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Comparative evaluation of biofilm suppression by plant extracts on oral pathogenic bacteria

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ABSTRACT

Microbial colonization as biofilm is one of the reasons for the emergence of drug resistant strains. In the oral cavity, drug resistant strains limit the efficacy of oral hygiene practices. *Enterococcus faecalis* and *Staphylococcus aureus* have been reported as drug resistant bacteria and producing oral biofilms in oral cavity. In this study we demonstrate the efficacy of aqueous extract of *Azadirachta indica*, *Mangifera indica*, *Piper betel* and *Pepper nigrum* for antibiofilm activity against *E. faecalis* and *S. aureus*. The aqueous extracts were obtained by cold percolation method. The antibiofilm activity of plants extract was evaluated at 30, 15 and 7.5 mg/ml concentration. The percentage yield of extract was maximum in *P. nigrum*. The aqueous extract of *A. indica* significantly suppressed *E. faecalis* and *S. aureus* biofilm at 7.5 mg/ml at $p<0.01$ and $p<0.001$ significance level. *P. betel* significantly ($p<0.001$) disintegrated the *E. faecalis* biofilm at 30 mg/ml and *S. aureus* at 15 mg/ml ($p<0.01$). *P. nigrum* disintegrated *E. faecalis* and *S. aureus* biofilm significantly ($p<0.05$ and $p<0.001$) at 30 and 15 mg/ml respectively. *M. indica* significantly ($p<0.05$) suppressed *S. aureus* biofilm at 30 mg/ml. These results clearly demonstrate the antibiofilm activity of plants extract against oral pathogens.

INTRODUCTION

Plant derived products or drugs have been used since time immemorial in health care for treatments of a variety of infectious diseases (Rios and Recio, 2005). In oral health care, use of plant twigs or leaves was in practice globally (Wu *et al.*, 2001). The introduction of allopathic or chemical based drugs caused the gradual decline in the use of herbal medicine. On the contrary the emergence of multidrug resistant microorganisms and their potential side effects urged in search for alternative medicine. So, in the past few decades the use of herbal medicine has gained importance to counteract the potential demerits of the allopathic system (Alviano and Alviano, 2009). *A. indica* (neem) belongs to Meliaceae family, and its importance has been recognized by the US National Academy of Sciences (1992) report entitled "Neem- a tree for solving global problem" (Hashmat *et al.*, 2012). The edible fruit of *M. indica* (mango) of family Anacardiaceae is an economically tropical fruit found throughout the world for its nutritional composition (Abdulla, 2007). *P. betel* (betel) is used in the Indian traditional system of medicine for its antioxidant and antimicrobial properties (Al-Adhroey, 2011). The leaves of betel are of medicinal, religious and ceremonial value in Southeast Asia. Chewing betel leaves prevent halitosis, improve the vocalization, hardens the gums, conserves the teeth and sweetens breath (Rai *et al.*, 2011). *P. nigrum* (black pepper) seed is popular among the spices and can be used for different purposes such as human dietaries, as medicine, as preservatives and as biocontrol agents (Ahmed *et al.*, 2012). Microbial biofilm is a specialized community of adherent microorganisms in a complex extrapolymeric matrix (Hall-Stoodleyl and Stoodleyl, 2005). Oral microbial biofilms are three- dimensional structured bacterial communities found attached to tooth enamel, root surface or dental implants (Zinge *et al.*, 2010). The structured organization of microbial cells reduces the efficacy of topically applied agents and thus has the direct implications for the successful treatment outcomes of oral diseases (Filoche *et al.*, 2010). *E. faecalis* is the main causative organism of failed root canal treatments (Mahmoudpour *et al.*, 2007); found to colonize, form biofilm even in medicated root canals of human teeth (Distel *et al.*, 2002). *S. aureus* is found to be present in substantial

Abbreviations: BHI: Brain Heart Infusion, MH: Muller Hinton

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numbers on teeth in geriatric and intensive care patients. The colonization of oral *S. aureus* is one of the major oral and dental risk factor for aspiration pneumonia in veteran residents (Terpenning *et al.*, 2001). The emergence of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Enterococcus faecalis* (VRE) in oral cavity (Love, 2001; Crusta *et al.*, 2010) pose a new threat to oral health. With this vision, this study was aimed to evaluate the efficacy of medicinal plants extract in suppressing *E. faecalis* and *S. aureus* biofilm.

MATERIALS AND METHODS

Collection and processing of plants

The fresh leaves of *A. indica*, *M. indica*, *P. betel* and *P. nigrum* free from insect infestation and infection, were collected from southern part of Karnataka state, India. The specimen samples were identified in the Department of Pharmacognosy, Sri Dharmasthala Manjunatheshwara Centre for Research in Ayurveda and Allied Sciences, Udupi, India. The collected plant's leaves were washed in tap water followed by distilled water and shade dried. The dried samples were powdered using a blender. The powdered materials were stored in an airtight container.

Microorganisms used for biofilm

The ATCC cultures of *E. faecalis* (29212) and *S. aureus* (25923) were obtained from the Nitte University Centre for Science Education and Research, Mangalore, India. *E. faecalis* and *S. aureus* were subcultured on Muller Hinton Agar Medium. The suspension culture was prepared in Brain Heart Infusion (BHI) broth and Mueller Hinton (MH) broth respectively.

Chemicals

The Mueller Hinton Agar, BHI broth and MH broth, 96 well microtiter plate and crystal violet stain were procured from Hi-media Laboratories, Mumbai. Crystal violet stain was prepared by dissolving 0.1g in 100 ml of double distilled water. Acetic acid was obtained from Merck and was diluted to 30% in double distilled water.

Aqueous extraction

The aqueous extraction of plant materials was done by cold maceration method (WHO, 1998). 10 g of the powdered sample was immersed in 100 ml of chloroform- water mixture (2.5:97.5) for 48 hrs and filtered through muslin cloth and filter paper. The filtrate was concentrated until it was free from moisture on a water bath, in an evaporating dish and stored in a desiccator. The yield was noted down and extracts were reconstituted in sterile double distilled water.

Biofilm suppression assay

Biofilm of microorganisms was grown in 96 well microtiter plate (O'Toole, 2011). The overnight cultures of *E. faecalis* and *S. aureus* were diluted in fresh BHI broth and MH broth respectively at 1:100 ratio and 100µl of this suspension was dispended into sterile flat-bottomed 96 well polystyrene microtiter plates. After 24 hrs of incubation broth was carefully drawn off by using multichannel pipette. Wells were washed three times with 100 µL phosphate buffer saline. After complete drying of the plates, biofilms were treated with aqueous extract of plants at 30, 15 and 7.5 mg/ml for 20 min. Extracts were carefully pipetted out and washed in distilled water. Microtiter plates were inverted on a blotting sheet and air dried. Biofilms were stained with 100 µl of 0.1% crystal violet stain for 15 min, and the wells were washed off using distilled water followed by air drying of the plates. In order to quantify the biofilm, 100 µl of 30% acetic acid was added to the each well to distain the biofilms. Thereafter, the optical density of resolubilized crystal violet stain was measured at 570 nm using LISA plus reader. The optical density readings of biofilms were statistically analyzed by one way ANOVA and Bonferroni test using Graphpad Prism at 5% level of significance.

RESULTS

The percentage yield of four medicinal plants aqueous extract is presented in Table1. The yield was more in *P. nigrum* and less in *M. indica*. The mean±SD optical density values of biofilms after treating with plants extracts is presented in Table 2 and 3 for *E. faecalis* and *S. aureus* respectively. Neem leaf extract suppressed the *E. faecalis* biofilm significantly ($p<0.01$) at 7.5 mg/ml. Betel and pepper leaf extracts at 30 mg/ml suppressed the *E. faecalis* biofilm at $p<0.001$ and $p<0.05$ significance level respectively. The Mango leaf extract did not suppress the *E. faecalis* biofilm significantly. Neem leaf at 7.5 mg/ml and pepper leaf extract at 15 mg/ml were highly significant ($p<0.001$) against *S. aureus* biofilm. The betel leaf extract was significant ($p<0.01$) in suppressing *S. aureus* at 15 mg/ml. The aqueous extract of mango leaves significantly ($p<0.05$) effective only against *S. aureus* biofilm at 30 mg/ml.

Table. 1: Percentage yield of aqueous extract of medicinal plants.

Medicinal plant	Percentage yield
Mangifera indica	10%
Azadirachta indica	28%
Piper Betel	16%
Piper nigrum	29.33%

Table. 2: Suppression of *E. faecalis* biofilm (0.219±0.03) by medicinal plants extract.

Medicinal plant	Concentrations		
	30 mg/ml	15 mg/ml	7.5 mg/ml
Azadirachta indica	0.135±0.04	0.138±0.0	0.145±0.02
Mangifera indica	0.183±0.07	0.197±0.0	0.205±0.01
Piper betel	0.135±0.02	0.161±0.0	0.168±0.03
Piper nigrum	0.148±0.04	0.165±0.0	0.167±0.01

Table. 3: Suppression of *S. aureus* biofilm (0.251±0.01) by medicinal plants extract.

Medicinal plant	Concentrations		
	30 mg/ml	15 mg/ml	7.5 mg/ml
Azadirachta indica	0.130±0.03	0.132±0.02	0.135±0.01
Mangifera indica	0.175±0.03	0.199±0.04	0.230±0.02
Piper betel	0.169±0.03	0.169±0.01	0.190±0.03
Piper nigrum	0.140±0.02	0.159±0.05	0.181±0.04

DISCUSSION

Oral biofilms distinctly contain more than 500 different bacterial taxa. Besides inter- individual variation in oral biofilm microbiome, heterogenesity of oral biofilm is reported on the same teeth (Hall- Stoodleyl and Stoodleyl, 2005). The thick exopolymer matrix of the biofilm prevents the penetration of antimicrobials and enables the microbial cells to be drug resistant. In addition to this, decreased growth rate and expression of possible resistant genes make antimicrobials ineffective to biofilm microbial cells (Lewis, 2001). Thus organized structure and mechanisms of biofilm are responsible for the emergence of drug resistant bacteria. The present allopathic formulations which used in oral care contain antibiotics, antimicrobial agents, surfactants and alcohol, are not efficient in eradicating oral pathogens completely; on the contrary they were found to be Cytotoxic (Walker, 1998; Flemingson *et al.,* 2008). So the plants derived product are of choice against oral pathogens. Hence, in this study aqueous extract of commonly available plant species in India, mango, neem, betel and pepper leaves were evaluated for their biofilm suppression activity. The leaf part of plants were chosen because leaves contain more secondary metabolites which are responsible for antimicrobial property (Maji *et al.,* 2010). Mango leaf and neem twigs were used by Indians as chewing sticks in oral care. Whereas betel leaves are used as a masticator along with lime and areca nut (Bissa *et al.,* 2007). Pepper leaves were also included in the study because it belongs to the Piperaceae family as betel, which is not explored for its medicinal value till date. So it is necessary to evaluate scientifically the antimicrobial property of these plants leaf extract against oral pathogens like *E. faecalis* and *S. aureus*, which are the most persistent bacteria in failed root canal treatment (Sundqvist *et al.,* 1998) and cause angular cheilitis, parotitis (Smith *et al.,* 2001) respectively. *E. faecalis* is reported to form biofilm successfully in extremely alkaline conditions (Manikandan *et al.,* 2013). Colonization of *S. aureus* in oral cavity is found to be a possible cause of endocarditis, a life threatening infection of heart (Ohara-Nemoto *et al.,* 2008). Among the four plants, neem leaf extract was more effective in reducing the biofilm of both the oral pathogens. Figure 1 and figure 2 shows the decrease in optical density of biofilms of *E. faecalis* and *S. aureus* after treating with plants extract, respectively. The aqueous extract of neem suppressed the *E. faecalis* and *S. aureus* biofilm at 7.5 mg/ml. In fact, the broad spectrum antimicrobial property of neem leaf has been recognized against Gram positive and Gram negative bacteria in different solvents (Timothy et al., 2011; Koona and Budida, 2011; Abalaka *et al.,* 2012). Supporting to our findings against oral pathogens, a study reported profound antimicrobial activity of neem leaf extract in different solvents, against a variety of bacterial strains causing dental caries (Lekshmi *et al.,* 2012). Betel and black pepper leaves, both were found to solubilize the *E. faecalis* and *S. aureus* biofilm at 30 mg/ml and 15 mg/ml respectively. To substantiate our results on betel leaves, the antimicrobial activity of betel leaf oil was reported against oral pathogens by disc diffusion method (Sugumaran *et al.,* 2011). It was observed that the betel leaf extract at 1 mg/ml coagulated the nucleoid material of *Streptococcus mutans*, one of the early colonizers of oral biofilm, into thick electron – dense filaments and destructed the plasma cell membrane and inner cell wall (Nalina and Rahim, 2007). In concurrence to our observations on black pepper, the acetone extract of black pepper seeds displayed excellent inhibition on the growth of *S. aureus* compared to other Gram positive bacteria (Karsha and Lakshmi, 2010). Although there are reports on antimicrobial activity of mango leaf extracts (Masibo, 2009; Alok et al., 2013) on a variety of pathogens, in our study we found it's antibiofilm activity only against *S. aureus*.

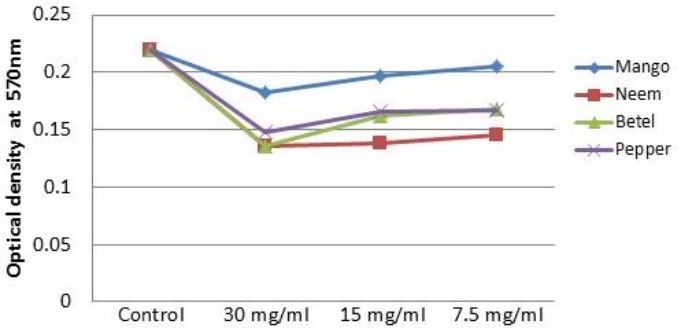

Fig. 1: Decrease in optical density of *E. faecalis* biofilm by plants extract at different concentrations.

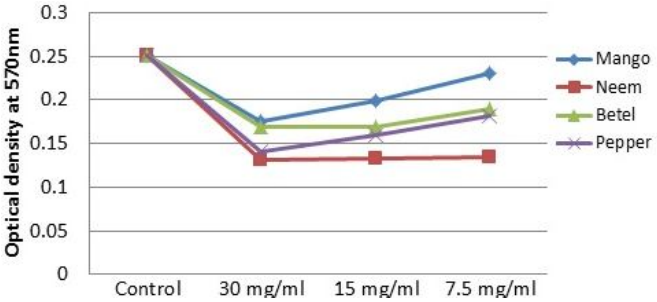

Fig. 2: Decrease in optical density of *S. aureus* biofilm by plants extract at different concentrations.

CONCLUSIONS

This is the first report on evaluation of plants extracts for their antibiofilm activity against oral pathogens. Our study results highlight the scientific evidence for the use of plants in oral care and treat the emergence of multidrug resistant microorganisms and potential side effects of allopathic health care products. Further this study result requires support by the evaluation of antimicrobial activity against drug resistant clinical isolates and cytotoxicity on human gingival fibroblast cells.

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