Isolation of Endophytic fungus from *Catharanthus roseus* (L.) G. Don. and their antibacterial activity

Ms. H. Jayasudha[1], Ms. Fathaunnisha. S[2], Dr.V.Hemamalini[3], Dr.M.Menaga[4], Mr. P. Sridhar[5]

[1,2]Research Scholar, Department of Plant Biology and Plant Biotechnology, Quaid-E-Millath College for women, Chennai.

[3]Assistant Professor, Department of Plant Biology and Plant Biotechnology, Quaid-E-Millath College for women, Chennai.

[4] Managing Director, Bioneemtec India Pvt.ltd Siruseri, Chennai.

[5]Research Scholar, Department of Education, Tamil Nadu Open University Chennai.

Abstract

Medicinal plants play an important role for health care. Medicinal have ability to cure both infectious and non-infectious diseases. . The capability of medicinal plant endophytic fungi for the production of bioactive secondary metabolites was evaluated under in vitro conditions. In the present investigation the diversity of endophytic fungi was studied associated with medicinally important plant *Catharanthus roseus.* A total of 14 fungal species from *Catharanthus roseus* were isolated and identified based on the morphology of the fungal culture and characteristics of the spores. Secondary metabolites were produce from isolated endophytic fungi to investigate their antibacterial activity. The fungal culture was extracted with ethyl acetate and crude extract was used to test against clinical pathogenic organisms. The bacterial strain used in this research work were Staphylococcus aureus (MTCCB 737), Escherichia coli (MTCCB 82), Bacillus (MTCCB 1272), Pseudomonas aeruginosa (MTCCB 741) were obtained from Microbial Type culture collection, Pune, India. Then by using agar diffusion assay antibacterial activity was estimated. The zone of inhibition were measured by scaling and represented by tables and graphs.

Keywords: Endophytic fungus, *Catharanthus roseus,* Disc Diffusion method, Antibacterial Activity.

1. INTRODUCTION

Endophytes are microbes that live in plant tissues for the whole or part of their life cycles without causing any harm to the host[1]. Endophytic fungi harbour bioactive compounds like alkaloids, steroids, triterpenoids, tannins, anthracenosides, reducing sugars, flavones, saponins which perform multiple functions including preventing plants from stress conditions like drought, heavy metals, competition,

herbivorous and promotion of growth fitness[2,3]. They are also known to produce natural bioactive compounds with great potential applications in agriculture, medicine, and food industry[4,5]. It's further known that endophytic fungi harboured in medicinal plants produce secondary metabolites with bioactive compounds similar to those of their hosts. They are thus vital in pharmaceuticals for the discovery of new drugs[6,1]. "Endophytic fungi are found in all kinds of plants, i.e. trees, grasses, algae and herbaceous plants. Dreyfuss and Chapela (1994)[7] estimated that could also be a minimum of a million species of endophytic fungi alone. Almost all plant species harbour one or more endophytic fungi[8]. Endophytic fungi are that live in plant tissues without any substantive harm or gaining benefit other than residency[9]. Fungal endophytes can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue[10].

Catharanthus roseus is an significant medicinal plant of family Apocynaceae. It is cultivated mainly for its alkaloids, which are having anticancer activities [11]. Antibacterial potential in crude extracts of various parts of C. roseus against clinically significant bacterial strains has been reported [12]. In the last few decades bacterial resistance to antibiotics has become a serious therapeutic problem and the rate at which new antibiotics are being produced is slowing[13]. Thus, the search for novel antimicrobial agents is of the utmost importance [14]. Worldwide attention has been shifted towards finding new herbal chemicals for the development of new drugs. These natural products can provide unique elements of molecular diversity and biological functionality, which is indispensable for novel drug discovery[15]. Several research groups have shown that Catharanthus roseus has a high potential for many varieties of medicinal properties, such as antibacterial [16] antifungal [17]and antiviral [18] More than 130 of different alkaloids are present in C. Roseus [19]As an important medicinal plant, it has a good antioxidant potential throughout its parts under drought stress. The present study was carried by using medicinal plant *Catharanthus roseus* belongs to Apocynaceae family. Endophytic fungus were isolated from healthy plants leaves and their secondary metabolites were obtained. Chosen fungal extract were analysed for antimicrobial activity against four different clinical bacterial pathogens.

2. MATERIAL AND METHODS

2.1. Collection of Plant Sample

Catharanthus roseus leaves were collected from women Biotech Park, Sipcot industrial estate, Siruseri, Chennai. The leaves of Superior portion of the plant (20cm above the soil) were collected.

2.2. Isolation of Endophytic Fungi

The plant material or leaves from the healthy plants were subjected to endophytic isolation soon after the collection according to the procedure given by Verma *et al.*, (2007)[20]. The healthy leaves collected were thoroughly washed with running tap water for 45 minutes to wash away all the soil particles and then air dried. All the work was performed under laminar air flow hood maintaining sterile conditions. The cleaned leaves after air drying were surface sterilized by immersion in 70% ethanol for 1 minute, followed by 2.5% sodium hypochlorite solution for 5 minutes, ethanol for 30 sec and then washed thrice (1 minute each time) with sterile distilled water and left for drying under sterilized condition. Both the borders of the sterilized leaf segments were cut off with the help of sterile blade and about 1cm of the plant material (leaf segment) was subjected to endophytic isolation.

The small leaf segments were placed on potato dextrose agar (PDA)[21] media plates supplemented with antibiotic streptomycin (100mg/ml) so as to avoid bacterial growth. Each plate was inoculated with seven leaf segments. All the plates were sealed and packed with parafilm and were incubated at 18ºC to 28ºC. The plates were observed daily for 15-20 days for emergence of endophytes. The emerging fungal hyphal tips from the plant leaf segments were picked and transferred on PDA plates to check purity of the culture.

Fungal out growth from the plant tissues were sub-cultured on fresh antibiotic- free medium for identification established on morphological examination and conidial characters. All the colonies were counted and expressed as CFU per gram of fresh tissue. The pure cultures were maintained on PDA slants at 4°C. Fungal mycelium and spores were stained with lacto phenol cotton blue reagent and examined with a bright-field microscope. Identification was based on morphological characteristics and spore dimensions.

2.3. Fermentation and Extraction

The PDA broth was prepared with all the optimized parameters with carbon sources, nitrogen sources, with prescribed pH. After prescribed duration the broth were taken and sacrificied. The broth was get filtered with whatmann filter paper to remove all the cell debris from the broth. The clarified broth was subjected to solvent extraction ethyl acetate to the broth double the amount of ethyl acetate was added kept stirring for four hours. After four hours ethyl acetate portion are separated from the water using separating funnel. This was done three times and all the ethyl acetate portion separated was clubbed together and distilled under rotor vaccum evaporation to get crude extract.

The crude extract was run in the TLC with solvent proportion of hexane: ethyl acetate (1:1) and dipped in the vanillin reagent to observe the various bands present in the crude extract.

2.4. Inoculum Preparation:

The fungal isolates used in this study was isolated from the plant Catharanthus roseus. The isolates separated was evaluated for its antibacterial activity and the best one strain was taken for the maximum production of secondary metabolites in broth in terms of yield and purity. The best isolates was cultivated on the potato dextrose broth medium using a 500ml Erlenmeyer flask at pH 6.5 and 37^{o}C for 24 hr, after 24 hr of incubation a loopful of culure was taken in Erlenmeyer flask and inoculated on PDA agar slants and incubated at 37^{o}C for 72 hr and used as inoculums.

2.5. Test Organism:

The antibacterial assay of *Aloe vera* and *Catharanthus roseus* endophytic extract were determined using some clinical pathogenic microorganism. The test microbes such as Staphylococcus aureus(MTCCB 737), Escherichia coli (MTCCB 82), Bacillus(MTCCB 1272), Pseudomonas aurginosa (MTCCB 741) were obtained from Microbial Type culture collection, Pune, India.

The bacterial cultures were maintained in nutrient agar slants at 37°C. Each of the microorganism was reactivated prior to susceptibility testing by transferring them into a separate test-tube containing nutrient broth and incubated overnight at 37 °C.

2.6. Antibacterial Screening Assay

Antibacterial activity of fungal endophytic extract was determined using a modified Kirby Bauer Disc Diffusion Method [22]. The antibacterial activity of *Catharanthus roseus* endophytic extract were determined using some clinical pathogenic microorganism. The test microbes such as Staphylococcus aureus (MTCCB 737), Bacillus (MTCCB 1272), Escherichia coli (MTCCB82), and Pseudomonas aeruginosa (MTCCB 741) were used for this study. All the isolated were checked for purity and were maintained in slants of nutrient agar. The antibacterial activity of the test samples was determined by the agar well-diffusion method on Nutrient agar (Hi Media, India) medium. Using a cork borer, wells (6 mm in diameter) were punched out in the agar medium and inoculants containing 10^5 CFU/ml of the each test bacteria were spread onto the surface

of the medium with a sterile spreader 50µl of the extract were pipette into the well. The agar plates were incubated at37ºC for 24 hours and the diameter of the zone of inhibition surrounding the wells were measured after incubation. The diameters of zone of inhibition due to extracts were compared with those produced by the commercial control antibiotics, Ampicillin (2mg/ml). Antibacterial tests were performed in triplicates and observed values of zone of inhibition were expressed as average value. Well diffusion assay was carried out by using standard protocol. Then 0.1g of the Crude extract was dissolved in 10ml DMSO(Di-methyl sulfoxide) to obtain the concentration of the extract. The active positive control were used Ampicillin 2mg/ml(2000 pap parts million). Muller Hinton agar was the media used as growth media prepared according to Manufacturer's instruction. (All media and other ingredients purchased from Hi-media Laboratories. India). The antagonistic positive endophytic extract from *Catharanthus roseus* (BNT 03, BNT 04) were taken for antibacterial screening assay.

3. RESULT AND DISCUSSION

Antibacterial Activity of *Catharanthus roseus* extract was evaluated against gram positive and gram negative bacteria. All the isolated strain showed an excellent inhibitory against clinical pathogens.

Among the test strain crude extract of *Catharanthus roseus* (BNT 04) have shown effective inhibition against all clinical pathogens. The extract of *Catharanthus roseus* (BNT 03) displayed a moderate inhibition against all clinical pathogen.

The present study reveals the antibacterial potential of crude extracts of C. roseus. Nearly all parts of the plant exhibited significant antibacterial activity. Nevertheless, leaf extracts demonstrated maximum antibacterial activity. In a comparable study, the leaf extracts of C. roseus was found to have significant antibacterial activity against Xanthomonas campestris[23]. Furthermore, Gram-positive bacteria were found to have more susceptibility as compared to Gram-negative bacteria species. It has been shown in various studies that polarity of antibacterial compounds is crucial for their activity[24]. Therefore it's obvious that extracts prepared using organic solvents were more active against bacterial species. Similar observations have been reported by Thongson et al., (2004)[25]. In a study with C. roseus it has been pointed out that the pattern of inhibition largely depends upon extraction procedure, plant part, physiological and morphological state of plant, extraction solvent and microorganism tested. It has been demonstrated that extracts prepared using dried plant material is much more effective than the fresh plant materials[24].The most active extract

can be subjected to column chromatography and carry out further pharmacological evaluation by several methods such as NMR, Mass spectrometry

ANTIBACTERIAL ASSAY:

TABLE.1 Antibacterial activity against crude extract of *Catharanthus roseus* (BNT 03, BNT 04)

TEST PATHOGEN	BNT 03			BNT 04		
	500 ppm	1000 ppm	Control Ampicillin	500 ppm	1000 ppm	Control Ampicillin
E.Coli	No Activity	9mm	15mm	9mm	12mm	15mm
S.Aureus	No Activity	8mm	13mm	10mm	15mm	15mm
Bacillus	No Activity	9mm	14mm	No Activity	10mm	14mm
P.Auruginosa	No Activity	9mm	15mm	No Activity	10mm	15mm

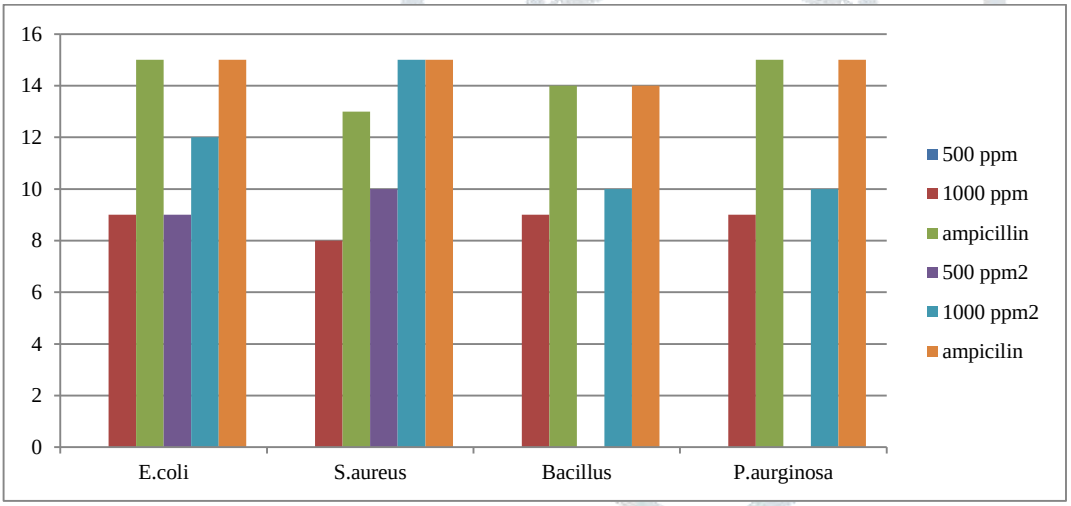
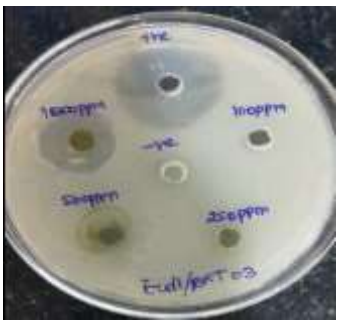


Fig. 1 *Catharanthus roseus* extract of BNT O3 tested with E.coli, P.aurginosa, Bacillus, S.aurginosa



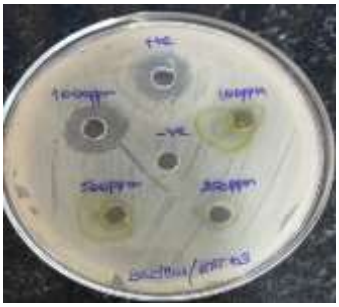
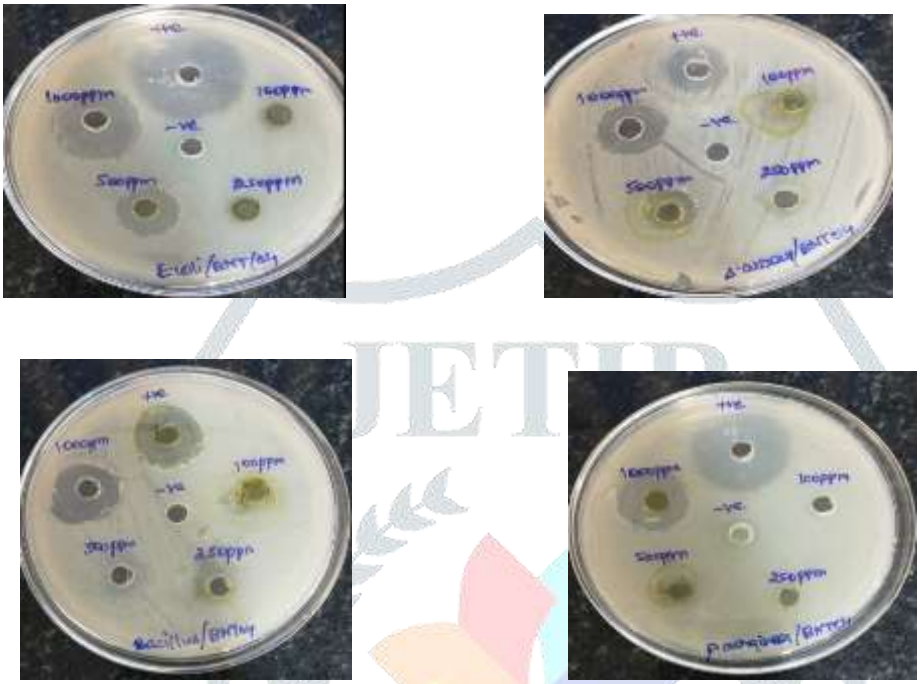


Fig. 2. *Catharanthus roseus* extract of BNT O4 tested with E.coli, P.aurginosa, Bacillus, S.aurginosa



4. CONCLUSION

This study has revealed that *Catharanthus roseus* extract possesses compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for therapy of infectious diseases in humans. The result of the present study thus explains the use of this plant in folk medicine for the treatment of various diseases whose symptoms might involve microbial infections and underline the importance of ethno botanical approach for the discovery of new bioactive compounds. These plants could be a source of new antibiotic compounds being non-toxic and less expensive than the allopathic drugs.

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REFERENCES:

1. Zhao J, Zhou L, Wang J, Shan T, Zhong L, Liu X and Gao X (2010) Endophytic fungi for producing bioactive compounds originally from their host plants. *Curr. Res. Technol. Educ. Trop. Appl. Microbiol. Microbial Biotechnol.* 1:pp. 567-576.
2. Omacini M, Chaneton EJ, Ghersa CM, and Müller CB (2001) Symbiotic fungal endophytes control insect host-parasite interaction webs. *Nature* 409:pp. 78-81
3. Waqas M, Khan AL, Kamran M, Hamayun M, Kang SM, Kim YH and Lee IJ(2012) Endophytic fungi produce gibberellins and indole acetic acid and promote host-plant growth during stress. *Molecules* 17: pp.10754-10773.
4. Stierle A, Strobel GA and Stierle D (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* 260 (5105):pp. 214-216.
5. .Verma VC, Kharwar RN and Strobel GA (2009) Chemical and functional diversity of natural products from plant-associated endophytic fungi. *Nat. Product. Commun*. 4: pp.1511-1532
6. Strobel GA, Miller RV, Martinez-Miller C, Condron MM, Teplow DB and Hess W M(1999) Cryptocandin, a potent antimycotic from the endophytic fungus Cryptosporiopsis cf. quercina. *Microbiol*. 145: pp.1919-1926
7. Dreyfuss M and Chapela I H (1994), "Potential of fungi in the diversity of novel, low molecular weight pharmaceuticals. In", The discovery of Natural Products with therapeutic Potential (ed Gullo, V.P.)Butterworth- Heinemann, Boston, pp. 49-80..
8. Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites. *National Product Reports* 18: pp.448-459
9. Kadouri, Daniel & Jurkevitch, Edouard & Okon, Yaacov. (2003). Involvement of the Reserve Material Poly- -Hydroxybutyrate in Azospirillum brasilense Stress Endurance and Root Colonization. Applied and environmental microbiology. 69. pp3244-50.
10. Hallmann, J., Kloepper, J.W., and Rodriguez Kabana, R.(1997a) Application of the Scholander pressure bomb to studies on endophytic bacteria of plants. Can J.Microbiol 43: 411-416

11. Jaleel CA, Gopi R, Manivannan P, Gomathinayagam M, Sridharan R and Panneerselvam R. 2008. Antioxidant potential and indole alkaloid profile variations with water deficits along different parts of two varieties of Catharanthus roseus. Colloids and Surf B: Biointerfaces;62:312–318.
12. Muhammad, L.R., N. Muhammad, A. Tanveer and S.N. Baqir.2009. Antimicrobial activity of different extracts of Catharanthas roseus. Clin. Exp. Med. J., 3: 81-85.
13. Russell, A.D., 2002. Antibiotic and biocide resistance in bacteria: Introduction. J. Appl. Microbial. Symp.Supply, 2: 176-181.
14. Gootz, T.D., 1990. Discovery and development of new antimicrobial agents. Clin. Microbiol. Rev., 2:176-181.
15. Nisbet, L.J. and M. Moore, 1997. Will natural products remain an important source of drug research for the future. Curr. Opin. Biotechnol., 8: 706-712.
16. Carew DP and Patterson BD.1970. The effect of antibiotics on the growth of Catharanthus roseus tissue cultures. Lloydia.;33:275–277.
17. Jaleel CA, Manivannan P and Sankar B. 2007. Induction of drought stress tolerance by ketoconazole in Catharanthus roseus is mediated by enhanced antioxidant potentials and secondary metabolite accumulation. Colloids and surf. B, Biointerfaces.;60:201–206.
18. Farnsworth NR, Svoboda GH and Blomster RN. 1968. Antiviral activity of selected Catharanthus alkaloids. J. Pharmacol. Sci: 57:2174–2175.
19. Pereira DM, Faria J, Gasparn L, Ferreres F,Valentao P, Sottomayor M and Andrade PB. 2010. Exploiting Catharanthus roseus roots:Source of antioxidants. J. Food Chem.;121:56–61.
20. Verma VC, Kharwar RN and Strobel GA 2009 Chemical and functional diversity of natural products from plant-associated endophytic fungi. *Nat. Product. Commun*. 4: 1511-1532.
21. Suryanarayanan T.S., Venkatesan G. and Murali T.S 2003. Endophytic fungal communities in leaves of tropical forest trees: Diversity and distribution patterns. *Curr. Sci*. 85: 489-493
22. A. W. Bauer, M.D., W. M. M. Kirby, M.D., J. C. Sherris, M.D., M. Turck, M.D., Antibiotic Susceptibility Testing by a Standardized Single Disk Method, *American Journal of Clinical Pathology*, Volume 45, Issue 4_ts, April 1966, Pages 493–496
23. Goyal P, Khanna A, Chauhan A, Chauhan G and Kaushik P (2008) In vitro evaluation of crude extracts of Catharanthus roseus for potential antibacterial activity. Int J Green Pharm 2:176-81.
24. Satish S, Raveesha KA and Janardhana GR (1999) Antibacterial activity of plant extracts on phytopathogenic Xanthomonas campestris pathovars. Lett Appl Microbiol 28:145-147
25. Thongson C, Davidson PM, Mahakarnchanakul W and Weiss J (2004) Antimicrobial activity of ultrasoundassisted solvent-extracted spi