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STUDIES ON ISOLATION AND CHARACTERIZATION OF PINK *CATHARANTHUS ROSEUS* ENDOPHYTES FOR THEIR ANTIPROLIFERATIVE ACTIVITIES.

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ABSTRACT

Five different types of fungal endophytes were isolated from the different parts of Pink Catharanthus roseus plant. Two endophytes from the leaves (Sclerotium rolfsii and Alternaria spp),one from Stem (Aspergillus nidulans) and two from roots (Colletotrichum spp. and Geotrichum spp.)were isolated from the plant parts for the study of their antiproliferative activities. The cytotoxicity of all isolates was tested on HeLa and MCF7(breast cancer cells) cell lines. Endophytic fungal extracts of pink Catharanthus roseus plant showed the cytotoxicity from 11% to 80%.The isolates from leaf-2 and root –2,shown the highest activity against HeLa cell line. The isolated endophytes had shown the cytotoxicity from 15 to 41% against MCF-7 cell line. The isolates from leaf-1 and leaf-2, shown the highest activity against MCF-7 cell line.

KEYWORDS: *characterization of Pink , antiproliferative activities , Catharanthus roseus .*

INTRODUCTION

Catharanthus roseus is a medicinal plant belongs to the family Apocynaceae, native and endemic to Madagascar. The plant is also known by the names such as *Vinca rosea, Ammocallis rosea* and *Lochnera rosea.* The plant has been put to traditional use for the treatment of a wide variety of ailments worldwide since ages.
Endophytic fungi grow inside the plant parts in the intercellular spaces of cells and as intracellular for at least part of their life cycle without propducing any disease symptoms in their host. Within hosts, fungi inhabit all available tissue including leaves with petioles, stems, twigs, bark, root, fruit, flower and seeds. Fungi have proven themselves as invaluable sources of natural products for the industrial as well as biomedical development for decades. A variety of relationships exist between fungal endophytes and their host plants. They improve the resistance of host plants to adversity by secretion of bioactive metabolites. It is cultivated mainly for its alkaloids, A number of endophytic fungi have been isolated from many plants for, antidaibetic, anti tumor, anticancer activity etc. (Jaleel *et al*., 2009). The extracts have demonstrated significant anticancer activity against numerous cell types (EL-Sayed and Cordell, 1981).

MATERIALS AND METHODS:

A) Collection of Plant Sample:

The mature White *Catharanthus roseus* plants were collected in sterile Polythene bags from the campus of Lokmangal Science and Entrepreneurship College, Wadala.Solapur.and used within two

hours. The plants were identified by the expertised Botanist. The plant sample was surface sterilized with two drops of Savlon, then washed frequently with tap water until the savlon removes. Then the plant was cut in to parts like root, stem and leaves .The explants were treated with mercuric chloride (0.1%, 0.5%, 1% for leaf, Stem and Root respectively) for two minutes, and finally rinsed with sterile distilled water for three times before sterilization separately.

B) Isolation of entophytes:

The leaf portion of healthy leaves, cut from the midrib (0.5 cm), stem and root segments were placed on 20 ml PDA medium in a Petri dish and incubated for 15days at 27^{0}C ± 1 ^{0}C .The fungal growth was observed on the culture medium after incubation period.

Total 05 different endophytes were isolated from Pink *Catharanthus roseus* plants. Table 1 showed the number of isolates, isolated from Leaf, Stem and Root explants of Pink *Catharanthus roseus* plants. Kumar et al. (2013) also isolated 52 endophytic fungi from the leaves of *Catharanthus roseus* plant which were unusual and slow growing. The isolates from the different plant parts are shown in the fig.1

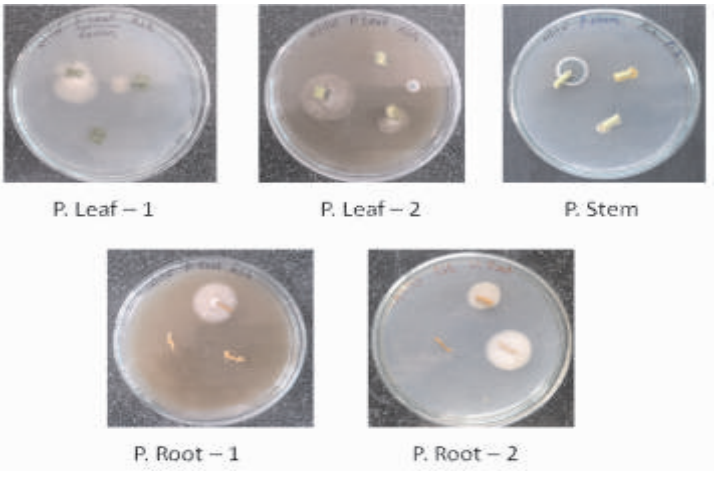

Plate 1: Isolates of Leaf, Stem and Root of Pink *Catharanthus Roseus* on Potato Dextrose Medium.

C) Identification and Characterization of isolates.

The slides of fungal species were prepared from fungal isolates by staining with Lacto phenol (Cotton Blue) and the identification was carried out using standard literature. All the isolates were identified by the expert mycologist, Plate-.1 shows the identified entophytic fungi from Leaf, Stem and Root and table -1 shows the list of isolates.

Characterization of 05 endophytic fungi was carried out by staining with Lactophenol Cotton Blue and observed under microscope. For this, Kumar et al. (2013) and Mahajan et al. (2014) method was used.

Table 1: Isolated endophytic fungi from Pink *Catharanthus roseus.*

Plant Material	No. of Isolates.	Name of Isolate Identified
Pink Leaf	2	*Sclerotium Rolsii*
		Alternaria spp.
Pink Stem	1	*Aspergillus nidulans.*
Pink Root	2	*Colletotrichum spp.*
		Geotrichum spp.

D) Production of Alkaloids from Entophytic Fungi-

The fungal isolates were used for the production of alkaloids by two stage fermentation method.

Stage I:

i) The fungal isolates were grown in 500 mL Erlenmeyer flasks (containing 100 mL MGYP medium composed of malt extract =0.3 %, glucose =1.0 %, yeast extract =0.3 % and peptone =0.5 %). The flasks were inoculated with the7 days old isolates (fungal mycelium) grown on PDA slants. The inoculated flasks were incubated at 25-27°C on a rotary shaker (240 rpm) for 7 days. These cultures were used as seed cultures.

Stage II:

i) 10 ml seed cultures were transferred to 500 mL flask (containing 100 mL vinca alkaloids (VM-1) medium.
ii)The flasks were incubated at 25-27oC on a rotary shaker (240 rpm) for 20 days
iii)After 20 days of incubation, the culture was harvested and passed through four layers of muslin cloth to separate the mycelia from the culture broth. The culture filtrates was lyophilized and extracted with equal volumes of ethyl acetate each time.

Alkaloids from Culture Filtrate:

All five endophytic fungi screened for *Vinca* alkaloids, the culture filtrates was extracted with equal volume of ethyl acetate yielded from the solvent, labeled and stored in vials. Plate- 2 shows different culture filtrate of Vinca alkaloids, which are extracted and stored in vials.

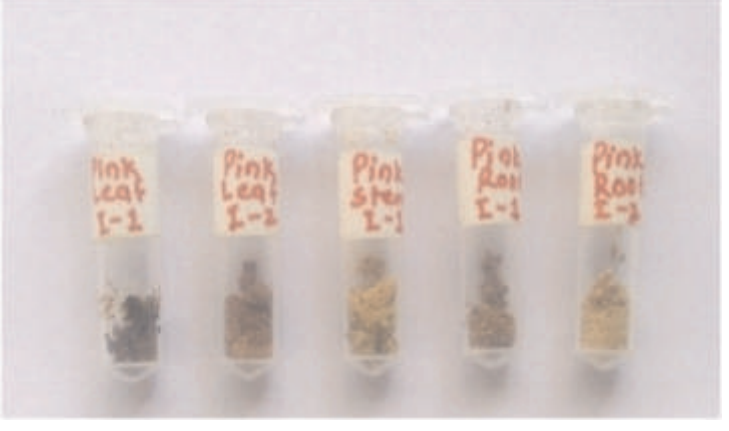

Plate- 2: Extraction of different isolates from Leaf, Stem and Root of Pink *Catharanthus roseus.*

E) Cytotoxicity assay:

Materials:

The two cell lines HeLa and MCF – 7 (human breast cancer), were purchased from NCL institute, Pune. These Cell lines were subcultured in Eagles minimal essential medium with 10% FBS and maintained in a 5% CO_2 incubator at 37°C.

MTT assay:

HeLa cell line and MCF-7 cell lines were maintained in Eagle's minimum essential medium (2mM L-glutamine and Earle's salts). The cytotoxicity was evaluated by MTT [3-4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] according to Mossmann et al. HeLa and MCF-7 cell cultures ($5x10^5$cells / mL) were cultured in 96-well flat bottomed microtitre plate and incubated for 48 h at 37 ^{0}C in a humidified 5% CO_2 incubator. Different concentrations of the endophytic extracts were added to the wells. The plate was incubated for 48 h at 37°C in a humidified incubator with 5% CO_2. MTT (5mg/ml) was prepared in phosphate

buffer saline (PBS). MTT (10 µl) was added to each well and incubated in dark for 4 hours in CO2 incubator. The supernatant was removed from the wells and the plate was washed three times with Dulbecco's formula PBS (pH 7.3). DMSO (100µl) was added to each well. The absorbance of the samples were measured at 570 nm after 30 min.

Cytotoxicity Assay:

The cytotoxicity of endophytic fungal extracts of Pink *Catharanthus roseus* was tested on HeLa and MCF7 (breast cancer cells) cell lines. Endophytic extracts showed the cytotoxicity from 11% to 80%. The isolates Pink leaf-2, Pink root – 2, shown the highest activity against HeLa cell line. The isolated endophytes had shown the cytotoxicity from 15% to 41% against MCF-7 cell line. Pink leaf-1, Pink leaf-2, shown the highest activity against MCF-7 cell line. Similar types of results were obtained by Kuriakose et al. (2014) when the endophyte *Fusarium solani* isolated from *Datura metel* and tested on human cancer cells. The results of cytotoxicity activity of leaf, stem and root of endophytic fungal extracts of Pink *Catharanthus roseus* is described in table 2, 3 and fig.1, 2.

Table.2- Cytotoxicity Assay of Pink *Catharanthus roseus* against HeLa cell line:

Extract	Percent Inhibition (%)			
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Pink Leaf – 1	18.67	20.48	25.30	34.93
Pink Leaf – 2	11.44	18.07	47.59	79.51
Pink Stem – 1	19.87	30.72	45.18	52.40
Pink Root – 1	41.56	42.77	50.00	63.85
Pink Root – 2	45.78	74.09	78.31	78.31

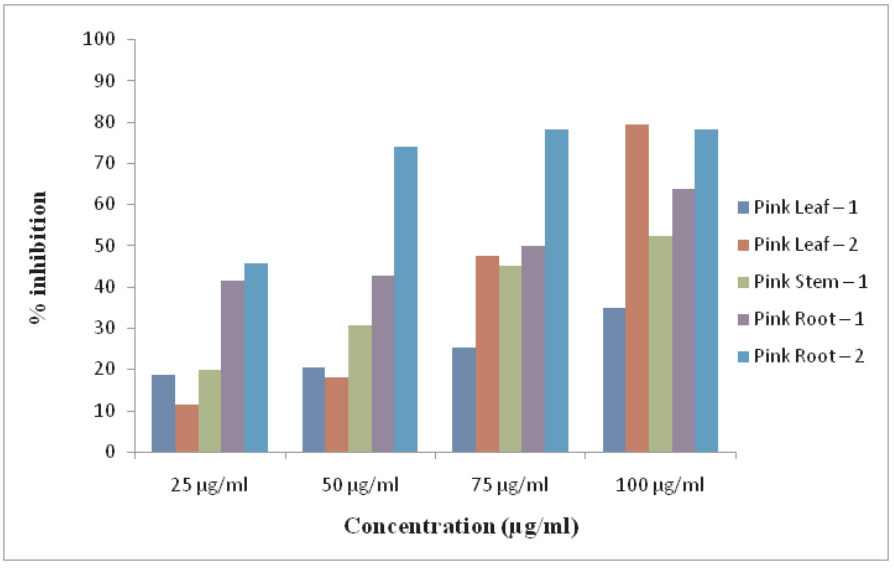

Fig 1: Cytotoxicity of endophytic fungal extract of leaf, stem and root of Pink *Catharanthus roseus* on HeLa cell line.

Table 3: Cytotoxicity of endophytic fungal extract of leaf, stem and root of Pink *Catharanthus roseus* on MCF 7 Cell line.

Extract	Percent Inhibition (%)			
	25ug/ml	50ug/ml	75ug/ml	100ug/ml
Pink Leaf – 1	32.15	36.74	39.92	41.34
Pink Leaf – 2	15.90	28.97	36.39	38.86
Pink Stem – 1	15.19	19.78	27.20	28.26
Pink Root – 1	26.14	28.97	30.74	32.86
Pink Root – 2	20.14	23.67	30.38	32.50

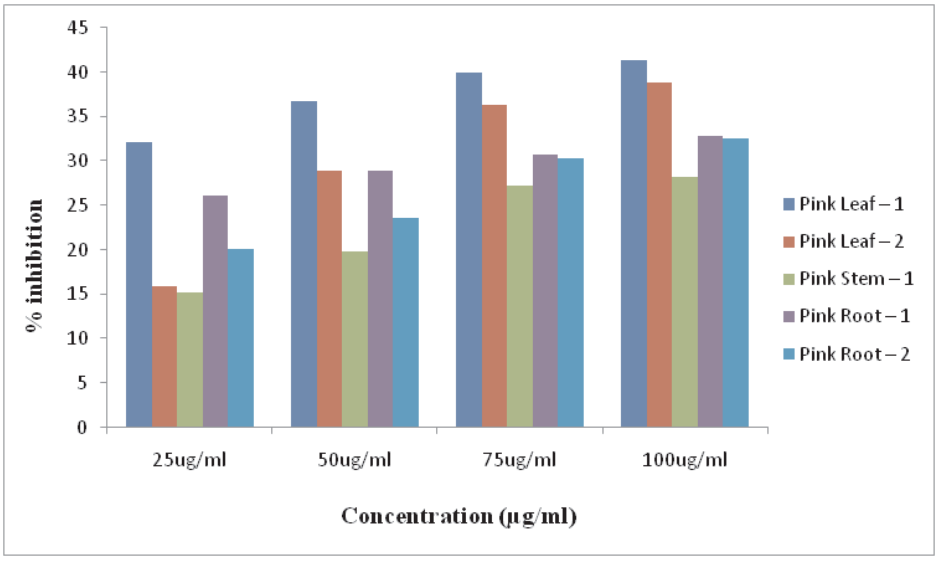

Fig 2: Cytotoxicity of endophytic fungal extract of leaf, stem and root of Pink *Catharanthus roseus* on MCF 7 Cell line.

Thus, all the endophytic extracts showed the potent cytotoxicity due to the presence of major vinca alkaloids like serpentine, catharanthine, ajmalicine, etc. along with flavonoids, glycosides and anthraquinones.

The Cytotoxicity of endophytic fungal extracts of Pink *Catharanthus roseus* was tested on HeLa and MCF7 (breast cancer cells) cell lines. Endophytic extracts showed the cytotoxicity from 11% to 80%.

RESULTS AND DISCUSSION:

The cytotoxicity of endophytic fungal extracts of Pink *Catharanthus roseus* was tested on HeLa and MCF7 (breast cancer cells) cell lines. Endophytic extracts of pink *Catharanthus roseus* plant showed the cytotoxicity from 11% to 80%. The isolates Pink leaf-2, pink root – 2, shown the highest activity against HeLa cell line. The isolated endophytes had shown the cytotoxicity from 15 to 41% against MCF-7 cell line. Pink leaf-1, pink leaf-2, shown the highest activity against MCF-7 cell line.

Cytotoxicity Assay of Pink *Catharanthus roseus* against HeLa cell line was max as 45.78 and min as 11.44 observed at 25µg/ml, whereas it was maximum as74.09 and min as 18.07 in at 50µg/ml in root-2 and leaf-2 respectively. Similarly in root-2 and leaf -1 it was max as78.31 and min as 25.30 at 50µg/ml respectively whereas it was maximum as78.31 and min as 34.93 in at 100 µg/ml in root-2 and leaf-1 respectively. Similar

results are also expressed graphically in fig.1

Cytotoxicity of endophytic fungal extract was observed maximum as 32.15 36.74, 39.92 and 41.34 in leaf -1 and minimum was observed as 15.19,19.78,27.20 and 28.26 in stem 1respectively at 25ug/ml, 25ug/ml,25ug/ml and 25ug/ml on MCF 7 Cell line. . Similar results are also expressed graphically in fig.2

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