

Biological screening of *Avicennia marina* for anticancer activity

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ABSTRACT

Avicenniaceae family is a member of true mangrove plants which has one genus, 11 species and several subspecies. *Avicennia marina* is the most current species among these plants in many forests. Regarding to the presence of many active biological constituents in this plant and their applications in traditional and alternative medicine, the in-vitro anticancer activity of its leaf extract on various cancer cell lines (HL-60, HepG2, NCI-H23 & HEK-293T) were determined by MTT bioassay. With use of MTT dye, % cell viability and % inhibition of the hit compounds was evaluated within respective wavelengths prior with standard compounds. Data obtained from MTT bioassay screening revealed that methanolic and aqueous extract of *Avicennia marina* shown cytotoxicity against HL-60 and NCI-H23 cell line with efficient IC₅₀ values and shown negligible toxicity against normal cell line (HEK-293T).

Keywords: *Avicennia marina*, MTT bioassay, anticancer activity, Avicenniaceae.

INTRODUCTION

With the emerging worldwide interest in adopting and studying traditional systems and exploiting their potential based on different health care systems, the evaluation of the rich heritage of traditional medicine is essential. Mangroves have long been a source of astonishment for the layman and of interest for scientist. Mangroves are biochemically unique, producing a wide array of novel natural products. Substances in mangroves have long been used in folk medicine to treat diseases. Although the chemical constituents of most mangrove plants still have not been studied extensively, investigations have led so far to the discovery of several novel compounds with prospective medicinal value for the discovery of new chemotherapeutic agents. It contains triterpenoids (betulic acid 0.3%, taraxerol 0.06% and taraxerone 0.05%) and traces of hydrocarbon, Sterols (β -sitosterol & stigmasterol), triterpene alcohols, iridoid glycosides and high amount of carbohydrates, lipids and proteins. [1,2,3,4,5,6,13,14]

It has been traditionally used for treatment of rheumatism, small pox, ulcers and other ailments. Bark is used as aphrodisiac, astringent, for scabies, antifertility agent and has tanning properties. Flowers used for perfumes. Leaves are aphrodisiac and used for toothache, Leaves and seeds forage for camels and animals. Wood was used as fuel and in traditional buildings. The plant is known for the quality of its honey and the charcoal has special uses. [7,8,9,14] Some of the complications occur during in-vivo cytotoxic screening that is intravenous administration of chemotherapeutic drugs cause significant individual differences in biotransformation and biodistribution. To overcome this problem, in-vitro cytotoxic screenings are used in which the effect of chemotherapeutic drug is being studied on the tumor cells in culture outside the body. There are two basic types of in-vitro cancer screening method - (a) chemo-sensitivity and (b) chemo-resistance. [10]

Common basic steps of in-vitro cytotoxic screening include: (a) isolation of cells, (b) incubation of cells with drugs, (c) assessment of cell survival and (d) interpretation of the result. The trypan blue dye exclusion assay is the most commonly accepted method for the measurement of cell viability. It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability. It is now well-

documented that apoptosis or programmed cell death is the key mechanism by which Chemotherapeutic agents exert their cytotoxicity. Colorimetric assay (MTT) is mainly useful in determination of cellular proliferation, viability and activation. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assays. Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts MTT are especially useful for assaying the quantification of viable cells. MTT works by being converted to a formazan dye only by metabolic active cells. Formazan dyes were solubilized and are directly quantified using an ELISA reader with their respective reference wavelengths. [11]

MATERIALS AND METHODS

Plant material

The authenticated sample was collected from Herbal Botanical garden, Bangalore, India and was further confirmed by the taxonomist.

Preparation of plant extracts [12]

Extraction with Alcohol:

Authenticated stem bark of *Avicennia marina* was shade dried at room temperature, pulverized, and 100g of the powder was extracted exhaustively with 95% ethanol at temperature 60°C, in a Soxhlet extractor. The extract was concentrated in a rotary flash evaporator; residue was dried in a dessicator over sodium sulfite.

Successive Extraction:

Another 100g of the powder was extracted exhaustively & successively with various solvents in an increasing order of polarity viz., Petroleum ether (40-60°C), Ethyl acetate, Alcohol and Water. Each extract was concentrated to a small volume and allowed to dry.

Media

Leibovitz L-15 Medium with L-Glutamine, FBS (Fetal Bovine Serum, South American origin), SFM HEK-293 (Serum Free Media), Thioglycollate medium (TGM), Tryptone soya broth (TSB) and Cell proliferation kit (MTT).

Cell lines

HEK-293T (Human embryonic kidney normal cell line), NCI-H23 (Human Non-Small Cell Lung cancer cell line), HepG2 (Human Hepatocellular carcinoma cell line) and HL-60 (Human promyelocytic leukemia cell line) were purchased from NCCS, Pune.

Microbial and fungal culture

Candida albicans, *Bacillus subtilis*, *Candida sporogenes* were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Subculture of adherent cell lines (HEK 293T, NCI-H23) [11]

Cultures were observed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with PBS without Ca²⁺/Mg²⁺ using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1 ml per 25 cm² of surface area. Flask was rotated to cover monolayer with trypsin. Flask was returned to the incubator and left for 2-10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were resuspended in a small volume of fresh serum containing HEK-293 medium. 100-200 µl was removed to perform a cell count. The required number of cells were transferred to a new labeled flask containing pre-warmed HEK-293 medium and incubated as appropriate for the cell line.

Determination of bacteria and fungi in normal and carcinoma cell lines [11]

Cell line was cultured in the absence of antibiotics at NCCS, Pune. Cell suspension was prepared by scrapping attached cells with the use of a cell scraper and maintained the pH 7.5-8.0. In 1.5 mL cell suspension, 2 mL thioglycollate medium (TGM) and 2 mL tryptone soya broth (TSB) were added and inoculated with two different strains; *Candida albicans* (0.1 mL) *Bacillus subtilis* (0.1 mL). Then in 1.5 mL cell suspension, 1 mL TGM was added and inoculated with 0.1 mL *Candida sporogenes* and 2 mL (TGM), 2 mL (TSB) were left uninoculated as negative controls. Broths were incubated at 32 °C. Test and Control broths were examined for turbidity after 14 days.

Anti-cancer Activity**MTT Assay: [11]**

The cells were preincubated at a concentration of 1×10^6 cells/ml in culture medium for 3 hrs at 37 °C and 6.5 % CO₂. Then, the cells were seeded at a concentration of 5×10^4 cells/well in 100 µl culture medium and at various concentrations (0.005-100 µM/ml) of standard doxorubicin and synthesized compounds (dissolved in 2 % DMSO (dimethylsulphoxide) solution) into microplates (tissue culture grade, 96 wells, flat bottom) and incubated for 24 hrs at 37 °C and 6.5 % CO₂. The cell proliferation is based on the ability of the mitochondrial succinate-terazolium reductase system to convert 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue colored formazan. The test denotes the survival cells after toxic exposure. Then, 10 µl MTT labelling mixture was added and incubated for 4 hrs at 37 °C and 6.5 % CO₂. Each experiment was done in triplicates. Then 100 µl of solubilization solution was added into each well and incubated for overnight. The spectrophotometric absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product in between 550 and 600 nm according to the filters available for the ELISA reader was used. The reference wavelength should be more than 650 nm.

IC₅₀, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of compound) VS % cell inhibition. A line drawn from 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC₅₀ value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where, At = Absorbance of Test,
Ab= Absorbance of Blank (Media),
Ac= Absorbance of control (cells)

$$\% \text{ cell inhibition} = 100 - \% \text{ cell survival}$$

RESULTS AND DISCUSSION**Total bacterial and fungal count**

The examination of the test and control broths after 14 days incubation confirmed the absence of turbidity. Absence of turbidity in the test broth means that there was no evidence of bacterial, fungal and cross contamination.

Cytotoxicity Assay

The effect of plant extract aliquots (test) and doxorubicin (standard) on the growth of HL-60, HepG2, HEK-293T and NCI-H23 cell lines were examined by the MTT assay. Dose response curves constructed between the range 0.005 – 100 µg/ml and 0.005 – 100 µM for compound aliquots and doxorubicin (control) respectively, express decreasing number of viable cells with increasing concentration of compounds aliquots as well as doxorubicin. Calculation of IC₅₀ value was done using *GraphPad Prism Software* (Figure 1, 2, 3 & 4). The susceptibility of cells to the compound aliquots and doxorubicin was characterized by IC₅₀ and R² values (Table 1 & 2). Results indicate that the cytotoxic effect steadily strengthens with increase in the concentration.

Table No. 1: IC₅₀ & R² values of Methanolic extract of *Avicenna marina*

Conc'n (µg/ml)	HL-60	NCI-H23	HepG2	HEK293
IC ₅₀	297.934	210.987	>1000	762.980
R ²	0.9452	0.9610	0.9712	0.9870

Table No. 2: IC₅₀ & R² values of Aqueous extract of *Avicenna marina*

Conc'n (µg/ml)	HepG2	HL-60	NCI-H23	HEK293
IC ₅₀	>1000	281.175	220.127	717.125
R ²	0.8089	0.9927	0.9863	0.9523

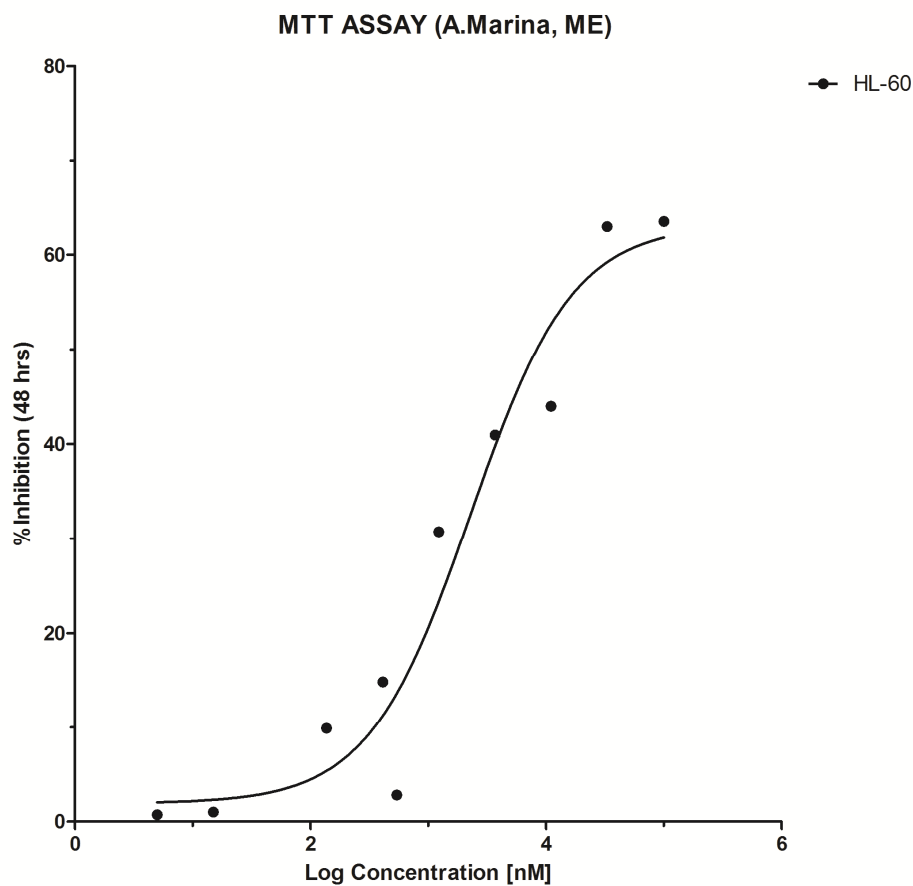


Fig. 1: % Inhibition v/s log conc (ng/ml) of Methanolic extract of *Avicenna marina* on HL60

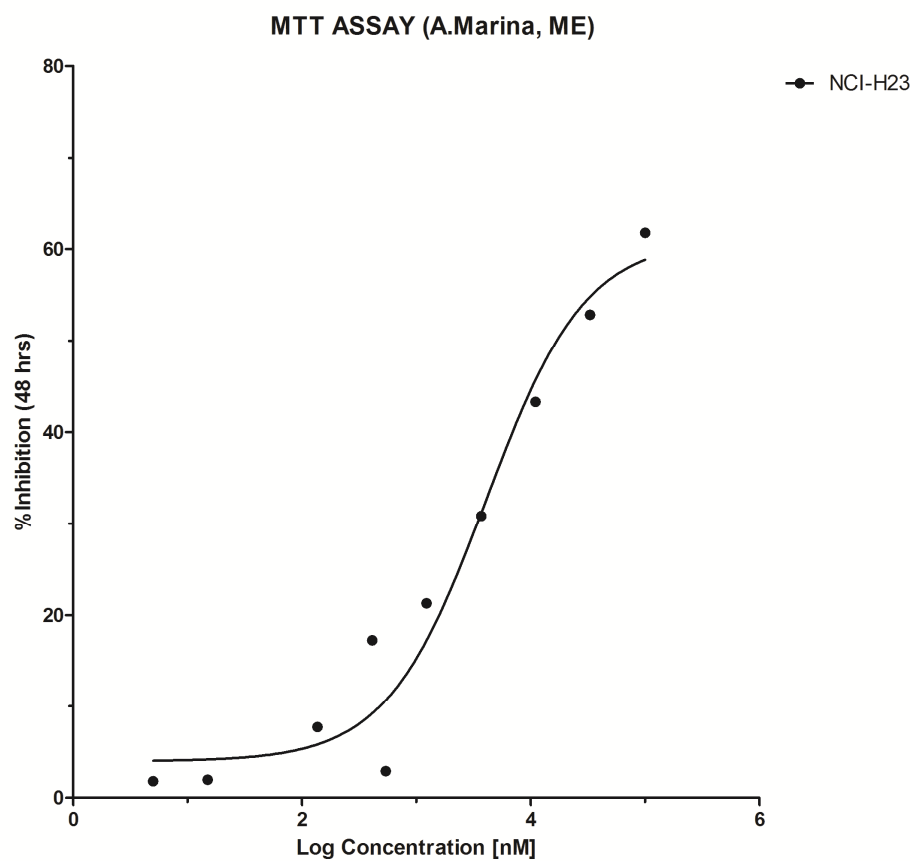


Fig. 2: % Inhibition v/s log conc (ng/ml) of Methanolic extract of *Avicenna marina* on NCI-H23

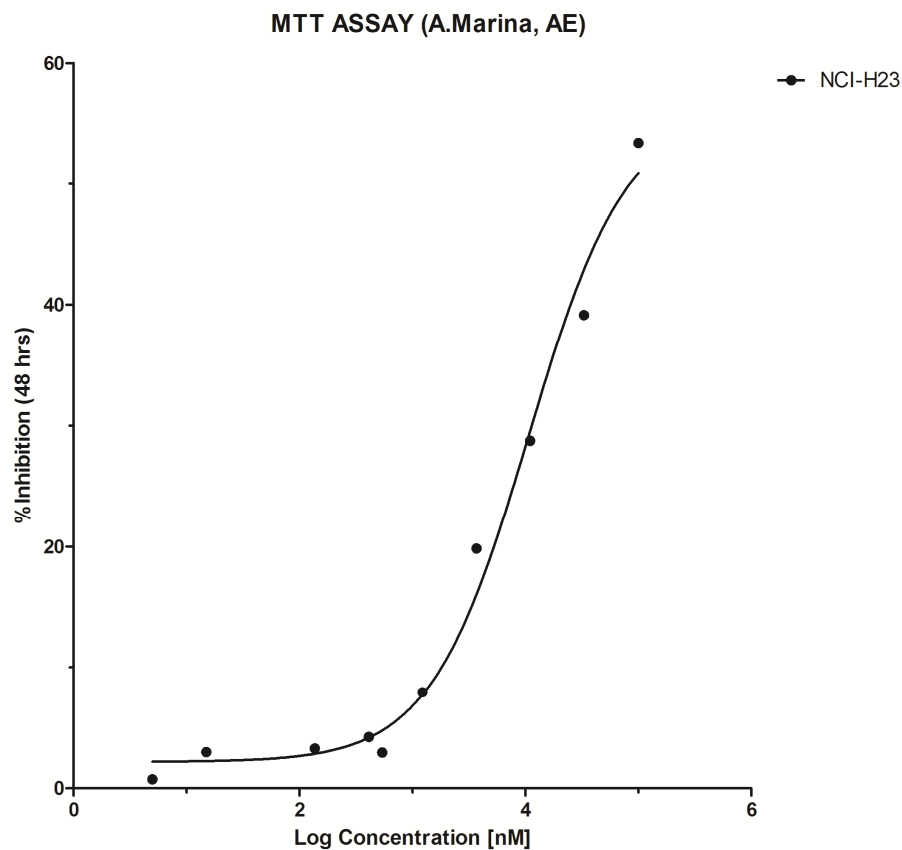


Fig. 3: % Inhibition v/s log conc (ng/ml) of Aqueous extract of *Avicenna marina* on NCI-H23

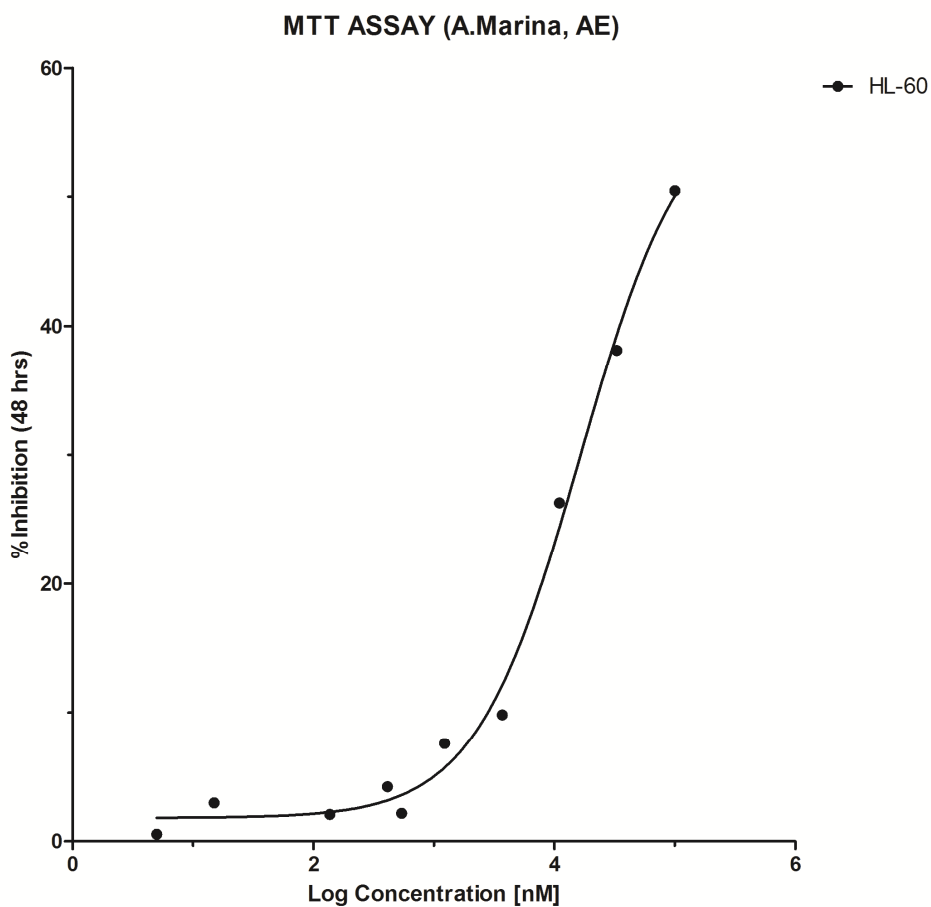


Fig. 4: % Inhibition v/s log conc (ng/ml) of Aqueous extract of *Avicenna marina* on HL-60

From the Table No. 1, we can see that highest activity of methanolic extract have found against NCI-H23 and HL-60 having IC₅₀: 210.987 and 297.934 respectively. But none of extract showed activity against HEK-293T and HepG2 (near to 1000 μ M; can be neglected).

The figure 1 & 2 for methanolic extract show the dose-effect co-relation with maximum linearity in case of NCI-H23 and HL-60 of the six cell lines at R² value being 0.9610 and 0.9452 respectively. The graphical correlation for HepG2 is non-linear. The other strains show insignificant regression with non linearity in the values of change of % inhibition with the increase in concentration. After evaluation, out of the four cell lines, NCI-H23 and HL-60 cell line showed best results in terms of IC₅₀ and regression.

Form the Table No. 2, we can see that highest activity of aqueous extract have found against NCI-H23 and HL-60 having IC₅₀: 220.127 and 281.175 respectively. But none of extract showed activity against HEK-293T and HepG2 (near to 1000 μ M; can be neglected).

The figure: 3 & 4 for aqueous extract show the dose-effect co-relation with maximum linearity in case of NCI-H23 and HL-60 of the six cell lines at R² value being 0.9863 and 0.9927 respectively. The graphical correlation for HepG2 is non-linear. The other strains show insignificant regression with non linearity in the values of change of % inhibition with the increase in concentration. After evaluation, out of the four cell lines, NCI-H23 and HL-60 cell line showed best results in terms of IC₅₀ and regression.

CONCLUSION

The methanolic and aqueous extracts of the plant part(s) used showed prominent anticancer activity having comparable cytotoxic IC₅₀ values with Doxorubicin against NCI-H23, HL-60 and HepG2 tumor cell lines. Further evaluation of cytotoxic activity of these compounds by *in-vivo* study should also be done for its cytotoxicity confirmation as well as ADME profiling. The results described indicate that these compounds could serve as the basis for the development of a new group of cancer chemotherapeutics and certainly holds great promise towards good active leads.

Acknowledgement

I owe a special word of thanks to Pharmacy department, JJT University, Jhunjhunu and Prof. Dr. G. Vidyasagar, Director and Principal, Veerayatan Institute of Pharmacy for providing necessary facilities and cooperation for this present research work.

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