

# Cytotoxic and Apoptotic Effects of *Cymodocea Serrulata* Extracts on MDA-MB-231 Breast Cancer Cells

V. Sundara Prabha<sup>1\*</sup>

<sup>1\*</sup>Assistant Professor, Department of Botany, S. T. Hindu College, Nagercoil-02 Affiliated to Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu.

---

## Abstract

*Cymodocea serrulata* is a seagrass that grows in the intertidal region of marine areas. It was analyzed for its efficiency as a source of phytochemical compounds as the potential medicines in the future. The ethanolic extracts of the samples were tested for in vitro cytotoxicity using MDA MB-231 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-2,5-diphenyltetrazolium bromide (MTT) assay, Mitochondrial Membrane Potential assay, DNA ladder assay, and Ethidium Bromide/Acridine Orange assay. The absorbance increased with a decrease in the concentration of the selected samples. The DNA ladder assay showed digestion of genomic DNA when cancer cells were treated with plant extracts. Human cancer cell lines incubated with plant extracts exhibited apoptotic DNA fragmentation profiles. This result clearly shows that it is effective in inducing cytotoxicity by interacting with nuclear DNA

**Keywords:** Cytotoxicity, MB-231 cells. DNA ladder and cancer

---

## INTRODUCTION

Seagrasses are one of the most widespread coastal vegetation types, which have developed unique physiological, morphological and ecological adaptations to grow and thrive in submerged marine conditions and provide essential ecological functions for the coastal and marine environment (Mtwana et al., 2016). Coastal marine ecosystems may provide shoreline protection (Spalding et al., 2014), maintain healthy and rich fish populations utilized by fisheries (Pauly and Zeller 2014), and provide opportunities for recreational activities (Brander et al . 2007). In India, the total seagrass cover is estimated to be 517 km<sup>2</sup> (Geevarghese et al ., 2017) with 14 reported species.

*Cymodocea serrulata* is a seagrass that grows on muddy sand, fine sand or sand with coral rubble substrates in the intertidal zone (Short et al ., 2010). *Cymodocea serrulata* has separate male and female plants. It has a creeping rhizome with scales and scale scars; shoots erect, with 2-6 leaves, and shoots covered by old decayed leaves.

Cancer remains a leading cause of death worldwide, with approximately 17.5 million new cases and 8.7 million cancer-related deaths in 2015 (Fitzmaurice et al., 2017). Cell cycle regulatory proteins control the entire process of cell proliferation to maintain the correct shape and size in animals. It has been found that failure in the regulatory mechanisms may lead a cell to divide for an indefinite period and finally lead to developing into cancer (Rowbotham and Kim, 2017).

In recent times, its treatment is among the most challenging task due to the involvement of several factors during carcinogenesis. Medicinal plants are playing an important role as a source of effective anticancer agents and it is significant that 60% of currently used anticancer agents are derived from natural sources including plants (Newman et al ., 2003). There is a growing interest in the pharmacological evaluation of various plants used in the Indian traditional system of medicine. To study new therapeutic approaches, cell lines are used to investigate novel compounds and their effects on tumour cells. Cell lines are used as cancer models to investigate in vivo anti-proliferative activities of novel compounds (Svejda et al, 2010)

Apoptosis is an active process which is a type of programmed cell death. It deals with a normal component of multicellular organisms' development and growth. The study of apoptosis is an important field of biological inquiry since a deficiency or an excess of apoptosis is one of the causes of cancers, autoimmune disorders, diabetes, Alzheimer's, organ and bone marrow transplant rejection, and many other diseases. Among the in vitro methods available to detect apoptosis (Frankfurt, 2004), dual Acridine orange/Ethidium bromide (AO/EB) fluorescent staining, visualized under a fluorescent microscope, has been used to identify apoptosis-associated changes in cell membranes during the process of apoptosis (Gherghi et al., 2003). They are used to differentiate cells in different stages of apoptosis. Other parameters include MTT, MMP and DNA fragmentation assay

## MATERIALS AND METHODS

*Cymodocea serrulata* was analyzed for their efficiency to serve as the source of phytochemical compounds as potential medicines in the future. *Cymodocea serrulata* was obtained from the Gulf of Mannar. Live

and healthy seagrass samples were collected during the low tide period. The samples were washed in seawater to remove epiphytes and extraneous matter. They were cleaned with fresh water to remove sand and salt to avoid the pumping of solvent during the extraction process. After draining off the water a known amount was taken for the preparation of the extract and the rest was pressed with blotting paper and shade dried at room temperature and used for dry extract preparation. The plant material was identified and authenticated by Dr. C. Murugan, Scientist-D, Botanical Survey of India, Southern Regional Centre, Coimbatore

#### **MTT Assay for Cell Cytotoxicity**

Cell culture 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% CO<sub>2</sub> at 37°C. The ethanolic extracts of the sample were tested for in vitro cytotoxicity, using MDA MB-231 cells by 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 1×10<sup>5</sup> 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. 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% CO<sub>2</sub> 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 IC<sub>50</sub> value were calculated using GraphPad Prism 6.0 software (USA).

#### **Measurement of Mitochondrial Membrane Potential (MMP)**

Cell culture MCF-7 cells (Human breast cancer cells) cell line was 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% CO<sub>2</sub> at 37°C. Measurement of mitochondrial membrane potential: The MDA-MBA-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% CO<sub>2</sub> 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 a 5% CO<sub>2</sub> 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).

#### **DNA Fragmentation Assay**

ctDNA from the treated cells was harvested by centrifugation for 5 minutes (3500 rpm). 875 µl of TE buffer was added to the cell pellet and cells were resuspended in the buffer by gentle mixing. 100 µl of 10% SDS and 5µl of Proteinase K were added to the cells. The above mixture was mixed well and incubated at 37°C for an hour in an incubator. 1ml of the phenol-chloroform mixture was added to the contents, mixed well by inverting and the samples were incubated at room temperature for 5 minutes. The contents were centrifuged at 10000 RPM for 10 minutes at 4°C. The highly viscous jelly-like supernatant was collected using cut tips and was transferred to a fresh tube. The process was repeated once again with a phenol-chloroform mixture and the supernatant was collected in a fresh tube. 100 µl of 5M sodium acetate was added to the contents and mixed gently. 2.0 ml of isopropanol was added and mixed gently by inversion till white strands of DNA precipitated out. The contents were centrifuged at 5000 RPM for 10 minutes. The supernatant was removed and 1ml of 70% ethanol was added. The above contents were centrifuged at 5000 RPM for 10 minutes. After air drying for 5 minutes, 200 µl of TE buffer or distilled water was added. Then an equal amount of each DNA sample was run at 0.8 % agarose gel electrophoresis along with a 1kb ladder. The image was captured by a gel documentation system.

## EtBr /AO STAINING

MDA-MB 231 (human breast carcinoma) cell line was purchased from NCCS, Pune and 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% CO<sub>2</sub> at 37 °C. EtBr/AO staining: Briefly,  $5 \times 10^5$  cells/ml of MDA MB 231 cells were plated into a 96-well tissue culture plate and incubated for 24 hr in a DMEM growth medium. After incubation, the cells were treated with 86.41 µg/ml of the sample in a serum-free DMEM medium. The plate was incubated at 37 °C in 5% CO<sub>2</sub> incubator for 24 hours. After incubation, 10 µl of 1 mg/ml acridine orange and ethidium bromide were added to the wells and mixed gently. Finally, the plate was centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour and at least 100 cells by a fluorescence microscope using a fluorescent filter.

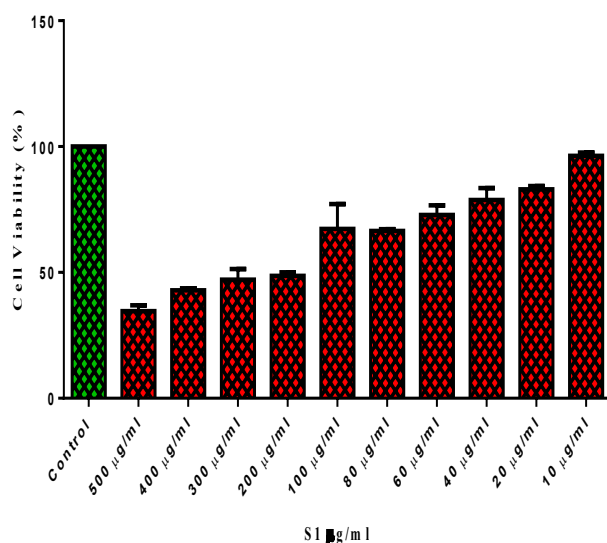
## RESULTS AND DISCUSSION

### MTT assay

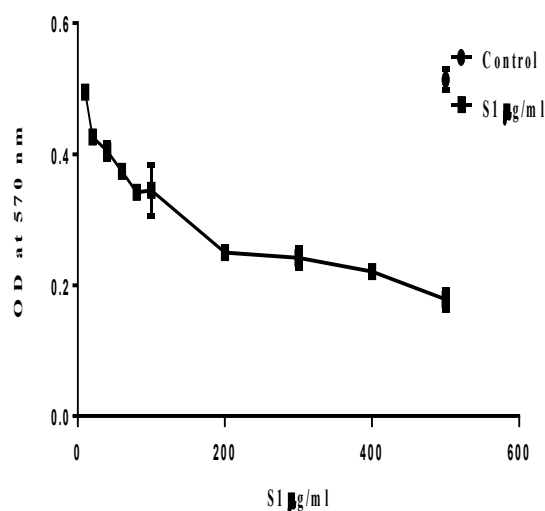
The cytotoxic activities of the ethanolic extracts of the selected seagrasses at various concentrations were determined against MDA MB 231 cells. The results showed an increase in the absorbance value with a decrease in the concentration of the plant extracts. The value ranges between 0.000 – 0.600 for both samples. The absorbance value was found to be the highest at the lowest sample concentration (10 µg/ml), with a value of 0.502, which was next to the control with 0.525. The least value was observed in the sample with the highest concentration of 500 µg/ml, with a value of 0.176. The cell viability percentage was analyzed for both the control and samples treated with various concentrations of plant extracts. The mean value of cell viability was found to be 100% for the control, next to the control, it was more in 10µg/ml concentration with a viability percentage of 95 % and the minimum mean value of viability was found in 500 µg/ml concentration which was about 33%.

The IC<sub>50</sub> value of *Cymodocea serrulate* was found to be 86.41 µg/ml, which was calculated using GraphPad Prism 6.0 Software. Results of the MTT assay were presented in Figure, 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, whose IC<sub>50</sub> value was found to be with significant cytotoxic properties.

MTT assay was performed using *Granulocystopsis* sps, microalgae extract to assess the cell viability in the lung cancer line and the normal cell line and their IC<sub>50</sub> 177 values were observed (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 IC<sub>50</sub> 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).



Mean value percentage of *Cymodocea serrulate* (S1)



Graph showing the results of the MTT assay for *Cymodocea serrulate* (S1)

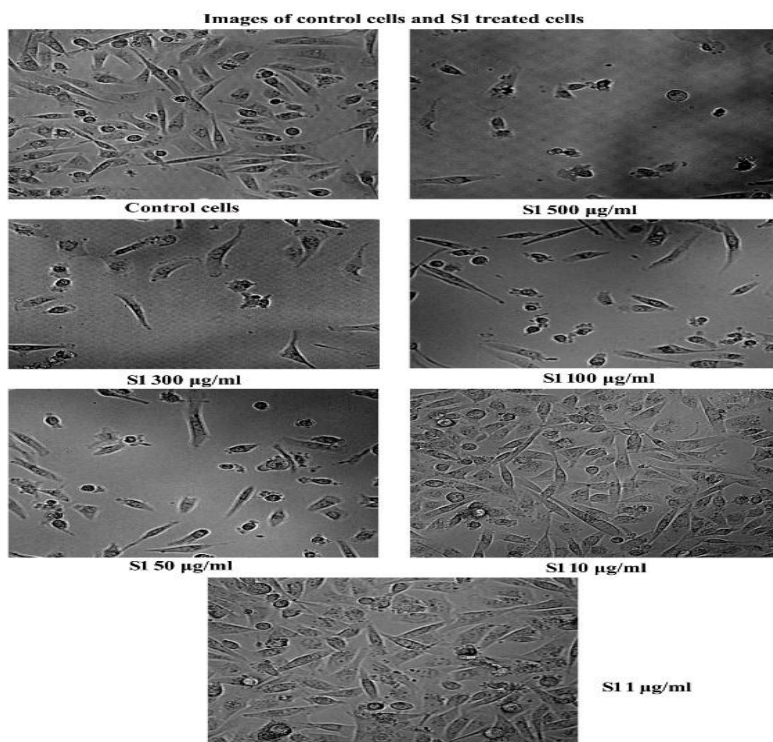


Figure 1. Results of 3-(4,5-dimethylthiazol-2-yl)-2,5-2,5-diphenyltetrazolium bromide (MTT) assay

#### Measurement of Mitochondrial Membrane Potential (MMP)

The MDA-MB 231 cells were used to measure the impact of the ethanolic extract of the experimental plants on cytotoxicity. 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 the strategy of preventing cell proliferation. In our experimental study also there is clear evidence that the membrane integrity is broken which leads to the failure of building the membrane potential across the membrane. When the cancer cells keep their mitochondrial membrane intact, the membrane potential will be built usual and the cells will proliferate in an uncontrolled manner. Cytotoxicity induction leads to the membrane breakdown and the vital enzymes will leached out of mitochondria that results in the failure of energy production. In the experiment, there is a clear indication that the ethanolic extracts of the plant have induced membrane breakdown that leads to cytotoxicity.

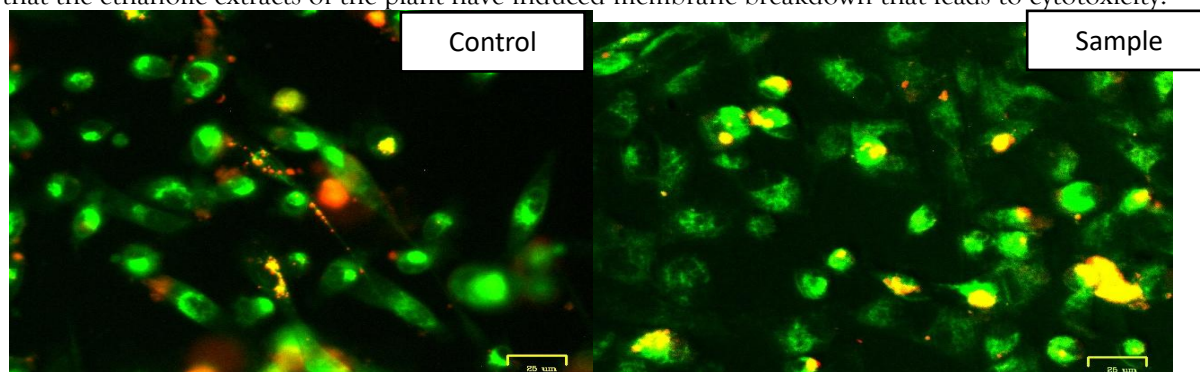


Figure 2. Results of Mitochondrial Membrane Potential with control and treated cells for *Cymodocea serrulate*

#### DNA ladder assay

Fragmentation of DNA by a specific nuclease called Caspase-activated DNase (CAD) is a distinctive biochemical feature of apoptosis. Activation of CAD is activated by the caspase cascade, which leads to specific cleavage of DNA at internucleosomal linker sites, generating fragments of ~200 base pairs known

as DNA ladders. Digestion of genomic DNA can be detected by using the classical method of 152 Agarose gel electrophoresis. This semi-quantitative method is a simple yet robust technique that provides a clear answer. In our experiments, the DNA ladder assay showed the digestion of genomic DNA when the cancer cells were treated with the plant extracts showing promising results. It was found that the human cancer cell lines incubated with the ethanolic extracts of the experimental plants showed apoptotic DNA fragmentation profiles. Nucleic acid fragmentation was not observed in untreated cells. Such a result has been reported with the four human cancer cell lines incubated with *T. peruviana* extract, which showed apoptotic DNA fragmentation profiles (Ramos Silva et al, 2017). Ethanolic extract of *Trachyspermum ammi* induced significant internucleosomal DNA fragmentation in the MCF-7 cell line as demonstrated by Ramya et al. (2017).

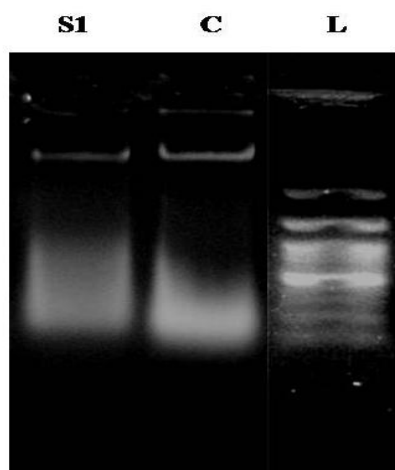


Figure 3. DNA fragmentation assay for *Cymodocea serrulate*

#### EtBr/AO staining assay

In control the percentage of early apoptotic cells was found to be just 1.5 and no late apoptotic and necrotic cells were observed. In the cells treated with *Cymodocea serrulata*, 51.5% of the cells were found to be in the early apoptotic stage and 45% was in late apoptotic condition and 3.5 % were necrotic.

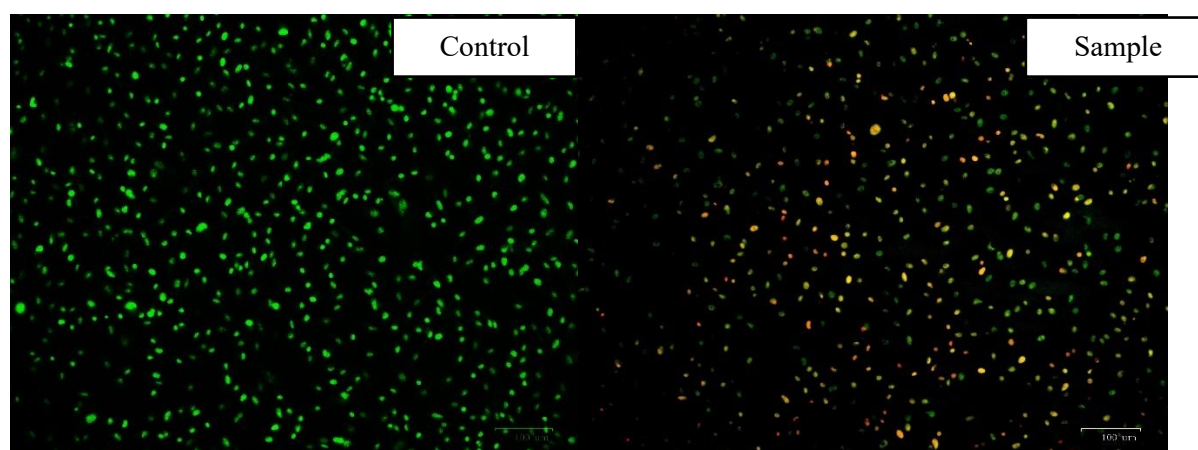


Figure 4. Results of Et Br/Ao staining assay for *Cymodocea serrulata*

The primary mechanism by which chemical compounds destroy the tumour cells is by inducing apoptosis. When the chemotherapeutic sensitivity of the cancer cells is more, high levels of apoptosis would be the result. Therefore, the main purpose of DSTs is to detect apoptosis. Even though multiple methods, such as PI, in situ nick translation, thermal denaturation, terminal deoxynucleotidyl transferase, acidic denaturation, and other assays have been developed and used, most of them can detect cell death rather than apoptosis (Lecoœur, 2002). Other drawbacks of these methods include the laborious protocols involved (Ribble et al ., 2005).

#### SUMMARY AND CONCLUSION

Coastal marine ecosystems are of great importance, not only from an ecological perspective but also in terms of their social, economic and medicinal value, by providing beneficial services for human

populations. Marine natural products have attracted the attention of scientists due to their precious and promising compounds related to the fight against several diseases. The results of -3-[4,5-dimethylthiazol-yl]-2, 5-diphenyl tetrazolium bromide assay show the absorbance value increases with decreases in the concentration of the selected samples *Cymodocea serrulata*, which showed the presence of viability values against MDA MB 231 Cells, which were incubated with the samples for 24 hrs. In the experimental study of mitochondrial membrane potential, results also have clear evidence that the membrane integrity is broken, which leads to the failure of building the membrane potential across the membrane. The DNA ladder assay showed the digestion of genomic DNA when the cancer cells were treated with the plant extracts. In the cells treated with *Cymodocea serrulata*, the cells were found to be in the early apoptotic stage and late apoptotic condition and necrotic cells were minimal. This result clearly shows the effect of the ethanolic extract of the plants in inducing cytotoxicity by interacting with the nuclear DNA.

## REFERENCE

1. Bendale, Y, Bendale, V & Paul, S 2017, „Evaluation of cytotoxic activity of platinum nanoparticles against normal and cancer cells and its anticancer potential through induction of apoptosis“, *Integrative Medicine Research*, vol. 6, vol. 2, pp.141-148
2. Brander LM, Van Beukering P & Cesar, HSJ 2007, ‘The recreational value of coral reefs: A meta-analysis’, *Ecol Econ*, vol. 63, pp.209-218
3. Fitzmaurice, C, Allen, C, Barber, RM, Barregard, L, Bhutta, ZA, Brenner, H, Dicker, DJ, Chimed-Orchir, O, Dandona, R, Dandona, L & Fleming T 2017, ‘Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: a systematic analysis for the global burden of disease study’, *JAMA Oncology* vol. 3, no. 4, pp. 524-48
4. Frankfurt, OS 2004, ‘Immunoassay for single-stranded DNA in apoptotic cells’, *Methods Mol Biol*, vol. 282, pp.85-101.
5. Geevarghese, GA, Akhil, B, Magesh, G, Krishnan, P, Purvaja, R & Ramesh, R 2017, ‘A comprehensive geospatial assessment of seagrass distribution in India. *Ocean Coast. Manag*, pp. 1-10
6. Gherghi, IC, Girousi, ST & Voulgaropoulos, A 2003, ‘Study of interactions between DNA-ethidium bromide (EB) and DNA-acridine orange (AO), in solution, using hanging mercury drop electrode (HMDE)’, *Talanta*, vol. 61, pp. 103-12
7. Lecoeur, H 2002, „Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases“, *Exp Cell Res.*, vol. 277, pp. 1-14.
8. Newman, DJ, Cragg, GM & Snader, KM 2003, ‘Natural products as sources of new drugs over the period 1981–2002’, *Journal of Natural Products*, vol. 66, no.7, pp.1022-1037
9. Nordlund, ML, Koch, EW, Barbier, EB & Creed, JC 2016, „Seagrass ecosystem services and their variability across genera and geographical regions“, *PLoS One*, vol.11, no.10, e0163091
10. Pauly, D & Zeller, D 2014, ‘Accurate catches and the sustainability of coral reef fisheries’, *Curr Opin Environ Sustain*, vol. 7, no. 44-51
11. Ramos-Silva, A, Tavares-Carreón, F, Figueroa, M, De la Torre-Zavala, S, Gastelum-Arellanes, A, Rodríguez-García A, Galan-Wong, LJ & AvilesArnaut, H 2017, „Anticancer potential of *Thevetia peruviana* fruit methanolic extract“, *BMC Complement Altern Med.*, vol.17, no.1, pp. 241.
12. Ramya, N, Priyadarshini, Prakash, R & Dhivya, R 2017, „Anti-cancer activity of *Trachyspermum ammi* against MCF7 cell lines mediates by p53 and Bcl-2 mRNA levels“, *The Journal of Phytopharmacology*, vol. 6, no. 2, pp. 78- 83.
13. Ribble, D, Goldstein, NB & Norris, DA 2005, „A simple technique for quantifying apoptosis in 96-well plates“, *BMC Biotechnol*, vol.5, no.12, pp. 1- 7.
14. Rowbotham, SP & Kim, CF 2017, ‘Don’t stop Re-healing!’ Cancer as an ongoing stem cell affair, *Cell*, vol. 169, pp. 563-565
15. Short, FT, Coles, R, Waycott, M, Bujang, JS, Fortes, M, Prathep, A, Kamal, AHM, Jagtap, TG, Bandeira, S, Freeman, A, Erfemeijer, P, Nafie YAL, Vergara, S, Calumpang, HP & Makm, I 2016, In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.
16. Spalding, MD, Ruffo, S, Lacambra, C, Meliane, I, Hale, LZ, Shepard, CC & Beck MW 2014, „The role of ecosystems in coastal protection: Adapting to climate change and coastal hazards“, *Ocean Coast Manag*, vol. 90, pp. 50-57
17. Svejda, B, Moser, VA, Sturm, S, Hoger, H, Ingolic, E, Siegl, V, Stuppner, H & Pfragner, R. 2010, ‘Anticancer Activity of Novel Plant Extracts from *Traillia doxagracilis*(W. W. Smith & Forrest) in Human Carcinoid KRJ-I Cells’, *Anticancer Res*, vol.30, no.1,pp.55-64.
18. Sylla, BS & Wild CP 2012, ‘A million Africans a year dying from cancer by 2030: what can cancer research and control offer to the continent’, *Int J Cancer*, vol. 130, no.2, pp.245-50
19. Thanuja, B, Parimalavalli, R, Vijayanand, S, Alharbi, RM, Abdel-Raouf, N, Ibraheem, IBM, Sholkamy, EN, Durairaj, K & Meansbo, K 2022, „Anticancer and Cytotoxicity Activity of Native and Modified Black Rice Flour on Colon Cancer Cell Lines.“, *Evid Based Complement Alternat Med.*, vol. 2022, no. 8575026, pp. 1-17.
20. Trease, G & Evans, SM 2002, ‘Pharmacognosy’, 15th ed. London: Bailer Tindal, pp. 23-67.
21. Wan, M, Zhang, L, Chen, Y, Li, Q, Fan, W, Xue, Q, Yan, F & Song, W 2019, “Synthesis and Anticancer Activity Evaluation of Novel Phenanthridine Derivatives“, *Front Oncol*. Vol.9, no. 274, pp.1-10.