Review of Research
International Online Multidisciplinary Journal
ISSN 2249-894X Impact Factor : 3.1402 (UIF) Volume - 5 | Issue -5 | Feb - 2016

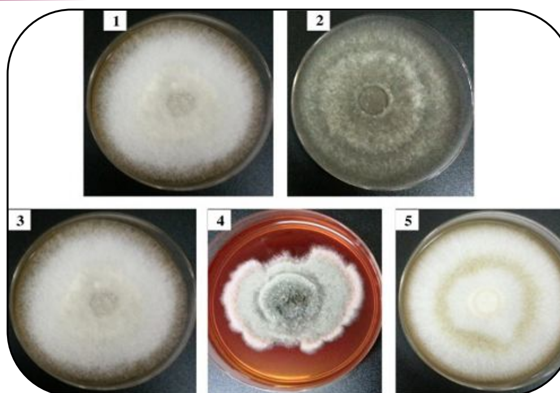
STUDIES ON ISOLATION AND CHARACTERIZATION OF WHITE *CATHARANTHUS ROSEUS* ENDOPHYTES FOR THEIR ANTIOXIDANT ACTIVITIES.

A. S. Gortyal[1] and N. S. Mali[2]
[1]Lokmangal Biotechnology College, Wadala. Dist. Solapur. (MS)
[2]Shankarao Mohite Mahavidyalaya, Akluj. Dist.Solapur. (MS)

Abstract:-
Total 8 different endophytes were isolated from *Catharanthus roseus* (white) plant; four from Leaf, one from Stem and three from root explants. From the leaves, the fungal isolates were mainly *Drechslera* sp.*Fusarium* sp and *Colletotrichum*,from the stem, the lone isolate was *Helminthosporium* sp isolated whereas from the roots, *Bispora* sp, *Alternaria* sp.and hypomycetes sp.of fungi were isolated. Identification of isolates, detection of antioxidant enzymes, culturing of entophytic fungi for Vinca alkaloid production,quantitative determination of antioxidant activities etc. were carried out.

Key words Isolates, Endophytes, Alkloides, Antioxidants.

INTRODUCTION:



Catharanthus roseus, L Family -Apocynaceae is an important medicinal plant and commonly known as Madagascar periwinkle because it has its origin from Madagascar. In India, it is commonly found in gardens and in warm conditions, it is found all over the world. It synthesizes useful secondary metabolites like-vendolin, vincristine and vinblastine, which were prevalently used in the treatment of cancer. Mycologists have detected the endophytic fungi from plant tissues. (Saikkonen *et al.*, 1998; Bacon and White, 2000). Endophytes isolated from *C. roseus* not only yield these valuable therapeutic molecules but have recently been studied to improve the plant content of terpenoid, indole alkaloids like serpentine, ajmalacine, vindoline and vinblastine. Vinblastin and Vincristine are the two alkaloids in *Vinca rosea*, which are prescribed for anticancer drug. The fungal endophytes have antibacterial, antifungal, antidiabetic, anticancer, antiviral, therapeutic, anti ulcer, anthelminthic, anti oxidant, anti diarrhoeal properties and also as phyto remediation, (Sain M. et al. (2013)

MATERIALS AND METHODS:

A) Collection of Plant Sample:

White *Vinca rosea* plant was collected from Department of Botany, New Arts Commerce and Science College, Ahmednagar in sterile ploythene bag and used for the further study within two hour. 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 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 ºC ± 1 ºC .The fungal growth was observed on the culture medium after incubation period.

C) Identification 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, New Arts, Commerce and Science College, Ahmednagar. Plate-.1 shows the identified entophytic fungi from Leaf, Stem and Root and table -1 shows the list of isolates.

D). Characterization:

Characterization of 8 entophytic fungi was carried out by staining with Lacto phenol Cotton Blue and observing under microscope (Kumar et al. 2013 and Mahajan et al.2014 method). The result of characterization is given in figure- 1 (photographs of entophytic fungi).

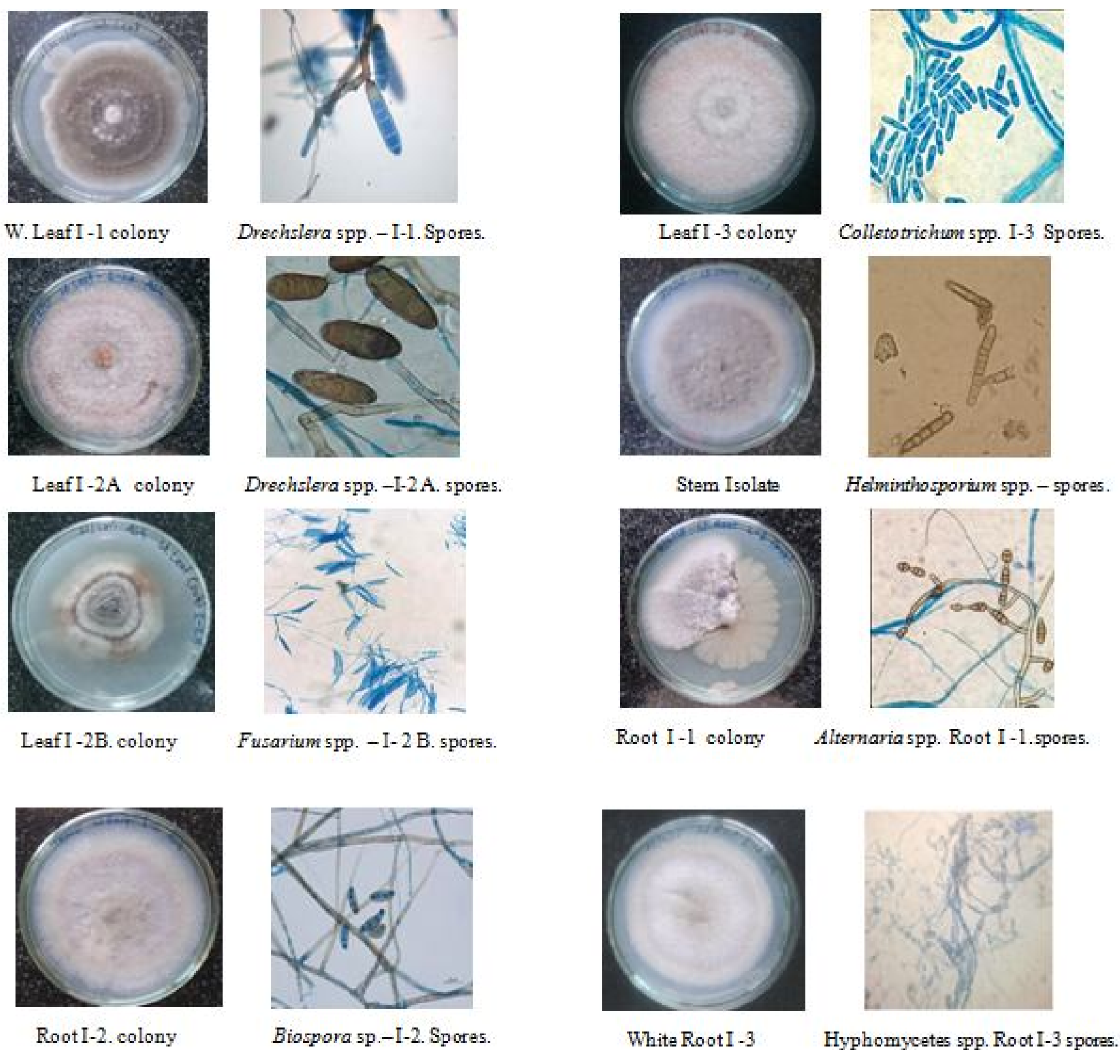


Plate - Culture plates of endophytes and their spores.

All the Isolates were identified by the expert mycologist, from department of Botany, New Arts, Commerce and Science College, Ahmednagar Table 1- shows the identified endophytic fungi from Leaf, Stem and Root, of White *Catharanthus roseus* plant.

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

Source of Isolates	Names of Entophytic Fungi
White Leaf – 1	*Drechslera* spp.
White Leaf – 2A	*Drechslera spp.*
White Leaf – 2B	*Fusarium spp.*
White Leaf – 3	*Colletotrichum spp.*
White Stem	*Helminthosporium spp.*
White Root – 1	*Bispora spp.*
White Root – 2	*Alternaria spp.*
White Root – 3	Dematious hyphomycetes.

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°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°C 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.

Spectrophotometrically analysis of isolated endophytes for different alkaloids was carried by= 0.2gm of extract was weighed and dissolved in methanol; the O.D. was measured in between 200 - 600nm.

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 to give water and oxygen;

Catalase

$2H_2O_2$ $2H_2O + O_2\uparrow$

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 $M^{-1}cm^{-1}$. (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)

Table-2. Catalase activity (µmole H_2O_2/min/mg protein) of leaf, stem and root of endophytic fungal extract of White *Catharanthus roseus.*

Extract	CAT activity (µmole H_2O_2/min/mg protein)
White Leaf – 1	361.25
White Leaf – 2A	705.25
White Leaf – 2B	292.42
White Leaf – 3	688.07
White Stem – 1	344.02
White Root – 1	189.22
White Root – 2	550.45
White Root – 3	258.02

The maximum Catalase activity µmole H_2O_2/min/mg protein was recorded as 705.25 from the leaf isolates 2-B and minimum was 189.22 from root-1isolate

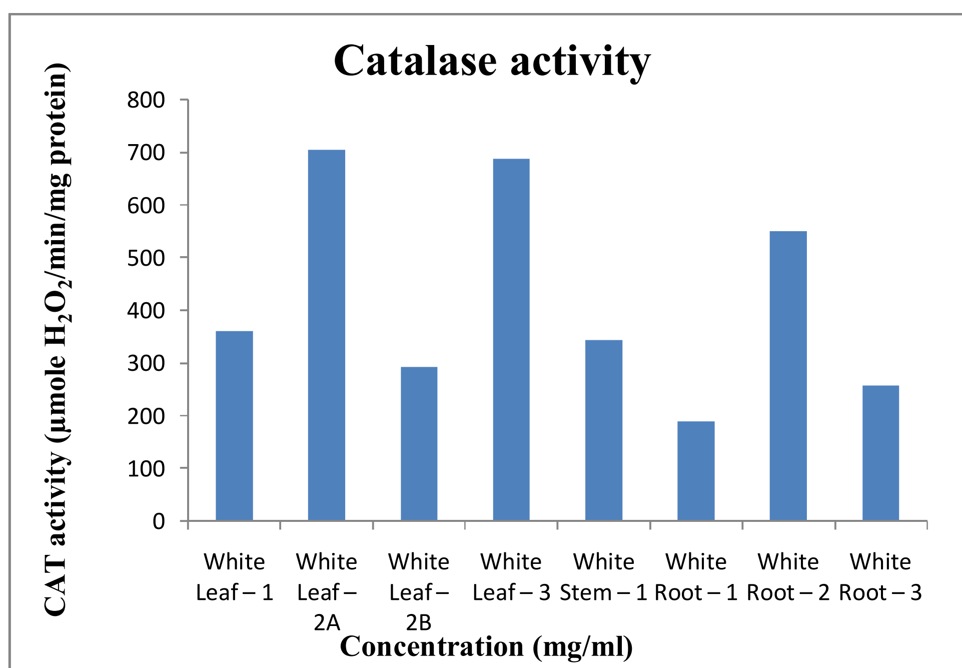


Fig.1: Catalase activity (µmole H_2O_2/min/mg protein) of leaf, stem and root of endophytic fungal extract of White *Catharanthus roseus.*

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 (%) = $[(A_{control} - A_{extract})/A_{control}] \times 100$ **(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 IC_{50} values were

calculated by using nonlinear regression analysis of dose dependent curves. IC_{50} 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. Among all endophytic fungal extracts. **(Table.3)**

Table-3. Showing antioxidant activities of endophytes.

Extract	Percent Inhibition (%)			
	50 µg/ml	100 µg/ml	250 µg/ml	500 µg/ml
White Leaf – 1	10.00	17.14	40.27	52.08
White Leaf – 2A	11.98	14.66	60.63	75.06
White Leaf – 2B	05.08	33.41	45.27	73.36
White Leaf – 3	04.34	13.91	14.78	38.47
White Stem – 1	03.60	16.10	21.18	48.72
White Root – 1	01.55	05.68	33.59	44.18
White Root – 2	02.89	14.21	40.52	56.31
White Root – 3	18.60	29.71	53.48	61.24
Ascorbic Acid	28.91	90.36	92.77	95.18
Gluthatione	0.9	29.71	47.16	55.18

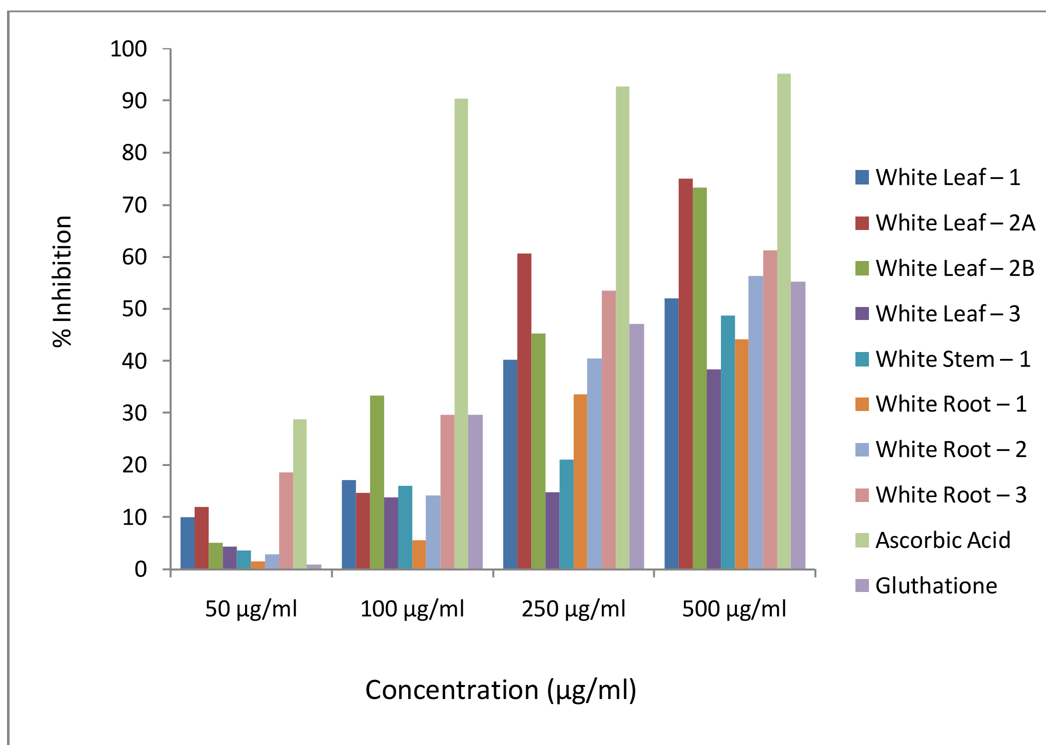


Fig 2: DPPH radical scavenging assay of White *Catharanthus roseus* endophytic extract with Ascorbic acid and Glutathione as a standard.

3) Reducing Power Assay:

The reducing power assay of *in vitro* cultures of *vinca rosea* 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. of White *Catharanthus roseus* and ascorbic acid as a standard.

Extract	Concentration (µg/ml)			
	50 µg/ml	100 µg/ml	250 µg/ml	500 µg/ml
White Leaf – 1	0.087	0.254	0.260	0.290
White Leaf – 2A	0.210	0.213	0.260	0.272
White Leaf – 2B	0.260	0.303	0.472	0.622
White Leaf – 3	0.348	0.382	0.599	0.682
White Stem – 1	0.237	0.257	0.267	0.279
White Root – 1	0.039	0.092	0.195	0.293
White Root – 2	0.400	0.425	0.600	0.684
White Root – 3	0.363	0.403	0.442	0.461
Ascorbic Acid	0.454	0.652	0.722	0.962

The presence of reducers i.e. antioxidants causes the reduction of the $Fe^{3+/}$ferricyanide complex to the ferrous (Fe^{2+}) form. Therefore, measuring at 700 nm can monitor the Fe^{2+}concentration. The reducing power of endophytic fungal extracts of leaf, stem and roots of Pink and White Catharanthus is shown in table 4 and fig.3. Ascorbic acid was used as reference compounds. The results showed that the reducing power of all the endophytic fungal extracts as well as ascorbic acid increased with an increase in their concentrations.

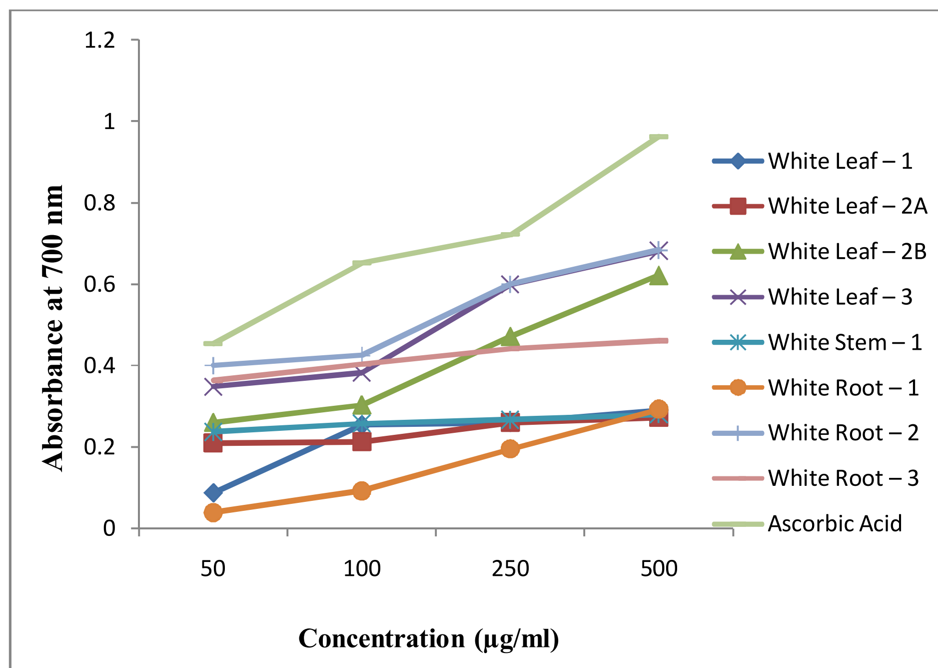


Fig 3: Reducing power of endophytic fungal extracts of leaf, stem and roots of White *Catharanthus roseus* and ascorbic acid as a standard.

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 3 min. The extinction coefficient of APO is 2.8 $mM^{-1}cm^{-1}$. (Table.5)

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

Extract	APO activity (µmole Ascorbate oxidised/min/mg protein)
White Leaf – 1	13.25
White Leaf – 2A	9.75
White Leaf – 2B	4.50
White Leaf – 3	10.00
White Stem – 1	9.75
White Root – 1	14.25
White Root – 2	14.00
White Root – 3	9.75

Ascorbate Peroxidase (APO) Activity:

Ascorbate peroxidase removes H_2O_2 to water via the Halliwell-Asada pathway in chloroplast and cytosole. One Ascorbate peroxidase is defined as a µmole of Ascorbate oxidized per min per mg protein. (Fig.4)

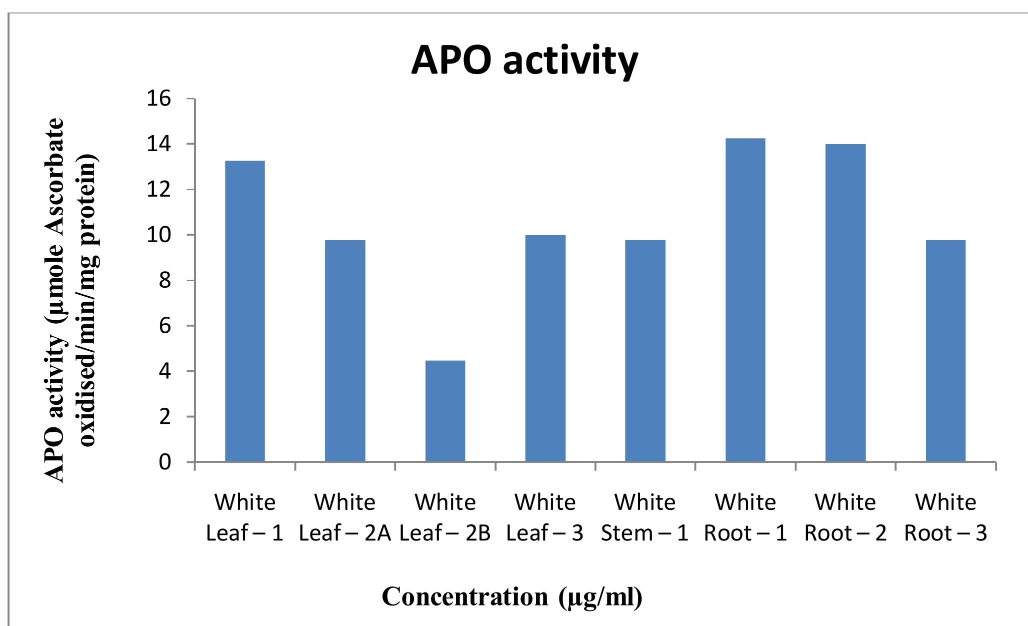


Fig.4: Ascorbate peroxidase activity (µmole ascorbate oxidized /min/mg protein) of endophytic fungal extracts of leaf, stem and roots of White *Catharanthus roseus*.

RESULTS AND DISCUSSION:

Isolation of Endophytes:

Total 8 different endophytes were isolated from White *Catharanthus roseus* plant; four from Leaf, one from Stem and three from Root explants. Table 1 shows the number of isolates, isolated from Leaf, Stem and Root explants of White *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.

Antioxidant Enzymes:

The Antioxidant enzymes, i.e. Catalase and Ascorbate Peroxidase (APO) were analyzed in endophytic fungal extracts of white *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 white *Catharanthus roseus* showed the presence of these two antioxidant enzymes.

One Catalase unit was defined as the amount of enzyme that would decompose 1 µmol of H_2O_2 per min per mg protein. The Catalase activity of white Leaf endophytic fungal extract showed highest Catalase activity, whereas white Stem, Root endophytic fungal extract showed less Catalase activity compared to Leaf extracts. Table 2 and figure 1 shows Catalase activities of endophytic fungal extracts of white *Catharanthus roseus* plants.

Quantitative Determination of Antioxidant Activity was done by DPPH Assay. Maximum Antioxidant activities of isolated endophytes was observed 18.60 from root - 3and minimum was observed as 01.55 from root-1 at 50 µg/ml concentration. At the 100 µg/ml Concentration, the maximum was33.41from leaf-2B and minimum was 05.68 from root-1**.**Again at 250 µg/ml conc.it was maximum as 60.63 from leaf-2B endophyte and minimum was 14.78 from leaf-3, at 500 µg/ml, the max. was 75.06 from leaf 2-A and min was 38.47 from leaf-3.This result indicates that the endophytes isolated from leaf show max. antioxidant activities as compared to root isolates.

The reducing power of endophytes. -Reducing power of endophytic fungal extracts in comparison with standard ascorbic acid was studied spectrophotometrically. The maximum reducing power of entophyte (**50 µg/ml**) was recorded as 0.400 from root-2 isolates and minimum as 0.039 from root-1 isolates. The maximum reducing power of entophyte **(100 µg/ml)** was recorded as 0.425 from root-2 isolates and minimum as 0.092 from root-1 isolates .The maximum reducing power of entophyte **(250 µg/ml)** was recorded as 0.600 from root-2 isolates and minimum as 0.195 from root-1 isolates. The maximum reducing power of entophyte (**500** µg/ml) was recorded as 0.684from root - 2 isolates and minimum as 0.272 from Leaf -2A isolates. This indicates that the maximum reducing power of endophyte is derived from the root -2 and minimum at root -1 isolates at various concentrations except leaf 2 A. APO Activity: Ascorbate peroxidase activity (µmole ascorbate oxidized /min/mg protein) of endophytic fungal extracts of leaf, stem and roots of White *Catharanthus roseus* was observed as 14.25 in root-1 isolates and minimum as 4.50 in leaf 2B isolates.(Table-5).The same result is observed in fig.4 except leaf-1 isolate. This indicates that APO Activity of the endophyte obtained from the root is maximum in comparison with stem and leaf endophytes except leaf-1 isolate

Acknowledgement:

The authors are grateful to the principal of New Arts, Commerce and Science College, Ahmednagar for providing the laboratory facilities.

REFERENCES:

1. Mahajan S., et al (2014), Isolation And Characterization Of Endophytes. International Journal of Latest Scientific Research and Technology, Volume1.
2. Jalgaonwala R.E. (2011) Isolation and Characterization of Endophytic Bacteria from Roots of *Pongamia glabra* vent. International Journal of Pharma and Bio Sciences, Volume1.
3. Pundir R.K. (2014) Bioprospecting potential of endophytic bacteria isolated from indigenous plants of ambala (Haryana, India). International Journal of Pharmaceutical Sciences and Research, Vol. *5(6): 2309-19.*
4. Powthong P. et al (2012) Evaluation of endophytic fungi extract for their antimicrobial activity from *Sesbania grandiflora (L.) Pers. Int. J. Pharm. Biomed Res* 2012, *3*(2), 132-136.

5. Patel C. et al (2013) Studies on Biodiversity of Fungal Entophytes of Indigenous Monocotaceous and Dicotaceous Plants and Evaluation of their Enzymatic Potentialities. *International Journal of Scientific and Research Publications, Volume 3, Issue 7.*
6. Jalgaonwala R.E. et al (2011) A review: Natural products from plant associated endophytic fungi. J. Microbiol. Biotech. Res., 2011, 1 (2): 21-32.
7. Koul M. et al (2013) *Catharanthus roseus* and prospects of its endophytes: a new avenue for production of bioactive metabolites. *International Journal of Pharmaceutical Sciences and Research. Vol. 4(7): 2705-2716.*
8. Selim K.A. et al (2012) Biology of Endophytic Fungi, *Current Research in Environmental & Applied Mycology Doi 10.5943/cream/2/1/3 32.*
9. Molina G. et al (2012) Application of fungal endophytes in biotechnological processes, *Chemical Engineering Transactions Vol. 27, 289-294.*
10. Ruma K. et al (2013), Antioxidant, Anti-inflammatory, Antimicrobial and Cytotoxic properties of Fungal endophytes from Garcinia Species. *International Journal of Pharmacy and Pharmaceutical Sciences, Vol 5, Suppl -3, 889-897.*
11. Vieira Fernandes M.R. et al (2009), Biological activities of the fermentation extract of the endophytic fungus *Alternaria alternata* Isolated from *Coffea arabica* L. *Brazilian Journal of Pharmaceutical Sciences vol. 45, no. 4.*
12. Malfanova N.V. et al (2010), Endophytic bacteria with plant growth promoting and biocontrol abilities. Published as a chapter in the book “Molecular Microbial Ecology of the Rhizosphere” (2013).
13. Selim K.A. et al (2012), Biology of Endophytic Fungi. *Current Research in Environmental & Applied Mycology. Pg no. 31-89.*
14. Tuppad D.S. et al (2014), Evaluation of endophytic fungi from *Butea monosperma* for antimicrobial and enzyme activity. *Journal of Medicinal Plants Studies 2014; 2(4): 38-45.*
15. Bharathidasan R. et al (2011), Isolation and identification of endophytic fungi from *Avicennia marina* in Ramanathapuram District, Karankadu, Tamilnadu, India. *European Journal of Experimental Biology, 2011, 1 (3):31-36.*
16. Arnold A.E. et al (2001), Fungal endophytes in dicotyledonous neotropical trees : patterns of abundance and diversity. *The British Mycological Society Mycol. Res. 105 (12): 1502-1507.*
17. Ahmed M. et al (2012), Isolation of microbial endophytes from some ethnomedicinal plants of Jammu and Kashmir. *J. Nat. Prod. Plant Resour*., *2012, 2 (2):215-220.*
18. Khan A.S. et al (2012), Isolation of Plant Growth Promoting Endophytic Fungi from Dicots Inhabiting Coastal Sand Dunes of Korea. *Pak. J. Bot., 44(4): 1453-1460, 2012.*
19. Prasad M.P. et al (2014), Identification and characterization of Endophytic fungus from fruits like Avacado and Black grapes. *Int. J. Curr. Microbiol. App. Sci.* (2014) 3(8) 937-947.
20. Pawle G. and Singh S. K. (2014), Antimicrobial, antioxidant activity and phytochemical analysis of an endophytic species of *Nigrospora* isolated from living fossil *Ginkgo biloba.* Current Research in Environmental & Applied Mycology 4 (1): 1–9 (2014).
21. Ting D. et al (2012), Studies on some active components and antimicrobial activities of the fermentation broth of endophytic fungi DZY16 Isolated from *Eucommia ulmoides* Oliv. *African Journal of Biotechnology vol. 12 (15), pp. 1862-1898.*
22. Kusari S. et al (2012), Chemical Ecology of Endophytic Fungi: Origins of Secondary Metabolites. *Chemistry & Biology 19, July 27, 2012.*
23. Kumar A. et al (2012), Cultural, Morphological and Molecular Characterization of Vinca Alkaloids Producing Endophytic Fungus *Fusarium Solani* Isolated from *Catharanthus Roseus. International Journal of Botany and Research (IJBR), Vol. 3, Issue 2, Jun 2013, 1-12*.
24. Tang Z. et al (2011), Effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus. Journal of Medicinal Plants Research Vol. 5(11), pp. 2192-2200, 4 June, 2011.*
25. Gurupavithra S. and Jayachitra A. (2013), Isolation and Identification of Endophytic Fungi from O*cimum sanctum* and Analyse its Antioxidant Properties. *International Journal of Research in Pharmaceutical and Biomedical Sciences, Vol. 4 (4) Oct – Dec 2013.*
26. Jalgaonwala R.E. et al (2014), Bioactive flavonoid of endophytic bacteria isolated from roots of *Pongamia glabra. Annals of Biological Research, 2014, 5 (5):38-44.*
27. Tiong S. H. et al (2013), Antidiabetic and Antioxidant Properties of Alkaloids from *Catharanthus roseus* (L.) G. Don. *Molecules 2013, 18, 9770-9784.*