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INHIBITORY EFFECTS OF PLANT EXTRACTS ON MULTI-SPECIES DENTAL BIOFILM FORMATION *IN-VITRO*

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ABSTRACT

Dental biofilms play an important role in development of caries and periodontal disease, therefore their removal is integral to maintenance of oral hygiene. In the present study, different extracts prepared from *Moringa oleifera, Murraya koenigii*, *Psidium guajava, Eclipta prostrata* and *Phyllanthus fraternus* were screened for their effect on the growth, adherence and co-aggregation of a consortium of dental biofilm isolates. Results indicate, the test consortium was most susceptible to Ethanolic extract of *M.koenigii* and Aqueous–Ethanolic extracts of *P.guajava* and *E.prostrata* at their minimum inhibitory concentrations of 0.5 mg/ml, 1 mg/ml and 5 mg/ml respectively and effectively suppressed adherence and co-aggregation of test consortium at sub-MIC concentrations. Subsequently, aqueous–ethanolic extracts of *P.guajava* and *E.prostrata* demonstrated a log reduction of 3.5 and 4.2 $\log_{10}$cfu/ml respectively, in the viable count of dental biofilm load in-vitro. Hence, this study justifies the paradigm shift in oral care towards the use of nature-based products.

KEYWORDS:Dental biofilm; anti-bacterial activity; anti-adherence activity; anti-co-aggregation activity; Biofilm load

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INTRODUCTION

Dental caries are one of the most prevalent diseases in humans, [1] the cause of which is believed to be "plaque"- a biofilm of commensal bacteria like *Streptococcus mutans*, *Streptococcus sanguinis, Streptococcus mitis* etc embedded in a matrix of bacterial and salivary polymers. [2] Dental biofilms develops naturally on teeth as a part of host defences against exogenous microflora. However, if left untreated, it leads to caries, gingivitis and periodontitis. The oral cavity may also disseminate pathogenic organisms to distant body sites, leading to systemic diseases like infective endocarditis, bacterial pneumonia, atherosclerosis, etc. [3] Changes in homeostasis of the oral cavity; with an overgrowth of *S.mutans* is recognized as the primary cause of cavities [1] making it the best studied example of cariogenic bacteria. Hence plant extracts that aim at either the elimination of this bacterium or suppression of its virulence have attracted the interest of many researchers. [5-11] However, it is imperative that efficacy of any extract be evaluated additionally on its ability to inhibit multi-species biofilms which are dynamic and possess variability of flora. Commercially available agents alter homeostasis of oral cavity and lead to development of resistance among flora. Prolonged use leads to side-effects like vomiting, diarrhoea, tooth staining. [4] Phytotherapy is now increasingly being looked at as an alternative. In the present study, five medicinal plants were investigated for their anti-biofilm activity. *Psidium guajava* Linn. (Family: Myrtaceae), is a small tree, various parts of which exhibit antioxidant, hepatoprotective, antimicrobial and antidiabetic properties. [12] *Phyllanthus fraternus* G.L. Webster (Family: Euphorbiaceae) is an annual herb, used for treatment of biliary and urinary tract conditions, bacterial and viral infections. [13] *Eclipta prostrata* Linn (syn. *Eclipta alba* Hassk.) (Family: Asteracea) is a small evergreen tree possessing anti-inflammatory, antimicrobial, antiviral, analgesic and immunomodulatory properties. [14] *Moringa oleifera* Lam (Family: Moringaceae) is a medium sized tree, known for antioxidant, antimicrobial, anticancer, antihyperlipidaemic and hepatoprotective properties. [15] *Murraya koenigii* Linn (Family: Rutaceae) is a small tree; the leaves of which are used for antioxidant, antidiabetic, antibacterial and cytotoxic effects. [16] Literature regarding activity of enlisted plants against multi–species dental biofilms is limited. Aim of the study was to evaluate the potential of enlisted plants to inhibit *in-vitro* biofilm formation by targeting cariogenic properties like growth, adherence and cellular co-aggregation.

MATERIALS AND METHODS

Brain Heart Infusion medium was purchased from Himedia Laboratories, Mumbai, India. 0.2% w/v Chlorhexidine gluconate (CHX), Hexidine® was obtained from ICPA Health products Ltd., Ankaleshwar, India. Chemicals used were of analytical grade purchased from Qualigens Fine Chemicals, Mumbai, India.

(i) Preparation of plant extracts

Young leaves of *M.oleifera*, *M. koenigii*, *E. prostrata P. fraternus* and *P. guajava* were collected from Mumbai, India. The plants were authenticated by The Blatter Herbarium, Mumbai, India. The leaves were washed, dried and pulverized into a powder. The powdered plant material was subjected hot and cold extractions using ethanol, water and aqueous-ethanol (1:1) as solvents. In cold extraction method, powdered plant material was macerated in the solvent with intermittent shaking for 2 days. In hot extraction method, soxhlation was done for 6 hours. The extracts were filtered, dried at 50^{o}C and stored at 4^{o}C.

(ii) Microorganisms

Streptococcus mutans MTCC#890 and *Streptococcus mitis* MTCC#2696 were obtained from Institute of Microbial Technology, Chandigarh, India. In order to prepare the test consortium for the study, plaque samples were collected from volunteers. 26 different isolates were obtained from plaque samples of 10 healthy volunteers, of which 6 isolates capable of forming biofilms were used as a part of the test consortium

along with standard strains - *S.mitis* and *S.mutans*. As per Bergey's manual of Systematic Bacteriology [17], isolates were identified using conventional biochemical tests up to species level as *Streptococcus salivarius, Streptococcus mitior, Streptococcus sanguinis* and *Streptococcus milleri* while two isolates were identified up to the genus level as *Streptococcus* spp.

(iii) Screening

Preparation of bacterial suspension: All strains of test consortium were sub-cultured onto fresh Brain Heart Infusion agar and incubated overnight at 37°C in candle jar. The inoculum was prepared by adjusting turbidity of suspension to 0.5 McFarland turbidity standards (≈10^8 cfu/ml). ***Preparation of extracts*:** All extracts were reconstituted in respective solvents to obtain a concentration of 100 mg/ml for screening.

***Antibacterial activity*:** Assay for antibacterial activity was performed by Agar well diffusion method [18] and the diameters of zones of inhibition were recorded.

***Anti-adherence activity*:** Adherence assay was performed by a method described by Islam et al [10] with slight modifications. BHI broth with 5% (w/v) sucrose and extract (final concentration of 5 mg/ml) were inoculated with overnight culture of test consortium. Tubes were inclined at 30° and incubated at 37°C for 24 hours. Degree of adherence was scored on a scale of 0 to 4+. [19]

***Anti-co-aggregation activity*:** Co-aggregation assay was performed by a method described by Prabhu et al [7] with slight modifications. BHI broth with 5% (w/v) sucrose and extract (final concentration of 5 mg/ml) were inoculated with overnight culture of test consortium. Degree of cell aggregation was observed after incubation at 37°C for 24 hours and culture fluid was scored on a scale of 0 to 4+. [19] For screening assays, solvent of each extract served as negative control while 0.2% w/v CHX served as positive control. Extracts demonstrating maximum activity were selected for determination of Minimum Inhibitory Concentration (MIC) by Agar well diffusion method and evaluation of anti-adherence and anti-co-aggregation activities at varying concentrations from 0.01 mg/ml – 15 mg/ml of extract.

(iv) Effect of extract on in-vitro biofilm formation

Extracts with lowest MIC, exhibiting substantial inhibition of adherence and co-aggregation were selected to evaluate anti-biofilm activity *in-vitro.*

***Preparation of saliva*:** Sterile saliva was prepared by a method described by Rahim et al [20] with slight modifications. Saliva was collected from a single volunteer into a chilled, sterile tube via expectoration. Saliva was centrifuged at 4,000*g* for 10 min. The supernatant was filter sterilized using sterile 0.45µ cellulose nitrate filter (Sartorius stedim biotech®, Germany) and stored at - 20°C till further use.

Substratum: Acrylic tooth was sterilised using 2% gluteraldehyde and washed with sterile distilled water before use.

Development of Biofilm: *1* ml of sterile saliva was added to a tube containing sterile tooth and kept undisturbed for 90 mins for conditioning. 3.9 ml of BHI broth with 1% (w/v) sucrose was then added followed by 100 µl of extract such as to obtain MIC of extract in each tube. The tube was inoculated with 0.05 ml of test consortium. Negative and positive controls were set up in a similar manner by substituting 100 µl of extract with 100 µl of solvent and 100 µl of 0.2% w/v CHX respectively. During standardization of the protocol, confluent growth of biofilm on tooth surface was obtained at the end of 7 hrs; hence incubation conditions for the study were set as 7 hrs at 37°C.

Assessment of microbial load in Biofilm

After 7 hrs, the tooth was placed in a sterile tube containing 1 ml phosphate buffered saline (PBS) and sonicated for 30 seconds to disrupt biofilm. Contents of the tube were further serially diluted using PBS and used to determine viable count. Effect of extract on biofilm formation was recorded in terms of Log reduction in viable count of biofilm, using the formula [Log (Mean viable count of negative control) – Log (Mean viable count of test)].

(v) Statistical analysis

Statistical analysis was performed using GraphPad Prism®5 software. Each experiment was carried out in duplicates and repeated in three independent experiments. Inter group difference was estimated by analysis of variance. Dunnett's multiple comparison test was performed to test significance of results, considering P value ≤ 0.05

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RESULTS

1. ***Screening:*** The results of screening of 30 extracts obtained from five plants for antibacterial, anti-adherence and anti-co-aggregation activities are depicted in Table 1.

Table 1
Screening of plant extracts for antibacterial, anti-adherence and anti-co-aggregation activities

	Anti-bacterial activity Zone of inhibition (mm) (Mean±S.D., n=6)	Anti-Adherence activity*	Anti-Co-aggregation activity*
CONTROL			
Ethanol	12	-	-
Aqueous-ethanol (1:1)	10	-	-
Water	10	-	-
CHX	25±0.7	+	+
EXTRACT			
M. koenigii			
HEE	14.6±0.55	-	-
HAEE	11.8±0.44	+	+
HAE	10	-	+
CEE	30.2±0.84	+	+
CAEE	10	+	+
CAE.	10	+	+
P. guajava			
HEE	15±0.7	+	+
HAEE	25±1.0	+	+
HAE	11.8±0.45	+	+
CEE	12	+	+
CAEE	18.4±1.14	+	+
CAE	21.2±0.84	+	+
E. prostrata			
HEE	25.2±0.84	+	+
HAEE	21.4±0.89	+	+
HAE	11.6±0.55	-	-
CEE	17.4±1.14	+	+
CAEE	25.2±0.44	+	+
CAE	19.6±0.55	+	+
M. oleifera			
HEE	24.2±0.45	-	+
HAEE	13.2±0.45	+	+
HAE	12.2±0.84	-	-
CEE	20.4±0.55	+	-
CAEE	20.2±0.84	+	+
CAE	10	+	+
P. fraternus			
HEE	24±0.7	-	+
HAEE	22.6±1.14	+	+
HAE	11.6±0.55	-	-
CEE	20±1.22	+	+
CAEE	25.8±0.84	+	+
CAE	20.6±0.55	+	+

**** + = presence of activity; - = absence of activity***

15 extracts (50%) showed zones of inhibition ≥ 20 mm for antibacterial activity; while 22 extracts (73.33%) showed complete inhibition of adherence and co-aggregation of test consortium. Results indicate that CEE of *M.koenigii,* HAEE of *P.guajava,* CAEE of *E.prostrata,* HEE of *E.prostrata* and CAEE of *P.fraternus* demonstrated maximum activity.

2. Antibacterial activity of plant extracts

When evaluated for anti-bacterial activity at varying concentrations (0.01-15 mg/ml), the

extracts exhibited antibacterial activity against test consortium with efficacy increasing with increase in concentration. The MIC values of extracts ranged from 0.5-15 mg/ml as shown in Table 2.

Table 2
MIC of plant extracts

EXTRACT	MIC (mg/ml)
M.koenigii CEE	0.5
P. guajava HAEE	1
E.prostrata CAEE	5
E. prostrata HEE	10
P. fraternus CAEE	15

3. Effect of plant extracts on sucrose-dependent adhesion and cellular co-aggregation

Interfering effect of various concentrations (0.01-15 mg/ml) of plant extract on adherence and co-aggregation was shown in Table 3.

Table 3
Effect of various concentrations of plant extracts on sucrose-dependent adherence and cellular co-aggregation

SCORE	EXTRACT	CONCENTRATION (mg/ml)									
		C	CHX	0.01	0.05	0.1	0.5	1	5	10	15
Visible adherence*	*P. guajava* HAEE	4+	0	4+	3+	2+	0	0	0	0	0
Visible co-aggregation†		4+	0	4+	3+	2+	0	0	0	0	0
Visible adherence	*E. prostrata* CAEE	4+	0	4+	3+	3+	0	0	0	0	0
Visible co-aggregation		4+	0	4+	3+	3+	0	0	0	0	0
Visible adherence	*E. prostrata* HEE	4+	0	4+	4+	4+	3+	1+	0	0	0
Visible co-aggregation		4+	0	4+	4+	4+	2+	1+	0	0	0
Visible adherence	*M. koenigii* CEE	4+	0	4+	4+	3+	0	0	0	0	0
Visible co-aggregation		4+	0	4+	4+	2+	1+	0	0	0	0
Visible adherence	*P. fraternus* CAEE	4+	0	4+	4+	3+	0	0	0	0	0
Visible co-aggregation		4+	0	4+	4+	3+	0	0	0	0	0

**** Scoring for adherence: 0, no visible adherence; 1+, few visible cells adhering to bottom of tube; 2+, thin confluent coat of cells on bottom of tube; 3+, thin confluent coat of cells on bottom and sides of tube; 4+, thick confluent coat of cells on bottom and sides of tubes.***

† Scoring for co-aggregation: 0, No visible aggregation; 1+, minute clumps of cells in turbid fluid; 2+, easily visible clumps of cells in turbid fluid; 3+, well defined clumps of cells in clear supernatant fluid and 4+, very large flocculent clumps of cells in clear supernatant fluid. (Murchison et. al, Loc. cit.)

HAEE of *P. guajava* and CAEE of *E. prostrata* substantially reduced adherence and co-aggregation up to 0.05 mg/ml while CAEE of *P. fraternus* and CEE of *M.koenigii* demonstrated similar results up to 0.1 mg/ml. HEE of *E. prostrata* showed inhibitory effects on both cariogenic properties only up to 0.5 mg/ml.

4. Effect of extract on in-vitro biofilm formation

CEE of *M. koenigii,* HAEE of *P. guajava* and CAEE of *E. prostrata* were selected to evaluate their inhibitory effect at MIC on *in-vitro* biofilm formation as depicted in Table 4 and Figure 1. Positive control, CHX, demonstrated 100% elimination of test consortium resulting in no biofilm formation.

Table 4
Effect of extract on in-vitro biofilm formation on sterile acrylic tooth

EXTRACT	INOCULUM CFU/ml	BIOFILM LOAD Negative Control CFU/ml	BIOFILM LOAD Test CFU/ml	LOG REDUCTION Log_{10}CFU/ml
E.prostrata CAEE (5mg/ml)	$4.967 \times 10^8 \pm 2.45$	$2.638 \times 10^5 \pm 1.28$*	$1.667 \times 10^1 \pm 0.76$*	4.206
P. guajava HAEE (1 mg/ml)	$3.645 \times 10^8 \pm 2.04$	$1.748 \times 10^5 \pm 1.06$*	$5.567 \times 10^1 \pm 2.67$*	3.4969
M. koenigii CEE (0.5 mg/ml)	$2.838 \times 10^8 \pm 2.02$	$2.52 \times 10^5 \pm 0.97$*	$4.117 \times 10^4 \pm 4.28$*	0.7869

Statistically significant results considering P value $\leq$ 0.05.

In-vitro biofilm formation on acrylic tooth

Figure 1

Visualization of biofilm post staining with 1% Crystal violet. Negative control (A) and Test (B) (CAEE of E. prostrata) show no biofilm formation at 0 hr. After 7hrs, note that negative control (C) shows confluent biofilm on tooth while Test (D) (CAEE of E. prostrata) shows inhibition of biofilm formation.

CAEE of *E. prostrata* exhibited maximum reduction of 4.2 log_{10} cfu/ml in viable count of the biofilm as compared to negative control while HAEE of *P. guajava* substantially reduced viable count by 3.5 log_{10} cfu/ml. CEE of *M. koenigii* caused a reduction of only 0.7 log_{10} cfu/ml in viable count.

DISCUSSION

Majority of literature on plaque control focuses on elimination of *S. mutans* [5-11], however, in the present study, plant extracts were evaluated for their anti-cariogenic activity against a consortium of dental biofilm isolates.CEE of *M. koenigii*, CAEE of *E.prostrata* and HAEE of *P. guajava* demonstrated maximum antibacterial activity. Evidence regarding anti-plaque activity of *E. prostrata* and *M. koenigii* is limited; however activity of *P. guajava* against oral bacteria has been documented. Prabhu et al (2006) isolated Guaijaverin from *P. guajava* having MIC of 2 mg/ml and 4 mg/ml against *S. mutans* of both clinical and type strain cultures respectively. [7] Fathilah et al (2011) demonstrated activity of aqueous extract of *P. guajava* (MIC values 2.61-4.69 mg/ml) against oral bacteria. [11] In this study, CAEE of *P. guajava* exhibited comparatively higher activity (MIC=1 mg/ml) against a multi-species consortium. *E. prostrata*, *M. koenigii* and *P. guajava* are rich in flavonoids, tannins and terpenes [12-16] which may be responsible for observed antibacterial activity against oral bacteria. [4] Sucrose metabolism by the test consortium promotes adherence through production of water-insoluble glucans by Glucosyltransferases (GTF) [19] while sucrose-dependent aggregation depends on synthesis of dextran by Dextransucrase. [7,21] Adhesion and co-aggregation allow bacteria to with stand mechanical forces exerted by cheek and tongue muscles and salivary flow. [22,23] Hence plant extracts that inhibit adherence and co-aggregation would prevent build up of plaque. Substantial anti-adherence and anti-co-

aggregation activities were demonstrated by extracts of *E. prostrata*, *P. guajava*, *M. koenigii* and *P. fraternus* at sub-MIC concentrations. Previous studies have highlighted the activity of plant extracts at sub-lethal concentrations. Nostro et al (2004) demonstrated that sub-MIC concentrations (7.81-31.25 µg/ml) of ethanolic extract of *Helichrysum italicum* were effective against adhesion and co-aggregation of *S. mutans* [5] while Prabhu et al (2006) demonstrated similar results with sub-MIC concentration (0.0078–2 mg/ml) of Guaijaverin. [7] Leaves of *P.guajava* and *E.prostrata* contain flavonoids like guaijaverin, apigenin respectively while *M. koenigii*, *P. guajava* and *P. fraternus* contain quercetin; which are known to have anti-GTF activity. [5,7,12-14,16,24,25] Inhibition of GTF by plant extracts may have interfered with ability of test consortium to adhere. Cellular co-aggregation dropped markedly in the presence of the extracts which suggest that bioactive compounds in the extracts may have interfered with co-aggregation by interacting with proteins involved in the process. [7] Effect of an anti-plaque agent is concentration dependent. In the mouth, the inhibitor will steadily be desorbed off and will operate at sub-lethal concentrations. [26] The present study has demonstrated that even at sub–MIC levels, extracts of *E. prostrata*, *P. guajava*, *M. koenigii* and *P. fraternus* can be effective by inhibiting adherence and co-aggregation. Use of agents at sub-lethal concentrations that disengage organisms from an intact biofilm, without affecting their viability, may prove clinically advantageous since selective pressure and overgrowth of resistant bacteria would be avoided. [23] The results obtained in *in-vitro* biofilm assay highlight the variability in susceptibility of test consortium as a biofilm towards plant extracts. CEE of *M. koenigii* demonstrated the maximum antibacterial activity against test consortium during screening; however it was ineffective in inhibiting biofilm formation *in-vitro*. Presence of multiple microbial interactions as well as structural and functional characteristics of a multi-species biofilm may have reduced the efficacy of CEE of *M. koenigii*. At concentrations evaluated, extracts did not show results comparable to CHX. CHX is capable of reducing percentage of viable microorganisms in the dental biofilm. However use of CHX for extended periods of time may lead to side-effects such as staining, perturbation of taste, burning sensations, mucosal erosion, etc. [4] Hence use of nature-based oral care products may prove to be a safer option for the long run.

CONCLUSION

HAEE of *Psidium guajava* and CEE of *Eclipta prostrata* proved to be most effective in retarding *in-vitro* dental biofilm formation by targeting growth, adherence and co-aggregation. The study demonstrated the efficacy of extracts even at sub-MIC concentrations suggesting that they may be formulated such that they inhibit plaque development without disruption of homeostasis of the oral cavity. The data gathered in this study may assist in formulation of these extracts as anti-plaque agents in oral care products. Such products that combine traditional therapy with the latest research will have larger markets due to its nature based approach and low cost.

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ABBREVIATIONS

MIC- Minimum inhibitory concentration, BHI-Brain Heart Infusion, MTCC- Microbial type culture collection (Chandigarh, India), CHX-0.2 % w/v Chlorhexidine gluconate, PBS-phosphate buffered saline, CAE- Cold aqueous extract, HAE- Hot aqueous extract, CEE- Cold ethanolic extract, HEE- Hot ethanolic extract, CAEE- Cold aqueous-ethanolic extract, HAEE- Hot aqueous-ethanolic extract, GTF– Glucosyltransferases, AE (1:1) – Aqueous-ethanol, E– Ethanol, C-Negative Control, CFUcolonyformingunitsf

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