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BIOPROSPECTING OF ENDOPHYTIC ACTINOMYCETES FROM *PLUMBAGO ZEYLANICA* (LINN.) FOR ANTIFUNGAL ACTIVITY

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ABSTRACT

The isolation of endophytic actinomycetes from surface sterilized parts (leave and stem) of *Plumbago zeylanica* (commonly known as Chitrak) was made using actinomycetes isolation agar. In the present study, two endophytic actinomycetes were isolated from root and stem and were identified as belong to *Saccharopolyspora* and *Nocardia* respectively. The *in vitro* antifungl activity of isolated endophytic actinomycetes has been investigated by measuring the Zone of inhibition (ZOI in mm) against the dermatophytic fungus *viz. Microsporum gypseum* and *Microsporum canis* performing different methods, as preliminary screening of actinomycetes by disk diffusion method, secondary screening by fermentation in shake flask and assay of antifungal activity from different fermented broth (GS, AGB and SCN) filtrate by well diffusion method. The two isolates of endophytic actinomycetes *Saccharopolyspora* and *Nocardia* showed significantly higher activity against both the fungus when performed by fermented broth filtrate method in case of GS and AGB which may be a good source of obtaining novel antimicrobials.

KEYWORDS: Antifungal activity, *Microsporum gypseum*, *Microsporum canis*, *Nocardia*, *Plumbago zeylanica*, *Saccharopolyspora*.

INTRODUCTION

The world is endowed with a rich wealth of medicinal plants. Medicinal Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. Natural products play an important role in drug development programmes in the pharmaceutical industry.[1] *Plumbago zeylanica* L. (Synonym: *P. viscosa* Blanco) commonly known as Chitrak, is a multipurpose medicinal herb of family *Plumbaginaceae*, is one of the common plants used in the Indian traditional system of medicine. Some parts of this plant species were used in various pharmacological activities.[2,3,4,5] Traditionally *P. zeylanica* is used as a stimulant digestant, expectorant, laxative and in the treatment of muscular pain and rheumatic diseases. Pharmacological studies have indicated that *P. zeylanica* extract consists of endophytic actinomycetes that have anti-plasmodial[6] anti-microbial[7] antifungal[8] anti-inflammatory[9] anti-hyperglycemic[10] hypolipidemic and anti-atherosclerotic activities. Endophytes colonizing inner tissues of plants usually draw nutrition and protection from host plants and in return, confer enhanced fitness to the host by producing a variety of bioactive metabolites and providing protection for the plant. Growth stimulation of plant by endophytes can be a consequence of nitrogen fixation or the production of phytohormones.[11,12,13] biocontrol of phytopathogens through production of enzymes, antibiotics or siderphores.[14,15,16,17] induction of systemic disease resistance.[18,19] Actinomycetes can occur in the plant rhizosphere soil and exercise an antagonistic and competitive effect on the microbial communities. They have the ability to produce active compounds, such as antifungal and antibacterial metabolites which have been developed for agricultural uses.[20,21] They have also been used as commercially formulated biocontrol agents of plant diseases such as *Streptomyces griseoviridis* cells used to protect crops against infections by *Fusarium sp.* and *Alternaria sp.*[22] In addition to their ability to inhibit plant pathogens, some actinomycetes are also known to form close associations with plants, colonize their internal tissues without causing disease symptoms, and promote their growth by producing plant growth regulators (PGRs),[23] The first identified endophytic actinomycetes capable of fixing molecular nitrogen belonged to the genus *Frankia*.[24] In the last decades other endophytic actinomycetes species of genera such as *Streptomycetes*, *Nocardia*, *Amycolatopsis*,

Micromonospora and *Microbiospora*, have been isolated from surface sterilized roots of various plant spp.[25,26,27] This study concentrated on bioprospecting of endophytic actinomycetes from *P. zeylanica* for its antagonistic activity against the dermatophytic fungus *Microsporum gypseum* and *Microsporum canis.*

MATERIALS AND METHODS

Sample collection

P. zeylanica was collected from herbal garden of Dehradun campus of Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences and Research and identified at Botanical Survey of India, Dehradun, Uttarakhand. Fresh plant materials were washed with running water and alcohol. Further they were transferred into sample bag and stored at 4ºC for further investigation. The stem and leave were excised and subjected to a three step surface sterilization procedure, a 60 second wash in 99% ethanol followed by a 6 minutes wash in 1% sodium hypochlorite solution, a 30 second wash in 99% ethanol and a final rinse in sterile water. The surface sterilized stem and leaves samples were then washed by being immersed in sterile distilled water 3 times to remove the surface sterilization agents.

Isolation of endophytic actinomycetes

After the disinfection, the stems and leaves of *P. zeylanica* were first fragmented into small pieces and then crushed which then transferred to petriplates containing the Actinomycetes isolation agar as a selection medium containing: Sodium caseinate 2.00 (gm/lt), L-aspargine 0.10 (gm/lt), Sodium propionate ($C_3H_5NaO_2$) 4.00 (gm/lt), Dipotassium phosphate (K_2HPO_4) 0.50 (gm/lt), Magnesium sulphate ($MgSO_4$) 0.10 (gm/lt), Ferrous sulphate ($FeSO_4$) 0.001 (gm/lt), Agar 15.0 (gm/lt) and pH ± 7.0 and supplemented with Cycloheximide (80 mg) and Nalidixic acid (15 mg) to suppress the growth of fungi and Gram-negative bacteria, respectively. Isolated colonies were picked and revived on Yeast Extract Malt Extract (YEME) (Hi MEDIA) agar slants for proper growth and maintenance. The agar slants were incubated at 28ºC±for 7 days.

Test organisms

Standard fungal cultures of *Microsporum gypseum* MTCC 2829 and *Microsporum canis* MTCC 2820 were used as test organisms. These cultures were revived on suitable media that is Potato Dextrose Agar (PDA) and Saboraud's Dextrose Agar (SDA) (Himedia) slants and incubated at 28ºC for 2-7 days and then stored at 4ºC as stock culture for further experimentation. These designated strains of fungal cultures were obtained from the Department of Microbiology Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences and Research, Balawala, Dehradun. These strains were identified according to the published guidelines.[28]

Preliminary antagonistic activity against microsporum spp

For preliminary test, the fungal cultures which were maintained on PDA slants were spread on the SDA plates. Then agar discs of actinomycetes growth which were incubated for 7 days at 28ºC were made with a sterile cork borer (6 mm) and placed on SDA plates seeded with the fungal culture using Disk diffusion method.[29] The plates were then incubated at 28ºC and observed for antibiosis after 24 hours. Inhibition zones were evaluated as follows: (<5 mm) no inhibition, (5-9 mm) weak inhibition, (10-19 mm) moderate inhibition, and (≥20 mm) strong inhibition.

Secondary screening by fermentation in shake flask Inoculum builds up in mother flask

For secondary screening well sporulated slant culture of endophytic actinomycetes were inoculated into shake flask. About 1cm² area of the growth of actinomycetes was scrapped off from the slants and inoculated into 40ml of mother flask medium (Nutrient broth) contained in 250 ml Erlenmeyer flask. The inoculated flasks were run on a rotatory shaker for 3 days at 28ºC (180 rpm).

Production of antifungal substance in shake flask

Five millilitre (10%) aliquot of inoculum from vegetative mother flask was transferred to 50 ml of AGB (Arginine Glycerol Salt Broth), SCN (Starch Casein Agar) and GS broth contained in 250 ml Erlenmeyer flask each. These flasks were incubated for 96 hrs on a rotatory shaker 200 rpm at 28ºC for the production of antifungal substance. The cultural filterate was collected by filtering the broth by using the Whatmann filter paper No.1. The cell biomass was separated from the broth culture; supernatant was used as a source of antifungal metabolite.

Assay for antifungal activity (agar well diffusion method)

Antifungal activity was performed by agar well diffusion method.[30] Diffusion method of quantitative determination of antifungal substance are based on the diffusion zone depends only on the nature of chemical substance diffused and on its concentration. The spore suspension (10^6 spores/ml) of fungal culture was made in distilled water and then spreaded over the presterilized SDA medium plates. Petriplates were allowed to dry at room temperature for 15 minutes. With the help of sterilized cork borer, 6mm wells were punched at equidistance. Nearly, six wells were made on each plate. 100µl of aliquot (filterate) was introduced in separate well. These plates were incubated at 28ºC ± for 96 hours. The results were obtained by measuring the diameter (ZOI) in (mm).

RESULTS

The large number of *Streptomyces* isolated from healthy plants show that there is a close relationship between these microorganism and tissues in which actinomycetes hyphal growth could have a favourable effect.[31] Only

limited attempts have been made to study endophytic actinomycetes and their metabolites in India.[32] Previous investigations proved that the ability of endophytic actinomycetes to inhibit phytopathogenic fungi is mainly by production of bioactive compounds, such as antibiotics and cell wall degrading enzymes and highlighted their importance as candidates for further investigation in the biocontrol of phytopathogens. The ability of endophytic actinomycetes to inhibit phytopathogenic fungi is mainly by production of bioactive compounds, such as antibiotics and cell wall degrading enzymes. In addition, endophytes are known to compete phytopathogens for nutrients.

In the present study, two different endophytic actinomycetes *viz. Saccharopolyspora* and *Nocardia* were identified which were grown in Actinomycetes isolation Agar with crushed stem and root material respectively and are subjected to preliminary and secondary screening (Table1).

In the preliminary screening, the *Saccharopolyspora* isolated from the stem of *P. zeylanica* showed a zone of inhibition of 15±0.57mm and 18±0.56mm against *Microsporum gypseum* and *Microsporum canis* respectively. The other actinomycetes *Nocardia*, isolated from the root of *P. zeylanica* showed a zone of inhibition of 10±2.3mm and 8±1.15mm against *M. gypseum* and *M. canis* respectively. The significance of differences was assessed with ANOVA (Analysis of Variance). TUKEY's Honest Significant Difference test (HSD) was applied for Post hoc analysis. T test for dependent variables allowed estimation of significant differences ($P<0.05$). A significant difference was found among the zone of inhibition against the *M. gypseum* formed by *Saccharopolyspora* isolated from the stem and the zone of inhibition against the *M. gypseum* formed by *Nocardia* isolated from the root of *P. zeylanica* F= 5.88, df= 5, $P<0.05$ (SAS,1995). Also significant difference was found among the zone of inhibition against the *M. canis* formed by *Saccharopolyspora* isolated from the stem and the zone of inhibition against the *M. canis* formed by *Nocardia* isolated from the root of *P. zeylanica* with F= 125, df= 5, $P<0.05$ (SAS, 1995). The paired t test at 95% confidence limits; $P<0.05$ showed that there is statistically significant difference between the zone formed against *M.* gypseum by *Saccharopolyspora* isolated from the stem and zone formed against *M. gypseum* by *Nocardia* isolated from the root of *P. zeylanica* with P=0.03. Also, the zone of inhibition formed against *M. canis by Saccharopolyspora* isolated from the stem and zone of inhibition formed against *M. canis by Nocardia* isolated from the root of *P. zeylanica,* a significant difference was found with P=0.005. The isolated endophytic actinomycetes, *Saccharopolyspora* and *Nocardia*, were then subjected to secondary screening to study the antifungal activity from the cultural filterate against *M. gypseum* and *M. canis.*

In case of GS broth, *Nocardia* and *Saccharopolyspora* showed a zone of inhibition of 20±1.5mm and 30±1.1mm against *M. gypseum* and a zone of inhibition of 24±01mm and 12±01mm against *M. canis.* Similarly in case of AGB broth, *Nocardia* and *Saccharopolyspora* showed a zone of inhibition of 30±1.7mm and 15± 01mm against *M. gypseum* and a zone of inhibition of 21±01mm and 17±01mm against *M. canis.* In case of SCN broth, there is no zone of inhibition showed by *Saccharopolyspora* against *M. gypseum* and *M. canis* but *Nocardia* showed a weak inhibition against *M. canis.* The significance of differences was assessed with ANOVA (Analysis of Variance). TUKEY's Honest Significant Difference test (HSD) was applied for Post hoc analysis. T test for dependent variables allowed estimation of significant differences ($P<0.05$). In case of GS broth, significant difference was found among the zone of inhibition against the *M. gypseum* formed by *Saccharopolyspora* isolated from the stem and the zone of inhibition against the *M. gypseum* formed by *Nocardia* isolated from the root of *P. zeylanica* with F= 64, df= 5, P=0.0004 at $P<0.05$ (SAS,1995). In case of AGB broth, statistically significant results were found among the zone of inhibition against the *M. gypseum* formed by *Saccharopolyspora* isolated from the stem and the zone of inhibition against the *M. gypseum* formed by *Nocardia* isolated from the root of *P. zeylanica* with F= 22.8, df= 5, P=0.0002 at $P<0.05$. Also significant difference was found in case of GS broth among the zone of inhibition against the *M. canis* formed by *Saccharopolyspora* isolated from the stem and the zone of inhibition against the *M. canis* formed by *Nocardia* isolated from the root of *P. zevlanica* with F= 216, df= 5, P= 0.021 at $P<0.05$. In case of AGB broth, the zone of inhibition against the *M. canis* formed by *Saccharopolyspora* isolated from the stem and the zone of inhibition against the *M. canis* formed by *Nocardia* isolated from the root of *P. zeylanica* with F= 13.5, df= 5, P= 0.0001 at $P<0.05$.

The paired t test at 95% confidence limits; $P<0.05$ showed that there is statistically significant difference between the zone formed against *M.* gypseum by *Saccharopolyspora* isolated from the stem and zone formed against *M. gypseum* by *Nocardia* isolated from the root of *P. zeylanica* with P=0.001 in case of GS broth and in case of AGB broth the P value was found to be 0.013. Also, in case of GS broth, the zone of inhibition formed against *M. canis by Saccharopolyspora* isolated from the stem and zone of inhibition formed against *M. canis by Nocardia* isolated from the root of *P. zeylanica,* a significant difference was found with P=0.002. In case of AGB broth, a P value of 0.04 was found which means there is a significant difference in the zone of inhibition formed against *M. canis by Saccharopolyspora* isolated from the stem and zone of inhibition formed against *M. canis by Nocardia* isolated from the root of *P. zeylanica.*

Similar results were reported in a study indicating that a small number of endophytic microorganisms had the

capability of producing broad-spectrum, antifungal compounds.[33] Mechanisms of action of endophytic actinomycetes are mainly focused on the production of bioactive compounds, such as antibiotics, cell wall degrading enzymes and competition for nutrients.[34]

Table 1: Showing zone of inhibition from preliminary and secondary screening of endophytic actinomycetes against *Microsporum spp.*

Plant material	Identified Genera	Preliminary screening of endophytic actinomycetes		Secondary screening of endophytic actinomycetes					
		Test organisms (zone of inhibition in mm)		Test organisms (zone of inhibition in mm)					
		Microsporum gypseum	*Microsporum canis*	*Microsporum gypseum*			*Microsporum canis*		
				GS	SCN	AGB	GS	SCN	AGB
Stem	*Saccharopolyspora*	15±0.57[a]	18±0.56[a]	20±1.5[b]	-	30±1.1[a]	24±01[a]	-	21±01[a]
Leave	*Nocardia*	10±2.3[b]	8±1.15[b]	30±1.7[a]	-	15±01[b]	12±01[b]	7±01	17±01[b]

± represents the standard deviation. ANOVA, standard deviation and TUKEY's honest test was done using SAS. Means with atleast one letter common are not statistically significant using TUKEY's Honest Significant Different at 5%. GS= Glycerol Salt Broth; SCN= Starch Casein Nutrient Broth; AGB= Arginine Glycerol Broth; -= no inhibition

DISCUSSIONS

There is a growing interest of researchers in bioprospecting of endophytic microbial communities inhabiting the plants from various ecosystems. Now a day there is severe need to find out new antibiotics. It is apparent that plants can serve as a reservoir of endophytic actinomycetes and evidence thus for indicates that the antibiotics from these sources are novel, interesting and hold pharmaceutical and agricultural promise.[35] It has been studied that the maximum endophytic actinobacteria have been recovered from roots followed by stems and least in leaves.[36,37] The use of antagonistic microorganisms such as endophytic *Streptomyces* is an ideal method of controlling plant diseases.[38,39,40] Flowers of *P. zeylanica* are used as digestant.[41] Leaves are caustic, vesicant, aphrodisiac, good for scabies stimulant and are also used in sore and swelling.[42] They are used to treat infections and digestive problems such as dysentery. Externally a paste is applied to painful rheumatic areas or to chronic and itchy skin problems.[43]

The main aim of this study was to study the antagonistic activity of Endophytic actinomycetes against pathogenic fungi. From the results it has been observed that the isolated endophytic actinomycetes showed a good antifungal activity in case of cultural filterate. As the cultural filterate show strong antagonistic activity it means that the antifungal metabolites are extracellular. Our study also supported the previous studies that showed that alcoholic extracts of *Plumbago zeylanica* showed strong antifungal against the pathogenic yeast, *Candida albicans* and dermatophytes, *Epidermophyton floccosum*, *Microsporum gypseum* and *Trichophyton rubrum* [8]. Thus, the metabolites obtained from these endophytic actinomycetes inhibit the phytopathogenic fungi and can be better and safer alternatives to the chemical fungicides, which pose potential environmental threat and mammalian toxicities.

CONCLUSIONS

It was concluded from this investigation that endophytic actinomycetes play an important role in human and pathogenic fungi, and are the rich and cost-effective source of numerous agro-based biological agents may be used at medicinal scales after being further studied and enabling the discovery of new antifungals and hence merit future studies.

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