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DIVERSITY OF ENDOPHYTIC FUNGI FROM ACACIA NILOTICA LINN., AND SCREENING THEIR BIO-ACTIVITY AGAINST ORAL PATHOGENS

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ABSTRACT

Endophytic fungi diversity from the leaves and barks of *Acacia nilotica* used in treating oral diseases were studied during four different seasons for about one year. Four hundred bark and leaf segments were analyzed and a total of 553 isolates representing 17 taxa and 3 morphotypes were isolated. *Aspergillus flavus* was found to be dominant in all seasons and the frequency of endophytic fungi was found to be more in leaves than in bark tissues with some tissue-specific endophytes. Ethyl acetate extracts of endophytic fungi isolated from *Acacia nilotica* were screened against major oral pathogens such as *Streptococccus mutans* and *Candida albicans* by agar well diffusion method in Mueller- Hinton Agar media. The crude extract of *Eupenicillium* sp., isolated showed maximum zone of inhibition against *S.mutans* (18.67±0.36 mm) and *C.albicans* (16.3±0.36 mm) and the isolate *Eupenicillium* sp., from *Acacia nilotica* could be further explored for its activity against oral pathogens for novel secondary metabolite isolation.

KEYWORDS: **Endophytic fungi, Diversity, Oral pathogens, Zone of inhibition.**

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INTRODUCTION

Endophytic fungi are microorganisms residing in the internal tissues of the plant without causing any harmful effects to the living host plant.[1] 1 million species of fungal endophytes have been estimated so farwhich represents an important component of fungal diversity as the distribution of endophytic fungi differs with the host.[2,3] Fungal endophytes serve as a repository for novel compounds with immense value in agriculture, medicine and industry.[4] Many ethno medicinal plants from unique environmental settings are likely to harbor distinct endophytes with novel biological properties.[5] Currently plants with immense medicinal values are explored for their fungal diversity which can produce distinct secondary metabolites with unique pharmacological values. Endophytic fungi are able to produce secondary metabolites with activities similar to or more than that of their respective hosts.[6] Hence search for novel compounds is aimed towards endophytic fungi for medicinal purpose. *Acacia nilotica,* nitrogen fixing legume tree widely known as Babul and Gum Arabic tree belongs to the family of Fabaceae.[7] The bark, root, gum, flowers and leaves of this flowering plant display a wide range of medicinal properties such as antidiabetic, antimicrobial, antioxidant, antispasmodic, antiplasmodic, antihypertensive and anticancer properties and for treating skin diseases, dental problems and oral candidiasis.[8] The tender twigs of *Acacia nilotica* are used as toothbrushes for its germicidal property.[9] *Acacia nilotica* is a rich source of tannins, glycosides, alkaloids and phenols which makes it a plant with high medicinal value.[10] Singh B and Kaur A (2016) exploited the anti-diabetic potential of a peptide from an endophytic *Aspergillus awamori* isolated from *Acacia nilotica*.[11] The human mouth is highly conducive for uncontrolled formation of natural microbial biofilms due to its diverse niches and ample supply of nutrients.[12] *Streptococcus mutans* are major cariogenic agents in dental caries whereas *Candida albicans* is the major causative agent for oral candidiasis.[13] Recent studies suggest that cariogenic development is mediated by interaction between oral bacteria and fungal pathogen, *Candida albicans* as there is high prevalence of *Streptococci* where *Candida* resides.[14,15] *Candida albicans* is the most frequently detected *Candida* species in human infections. It has become very difficult to control these organisms because of their tolerance towards various antimicrobial agents in routine use during the course of therapy.[16] Natural products make excellent leads for new drug development which are safer and biodegradable.[17] The present study is aimed to explore the diversity of endophytic fungi present in *Acacia nilotica* from leaf and bark tissues in four different seasons and examine the activity of isolated endophytes against major human oral pathogens.

MATERIALS AND METHODS

i) Collection of plant material

The host plant *Acacia nilotica* is a tropical tree growing in the drier parts of India. The plant has yellow colored flowers and long grey pods. It has dark barks and branches which bear spikes of about 2 cm long. The leaves are bipinnate with 10-20 pairs of leaflets in them.[18] Babul plant can withstand daily maximum temperature of about 50 °C. The plant samples were collected in the month of May from Alagarkovil hills near Madurai, Tamilnadu. The plant was authenticated by Dr.G.Kathiravan, Associate Professor, Department of Biotechnology, Vels University, Chennai, Tamilnadu, India. with the voucher specimen number VUCC0017 (Vels University Culture Collection) was deposited for future reference. Fresh and healthy leaves and barks of *Acacia nilotica* were cut with a sterile scalpel and stored in a sterile polythene bag at 4°C.

ii) Isolation and identification of endophytic fungi

About 400 leaf and 400 bark segments were collected and washed thoroughly with sterile distilled water and air dried before they are processed and surface sterilized by immersing them sequentially in 70% ethanol for 5 sec and 4% sodium hypochlorite for 1 min and rinsed with sterile distilled water.[19] The excess water was dried under laminar airflow chamber. Each plant sample was cut aseptically into 0.5 cm long segment and placed on petri dishes containing potato dextrose agar (PDA) supplemented with chloramphenicol (50 μg/ml) to suppress bacterial growth.The plates were sealed using parafilm and incubated at 25°C in a light chamber with 12 hours of light followed by 12 hours of dark cycles.[20] Fungal hyphae tips growing out from sterile segments were sub cultured on fresh slants.The petri dishes were monitored for the growth of endophytic fungal colonies. Isolated fungal endophytes were identified macroscopically based on colony morphology and microscopically by lactophenol cotton blue staining method.[21]

iii) Determination of species diversity

Colonization rate (CR) was calculated as the total number of plant tissue segments infected by fungi divided by the total number of segments incubated.[22] Isolation rate (IR) was determined as the number of isolates obtained from plant segments divided by the total number of segments incubated. Colonization frequency (CF) was calculated as the number of plant segments colonized by a single endophyte divided by the total number of segments observed × 100. Isolation frequency (IF) was calculated as the total number of isolates of one speciesdivided by the total number of isolates in that sample × 100. Simpson dominance index and Shannon–Wiener's diversity index were calculated for fungal diversity.[23,24] Simpson's index of diversity was calculated using formula 1-D as given in the following equation:

$$D= \Sigma n(n-1) / N(N-1)$$

where n is the total number of organisms of a particular species and N is the total number of organisms of all species. Shannon-Wiener diversity index was calculated using the following equation:

$$H_s = -\sum_{i=1}^{S} (P_i)\ (\ln P_i)$$

where H_s is the symbol for the diversity in a sample of s species or kinds, s is the number of species in the sample, P_i is the relative abundance of i^{th} species where P_i= n/N, N is the total number of individuals of all kinds, n_i is the number of individuals of i^{th} species, and ln is the log to base 2.

iv) Test organisms used

The test organisms used in this study were two major oral pathogens, *Streptococcus mutans* and *Candida albicans*. The oral pathogens were obtained from the laboratory of Billroth hospitals, Chennai which were isolates of dental caries affected patients. *Streptococcus mutans,* a gram positive bacterium was maintained on brain heart infusion broth and the yeast *Candida albicans* was cultured on Sabouraud's Dextrose broth.

v) Extraction of secondary metabolites

Extraction of secondary metabolites was carried out by the method described by Radji et al. (2011). Endophytic fungi isolated were grown on 100 ml of Potato Dextrose broth inoculated with a mycelial agar block taken from an actively growing colony on Potato Dextrose agar plate.The flask cultures were incubated at 25°C under light and dark conditions of 12 hours. They were grown for about 21 days for the extraction of secondary metabolites.The fungal broth culture was filtered and to the filtrate equal volume of ethyl acetate was added and mixed vigorously for 10 minutes. They were kept still till two immiscible clear layers were formed. The upper layer with the extracted compounds was separated using separating funnel. The mycelium was grinded using ethyl acetate and filtered using cheese cloth. Both culture filtrate and mycelia extracts were pooled and dried in hot air oven. The dried residue was dissolved in dimethylsulfoxide and stored at 4°C for further use.[25]

vi) Assessment of antimicrobial assay

Antimicrobial assay was done by agar well diffusion method in Muller Hinton agar as described by NCCLS 172056.[26] Inoculum was standardized by picking six colonies of *S.mutans* and *C.albicans* in to their respective media and incubated at 37°C for 18-24 hours. Inoculum of the test microorganism was adjusted to a 0.5 McFarland standard (10^8 Cfu/ml) and 100 µl of the standardized inoculum was swabbed on to Muller-Hinton agar for antimicrobial assay by agar well diffusion method. The wells were punched using sterile cork borer and 100 µl of 100 µg/ml concentration of fungal extracts were loaded on to the agar wells. 2% chlorhexidine was used as positive control. The plates were incubated at 37°C for 24 hours. The diameter of inhibition zone around the well was measured in millimeter. Secondary screening of positive fungal endophytes with more antimicrobial activity were done by loading different concentrations (25 µg/ml, 50 µg /ml, 75 µg/ml and 100 µg/ml) on the wells and the zone of inhibition was measured (Fig 2 and Fig 3).Experiments were carried out in triplicates and the statistical analysis was done. The values of antimicrobial activity were expressed as mean± standard error mean (n=3) for each sample.

RESULTS AND DISCUSSION

1. Endophytic fungi diversity and species richness

In the present study on *Acacia nilotica*, endophytic fungi colonize both leaf and bark tissues in four different seasons of the year. A total of 553 isolates were obtained from 800 tissue segments of *Acacia nilotica* of which 299 isolates from leaf and 254 from bark segments were obtained.Twenty fungal taxa were identified with *Aspergillus flavus* as the dominant species. *Nodulisporium, Aspergillus, Colletotrichum, Eupenicillium, Bipolaris* and *Trichoderma* were the most frequently isolated species with two sterile forms as shown in Table 1. All the endophytes isolated were identified based on their morphological characters and the identity of fungal cultures was confirmed by Agharkar research institute, NFCCI, Pune.

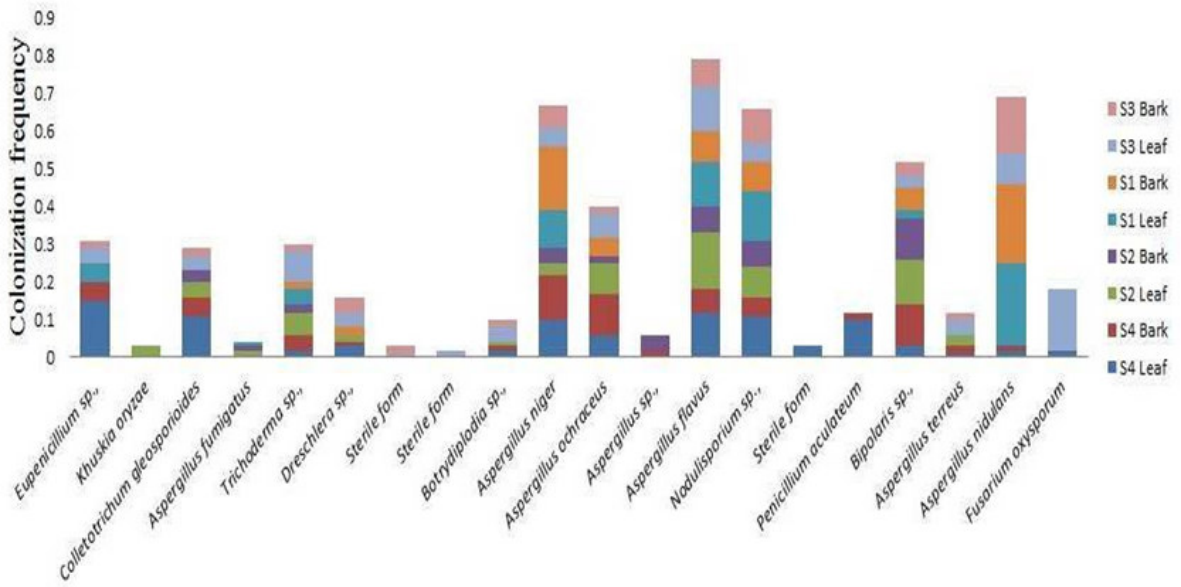
Table 1

Colonization frequency of endophytes isolated in different seasons from leaf and bark tissues of Acacia nilotica

Endophytes	Season 1 (March - May) 2014 (Summer)		Season 2 (June -Aug) 2014 (Pre- monsoon)		Season 3 (Sep-Nov) 2014 (Post-monsoon)		Season 4 (Dec-Feb) 2015 (Winter)		Total Colonization Frequency	Total number of isolates
	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark	C.F%	
M1 (*Eupenicillium* sp.,)	5	-	-	-	4	2	15	5	3.875	31
M2 (*Khuskia oryzae*)	-	-	3	-	-	-	-	-	0.3	3
M3 (*Colletotrichum gleosporioides*)	-	-	4	3	4	2	11	5	3.625	29
M4 (*Aspergillus fumigatus*)	1	-	2	1	-	-	-	-	0.5	4
M5 (*Trichoderma* sp.,)	4	2	6	2	8	2	2	4	3.75	30
M6 (*Dreschlera* sp.,)	-	2	2	-	4	4	3	1	2	16
M7 (Sterile form)	-	-	-	-	1	2	-	-	0.375	3
M8 (Sterile form)	-	-	-	-	2	-	-	-	0.25	2
M9 (*Botrydiplodia* sp.,)	-	-	1	-	4	2	2	1	1.25	10
M10 (*Aspergillus niger*)	10	17	3	4	5	6	1	12	7.25	67
M11 (*Aspergillus ochraceus*)	-	5	8	2	6	2	6	11	5	40
M12 (*Aspergillus* sp.,)	-	-	-	4	-	-	-	2	0.75	6
M13 (*Aspergillus flavus*)	12	8	15	7	12	7	12	6	9.875	79
M14 (*Nodulisporium*sp.,)	13	8	8	7	5	9	11	5	8.25	66
M15 (Sterile form)	-	-	-	-	-	-	3	-	0.375	3
M16 (*Penicillium aculeatum*)	-	-	-	-	2	-	10	-	1.5	12
M17 (*Bipolaris* sp.,)	2	6	12	11	3	4	3	11	6.5	52
M18 (*Aspergillus terreus*)	-	-	3	1	4	2	1	2	1.625	13
M19 (*Aspergillus nidulans*)	22	21	-	-	8	15	2	1	8.625	69
M20 (*Fusarium oxysporum*)	-	-	-	-	16	-	2	-	2.25	18

Graph 1

Colonization frequency of endophytic fungi isolated in for different seasons with respect to leaf and bark tissues where S1, S2, S3 and S4 represents Season 1, Season 2, Season 3 and Season 4 respectively.



A graph showing the colonization frequency of endophytic fungi isolated in four different seasons with respect to leaf and bark tissues was shown. The colonization rate (CR) of leaf and bark tissues (60.75% and 53.75%) as well as the isolation rates (IR) of bark and leaf tissue (74.75% and 63.5%) indicate large variation in the endophytic assemblages of the tissues. Table 2) The endophytes *Fusarium oxysporum, Khuskia oryzae* and *Penicillium aculateum* were the leaf specific (endophytes isolated. As shown in table 3, the difference in endophytes is tissue specific due to differences in their anatomical structure.[27] The study of endopyhte differences in *Quercus Ileus* indicates that the commonest endophytes were found in leaves, whereas the remaining were found in both leaves and twig and no twig specific endophytes were found.[28]

Table 2
Colonization rate and isolation rate of endophytes isolated

	Leaf	Bark	Total
No. of samples	400	400	800
No. of samples yielding fungi	243	215	458
No. of isolates	299	254	553
% CR	60.75%	53.75%	57.25%
% IR	74.75%	63.5%	69.1%

The overall endophytic population during winter was higher when compared to other seasons.In many instances more number of endophytes were harbored during winter than in dry season. Seasonal variation plays a significant role in endophytic diversity where significant difference was observed in CR and IR of the endophytes isolated. Environmental conditions during winter season pave the way for higher number of endophytes which is suitable for the symbiotic microbes to survive. It has been found that precipitation may be a major factor for the prevalence of endophytes.[29] Shannon Wiener index (Table 3) shows that the endophyte frequency is higher in leaves (3.00) than in bark (2.77) tissues as more number of endophytes has been isolated from leaves. Simpson index of diversity is higher in leaves when compared to bark which indicates the species specificity with leaves as *Trichoderma* sp., and *Fusarium oxysporum* have been studied with more number of isolates (20 and 18) respectively. In this case *Fusarium oxsporum* is completely absent in bark tissue. Evenness ratio which measures the equitability between the species is higher for bark than leaf tissues due to the frequent isolation of *Aspergillus niger, Aspergillus flavus, Bipolaris* sp., *Nodulisporium* sp., *Aspergillus nidulans*and some rare sterile forms from the leaves. There is even distribution of fungal endophytes in bark than in leaf tissues. Diversity index of leaves in different seasons studied is higher in leaves than in bark tissues as shown in Table 4. Evenness ratio is much higher in winter season as there is uniform distribution of endophytes in bark tissue during winter than in summer.

Table 3
Species diversity in terms of dominance, richness and evenness of endophytic assemblages of Acacia nilotica during different seasons

	Season 1		Season 2		Season 3		Season 4	
	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark
Shannon-Wiener's index (H)	1.75	1.76	2.23	2.07	2.39	2.33	2.35	2.44
Simpson's index of diversity (1-D)	0.818	0.817	0.883	0.868	**0.921**	0.886	0.907	0.90
Evenness ratio (J')	0.413	0.416	0.53	0.552	0.563	0.54	0.519	0.579

Table 4
Species diversity in terms of dominance, richness and evenness of endophytic assemblages in different tissues of Acacia nilotica

Tissue segments	Shannon-Wiener's index (H)	Simpson's index of diversity (1-D)	Evenness ratio (J')
Leaf	3.00	0.911	0.526
Bark	2.17	0.901	0.932

Table 5
Species diversity in terms of dominance, richness and evenness of endophytic assemblages of Acacia nilotica during different seasons

	Season 1		Season 2		Season 3		Season 4	
	Leaf	Bark	Leaf	Bark	Leaf	Bark	Leaf	Bark
Shannon-Wiener's index (H)	1.75	1.76	2.23	2.07	2.39	2.33	2.35	2.44
Simpson's index of diversity (1-D)	0.818	0.817	0.883	0.868	**0.921**	0.886	0.907	0.90
Evenness ratio (J')	0.413	0.416	0.53	0.552	0.563	0.54	0.519	0.579

Table 6
Species diversity in terms of dominance, richness and evenness of endophytic assemblages in different tissues of Acacia nilotica

Tissue segments	Shannon-Wiener's index (H)	Simpson's index of diversity (1-D)	Evenness ratio (J')
Leaf	3.00	0.911	0.526
Bark	2.17	0.901	0.932

2. Effect of secondary metabolites of endophytic fungi on major oral pathogens.

The endophytic fungi isolated were evaluated for their antimicrobial activity against major oral pathogens such as *Streptococcus mutans* and *Candida albicans*. The ethyl acetate extracts of 20 different endophytic fungi were screened for their activity against the oral pathogens. (Table 7) Among the isolates, 80% and 70% of the isolates showed activity against *S.mutans* and *C.albicans* respectively. *Eupenicillium* sp., *Aspergillus niger*, *Aspergillus ochraceus* and *Aspergillus nidulans* isolated from both leaves and barks were found to have good activity against the oral pathogens with a zone of inhibition of more than 15 mm. *Aspergillus terreus*, *Aspergillus fumigatus* and *Botrydiplodia* sp., were found to produce zone of inhibition of about 15 mm against *S.mutans* and about10-15 mm against *C.albicans*. The endophytes *Khuskia oryzae* and *Penicillium acculateum* isolated from leaf tissues and M15 Sterile form were found to be active only against *S.mutans* whereas they don't have any activity against *C.albicans*.

Table 7
Screening of endophytic fungi against S.mutans and C.albicans

No	Endophytes	Zone of inhibition for *S.mutans* (mm)	Zone of inhibition for *C.albicans* (mm)
M1	Eupenicillium sp.,	18.67±0.36	16.36±0.36
M2	Khuskia oryzae	12.5±0.39	-
M3	Colletotrichum gleosporioides	-	10.3±0.29
M4	Aspergillus fumigatus	12.6±0.42	10.77±0.29
M5	Trichoderma sp.,	10.26±0.36	12.5±0.5
M6	Dreschlera sp.,	-	-
M7	Sterile form	-	-
M8	Sterile form	10.67±0.36	12.6±0.36
M9	Botrydiplodia sp.,	12.47±0.5	13.53±0.43
M10	Aspergillus niger	13.4±0.51	16.4±0.51
M11	Aspergillus ochraceus	16.67±0.36	10.06±0.29
M12	Aspergillus sp.,	10.33±0.36	8.46±0.5
M13	Aspergillus flavus	11.4±0.51	12±0.57
M14	Nodulisporium sp.,	8.46±0.5	-
M15	Sterile form	10.2±0.42	-
M16	Penicillium aculeatum	12.4±0.28	-
M17	Bipolaris sp.,	8.6±0.42	10.47±0.5
M18	Aspergillus terreus	12.47±0.5	15.5±0.5
M19	Aspergillus nidulans	18.53±0.22	14.5±0.5
M20	Fusarium oxysporum	-	12.13±0.5

Values are expressed as mean ± standard error mean (SEM) of the three replicates.

Trichoderma sp., M8 Sterile form isolated during the Pre-monsoon period and *Aspergillus* sp., which is specific to bark tissue have moderate activity against both the pathogens. *Nodulisporium* sp., which is the second dominant endophyte isolated have very least activity (8.6±0.42 mm) against *S.mutans* and no activity against C.albicans. *Colletotrichum gleosporioides* isolated from leaf and bark tissues and *Fusarium oxysporum* specific to leaf tissue have less activity of less than 10 mm zone of inhibition against *C.albicans* and no activity against *S.mutans*. Fabry et al. (1998) reported that endophytic fungi with low antimicrobial activity might possess the active compounds in a low concentration and the amount of these compounds could be enhanced by optimizing some of the factors of fermentation processes such as inoculum concentration, solvents used for extraction and method of purification processes.[30] *Dreschlera* sp., and M7 sterile form have no activity against both the pathogens. Among all the isolates, *Eupenicillium* sp., (Fig 1) have shown maximum zones of inhibition against both the pathogens and this endophyte was further explored for its activity against oral pathogens with different concentrations of extracts as shown in Table 6.

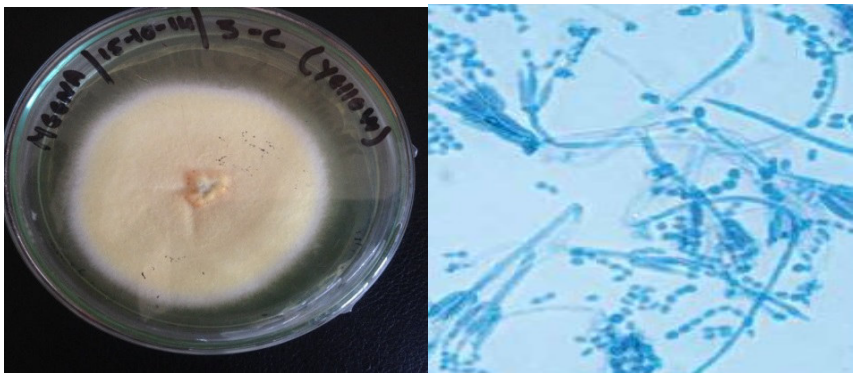


Figure 1
Macroscopic and microscopic view of Eupenicillium sp.,

Table 8
Secondary screening of pathogens using different concentrations of ethyl acetate extracts of Eupenicillium sp.,

Concentration of ethyl acetate extracts of Eupenicillium sp., (µg/ml)	25	50	75	100	Positive control (2% chlorhexidine)
Zone of inhibition for S.mutans (mm)	10.5±0.5	11.5±0.5	15.4±0.51	18.67±0.36	11.4
Zone of inhibition for C.albicans (mm)	11.26±0.36	13.3±0.36	16.4±0.28	16.3±0.36	13.5

Values are expressed as mean ± standard error mean (SEM) of the three replicates.

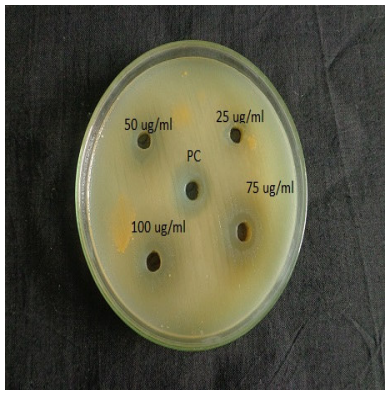


Figure 2
Screening of ethyl acetate extract of Eupenicillium sp., by agar well diffusion method against S.mutans

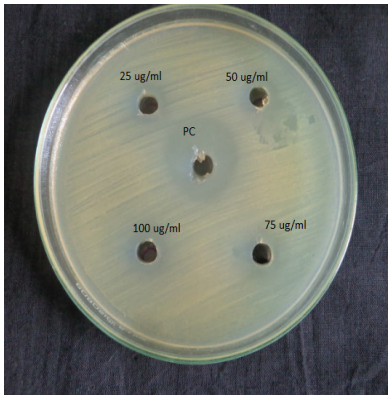


Figure 3
Screening of ethyl acetate extract of Eupenicillium sp., by agar well diffusion method against C.albicans

CONCLUSION

The present study on endophytic fungi from Acacia nilotica helps us to understand the species richness of endophytes in two different parts of the plant. More number of endophytes was isolated during winter season which provides favorable conditions for the growth of the fungi. Though more studies have been done on endophytic fungal diversity, each study is unique resulting in the isolation of newer taxa. The antimicrobial studies on the endophytes isolated gives varying degree of activity against the oral pathogens.

Eupenicillium sp., a telomorphic form of *Penicillium* species has maximum antimicrobial activity and this fungus could be further studied for the presence of active compound and its purification.

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