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RESEARCH ARTICLE

Biofilm Inhibition Efficiency of Endophytic Fungi Isolated from *Acacia nilotica* against Oral Pathogens

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ABSTRACT:

The aim of the study was to investigate the biofilm inhibition efficiency of endophytic fungi isolated from *Acacia nilotica* Linn. which is a traditional plant used for oral ailments. About 10 oral pathogenic strains of *Streptococcus mutans* and *Candida albicans* were collected from patients with dental caries and tested for their biofilm forming ability by crystal violet assay. Endophytic fungi were isolated from the leaves of *Acacia nilotica* and secondary metabolites were extracted using ethyl acetate as the solvent. The crude extracts of endophytic fungi at different concentrations (0, 10, 25, 50 and 100 µg) were then subjected to biofilm inhibition studies against the biofilm forming oral strains of *Streptococcus mutans* and *Candida albicans* with chlorhexidine as the positive control. The percentage of biofilm inhibiton efficiency of endophytic fungal extracts were calculated from the optical density values at 570 nm. The isolates of *Streptococcus mutans* (SM08) and *Candida albicans* (CA09) showed stronger biofilm formation and was further used for biofilm inhibition studies. The endophytic fungi *Eupenicillium* sp. and *Aspergillus nidulans* were isolated in dominant numbers and at 100 µg concentration of extracts, the percentage of *Candida albicans* biofilm inhibition efficiency of extracts of *Eupenicillium* sp. and *Aspergillus nidulans* were 79.53% and 74.86% respectively. Similarly, at 100 µg concentration of *Eupenicillium* sp. and *Aspergillus nidulans* extract, there was 55.94% and 51.49% of biofilm inhibition of *Streptococcus mutans*.The present study proved the potential of endophytic fungi isolated from *Acacia nioltica* for their biofilm inhibition of oral pathogens and the compounds in the endophytic fungal extracts would further serve as the leads of drugs for oral ailments.

KEYWORDS: *Streptococcus mutans*, *Candida albicans*, *Acacia nilotica*, Endophytic fungi, Biofilm inhibition**.**

INTRODUCTION:

The human mouth is highly conducive for uncontrolled formation of natural microbial biofilms due to its diverse niches and ample supply of nutrients. Biofilms are formed by the attachment of microbes to solid surfaces and and they are surrounded by extracellular matrix providing structure and protection to the community. Mature biofilms are resistant to antibiotic therapy and to immune system effectors. Dental plaques are characterized by the formation of biofilms by oral pathogenic strains predominantly by Streptococcus mutans. The dental implants and the medical devices of immune compromised people are dominated by *Candida albicans* biofilm and produce serious consequences

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when they enter the blood stream[1]. Recent studies suggest that cariogenic development is mediated by interaction between oral bacterial and fungal pathogenas there is high prevalence of *Streptococci* where *Candida* resides[2]. 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. Natural products make excellent leads for new drug development which are safer and biodegradable. The medicinal plant *Acacia nilotica* is a xerophytic plant used in traditional medicine for various health problems. *Acacia nilotica*, nitrogen fixing legume tree widely known as Babul and Gum Arabic tree belongs to the family of Fabaceae. The tender twigs of *Acacia nilotica* are used as toothbrushes for its germicidal property[3]. The extracts of *Acacia nilotica* have good antimicrobial activity especially against oral pathogens. A remarkable antibacterial activity against the dental caries pathogen *Streptococcus mutans* was seen in the stem bark extract of the plant[4]. Dried fruits of *Acacia* species inhibit *Candida albicans* and are used in traditional medicine for oral candidiasis.

Fungal endophytes serve as a repository for novel compounds with immense value in agriculture, medicine and industry. Many ethno medicinal plants from unique environmental settings are likely to harbor distinct endophytes with novel biological properties. Currently plants having medicinal values are studied for their endophytic fungal diversity for the synthesis of distinct secondary metabolites with unique pharmacological values. Endophytes are less studied group of microorganisms that have an enormous potential to be explored in the field of medicinal, agricultural and industrial arenas[5]. Thus, exploring the endophytes in the hotspots of biodiversity is considered important to produce plethora of useful natural products.

As there are no reports available on the biofilm inhibition efficiency of endophytic fungi in general and particularly from *Acacia nilotica* plant, this study would be an initiative of exploring antibiofilm activity of endophytic fungi against oral biofilm forming pathogens. In the present study, the fungal endophytes isolated from *Acacia nilotica* were tested for their biofilm inhibition efficiency against oral pathogens *Streptococcus mutans* and *Candida albicans*.

MATERIALS AND METHODS:

Collection of oral pathogens and their biofilm formation ability:

Pure cultures of *Streptococcus mutans* and *Candida albicans* were obtained from the laboratory of Billroth hospitals, Chennai which were isolates from patients with dental caries. The cultures were identified and authenticated by Dr. Kayalvizhi, Microbiologist, Billroth Hospitals, Chennai. *Streptococcus mutans*, a gram positive bacterium was cultured on Brain Heart Infusion Broth (BHIB) (HiMedia Labs.) and the yeast *Candida albicans* was cultured on Sabouraud Dextrose Broth (SDB) (HiMedia Labs.). Both the cultures were maintained on their respective agar slants at 4°C for further use.Biofilm formation of 10 oral pathogenic strains of *Streptococcus mutans and Candida albicans was assessed by* crystal violet assay and only the broth without cell suspension served as control[6]. The value of optical density at 570 nm was considered as an index of forming biofilms. The mean OD value of the number of experiments performed was considered. The difference between the OD values of control and their respective test organisms was calculated[7]. If the difference is less than 0.1, it indicates weak biofilm formation and for values between 0.1 to 0.3, it indicates moderate biofilm formation. Isolates with OD values greater than 0.3, were considered as forming stronger biofilms[8].

Isolation of endophytic fungi:

The endophytic fungi used in this present work was isolated from the leaves of *Acacia nilotica* collected from reserved forest near Madurai, Tamilnadu, India. The segments measuring about 0.5x0.5 cm^2 were cut from the plant samples using sterile scalpel. Leaflets were collected and washed thoroughly with sterile distilled water and surface sterilized by immersing sequentially in 70% ethanol for 5 seconds and 4% sodium hypochlorite for 1 min and rinsed with sterile distilled water 2-3 times[9]. The surface sterilized segments were inoculated on to petridishes containing Potato Dextrose Agar (PDA) media supplemented with chloramphenicol (50 µg/ml) to suppress bacterial growth. The plates were incubated at 25°C in a light chamber with 12 hours of light followed by 12 hours of dark cycles for 3-6 days[10]. Fungal hyphae tips growing out from sterile segments were sub cultured and the pure fungal isolates were transferred to PDA slants and stored at 4°C for further use. The endophytes were identified and authenticated by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India.

Extraction of crude metabolites:

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 25^0C 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 dimethyl sulfoxide (DMSO) and stored at 4^0C for further use[11].

Biofilm inhibition study using extracts of endophytic fungi:

Biofilm inhibition assay of *Streptococcus mutans* and *Candida albicans* were performed using the ethyl acetate extracts of the dominant endophytic fungi isolated. The fungal extracts were tested for their biofilm inhibition efficiency against their respective pathogens. Different concentrations of the ethyl acetate such as 0, 10, 25, 50 and 100 µg of both the endophytes were prepared. 20 µL of overnight grown *Streptococcus mutans* and *Candida albicans* cells were seeded in 96 well plate with 180 µL of their respective media. Ethyl acetate extracts were added in different concentrations to the wells (0, 10, 25, 50 and 100 µg) containing *Streptococcus mutans*and*Candida albicans* cells. The microtitre plate was incubated at 25°C for 24 hrs. Culture medium was removed from the 96 well plate and the wells were washed with 200 µL of Phosphate Buffer Saline (PBS). PBS was removed and the wells were stained with 1% Crystal violet solution to stain the polysaccharides of the biofilm. It was incubated for 10 minutes at room temperature. The plate was washed in tap water and drained upside down on paper towels and % SDS was added to solubilize the stain. The plate was agitated on orbital shaker until color is uniform with no areas of dense coloration in bottom of wells. Absorbance of each well was measured at 570 nm. Chlorhexidine (0.12%) was used as a positive control which inhibits biofilm formation. Culture without adding fungal extract with the media and the oral pathogenic cells alone served as control [12].The percentage of biofilm inhibition was calculated using the following formula:% of biofilm inhibition = ([Control $OD_{570\ nm}$- Test $OD_{570\ nm}$] / Control $OD_{570\ nm}$) × 100.

RESULTS:

The isolates of *Streptococcus mutans* and *Candida albicans* collected were given codes from SM 01 to SM 10 and CA 01 to CA 10 respectively. The isolate SM 08 showed strong biofilm activity (OD > 0.3) with optical density value of 0.45 assessed by crystal violet assay. Similarly, the biofilm formation of the strain CA 09 was stronger with optical density value of 0.53. Thus, the strains SM 08 and CA 09 were selected for further study (Table 1 and Table 2).

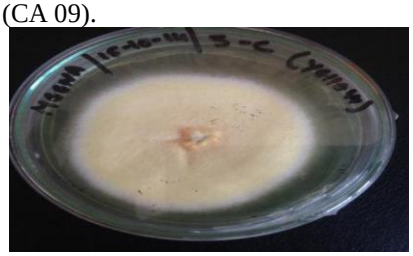
Table 1: Screening of *Streptococcus mutans* for biofilm formation

S.No.	Codes of Streptococcus mutans isolates	Quantitative assay (OD at 570 nm)	Biofilm formation
1	SM 01	0.231	Moderate
2	SM 02	0.354	Strong
3	SM 03	0.141	Moderate
4	SM 04	0.362	Strong
5	SM 05	0.014	Weak
6	SM 06	0.330	Strong
7	SM 07	0.264	Moderate
8	SM 08	0.45	Strong
9	SM 09	0.002	Weak
10	SM 10	0.112	Moderate

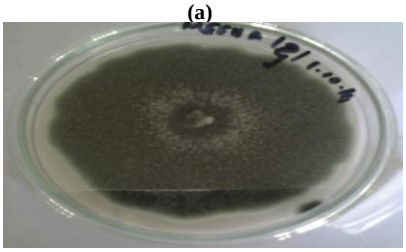
Table 2: Screening of Candida albicans for biofilm formation

S.No.	Codes of *Candida albicans* isolates	Quantitative assay (OD at 570 nm)	Biofilm formation
1	CA 01	0.421	Strong
2	CA 02	0.245	Moderate
3	CA 03	0.132	Moderate
4	CA 04	0.012	Weak
5	CA 05	0.142	Moderate
6	CA 06	0.210	Moderate
7	CA 07	0.462	Strong
8	CA 08	0.162	Moderate
9	CA 09	0.530	Strong
10	CA 10	0.228	Moderate

A total of 20 different endophytic fungi were isolated from the leaflets and bark tissues of *Acacia nilotica* and tested for their antimicrobial property against the oral pathogens[13]. The dominant endophytic fungi with potent antimicrobial activity were identified as *Eupenicillium* sp. and *Aspergillus nidulans* (Eidam) G. Winterand authenticated by National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India. The growth of endophytic fungi on PDA plates is shown in fig. 1. These two endophytes were tested for their inhibition activity towards the biofilm forming strains of *Streptcoccus mutans* (SM 08) and *Candida albicans* (CA 09).



(a)



(b)

Fig. 1: Macroscopic view of a) Eupenicillium sp. and b) Aspergillus nidulans:

The ethyl acetate extracts of the endophytes *Eupenicillium* sp. and *Aspergillus nidulans* were used to inhibit the biofilm formation of *Streptococcus mutans* and *Candida albicans* at different concentrations (0, 10, 25, 50 and 100 µg). 0.12% chlorhexidine was used as positive control. Muller Hinton broth was used as negative control for *Streptococcus mutans* biofilm inhibition assay where as Sabouraud's dextrose broth was used as negative control for *Candida albicans* biofilm inhibition assay. At 100 µg concentration of *Eupenicillium* sp. and *Aspergillus nidulans* extract, there was 55.94% and 51.49% of biofilm inhibition of *Streptococcus mutans*. The ethyl acetate extract of *A.nidulans* at different concentrations inhibits *Streptococcus mutans* biofilm formation better than that of *Candida albicans*. At concentrations lesser than 100 µg, the biofilm inhibiton efficiency of both the endophytic extracts was minimal.

Fig. 2 shows the percentage of biofilm inhibiton of *Streptococcus mutans* using the ethyl acetate extracts of *Eupenicillium* sp. and *Aspergillus nidulans*.

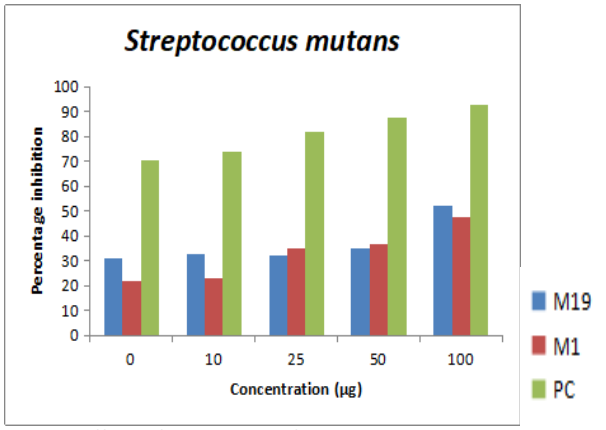


Fig. 2: Effect of endophytic fungal extracts on *Streptococcus mutans* biofilm formation
M1 – *Eupenicillium* sp. extract, M19 – *Aspergillus nidulans* extract, PC- Positive control

Similarly, the *Candida albicans* biofilm inhibition assay was performed and the percentage inhibition efficiency of extracts of *Eupenicillium* sp. and *Aspergillus nidulans*were 79.53% and 74.86% respectively at 100 µg concentration of extracts. At about 50 µg concentration of *Eupenicillium* sp. extract, there was about 50% inhibition of *Candida albicans* biofilm. The biofilm inhibition efficiency of *A.nidulans* extract was higher when compared to that of *Eupenicillium* sp., extract. (Fig. 3)

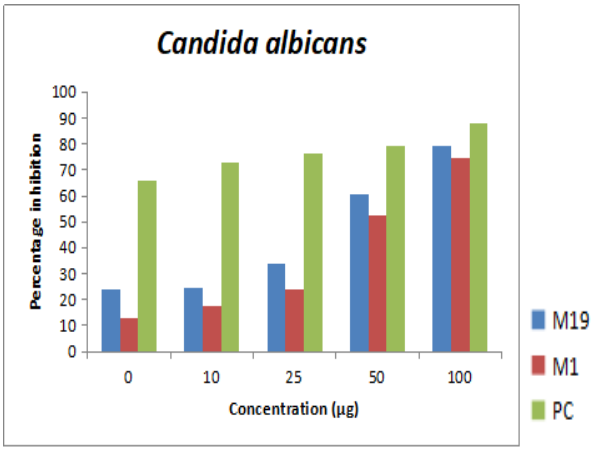


Fig. 3: Effect of endophytic fungal extracts on *Candida albicans* biofilm formation
M1 – Eupenicillium sp. extract, M19 – Aspergillus nidulans extract, PC- Positive control

DISCUSSION:

Streptococcus mutans and *Candida albicans*are found to be the major organisms residing in the oral biofilms and both the pathogens are benefitted mutually as a result of their associations[14]. The hypothesis of interaction between *Streptococcus mutans* and *Candida albicans* depends on their virulence mechanism and host factors which provide favorable oral environment for both the organisms[15]. In the present study, the pathogens *Streptococcus mutans* and *Candida albicans* isolated from the dental caries of infected patients formed strong biofilms which was evident from the optical density values at 570 nm. The difference between the OD values of the control and the test organism served as the index of forming biofilm which was greater than 0.1 for both the oral pathogens. Similar study was done by Jadhav and Tale (2015) in which several isolates of *Streptococcus* sp. from oral cavity of cancer patients were isolated and tested for their biofilm formation[16].

Biofilm inhibition studies on *Streptococcus mutans* and *Candida albicans*by plant extracts have gained focus because of the adverse effects of long term use of commercial drugs. Similarly, cardamom extracts have been proved for their antimicrobial activity against oral pathogens[17]. Teanpaisan *et al*., (2016) screened various Thai medicinal plants against the oral microbes for their biofilm inhibition[18].In the present investigation, the extracts of different concentrations of *Eupenicillium* sp. and *Aspergillus nidulans* isolated from the medicinal plant *Acacia nilotica* exhibited antibiofilm activity against *Streptococcus mutans* and *Candida albicans*. Similar studies were donein which the extracts of marine endophytes such as *Fusarium* sp., *Khuskia* sp., *Epicoccum* sp. and *Sarocladium* sp. have good quorum sensing inhibitor activity which in turn inhibits bacterial biofilm[19]. Estrela and Abraham also reported the biofilm

inhibition efficiency of several fungal metabolites that inhibit quorum sensing receptors and cell wall synthesizing enzymes of the bacteria, thus preventing their biofilm formation[20].

In the current study, the biofilm inhibition efficiency of *A. nidulans* extract was better than the extract of *Eupenicillium* sp. Some metabolites such as terreic acid from *A. terreus* and flavipesin from *A. flavipes* have inhibitory activity towards various bacterial and fungal biofilms[21,22]. The current study indicated that theendophyte *Eupenicillium* sp. and *Aspergillus nidulans* isolatedfrom the traditionally used oral ailments preventive plant *Acacia nilotica* was effective in treating biofilm forming oral*Streptococcus mutans* and *Candidaalbicans*. To conclude, the secondary metabolites of these endophytic fungicould be used for the prevention of oral infections and their biofilm-mediated diseases. Further, the compounds in the extract have to be isolated which provides a scope for the target specific inhibition of oral *Streptococcus mutans* and *Candida albicans*.

CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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