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

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**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

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**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 tranquilizer 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 (Iyaparaj 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 authenticated 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 Men sor et al. The 2,2 azino bis (ethylenothiazoline-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 10^4 cells/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 different concentrations of the hydro alcoholic extract of cymodocea serrulata (25,50,100,200, 400μg/ml) for 24 hours. After the incubation, the medium
RESULTS AND DISCUSSION

Qualitative analysis

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

Flavonoids, Terpenoids shows higher concentration than the other phytoconstituents with hydroethanolic extraction. Phenolic compound play an important role in the defense mechanism, can act against pathogens. Phenolic acid also acts as a chemoprotective agent which can prevent the formation of cancer cells. They inhibit the signaling pathway thereby reducing
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 properties when it is extracted with acetone, ethanol and methanol (Iyapparaj et al., 2014). Flavonoids acts as a free radical 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 Superoxide, 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 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 (Palanippan et al., 2015). A compound which may include Quercetin, chlorogenic 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 Cymodocea 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\textsubscript{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\textsubscript{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\textsubscript{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 cytotoxicity HepG2 Cells were more susceptible to 2011 high IC\textsubscript{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.

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