAGGCATGCAACACAAG.

Results and Discussion

The phytochemical analysis of *S. wightii* revealed the presence of flavonoids, tannins, glycosides, terpenoids, carbohydrates, fats, resins, and phytosterols in the ethyl acetate, ethanol and methanol extracts; however, terpenoids, saponins, carbohydrates, proteins, and resins were not detected in hexane and DCM extracts [Table 1]. The abundance of phytoconstituents in *S. wightii* ethyl acetate extract (SEA) may be attributed to the mid-polar nature of the solvent. In previous studies, the non-polar solvents such as benzene, chloroform, and petroleum ether were used for extracting *S. wightii*,^[15] but extraction with ethyl acetate increased the concentration of the phytoconstituents than the non-polar solvents.

Table 1 shows the results of preliminary phytochemical analysis of *S. wightii* by sequential extraction method. The phytoconstituents were found in different concentration in each extract.

The quantification of polyphenols [Figure 1a] in the algal extracts showed the highest concentration (266.6 $\mu\text{g}/100\text{ mg}$) in SEA, followed by ethanol (215 $\mu\text{g}/100\text{ mg}$), DCM (159 $\mu\text{g}/100\text{ mg}$), methanol (148 $\mu\text{g}/100\text{ mg}$), and hexane (122 $\mu\text{g}/100\text{ mg}$) extracts. As SEA showed the highest concentration of polyphenols which has various biological properties, it was utilized for further studies. Polyphenols form the major group of natural antioxidants. The polyphenol phlorotannin in brown algae is found to confer protection from ultraviolet radiation. Further, polyphenols are reported to possess various therapeutic properties such as antibacterial, antiviral, anticancer, and antidiabetic activities.^[16] The HPLC analysis [Figure 1b] showed the presence of five major compounds in the SEA with RT 16.742, 18.907, 19.989, 21.539, and 22.684 min, respectively. The results of FTIR analysis [Figure 1c and Table 2] enabled the identification of functional groups such as alcohol, alkenes, and aliphatics in the extract at varying concentration. The presence

of these functional groups may account for the biological properties of *S. wightii*.

The FTIR analysis showed many peaks which represent the functional groups that are present in the ethyl acetate extract of *S. wightii*.

The mass chromatogram of SEA revealed the presence of two major compounds. Based on the ion pattern, the two components were identified, namely, n-hexadecanoic acid with the molecular formula $\text{C}_{16}\text{H}_{32}\text{O}_2$ and molecular weight 256.4 [Figure 2a] and l-(+)-ascorbic acid 2,6-dihexadecanoate with the molecular formula $\text{C}_{38}\text{H}_{68}\text{O}_8$ and molecular weight 652.9 [Figure 2b]. Based on the ion pattern and the mass of parent peak, the following structures are proposed [Figure 2c and d]. The presence of strong peak at 3272 cm^{-1} in FTIR correlates with the carboxylic groups in both these compounds, and further, the peaks at 1654 cm^{-1} , 1086 cm^{-1} , and 1044 cm^{-1} coincide with the C=C, C-O-C, and C-OH stretching in l-(+)-ascorbic acid 2,6-dihexadecanoate. Hence, the results of FTIR support the MS data and the proposed structures of both compounds. These results correlate well with the previous

Table 1: Preliminary phytochemical screening of crude extracts of *S. wightii*

Phytoconstituent	Hexane	DCM	Ethyl acetate	Ethanol	Methanol
Tannins	++	++	+++	++	+
Flavonoids	++	++	+++	++	+++
Terpenoids	-	-	+++	++	+++
Saponins	-	-	-	-	-
Glycosides	+	+	+++	+++	+++
Carbohydrates	-	-	++	+++	+++
Fats and oils	-	-	++	+++	+++
Resins	-	-	+++	++	+
Phytosterols	++	++	+++	++	+
Protein	-	-	++	+	+

+: Presence, ++ and +++: Abundance, -: Absence, *S. wightii*: *Sargassum wightii*

Table 2: FTIR spectra of *S. wightii* ethyl acetate extract

Absorbance (cm^{-1})	Functional groups	Compounds
3272	O-H stretching of carboxylic acid or enolic OH or alcohol	Polysaccharides/alcohols
2970.5	C-H stretching of CH_3 , $\text{CH}_2/\text{N-H}$ stretching	Aliphatic compounds
1654.2	C=C	Alkene
1086	C-O-C stretch	Starch polysaccharides
1044	C-OH stretch	Alcohol

S. wightii: *Sargassum wightii*, FTIR: Fourier-transform infrared

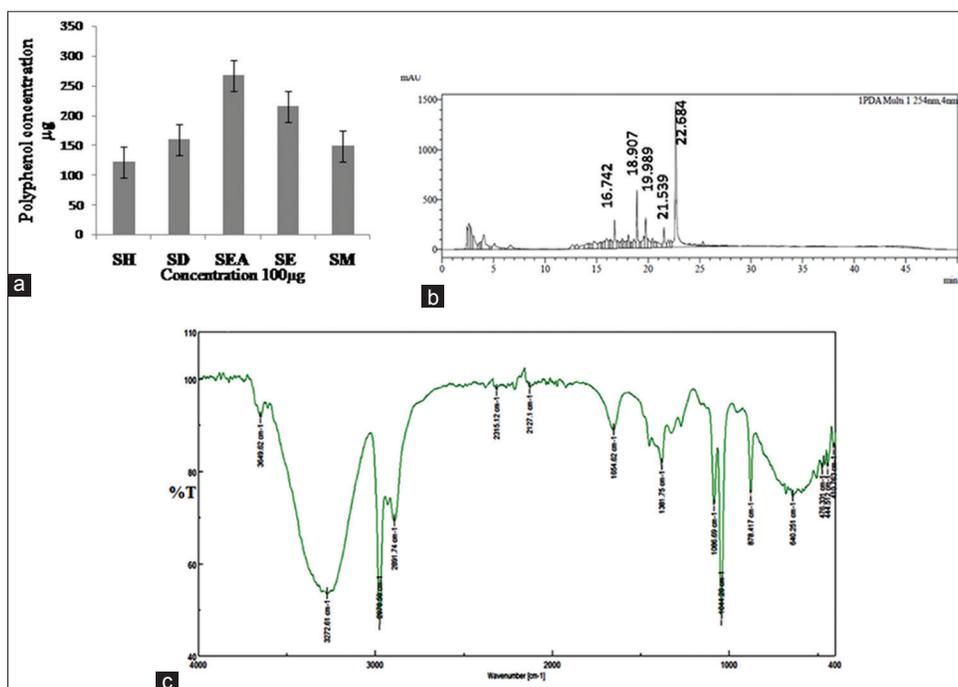


Figure 1: (a) Estimation of total phenolic content. The crude extracts of *Sargassum wightii* were analyzed for their total phenolic content. The ethyl acetate extract showed more polyphenol content than the other extracts, (b) high-performance liquid chromatography chromatogram of ethyl acetate extract, (c) Fourier-transform infrared spectra of ethyl acetate extract

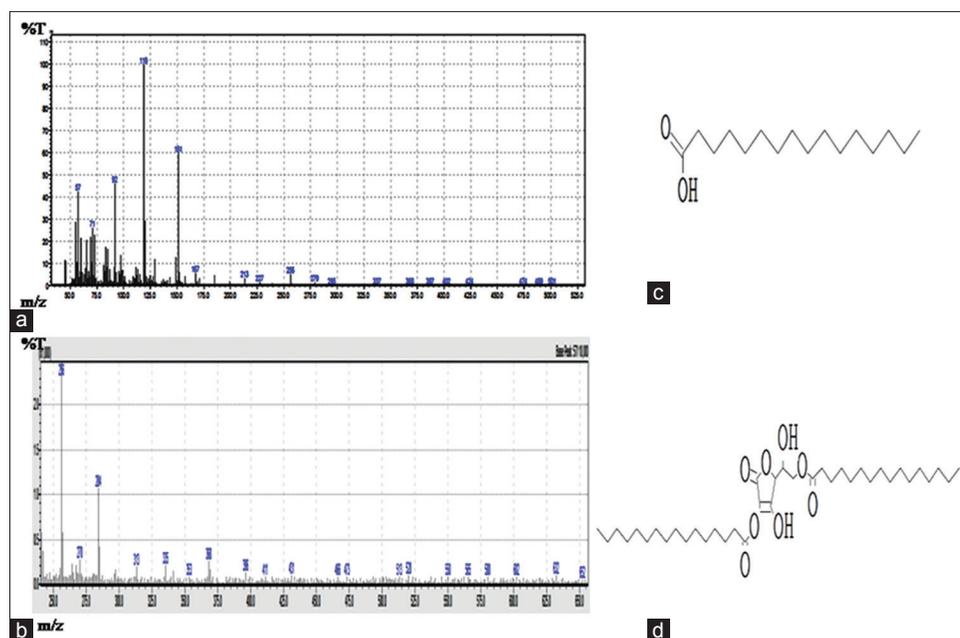


Figure 2: Mass spectrometry analysis of SEA. (a) Mass chromatogram of n-hexadecanoic acid, (b) mass chromatogram of l-(+)-ascorbic acid 2, 6 dihexadecanoate, (c) structure of n-hexadecanoic acid, (d) structure of l-(+)-ascorbic acid 2, 6 dihexadecanoate

studies of *S. wightii*.^[12] These compounds were previously reported in macroalgae.^[17]

However, this is the first study to report the occurrence of l-(+)-ascorbic acid 2, 6 dihexadecanoate in *S. wightii*. The compound n-hexadecanoic acid is used in the manufacture of artificial flavors and is reported to delay aerobic deterioration of silages.^[18] Ascorbic acid is a natural hydrophilic antioxidant. The analogs of ascorbic acid formed by esterification and transesterification with fatty acids are shown to enhance the antioxidant, anti-inflammatory, and antitumor properties of ascorbic acid.^[19] The presence of l-(+)-ascorbic acid 2, 6 dihexadecanoate in SEA which contains two hexadecanoates esterified with ascorbic acid may contribute to the enhanced antioxidant activity of *S. wightii*. l-(+)-ascorbic acid dihexadecanoate is reported to possess antibacterial, antitumor, and wound healing properties^[20,21].

The compounds identified by mass spectrometry were subjected to molecular docking study with the cell survival gene PI3K. The structure of the protein PI3K and SEA compounds identified by MS analysis are depicted in Figure 3. The interaction study revealed that n-hexadecanoic acid interacted with glutamate 563 of PI3K. Similarly, l-(+)-ascorbic acid 2, 6 dihexadecanoate interacted through threonine 560 and serine 535 of PI3K [Figure 3d and e]. Apart from these residues, the interaction was stabilized through many hydrophobic bonds. Table 3 shows the interacting residues and bond distance formed between the ligands and receptors. In one-third of human cancers, PI3K gene is aberrantly expressed which contributes to the uncontrolled proliferation and evasion of apoptosis in cancer cells.

^[22]The compounds interacted with the target protein with a consensus scoring (C score) of 5 which showed the specificity and stability of the interaction. As the algal compounds showed interaction with PI3K by *in silico*, *in vitro* studies were carried out in A549 cells.

Table 3: Interaction results of n-hexadecanoic acid and l-(+) - ascorbic acid 2, 6 dihexadecanoate with PI3K

Category	n-hexadecanoic acid with PI3K	-(+) - ascorbic acid 2, 6 dihexadecanoate with PI3K
Total score	54.3789	82.063
C score	5	5
Interacting residues and bond length	GLU563 3Å	THR 560 2.6Å SER 535 3.3Å

Table 1 shows the interaction results of n-hexadecanoic acid and l-(+)-ascorbic acid 2, 6, dihexadecanoate with PI3K. N-hexadecanoic acid interacted with glutamic acid in the receptor PI3K with a C score of 5. The bond distance was 3Å. The ligand l-(+)-ascorbic acid 2, 6, dihexadecanoate interacted with threonine 560 and serine 535 in PI3K with a C score of 5. The length of bond between the ligand and THR was 2.6Å and that of SER was 3.3Å.

The results of MTT assay [Figure 4a] showed the ability of SEA to induce cytotoxicity in A549 cells. The treatment with different concentrations of SEA showed an increase in the cytotoxicity with increasing concentration. The IC₅₀ was determined as 1500 µg/ml. SEA inhibited the proliferation of A549 cells in a dose-dependent manner. The highest inhibition (77.84%) was observed at 2000 µg/ml.

The fluorescent microscopy analysis [Figure 4b] by DAPI staining detected the sublobe formation and fragmentation and blebbing of nuclei. To differentiate the live and apoptotic cells, acridine orange and ethidium bromide staining were carried out. The live cells showed green fluorescence with an unaltered morphology, whereas the early and late apoptotic cells showed orange fluorescence with rounding of the cells. The DAPI staining showed the induction of nuclear fragmentation in A549 cells treated with SEA, whereas the control

cells showed intact nuclei. The results of agarose electrophoresis [Figure 4c] showed the fragmentation of DNA isolated from SEA-treated cells, whereas the control cells showed intact DNA. The real-time expression of gene encoding PI3K [Figure 4d] showed the

downregulation in SEA-treated cells. The electrophoretic separation of the real-time product showed the absence of DNA band in the treated group [Figure 4e] compared to the control. The antiproliferative activity of SEA may be due to the presence of the newly identified

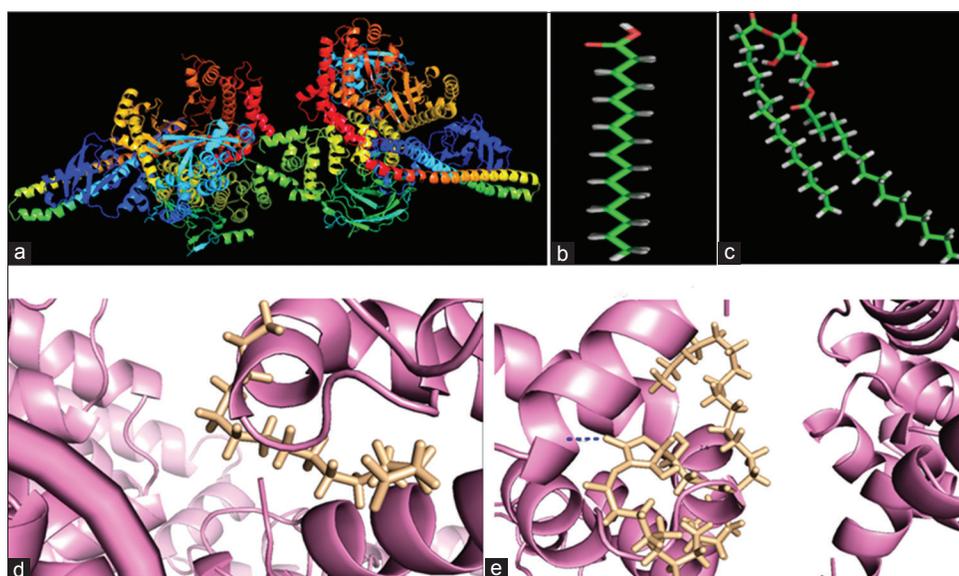


Figure 3: Molecular Docking analysis. (a) Structure of PI3K, (b) structure of n-hexadecanoic acid, (c) structure of l-(+)-ascorbic acid 2, 6 dihexadecanoate, (d) interaction of PI3K with n-hexanoic acid, (e) interaction of PI3K with l-(+)-ascorbic acid 2, 6 dihexadecanoate. The interaction studies were done using SYBL X 1.3

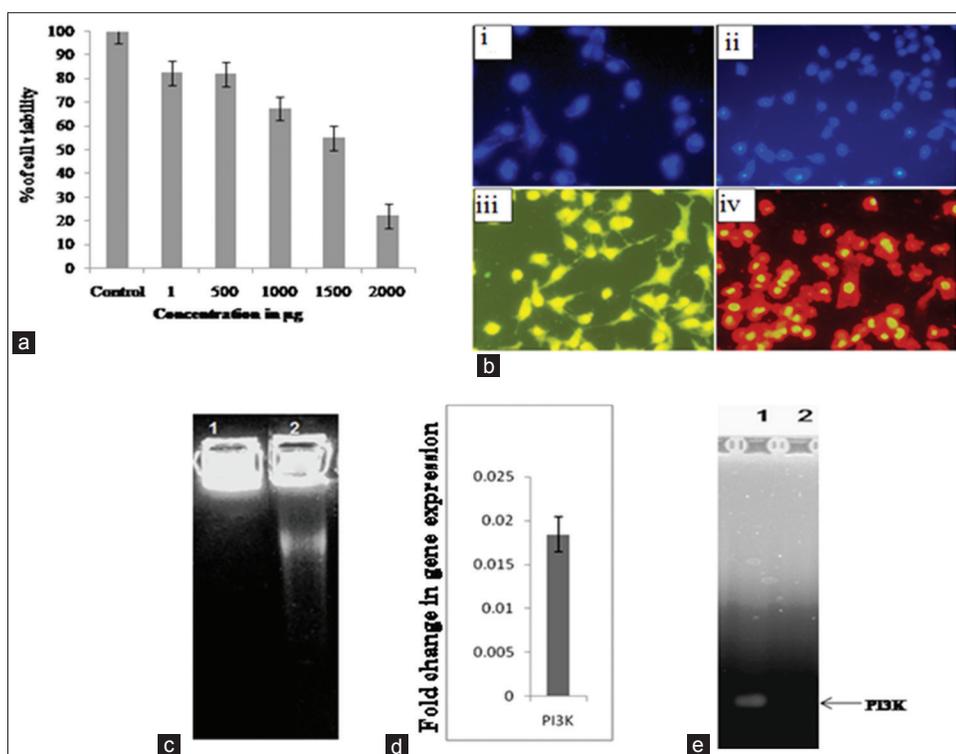


Figure 4: (a) Cytotoxic activity of SEA against A549 cells. The A549 cells were incubated with SEA at varying concentrations (1, 500, 1000, 1500, and 2000 µg/ml) for 24 h and the cytotoxicity was assessed by MTT assay. The IC₅₀ was found as 1500 µg/ml. Control cells were treated with ethyl acetate. The data presented are mean ± SD, (b) analysis of apoptosis by fluorescent microscopy. DAPI staining of control (i) and treated (ii) showing fragmented and lobbed nuclei. Acridine orange and ethidium bromide staining of control (iii) and treated (iv) showing live and apoptotic cells. The experiment was done in triplicate, (c) agarose gel electrophoresis showing intact DNA (lane 1) and fragmented DNA (lane 2), (d) fold change in PI3K gene expression in treated A549 cells, (e) agarose gel electrophoresis showing real-time polymerase chain reaction products

algal compounds 1-(+)-ascorbic acid 2, 6 dihexadecanoate and n-hexadecanoate.

Conclusion

The inhibition of proliferation in cancer cells is an essential criterion for an anticancer drug. In the current study, SEA inhibited cell proliferation through the induction of apoptosis. This suggested that the algal bioactives possess anticancer activity which can be further studied for the development of effective anticancer drug in the management of NSCLC. The presence of polyphenols, flavonoids, and phytosterols and 1-(+)-ascorbic acid dihexadecanoate in SEA may contribute to its antiproliferative nature. The identification of nuclear fragmentation, blebbing, and late apoptotic cells in SEA-treated cells by fluorescent microscopy confirmed the ability of SEA compounds to induce apoptosis. The real-time analysis of expression of gene PI3K showed that the inhibition of cell proliferation is mediated by the induction of apoptosis, through suppression of PI3K gene expression in the treated cells. The results of the *in vitro* study coincide with the *in silico* interaction study where the algal compounds showed good interaction with PI3K protein. As the outcomes of the current study highlight the downregulation of cell survival gene, the compounds in SEA may contribute to this effect. As marine natural products are gaining importance in the field of anticancer research, this study opens up a new avenue about marine algae which can be utilized effectively in the field of biomedical research.

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