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Research Article

IN VITRO ANTICANCER ACTIVITY OF METHANOL EXTRACTS OF *AVICENNIA MARINA* (FORSSK) VIREH AGAINST HEPG2 LIVER HEPATOCELLULAR CARCINOMA CANCER CELL LINE

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ABSTRACT

Anticancer activity of *Avicennia marina* against HepG2 cell line was studied. During the present investigation, crude methanol extracts of *A.marina* were prepared using soxhlet apparatus. *In vitro* anti cancer activity of mangrove plant extracts at Eight different concentrations (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/ml) of methanol extract were applied against the chosen cell line using MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). The various concentrations of methanolic extracts showed anti cancer activity. The most potent antitumor activity has been shown at concentrations 7.8 µg/mL and 500 µg/mL of methanol extract of *A. marina* on Hep G2 cell lines, respectively. The present study indicates that the methanolic mangrove plant extracts were effective against tumor cells and possess anticancer activity.

INTRODUCTION

Man has been using herbs and plant products for combating diseases since times immemorial. Indian systems of medicine have a deep root in our cultural heritage and cater to the Medicare of large sections of our population. Natural products have been used as a major tool for discovery of drugs for pharmaceutical importance. In recent years, antimicrobials derived from the plants have received increasing attention, as the synthetic antibiotics have shown the ineffectiveness against several pathogenic organisms, due to increasing drug resistance (1Teiten *et al*., 2013). Natural products and related drugs are used to treat 87% of all categorized human diseases, including bacterial infection, cancer and immunological disorders (2Newman and Cragg, 2007).

Cancer is a notorious disease that now becomes the major cause of human mortality in the world. Almost half of the incidence and mortality happen in Asia, with lung and bronchus, breast, and colorectal cancers in women to be the most common fatal cancers (3Parkin *et al*., 2005). Liver disease or more known as hepatitis is an inflammation of the liver tissue. The caused of liver function damage can be caused by viruses, bacteria aflatoxin, prolonged consumption of alcohol and drugs that enter the body with a variety of mechanisms (4Nugroho *et al*., 2008). The liver is the very important organ and has a variety functions in the metabolic process so that these organs are often exposed to chemicals. These chemicals will have detoksication and inactivation so that be not harmful for body. Chemotherapy, radiotherapy, hormone therapy, immune therapy and surgery have shown limited success in the treatment of cancer (5 Mishra *et al*., 2013); however, this kind of treatments triggers enormous side effects. Regarding this dilemma, ongoing research on natural medicine sources as practical sustenances or Nutraceuticals has been pulling in numerous researchers. Plants assume an essential part in the improvement of anti-cancer drugs. As indicated by the conventional suggestions and exploratory examinations, various medicinal plants have been accounted to have anticancer effect (Hosseini and Ghorbani., 2015).

Medicinal plants represent an indispensable resource of pharmacologically active compounds with complex molecular structures. The cytotoxic and antitumor activity of these compounds results from various mechanisms, such as their activity on cytoskeletal proteins, which play a main role in cell division, inhibition of DNA topoisomerases, anti-protease or

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antioxidant activity and many others. (7Abu-Darwish *et al*., 2018). Complex and highly chiral structures have been optimized by high salt concentrations and high pressure environment over millions of years, which confers marine organisms the potential to produce valuable therapeutic entities (8Molinski *et al*., 2009). Emerging evidence suggests that marine natural products, especially the secondary metabolites from marine organisms, are far more likely to yield anticancer drugs than terrestrial sources (9Simmons *et al*., 2005). Recent research evidences suggest Indian mangrove plant species have antibacterial and anticancer activities (10Arivuselvan *et al*., 2011). The potential of mangrove plants as a source of new bio active principles is still unexplored.

Avicennia marina (Forssk) Vireh (Avicenniaceae) is commonly known as a gray mangrove tree. It is a high salinity tolerant plant and is found on the coastline. Recent research also suggests *A.marina* plant species have antibacterial and anticancer activities.

A.marina is also one of the plants they have anticancer property. So far mechanism of anticancer activity of *A. marina* extract has not been investigated against liver cancer cell lines. Herbal based therapeutics for liver disorders have been practiced in India for a long time and popularized globally by leading pharmaceutical companies.But limited studies are available on the use of marine halophytes for the management of liver diseases.. The present study aims to determine the anticancer and the cytotoxicity potential of the crude methanol leaf extracts from *Avicennia marina*.

MATERIALS AND METHODS

Collection of materials

Avicennia marina collected from the Pichavaram mangroves in Tamil Nadu. The plant was identified at CAS in Marine Biology, Annamalai University, Parankipettai. The materials were then thoroughly washed in tap water followed by distilled water to remove the salt on the surface of the leaves. For drying, washed specimens were placed on blotting paper and spread out at room temperature in the shade. The shade dried samples were grounded into fine powder using a tissue blender. The powdered samples were then stored in the refrigerator for further use.

Reagents

MEM was purchased from Hi Media Laboratories Fetal Bovine Serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyldiphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

Extraction of sample

The dried and powdered materials (5 g) were extracted successively with 250 mL of methanol by using a Soxhlet extractor for 8 hrs at a temperature not exceeding the boiling point of the solvent. The aqueous extracts were filtered by using Whatman filter paper (No: 1) and then concentrated in vacuum at the 40°C using Rotary evaporator. The residues obtained were stored in a freezer -20°C until further tests.

Cell line and cell culture

The Hep G2 cell lines were obtained from King Institute, Guindy, Chennai. The cancer cells were maintained in Minimal Essential Medium supplemented with 10% FBS, Penicillin (100 U/mL) and Streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37°C. Cells were fed with fresh cultured medium every 2-3 times per week and subcultures when 80% confluent. All cultures were free of Mycoplasma.

Evaluation Cytotoxicity activity by MTT assay (Mosmann, 1983)

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37°C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100µL/well (5mg/mL) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570 nm was measured with a UV-spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100

Graphs are plotted using the % of Cell Viability at Y-axis and the concentration of the sample in X-axis. Cell control and sample control are included in each assay to compare the full cell viability assessment.

Statistical analysis

The data on cell viability were analyzed by using the one way ANOVA followed by the Dennett's multiple comparison tests with equal sample size by using SPSS 17.0. The difference was considered significant when $p<0.005$. All the values were expressed as mean ± standard deviation (S.D). Triplicate assays were performed for each set of test conditions.

RESULTS

In this study, we used the MTT test to evaluate the cytotoxic effect of the methanol plant extract on HepG2 liver hepatocellular carcinoma cancer cell line, using a MTT assay. The metabolic activity or cytotoxic activity of sample on cells by measuring the activity of mitochondrial enzyme succinate dehydroginase was measured by Micro culture tetrazolium (MTT) assay. While cytotoxic activity is determined as a decreased cell number because of cell death, cell-growth inhibition (CGI) or cytostatic activity is determined as a suppression of the increase of cell number without causing cell death. The variation in the activity among the methanol extract of *A. marina* was indicated by determining the IC_{50} of each extract against the particular cell line. Cytotoxic activity of methanol extracts of *A. marina* against human Hep G2 cell line showed a dose and time-dependent inhibitory effect. IC_{50} was 27.11 g/mL, was observed in methanol extracts of *A. marina,* respectively. The methanol extracts of *A. marina s*howed a potential inhibitory effect as compared to the cell control.

MTT assay was carried out to investigate the inhibitory effects of methanol extracts of *A. marina* on the growth of Hep G2 cells and the results are represented in Fig.1; Plate.I. Eight different concentrations (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/mL) of methanol extract were applied. Plate.I shows the percentage of growth inhibition against the methanol extracts of *A. marina*. The highest percentage (67.57%) of growth inhibition was observed with the treatment using 7.8 µg/ml of methanol extract. This was followed by the treatment using methanol extracts at 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg/mL with 7.07,15.82, 20.89, 27.92, 41.79, 53.71 and 58.98 and of growth inhibition, respectively. Treatments with 500 and 1000 µg/mL methanol extracts did not show any growth inhibition. The methanol extract of *A. marina* significantly inhibited the proliferation of Hep G2 cells in a dose dependent manner. The cell control showed 100% protection.

For the treatment with various concentration of methanol extracts from *A. marina*, at 1000 µg/mL exhibited development inhibition (7.03%) as given in Table.1. The increment of methanol extract concentrations resulted in the decrement of growth inhibition as seen with 250 and 500 µg/ml of extracts demonstrated 20.89 and 15.82% of growth inhibition, respectively.

The results indicated that the methanol extracts have positive inhibition on the Hep G2 cell lines. The results of our study display that mangrove methanol extracts have a cytotoxic effect against Hep G2 liver hepatocellular carcinoma cells in a concentration dependent manner, the methanol extracts of *A. marina* showed a high therapeutic value against Hep G2 liver Hepatocellular carcinoma cells with 53.71 µg/mL.

The morphological studies also confirmed that the methanol extract of *A. marina* showed potent cytotoxic effect. It was observed that exposure to methanol extracts of *A. marina* for 24 h, Hep G2 cell growth was inhibited as it is clear from MTT assay and direct cell count (Fig.1). The morphological changes in cells clearly indicate that cells undergo apoptosis at 24 hrs after incubation with the concentration of methanol extracts of A.marina chosen based on the MTT assays (Plate.I). This indicates that methanol extracts of A.marina decrease the potential of individual cells to form a colony and thereby acts as an anticancer drug. This finding well corroborated with our cell proliferation and cell count studies.

Table.1 Effect of methanol extracts on human liver cancer cell line (Hep G2)

S.No	Concentration (µg/mL)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.036	7.03 ± 0.00^{a}
2	500	1:1	0.081	15.82 ± 0.00^{b}
3	250	1:2	0.107	20.89 ± 0.01^{c}
4	125	1:4	0.143	27.92 ± 0.01^{d}
5	62.5	1:8	0.214	41.79 ± 0.01^{e}
6	31.2	1:16	0.275	53.71 ± 0.00^{f}
7	15.6	1:32	0.302	58.98 ± 0.01^{g}
8	7.8	1:64	0.346	67.57 ± 0.01^{h}
9	Cell control	-	0.512	100
IC_{50}				88 µg/mL
P-Value				0.0000
F-Value				2340000

The results of the present study suggest that the methanol extract of *A. marina* may be used as an anticancer drug in the near future. However, further study needs to elucidate the chemical nature and the active principle of *A. marina* which is responsible for its activity.

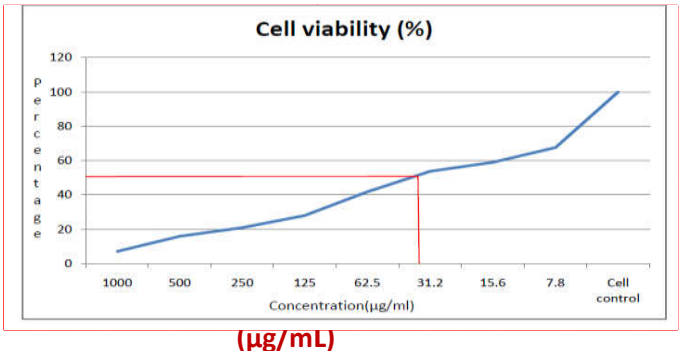

Fig 1 Percentage of growth inhibition of Hep G2 cell lines in the presence of methanol extracts of *A. marina.*

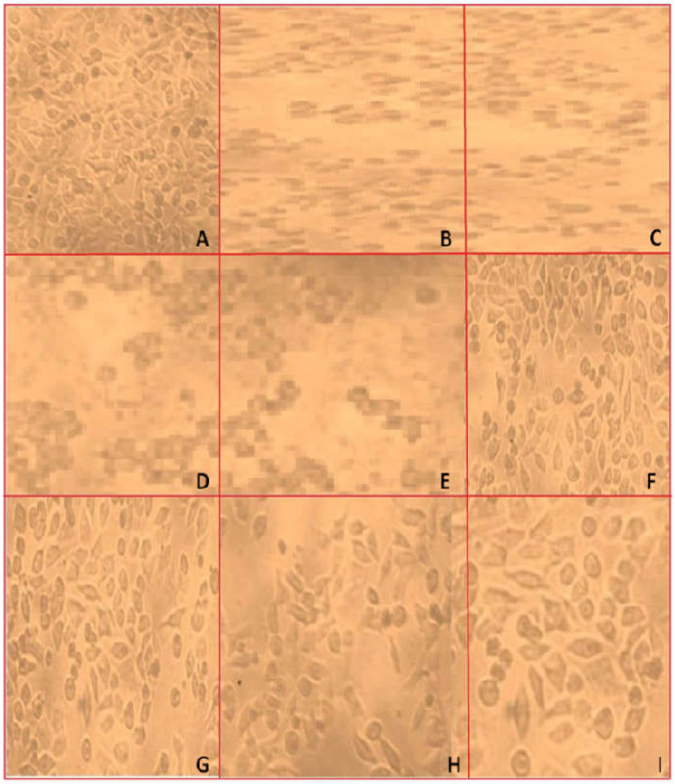

Plate I Inhibition of cell proliferation by methanol extracts of A. marina

A -Normal Hep G2 cell lines Cell line
B) Methanol extract of HT-29 cell line treated with 1000 µg/mL of *A. marina.*
C) Methanol extract of HT- 29 cell line treated with 500 µg/mL of *A. marina.*
D - Methanol extract of HT-29 cell line treated with 250 µg/mL of *A. marina.*
E - Methanol extract of HT-29 cell line treated with 125 mg/mL of *A. marina.*
F - Methanol extract of HT-29 cell line treated with 62.5 µg/mL of *A. marina.*
G - Methanol extract of HT-29 cell line treated with 31.2 µg/mL of *A. marina.*
H - Methanol extract of HT-29 cell line treated with 15.6 µg/mL of *A. marina.*
I - Methanol extract of HT-29 cell line treated with 7.8 µg/mL of *A. marina.*

DISCUSSION

The parts of selected mangrove plants have been commonly used in traditional medicine for the treatment of human diseases. It is believed that the extracts of these plants contain a wide variety polyphenolic compounds which might possess cancer preventive, antioxidant and therapeutic properties.

The major aims of the present study were to determine whether the crude methanol extract of the leaf exerted an inhibitory effect on cancer cell proliferation and caused cell death. The result of the present study suggests that methanol leaf extract of *A. marina* posses the strongest cytotoxic effect on HePG2 cell lines which was compared with normal cell lines. To be a good drug, the IC_{50} values of such agent should be sufficiently low to avoid any possible unspecific effects. The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product exerts an IC_{50} value <30 µg/mL (11Suffness and Pezzuto, 1990).

The present study result shows IC_{50} values against Hep G2 cell line is 88 µg/mL. The IC_{50} values of the extracts using the MTT assay against Hep G2 cell lines revealed that the methanol extract of *A. marina* exerted a significant antiproliferative effect on the Hep G2 cell lines. This indicates that the specific inhibitory effect may be due to the apoptosis-inducing ability of the methanol extracts of *A. marina* in response to the defective gene expression in cancer cell lines rather than the normal cell line.So leaf extract of *A. marina* used as promising anticancer agent. Similar type of Cytotoxic effect against Helen and HePG2 cell lines were reported in *Sophora* interrupted by 12Vithya *et al*., (2012).

The Hep G2 cells at exponential phase were treated with methanol extracts of *A. marina* and the suitable duration of exposure to this cytotoxic agent was determined. Based on this study, the *in vitro* cytotoxicity of *A. marina* was determined using 3-(4, 5-dimethylthiasol-2-yl)-2, 4,-di phenyl tetrazoliumbromide (MTT) assay. The MTT cell viability test shown that various concentrations of the methanol extract from *A. marina* inhibited the growth of the Hep G2 cells for 24 hrs incubated with methanol extracts from *A. marina* respectively. The cell viability percentage decreased gradually when the doses are increasing for 24 hrs. On the other hands, cell viability of fibroblast did not decrease even though the highest dose of 1000 mg/mL was applied. Consequently, the percentage of various cancer cell viability decreased in a dose dependent manner yet to prevent fibroblast, human normal cell lines from elimination. The most effective concentration to inhibit cell growth was found to be 31.2 µg/mL of methanol extract of *A. marina* with 53.7% of growth inhibition, respectively. As the concentration of the *A. marina* extract increases, the number of viable cells decreased. 13Joshi and Srisudha (2012) reported that extract of *A. marina* exhibited cytotoxic activity against Hep G2 cells *in vitro* with a CD50 of 250 µg/mL. Thus, the bioactive compounds of *A. marina* can be considered as a chemotherapeutic agent against cancer. 7.8 µg/mL of the mangrove extract exhibited 67.57% cell viability whereas 1000 µg/mL of the seaweed extract showed 7.03% cell viability. Increasing concentration of *A. marina methanolic* extract from 7.8 to 1000 µg/mL leads to 67.57% decrease in cell viability of Hep G2 cell lines.

Previous phytochemical and bio-efficacy studies on plants confirmed cytotoxic properties of alkaloids (14,15,16,17Griffin *et al.,* 2007; Chougule *et al.,* 2010; Suresh *et al.,* 2011., Shanthi *et al*., 2017). Recently 18 Altemimi *et al*, 2017 reported the synthetic phenolic antioxidants replacement of these natural antioxidant extractionsfrom various foods .According to the 9Suffness and Pezzuto (2009), a crude extract can be considered as cytotoxic against cancer cells *in vitro* and can be used for anticancer drug development if the standard IC_{50} value is less than 30 µg/mL. Based on the results, *A. marina* is considered as highly cytotoxic against Hep G2 cell lines.Although the metabolites responsible for the antiproliferative action of mangrove species studied have not been chemically characterized in this study, the data suggest the occurrence of several secondary compounds with low polarities which are spread more easily in cell membranes than the more polar (19 and 20Moo-Puc *et al.,* 2009; 2011) once the crude extracts of dichloromethane, ethanol and chloroform fraction concentrated the substances responsible for the most significant cytotoxic activity. In this context methanol extract from *A. marina* may be used as a drug to inhibit the tumour cells. One of the vital criteria for an anticancer drug is to have minimum or no side effects on normal cells. Thus, the drug should not only have a high potent anticancer activity, but also should exhibit a high degree of selectivity (21 Badisa *et al.,* 2009).

CONCLUSION

The results of the present study suggest that the methanol extract of *A. marina* had high anticancer activity as evidenced from the MTT assay in a concentration dependent manner may be used as an anticancer drug in the near future. The methanol extract of *A. marina* showed notable cell death against the Hep G2 cancer cell line. However, further study needs to elucidate the chemical nature and the active principle of *A. marina* which is responsible for its activity. The outcome of the present study encourages carrying out further studies to be extended for other cell lines and *in vivo* cytotoxicity investigation and required to identify anticancer activity.

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