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REVIEW OF RESEARCH

ISOLATION AND CHARACTERIZATION OF PINK *CATHARANTHUS ROSEUS* ENDOPHYTES FOR THEIR ANTIOXIDANT ACTIVITIES.

Fig.: Pink *Catharanthus Roseus.*

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ABSTRACT:-

Five different types of fungal endophytes were isolated from the different parts of Pink Catharanthus roseus plant. Two endophytes (Sclerotium rolfsii and Alternaria spp)were isolated from the leaves,one from Stem (Aspergillus nidulans) and two from roots (Colletotrichum spp. and Geotrichum spp.).They were further used for their study of antitoxidant activities. Identification of isolates, detection of antioxidant enzymes, culturing of entophytic fungi for Vinca alkaloid production, quantitative determination of antioxidant activities etc. were carried out. DPPH radical scavenging activity: and determination of catalase and ascorbate Peroxidase activities was carried out prior to study.

KEY WORDS: *endophytes, antioxidant, DPPH, Catalase, peroxidase.*

INTRODUCTION-

The term Endophyte (Gr. *endon*, within; *phyton*, plant) was first coined by De Bary (1866) .The bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing in the tissues of host plants (as inter-or intra-cellular) typically causing no apparent symptoms of disease (Wilson, 1995). Fungi are heterotrophic eukaryotes with unique characteristics that set them well apart from both plants and animals. Fungi are so versatile in adapting themselves that they could occupy a variety of ecological habitats. They can be isolated from unusual habitats such as peat, soil, petroleum polluted soils, from the tissues of plants and animals. Fungi are also known to establish themselves in healthy plant tissues without causing any "injury or visible symptoms. Such an association is generally termed as endophytic association. Present study was carried out to isolate and characterize endophytic fungi from *C. roseus* and to screen out valuable antioxidant activity. *Catharanthus roseus* (L.) G. Don, is a tropical plant that belongs to the Apocynaceae family. The plant is of interest because it is capable of synthesizing useful secondary metabolites, including vendolin, vincristine and vinblastine, which were prevalently used in the treatment of cancer (Lounasmaa et al.1989). The fungal endophytes have antibacterial, antifungal, antidaibetic,anticancer,antiviral,therapeutic,anti ulcer,anthelminthic,antioxidant, anti diarrheal properties.

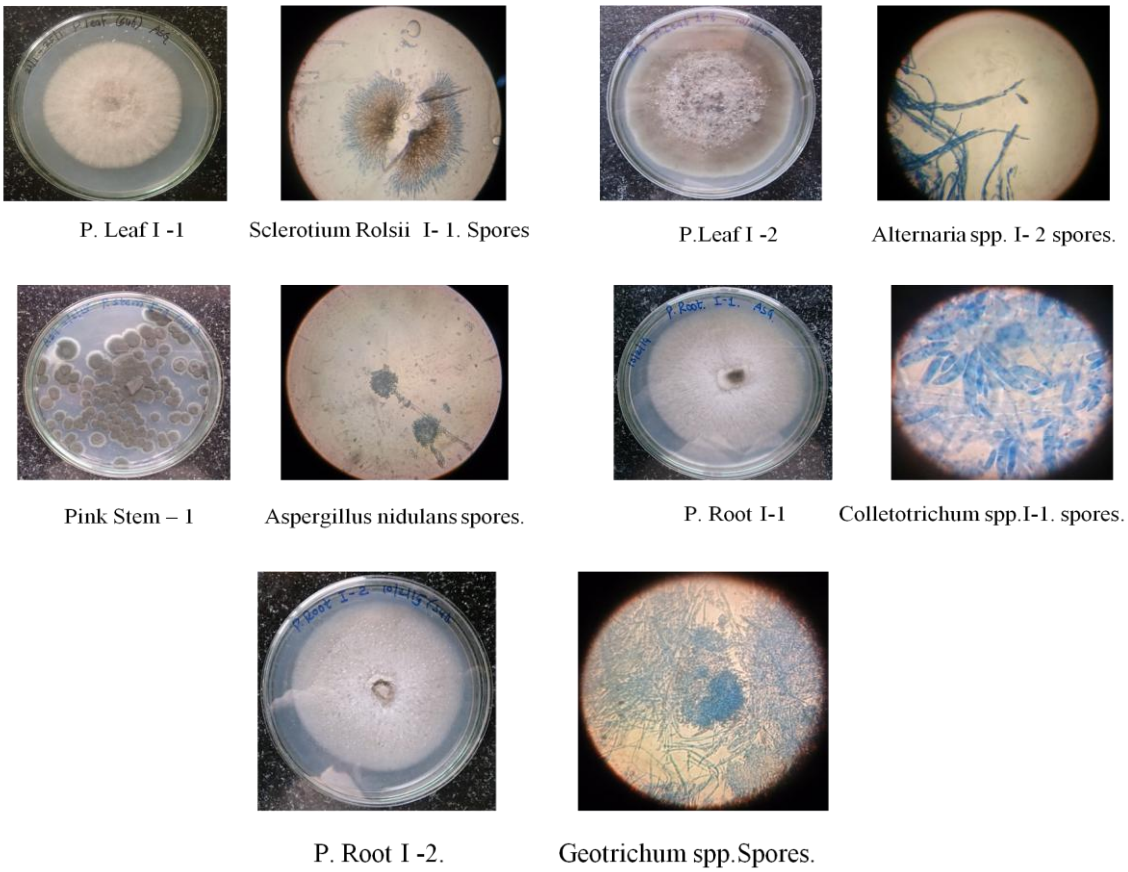
MATERIALS AND METHODS:

A) Collection of Plant Sample:

Pink *Catharanthus roseus* plant was collected from the college campus of Lokmangal Science and Entrepreneurship College, Wadala. The plant was collected in sterile ploythene bag and used for the further study within two hours. The plant was surface sterilized with two drops of savlon, then washed frequently with tap water until the savlon removes. Then the plant was cut in two parts like root, stem and leaves .The explants of leaf, stem and root were treated with mercuric chloride 0.1%, 0.5%, 1% for surface sterilization respectively for two minutes, and finally rinsed with sterile distilled water for three times before sterilization separately.

B) Isolation of entophytes:

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



P. Leaf I -1 | Sclerotium Rolsii I- 1. Spores | P.Leaf I -2 | Alternaria spp. I- 2 spores.

Pink Stem – 1 | Aspergillus nidulans spores. | P. Root I-1 | Colletotrichum spp.I-1. spores.

P. Root I -2. | Geotrichum spp.Spores.

Plate-1: Agar plates showing endophytic isolated fungi from Pink *Catharanthus roseus.* After the incubation period the fungal growth was observed in each plate (Plate-1)

C) Identification and of Isolates:

The slides of fungal isolates were prepared 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, from department of Botany. Plate-.1 shows the identified entophytic fungi from leaf, stem and root Table -1 shows the list of fungal isolates.

Table 1: List of isolated entophytic fungi from Pink *Catharanthus roseus*

Sr.No.	Isolate	Endophytic Fungi
	Pink Leaf -1	*Sclerotium rolsii*
	Pink Leaf – 2	*Alternaria spp.*
	Pink Stem - 1	*Aspergillus nidulans.*
	Pink Root – 1	*Colletotrichum spp.*
	Pink Root – 2	*Geotrichum spp.*

D). Characterization:

Characterization of five entophytic fungi was carried out by staining fungi with Lacto phenol Cotton Blue and observing under the microscope (Kumar et al. 2013 and Mahajan et al.2014 method).On the basis of morphology and reproductive characters, the endophytes were identified and listed as in table-1. The extraction of different isolates has shown in plate-3.

E) 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 % + 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^{o}C on a rotary shaker (240 rpm) for 7 days. These cultures were used as seed cultures.

Stage II:

i) 10 ml of seed cultures were transferred to 500 mL flask (containing 100 mL vinca alkaloids (VM-1) medium.

ii) The flasks were incubated at 25-27^{o}C on a rotary shaker (240 rpm) for 20 days. **(Plate-2)**

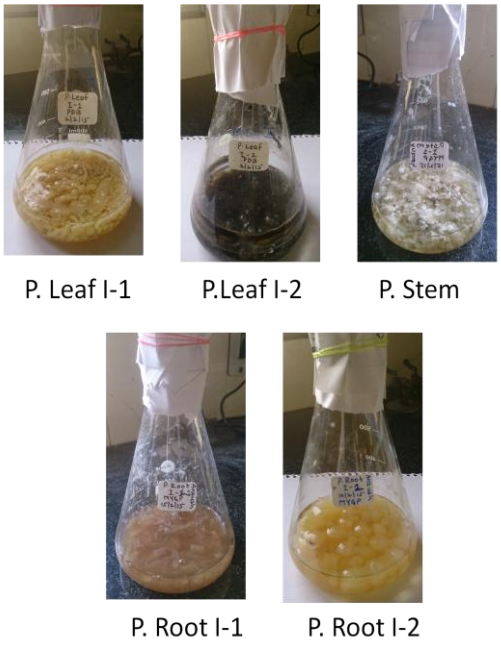


Plate-2. : Production of Alkaloids from Entophytic Fungal Isolates from *Catharanthus Roseus.*

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.

Plate- 3: Extraction of different isolates from Leaf, Stem and Root of Pink *Catharanthus roseus.*

F) Detection of Antioxidant Enzymes:

1. Determination of Catalase:

Catalase converts H_2O_2 into oxygen and water. Catalase has a double function as it catalyses the following reactions; (Sadasivam and Manickam (2008). Splitting of hydrogen peroxide in to water and oxygen;

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2\uparrow$$

Catalase (EC 1.11.16) activity was assayed by measuring absorbance of H_2O_2 at 240 nm. The 0.2 gm of fresh samples were ground with 1 ml 50 mM phosphate buffer (pH 7.4) in prechilled morter and pestle at -4°C. The mixture was centrifuged at 200×g at 4°C for 10 min. The reaction mixture contained 3 ml of reaction buffer (19 mM H_2O_2 in 50 mM phosphate buffer, pH 7) and 04 ml of sample extracts. The changes in absorbance of the reaction were monitored at 240 nm for 3 min. the extinction coefficient of H_2O_2 is 43.6 M-1cm-1. (Table-2)

Catalase Test:

The endophytic fungal leaf extract showed highest Catalase activities whereas both stem, root endophytic fungal extract showed less Catalase activities (fig.1)

A) Catalase Test of endophytes:

Table 2: Catalase activity (µmole H_2O_2/min/mg protein) of leaf, stem and root of endophytic fungal extract.

Extract	CAT activity (µmole H_2O_2/min/mg protein)
Pink Leaf – 1	567.08
Pink Leaf – 2	378.45
Pink Stem – 1	309.62
Pink Root – 1	137.6
Pink Root – 2	477.76

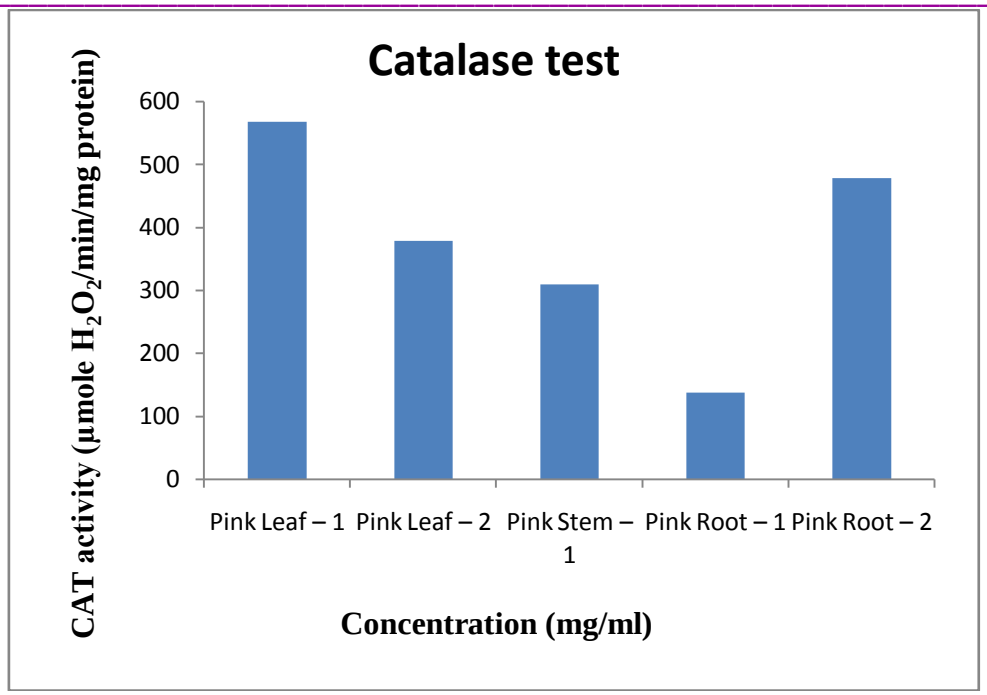


Fig. 1: Catalase activity (µmole H_2O_2/min/mg protein) of leaf, stem and root of endophytic fungal extract of Pink *Catharanthus roseus.*

2) DPPH radical scavenging activity:

DPPH radical scavenging activity of extract was measured by the method described by Brand-Williams *et al* (1995). The sample stock solution (1 mg/ml) was diluted to final concentrations of 500, 250, 100 and 50 µg/ml in methanol. A total of 1 ml of a freshly prepared DPPH solution (20 mg/l) was added to 500 µl of sample solution of different concentrations and allowed to react at room temperature for 30 minutes in dark condition. After 30 min., the absorbance was measured at 517 nm and converted into the percentage antioxidant activity using the following formula:

DPPH radical scavenging activity (%) = [(Acontrol – Aextract)/Acontrol] ×100 **(Fig 2)**

Methanol plus plant extract solution was used as a blank while DPPH solution plus methanol was used as a negative control. The positive control was DPPH solution plus glutathione. The IC50 values were calculated by using nonlinear regression analysis of dose dependent curves. IC50 value is the sample concentration required to scavenge 50% of the DPPH free radicals (Mosquera *et al.,* 2007).

Quantitative Determination of Antioxidant Activity:

DPPH Assay:

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Oxidation reactions can produce free radicals. DPPH is purple in color. When the O.D. is measured at 517 nm, the colour turns from purple to yellow as the molar absorptivity of the DPPH radical is at 517 nm. **(Table.3)**

Table - 3. DPPH radical scavenging assay of Pink *Catharanthus roseus* endophytic extract with Ascorbic acid and Glutathione as standards.

Extract	Percent Inhibition (%)			
	50 µg/ml	100 µg/ml	250 µg /ml	500 µg/ml
Pink Leaf – 1	78.44	82.63	93.41	95.80
Pink Leaf – 2	80.83	85.62	95.80	98.20
Pink Stem – 1	61.49	77.01	84.47	93.16
Pink Root – 1	32.91	39.13	63.35	78.26
Pink Root – 2	10.17	10.17	32.33	38.92
Ascorbic Acid	28.91	90.36	92.77	95.18
Glutathione	0.9	29.71	47.16	55.18

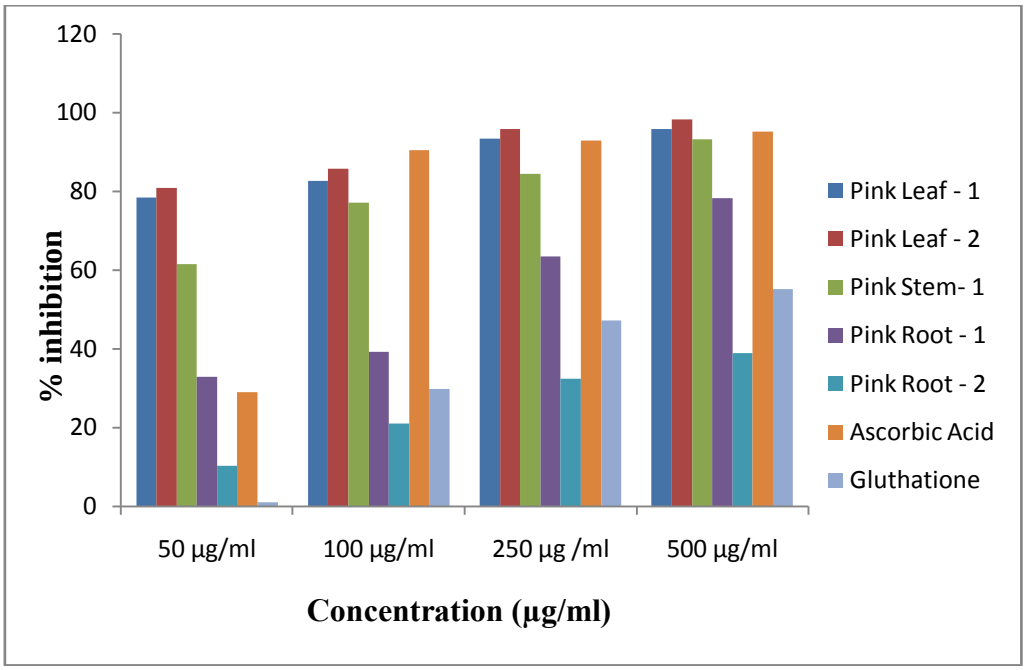


Fig 2: DPPH radical scavenging assay of Pink *Catharanthus roseus* endophytic extract with Ascorbic acid and Glutathione as a standard.

3) Reducing Power Assay:

The reducing power assay of *in vitro* cultures of *Catharanthus roseus* was carried out as described by Yildirim *et al*. (2001). Different concentrations of endophytic fungal extract of leaf, stem and root suspension culture (50-500 µg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricynide (2.5 ml, 1%). The mixture was incubated at 50ºC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) and the absorbance was measured at 700 nm and compared with ascorbic acid as a standard. Increased absorbance of the reaction mixture indicated increased reducing power.

Table.4. Showing reducing power of endophytic fungal extracts of leaf, stem and roots. ascorbic acid as a standard.

Extract	Concentration (µg/ml)			
	50 µg/ml	100 µg/ml	250 µg /ml	500 µg/ml
Pink Leaf – 1	0.067	0.073	0.121	0.194
Pink Leaf – 2	0.009	0.030	0.077	0.098
Pink Stem – 1	0.093	0.199	0.134	0.245
Pink Root – 1	0.009	0.016	0.041	0.064
Pink Root – 2	0.017	0.026	0.034	0.044
Ascorbic Acid	0.454	0.652	0.722	0.962

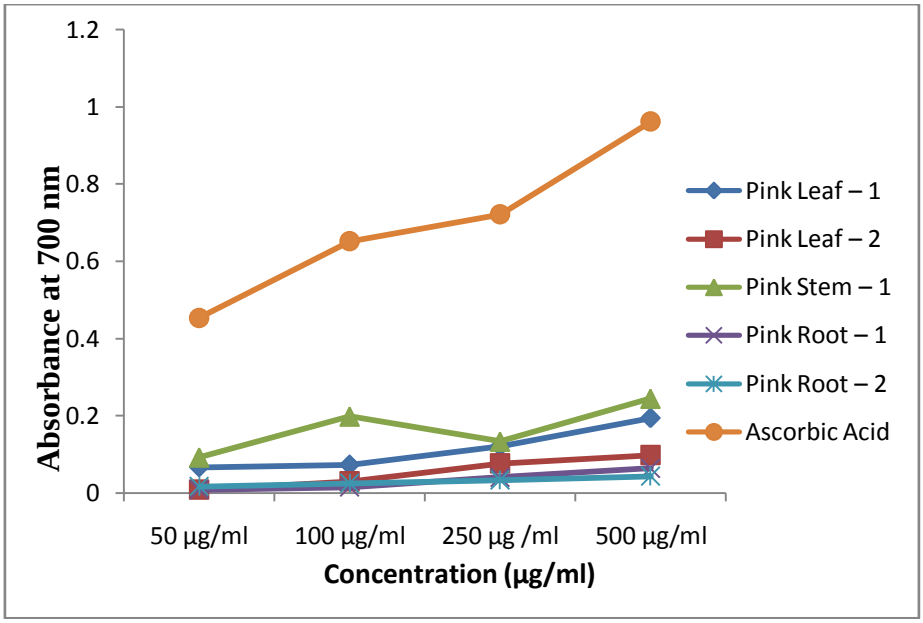


Fig 3: Reducing power of endophytic fungal extracts of leaf, stem and roots of Pink *Catharanthus roseus* using ascorbic acid as a standard.

4) Determination of Ascorbate Peroxidase:

Ascorbate peroxidase (APO) (EC 1.11.17) activity was assayed according to the method of Nakano and Asada (1980) and Sairam *et al.* (1998) with a slight modification by monitoring the decrease in ascorbic acid content at 290 nm as ascorbic acid was oxidized. The 0.2 gm of fresh samples were ground with 2 ml 50 mM phosphate buffer (pH 7.4 containing 1% polyvinyl pyrrolidone and 1 mM ascorbate) in prechilled morter and pestle. The reaction mixture contained 1.7 ml of 50 mM potassium phosphate buffer (pH 7), 0.5 ml 3 mM ascorbic acid, 0.1 ml of 3 mM EDTA, 0.5 ml of 1 mM H_2O_2 and 0.2 ml of crude enzyme extract making up a total volume of 3 ml. The reaction was started with the addition of H_2O_2 and the absorbance was measured at 290 nm spectrophotometrically for 3min. The extinction coefficient of APO is 2.8 mM-1cm- 1. (Table.5)

Table 5: Showing ascorbate peroxidase activity (µmole Ascorbate oxidized /min/mg protein) of endophytic fungal extracts.

Extract	APO activity (µmole Ascorbate oxidized /min/mg protein)
Pink Leaf – 1	9.75
Pink Leaf – 2	6.25
Pink Stem – 1	25.0
Pink Root – 1	20.5
Pink Root – 2	4.25

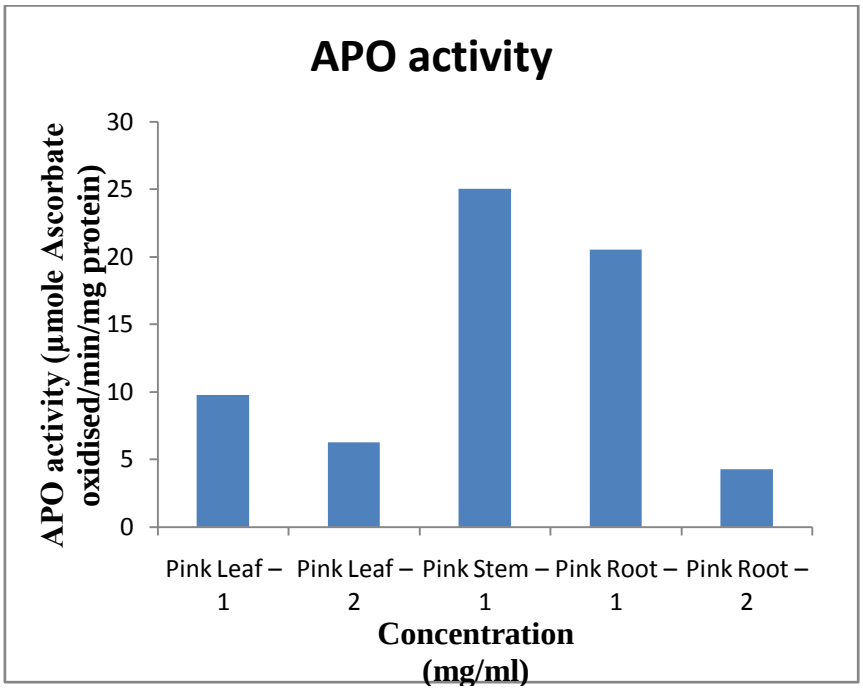


Fig. 4: Ascorbate peroxidase activity (µmole ascorbate oxidized /min/mg protein) of endophytic fungal extracts of leaf, stem and roots of Pink *Catharanthus roseus*.

RESULTS AND DISCUSSION:

Total 5 different endophytes were isolated from Pink *Catharanthus roseus* plant; two from Leaf, one from Stem and two from Root explants.

The Antioxidant enzymes, i.e. Catalase and Ascorbate Peroxidase (APO) were analyzed in endophytic fungal extracts of Pink *Catharanthus roseus*. The two enzymes react rapidly with hydrogen peroxidase (H_2O_2), which is produced by all living beings by oxygen metabolism. All the endophytic fungal extract of Pink *Catharanthus roseus* showed the presence of these two antioxidant enzymes. The maximum Catalase activity µmole H_2O_2/min/mg protein was recorded as 567.08 from the pink leaf isolates-1 and minimum was 137.6 from pink root Isolate-1. (Table-2) The same result has shown graphically in Fig.1 Kumar et al. (2013) also isolated 52 endophytic fungi from the leaves of *Catharanthus roseus* plant which were unusual and slow growing.

When percent inhibition of pink leaf-2 endophytes was measured at 517 nm,it was found maximum as 80.83,85.62, 95.80, 98.20 percent respectively 50,100,250 and 250mg/ml with increase in order. Similarly it was found minimum at 517 nm as10.17, 10.17, 32.33, 38.92 percent respectively 50,100,250 and 250mg/ml of the root-2 isolates with increase in order. Similar results have also been expressed in figure no.2

Reducing power of endophytic fungal extracts in comparison with standard ascorbic acid was studied spectrophotometrically reducing power of endophytic fungal extracts of leaf, stem and roots was measured

using ascorbic acid as a standard. The maximum was observed as0.093 in leaf-2 and minimum as 0.009 in stem-and root -1 isolates, 0.199 as max in stem-1 and minimum as 0.016 in root-1isolates,as max,. 0.134 in stem-1 and min as 0.034 in root-2,again max as 0.194 in leaf-1 and min as 0.044 in root -2 isolates respectively 50,100,250 and 500 mg/ml concentration.(Table-4) The same result has expressed in fig-3

When the ascorbate peroxidase activity (µmole Ascorbate oxidized /min/mg protein) of endophytic fungal extracts was carried out, the max APO activity was obtained as 25.0 in stem-1 isolates and min as 4.25 in root-2 isolates (Table-5).similar results are also obtained when graphically expressed(Fig. 4).

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