The in vitro anti-tumor efficacy of methanolic leaf extract of Exoecaria Agallocha in cancer cell lines of different tissue origin

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Abstract **- The main goal of this study was to evaluate the antiproliferative activities of leaf extracts of Exoecaria agallocha on cancer cell lines of different tissue origin i.e. Breast, Ovary, Cervix, Melanoma, Colon, and Glioma. The methanolic leaf extract was concentrated to yield the crude extract, which was diluted in DMSO as per the desired concentration. The cytotoxic potential of the extracts was determined by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Assay. The leaf extract exhibited significant cytotoxicity *in vitro* in majority of the cancer cell lines taken for study with IC_{50} values ranging from 13 - 20µg/ml except melanoma cells. Concurrently the cells were exposed to Adriamycin, a chemotherapy medication that is in use for treatment of cancer and both IC_{50} value was compared to evaluate the efficacy of the leaf extract. The result suggests more study on the phytoconstituent of this plant to explore promising anticancer drugs.**

Index Terms **- cytotoxicity, Cancer cell line, MTT Assay. Exoecaria agallocha.**

INTRODUCTION

Cancer, a silent killer, has become a metaphor of anguish and pain in recent times. It sneaks into us sans a warning, and eventually fuels untold miseries and desperation. International Agency for Global Cancer Statistics and Research on Cancer revealed that the estimated new cancer case is around 19.3 million and approximately 10 million deaths in the year 2020 due to cancer. The main types of cancers are Lung, Stomach, Colorectal, Liver, Breast and ovary. Different therapeutic strategies have been recommended which aims to reduce mortality rates, including surgery, radiotherapy and adjuvant chemotherapies, and hormonal therapies. In spite of all these treatment options, treatment of breast and ovarian cancer is very thought provoking. These therapies are linked with quite a lot of side effects, ranging from nausea, vomiting to bone marrow failure and development of multidrug resistance. Therefore, finding natural compounds from plants may provide an alternative cancer treatment. Hence, patients are now turning to complementary and alternative medicine (CAM) to treat the disease. The CAM encompasses medical herbs and plant foods such as fruits, vegetables and spices having many biologically active phytochemicals with various health promoting effect. Natural compounds are not only sources of drugs or drug templates but in many instances, they had been the basic sources of discovery of novel pathway involved in the disease processes. Majority of anti-cancer drugs used today are derived from natural compounds. It has been claimed that drugs derived from natural compounds are more efficacious for cancer patients than those manufactured synthetically. It is known that approximately 10000 out of 500,000 plant species are likely to have medicinal substances of which most located in the rain forests, grasslands and fields. However, only a fraction of these plants have been analyzed and investigated for their therapeutic potential. Majority of the mangrove plants possess the medicinal and commercial importance, such as Rhizophora, Bruguiera, Excoecaria, xylocarpus, Avicennia etc.

Excoecaria agallocha L. (Euphorbiaceae) is a mangrove species widely distributed in marine coastal environments. These multi-stemmed shrubs produce a profuse amount of white and poisonous latex, which causes blistering in skin and temporary blindness when in contact. The bark is grayish with vertical fissures and lenticels, leaves are opposite, ovate or elliptic with a toothed margin and have two basal glands. The woody plants grow up to one half meter in height, normally grows above mean sea level in the

intertidal zone of marine coastal environments and estuarine margins.

The plant is rich sources of primary and secondary metabolites and are endowed with several phytochemicals having pharmaceutical properties. The leaf extract of E. agallocha used for rheumatism, paralysis, cutaneous infection and abortifacient. Alkaloids, carboxylic acid, flavonoids, phenol, saponin, resins, steroids, tannin and sugars from seeds of E. agallocha exhibited anti-inflammatory and analgesic activity [1]. Several Preclinical trials carried out on Secondary metabolites of E. agallocha showed its potential as anti-HIV, anticancer, antibacterial, antidiabetic activities and antiviral agent. Earlier investigations has revealed the presence of diterpenoid, triterpenoids, flavonoids, alkaloids, anthraquinone, phytosterol, fixed oil, tannin , phorbol esters ,free aminoacids, mucilages, glycosides, carbohydrates and lignin [3, 6] Numerous medicines are derived from mangrove and are used for curing several diseases i.e. elephantiasis, abdominal troubles, and skin diseases, sores, leprosy, rheumatism, snakebites, boils, ulcers, diarrhea and hemorrhages. It is a potential source of mosquito repellents, larvicides and for antiviral drug formulations especially against AIDS and jaundice. Several Preclinical trials carried out on Secondary metabolites of E. agallocha showed its potential as anti-HIV, antibacterial, antidiabetic, antiviral activities, but anticancer activity of this plant has not been fully explored. Since cancer is a major health issue in recent time all round the world, it is necessary to explore new anticancer agents from natural sources. Hence, an attempt has been taken to investigate the activities of methanolic leaf extract of E.agallocha in breast and ovarian cancer cell lines.

MATERIALS AND METHODS

Fresh E.agallocha plant material was collected from Bhitarkanika Mangrove Forest, Odisha and transported to the laboratory within 6 hrs. The experts identified the plant and voucher specimen of the whole plant (E.A/07/21) was deposited at the herbarium unit of the KISS, Deemed to be University. The leaves were collected and washed thoroughly with tap water and shade dried (Temp: 41ºC, Relative humidity: 76%), the drying was prolonged for 20-25 days. The dry plant material was coarsely ground in a mixture and distributed equally in conical flask for extraction (percolation method at room temperature).

EXTRACTION OF THE PLANT EXTRACT

The dry leaf material of was soaked with in methanol for three days. On the third day, they were decanted and filtered using bogen sheets. The process was repeated thrice to get the maximum yield of the extract. The pooled filtrate was condensed in a rotary evaporator (Buchi R-200 rota vapor) and dried under air draft for three days to obtain the crude extract. The crude extract was stored at 5ºC until usage.

Cell Culture Conditions and preliminary screening:

Cell lines used for experiments were procured from NCCS, Pune and NCI, Bethesda and maintained in appropriate media and cryo- preserved in liquid Nitrogen. All the cell lines were maintained in RPMI-1640 and DMEM medium supplemented with 10%FCS and used for the experiment. Cells were maintained in 5% CO2. All experiments were carried out in pathogen free conditions in sterile tissue culture room.

The National Cancer Institute (NCI) has played a pivotal role in cancer drug discovery and development. As the drug discovery and developmental arm of NCI, the Developmental Therapeutic Program (DTP) facilitates development of therapeutic agents for cancer and AIDS. The in vitro anticancer drug cell line screen established at DTP is unique in several aspects. The standard NCI protocol was followed for screening of the plant extract. The preliminary screening used by NCI includes three cell lines (MCF 7, NCI H460 and SNB19) of three different tissue origin.

Cell Proliferation Assay:

Approximately 2500 cells per well were plated in 96 well plate and incubated for 24 hours to study the cytotoxicity. The plant extract were dissolved in DMSO and further diluted to get required concentrations for the experiment (10µg to 140µg/ml). Cells were exposed to different concentrations of the plant extracts for 48 hours. After the exposure, cells were taken for cytotoxicity assay with Cell Titer 96® AQueous one Solution which was obtained from Promega Corporation, USA. Cell Titer 96® AQueous one Solution Cell Proliferation Assay is a colorimetric

method for determining the number of viable cells. The solution contains a tetrazolium compound MTS ([3-(4, 5-dimethylthiozol-2-yl)-5-(3-carboxy methoxy phenyl)-2-(4-sulphonyl)-2H-tetrazolium]) and an electron coupling reagent (Phenazine ethosulphate, PES). PES in combination with MTS forms a stable solution. The MTS tetrazolium compound is bio reduced by cells into a colored formazon product that is soluble in tissue culture medium. NADPH produced by the dehydrogenase enzymes in metabolically active cells accomplishes this conversion. Cells were exposed to the solution for three hours and the OD was taken at 492 nm using the Elisa Reader (Thermo Electron Corporation, Finland).

STATISTICAL ANALYSIS

Student's t-test was used to analyze intergroup differences. Experiments were repeated at least three times and data represented as the mean ± SD. p - value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Table 1 -3, depicts the IC50 value of plant extract exposed to different cancer cell line and IC50 values obtained from Adriamycin treated cells. Fig A-F shows the survival percentage of different cells at varied concentrations on the antiproliferative effect of plant extract on cancer cell lines of different tissue origin as evaluated through micro-culture tetrazolium assay (MTT). The multiple concentrations of methanolic leaf extract of E.agallocha was used. Inhibition of cell growth was observed on preliminary screening (recommended cell lines, NCI protocol), based on which the screening was performed in other cell lines of different tissue (Breast, Glioma, Colon, Melanoma, cervix and ovary) origin. Adriamycin, an established therapeutic agent in cancer treatment, was used as positive control. Table 1 depicts the IC50 values of cells with varied concentrations ranging from 10 µg/ml to 140 µg/ml. The experiments were carried out in triplicates and IC50 value was calculated for each set of experiment. IC50 value revealed that the leaf extract exposed to Breast and Ovarian cancer cells was very effective however in BT549 (Breast cancer cells) and Ovcar-3 (Ovarian cancer cells) the effect is not prevalent. On Glioma cells remarkable growth inhibition effect was also observed (IC50 19-20 µg/ml). In colon cancer cells, the effect was as par with Glioma except HCT15 on which the effect was not conspicuous. All the treated groups were compared with control 293 HEK (human embryonic kidney cells), and there was significant inhibition of cell growth observed ($p<0.05$) in the cells exposed to plant extract of different concentrations.

In our study, we used methanol, as it was recognized as the most effective solvent for the extraction and have highest extraction yield of various secondary metabolites present in plant parts [2]. Stock solution of the extract was prepared in DMSO at concentrations (100mg/ml) and serially diluted to make sure that the final concentration of DMSO were not more than 0.2% and not toxic to the cells. Therefore, the cytotoxic effect observed in this experiment was due to the test material but not due to the solvent.

Natural products have played a dominant role by contributing potential bioactive novel products, which are in use for treatment and prevention of cancer. Compounds such as Taxol, Vinblastine and Camptothecin are of plant origin and these compounds are reported to improvise the chemotherapy of some cancers [5]. In general, natural product research is a powerful approach to ascertain biologically active compounds with unique structures and mechanism of action. Given the immeasurable diversity of nature, it is rational to screen the plant parts by which chemical leads can be generated that may have efficacy to interact with most or possibly all therapeutic targets. Hence, continuous effort to search new anti-cancer compounds in plant medicines and tradition foods is a realistic and promising strategy for cancer treatment and prevention.

In this study E. agallocha leaf extract was evaluated and found to be cytotoxic in all types of cancer cell taken for study except melanoma. The American National cancer Institute (NCI) set the limit of activity for crude extracts at 50% inhibition (IC50) as new anti-cancer agent proliferation of less than 30 µg/ml of 48 hrs. However, a crude extract with IC50 less than 20 µg/ml is considered highly cytotoxic. The results of the present study showed potent cytotoxic effects on cancer cells of different issue origin. It may be due to existing phytochemicals in the extract as mentioned in earlier reports of several investigators. In another report the cytotoxic effect of Ethnolic stem extract of E.agallocha has been observed in different cancer cell

lines i.e. Miapaca-2,BxPC-3,PANC-1 and Capan -1 [4] which is in accordance with the present study.

CONCLUSION

This finding recommends for further studies on the active compounds for proper assessment of their chemotherapeutic properties as well as their possible development as promising anticancer drug.

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Table 1-3: IC 50 values of leaf extract of E.agallocha and Adriamycin in cancer cell lines

Table -1:

Cancer types	**Melanoma**							**CNS**	
Cell line	M14	SKMEL5	SKMEL2	SKMEL28	MALME3M	UACC62	UACC257	U251	SNB19
IC50(µg/ml) of Plant extract	47.5± 4.12	89.2±4.62	30.0±2.12	29.8±2.46	35.0±3.12	34.0±2.90	26.0±3.12	19.44±1.14	19.84±1.60
IC50 of Adriamycin(µg/ml	4.50±0.80	3.38±0.65	4.20±0.48	3.90±0.30	3.36 ±0.50	4.40± 0.46	4.10 ± 0.60	4.46±0.68	4.23±0.82

Table – 2:

Cancer types	Breast				Ovary		Colon			
Cell line	MDAMB231	MCF7	T47D	BT549	Ovcar 3	Ovcar5	HCC2998	Colo205	HCT15	KM12
IC50(µg/ml) of Plant extract	15.56 ± 1.14	20.24 ± 1.16	19.20 ± 0.15	63.42 ± 5.12	39.44 ± 1.14	19.84 ± 0.60	20.28 ± 0.56	12.0 ± 2.12	39.0 ± 3.82	20.0 ± 1.70
IC50 of Adriamycin(µg/ml	5.60 ± 0.62	4.66 ± 0.72	3.40± 0.42	3.46 ± 0.92	4.15 ± 0.82	4.2 ± 0.60	6.70 ± 0.90	4.60 ± 0.18	4.80 ± 0.20	4.25± 0.34

Table – 3:

Cancer types	Cervix		
Cell line	HeLa	SIHA	C33A
IC50(µg/ml) of Plant extract	18.60 ± 2.20	23.42 ±1.90	39.9 ± 3.10
IC50 of Adriamycin(µg/ml	4.40 ± 0.40	4.10±0.55	6.80 ± 0.46

Figure A-F: Percentage of cell survival of different cancer cell lines upon the exposure of plant extract.