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IN VITRO SCREENING OF SYRINGODIUM ISOETIFOLIUM AGAINST HUMAN BREAST CARCINOMA UTILIZING CELL VIABILITY

V. Sundara Prabha[1*], J. L. Lekshmi[2]

[1*]Assistant Professor, Department of Botany, S. T. Hindu College, Nagercoil-02 Affiliated to Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu. Email: prabhavasu90@gmail.com; Orcid Id: 0009-0007-5601-3301
[2]Assistant Professor, Department of Botany, S. T. Hindu College, Nagercoil-02 Affiliated to Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu. Email: mathulekshmi@gmail.com
***Corresponding author**: V. Sundara Prabha, e-mail: prabhavasu90@gmail.com

ABSTRACT
This study evaluated the cytotoxic profile of Syringodium isoetifolium ethanolic extracts against the MDA-MB-231 human breast cancer cell line, underscoring the therapeutic potential of marine seagrasses in oncology. A series of MTT assays evaluated cell viability across various concentrations, revealing a notable decrease in cell viability with an IC50 of 54.55 µg/ml, indicating significant cytotoxicity at lower concentrations. Additionally, the impact of the extracts on Mitochondrial Membrane Potential was assessed, where the early and late apoptotic responses were characterized by alterations in membrane integrity. These results show that Syringodium isoetifolium has promising anticancer properties, highlighting the potential of marine plants in cancer drug discovery.

KEYWORDS: MDA-MB-231, Syringodium isoetifolium, cancer. s

INTRODUCTION
Seagrasses are an important habitat linked to the productivity of our abundant fisheries (Ogden et al., 1977). Approximately 40 percent of all primary energy production or photosynthesis, occurs in the seas. In the process, oceanic plants (phytoplankton, seaweed and seagrasses) take up carbon dioxide and convert it into organic carbon (primarily sugars) and oxygen using light from the sun as an energy source. The Gulf of Mannar, Palk Bay, Andaman and Nicobar Islands and Lakshadweep Islands are known for their seagrass resources. Seagrasses take up nutrients from the sediments, transporting them through the plant and releasing them into the water column through the leaves. The environment of seagrasses is highly saline, relatively anoxic, and frequently disturbed by waves and tides, seagrasses have evolved unique morphological structures unknown in terrestrial monocotyledons or freshwater submerged monocotyledons (Kuo et al., 2018)
Syringodium isoetifolium leaves are tubular, narrowed at the base and pointed at the apex. They are herbaceous plants; rhizomes creeping; shoots erect, branched, bearing 2-3 leaves; rhizomes and shoots have scars; rhizomes produce branched roots at each node. It generally grows well on coral flats, but also grows on sandy to muddy bottoms up to 15 m depth. It is not seen in backwaters and estuaries.
Syringodium isoetifolium has leaves that are circular in cross-section and taper to a smooth pointed tip (Waycott et al., 2004). It is widely distributed across tropical and subtropical coastal marine environments in South Africa, Indo-Pacific, and Australia (Kuo & den Hartog, 2001). Medicinal plant-derived products are used either as such or as templates in the discovery of new drugs (Fakim, 2006 & Oliveira et al., 2009). Plants are continuously serving as possible sources for new therapeutic agents. Several biologically active compounds such as carbohydrates, proteins, enzymes, fats, oils, minerals, vitamins, alkaloids, terpenoids, quinones, flavonoids, carotenoids, sterols, tannins, simple phenolic glycosides, saponins, and polyphenols have been used for their medicinal values. Metabolomics is used to identify and quantify the metabolites present in biological samples using targeted and non-targeted approaches.
Cancer is a global health threat and a leading cause of death worldwide, driven by failed cell cycle regulation that triggers unchecked cell division (Rowbotham and Kim, 2017). Developing effective treatments remains highly challenging due to the multifactorial nature of carcinogenesis. Because traditional therapies face these hurdles, there is escalating interest in medicinal plants as natural sources, which currently supply 60% of active anticancer agents (Newman et al., 2003). Researchers heavily rely on cancer cell lines as models to perform the initial screening of these novel plant-derived compounds (Svejda et al., 2010 & Erel et al., 2014). To establish the safety and preliminary efficacy of any new drug candidate, scientists prioritize cell viability and oxidative stress tests.
Mitochondria are unique organelles of eukaryotic cells with their own independent genetic system. Because they provide energy for cellular life activities, they are known as the “power house”. The plant mt genome encodes genes related to oxidative phosphorylation and respiratory metabolism, including cytochrome complex I-V subunit genes, cytochrome C synthesis-related genes, ribosomal protein-related genes, tRNAs, rRNAs, and some open reading frames of unknown function (Millar et al.,2011).

The MTT assay evaluates cytotoxicity by leveraging cellular oxidoreductase enzymes (Berridge et al., 2005). Active, healthy cells reduce yellow tetrazolium dye into a purple formazan product dissolved in DMSO. Measuring light absorbance at 540 nm provides a direct, linear correlation to the number of viable cells.

MATERIALS AND METHODS

The botanical aspects of each species can differentiate it from other species of seagrasses. Their habitats are normally seagrass meadows that survive a range of environmental conditions in coastal bays, reefs, estuarine and deepwater habitats. Changes in the light climatic conditions and light attenuation may lead to fluctuations in photosynthetic performance (Campbell et al., 2007)

Among all the seagrasses, one of the major tropical seagrasses is Syringodium isoetifolium. Syringodium belongs to the family Cymodoceaceae. It was described as a genus in 1860. Syringodium isoetifolium is otherwise called Noodle grass. It can be found in tropical and subtropical marine environments. It is rarely found in shallow intertidal areas. It appears to respond rapidly to increased nutrient availability. In Tamil Nadu, S.isoetifolium is commonly called Neer pasi, Oosi korai, or Nool pasi. Long spaghetti-like leaves can be seen in S.isoetifolium, and they can absorb nutrients and gases through thin cuticles since they lack stomata.

MTT Assay for Cell Cytotoxicity

The MDA-MB-231 (Human breast cancer cells) cell line was cultured in liquid medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin, and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO2 at 37ºC. The ethanolic extracts of the sample were tested for in vitro cytotoxicity using MDA MB-231 cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5- 2,5- diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured MDA-MB-231 cells were harvested by trypsinization and pooled in a 15 mL tube. Then, the cells were plated at a density of 1×105 cells/ml cells/well (200 µL) into a 96-well tissue culture plate in a DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hours at 37°C. 79 The wells were washed with sterile PBS and treated with various concentrations of the Sorafenib tosylate sample in a serum-free DMEM medium. Each sample was replicated three times, and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (20 µL of 5.0 mg/ml) was added into each well and the cells were incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) was aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value were calculated using GraphPad Prism 6.0 software (USA).

Measurement of Mitochondrial Membrane Potential (MMP)

MCF-7 cells (Human breast cancer cell line) were purchased from NCCS, Pune and were cultured in liquid medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO2 at 37ºC. 81 Measurement of mitochondrial membrane potential: The MDA-MB-231 cells (20,000–50,000 cells/well) were plated to a 24-well plate and incubated for 24 hr in a DMEM growth medium. After incubation, the plate was washed with PBS and with 47.89 µg/ml of samples in a serum-free DMEM medium. Again, the plate was incubated at 37°C in a humidified 5% CO2 incubator for 24 hrs. The measurement of mitochondrial membrane potential for the treated and control cells was carried out according to the manufacturer's instructions. Briefly, the cells were incubated with 100 µl/well of JC-10 dye loading solution and the plate was protected from light. The plate was incubated for 30 – 60 minutes in 5% CO2 at 37°C. After incubation, 100 µl/well assay buffer B was added to each sample/well. Finally, the plate was centrifuged at 800 rpm for 2 minutes and the fluorescence was observed using the Fluorescent Cell Imaging System (Bio-Rad, USA).

RESULTS AND DISCUSSION

The cytotoxic activities of the ethanolic extracts of the selected seagrasses at various concentrations were determined against MDA MB 231 cells. The results showed an increase in absorbance with decreasing extract concentration for Syringodium isoetifolium, with values ranging from 0.000 to 0.600. The absorbance value was highest at the control (0.596), followed by the lowest sample concentration (10 µg/ml) at 0.585, and lowest at the highest concentration (500 µg/ml) at 0.078. The cell viability percentage was calculated using the standard formula along with the mean value percentage. The cell viability percentages were analyzed for both the control and samples treated with various concentrations of plant extracts. For the cells treated with the extracts, the value was found to be 100% for the control, next to the control it was more in 10µg/ml concentration with 94 % and the lowest mean value (%) was found at the 500 µg/ml concentration with 13%. The IC50 value of Syringodium isoetifolium was found to be 54.55 µg/ml, which was calculated using GraphPad Prism 6.0 Software. Results of the MTT assay were presented in Figure 1, which shows the presence of viability values against MDA MB 231 cells, which were incubated with the samples for 24 hrs.

Thaniya et al. (2022) intended to evaluate the cytotoxicity of black rice flour against colon cancer cell lines and mouse embryo cell lines using an MTT assay and the IC50 value was found to indicate significant cytotoxic properties. MTT assay was performed using Granulocystopsis sps, a microalgae extract to assess cell viability in the lung cancer line and the normal cell line and their IC50 values were observed to be 177 (Tavares- Carreon et al., 2020). The synthesized novel Phenanthridine was analyzed for its cytotoxic activity against five selected cell lines via MTT assay, where the IC50 value was calculated as 0.28 µM (Wan et al., 2019). Not only plant extracts, but even Platinum nanoparticles also exerted cytotoxic effects on the cell lines (Bendale et al., 2017).

Table 1. Results of the MTT assay for ethanolic extract of Syringodium isoetifolium

S. No	Tested sample concentration (µg/ml)	OD Value at 570 nm (in triplicates)			Mean value (%)
1.	Control	0.596	0.575	0.565	100
2.	500 µg/ml	0.064	0.094	0.078	13.630464
3.	400 µg/ml	0.116	0.125	0.122	20.931713
4.	300 µg/ml	0.157	0.128	0.146	24.81462
5.	200µg/ml	0.149	0.140	0.155	25.593818
6.	100 µg/ml	0.290	0.218	0.221	41.895269
7.	80 µg/ml	0.231	0.245	0.249	41.812627
8.	60 µg/ml	0.283	0.272	0.266	47.289072
9.	40 µg/ml	0.317	0.308	0.293	52.870515
10.	20 µg/ml	0.539	0.526	0.495	89.841707
11.	10 µg/ml	0.585	0.531	0.522	94.29719

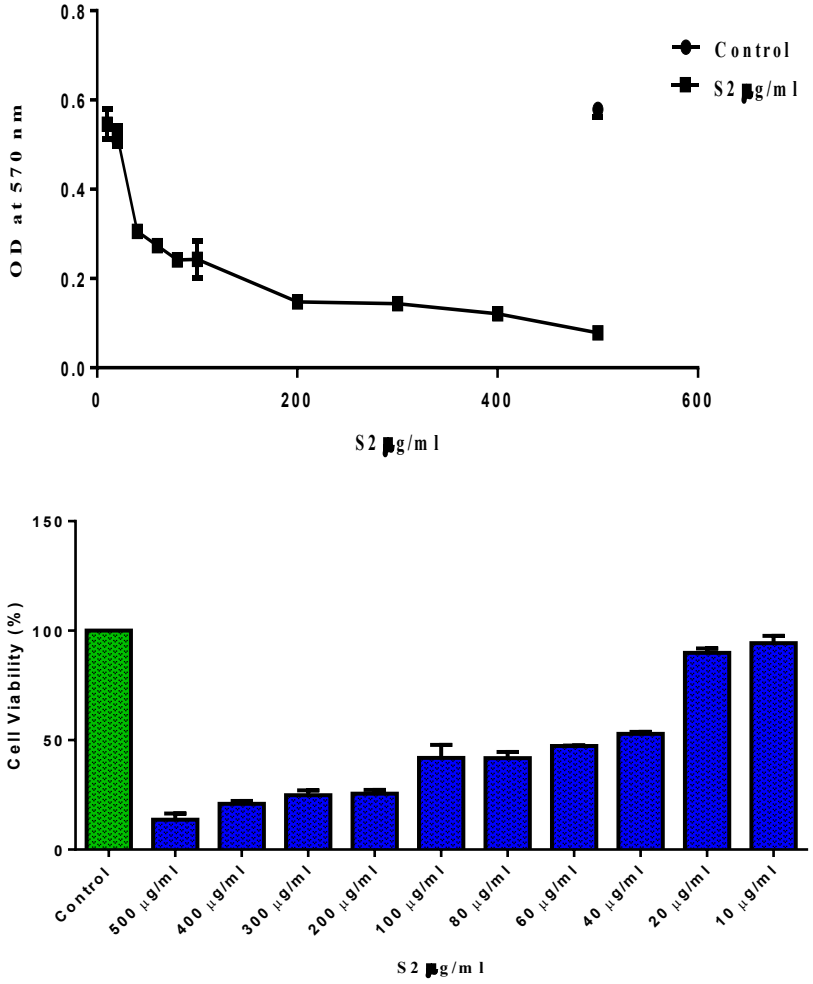

Figure 1. Graph showing the results of the MTT assay for ethanolic extract of Syringodium isoetifolium

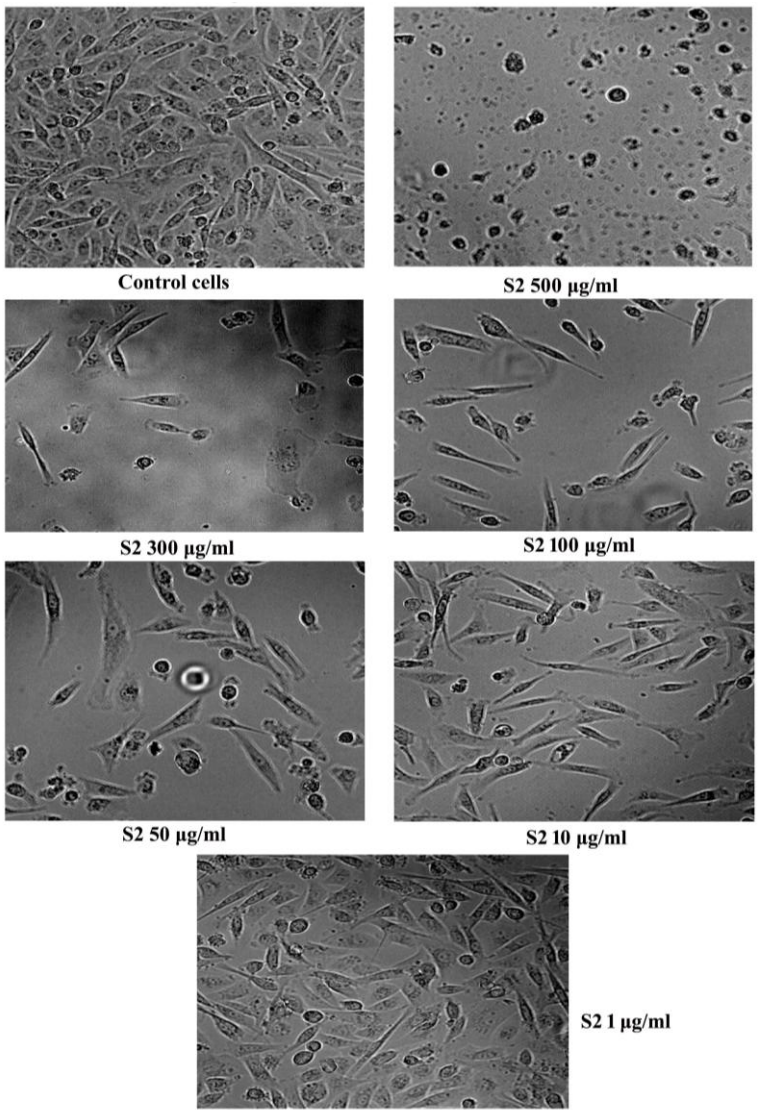

Figure 2. Images of control cells and Syringodium isoetifolium extract-treated cells

Measurement of Mitochondrial Membrane Potential

The MDA-MB 231 cells were used to measure the impact of the ethanolic extract of the experimental plants. The standard protocol was followed to measure the mitochondrial membrane potential. The cells untreated with the plant extract showed intact membranes, as it was evident from the orange light emitted from the cells. Orange light shows that the mitochondria have a high degree of polarization and aggregation of the dye J-10 in such mitochondria.

However, in the cancer cells treated with the plant extracts, greenish-orange or reddish colour was observed, showing that the cells were undergoing early or late apoptosis. Anti-cancer drugs induce multiple mechanisms of response from the cancer cells and most of them show apoptosis as a strategy of preventing cell proliferation. In our experimental study also there is also clear evidence that membrane integrity is broken, which leads to the failure to build the membrane potential across the membrane. Results of Mitochondrial Membrane Potential are shown in Figure 3.

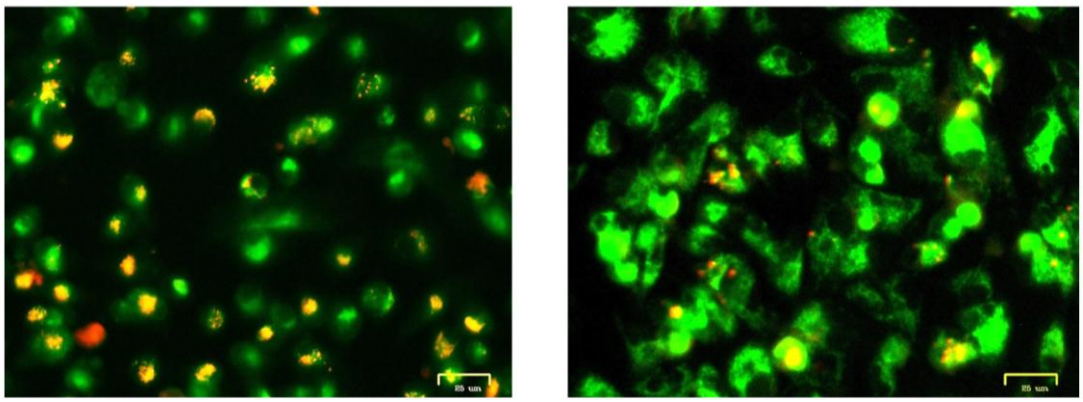

Figure 3. Results of Mitochondrial Membrane Potential with Control and treated Cells for Syringodium isoetifolium

When the cancer cells keep their mitochondrial membrane intact, the membrane potential will be built normally, and the cells will proliferate in an uncontrolled manner. Cytofoxicity induction leads to membrane breakdown and vital enzymes are leached out of mitochondria, that fails in energy production. In our experiment, there is a clear indication that the ethanolic extracts of both plants have induced membrane breakdown that leads to cytotoxicity

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