

In vitro anticancer activity of *Sargassum* sp. polysaccharides against MCF-7 cell lines

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Seaweed polysaccharides are compounds with promising chemopreventive and chemotherapeutic activities. In this study, polysaccharides isolated from *Sargassum* seaweeds were tested for their efficiency in cancer therapy. Polysaccharides were isolated, purified, and characterized using nuclear magnetic resonance (NMR); and Fourier transform infrared (FT-IR) spectroscopic and matrix-assisted laser desorption ionization mass spectrometric (MALDI-MS). It was found that the polysaccharides from *Sargassum* sp. branches are sulfated galactose–fucose disaccharides and sulfated galactose monosaccharides attached to the main chain through (1–4) linkages. Further, the polysaccharides were tested for cytotoxicity and anticancer activity against human breast adenocarcinoma cell line (MCF-7) using the Annexin-V/propidium iodide (PI) staining method.

[Keywords: Polysaccharides; Spectroscopy; Apoptosis; *Sargassum* sp.; Adenocarcinoma]

Introduction

The drugs currently available for cancer therapy are not only expensive but also toxic as they affect not only the cancer cells but also normal cells. Thus, finding novel, effective, and nontoxic compounds from natural sources is more crucial now than ever before. Marine macroalgae (seaweeds) are rich in bioactive compounds that could potentially be exploited as functional ingredients for both human and animal health applications¹. Among the seaweeds, brown seaweeds (Phaeophyta) contain rich source of structurally diverse polysaccharides with valuable biological activities². Polysaccharides are macromolecules with polymeric carbohydrate structures. *Sargassum* sp. is one of the industrially important brown algae occurring in Rameswaram, east coast of Tamil Nadu, India³. Large amount of polysaccharides is present in the seaweed cell walls conferring strength and flexibility. Generally, *Sargassum* contains more alginate⁴ and less fucodian and laminaran. However, a large number of bioactive polysaccharides found in *Sargassum*^{5,6} have not been characterized. Water-soluble sulfated polysaccharides from brown seaweeds have been extracted, purified, and investigated for their anticancer potential^{3,7,8}. *In vitro* and *in vivo* studies suggested that *Sargassum* sp. had anticancer, antiviral, and antidiabetic activity^{9,11}.

Further, the hexane fraction of methanol extract of *Sargassum* sp. had *in vitro* cytotoxicity against Caco-2 and T47D cells and increased the percentage of apoptotic cells among these cells¹². Apoptosis is a gene-regulated phenomenon which is induced by many chemotherapeutic agents in cancer treatment¹³.

In the present work, polysaccharides isolated from *Sargassum* sp., i.e., *Sargassum swartzii* and *S. wightii* were tested for anticancer activity against human breast adenocarcinoma cell line (MCF-7) using the Annexin-V/propidium iodide (PI) staining method.

Materials and Methods

All the chemicals used in the procedure were of analytical grade and obtained from Merck, India.

The brown seaweeds *S. swartzii* and *S. wightii* were collected off Mandapam (78°8'E, 9°17'N) in the Gulf of Mannar, Tamil Nadu, India. The collected seaweed was washed by seawater to remove sand particles and epiphytes. Then it was washed thoroughly using double-distilled water. The algae were air-dried in the shade at room temperature and ground well for further use, by using a mixer grinder.

Polysaccharides were extracted from *S. swartzii* and *S. wightii* following Chandia *et al.*¹⁴ About 40 g of algae powder was suspended in 0.25 M NaCl and the pH was adjusted to 8.0 with NaOH. Protease 2

mg, an alkaline protease from *Esporobacillus* (Bio Bras, Montes Claros, MG, and Brazil), was then added to the mixture for proteolytic digestion. After incubation for 24 h at 60 °C under agitation and periodic pH adjustments, the mixture was filtered through cheesecloth and precipitated with increasing amount of ice-cold acetone (0.5 v and 1.0 v) under gentle agitation at 4 °C¹⁵.

The precipitates were collected by centrifugation at 10,000 rpm for 20 min and dried at room temperature. Completely dried pellets were packed in an airtight container for further use.

About 5 g of extracted polysaccharide was dissolved with 10 mL of Millipore water (Milli Q) and used for further processing. After hydrolysis (with 2 M HCl), the fraction of total monosaccharide composition was determined by column chromatography (CC).

Characterization of seaweed polysaccharide

IR spectroscopic measurements of *S. swartzii* and *S. wightii* seaweed extracts were obtained using a Shimadzu IR affinity-1S spectrophotometer. The readings were recorded between 400 and 4000 cm⁻¹. After hydrolysis, the purified polysaccharide was dissolved in 400 µL D₂O (Deuterium oxide). The proton number and carbon number of the polysaccharide were identified and confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) experiments using a Bruker Biospin Avance III 800 NMR spectrometer (¹H frequency ¼ 400.13 MHz, ¹³C frequency ¼ 100.62 MHz) at 298 K using a 5-mm broadband inverse probe head equipped with a shielded gradient and XWIN-NMR software version 3.5 using TMS as internal reference. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) in both positive and linear modes using sinapic acid shows the polysaccharides to have an *m/z* ratio of around 1150. The MALDI-MS spectra were taken with the help of a 2,5-Dihydroxybenzoic acid matrix. A pulsed nitrogen laser of 337 nm was used for the MALDI-MS studies.

Cell lines and culture medium

The breast cancer cell line (MCF-7) was obtained from the National Centre for Cell Sciences, Pune, India. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, high glucose) containing 10% fetal bovine serum (FBS), 1% glutamine, and 100 IU of streptomycin/penicillin (Sigma) maintained at 37 °C. Cultures were maintained in a

T25 mm cell culture flask (Corning) and passaged every 3 days. After removal of the media, the cells were washed in Dulbecco's Phosphate-Buffered Saline 1× (DPBS, Sigma- Aldrich) to remove residual media that could inactivate the try psin (Sigma-Aldrich). DPBS was completely removed and trypsin (0.5 mL of 0.25% trypsin) was added to the cells to release them from the flask. After incubation at 37 °C for 5 min, 5 mL of media was added to the dissociated cells. Cells were spun down at 1500 rpm for 5 min at room temperature. The cells were resuspended in 1 mL of complete medium and counted. An aliquot of the counted cells was placed in a new T25 mm cell culture flask and cultured under described conditions.

The cytotoxicity profiles of the extracted *Sargassum* sp. polysaccharides were assessed using the MTT¹⁶ assay in the MCF-7 cell line. Briefly, cells (5×10³ cells/well) were incubated with various concentrations (3.125–200 µg/mL) of drug plated out in triplicates. Each plate included extracted *Sargassum* sp. polysaccharides treated, untreated cell controls, and a blank cell-free control. After 24 h of incubation, the culture media were removed and the cells were incubated with 5 mg/mL MTT in fresh medium at 37 °C for an additional 4 h. After this period, the supernatants were removed and 100 µL DMSO was added to each well to dissolve the formazan crystals. The plates were read on a microplate reader using a MultiSkan GO, Thermo Scientific spectrophotometer at a test wavelength of 540 nm and a reference wavelength of 650 nm. The percentage cellular viability was calculated with appropriate controls. The concentration which inhibited 50% of the cellular growth (IC₅₀ value) was determined.

Morphological changes of cells

Cells were grown to 1×10⁵ cells/well in 12-well plates and incubated with indicated concentrations of extracted *Sargassum* sp. polysaccharides (IC₅₀ 39.46 mg/mL) at 37 °C with 5% CO₂ for 24 h. After incubation, the cells were trypsinized, washed with PBS, and checked for morphological evidence of apoptosis. Cells were fixed with 4% paraformaldehyde for 20 min and washed with PBS, and then incubated with DAPI for 10 min at room temperature. After incubation, the cells were washed twice with PBS and examined in fluorescent microscopy (Carl Zeiss, Germany) following the procedure with slight modifications.

Annexin V/PI double staining assay

Apoptosis-mediated cell death of tumor cells were examined using Fluorescein isothiocyanate (FITC) labeled Annexin V/ PI according to the manufacturer's protocol (In vitrogen, USA). Cells ($1-2 \times 10^5$ cells/well) were incubated at 37 °C with 5% CO₂ for 24 h in the presence or absence of extracted *Sargassum* sp. polysaccharides at IC₅₀ 39.46 mg/mL. Cells were trypsinized, washed twice with HBSS, resuspended in 100 µL Annexin V binding buffer and then incubated for 20 min in 5 µL of Annexin V FITC. About 1 µL of 1 mg/mL PI solution was added to each cell suspension and run immediately in a flow cytometer (FACS Calibur, Becton Dickinson, USA) and analyzed in CellQuest software as described in the statistical analysis.

Results and Discussion

Fourier transform infrared (FT IR) spectrum recorded for *S. swartzii* polysaccharides reveals the presence of different functional groups (Fig. 1a). The peak at 3315 cm⁻¹ indicates the role of -OH or -COOH groups. The peak at 2360 cm⁻¹ indicates that the -CH₂ group present in the seaweed extract would have caused the reduction reaction. The peak at 1402 cm⁻¹ indicates the C-O stretch group in the reaction¹⁷. The peak at 1664 cm⁻¹ indicates -NH₂ stretch group in the reaction. Figure 1b illustrates the FT IR spectra of the different functional groups in *S. wightii* polysaccharides. The peak at 3315 cm⁻¹ indicates the role of -OH or -COOH groups. Similarly, the peaks at 2926 cm⁻¹ indicate that the -CH₂ group present in the seaweed extract would have caused the reduction

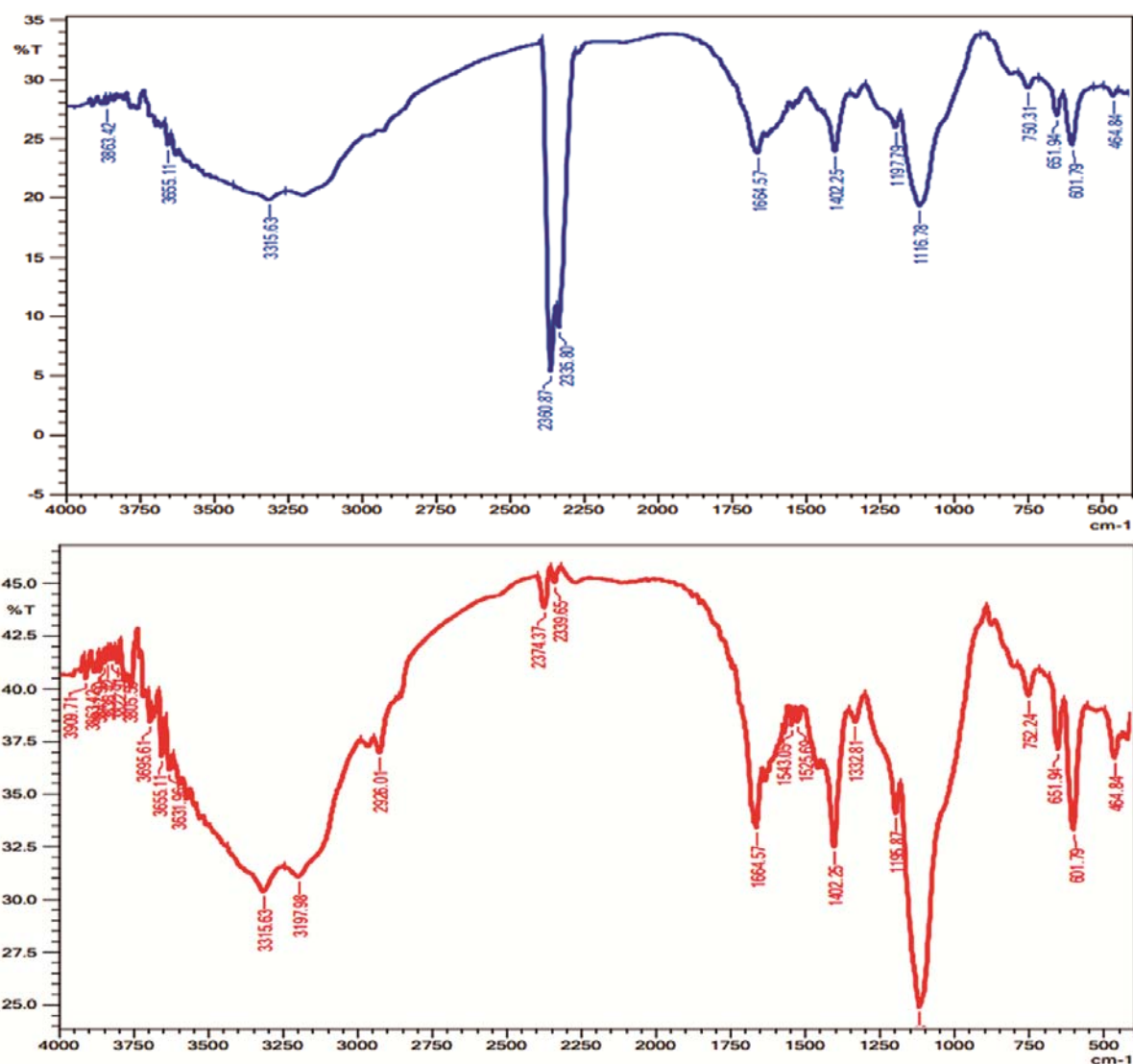


Fig. 1 — (a) FT IR spectra of extracted *S. swartzii* polysaccharides and (b) FT IR spectra of extracted *S. wightii* polysaccharides.

reaction. The peak at 1402 cm^{-1} indicates the C–O stretch group in the reaction. The peak at 1664 cm^{-1} indicates the –NH_2 stretch group in the reaction.

NMR analysis of *S. swartzii* polysaccharides is a convenient method to obtain structural information on the compounds. NMR spectroscopy was used to verify the linkages. The signal at 1.27 ppm was

attributed to the –CH_2 groups. A group of signals at 3.8–4.4 ppm were produced by $\text{C}^2\text{–C}^6$ protons, but the signal at 3.94 ppm shows the –CH_3 groups. No signal at 9–14 ppm in proton NMR indicates the absence of benzene or phenyl groups (Fig. 2a). Similarly, *S. wightii* polysaccharides showed linkages as the signal at 1.26 ppm which may be attributed to the –CH_2

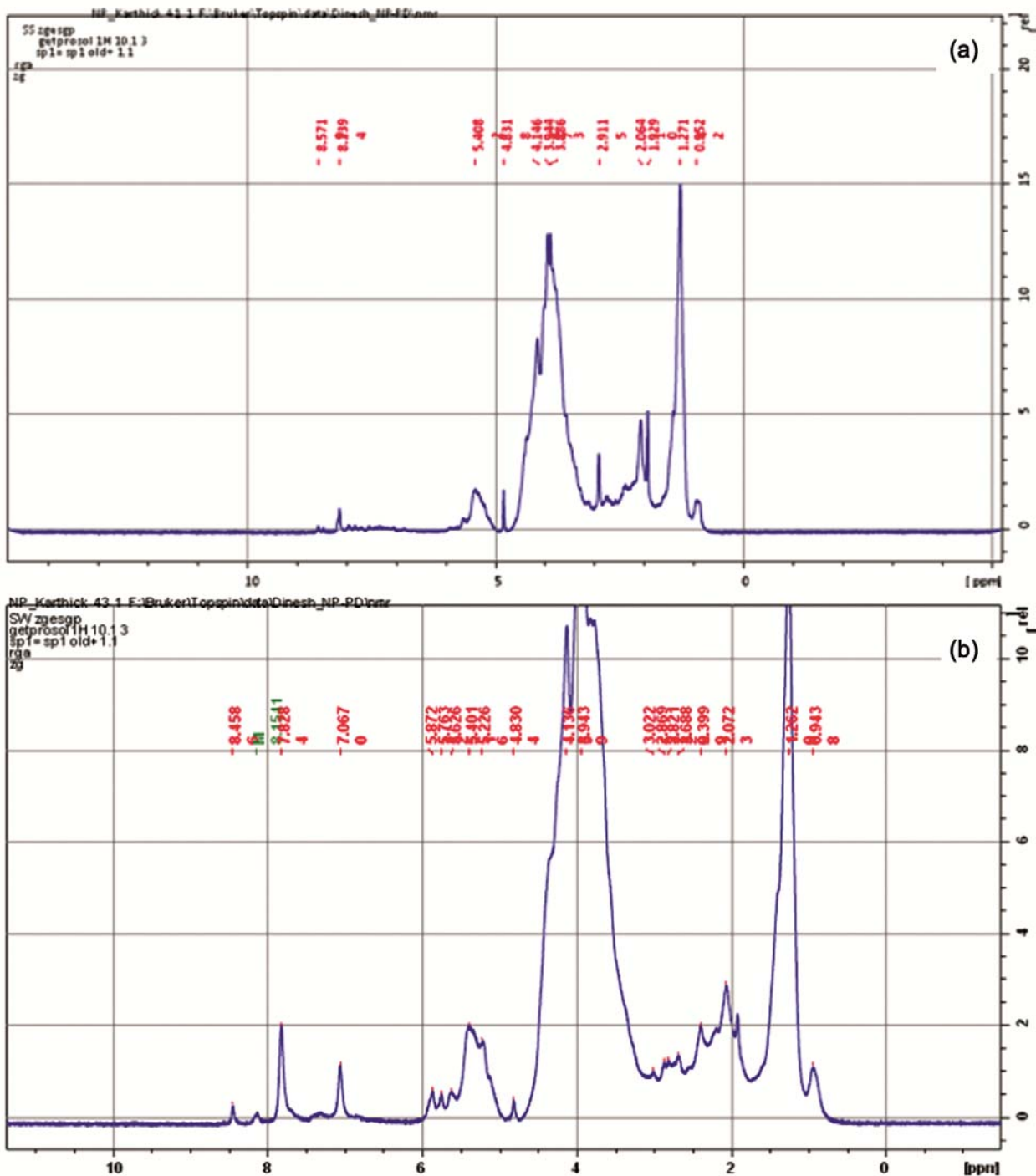


Fig. 2 — (a) ^1H NMR spectrum of extracted *S. swartzii* polysaccharides and (b) *S. wightii* polysaccharides.

groups (Fig. 2b). A group of signals at 3.8–4.4 ppm were produced by C²–C⁶ protons, but the signal at 4.13 ppm shows the –CH₃ groups. No signal at 9–12 ppm in proton NMR indicates the absence of benzene or phenyl groups. From the results of the chemical and spectroscopic analyses, we could conclude that the polysaccharide from *S. wightii* and *S. swartzii* differed in monosaccharide composition, degree of methylation, position of methyl groups in the backbone of the polysaccharide,¹⁸ as well as in the structure of their main chain.

Figure 3a illustrates the extracted *S. swartzii* in which the first distinct *m/z* peak is due to the charged group which has an *m/z* of 6349. The parent peak has then split into several fragments¹⁹. Figure 3b illustrates the extracted *S. wightii*, in which the first distinct *m/z* peak is due to the charged group which has an *m/z* of 6873. The parent peaks have then split into several fragments. In an earlier study, *Saccharina*

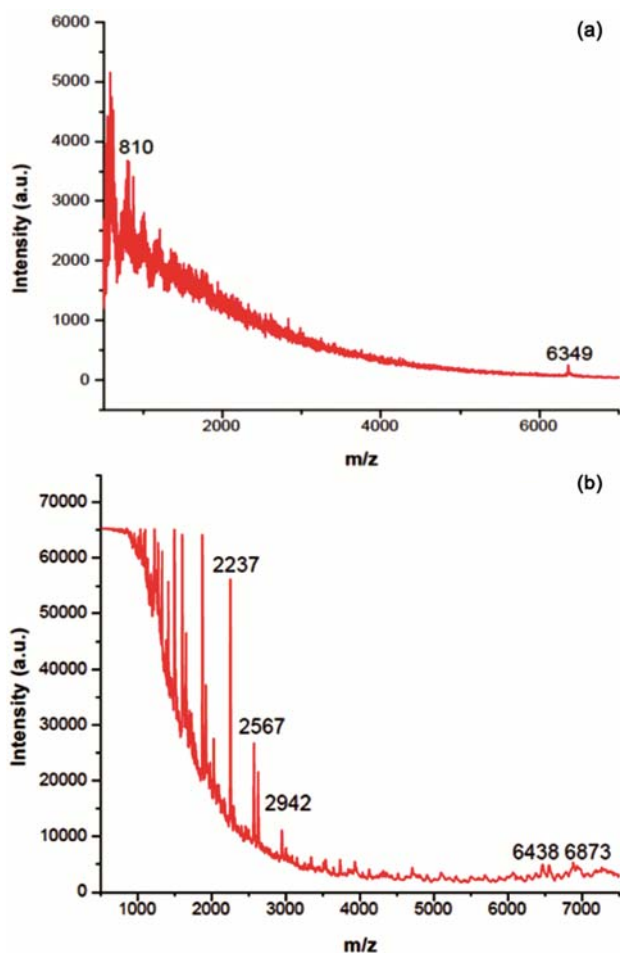


Fig. 3 — (a) MALDI analysis of extracted *S. swartzii* polysaccharides and (b) spectra recorded for *S. wightii* polysaccharides

cichorioides was analyzed by MALDI at a concentration of 0.1 mg/mL using arabinosazone as a matrix. Surprisingly, major signals were observed at *m/z* 243.0 and 344.9 for all samples, being those of mono and disulfated fucose residues, respectively²⁰.

MTT test was performed to determine the cytotoxicity range of both the polysaccharides in MCF-7 cell lines. When the polysaccharides were tested, *S. swartzii* polysaccharides did not show significant effect, and hence the *S. wightii* polysaccharides were taken for further studies. As shown in Figure 4, the cytotoxicity effect on MCF-7 cell lines was increased with increased concentration of the extracted *S. wightii* polysaccharides.

However, the extracted *S. wightii* polysaccharides inhibited cell viability at the highest dose that was tested (200 µg/mL) after 24 h incubation. Hence, the inhibitory concentration at 50% (IC₅₀) was fixed at 39.46 mg/mL. Thus, doses of extracted *S. wightii* polysaccharides at the concentration that gave maximum inhibitory concentration at 50% were used to treat the Inhibitory effect of the drug on the

Under the fluorescence microscope, MCF-7 cells treated with different concentrations of drug for 24 h presented the morphological features of early apoptotic cells, such as bright nuclear condensation (identified by DAPI staining). The apoptotic body appeared with increasing concentration of the drug (Figs 5a and b). In general, morphological alterations during apoptosis including chromatin condensation²¹, nuclear remodeling and membrane blebbing were determined by the interplay of caspase substrate cleavage.

To further confirm the apoptosis induced by drug Annexin V/PI, a staining assay was used²². The results

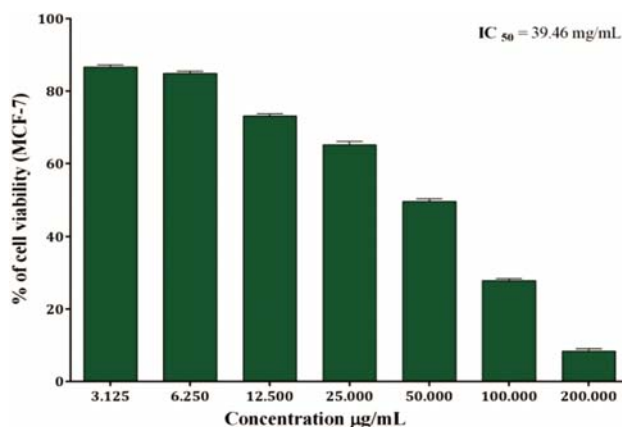


Fig. 4 — Cell viability using MTT assay for *S. wightii* polysaccharides

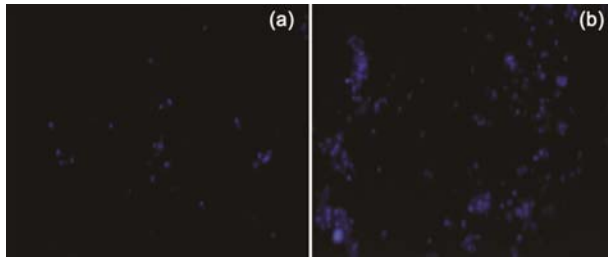


Fig. 5 — (a) Control and (b) Morphological changes in MCF-7 cells. MCF-7 cells treated with extracted *S. wightii* polysaccharides IC₅₀ 39.46 mg/mL for 24 h

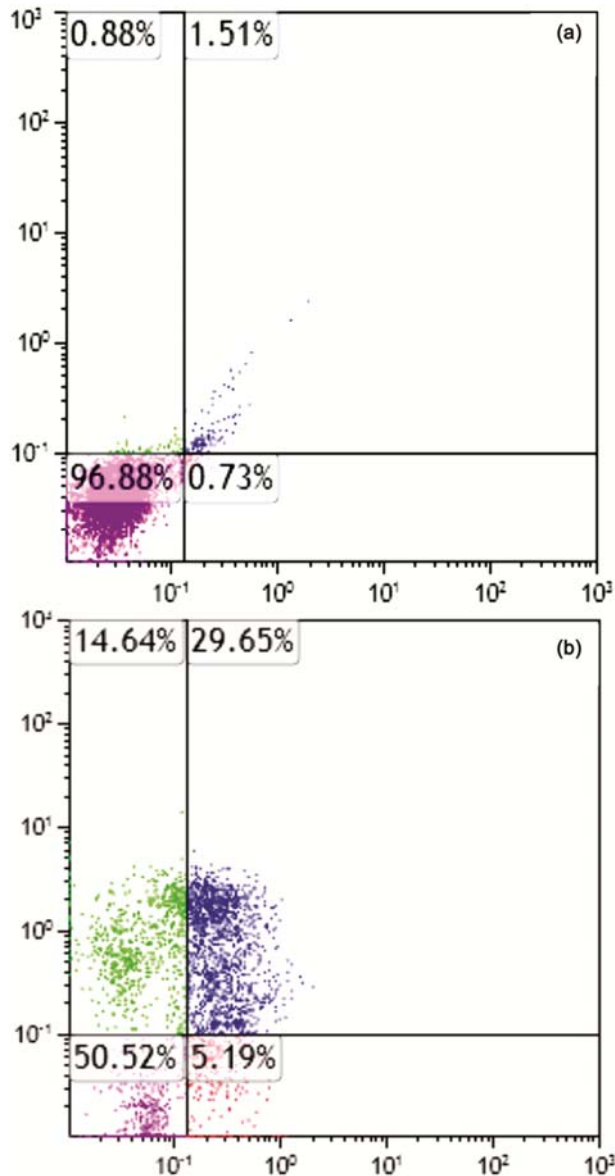


Fig. 6 — Effect of *S. wightii* polysaccharide-induced apoptosis in HeLa cells using Annexin V-FITC and PI double-staining method showed that after treatment with IC₅₀ 39.46 mg/mL for 24 h, the early and median apoptotic cells (left

lower section of fluorocytogram) increased (5.19%) and the late apoptotic and necrotic cells (left upper section of fluorocytogram) increased slightly (Figs 6a and b)²³. These results indicate the polysaccharides role in the apoptosis mediated cell death.

Conclusion

The present work demonstrates the extraction and purification of polysaccharides from the marine algae *S. swartzii* and *S. wightii*. The purification was aided by column chromatography. The components were also identified using FT IR, NMR, and MALDI. The study measured the potential role of oxidative stress as a mechanism of polysaccharide-induced apoptosis via the mitochondrial pathway. Collectively, these findings provide *in vitro* evidence of polysaccharides for effective utilization in colloidal drugs as an anticancer agent. With the help of MALDI-MS, the compositions of the two polysaccharides have been confirmed and have an *m/z* ratio of around 6349 and 6873. In conclusion, these polysaccharides have been identified to possess anticancer potential for future medical applications.

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