ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Phytochemical Screening and Effect of *Cymodocea serrulata on* HepG2-Human Hepatocellular Carcinoma Cell Line

Kalpana C S, Kalaivani P, Vanitha V*

Department of Biochemistry, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai-600117, Tamilnadu, India

Article History:

Received on: 30 Dec 2019
Revised on: 04 Feb 2020
Accepted on: 07 Jan 2020

Keywords:

Cymodocea serrulata, phytoconstituents, antioxidant, anticancer, hepatocellular carcinoma, cell line, MTT Assay

ABSTRACT

Cymodocea serrulata, a seagrass commonly known as *karumbupassi* has been used as a food and also as a medicine by coastal region people and by fishermen while traveling in the sea. It is used as a tranquilizer for babies as it has a soothing quality, it helps during pregnancy and against cough and malaria. *C.serrulata* is seen abundant in South Indian coastal region. Although there is a report on the antibacterial, antioxidant, and anti-inflammatory property of *C.serrulata*, there are no evident details on phyto-compounds present in it. *Invitro* antiproliferative test was performed by MTT (methylthiozoltetrazolium) assay method against HePG2 celline of liver cancer cells. MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by liver cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

*Corresponding Author

Name: Vanitha V
Phone: 9941709668
Email: vanitha.sls@velsuniv.ac.in

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v11i2.2058

Production and Hosted by

IJRPS | www.ijrps.com

INTRODUCTION

The synthetic drugs have an adverse effect leading to lack of resistance. (low immunity levels!?). To avoid such lack and to overcome the disease, the marine plants are used. Cymodocea serrulata, marine plant which is a flowering plant that comes under the family Cymodoceaceae. They can be easily recovered (collected) with the cyclone disturbances (Bharathi *et al.*, 2019). *Cymodocea serrulata* are the marine plants which are never seen in deeper area and are available only on the intertidal area as shown in Figure 1, (Hena *et al.*, 2001). Phenol, Flavones, alkaloids, tannin, glycosides saponins and anthraquinones are the phytochemicals present in the seagrass *Cymodocea serrulata* they acts as a potential antioxidant (Ramalingam *et al.*, 2013). *Cymodocea serrulata* used for various remedial purposes such as, muscle pain, fever, stomach problem, wounds and skin disease (Kannan *et al.*, 2013). During pregnancy it helps in the cure of malaria cough, and also used as a transquilizer for babies. HeLa cancer cells can be killed by the leaves of *C.serrulata.* They have cytotoxic assay and high free radical scavenging activity which may help to find the potential drug (Bharathi *et al.*, 2019). Analysing by FESEM *C.serrulata* shows spherical in shape. It shows good inhibition rate on cytotoxic assay on cervical cancer (Chanthini *et al.*, 2015). Towards cancer therapy *C.serrulata* generate ecofriendly bioactive silver nano particles. Synthesized AgNPs shows high

cytotoxic effect on Lung cancer. They also act as a potential bio-reactant (Palaniappan *et al.*, 2015). On the extraction with organic solvent like acetone, methanol, it shows anti micro-fouling activity (Iyapparaj *et al.*, 2014).

MATERIALS AND METHODS

Collection of Sample

The fresh seagrass *C.serrulata* was collected from Thirupalaikudi, Ramanathapuram district, coastal region during June by skilled divers. It was identified and authentified by Dr. N.Kaliaperumal, Former Principal Scientist, CMFRI (ICAR, Govt. of India). The collected seagrass was washed thoroughly and dried in shade. Then, the dried *C. serrulata* was powdered and preserved in an airtight container.

Extraction

1kg of dried, powdered plant material is extracted with 30:70 proportion of hydroethanol for maceration periods (24hrs). The extraction was carried at room temperature with 150 rpm agitation. The extracts were filtered through Whatman filter paper after the maceration period. As per the previous study, hydroethanol extract of *C. serrulata* shows an efficient antioxidant activity.

PHYTOCHEMICAL SCREENING

Qualitative Analysis

Preliminary phytochemical analysis was carried out by utilizing standard technique (Sofowora, 1993; Trease and Evans, 1980; Harborne, 1973).

Quantitative Analysis

The Total Phenol content of *C.Serrulata* was determined by Folin-Ciocalteu's method and the values were expressed as gallic acid equivalence (mg/ml). The total Flavonoids were measured by following standard method Singh *et al* (mg/ml). The total tannin value was determined by Folin Denis method (mg/ml).

Antioxidant Assay

DPPH (2, 2 diphenyl -1-picrylhydrazyl) scavenging assay was determined by the basic protocol of Mensor *et al*. The 2,2 azino bis (ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was determined by the Re *et al*. Nitric oxide (NO) scavenging activity of *C.Serrulata* was determined by basic method Tsai *et al*. Superoxide (SO) anion radicals was measured by the standard method of Liu *et al*.

Cell lines and Culture

Flow Cytometer is the method used for Cell Cycle Analysis. Propidium Iodide is the most widely used dye which has red fluorescence and is excited at 448nm. But this PI has two disadvantages; it stains all double stranded nucleic acids; so, the cells have to be incubated with RNase to remove any double stranded RNA and the dye is excluded by the plasma membrane so that the cells have to be fixed or permeabilised before adding the dye.

Cells were cultured in a 6 well plate at a density of 22 x 10^5 cells/2 ml and incubate in a CO_2 incubator overnight at 37°C for 24 hours. After aspirating the spent medium, the cells were cultured and incubated for 24 hours. Finally, at the end of the treatment medium is removed from all the wells and PBS wash is given and all tubes were centrifuged for 5 minutes at 300 x g at 25°C. 1ml of cold 70% ethanol was added drop wise to the cell pellet and the specimen left at this stage for several weeks. Ethanol fixed cells require high centrifugal speed when compared to unfixed cells.

MTT Assay

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colored water soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

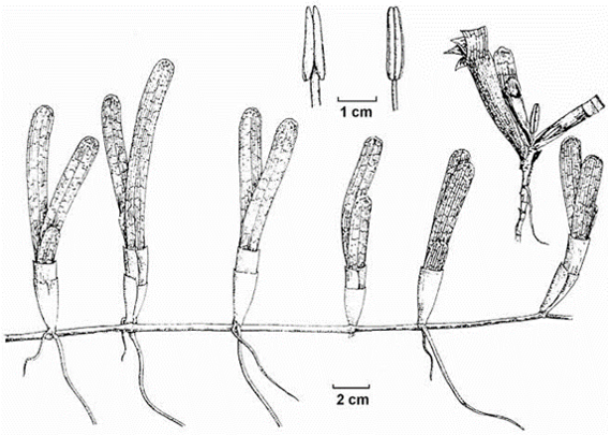

Figure 1: Morphological structure of *Cymodocea serrulata*

The Hep G2 cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2X 104cells/well and allowed to attach overnight at 37°C. The medium was carefully taken and discarded from wells without disturbing the cells and then cells were incubated with differ-

Table 1: Phytochemicals present in *Cymodocea serrulata*

S.No	Compounds	Hydro ethanol
1	Tannin	+
2	Carbohydrates	+
3	Steroids	+
4	Flavonoids	++
5	Glycosides	-
6	Terpenoids	++
7	Saponin	+
8	Quinone	+
9	Phenols	+
10	Alkaloids	-

+ - Present - - Absent

Table 2: Quantitative analysis of Total phenol, Total flavonoids and Total tannin content of Hydroethanolic extract of *Cymodocea serrulata*

Sample	Total Phenol (mg/g)	Total Flavonoid (mg/g)	Total Tannin (mg/g)
Cymodocea serrulata	250.85	40.56	30.45

Table 3: Percentage of cell viability of hydro alcoholic extract of *Cymodaecea serrulata* on Hep G2 cell lines

Positive Control	Hydro alcoholic extract of *Cymodaecea Serrulata* (μg)				
CPT (25μg)	25	50	100	200	400
Cymodaecea Serrulata	89.13	62.07	34.66	21.25	16.42

Table 4: Table showing the IC_{50} Concentrations of the Test Compound(CS) against the Drug Treated HepG2 Cells compared to the standard drug Camptothecin at 25uM (8uG/mL) and results plotted in Bar graph as below

S.No	Sample Code	IC_{50} (uG/mL)-HepG2
1	CS	82.92

ent concentrations of the hydro alcoholic extract of *cymodaecea serrulata* (25,50,100,200, 400μg/ml) for 24 hours. After the incubation, the medium was discarded and 100μl fresh medium was added with 10μl of MTT (3-(4, 5-di- methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5mg/ml concentration. The medium was discarded and 100μl of DMSO was added to dissolve the formazan crystals after 4 hours. Then, the absorbance was read at 570nm in a microtitre plate reader. Camptothecin was used as a positive control.

Cell survival was calculated by the following formula,

$$Cell\ Viability\ \% = \frac{Test\ OD}{Control\ OD} \times 100$$

Cytotoxicity % = 100 – Viability%

RESULTS AND DISCUSSION

Qualitative analysis

Hydroethanolic extract of *C. serrulata* shows the presence of phytocompounds such as flavonoids, phenols, quinones, tannin, carbohydrates, and steroids as shown in Table 1.

Flavonoids, Terpenoids shows higher concentration than the other phytoconstituents with hydroethnaolic extraction. Phenolic compound play an important role in the defense mechanism, can act against pathogens.

Phenolic acid also acts as a chemoprotective agent

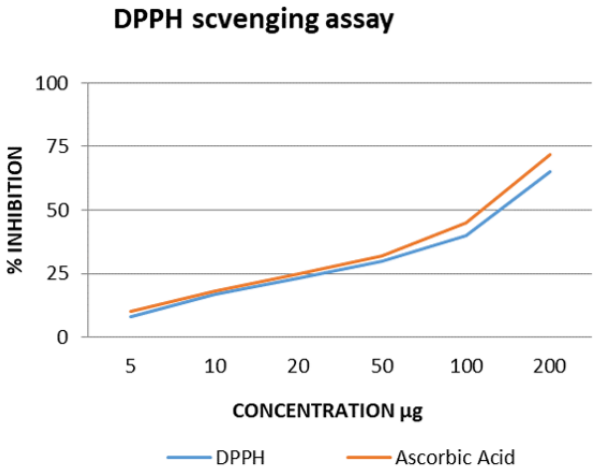

Figure 2: DPPH scavenging activity of *C.serrulata*

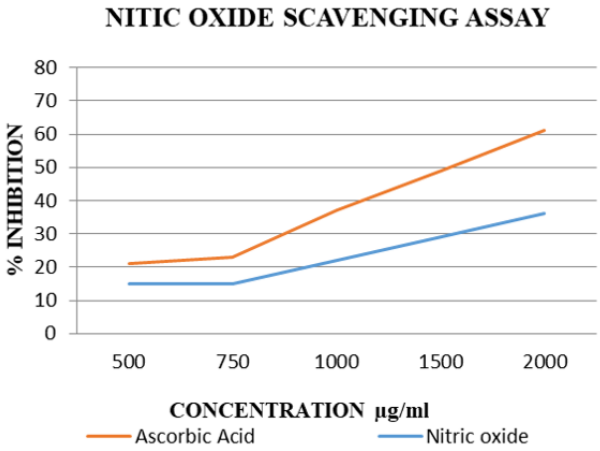

Figure 3: NO scavenging activity of *C.serrulata*

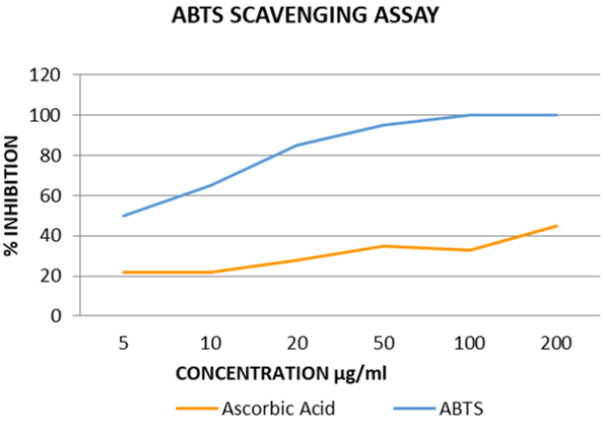

Figure 4: ABTS scavenging activity of C.*serrulata*

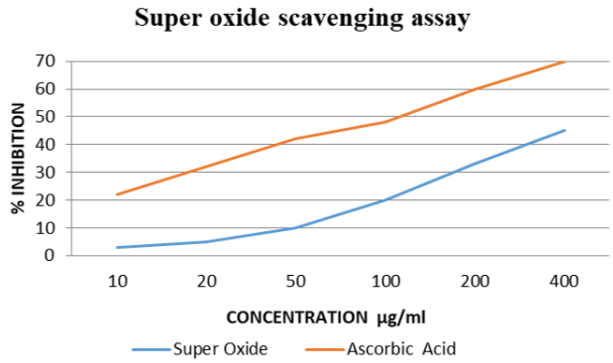

Figure 5: Super oxide scavenging activity of *C.serrulata*

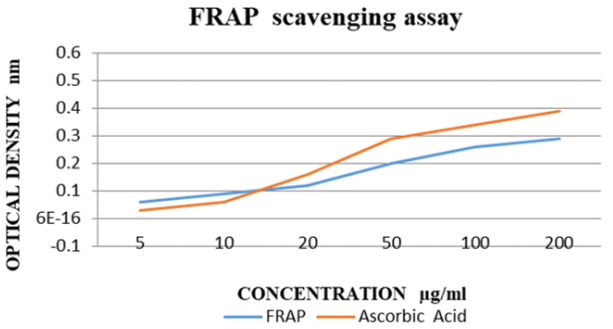

Figure 6: FRAP scavenging activity of *C.serrulata*

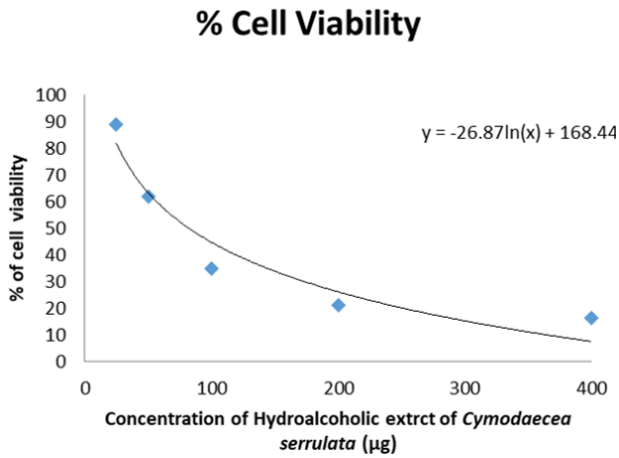

Figure 7: Percentage of Cell Viabilty of *Cymodocea serrulata*

which can prevent the formation of cancer cells. They inhibit the signaling pathway thereby reducing the uncontrolled production of cancer cells. Dietary intake of phenol may prevent the metastasis (Weng and Yen, 2012).

Widely present land plants are the seagrass which predominantly has phenolic acid. They are the potent antioxidant, which can scavenge the free radicals (Zapata and McMillan, 1979)*C.serrulata* shows antifouling and also toxic properities when it is extracted with acetone, ethanol and methanol (Iyapparaj *et al.*, 2014). Flavonoids acts as a free radi-

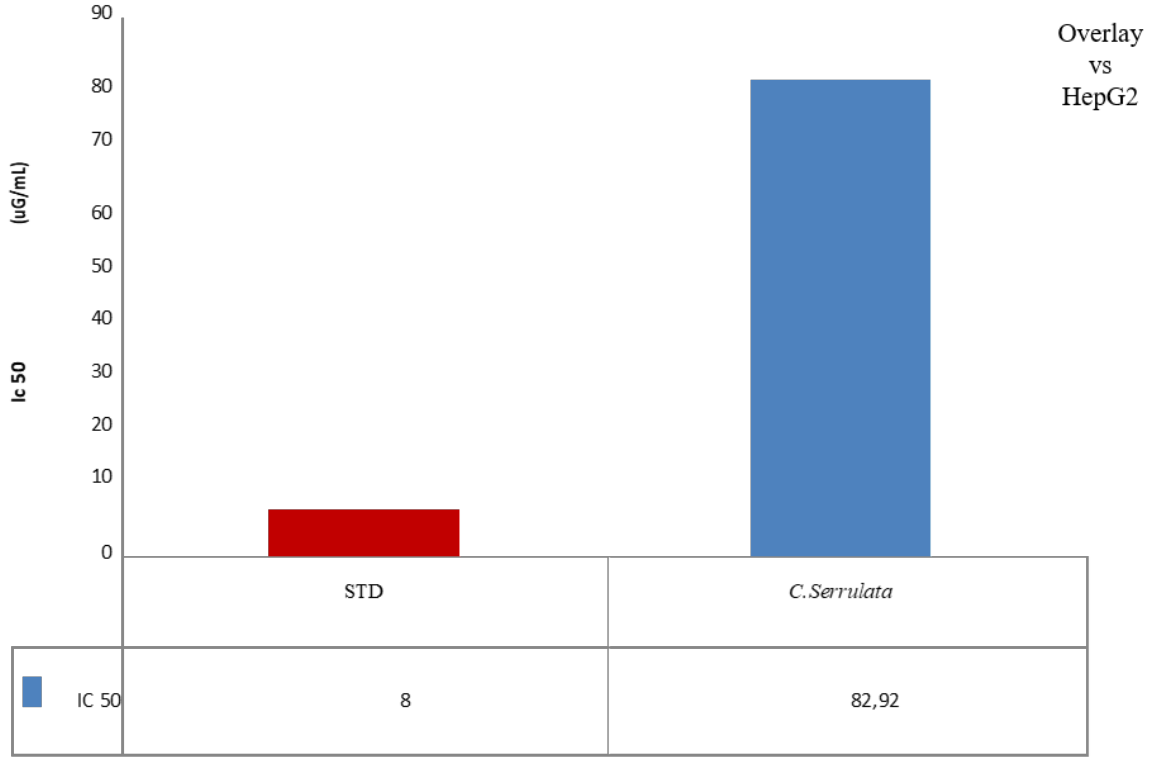

Figure 8: Results of plotted in Bar graph *Cymodocea serrulata*

cal scavenger hence it has a good antioxidant property (Bors *et al.*, 1990).

Quantitative Analysis

Total Phenol, Total Flavonoid and total Tannin were assessed by quantitative analysis and shown in Table 2. The extraction of *Cymodocea serrulata* shows high phenol content 250.85 mg/g. Phenol has the following pharmacological action which may include cytotoxic, anticancer, anti inflammatory, antioxidant activity. The hydroethanolic extract of *Cymodocea serrulata* exhibits 40.56 of total flavonoid content. Flavonoids can inhibit lipid peroxidation, they are metal chelators and have potent antioxidant property. Flavonoids can be excreted through urine and are absorbed by the gastrointestinal tract. They also have ability to cure the coronary heart disease (Cook and Samman, 1996).

Tannin usually binds with lipids or proteins and shows high molecular weight. In this extraction it shows 30.45 (mg/g) of total tannin content. Tannin has wound healing effects because it acts as astringents and present almost in all medicinal plants. (Tsala *et al.*, 2013).

Antioxidant Assay

DPPH has delocalization property and can donate a hydrogen ion. It also has dimerization capacity and can be stable at ambient temperature. Hydroethanolic extract of *Cymodocea serrulata* has high DPPH scavenging activity and very near to the standard ascorbic acid value as shown in Figure 2. DPPH (2, 2 diphenyl-1-picrylhydrazyl) has ability to scavenge the free radicals and commonly called as an antioxidant assay. Most commonly ascorbic acid, BHT and propyl gallate are the standards used. In this study, ascorbic acid is used as standard (Sharma and Bhat, 2009). Biological functions which may include vascular homeostasis, neuro-transmission, anti-tumor activity needs the presence of Nitric Oxide. Peroxynitrite anionic compound is formed due to the combination of Nitric Oxide with Superoxide which has ability to decompose and produce OH & NO radical (Patel and Patel, 2011).

NO activity of hydroethanolic extracts show less inhibitory effect with the standard ascorbic acid as shown in the Figure 3. ABTS shows maximum scavenging activity and it shows equal value of standard ascorbic acid as shown in Figure 4, and Figure 5 shows moderate scavenging activity of Super oxide, which is the strongest reactive oxygen species. Presence of tannin is responsible for the superoxide scavenging activity.

FRAP has the ability to break free radicals by donating the hydrogen ions. FRAP has a good reducing power which has ability to scavenge free radicals and it is a good reliable method to identify the antioxidant property (Jeyapragash *et al.*, 2016). Comparative graph of FRAP scavenging activity with

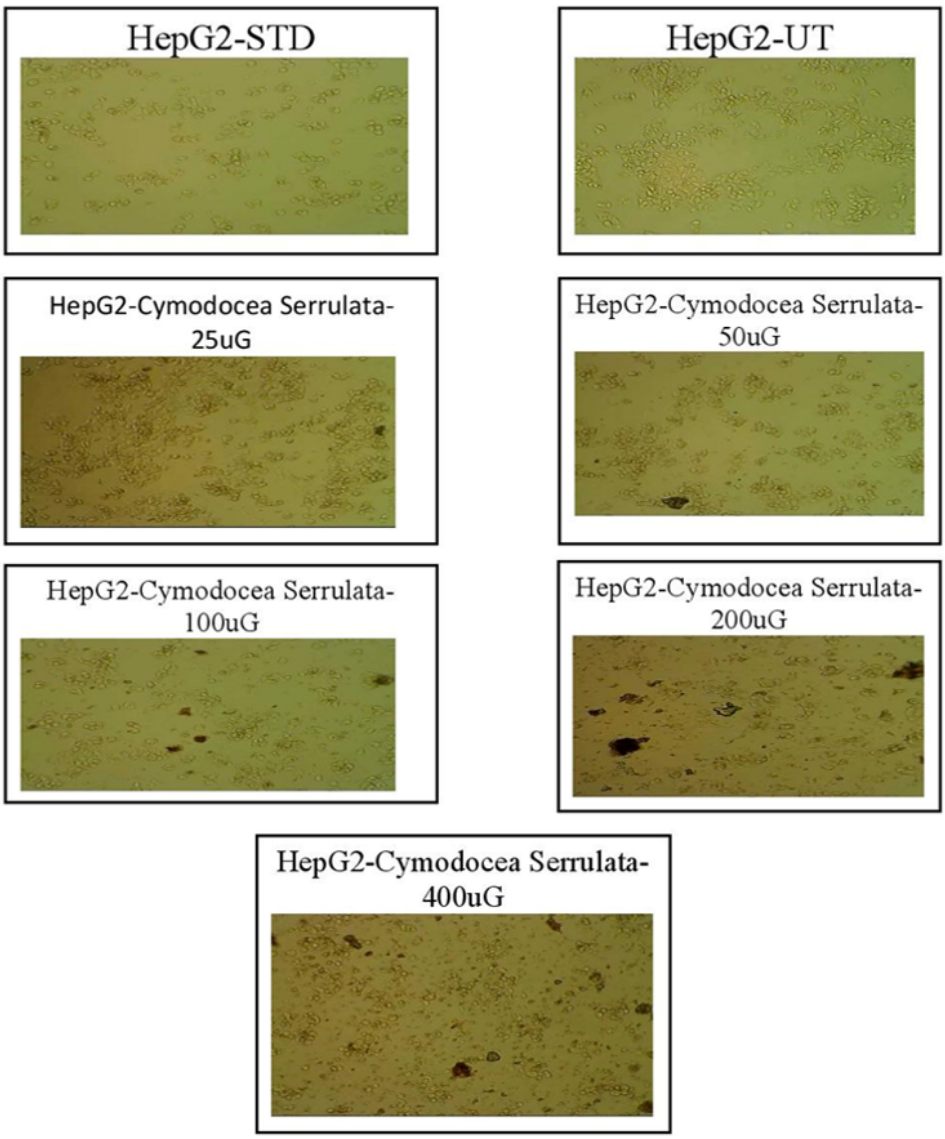

Figure 9: The direct Microsciopic Observations of Drug Treated Images of HePG2 Cell lines. Biological Microscope after incubation of 24hours.

the standard ascorbic acid is shown in the Figure 6.

Cell Cycle

In this study, 1 Test Compound (LP) with 2 Controls is used to check the Cell Cycle Study. A standard Camptothecin drugs were used because of its antiproliferative activity.

The used Concentrations of the compound to treat the cells are shown in Table 3

Concentration of standard Camptothecin 25uM and the concentration of the samples *C.serrulata* were shown in the Table 3. The valuable bio-source *C.serrulata* is a potent bio-reductant, which can generate an ecofriendly bioactive AgNps towards cancer therapy. It shows a good cytotoxic effect on human lung cancer cell A549 cells (Palaniappan *et al.*, 2015). A compound which may include Quercetin, cholorogenic acid induces the apoptosis pathway where it arrests the G1 phase thereby reducing the cell viability of HepG2 cells. Hence the production of cancer cell is reduced due to programmed cell death (Ramos *et al.*, 2005). For an artificial tissue construction, three dimensional high density cell culture techniques used to study the hepatoblastoma cell line, HepG2. Results of this study show that, cells remain normal in the Go/ G1 Phase (Hongo *et al.*, 2005).

Calcium carbonate is an excellent drug because it has good pH sensitivity and also commercially available drug. An anticancer drug Camptothecin was used for absorption and diffusion and this can be given at various concentrations to check the cell viability (Qiu *et al.*, 2012). And the percentage of cell viability is shown in Figure 7. Camptothecin has the

ability to control the cell signaling pathways which can reduce the neuronal apoptosis. This drug used for the treatment of cancer where it has ability to promote the programmed cell death pathway (Park *et al.*, 1997).

Study of the Test Compound *Cymododea serrulata* against HepG2 Cell lines by MTT Assay

The MTT assay is based on the reduction of a yellow tetrazolium dye to a purple formazan product by live cells which in turn gives the count of live cells measurement of purple colour. The hydroethanolic extract of *Cymodocea serrulata* obtained were subjected to MTT assay against HepG2 cell lines and the cell viability and cytotoxicity were represented Table 3 and Table 4. The results were plotted in the graph and shown in Figure 8. Observation of morphological changes in cells indicated that the extracts inhibited proliferation of the HepG2 cancer cell lines in a dose dependent manner. No toxicity was seen in the normal liver cell lines. The IC_{50} value calculated for the ethanolic extract in the cancer cell lines (HepG2) after treatment for 24 hrs.

The Observation in Statistical data of Cell Cytotoxicity Study by ELISA Reader suggesting that in HepG2 cells, *Cymodocea serrulata* shows IC_{50} value at 82.92uG/mL as shown in Table 4. This means that only 82.92ug of the extract was needed to inhibit 50% of the HepG2 cancer cells which shows that it is quite potent in its inhibitory effect. Against various concentrations, the Camptothecin drug was treated with *Cymodocea serrulata*. Comparatively it shows high IC_{50} value. It includes medium control (without cells), Negative control (medium with cells but without drug) and Positive control (medium with cells and 25 uM of Camptothecin). It does not show any toxicity in normal liver cells, but in case of hepatocellular carcinoma cells, the cell viability is more. When these treated with the drug compound Camptothecin with different concentrations shows a good response.

MTT Assay used to determine the mitochondrial activity; thereby, it measures the cytotoxic effects of drugs on cell line. Drug used here to determine the cytotoxicity is the camptothecin (Meerloo *et al.*, 2011). Copper is highly toxic, when it is tested for cytotoxicity HepG2 cells were more susceptible to cause damage to the liver cells. And it gets protected against the drug Camptothecin (Seth *et al.*, 2004). CPT is as anticancer drug and the target enzyme where the drug act is topoisomerase. A patient with lung carcinoma, cervix, ovary and colorectal cancer has anti-tumour activity due to the CPT-11 drug (Slichenmyer *et al.*, 1993). Significant variation in IC_{50} Concentration was seen with the comparison of CCRF-CEM and K562 human cell line (Marks *et al.*, 1992). Figure 9 shows the Microscopic examination of *cymodocea serrulata* at different concentration.

CONCLUSION

Cymodocea serrulata showing IC_{50} concentration (The Concentration of the Compound have the capacity to kill 50% of Viable Cells) against the HepG2 Cells at the 82.92uG/mL after the treatment of 24hours of incubation at 37^{o}C temperature. The obseravation strongly suggests that *Cymodocea serrulata* have possible therapeutic potential against Liver cancer cells based on the dosage given. It has antiproliferative effect on liver cancer cells thereby inhibiting the cell signaling pathway.

REFERENCES

Bharathi, N. P., Jayalakshmi, M., Amudha, P., Vanitha, V. 2019. Phytochemical screening and in vitro antioxidant activity of the seagrass Cymodocea serrulata. *Indian Journal of Geo-Marine Sciences*, 48(08):1216–1221.

Bors, W., Heller, W., Michel, C., Saran, M. 1990. Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *In Methods in Enzymology*, 186:343–355.

Chanthini, A. B., Balasubramani, G., Ramkumar, R., Sowmiya, R., Balakumaran, M. D., Kalaichelvan, P. T., Perumal, P. 2015. Structural characterization, antioxidant and in vitro cytotoxic properties of seagrass, Cymodocea serrulata (R.Br.) Asch. & Magnus mediated silver nanoparticles. *Journal of Photochemistry and Photobiology B: Biology*, 153:145–152.

Cook, N. C., Samman, S. 1996. Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of nutritional biochemistry*, 7(2):66–76.

Harborne, J. B. 1973. Phytochemical methods: A guide to modern techniques of plant analysis. 2nd Edition. pages 49–188.

Hena, M. K. A., Misri, K., Sidik, B. J., Hishamuddin, O., Hidir, H. 2001. Photosynthesis of seagrass Cymodocea serrulata in field and laboratory. *Indian journal of Marine Sciences*, 30(4):253–256.

Hongo, T., Kajikawa, M., Ishida, S., Ozawa, S., Ohno, Y., Sawada, J. I., Honda, H. 2005. Three-dimensional high-density culture of HepG2 cells in a 5-ml radial-flow bioreactor for construction of artificial liver. *Journal of Bioscience and Bioengineering*, 99(3):237–244.

Iyapparaj, P., Revathi, P., Ramasubburayan, R.,

Prakash, S., Palavesam, A., Immanuel, G., Hellio, C. 2014. Antifouling and toxic properties of the bioactive metabolites from the seagrasses Syringodium isoetifolium and Cymodocea serrulata. *Ecotoxicology and Environmental Safety*, 103:54–60.

Jeyapragash, D., Subhashini, P., Raja, S., Abirami, K., Thangaradjou, T. 2016. Evaluation of In-vitro Antioxidant Activity of Seagrasses: Signals for Potential Alternate Source. . *Free Radicals and Antioxidants*, 6(1):77–89.

Kannan, R. R. R., Arumugam, R., Thangaradjou, T., Anantharaman, P. 2013. Phytochemical constituents, antioxidant properties and p-coumaric acid analysis in some seagrasses. *Food Research International*, 54(1):1229–1236.

Marks, D. C., Belov, L., Davey, M. W., Davey, R. A., Kidman, A. D. 1992. The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. *Leukemia Research*, 16(12):1165–1173.

Meerloo, J. V., Kaspers, G. J. L., Cloos, J. 2011. Cell Sensitivity Assays: The MTT Assay. Cancer Cell Culture. *Methods in molecular biology*, 731:237–245.

Palaniappan, P., Sathishkumar, G., Sankar, R. 2015. Fabrication of nano-silver particles using Cymodocea serrulata and its cytotoxicity effect against human lung cancer A549 cells line. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 138:885–890.

Park, D. S., Morris, E. J., Greene, L. A., Geller, H. M. 1997. G1/S Cell Cycle Blockers and Inhibitors of Cyclin-Dependent Kinases Suppress Camptothecin-Induced Neuronal Apoptosis. *The Journal of Neuroscience*, 17(4):1256–1270.

Patel, R. M., Patel, N. 2011. Vitro antioxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging methods. *Japer*, 1:52–68.

Qiu, N., Yin, H., Ji, B., Klauke, N., Glidle, A., Zhang, Y., Wang, W. 2012. Calcium carbonate microspheres as carriers for the anticancer drug camptothecin. *Materials Science and Engineering: C*, 32(8):2634–2640.

Ramalingam, N., Ramakrishnan, K., Krishnan, C., Sankar, S. 2013. Phytochemical analysis, HPTLC finger printing, in vitro antioxidant and cytotoxic activity of Cymodocea serrulata. *Pharmacognosy Journal*, 5(5):238–241.

Ramos, S., Alía, M., Bravo, L., Goya, L. 2005. Comparative Effects of Food-Derived Polyphenols on the Viability and Apoptosis of a Human Hepatoma Cell Line (HepG2). *Journal of Agricultural and Food Chemistry*, 53(4):1271–1280.

Seth, R., Yang, S., Choi, S., Sabean, M., Roberts, E. 2004. In vitro assessment of copper-induced toxicity in the human hepatoma line, Hep G2. *Toxicology in Vitro*, 18(4):501–509.

Sharma, O. P., Bhat, T. K. 2009. DPPH antioxidant assay revisited. *Food Chemistry*, 113(4):1202–1205.

Slichenmyer, W. J., Rowinsky, E. K., Donehower, R. C., Kaufmann, S. H. 1993. The Current Status of Camptothecin Analogues as Antitumor Agents. *JNCI Journal of the National Cancer Institute*, 85(4):271–291.

Sofowora, A. 1993. Medicinal Plants and Traditional Medicine in Africa. *Spectrum Books Ltd*, pages 191–289.

Trease, G. E., Evans, W. C. 1980. Pharmacognosy, 11th ed. *Journal of Pharmaceutical Sciences*, 69(5):619–620.

Tsala, D., Emery, Amadou, Dawe, Habtemariam, S. 2013. Natural wound healing and bioactive natural products. *Phytopharmacology*, 4(3):532–560.

Weng, C. J., Yen, G. C. 2012. Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: Phenolic acids, monophenol, polyphenol, and their derivatives. *Cancer Treatment Reviews*, 38(1):76–87.

Zapata, O., McMillan, C. 1979. Phenolic acids in seagrasses. *Aquatic Botany*, 7:307–317.