PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.3011

HORIZON
e-Publishing Group
HePG

RESEARCH ARTICLE

Exploring the *in vitro* antimicrobial, antioxidant, and anticancer potentials of *Spinifex littoreus* Burm f. Merr. against human cervical cancer

J Vedhamani[1], I Paul Ajithkumar[1*] & E. Angel Jemima [2]

[1] Department of Botany, Bishop Heber College (Autonomous), Affiliated to Bharathidasan University, Tiruchirappalli 620 017, Tamil Nadu, India

[2] Trichy Research Institute of Biotechnology Private Limited, Tiruchirappalli 620 018, Tamil Nadu, India

*Email: paulajith@bhc.edu.in

OPEN ACCESS

ARTICLE HISTORY

Received: 21 November 2023
Accepted: 17 February 2024

Available online
Version 1.0 : 07 March 2024

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Vedhamani J, Ajithkumar I P, Jemima E A. Exploring the in vitro antimicrobial, antioxidant, and anticancer potentials of Spinifex littoreus Burm f. Merr. against human cervical cancer. Plant Science Today (Early Access). https://doi.org/10.14719/pst.3011

Abstract

Spinifex littoreus Burm f. Merr. is considered as a pharmaceutically important coastal weed whereas, therapeutical applications of this plant are least studied. The current study aimed to identify the antibacterial, antioxidant, and anticancer properties in the chloroform extract of *Spinifex littoreus*. Six bacterial and 4 fungal human infections were tested for antibacterial activity in the chloroform extract of *S. littoreus* (SL-CH). The antioxidant ability of SL-CH was screened using the DPPH, ABTS, and H_2O_2 assays. MTT assay was used to investigate the anticancer activity of SL-CH and ROS, Annexin V, PI, and cell cycle analysis were used to determine its efficacy. The bacterial strain *Propionibacterium acnes* and the fungal strain *Sporothrix schenckii* were found to have the largest zones of clearance when the SL-CH extract was used at a concentration of 500 µg/ml. The antioxidant potentials of SL-CH extract showed the maximum radical scavenging activity against ABTS (83.98%), H_2O_2 (63.73%), and DPPH (52.02%) and their IC_{50} value was 31.74 µg/ml, 53.93 µg/ml, and 139.6 µg/ml respectively. The IC_{50} concentration for the chloroform extract of *Spinifex littoreus* was determined to be 117.5 µg/ml and it showed concentration-dependent anticancer activity against HeLa cells. The ROS fluorescence staining result of SL-CH demonstrated the production of reactive oxygen species, which caused HeLa cells to undergo apoptosis. It was demonstrated by annexin V staining, PI staining technique, and cell cycle analysis that SL-CH extracts caused apoptosis in HeLa cells, preventing their growth. Taken together, these results showed that *Spinifex littoreus* chloroform extract is rich in antimicrobial, antioxidant, and anticancer potentials. Therefore, *Spinifex littoreus* extract can treat and prevent various ailments.

Keywords

Spinifex littoreus Burm f. Merr., antimicrobial, antioxidant, anticancer, HeLa cell line.

Introduction

Currently, cancer is a significant global health issue, affecting 182 per 100000 people annually and causing 102 deaths. The World Health Organization reports 14 million cases and 8 million deaths worldwide (1). Fatality associated with cancers continues to rise all over the globe, and it has been estimated that a total of 13.1 million deaths are expected as a result of cancer globally by 2030 (2). Nowadays, numerous approaches for cancer treatment are employed, such as chemotherapy, however, due to the non-selectivity of drugs, a substantial number of healthy cells are lost along with malignant cells in this procedure (3). Antioxidant plays a vital

role in the prevention of cancer cells and in treatment procedures (4). Therefore, the interest in the medicinal herb increased extensively. Over the past 30 years and more, chemicals found in nature have drawn significant interest due to their potential as cutting-edge cancer-preventative and therapeutic agents (5). Most medications used to treat cancer today were extracted from natural products—roughly 60% (6). Since natural herbal remedies have many benefits, the ongoing quest for anticancer drugs or molecules from plants plays a significant role in determining alternative ways to be safe and reduce the negative effects caused by chemotherapy.

Plants have been a major source of food and medicine to living beings from the dawn of life. In ancient days, people have utilized plants as medication to treat contagious illnesses and some of those plants are being employed today in traditional home remedies and ethnomedicine. The coastal regions are popular for their specific environmental conditions such as higher humidity, diurnal variations, light intensity, and alkaline pH with higher vapor pressure deficits (7). These parameters most likely play a vital role in the production of significant secondary metabolites in plants to fit them with their adverse climatic conditions. These secondary metabolites will be a good medication to treat different kinds of disorders (8).

Spinifex littoreus Burm f. Merr. is a coastal grass also known as Ravana-moustache which belongs to the family Poaceae. It is a colony-forming, psammophilous grass with bent, rigid, sharp leaf blades and prominent, globose, wind-dispersed infructescence. It is a diecious shrub that grows on the dune of the seashore (9). *S. littoreus* may adapt to total and extensive sand burial during the growing season by quick stolon growth, profuse growth of adventitious roots around the stolon base, and increased leaf germination on the stolon top (10). *S. littoreus* showed therapeutic properties such as antibacterial (8) and antioxidant (11) activities.

Keeping this in consideration, the present investigation intends to look for phytochemical components in a chloroform crude extract of *Spinifex littoreus* to find an effective cancer medication and also to analyze their biological activities such as antioxidant and antimicrobial resistance.

Materials and Methods

Chemical Reagents

Both the potato dextrose agar and the nutrient agar medium were bought from Merck in Germany. Penicillin/ Streptomycin antibiotic solution, DMEM medium, Foetal Bovine Serum (FBS), and Trypsin-EDTA were all acquired from Gibco (USA). The following were purchased from Sigma, in the USA: DMSO (Dimethyl sulfoxide), MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml), Propidium iodide (100 µg/ml) and Ribonuclease I (stock 100 mg/ml). Himedia, an Indian company, sold 1X PBS. Thermo Scientific (USA) sold the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit.

Microorganism

The bacterial strains of *Escherichia coli* (MTCC 443), *Propionibacterium acnes* (MTCC 1951), *Staphylococcus aureus* (MTCC 902), *Aeromonas hydrophila* (MTCC 12301), *Streptococcus faecalis* (MTCC-439), *Bacteroids fragilis* (ATCC 25285) and the fungal strains of *Cryptococcus neoformans* (ATCC 32045), *Aspergillus fumigatus* (MTCC 343), *Aspergillus niger* (MTCC 281), *Sporothrix schenckii* (ATCC 26327) were purchased from Microbial Type Culture Collection, Chandigarh, India.

Cell culture

HeLa (Human Cervical Cancer Cells) were bought from the National Centre for Cell Science (NCCS) in Pune and cultivated in liquid medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, all while being kept at 37 °C in a 5% CO_2 atmosphere.

Plant Collection, Identification and Extraction

The *Spinifex littoreus* plant was collected in Kunthukal Beach in Pamban, Tamil Nadu, India (latitude: 9.25323; longitude: 79.21829). It is a species of coastal grass. The Rapinat Herbarium and Centre for Molecular Systematics Director, Rev. Dr. L. John Peter Arulanandam SJ, St. Joseph's College, Tiruchirappalli, authenticated the collected plants (authentication numbers JV001 and JV002). *Spinifex littoreus* leaves were cleaned twice or more with running water before being left to air dry. The dried leaves of *Spinifex littoreus* were electronically crushed into powder. The extraction of *S. littoreus* leaf powder was done by soxhlet equipment and the solvent system used was chloroform (12).

Antibacterial activity of *Spinifex littoreus* chloroform extract

By using bacterial species like *Escherichia coli, Propionibacterium acnes, Staphylococcus aureus, Aeromonas hydrophila, Streptococcus faecalis,* and *Bacteroids fragilis,* the antibacterial capacity of the chloroform extract of *Spinifex littoreus* was examined (13). The nutrient agar medium was added to the petri plates in an amount of around 20 ml. The surface of the solidified agar medium was injected with the chosen bacterial species. Then wells were cut and loaded with four different concentrations such as 500, 250, 100, and 50 µg/ml of chloroform extract of *S. littoreus*, positive control of Gentamicin (antibacterial drug), and negative control (Chloroform). This set-up was kept undisturbed for 24 h at 37 °C. Then the zone of clearance around the well was measured.

Antifungal activity of *Spinifex littoreus* chloroform extract.

The antifungal activity of the chloroform extract of *Spinifex littoreus* was also studied by agar well diffusion method (13) with the fungal strains of *Cryptococcus neoformans, Aspergillus fumigatus, Aspergillus niger and Sporothrix schenckii.* Approximately 20 ml of PDA medium was poured into the Petri plates. The surface of the solidified agar media was injected with the chosen fungal strains. The *S. littoreus* chloroform extract was sliced into wells and

loaded with various quantities (500, 250, 100, and 50 µg/ml) as well as a positive control of the commercial antifungal drug Amphotericin-B and a negative control (Chloroform). At 28 °C, the plates were incubated for 72 h. After that, the clean zone around the well was measured.

Free Radical Scavenging Assays for Antioxidant Activity of *Spinifex littoreus* chloroform extract

DPPH Radical Scavenging Examination

DPPH (1,1-Diphenyl-2-Picryl-Hydrazyl) free radical activity is to scavenge radicals. The antioxidant activity of the S-CH extract and the standard were assessed using a modified approach (14). Exactly 0.1 mM of DPPH solution in methanol (100 µl) was added to the various concentrations (500, 250, 100, 50, and 10 µg/ml) of SL-CH extract. After giving the mixture a thorough shake, the mixture must stand at room temperature for 30 min. The absorbance at 517nm must then be measured with a UV-VIS spectrophotometer. Enhanced radical scavenging activity was shown by the reaction mixture's lower absorbance values. The following formula is used to determine the ability to scavenge the DPPH radical.

$$(\%) = Abs_{control} - Abs_{sample} / Abs_{control}$$

$Abs_{control}$ is the absorbance of ABTS radical in methanol;

Abs_{sample} is the absorbance of ABTS radical solution mixed with sample extract/standard.

ABTS Assay

The protocol for the ABTS assay was modified from the approach used (15). Both 2.4 mM and 7 mM potassium persulfate solutions were used as the stock solutions. Then, the 2 stock solutions were combined in equal parts and allowed to react for 14 h at room temperature and in the dark before being turned into the working solution. An absorbance of 0.706 ± 0.01 units at 734 nm was then obtained using a spectrophotometer by diluting the solution by combining 1 ml of ABTS solution with 60 ml of methanol. SL-CH (500, 250, 100, 50, and 10 µg/ml) were allowed to react with 1 ml of the fresh ABTS solution for each experiment. The absorbance was measured at 734 nm after 7 min using a spectrophotometer. The % of inhibition was obtained after comparing the extract's ability to scavenge ABTS to that of ascorbic acid. The absorbance of an ABTS radical solution combined with a sample extract or standard is called an abs sample. Each analysis was carried out in three instances (n = 3). The following formula helps to calculate the rate of ABTS scavenging.

$$(\%) = Abs_{control} - Abs_{sample} / Abs_{control}$$

Hydrogen Peroxide Assay

With a few minor adjustments, the procedure outlined was employed to ascertain the hydrogen peroxide-scavenging ability of plant extracts (16). A hydrogen peroxide (43 mM) solution is created in phosphate buffer (1 M pH 7.4). Different quantities of the sample SL-CH (500, 250, 100, 50, and 10 µg/ml) were added to a hydrogen peroxide solution (0.6 ml, 43 mM). The absorbance of hydrogen peroxide at 230 nm was assessed after 10 min in comparison to a phosphate buffer-based blank solution. The positive control was ascorbic acid. The formula for the inhibition % below was used to determine the free radical scavenging activity.

$$(\%) = Abs_{control} - Abs_{sample} / Abs_{control}$$

Anticancer Analysis

Cytotoxicity Assay

The anticancer property of *Spinifex littoreus* was preliminarily assessed by the cell viability index. The chloroform extract of *S. littoreus* was *invitro* screened against HeLa cells. The cytotoxicity was carried out by MTT assay (17). MTT is a yellow tetrazolium salt, which is converted into a purple formazan crystal by the metabolic action of viable cells. In brief, 1×10^5 cells/ml cells/well (200 µl) of well-grown HeLa cells were harvested and seeded in a 96-well tissue culture plate containing DMEM medium with 10% FBS and 1% antibiotic solution and kept undisturbed for 24-48 h at 37 °C. Then the plates were rinsed with sterile PBS and treated with various concentrations of chloroform extract of *S. littoreus*. This was incubated in a humidified 5% CO_2 incubator for 24 h at 37 °C temperature. 20 µl of MTT at a concentration of 5 mg/ml were added to each well after the initial incubation period, and the cells were then left to continue incubating for an additional 2-4 h until purple precipitates could be seen under an inverted microscope. The absorbance for each well at 570 nm was measured using a microplate reader (Thermo Fisher Scientific, USA) and the percentage of cell viability and IC_{50} value were calculated using Graph Pad Prism 6.0 software [USA]. The following formula was used to determine the cell viability:

Cell viability % = Test OD/Control OD X 100

Reactive Oxygen Species

The SL-CH extract was tested for its ability to produce reactive oxygen species (ROS) in HeLa cells. In summary, trypsinization was used to harvest the grown HeLa cells, which were then gathered in a 15 ml tube. After that, the cells were cultured for 24 h at 37 °C in DMEM media with 10% FBS and 1% antibiotic solution at a density of 1×10^5 cells/ml in a 96-well tissue culture plate. The wells were washed and treated with the IC_{50} concentration of 117.5 µg/ml of SL-CH extract in a serum-free DMEM medium and incubated at 37 °C for 24 h. After 24 h, 100 µl of 1X assay staining solution and 1 ml of the ROS assay buffer were added to the wells and gently mixed. Following that, the plate was allowed to incubate for 60 min at 37 °C with 5% CO_2. Immediately following the incubation time, the ROS production was assessed using a fluorescence imaging system (ZOE, BIO-RAD) (18).

Apoptosis Assay

Hela cells were cultivated in a 96-well tissue culture plate for 24-48 h at 37 °C in a medium containing 10% FBS and 1% antibiotic solution at a density of 5×10^5 cells/ml. The wells were cleansed with sterile PBS, placed in a serum-free DMEM medium, and treated with 117.5 µg/ml of SL-CH extract before being incubated for 24 h at 37 °C with 5% CO_2. For the apoptosis analysis, 400 µl of 1x Annexin binding buffer was added and gently mixed, and

then 10 µl each of Alexa Fluor and PI were added to the wells. The plate was spun for 2 min at 800 rpm after 15 min of incubation and the cells were examined using a fluorescence imaging device (ZOE, Bio-Rad, USA) (19).

Cell Cycle Assay

Utilizing flow cytometry, the cell cycle was examined. The growing HeLa cells were collected in a 15 ml tube. The cells were then cultured for 24-48 h at 37 °C in DMEM media with 10% FBS and 1% antibiotic solution, seeded into a 6-well tissue culture plate at a density of 1×10^6 cells/ml. After being treated with 117.5 µg/ml of SL-CH sample in a serum -free DMEM medium and being rinsed with sterile PBS, the wells were placed in an incubator and incubated for 24 h at 37 °C with 5% CO_2. The cells were centrifuged with PBS for 5 min at 1500 rpm following incubation. Furthermore, the cells were fixed for 30 min at 4 °C using cold 70% ethanol, which was applied drop wise to the cell pellet. Then the cells were again centrifuged twice for 5 min at 1500 rpm with sterile PBS and the supernatant was discarded. The cell pellet was then treated with 500 µl of PI (100 µg/ml) and 50 µl of RNase (100 µg/ml) before being stored at 4 °C for flow cytometry analysis. A flow cytometer was used to sort the cells (BD Biosciences) (20).

Results

Antimicrobial Activity of *Spinifex littoreus* chloroform extract.

S. littoreus chloroform extract exhibited antibacterial activity against bacterial strains such as *Staphylococcus aureus* (10.25 mm), *Aeromonas hydrophila* (11.25 mm), *Streptococcus faecalis* (12.5 mm), *Bacteroids fragilis* (12.75 mm), *Escherichia coli* (11.5 mm), *Propionibacterium acnes* (13.5 mm), *Cryptococcus neoformans* (9.5 mm), *Aspergillus fumigatus* (12.75 mm), *Sporothrix schenckii* (17.5 mm) and *Aspergillus niger* (11.5 mm) by agar well diffusion method. The maximum inhibition was recorded at 500 µg/ml of SL-CH extract for *Propionibacterium acnes* with a 13.5 mm diameter (Table 1). Antibacterial activity of SL-CH extract). Similarly, the maximum antifungal activity was observed in *Sporothrix schenckii* with a 17.5 mm diameter in antifungal activity of SL-CH extract (Table 2). The pictorial results of the antibacterial and antifungal activity are shown in Fig. 1 and 2 respectively.

Antioxidant Activity of *Spinifex littoreus* chloroform extract

The free radicals scavenging activity of SL-CH extract was confirmed by the antioxidant assays namely DPPH, H_2O_2, and ABTS. The percentage of inhibition of DPPH, H_2O_2, and ABTS are in Fig. 3). Antioxidant activity of SL-CH extract at various concentrations was analyzed using different antioxidant assays ABTS assay, Hydrogen peroxide assay, and DPPH assay of SL-CH extract with their respective standard (Ascorbic acid). The figures show the mean ± SD of the 3 replications). Antioxidant assays for SL-CH extract showed the concentration-dependent inhibition of free radicals. The maximum inhibition was observed at 500 µg/ml for all the tested assays. The DPPH assay results for SL-CH extract showed 52.02% of inhibition and the IC_{50} value was formed to be 139.6 µg/ml. Similarly, the ABTS assay exhibited 83.98% maximum inhibition with an IC_{50} value of

Table 1. Antibacterial activity of SL-CH extract

Sl. No.	Name of the test organism	Zone of inhibition (mm) Mean ± SD				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Staphylococcus aureus*	10.25±0.35	6.25±0.35	5.25±0.35	4.25±0.35	16.5±0.35
2	*Aeromonas hydrophila*	11.25±0.35	9.25±0.35	6.25±0.35	5.25±0.35	17.5±0.7
3.	*Streptococcus faecalis*	12.5±0.7	10.25±0.35	9.25±0.35	5.25±0.35	11.5±0.7
4.	*Bacteroids fragilis*	12.75±1.06	11.25±0.35	10.25±0.35	9.25±0.35	14.5±0.7
5.	*Escherichia coli*	11.5±0.7	5.5±0.7	0	0	14.5±0.7
6.	*Propionibacterium acnes*	13.5±0.7	11.5±0.7	0	0	17.5±0.7

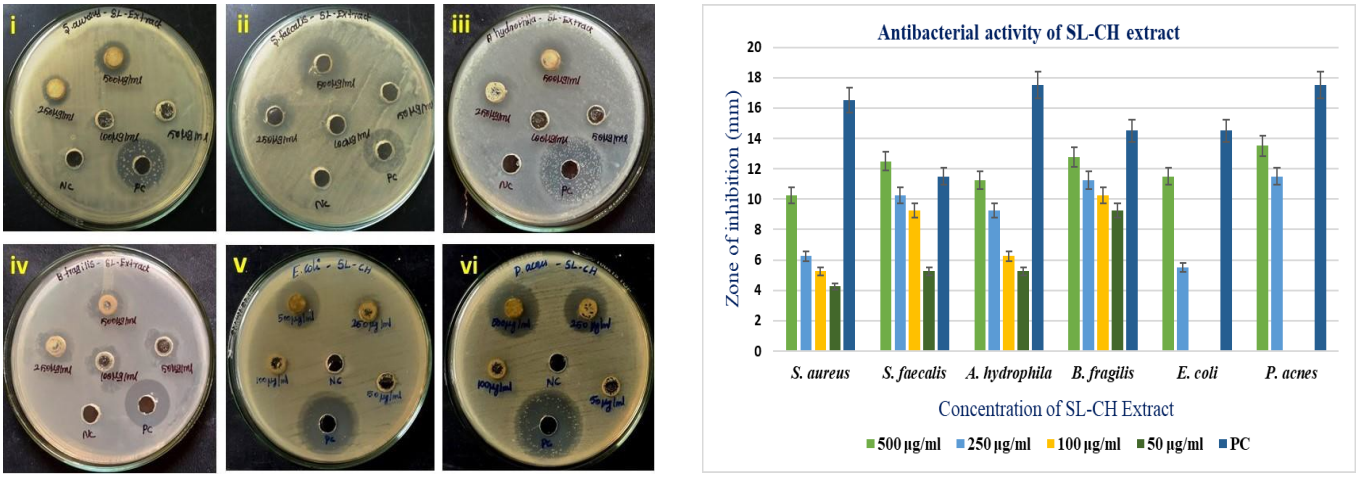

Fig. 1. Antibacterial assay by agar well diffusion method of SL-CH extract and Gentamicin as control indicated by the zone of inhibition against (i) *Staphylococcus aureus*, (ii) *Streptococcus faecalis*, (iii) *Aeromonas hydrophila*, (iv) *Bacteroids fragilis*, (v) *Escherichia coli*, (vi) *Propionibacterium acnes*. The results were shown as independent experiments in triplicates.

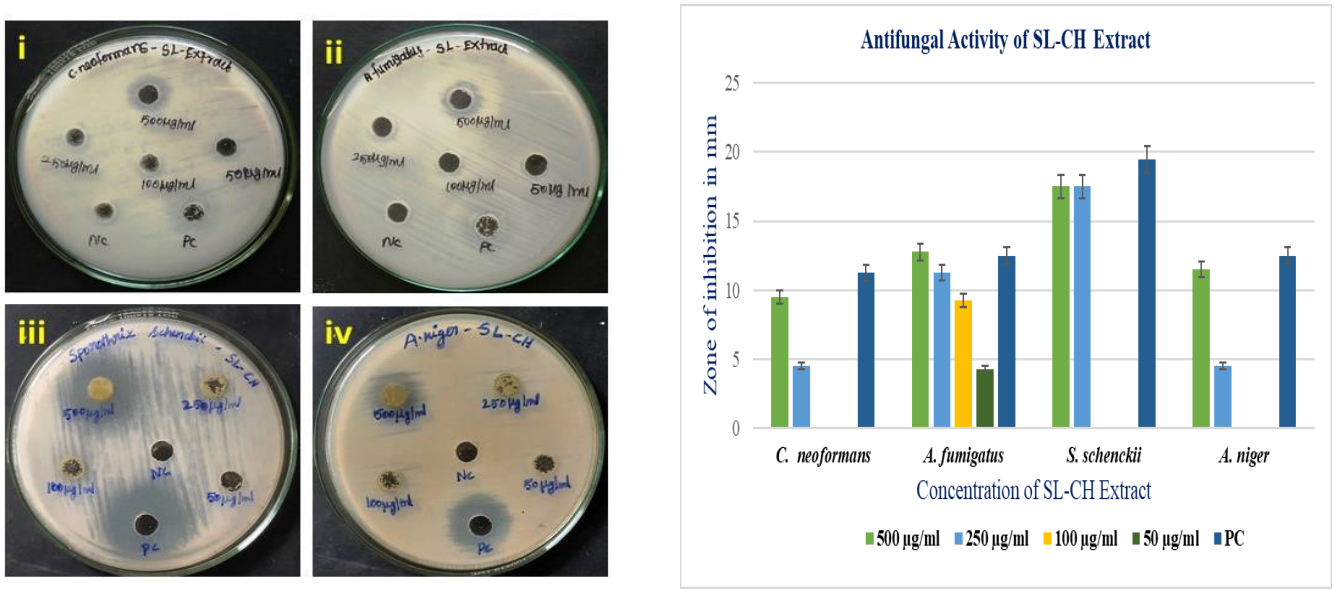

Fig. 2. Antifungal activity of SL-CH extract and Amphotericin-B as control showed by a zone of inhibition against (i) *Cryptococcus neoformans,* (ii) *Aspergillus fumigatus,* (iii) *Sporothrix schenckii,* (iv) *Aspergillus niger*. The results were shown as independent experiments in triplicates.

Table 2. Antifungal activity of SL-CH extract

Sl.No.	Name of the test organism	Zone of inhibition (mm) Mean ± SD				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Cryptococcus neoformans*	9.5±0.7	4.5±0.7	0	0	11.25±0.35
2.	*Aspergillus fumigatus*	12.75±1.06	11.25±0.35	9.25±0.35	4.25±0.35	12.5±0.7
3.	*Sporothrix schenckii*	17.5±0.7	4.5±0.7	0	0	19.5±0.7
4.	*Aspergillus niger*	11.5±0.7	4.5±0.7	0	0	12.5±0.7

31.74 µg/ml. The hydrogen peroxide test showed 63.73% of inhibition with IC_{50} of 53.93 µg/ml. Table 3 visualizes the antioxidant scavenging rate of *S. littoreus* chloroform extract in total antioxidant assays.

Anticancer Analysis of *Spinifex littoreus* chloroform extract

The anticancer capability of the plant SL-CH extract was investigated using MTT assay, ROS, Annexin V, and PI and cell cycle analysis. The antiproliferative activity of SL-CH extract by MTT assay showed a dose-dependent inhibition in HeLa cell lines. The maximum inhibition of HeLa cell viability was observed in the concentration of 500 µg/ml with 44.35% when compared to the control were shown in Table 4. Fig. 4 pinned the pictures of untreated cells and cells after treatment and the percentage of cell viability of the different concentrations of chloroform extract of *S. littoreus*.

SL-CH-induced Reactive Oxygen Species were confirmed by the ROS fluorescent imaging method. Induction of intracellular ROS has been implicated in the

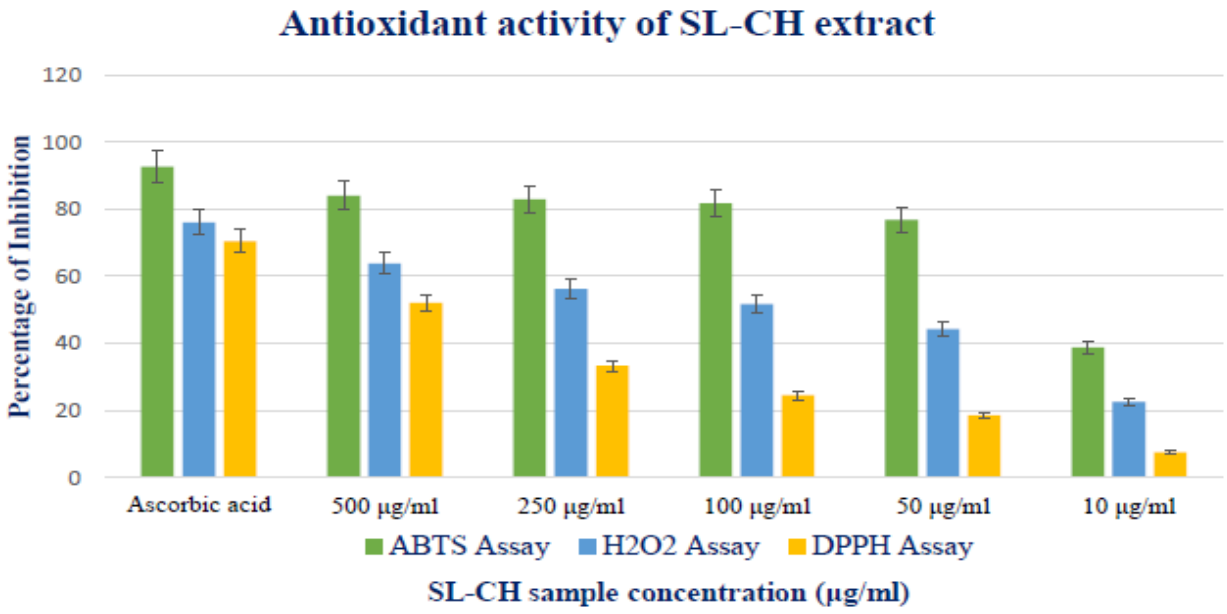

Fig. 3. Antioxidant activity of SL-CH extract at various concentrations was analyzed using different antioxidant assays ABTS assay, Hydrogen peroxide assay, and DPPH assay of SL-CH extract with their respective standard (Ascorbic acid). The figures show the mean ± SD of the three replications.

Table 3. Antioxidant scavenging rate of *S. littoreus* chloroform extract

Sl. No	Sample concentration	Antioxidant assays Mean ±SD		
		ABTS Assay	H_2O_2 Assay	DPPH Assay
1	Ascorbic acid	92.63±4.61	75.99±0.55	70.40±1.77
2	500 µg/ml	83.98±0.91	63.73±0.69	52.02±5.59
3	250 µg/ml	82.89±0.23	56.20±0.84	33.23±8.46
4	100 µg/ml	81.75±0.63	51.61±1.84	24.40±2.16
5	50 µg/ml	76.82±0.45	44.09±4.48	18.58±5.80
6	10 µg/ml	38.67±1.88	22.49±3.17	7.47±4.71

cytotoxicity of cancer cells (21). The outcome obtained from this study showed the SL-CH treated HeLa cells illuminated higher intensity of green dye compared to untreated cells were shown in Fig. 5. These results confirm that SL-CH-induced ROS generation in HeLa cells.

The apoptotic rate of the HeLa cell line that is treated with SL-CH was examined with the help of Annexin -V and PI staining kits. HeLa cells after being exposed to the SL-CH extract for 24 h were depicted in Fig. 6 with a variety of coloured cells, including yellow-green fluorescence early apoptotic cells, orange yellow of late apoptotic cells was identified by combining images obtained with green and red filter (yellow) (22). The results

Table 4. Percentage of cell viability in different concentrations of *S. littoreus* chloroform extract

Sl. No	Tested sample concentration (µg/ml)	Cell viability (%) Mean ± SD
1	Control	100±0
2	500 µg/ml	44.35±1.37
3	400 µg/ml	53.49±4.24
4	300 µg/ml	59.52±0.24
5	200 µg/ml	64.25±3.16
6	100 µg/ml	75.11±0.38
7	80 µg/ml	78.89±1.68
8	60 µg/ml	83.51±0.64
9	40 µg/ml	87.29±3.13
10	20 µg/ml	95.22±0.90
11	10 µg/ml	99.58±1.10

obtained from this analysis indicate that cell contraction along with membrane blebbing are characteristic signs of cell apoptosis.

The cell cycle is a series of processes that regulate cell duplication (23). The effect of SL-CH extract on the cell cycle of the HeLa cell line was analyzed by flow cytometry using the PI staining method. According to the analysis findings, SL-CH extract-treated HeLa cells experienced

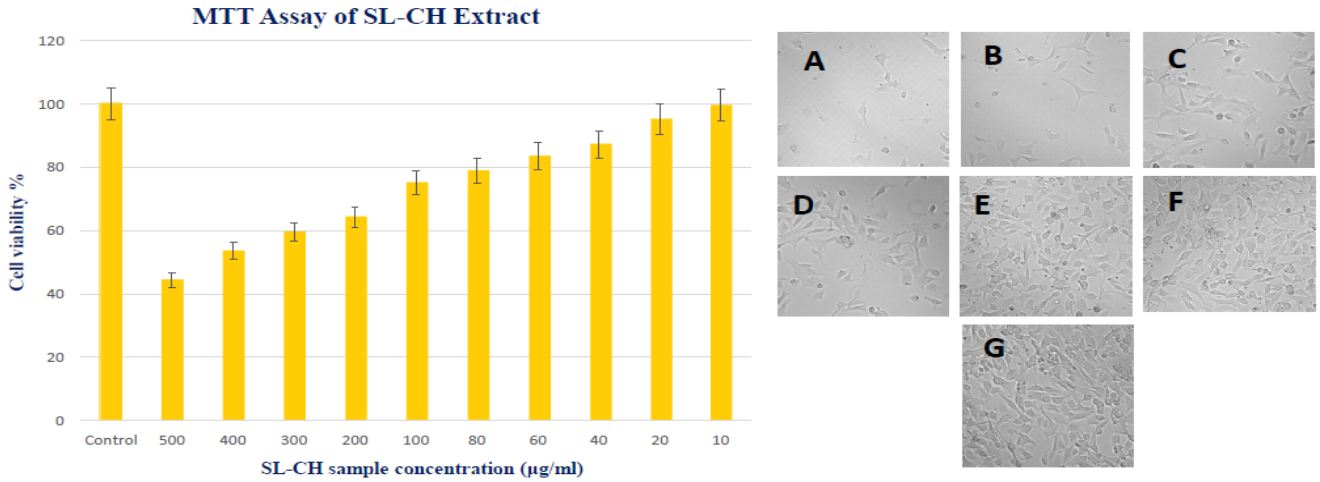

Fig. 4. MTT assay exploring the anticancer activity of SL-CH extract in HeLa cell. (A-F) SL-CH extract showed dose-dependent cell viability inhibition in the HeLa cell line and (G) untreated control cells showed cell proliferation.

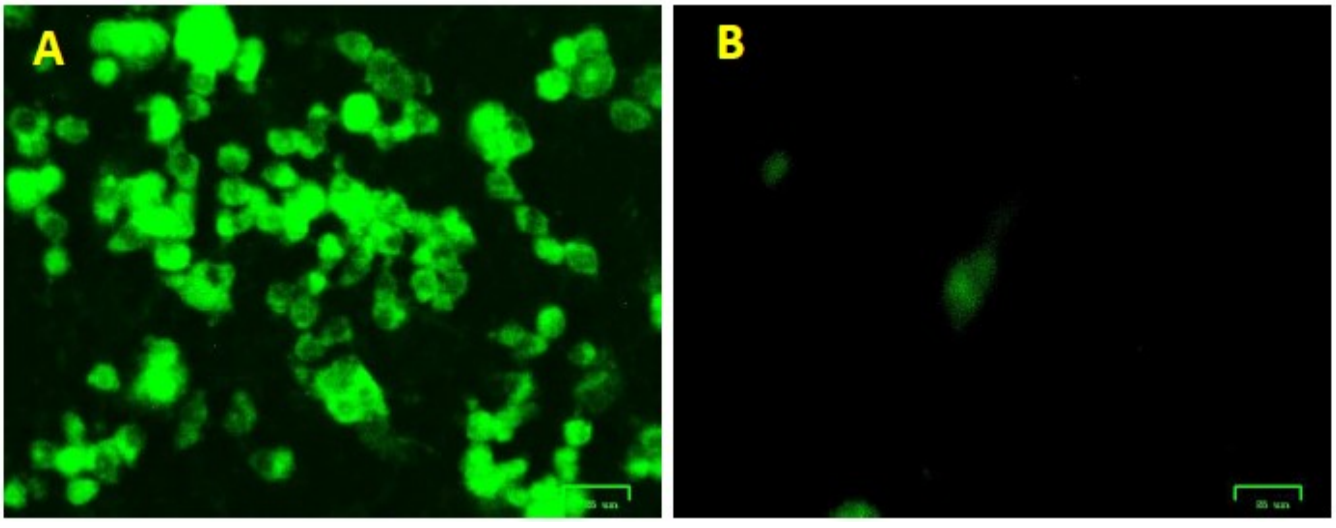

Fig. 5. The fluorescence imaging approach was used to determine intracellular ROS in HeLa cells. (A) treatment with 117.5 µg/ml of SL-CH-induced intracellular ROS production was shown as green fluorescence emission in HeLa cells. (B) Control cells.

86.66% of apoptosis, 9.8% of the cells in the G_1/G_2 stage, 2.96% of cells entered the S phase, and 0.56% of cells were observed in the G_2/M phase (Fig. 7). These results showed that SL-CH caused cell death by generating ROS which leads to apoptosis of HeLa cells.

Discussion

Coastal plants are subjected to unfavorable environments, which result in the production of stress-related bioactive compounds, the majority of which are unknown (24). This current study was designed to search for a natural antimicrobial, antioxidant, and anticancer source from a coastal grass *Spinifex littoreus.* Secondary metabolites are plant-derived derivatives of primary metabolites that enhance plant development and existence under a variety of environmental situations while also acting as essential primary metabolites (25). Secondary plant metabolites play an important role in the treatment of many diseases in both traditional medicine and common practice. They provided lead compounds for the production of medicines used in contemporary medicine to treat conditions ranging from cancer to migraine (26).

In the present study, the finding of antimicrobial activity demonstrates that chloroform extract of *S. littoreus* at a higher concentration (500 mg/ml) was effective in combating the *Staphylococcus aureus* (10.25 mm), *Aeromonas hydrophila* (11.25 mm), *Streptococcus faecalis* (12.5 mm), *Bacteroids fragilis* (12.75 mm), *Escherichia coli* (11.5 mm), *Propionibacterium acnes* (13.5 mm), *Cryptococcus neoformans* (9.5 mm), *Aspergillus fumigatus* (12.75 mm), *Sporothrix schenckii* (17.5 mm) and *Aspergillus niger* (11.5 mm). This also suggests that *S. littoreus* chloroform extract might work well as a treatment for bacterial and fungal disorders. This may be due to the presence of the secondary metabolites in *Spinifex littoreus*, such as alkaloids, flavonoids, tannins, terpenoids, and saponins (8). There was a study documenting that antimicrobial properties of *Spinifex littoreus* aqueous extract and his work suggests it can be a good antimicrobial agent (27). Seaweeds such as *Kappaphycus alvarezii* and *Sargassum ilicifolium* have been recorded as a potential antimicrobial agent for 18 different human pathogens (28, 29). *Acanthus ilicifolius* is one of the

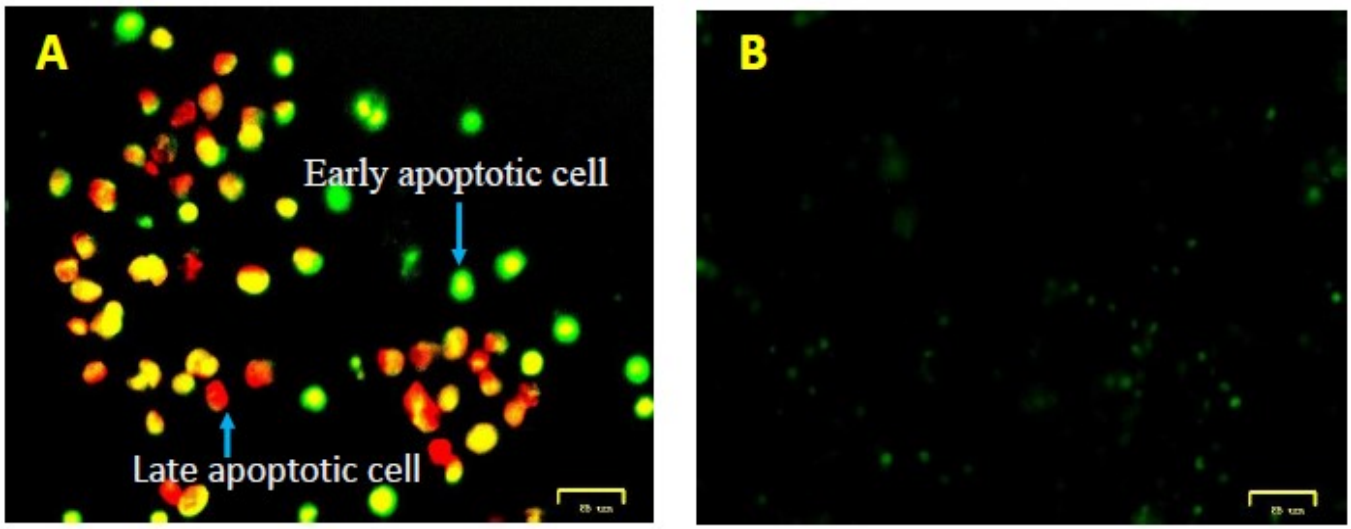

Fig. 6. (A) SL-CH treated and Annexin V-PI counterstained HeLa cells showed late apoptotic cells (orange-red) and early apoptotic cells (yellow-green) after 24 h of incubation (B) untreated HeLa cells.

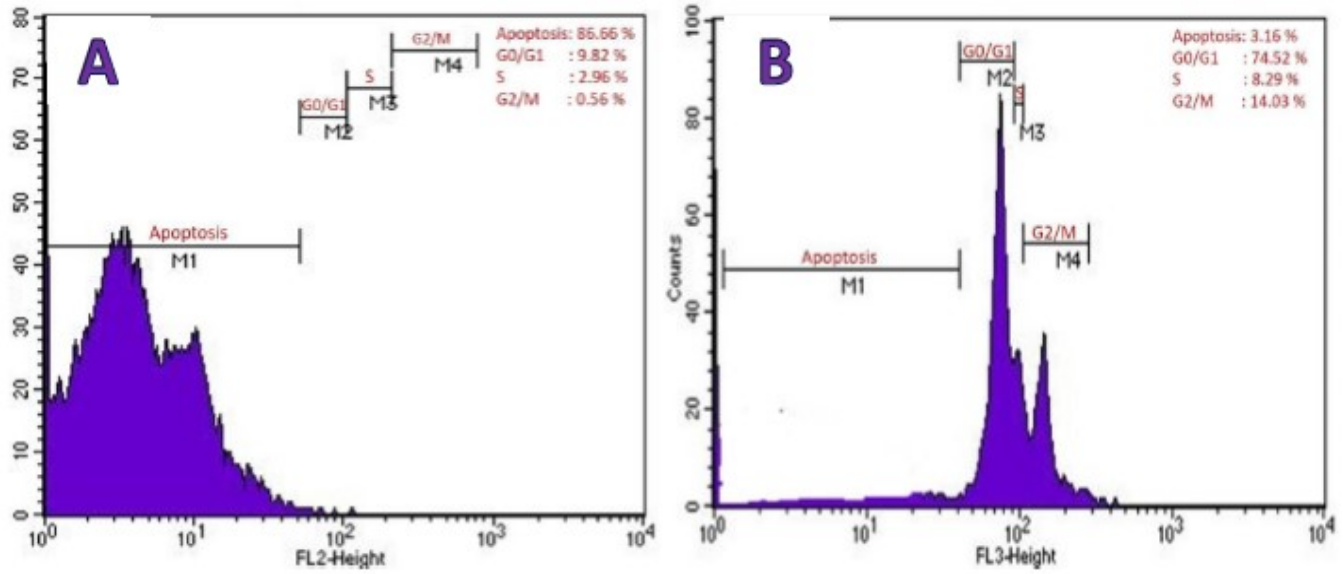

Fig. 7. Population histogram of cell cycle assay. (A) SL-CH-treated HeLa cells showed the maximum (86.66%) accumulation in the M_1 phase which is the apoptotic stage of the cell cycle. (B) The untreated control cells largely accumulated in the G_0/G_1 growth phase.

mangrove plants that has exhibited antimicrobial properties against four bacterial and 3 fungal species (30).

The current work exposes the antioxidant property of the coastal dune grass *S. littoreus* by DPPH free radical scavenging, ABTS, and hydrogen peroxide assays. The plant extract showed strong antioxidant activity on ABTS with an IC_{50} value of 31.74 µg/ml, moderate antioxidant properties on H_2O_2 with an IC_{50} value of 53.93 µg/ml, and weak antioxidant on DPPH assay (139.6 µg/ml). The active chemicals found in plants are the key contributors to their antioxidant activity. The activity of antioxidants is dependent on the redox properties of plant extracts which aid their ability as reducing agents associated with the involvement of reductants to disrupt the chain of free radicals by donating a hydrogen atom or prevent the production of peroxide to produce antioxidant activity (31). The antioxidant activity of *S. littoreus* ethanolic extract was studied through DPPH assay (32). Her results revealed the presence of flavonoids with antioxidant activity. The chloroform extract of *Globularia alypum* showed an IC_{50} value of 69.8 µg/ml in the DPPH assay and 114 µg/ml in the ABTS assay (33). Additionally, it was investigated the antioxidant activity of twelve Yucatan coast plant species. Among 12 tested plants, *Manilkara zapota* showed maximum antioxidant activity (34).

In addition, the MTT assay revealed the cytotoxicity property of the chloroform extract of *S. littoreus.* Treatment with the highest concentration (500 µg/ml) of SL-CH extract reduced the % of viable cells to 44.3%. The quantity of viable cells decreases as sample concentration increases and the IC_{50} value of the chloroform extract of *S. littoreus* was 117.5 µg/ml. The National Cancer Institute and Geran protocol stipulate that extracts with IC_{50} values of 20 g/ml or above are extremely cytotoxic, those between 21 and 200 µg/ml are moderately cytotoxic, those between 201 and 500 µg/ml are weakly cytotoxic and those above 501 µg/ml are not cytotoxic (35, 36). According to this statement, the current investigation showed moderate cytotoxicity to the HeLa cell line. Similar results were recorded in *Aspilia africana* and it showed that the chloroform extract of the leaf was more toxic when compared to ethanolic and aqueous extracts (37). Likewise, the ethanolic extract of *Moringa oleifera* had a weak cytotoxic effect >250 µg/ml as an IC_{50} value against HeLa cell lines (38).

In addition, our study demonstrates the cytotoxic effect of the plant *Spinifex littoreus* in HeLa cell lines by inducing Reactive Oxygen Species. The extremely reactive molecules and radicals known as ROS (O_2, O_2•, H_2O_2, HO•) are continuously produced during aerobic metabolism. However, free radicals present in large concentrations can cause protein and DNA oxidation, protein cross-linking, and cell death. To better understand the mechanisms underlying cell death or disease processes, it is crucial to estimate the levels of ROS in cell culture (39). SL-CH extract induced Reactive Oxygen Species at the IC_{50} concentration of 117.5 µg/ml and the green fluorescent emission was observed as shown in Fig. 5. Likewise, ethanolic extract of the plant *Cremanthodium humile* proved ROS production in HeLa cells after 24 h of incubation (40).

Apoptosis is a highly regulated biological process used to eliminate damaged or abnormal cells in multicellular organisms (41). In this current investigation, the Annexin V and PI stain help to unveil the different stages of HeLa cells after being treated with SL-CH extract. Cells labeled with annexin V look green under fluorescence microscopy which was in the early apoptotic stage (42), PI marks dead cells with red fluorescence and binds strongly to the nucleic acids in the cell, but is impermeable to living and apoptotic cells (43). Similar to this, *Pterocarpus santalinus* methanolic extract inhibits HeLa cell proliferation by inducing apoptosis (44).

In the cell cycle assay of our study, it was found that the majority of HeLa cells treated with SL-CH extract (86.66%) accumulated at the M1 stage, which is the apoptotic stage. It gives an organism the ability to get rid of undesirable and damaged cells through a controlled process of cellular disintegration, which has the benefit of not triggering an unfavorable inflammatory response (45). The majority of the cells in the untreated control cells accumulated in the G0/G1 phase. The G0/G1 phase has been the longest that cells had to go through, which is why cells congregated there (46). It was reported that the *plant Primula vulgaris* can lead to cell cycle arrest and apoptosis in HeLa cells (47).

Conclusion

The chloroform extract of *Spinifex littoreus* exhibits potential antimicrobial properties against *Staphylococcus aureus, Aeromonas hydrophila*, *Streptococcus faecalis*, *Bacteroids fragilis, Escherichia coli*, *Propionibacterium acnes*, *Cryptococcus neoformans, Aspergillus fumigatus, Sporothrix schenckii* and *Aspergillus niger* due to the reserve of the secondary metabolites in the plants. In this study, free radical scavenging results prove the chloroform extract of *S. littoreus* has an antioxidant potential. Free radicals and antioxidants must coexist in equilibrium for essential physiological processes to occur. Therefore, applying antioxidants from an external source can help people cope with disorders brought on by oxidative stress. MTT experiment of SL-CH extract has an antiproliferative effect on human cervical cancer cell lines. In addition, SL-CH extract exhibited good apoptotic effects in the cell cycle and annexin V and PI assays due to the existence of biologically active phytoconstituents. Hence, this study suggests the *Spinifex littoreus* chloroform extract could be a potent therapeutical remedy for providing new insights into biomedical applications.

Acknowledgements

We the authors sincerely give our gratitude to the management of Trichy Institute of Biotechnology Pvt. Ltd. for their support in conducting all these experiments.

Authors' contributions

PA and JV participated in the design of the study; JV carried out all the experiments. JV and AJ drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: All the authors declare that they have no conflicts of interest.

Ethical issues: None

References

1. de Martel C, Georges D, Bray F, Ferlay J, Clifford GM. Global burden of cancer attributable to infections in 2018: A worldwide incidence analysis. The Lancet Global Health. 2020;8(2):180-90. https://doi.org/10.1016/S2214-109X(19)30488-7
2. Mousavi SM, Gouya MM, Ramazani R, Davanlou M, Hajsadeghi N, Seddighi Z. Cancer incidence and mortality in Iran. Annals of Oncology. 2009;20(3):556-63. https://doi.org/10.1093/annonc/mdn642
3. Rafieian-Kopaie M, Nasri H. On the occasion of World Cancer Day 2015; the possibility of cancer prevention or treatment with antioxidants: The ongoing cancer prevention research. International Journal of Preventive Medicine. 2015;6. https://doi.org/10.4103%2F2008-7802.169077
4. Luo M, Zhou L, Huang Z, Li B, Nice EC, Xu J, Huang C. Antioxidant therapy in cancer: Rationale and progress. Antioxidants. 2022;11(6):1128. https://doi.org/10.3390/antiox11061128
5. Newman DJ. Natural products as leads to potential drugs: An old process or the new hope for drug discovery. Journal of Medicinal Chemistry. 2008;51(9):2589-99. https://doi.org/10.1021/jm0704090
6. Gordaliza M. Natural products as leads to anticancer drugs. Clinical and Translational Oncology. 2007;9:767-76. https://doi.org/10.1007/s12094-007-0138-9
7. Gulzar S, Khan MA. Diurnal water relations of inland and coastal halophytic populations from Pakistan. Journal of Arid Environments. 1998;40(3):295-305. https://doi.org/10.1006/jare.1998.0447
8. Chandran M, Vivek P, Kesavan D. Phytochemical screening and anti-bacterial studies in salt marsh plant extracts (*Spinifex littoreus* (Burm. f) Merr. and *Heliotropium curassavicum* L.). Recent Trends in Biotechnology and Chemical Engineering. 2014;6(9):4307-11.
9. Connor HE. Breeding systems in Indomalesian *Spinifex* (Paniceae: Gramineae). Blumea: Biodiversity, Evolution and Biogeography of Plants. 1996;41(2):445-54.
10. Song S, Du J, Wu Q, Ni M, Wang Y, Zhang Y. The responses of *Spinifex littoreus* to sand burial on the coastal area of Pingtan Island, Fujian Province, South China. Ecoscience. 2021 Apr 3;28(2):149-58. https://doi.org/10.1080/11956860.2021.1888523
11. Masuda T, Yonemori S, Oyama Y, Takeda Y, Tanaka T, Andoh T *et al*.. Evaluation of the antioxidant activity of environmental plants: Activity of the leaf extracts from seashore plants. Journal of Agricultural and Food Chemistry. 1999 Apr 19;47(4):1749-54. https://doi.org/10.1021/jf980864s
12. Geetha TS, Geetha N. Phytochemical screening, quantitative analysis of primary and secondary metabolites of *Cymbopogan citratus* (DC) Stapf. leaves from Kodaikanal hills, Tamil Nadu. International Journal of Pharmtech Research. 2014;6(2):521-29.
13. Hufford CD, Funderburk MJ, Morgan JM, Robertson LW. Two antimicrobial alkaloids from the heartwood of *Liriodendron tulipifera* L. Journal of Pharmaceutical Sciences. 1975;64(5):789-92. https://doi.org/10.1002/jps.2600640512
14. Braca A, Sortino C, Politi M, Morelli I, Mendez J. Antioxidant activity of flavonoids from *Licania licaniaeflora*. Journal of Ethnopharmacology. 2002;79(3):379-81. https://doi.org/10.1016/S0378-8741(01)00413-5
15. Arnao MB. Some methodological problems in the determination of antioxidant activity using chromogen radicals: A practical case. Trends in Food Science and Technology. 2000;11(11):419-21. https://doi.org/10.1016/S0924-2244(01)00027-9
16. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989;10(6):1003-08. https://doi.org/10.1093/carcin/10.6.1003
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983;65(1-2):55-63. https://doi.org/10.1016/0022-1759(83)90303-4
18. Nguyen NH, Ta QT, Pham QT, Luong TN, Phung VT, Duong TH, Vo VG. Anticancer activity of novel plant extracts and compounds from *Adenosma bracteosum* (Bonati) in human lung and liver cancer cells. Molecules. 2020;25(12):2912. https://doi.org/10.3390/molecules25122912
19. Choudhari AS, Suryavanshi SA, Kaul-Ghanekar R. The aqueous extract of *Ficus religiosa* induces cell cycle arrest in human cervical cancer cell lines SiHa (HPV-16 positive) and apoptosis in HeLa (HPV-18 positive). PLoS One. 2013;8(7):70127. https://doi.org/10.1371/journal.pone.0070127
20. Darzynkiewicz Z, Bedner E, Smolewski P. Flow cytometry in analysis of cell cycle and apoptosis. InSeminars in Hematology. 2001;38(2):179-93. https://doi.org/10.1016/S0037-1963(01)90051-4
21. Rahman MA, Akhtar J. Evaluation of anticancer activity of *Cordia dichotoma* leaves against a human prostate carcinoma cell line, PC3. Journal of Traditional and Complementary Medicine. 2017;7(3):315-21. https://doi.org/10.1016/j.jtcme.2016.11.002
22. Koç E, Çelik-Uzuner S, Uzuner U, Çakmak R. The detailed comparison of cell death detected by annexin V-PI counterstain using fluorescence microscope, flow cytometry and automated cell counter in mammalian and microalgae cells. Journal of Fluorescence. 2018;28:1393-404. https://doi.org/10.1007/s10895-018-2306-4
23. MacLachlan TK, Sang N, Giordano A. Cyclins, cyclin-dependent kinases and cdk inhibitors: Implications in cell cycle control and cancer. Critical Reviews™ in Eukaryotic Gene Expression. 1995;5(2):127-56. https://doi.org/10.1615/CritRevEukarGeneExpr.v5.i2.20
24. Qasim M, Abideen Z, Adnan MY, Gulzar S, Gul B, Rasheed M, Khan MA. Antioxidant properties, phenolic composition, bioactive compounds and nutritive value of medicinal halophytes commonly used as herbal teas. South African Journal of Botany. 2017;110:240-50. https://doi.org/10.1016/j.sajb.2016.10.005
25. Achakzai AK, Achakzai P, Masood A, Kayani SA, Tareen RB. Response of plant parts and age on the distribution of secondary metabolites on plants found in Quetta. Pak J Bot. 2009;41(5):2129-35.
26. Hussein RA, El-Anssary AA. Plants secondary metabolites: The key drivers of the pharmacological actions of medicinal plants. Herbal Medicine. 2019;1(3):11-26. https://dx.doi.org/10.5772/intechopen.76139
27. Thirunavukkarasu P, Ramanathan T, Ramkumar L, Balasubramanian T. Antimicrobial effect of a coastal sand dune plant of *Spinifex littoreus* (Burm. f.) Merr. Current Research Journal of Biological Sciences. 2010;2(4):283-85.

28. Alwarsamy M, Ravichandran R. *In-vitro* antibacterial activity of *Kappaphycus alvarezii* extracts collected from Mandapam coast, Rameswaram, Tamil Nadu. International Journal of Innovative Research in Science, Engineering and Technology. 2014;3 (1):8436-40.
29. Rebecca LJ, Dhanalakshmi V, Shekhar C. Antibacterial activity of *Sargassum ilicifolium* and *Kappaphycus alvarezii*. Journal of Chemical and Pharmaceutical Research. 2012;4(1):700-05.
30. Khajure PV, Rathod JL. Antimicrobial activity of extracts of *Acanthus ilicifolius* extracted from the mangroves of Karwar coast Karnataka. Recent Research in Science and Technology. 2010;2(6).
31. Taghzouti OK, Balouirib M, Ouedrhiric W, Chahadd AE, Romanea A. *In vitro* evaluation of the antioxidant and antimicrobial effects of *Globularia alypum* L. extracts. J Mater Environ Sci. 2016;7:1988-95.
32. Shakila K. Bioactive flavonids of *Spinifex littoreus* (Burm. f.) Merr. Investigation by Elisa method. Journal of Stress Physiology and Biochemistry. 2020;16(4):107-12.
33. Asraoui F, Kounnoun A, Cadi HE, Cacciola F, Majdoub YO, Alibrando F *et al*. Phytochemical investigation and antioxidant activity of *Globularia alypum* L. Molecules. 2021;26(3):759. https://doi.org/10.3390/molecules26030759
34. Rodríguez-García CM, Ruiz-Ruiz JC, Peraza-Echeverría L, Peraza-Sánchez SR, Torres-Tapia LW, Pérez-Brito D *et al*. Antioxidant, antihypertensive, anti-hyperglycemic and antimicrobial activity of aqueous extracts from twelve native plants of the Yucatan coast. PLoS One. 2019;14(3):e0213493. https://doi.org/10.1371/journal.pone.0213493
35. Grever MR, Schepartz SA, Chabner BA. The National Cancer Institute: Cancer drug discovery and development program. InSeminars in Oncology. 1992;19(6):622-38. https://www.altmetric.com/details/45942726
36. Goldin A, Venditti JM, Macdonald JS, Muggia FM, Henney JE, Devita Jr VT. Current results of the screening program at the Division of Cancer Treatment, National Cancer Institute. European Journal of Cancer (1965). 1981;17(2):129-42. https://doi.org/10.1016/0014-2964(81)90027-X
37. Ala AA, Olotu BB, Ohia CM. Assessment of cytotoxicity of leaf extracts of *Andrographis paniculata* and *Aspilia africana* on murine cells *in vitro*. Archives of Basic and Applied Medicine. 2018;6(1):61.
38. Hermawan A, Nur KA, Dewi D, Putri P, Meiyanto E. Ethanolic extract of *Moringa oleifera* increased cytotoxic effect of doxorubicin on HeLa cancer cells. Journal of Natural remedies. 2012;12(2):108-14.
39. Md Yusof EN, M Latif MA, M Tahir MI, Sakoff JA, Simone MI, Page AJ *et al*. o-Vanillin derived Schiff bases and their organotin (IV) compounds: Synthesis, structural characterisation, *in-silico* studies and cytotoxicity. International Journal of Molecular Sciences. 2019;20(4):854. https://doi.org/10.3390/ijms20040854
40. Li H, Wang LJ, Qiu GF, Yu JQ, Liang SC, Hu XM. Apoptosis of HeLa cells induced by extract from *Cremanthodium humile*. Food and Chemical Toxicology. 2007;45(10):2040-46. https://doi.org/10.1016/j.fct.2007.05.001
41. Yi JL, Shi S, Shen YL, Wang L, Chen HY, Zhu J, Ding Y. Myricetin and methyl eugenol combination enhances the anticancer activity, cell cycle arrest and apoptosis induction of cis-platin against HeLa cervical cancer cell lines. International Journal of Clinical and Experimental Pathology. 2015;8(2):1116.
42. Atale N, Gupta S, Yadav UC, Rani V. Cell-death assessment by fluorescent and nonfluorescent cytosolic and nuclear staining techniques. Journal of Microscopy. 2014;255(1):7-19. https://doi.org/10.1111/jmi.12133
43. Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death. Journal of Visualized Experiments. 2011;24(50):2597. https://doi.org/10.3791/2597-v
44. Kwon HJ, Hong YK, Kim KH, Han CH, Cho SH, Choi JS, Kim BW. Methanolic extract of *Pterocarpus santalinus* induces apoptosis in HeLa cells. Journal of Ethnopharmacology. 2006;105(1-2):229-34. https://doi.org/10.1016/j.jep.2005.10.025
45. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell. 1997;88(3):347-54. https://doi.org/10.1016/S0092-8674(00)81873-5
46. Kumar RR, Ramesh R, Małecki JG. Synthesis and structure of arene ruthenium (II) benzhydrazone complexes: Antiproliferative activity, apoptosis induction and cell cycle analysis. Journal of Organometallic Chemistry. 2018;862:95-104. https://doi.org/10.1016/j.jorganchem.2018.03.013
47. Demir S, Turan I, Aliyazicioglu R, Yaman SO, Aliyazicioglu Y. *Primula vulgaris* extract induces cell cycle arrest and apoptosis in human cervix cancer cells. Journal of Pharmaceutical Analysis. 2018;8(5):307-11. https://doi.org/10.1016/j.jpha.2018.05.003