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In vitro anticancer activity of ethanolic extract of *Stoechospermum marginatum* against HT-29 human colon adenocarcinoma cells

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Colorectal cancer is a one of the leading causes of death globally and its clinical management of cancer involves chemotherapy. Increase in the development of resistance to the drugs used in the cancer treatment and serious side effects associated with chemotherapeutic drugs are the major limitations in cancer therapy. Hence, there exists a huge need to develop safer natural therapeutic products for cancer therapy. In this study, ethanolic extract of *Stoechospermum marginatum* was evaluated for its anticancer activity. The cytotoxicity of *S. marginatum* extract was evaluated on HT-29 cells by MTT assay. Trypan blue cell viability was also carried out to evaluate cytotoxicity and antiproliferative effect. The apoptosis-inducing potential of the extract was analyzed by acridine orange and ethidium bromide dual staining method, mitochondrial membrane potential assay and FITC Annexin V-Propidium iodide staining method. The ethanolic extract of *S. marginatum* showed significant dose-dependent cytotoxicity in HT-29 cells Treatment with *S. marginatum* extract increased number of apoptotic cells in HT-29 cells and caused damage to mitochondrial membrane potential. The findings of the present study confirmed *in vitro* anticancer activity of ethanolic extract *S. marginatum*

Keywords: Antiproliferative, Apoptosis, Brown algae, Chemotherapeutic, Cytotoxicity, Seaweed extract

Colorectal cancer is one of the most commonly diagnosed and its incidence is increasing due to changing life styles, dietary habits and environmental factors *etc*.[1]. According to the estimates of World Health Organization, colorectal cancer is the third most commonly diagnosed malignancy and the second leading cause of cancer related deaths in the world with an estimated global incidence of 1.9 million new cases in 2020[2]. Majority of drugs used in cancer chemotherapy are cytotoxic and produce various side effects including vomiting, in appetence, loss of hair and impairment of immune system, *etc*.[3]. The high cost of chemotherapeutic drugs, potential side effects, resistance development and non-availability of the drugs in developing countries like India are the major challenges faced in cancer treatment[4]. Hence, there is a constant search for an alternative source of drug without side effects and at affordable cost. Natural products derived from plants, microorganisms and marine organisms rich in bioactive molecules, can be a suitable alternative, and hence their potential for cancer treatment requires to be tapped[5,6].

Stoechospermum marginatum, brown marine macroalgae of class phaeophyceae (Family Dictyotaceae) is widely distributed in Eastern coastal regions of Tamil Nadu[7]. Phytochemicals such as triterpenoids, sulfated polysaccharides, phenols, flavonoid, tannin, saponin, sterols, steroids, glycosides were reported in *S.* marginatum[8,9]. *S. marginatum* were reported to have antioxidant, antiviral, antifungal, antibacterial, cytotoxic and antiproliferative activities[7]. Though the seaweed has significant antioxidant and cytotoxicity activity, its anticancer activity against colon carcinoma has not been studied until now.

Cell based assays are widely used technique for various investigations on cancer. HT-29, human colorectal adenocarcinoma cell line is an epithelial cell line and it is characterized by invasion and migration. It is a xenograft tumour model for colorectal cancer and it is extensively used in cancer studies for *in vitro* screening of anticancer activity of drugs and natural products[10,11].

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With this background, the present was initiated to evaluate the *in vitro* anticancer activity of ethanolic extract of brown seaweed, *Stoechospermum marginatum* against HT-29 human colorectal cancer cell line.

Materials and Methods

Chemicals

Trypan blue dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-fluoro-uracil were obtained from Sigma-Aldrich, USA. Dulbecco's modified Eagles medium (DMEM) was obtained from Lonza Biosciences. Phosphate buffered saline (PBS), fetal bovine serum (FBS) and 0.25% trypsin EDTA were obtained from Hi Media Laboratories, Mumbai. Antibiotic antimycotic solution was obtained from Gibco, USA. All other chemicals and solvents used throughout the study were of analytical and molecular biology grade.

Collection and authentication of seaweed

Fresh thallus of *Stoechospermum marginatum* was collected from the Gulf of Mannar Region of Mandapam Coast (Lat 09° 17`N, Long 79° 07`E), Tamil Nadu, South-East coast of India and authenticated by the botanist of Botanical Survey of India, Coimbatore.

Preparation of seaweed extract

The sea weeds were washed thoroughly, air dried and powdered. The ethanolic extract of the seaweed were prepared by continuous hot percolation at 55°C in soxhlet apparatus[12]. The extracts were then vacuum concentrated, air dried and stored at 4°C.

Cell line and culture

HT-29 (human colon adenocarcinoma) cells obtained from Centre for Cellular and Molecular Biology, Hyderabad were used for *in vitro* studies. The cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L) and supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution. Cells in passage number 12-15 were used for the experiments.

MTT cell viability assay

The cytotoxic property of seaweed extract was evaluated by MTT assay according to Kowalczyk *et al.*[13] in HT-29 cells thatwere maintained as a monolayer in DMEM, supplemented with 10% FBS. Cells were trypsinized and the cell count was adjusted to 1.0×10^6 cells/mL using DMEM containing 10% FBS. To each well in the 96-well microtiter plate, 0.1 mL of the diluted cell suspension was added and incubated overnight. After 24 h of incubation, media was removed from the well and 100 µL of different concentration of seaweed extracts (600, 300, 150, 75, 37.5 and 18.75 µg/mL) were added. Serial double dilution of extracts with highest concentration of 600 µg/mL was used in the study. Untreated cells (without extract) served as negative control whereas cells treated with DMSO (0.1% v/v) were used as vehicle control. The plates were incubated at 37°C for different time periods (24 h and 48 h). After incubation, media were removed and to each well, 50 µL of MTT in PBS (5 mg/mL) and 50 µL fresh media were added. The plates were further incubated at 37°C for 4 h. To each well, 150 µL of DMSO was added and the plates were gently shaken to solubilize the formazan crystals and the absorbance was measured at 595 nm[13] in an ELISA plate reader (Tecan, Infinite M200 Pro, Austria) Each experiment was performed in triplicate. Concentration of the extract needed to inhibit cell growth by 50% (IC_{50}) was calculated by probit analysis using IBM SPSS version 20.0.

Trypan blue dye exclusion assay

The cytotoxic property of seaweed extract was also evaluated by trypan blue dye exclusion assay according to the method of Soumya *et al.*[15]. Cells were seeded in 6 well plates at a concentration of 5×10^4 cells /well and incubated overnight. After 24 h, culture media was gently aspirated and fresh media containing 75 µg/mL and 150 µg/mL of ethanolic extract of *S. marginatum* were added. The plates were incubated at 37°C and 5% CO_2 for 48 h. Adherent cells were removed by trypsinization using 0.25 trypsin-EDTA solution. Cells were centrifuged at 2000 rpm for 5 min and supernatant was discarded to pellet the cells. Cell pellets were resuspended in 0.5 mL of PBS and from which 0.2 mL of cell suspension was mixed with 0.2 mL 0.4% trypan blue solution. 20 µL of this suspension was placed in a haemocytometer chamber and examined immediately. Live cells with intact cell membrane will not take up the dye whereas dead cells will be stained blue. Both viable and dead cells were counted and the cell viability was calculated using the formula given below. Cells without any treatment served as control and the cells treated with 0.1% DMSO served as vehicle control. Cells treated with 50 µg/mL 5-fluorouracil served as a positive control.

$$\% \text{ viability} = \frac{\text{Total no. of unstained cells}}{\text{Total no. of stained cells} + \text{Total no. of unstained cells}} \times 100$$

Morphological analysis by phase contrast microscopy

Cells were seeded in 24-well plates and treated with *S. marginatum* extract for 24 and 48 h. The morphological alterations of cells were visualized under the phase contrast microscope.

Analysis of apoptosis

To detect the apoptosis induction in HT-29 cells, cells were treated with ethanolic extract *S. marginatum* at the 75 and 150 µg/mL for 48 h.

Acridine orange (AO) - Ethidium bromide (EB) double staining

A double staining with acridine orange and ethidium bromide was performed as reported by Behzad *et al*[16]. At the end of the treatment period, cells were harvested, centrifuged and resuspended in PBS. To 20 µL of cell suspension, 2 µL acridine orange-ethidium bromide working solution (1.0 µL of 5 mg/mL acridine orange and 1 µL of 3 mg/mL ethidium bromide was added to 1.0 µL of PBS) was added and mixed. 25 µL of the suspension was then placed on a glass slide, covered with cover slip and examined under florescence microscope (Olympus BX60F5, Japan) with an excitation maximium at 495nm and emission maximum at 515 nm. 300 cells from randomly selected fields were counted and scored as viable, apoptotic or necrotic. Acridine orange is a vital dye that stains both live and dead cells and gives a green florescence, whereas ethidium bromide will stain only those cells that have lost their membrane integrity and gives a red florescence. Cells were visualized under microscope as viable cells (green nuclei), apoptotic cells (nucleus condensed and orange colour), and necrotic cells (red nuclei). The apoptotic index (percentages of apoptotic cells) was then calculated.

Mitochondrial membrane potential assessment

Mitochondrial membrane potential was assessed using the fluorescent potentiometric dye JC-1 as described by Wang *et al*.[17]. Briefly, at the end of the treatment period, cells were trypsinized, harvested and washed twice with PBS. Then, 100 µL of JC-1 staining solution (diluted 1:10 with culture media) was added to the cell suspension and mixed gently and incubated in CO_2 incubator at 37ºC for 15-30 min in dark. After staining, cells were centrifuged at 2500 rpm for 5 min and then resuspended in PBS. Cell suspension placed on a glass slide, covered with cover slip and examined under confocal fluorescent microscope (Zeiss LSM700, Germany).

FITC-Annexin V--Propidium iodide (PI) method

The extent of apoptosis and / or necrosis was measured using an FITC-annexin V-propidium iodide assay as described by Badmus *et al*.[18]. At the end of treatment cells were collected and 5×10^5 cells were resuspended in 500 µL of 1X binding buffer. The cells were double stained with FITC-annexin V (5 µL) and PI (5 µL) for 5 min in the dark. Stained cells are analyzed by flow cytometer (Cytoflex Beckman Coulter, Germany) with excitation wavelength of 488 nm and emission wavelength of 530 nm. Differential analysis was done based on annexin V and propidium iodide uptake by the cells. Gating was performed with reference to unstained control cells. Forward scatter, side scatter, annexin V-FITC channel and propidium iodide channel compensations were set for the unstained control cells, annexin V and propidium iodide positive controls. A total of 5000 cells were analyzed for each sample. The normal live cells are annexin negative and PI negative. Early apoptotic cells are annexin positive and PI negative and the late apoptotic cells are annexin positive and PI positive.

Statistical analysis

The results were expressed as Mean ± SD. The data were analyzed by one way ANOVA with Duncan's posthoc analysis using statistical software IBM SPSS version 20.0 for windows. P values <0.05 or <0.01 were considered as statistically significant

Results

Effect of *S. marginatum* extract on viability of HT-29 cells by MTT assay

Cells treated with *S. marginatum* extracts showed significant reduction in cell viability and this reduction were both concentration and time-dependent with an IC_{50} of 166.05 µg/mL and 78.88 µg/mL after 24 hours and 48 hours of treatment, respectively (Table 1, Fig. 1).

Effect of *S. marginatum* extract on viability of HT-29 cells by Trypan blue dye exclusion assay

The effect of *S. marginatum* extract on cell proliferation and cell viability was assessed by trypan blue dye exclusion method in HT-19 cells (Table 2). Total cell count was significantly (P <0.01) decreased with *S. marginatum* treatment and positive control 5-FU when compared to negative control). Maximum reduction in cell proliferation was noticed in the

Table 1 — Effect of *Stoechospermum marginatum* ethanolic extract on cell viability (%) by MTT assay in HT-29 cells after 24 and 48 h of treatment

Treatment period	Concentration (µg/mL)						5-FU (50 µg/mL)
	18.75	37.5	75	150	300	600	
24 h	83.27 ±0.49	74.37 ± 0.22	55.12 ± 0.22	37.69 ±0.33	25.09 ±0.24	18.84 ±0.35	51.71 ±0.26
48 h	69.18 ± 0.33	66.71 ± 0.22	43.87 ± 0.54	23.50 ± 0.24	13.87 ± 0.23	1.70[a] ± 0.25	30.59 ± 0.18

[Values are expressed as mean ± S.D (n=6)]

Table 2 — Effect of *Stoechospermum marginatum* extract on proliferation and viability of HT-29 cells by Trypan blue dye exclusion method

Treatment group	Concentration	Total cell count (10^5 cells/mL)	Cell viability (%)
Negative control	-	3.41[c]±0.10	97.07[d]±1.31
Solvent Control	0.1% DMSO	3.49[c]±0.23	96.41[d]±0.79
S. marginatum extract 75 µg/mL	75 µg/mL	1.59[b]±0.09	58.29[c]±3.04
S. marginatum extract 150 µg/mL	150 µg/mL	1.09[a]±0.04	36.79[b]±3.35
5-FU (positive control)	50 µg/mL	1.05[a]±0.04	30.82[a]±1.54

[Values are expressed as mean ± S.D (n=6). Means in same column bearing different superscript differ significantly (P <0.05)]

positive control, which differs significantly (P <0.01) from negative control, solvent control and *S. marginatum* treatment at 75 µg/mL. There was no significant difference between positive control and SM150. There was significant (P <0.01) decrease in cell viability in the treatment groups SM75, SM150 and positive control when compared to negative control. Marked reduction in cell viability was observed in positive control and it differs significantly from all the treatment groups.

Effect of *S. marginatum* extract on morphology of HT-29 cells

Negative control cells displayed normal cellular morphology (polygonal shaped) with flat cell bodies containing several nucleoli (Fig. 2). The cells treated with *S. marginatum* extract at 150 µg/mL showed typical apoptotic features such as rounding, shrinkage, blebbing of membrane and detachment from the substratum. Condensed and vacuolated nucleus, loss of contact with adjacent cell, detachment from the substratum and clumping was also observed. These changes were minimal in cells treated with 75 µg/mL of *S. marginatum* extract. All these changes were intense after 48 h of treatment. The morphological features of cells treated with 150 µg/mL of *S. marginatum* extract were comparable to that of positive control treated cells.

Detection of apoptosis by Acridine orange-Ethidium bromide staining

The per cent of live cell population was significantly decreased in treatment groups SM75, SM150 compared to untreated negative control. Increased apoptotic index was observed in cells treated with *S. marginatum* extract at 75 and 150 µg/mL compared to untreated negative control group (Table 3, Fig. 3A).

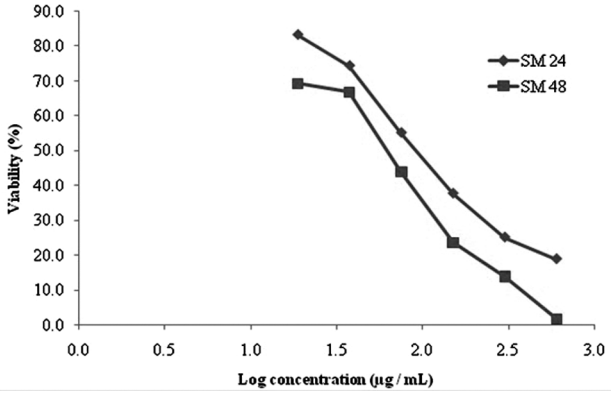

Fig. 1 — *In vitro* cytotoxity of *Stoechospermum marginatum* in HT-29 cells by MTT assay after 24 and 48 h of treatment

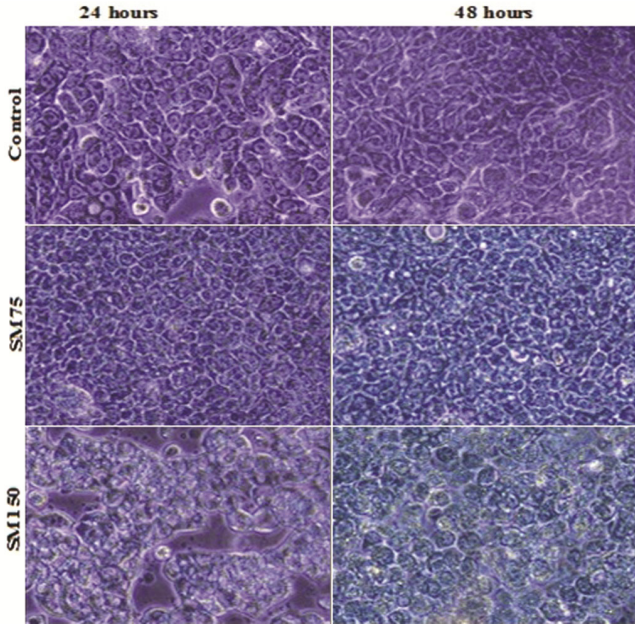

Fig. 2 — Microscopic images (40X) of control cells, cells treated with *S. marginatum* extract at 75 (SM75) and 150 µg/mL M150). 5-Fluorouracil (5-FU) was used as positive control.

Table 3 — Effect of *Stoechospermum marginatum* extract on induction of apoptosis in HT-29 cells by acridine orange/ethidium bromide staining

Treatment group	Concentration	Live cell (%)	Apoptotic cell (%)	Necrotic cell (%)
Negative control	-	89.33^{d}±1.51	6.50^{a}±1.38	4.17^{a}±0.41
S. marginatum extract 75 µg/mL	75 µg/mL	67.50^{c}±1.52	26.17^{b}±0.75	6.33^{b}±1.03
S. marginatum extract 150µg/mL	150 µg/mL	58.50^{b}±1.22	33.67^{c}±0.52	7.83^{c}±0.75
5-FU (positive control)	50 µg/mL	47.00^{a}±1.26	38.83^{d}±1.17	14.17^{d}±0.75

[Values are expressed as mean ± S.D (n=6). Means in same column bearing different superscript differ significantly (P <0.05)]

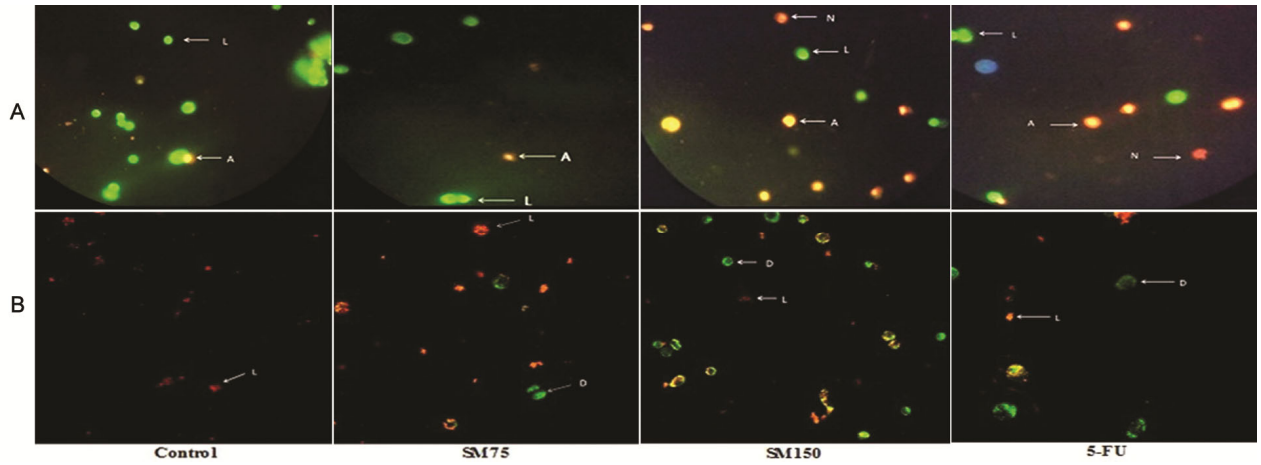

Fig. 3 — (A) Dual AO/EB staining images; and (B) JC-1 staining images of HT-29 cells treated with *S. marginatum* ethanolic extract at 75 (SM75) and 150 µg/mL (SM150). [5-Fluorouracil (5-FU) was used as positive control. L, Live cells; A, Apoptotic cells; N, Necrotic cells; and D, cells with altered mitochondrial membrane potential]

Detection of apoptosis by Mitochondrial Membrane potential assay

Control cells emitted orange red fluorescence due to high mitochondrial potential. Cells treated with *S. marginatum* extract showed progressive loss of orange J-aggregate fluorescence and cytoplasmic diffusion of green monomer fluorescence due to mitochondrial transmembrane depolarization. Cells treated with *S. marginatum* extract at 150 µg/mL and 5-fluorouracil showed intense green fluorescence compared to control (Fig. 3B)

Detection of apoptosis by FITC Annexin V-Propidium iodide method

The effect of *S.marginatum* extract on induction of apoptosis was evaluated using FITC Annexin V– Propidium binding by flow cytometry (Fig. 4). The results of the study showed considerable decrease in the percentage of viable cells between the treatment group SM75 (64.86%), SM150 (31.86%) and positive control 5-FU (53.66%) compared to control (82.92%). There was also considerable increase in the cells showing early apoptosis between the treatment groups SM75 (20.20%), SM150 (57.46%) and positive control 5-FU (33.66%) compared to negative control (1.96%). The percentage of cells showing late apoptosis was highest in SM75 (14.32%) followed by positive control 5-FU (11.46%) and SM150 (10.56%).

Discussion

The ethanolic extract of *Stoechospermum marginatum* was found to be cytotoxic against HT-29 cells and it markedly reduced the cell viability in a concentration and time-dependent manner. Similarly in the trypan blue assay, *S. marginatum* exhibited significant cytotoxic and antiproliferative activity as evidenced by reduced total cell count and viable cell count. This reduction on cell proliferation was found to be dose-dependent. The results of the study confirmed the antiproliferative and cytotoxic activity of *S. marginatum*, which could be due to the presence of phenols, terpenoids, flavonoids and poly-saccharides present in the extract[3,19].

Cancerous cells are fast growing and they resist apoptosis. Apoptosis is a programmed cell death that is essential for normal development and for maintenance of homeostasis by destroying injured or abnormal cells. Deregulation of apoptosis in cancerous cells results in unchecked cell proliferation and is associated with tumour development, tumour progression and development of resistance to chemotherapy[20]. Apoptosis is one of the important and reliable markers for the evaluation of anticancer activity[16,21]. Hence, the ability of *S. marginatum* extract to induce apoptosis was investigated by

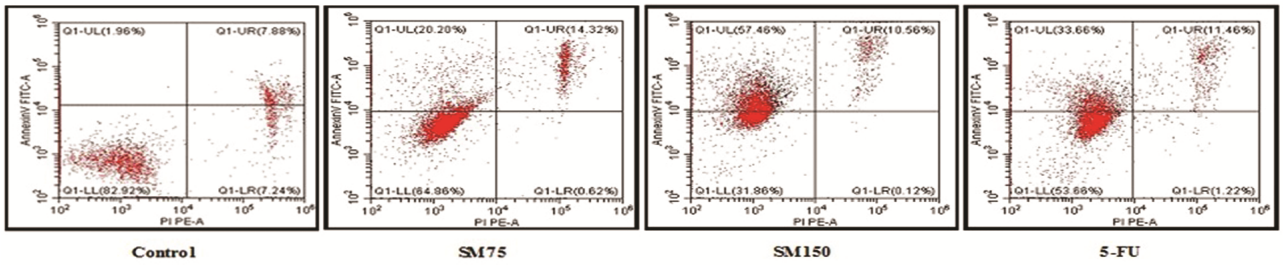

Fig. 4 — FITC-annexin V-propidium iodide staining dot plot analysis of HT-29 cells treated with *S. marginatum* ethanolic extract at 75 (SM75) and 150 µg/mL (SM150) by Flow Cytometry. [5-Fluorouracil (5-FU) was used as a positive control]

acridine orange/ethidium bromide staining, mitochondrial membrane potential assay and FITC Annexin V-Propidium iodide methods.

Double staining with acridine orange and ethidium bromide is widely employed in different cell populations[16]. In the present study, more than 30% of the cells treated with *S. marginatum* showed orange fluorescence indicating apoptosis. The percentage of cellular apoptosis caused by *S. marginatum* at 150 µg/mL was comparable to that of positive control 5-FU.

Death receptor and mitochondrial (intrinsic) pathways are the two signaling pathways involved in cell apoptosis. Loss of mitochondrial membrane potential and the redistribution of Cytochrome C release are early steps in apoptotic cascade[16]. Apoptosis induced by anticancer compounds were associated with the rapid collapse of mitochondrial membrane[22,23].

The effect of *S. marginatum* on mitochondrial membrane potential was investigated using fluorescent dye JC-1. Upon staining with JC-1, the loss of mitochondrial membrane potential was indicated by the decrease of red fluorescence and the increase of green fluorescence showing that the cells treated with *S. marginatum* extract at 150 µg/mL indicated that apoptosis induced by *S. marginatum* extract was related to the collapse of mitochondrial membrane potential.

The flow cytometric method was done for analysis of apoptosis by dual-staining annexin V-FITC and PI. In normal cells, phosphatidyl serine is predominantly located in the innermost layer of the plasma membrane. In early stages of apoptosis, phosphatidyl serine gets translocated from inner part of plasma membrane to outer part. Annexin V binds with phosphatidyl serine with high affinity and makes it possible to detect early apoptosis in cells. Propidium iodide enters the cell during later apoptosis or necrosis. Cells in early stages of apoptosis binds only with annexin V while the cells in late apoptosis binds with both annexin V and propidium iodide. Necrotic cells binds only with propidium iodide[17]. A higher percentage of annexin V positive cells were observed with *S. marginatum* compared to control indicating the potential of the extract to induce apoptosis, suggesting potential anticancer activity.

Resistance to apoptosis is one of the hallmarks of cancerous cells. Compounds that induces apoptosis or restores the sensitivity of cancerous cells to apoptosis could be promising drug candidate in the treatment of cancer[24]. The findings of the present provide evidence for the cytotoxicity and apoptosis induction potential of ethanolic extracts of *S. marginatum* and could be a potential source for bioactive molecules. Hence, further studies are warranted for isolation and characterization of bioactive molecules.

Conclusion

The ethanolic extract of *Stoechospermum marginatum* showed significant cytotoxic effect on HT-29 cancer cells with 48 h IC_{50} of 78.88 µg/mL. Acridine orange ethidium bromide staining revealed that *S. marginatum* induced apoptosis in 26-36% cells. The apoptosis induced by *S. marginatum* extract was related to the collapse of mitochondrial membrane potential FITC-Annexin V assay also confirmed that *S.* marginatum caused dose-dependent increase early apoptosis. The apoptosis induction potential of the extract was confirmed in the present and the compound which induces apoptosis are considered to be promising source for the development of new drug molecules for cancer treatment. Hence, *S. marginatum* could be considered as a promising candidate for further studies are required for isolation and development of bioactive molecule.

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Conflict of Interest

Authors declare no competing interests.

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