



## Evaluation of Anticancer Activity of *Alphonsea Sclerocarpa* Crude Extract against HepG2 Cell Lines by Inducing Nuclear Morphology Changes and DNA Fragmentation

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### ABSTRACT

The present study was conducted to evaluate the anticancer activity of *Alphonsea sclerocarpa* with a focus of its cytotoxicity on HepG2 cancer cell lines. Crude extract was prepared by soxhlet extraction. HepG2 cell lines were assessed by MTT assay after treatment with various concentrations (0.78-500 µg/ml) for 48 h shows IC50 at 199.98 µg/ml indicates dose dependent manner. Further, nuclear morphology changes were carried by DAPI shows changes in morphology, apoptotic body formation. Apoptosis which was further confirmed through increasing nuclear DNA damage. In conclusion, the present findings clearly indicated that *Alphonsea sclerocarpa* showed dose dependant cytotoxicity.

**Keywords:** HepG2 cell lines; MTT assay; DNA damage; DAPI (4',6-diamidino-2-phenylindole); Apoptosis

### INTRODUCTION

Cancer is the third leading cause of death worldwide and an aggressive killer worldwide which is an economic burden worldwide. There are 100 different known cancers which are classified by the cell that is initially affected. By 2030, it is predicted that there will be 26 million new cancer cases and 17 million cancer deaths per year [1]. More than 30% of cancers are caused by modifiable behavioural and environmental factors. Cancer involves abnormal cell growth which invades and spread to other parts of the body. Biological symptoms of cancer includes proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, induction of angiogenesis, activating invasion and metastasis [2,3]. A great effort has been given towards the research on complementary and alternative medicine that deals with cancer treatment by ethnomedical approach from natural sources. Among natural sources, plants have played an important role as a source of effective anticancer agents [4-6] with biologically active compounds. Constituents of medicinal plants include bioactive compounds are the potent sources which play a significant role in cancer control through the regulation of genetic pathways without any side effect [7]. More than 60% of drugs for cancer treatment are derived from plants. Easy availability, low cost, minimal side effects, makes the herbs play a vital role in the prevention and treatment of cancer. Plants are the natural sources have good anticancer activities with their bioactive compounds by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumor cells has become an indicator of the tumor treatment response [8-10]. In the present study, *in vitro* anticancer activity of *Alphonsea sclerocarpa* crude extract has been investigated against HepG2 cell lines by morphology changes and DNA fragmentation induction.

## MATERIALS AND METHODS

### Determination of *In Vitro* Assay of Anticancer Activity

#### Cell lines and culture:

Human hepato cellular carcinoma cell lines (HepG2) that is liver cancer cell lines were procured from national centre for cell sciences (NCCS), Pune. Stock cells were cultured in dulbecco's modified eagle's medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin, streptomycin in a humidified atmosphere with 70-80% confluency. After receiving cells, culture was trypsinized and grown in fresh media of t-culture flasks. The t- culture flask was then marked with the seeding date, cell line, and the passage number. This cell suspension was then transferred to a new t-culture flask and allowed for incubation in 5% CO<sub>2</sub> at 37°C.

#### Preparation of Test Solutions for Cytotoxicity Studies

Powdered leaves were extracted with methanol in soxhlet. Extracts were separately dissolved in 1% DMSO and volume was made with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration which are used to carry out cytotoxic studies [11].

#### Cytotoxicity Assay (MTT Assay)

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to screen the cytotoxic activity of plant extract. The HepG2 cells were grown overnight on 96-well flat bottom cell culture plates, and were then exposed to different concentrations include 0.78, 1.56, 3.12, 6.25, 12.50, 25, 50, 100, 200, 300, 400, 500 and 1000 µg / ml of plant extract for 24hours. DMSO is used as negative control. 10µl MTT reagent prepared in 5.0 mg/ml phosphate buffered saline (PBS) was added to each well and incubated for 3 hours at 37°C. Finally, the medium with MTT solution was removed, and 200 µl of DMSO were added to each well and further incubated for 20 minutes. The optical density (OD) of each well was measured at 560 nm by using spectrophotometer. Results were generated from three independent experiments and each experiment was performed in triplicate. The cell viability ratio was calculated by the following formula:

$$\text{Inhibitory ratio (\%)} = (\text{OD control} - \text{OD treated}) / \text{OD control} \times 100.$$

Cytotoxicity was expressed as the concentration of extract inhibiting cell growth by 50% (IC<sub>50</sub> value) [12].

#### Detection of Apoptosis by DAPI, DNA Fragmentation Assay

##### Detection of nuclear morphological changes using DAPI fluorescent dye staining assay:

A DAPI staining assay was performed to identify the nuclear morphological changes. The cells were seeded in 6-well plates in the medium and incubated for 24 hours. The cells were then treated at IC<sub>50</sub> concentration of crude, nano extract of *Alphonsea sclerocarpa* and *Gyrocarpus asiaticus* for 24hours. Untreated cells were used as controls which contains only the complete medium and cells. After treatment, the cells were washed with 1X PBS once and harvested using centrifugation at 4000 rpm at 4°C for 5 minutes. The treated cells were fixed at -20°C for 10minutes with 50 µl of methanol and water (1:1). The 100 µl (1 µg/ml) of DAPI dye was added to the frozen cells and the mixture kept at 37°C for 30 minutes for staining protected from light. Excess DAPI was then removed with the supernatant by centrifugation at 4000 rpm at 4°C for 5 minutes and then observed under 40 × magnification using fluorescent microscope [13-15].

#### DNA Fragmentation Detection using DNA Laddering Assay

##### DNA extraction:

HepG2 Cells were seeded in t-25 flask containing in DMEM medium and incubated at 37°C in 5% CO<sub>2</sub>. The treated and control cell samples were collected in to centrifuge tubes, washed with PBS and resuspended in EDTA buffer with pH 8.0 (10 mM Tris, 1mM EDTA). Then they were incubated in ice for 30minutes with lysis buffer (pH 8.0) containing 20 mM EDTA, 50 mM Tris, 1% Sodium Dodecyl Sulfate (SDS).After incubation cells were treated with the IC<sub>50</sub> doses of plant extract along with a control (untreated cells).Centrifuge cells at 12000 rpm at 4°C for 10 minutes and discard supernatant. To the pellet add 0.5 ml volume of ice- cold 1 M NaCl, 0.7 ml of ice-cold isopropanol and vortex vigorously. Allow precipitation to proceed overnight at - 20°C. After, precipitation, recover DNA by centrifugation at 2700 rpm and the pellet was washed with 70% alcohol and dissolved in TE buffer. The solution was then incubated at 37°C for 30 minutes with ribonuclease A (RNase A). To the pellet containing DNA add 70% alcohol and finally dissolved in TE buffer.

**Agarose gel electrophoresis:**

For casting 1% Agarose gel add 0.8 gm of agarose in 80 ml of diluted 1X TBE buffer with the addition of 0.5 mg/ml of ethidium bromide. The agarose mixture was poured into an electrophoresis chamber, and a gel comb was inserted to create wells for samples. Once solidified, the gel was transferred into a gel buffer tank. 5  $\mu$ L of DNA 100 bp ladder used as marker, samples were carefully loaded into the wells. The electrophoresis was run at a constant  $\sim$ 50 volt until the dye front has reached 1-2 cm from the bottom of the gel. The gel was then examined through UV illumination for the detection of DNA fragments [16].

**RESULTS AND DISCUSSION****Evaluation of Anticancer Activity of *Alphonsea Sclerocarpa* Crude Extract**

The IC<sub>50</sub> value was graphically obtained by plotting the percentage of growth inhibition against different concentrations of the crude extract of *Alphonsea sclerocarpa* shown in Figure 1. A decrease in the viable cells were observed with the increase in the concentration of the extract. There was a dose depended increase in the cytotoxic activity. The crude extract at low concentration (0.78  $\mu$ g/mL) showed 98.624% cell viability and at high concentration (500  $\mu$ g/mL) 27.162% cell viability. The extract shows IC<sub>50</sub> value 199.87  $\mu$ g/ml which corresponds to cytotoxicity of approximately 200  $\mu$ g/ml concentrations.

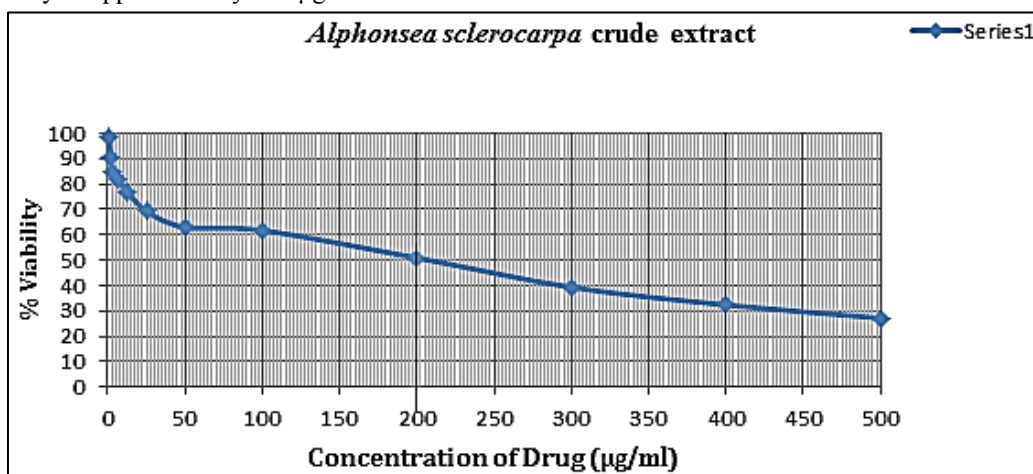


Figure 1: Cytotoxic activity of methanolic *alphonsea sclerocarpa* methanolic crude extract on HepG2 cell lines treated with different concentrations

**DAPI Fluorescent Dye Staining Assay**

Cells were stained with DAPI fluorescent dye is to assess apoptosis after treatment of cell lines with plant extract with IC<sub>50</sub> concentration of *Alphonsea sclerocarpa* crude extract (200  $\mu$ g/ml) observed under microscope. Plant extract were observed to cause alterations of nuclear morphology by cell shrinkage, membrane blebbing, and apoptotic bodies shown in Figure 2.

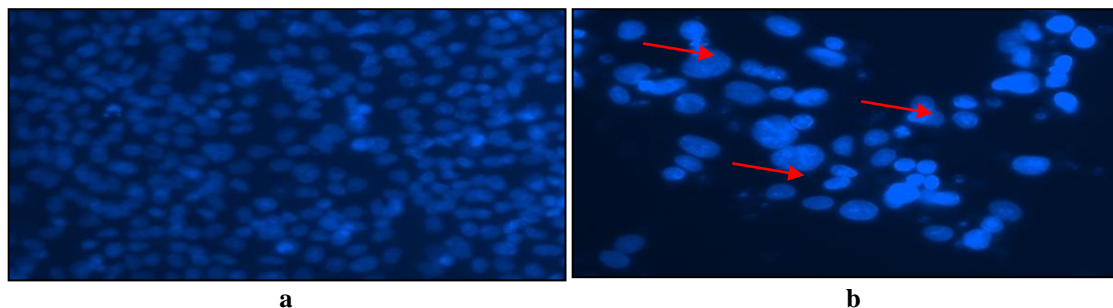
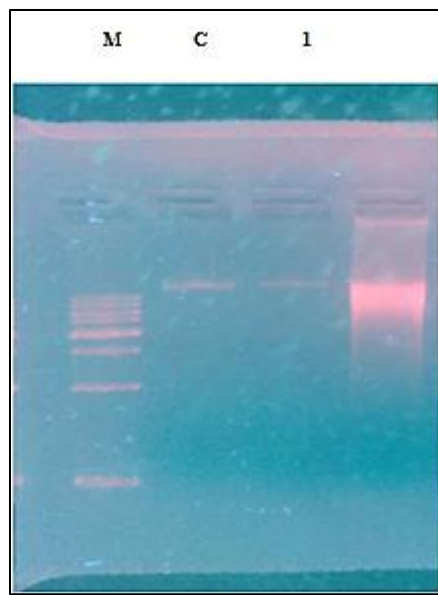


Figure 2: Nuclear morphological change of HepG2 cells stained with DAPI fluorescent dye at 24 hours. a) untreated HepG2 cells b) HepG2 cells treated with 200  $\mu$ g/ml crude extract. Arrows indicate apoptotic nuclei by DNA condensation, apoptotic bodies

### DNA Fragmentation

Plant extracts damage the genomic DNA shown in Figure 3 indicating a clear smear which indicates extensive DNA fragmentation, whereas the control do not show smear of DNA. MTT assay is a colorimetric approach used to determine cell growth and cell cytotoxicity, It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction of MTT to formazan. The amount of formazan crystals produced by MTT is directly proportional to the number of viable cells. % of viability was decreased as the concentration increases. Fragmentation induction is the hall mark of apoptosis. *Alphonsea sclerocarpa* induces cytotoxicity by inducing apoptosis in HepG2 cell lines.



**Figure 3: DNA fragmentation of *Alphonsea sclerocarpa* crude extracts**

M: DNAmarker ladder, C: Control, I: Cell lines treated with *Alphonsea sclerocarpa* crude extract

### CONCLUSION

Results from the present study show a significant decrease in viability of HepG2 cell lines by dose dependent manner. Extract treated cells shows nuclear morphology changes and induces DNA fragmentation. The DNA fragmentation is the separation or breaking of DNA strands into pieces which is a biochemical hallmark of apoptosis which is a direct or indirect outcome of cell death. The present study concludes that plant acting as an efficient anticancer source.

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