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Deciphering the Effect of Hydro-alcoholic extract of Cymodocea serrulata on the Cell Cycle Arrest and Apoptosis

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ABSTRACT

Liver cancer is the most common cancer both in men and women. Hepato carcinoma is caused due to chemicals, food and also by even some drugs. Many therapies and therapeutic agents show side effects of liver cancer. A natural medicine *Cymodoceae serrulata,* seagrass under Cymodoceaceae family, found in the subtidal region may prove a source of benefit to avoid such side effects. Hydro ethanol extract of *Cymodoceae serrulata* shows high antioxidant and pharmacological activities, which may be used for the treatment of liver cancer. The earlier stage of Apoptosis can be identified by the nuclear changes such as DNA fragmentation. Propidium iodide can determine the proportion of cell cycle analysis—Annexin V conjugated to fluorochromes, including FITC. When the HepG2 test sample treated with the drug *Cymodoceae serrulata*, viable cell growth is significantly reduced compared to the standard camptothecin, which is an antitumor agent. Percentage of viable cell count declined significantly to 5.38%, and the percentage of the late apoptotic cell increased impressively 42.08%.

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INTRODUCTION

Liver is the metabolic engine room of the body where all food, water constituents and drugs are detoxified. Liver disease such as Hepatitis, cirrhosis, Jaundice and liver cancer can be caused by chemicals, food and may even by drugs (Bosch *et al.*, 2004).

From the year 2000, Liver cancer is the most common malignancy in both Men and Women. Yearly 5, 64,000 peoples get affected due to liver cancer and mostly men are affected than the women. This Liver cancer can be treated with hepatitis B and C vaccines (Govind, 2011). Apart from the drugs used nowadays, medicinal plants play a vital role in the treatment of cancer. Especially, in seagrass *Cymodoceae serrulata* shows more efficacies and also decrease the side effects during the liver cancer therapy (Ram and Kumari, 2001).

The Marine algal composition was found to be more in the main Indian coast, Lakshwadeep and Andaman Islands due to its high salinity, sandy substratum and clarity of water. Approximately twelve numbers of species and seven genera was found from the intertidal to subtidal region (Jagtap, 1991).

Among all the seagrass most dominant seagrass present in Lakshwadeep island is *Cymodocea serrulata* as shown in Figure 1 , (Nobi *et al.*, 2011, 2012).

Calculated and state that the sea grass *Cymodocea serrulata* shows high calorific content. High antioxidant and cytotoxic activity were found to be present in the methanol extract of *Cymodocea serrulata* (Ramalingam *et al.*, 2013).

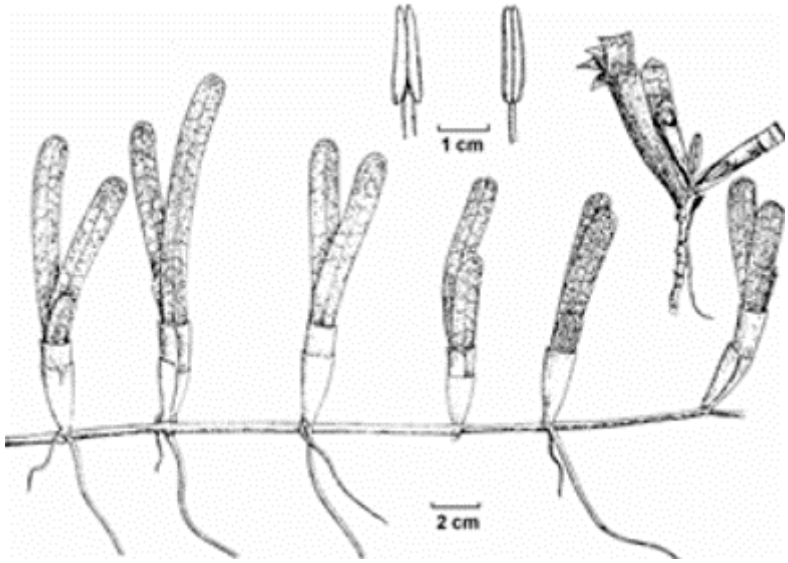


Figure 1: Morphological structure of Cymodoceae serrulata

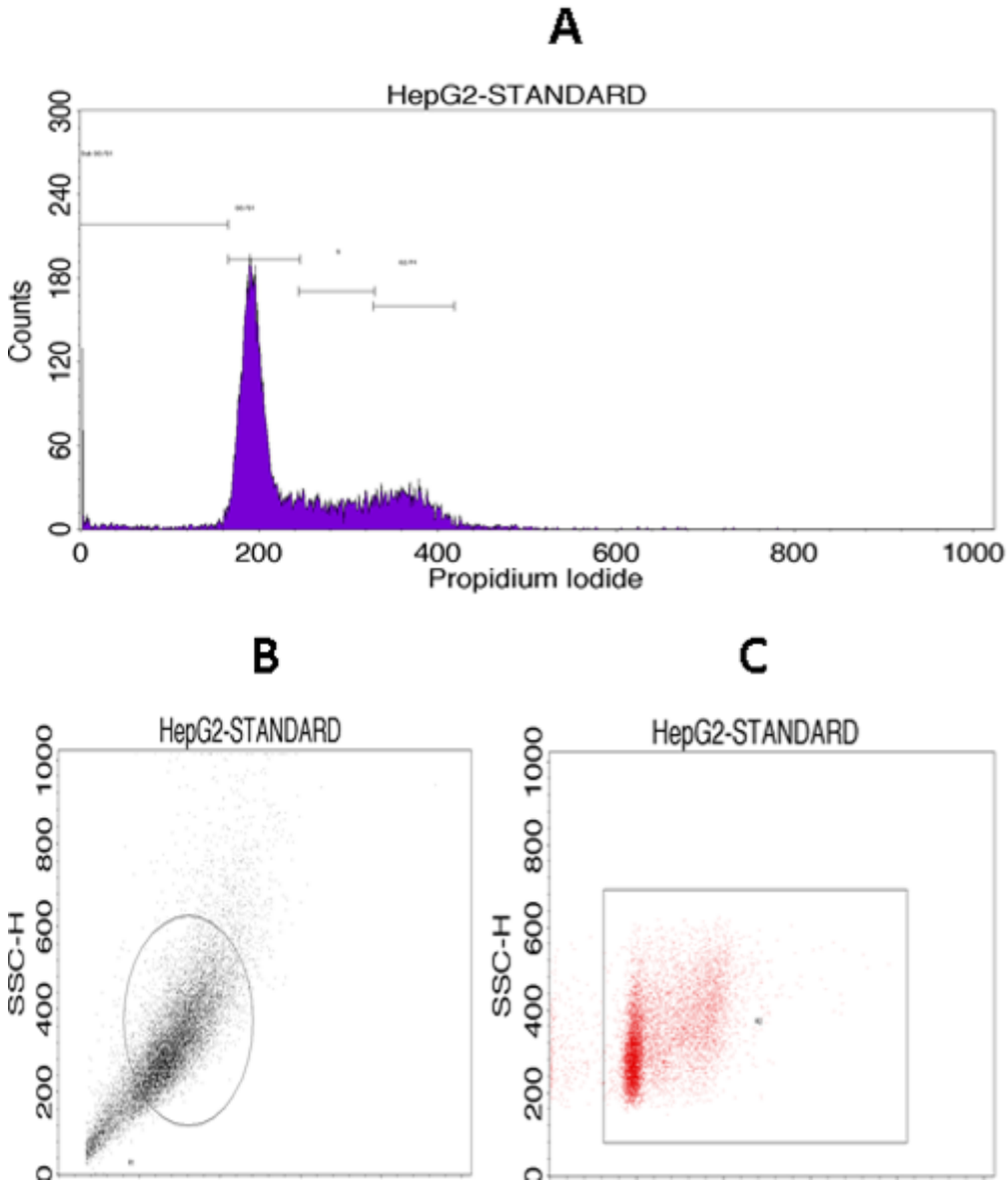


Figure 2: Cell growth on treating with standard

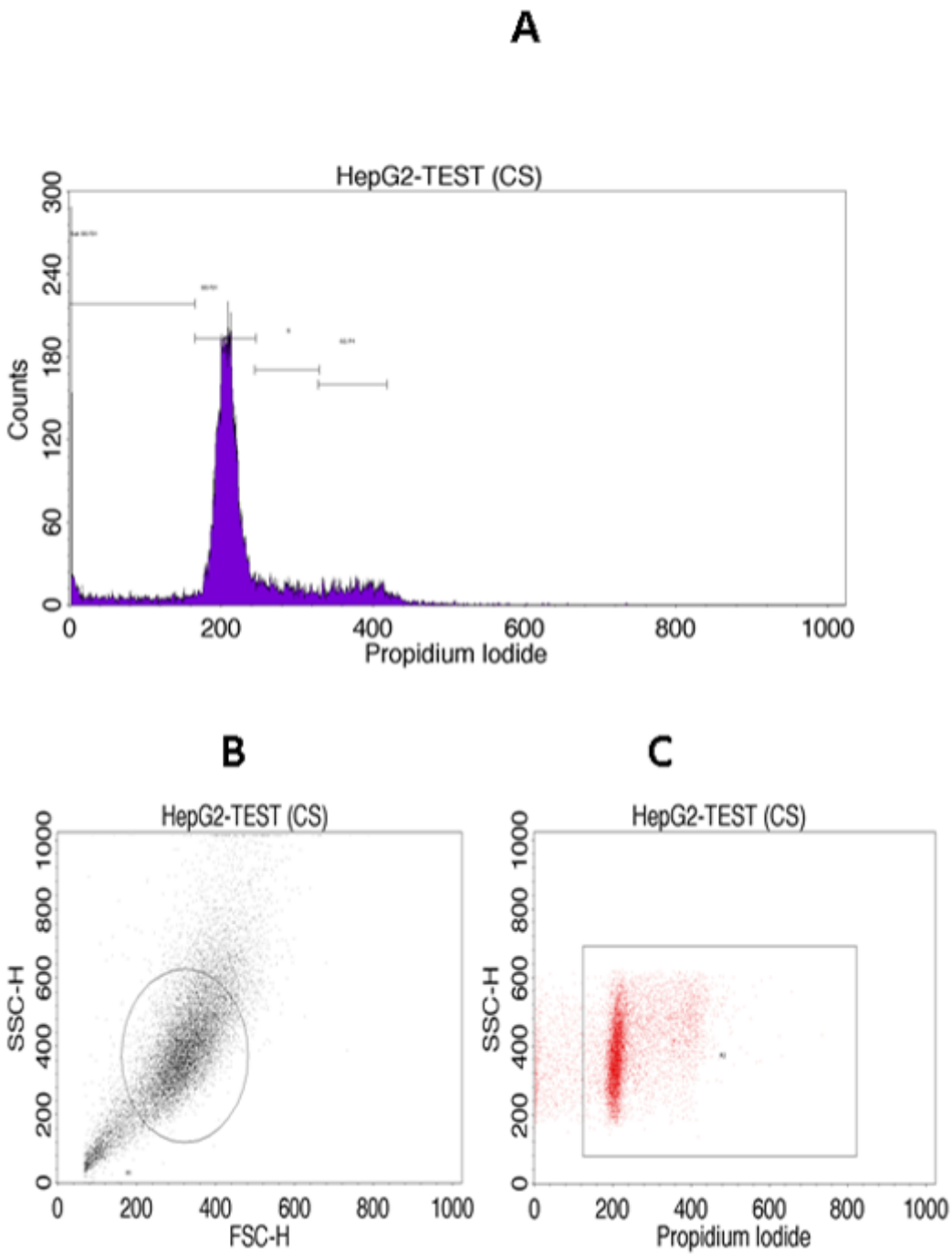


Figure 3: HepG2 cell growth declines on treating with drug cymodoceae serrulata

Table 1: Details of Drug Treatment to respective Cell line for Cell Cycle Study

Sl. No	Test Compounds	Cell Line	Concentration Used
1	Untreated	HepG2	No Treatment
2	STD (Camptothecin)	HepG2	25uM
3	Test-1 (CS)	HepG2	82.92uG/mL

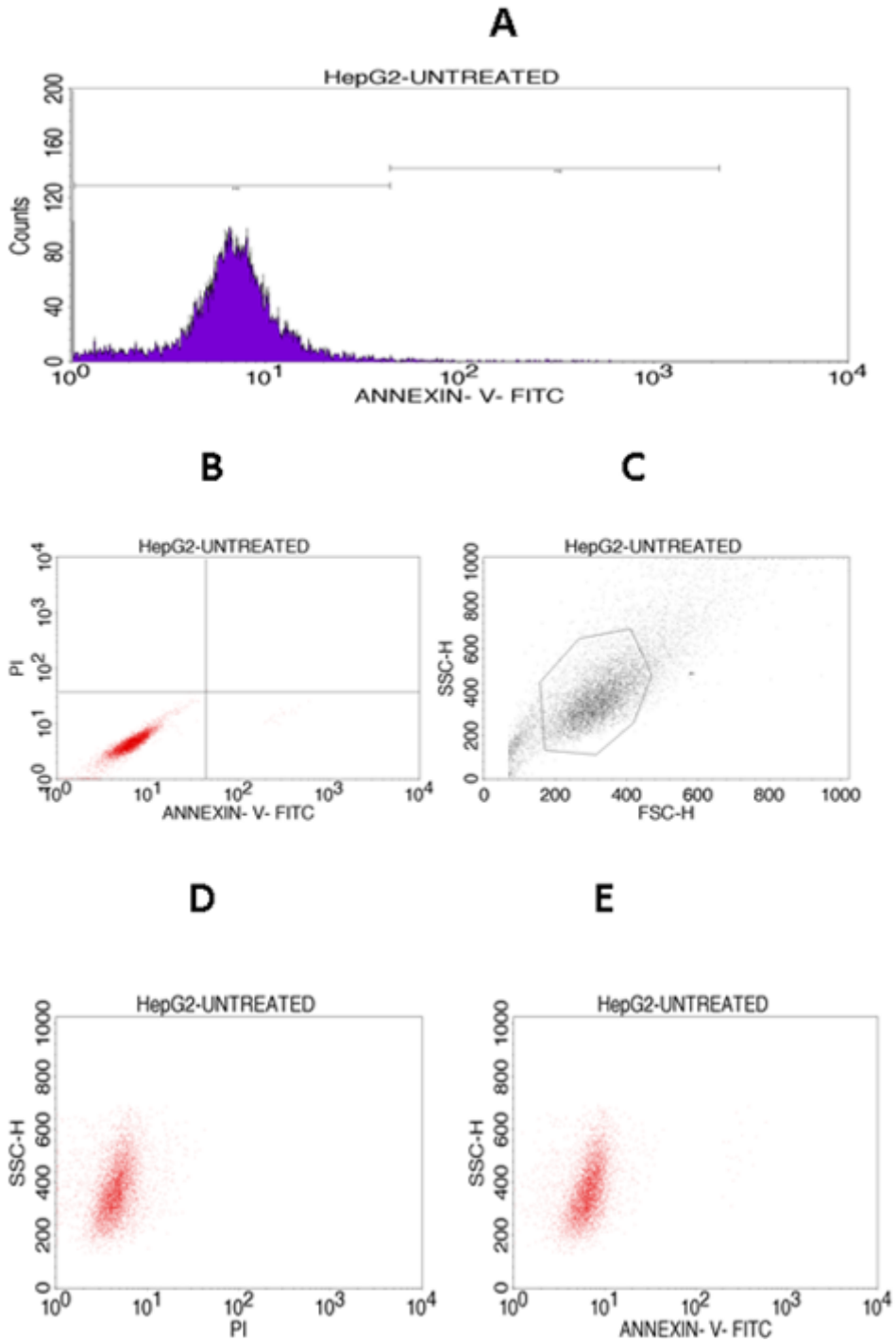


Figure 4: Untreated HepG2 cells using Annexin-V

High flavonoid content is observed when it is treated with the aqueous methanol extract and shows high scavenging activity to prevent oxidative stress (Kannan *et al.*, 2013).

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 has been identified and authenticated by Dr. N.Kaliaperumal, Former Principal Scientist, CMFRI (ICAR, Govt. of India).

The collected seagrass was washed thoroughly and shade dried. Then, the dried *C. serrulata* was powdered and preserved in an airtight container.

Extraction

1kg of dried, powdered plant material is extracted

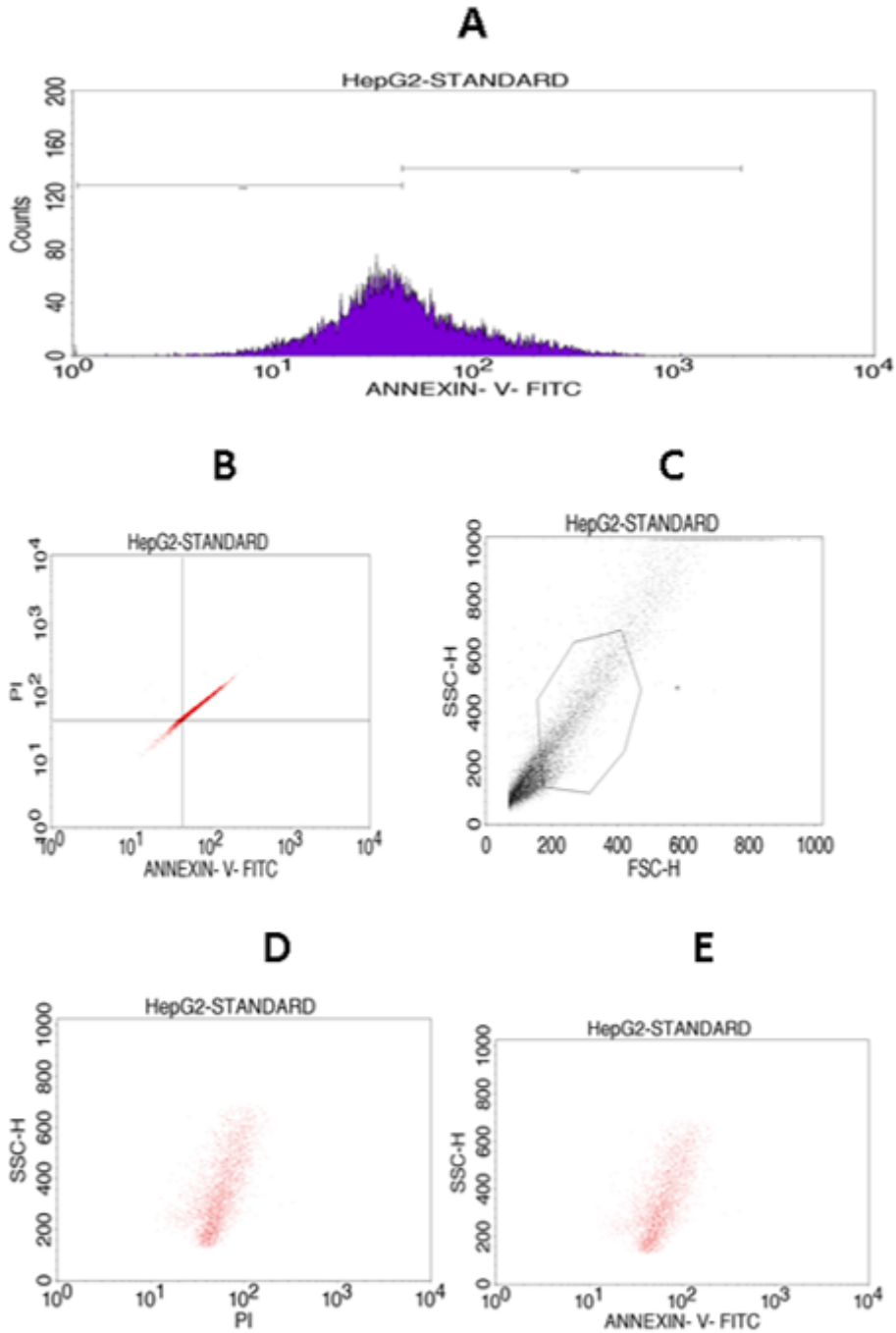


Figure 5: HepG2 cells on treating with Standard Camptothecin

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 macretion period. As per the previous study, hydroethanol extract of *C. serrulata* shows an efficient antioxidant activity.

Cell Cycle Analysis

To identify the proportion of cells at different stages of cell cycle using Propidium Iodide (PI). To assess the cell cycle distribution the whole cell population was fixed with 70% ethanol.

Cell cycle distribution of GFP transfected cells can be assessed by this method. Compound Spectrin provides an excellent result.

In a 6-well plate, the cells were cultured at a density 2×10^5 cells / 2ml. The cultured cells were incubated overnight at 37º C for 24hrs in a CO_2 incubator.

By aspirating the spent medium, the cells were treated with required concentration of experimental compounds and controls.

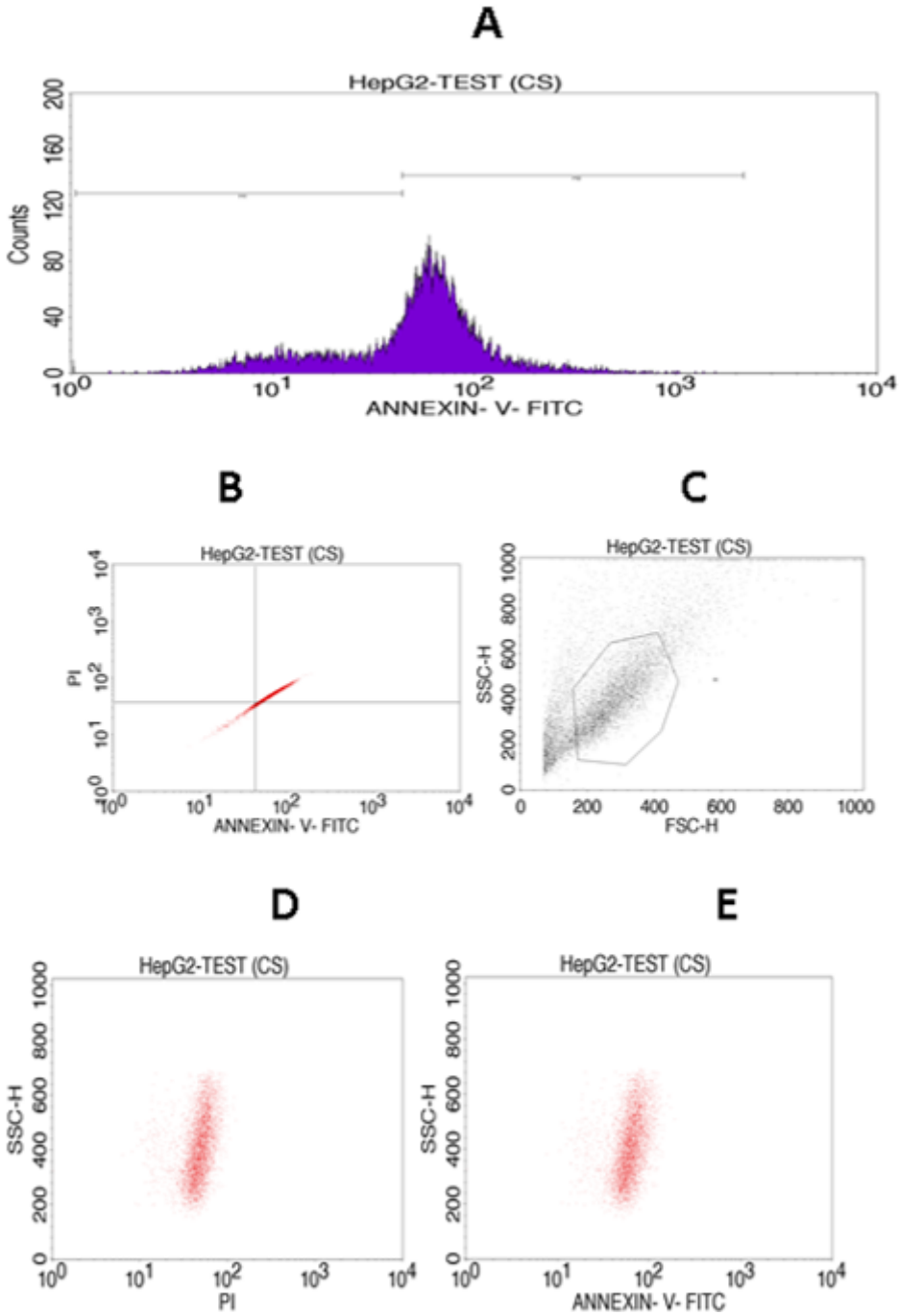


Figure 6: HepG2 Test sample with the drug cymodoceae serrulata

After washing the cells with PBS add 200 μl of trypsin- EDTA solution. Now it is incubated for 3-4 minutes at 37 º C. 2ml of cultured medium was added and the cells are harvested directly into 12×75mm polystyrene tubes.

Centrifuge the tubes for 5 minutes at 300×g at 25ºC and the supernatant was decant. PBS wash is repeated again. Dropwisely, add 1ml of cold 70% ethanol to the cell pellet which has ability to minimize clump formation.

Now the specimen kept in ice for 30 mins. Pellet cells washed twicely with PBS and stained with PI solution. Finally the cells are incubated 5 to 10 minutes at room temperature and analysed by using Flow cytometry.

Apoptosis Cell line study by Flow Cytometry

Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenance of tissue homeostasis. The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment.

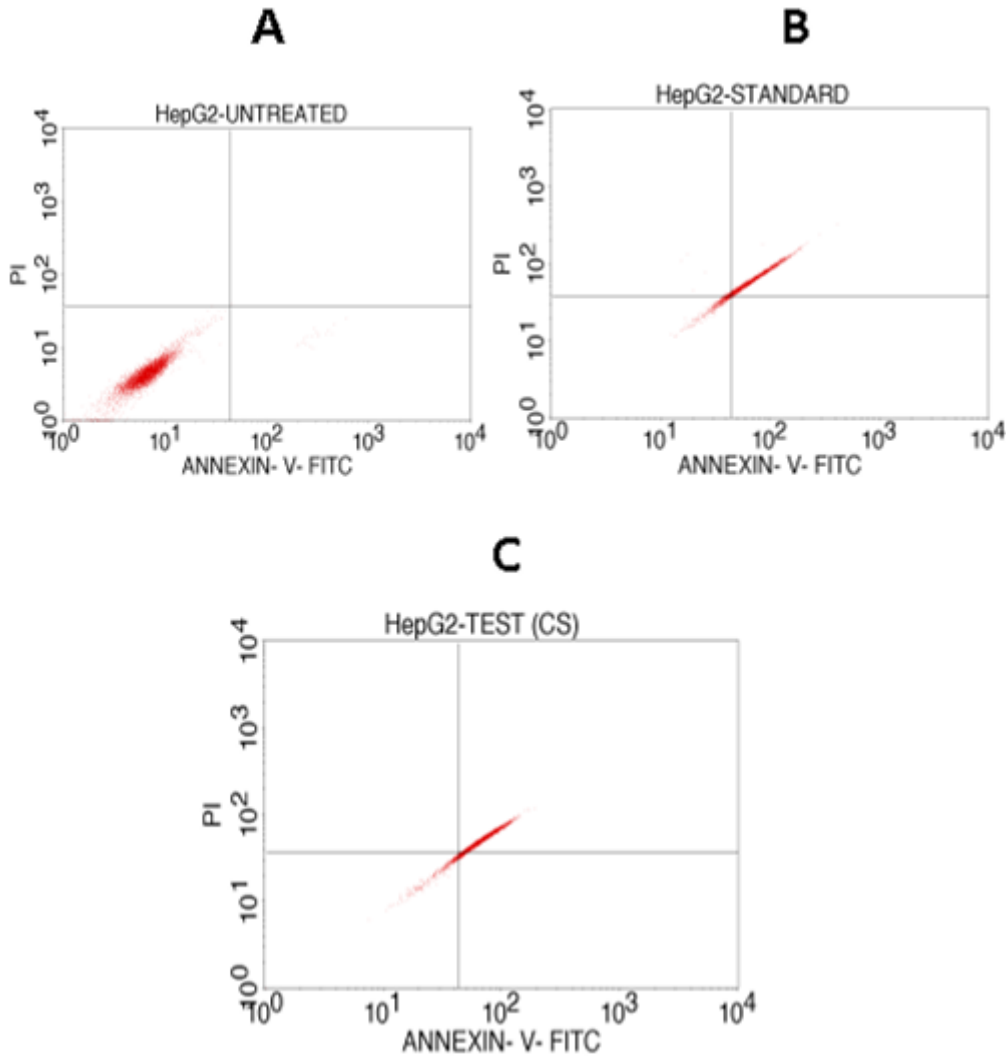


Figure 7: Annexin V-PI expression Study of the Test Compound-1 (CS) against HepG2 Cell line

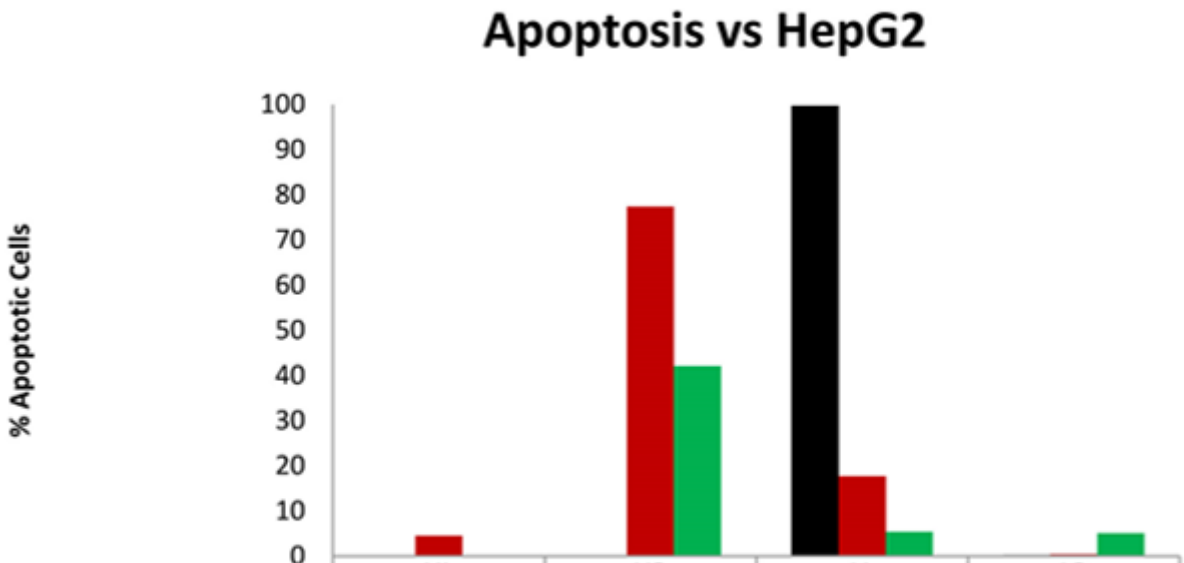
Table 2: Details of Drug Treatment to respective Cell line for the Apoptosis

Sl. No.	Test Compounds	Cell Line	Concentration Treated to Cells
1	Untreated	HepG2	No treatment
2	STD (Camptothecin)	HepG2	25uM
3	Test-1 (CS)	HepG2	82.92uG/mL

Condensation of the cytoplasm and nucleus, and inters nucleosomal cleavage of DNA. Loss of Plasma membrane is one of the earliest features. Phosphatidylserine is exposed to the external cellular membrane during apoptosis, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. FITC and Annexin V may conjugate to fluorochromes.

At earlier stage of apoptosis externalization of PS occurs. The stain due to Annexin V can identify apoptosis at an earlier stage. The stain can precedes the loss of membrane integrity. And they can able identify. Therefore, staining with FITC Annexin V is typically used in conjunct the latest stage of cell death process. The dye propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) can investige and identify the early apoptotic cells. Viable cells can intact membranes and incase of damaged cells, they are permeable to PI.

In early apoptosis with FITC Annexin V the cells show positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive.



	UL	UR	LL	LR
Untreated HepG2 Cells	0	0.01	99.72	0.27
STD (Camptothecin)	4.51	77.38	17.71	0.39
Test Compound-1 (CS)	0	42.08	5.38	5.09

Figure 8: Overlay of the results plotted in Bar graph

The dead cells are staind by both Annexin and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable), to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death).

The movement of cells through these three stages suggests apoptosis. A single indication of cell shows that both FITC Annexin V and PI positive.

Table 3: Meaning of the Quadrant layout in Apoptosis Study

Lower Left Quadrant	Upper Right Quadrant
UL	UR
% of Necrotic Cells	% Late Apoptotic Cells
LL	LR
% Viable Cells	% of Early apoptotic cells

Same as cell cycle analysis with PI, the cells were cultured in a 6-well plate at a density of 3 x 105 cells/2 ml and incubate in a CO2 incubator overnight at 37°C for 24 hours. Then the spent medium is aspirated and taken as control in 2 ml of culture medium.

Now incubate the cells for 24 hours. Medium is removed from all the well and washed with PBS then add 200 μl of trypsin-EDTA solution. After incubation for 3-4 minute at 37°C, add 2 ml of culture medium and harvest the cells directly into 12x 75 mm polystyrene tubes.

And centrifuge the tubes for five minutes at 300 x g at 25°C and carefully decant the supernatant. Repeat the steps twice and decant the PBS completely. Now 5 μl of FITC Annexin V was added and incubate for 15 min at 25°C in the dark. Finally add 5 μl of PI and 400 μl of 1X Binding Buffer to each tube and vortex gently and analyze by flow cytometry immediately after addition of PI.

RESULTS AND DISCUSSION

Cell cycle Study with Propidium Iodide

In this study, 1 Test Compound (LP) with 2 Controls is used to check the Cell Cycle Study. The used Concentrations of the compound to treat the cells as follows in (Table 1).

Camptothecin acts as an antitumour agent which has ability to inhibit DNA relaxation by topoisomerase I (Thomas *et al.*, 2004).

Binding and labeling of DNA can be carried out by Propidim Iodide. By this analysis hypodiploid cells can be identified and this PI is mainly used for measure of rapid cell apoptosis (Riccardi and Nicoletti, 2006).

At the same time, in case of cyclin, otherwise called as PCNA (Proliferation cell nuclear antigen in the PI iodide was used to stain the DNA (Kurki *et al.*, 1986).

Table 4: The % of Cells of arrested indifferent stages of Apoptosis in the Untreated, Standard and Test Compound namely CS treated HepG2 Cells

Quadrant	% of Necrotic Cells	% Late Apoptotic Cells	% Viable Cells	% of Early apoptotic cells
Label	UL	UR	LL	LR
Untreated Cells	0	0.01	99.72	0.27
STD (Camptothecin)	4.51	77.38	17.71	0.39
Test Compound-1 (CS)	0	42.08	5.38	5.09

Annexin V-PI expression Study of the Test Compound-1(CS) against HepG2 Cell line

In this study, the camptothecin compound with the concentration of 25uM was used for cell cycle analysis. Figure 2(A, B, C) shows the Hep G2 cell growth when it is treated with standard. When the Hep G2 cell treated with the drug *Cymodoceae serrulata* the growth of cell declines as shown in Figure 3(A, B, C).

Apoptosis Study on Cell line Study

In this study, Test Compound-1 namely CS with (IC50) is used to check the Annexin V – PI expression Study on the 1 cell line namely, HepG2. The used Concentrations of the compound to treat the cells as follows: (Table 2). Apoptosis is programmed cell death, cell growth increases when there is defect in this process which may lead to abnormal growth of cell called as cancerous cell (Reed, 1999).

5, 7- Dimethoxyflavone possess high anticancer activity can cause cell cycle arrest which is a natural flavonoid against Hep G2 cell line (Hengping *et al.*, 2017). The cell cycle may be arrested and also lead to cell death by many genotoxic drugs with their cytotoxix effects (Vermund and Gollin, 1968).

In this study, Annexin V is used which has ability to bind with the cells. By *invitro* IP6 acts as a tumour marker and has ability to inhibit the proliferation of human liver cancer cell (Shamsuddin *et al.*, 1997). A sensitive and easy assay is due to Annexin V, which inturns used to detect the apoptosis process at different phases before cell death (Vermes *et al.*, 1995). One of isoflavomes namely, genistein has ability to inhibit the number of cell line growth and the compound shows high effect on HepG2 Human hepatocarcinoma cell (Chodon *et al.*, 2007). Javier Matin-Renedo et al., narrates the melatonin has ability to arrest the cell cycle by reducing the cell proliferation. Administration of melatonin on HepG2 cell can analyse the Mitogen activated protein kinase (MAPK), apoptosis and cellcycle arrest. Incase of hepatocarcinoma therapy, melatonin used a novel drug (Martín-Renedo *et al.*, 2008). Figure 4 shows the untreated HepG2 cells using Annexin-V, Figure 5 HepG2 cells on treating with Standard Camptothecin, figure 6 shows HepG2 Test sample with the drug *cymodoceae serrulata* when it is treated with the drug the growth of live cells reduced and Figure 7 shows Annexin V-PI expression Study of the Test Compound-1 (CS) against HepG2 Cell line.

Lower Left quadrant shows the percentage of viable cell which in turn reduced when the concentration drug, test sample (cs) increases as shown in Table 3. Continuous exposure on phosphatidylserine may cause immediate cellcylce arrest. Apoptosis or celldeath can easily be identified due to the strong interaction between the Annexin V and Phosphotidylserine (van Engeland *et al.*, 1998).

Annexin V-PI expression Study of the Test Compound-1 (CS) against HepG2 Cell line. Precentage of viable cell is 99.72with untreated cells and with standard camptothecin reduced to 17.71. With the drug *Cymodocea serrulata* the percentage of viable cell greately reduced to 5.38. Early stage of living and unfixed cells can be identified using Annexin V, thus it acts as an early marker (Zhang *et al.*, 1997). Table 4 shows the percentage of Cells of arrested in different stages of Apoptosis in the Untreated, Standard and Test Compound namely CS treated HepG2 Cells. And the overlay results between apoptosis and HepG2 cell was plotted and shown in Figure 8.

Resveratrol show high antioxidant effects, also acts as a hormone receptor binding, interact directly and indirectly with nucleic acid. The cell proliferation is greatly reduced by resveratrol. By inducing apoptosis, the production of reactive oxygen species reduced and the cell cycle arrest occurs at G1 and G2/M phase (Notas *et al.*, 2006). By activating caspase ergone induces apoptosis. Treatment of ergone with HepG2 cells exhibit both intrinsic and extrinsic pathway (Zhao *et al.*, 2011).

CONCLUSIONS

Cymodoceae serrulata is a potent hepatoprotective drug which has ability to reduce the growth of viable cells. Annexin V and Propidium Iodide were used to identify the different stages of Apoptosis. *Cymodoceae serrulata* drug is cost effective and has all the phytochemicals with high concentration. They show high antioxidant, anti-inflammatory, antiviral and other pharmacological activities. The overlay graph shows the percentage of apoptosis, when the HepG2 cell treated with the drug, it shows high effect where the growth of live cell is greatly reduced. Thus the paper conclude that the drug *Cymodoceae serrulata* greatly induce the process of apoptosis and thus used in the treatment of human heapatocarcinoma (HepG2 cell).

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Conflict of Interest

The authors declare that there is no conflict of interest.

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