European Journal of Medicinal Plants

28(4): 1-11, 2019; Article no.EJMP.51126
ISSN: 2231-0894, NLM ID: 101583475

Antimicrobial and Antibiofilm Activity of Essential Oil of *Cymbopogon citratus* against Oral Microflora Associated with Dental Plaque

Sonia V. Ambade[1*] and Neelima M. Deshpande[2]

[1]Department of Microbiology, H V Desai College, Pune 411002, India.
[2]Department of Microbiology, Abasaheb Garware College, Pune 411004, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author NMD supervised and guided the entire study. Author SVA concept, conducted the study, review of literature and wrote the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2019/v28i430143
Editor(s):
(1) Dr. Ghalem Bachir Raho, Sidi Bel Abbes University, Algeria.
(2) Dr. Marcello Iriti, Professor, Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
(1) Ahmed Tabbabi, Jichi Medical University, Japan.
(2) Eray Tulukcu, University of Selcuk, Turkey.
Complete Peer review History: http://www.sdiarticle3.com/review-history/51126

Original Research Article

Received 20 June 2019
Accepted 28 August 2019
Published 02 September 2019

ABSTRACT

Aims: Dental biofilms are complex, multi-species microorganism communities that inhabit the oral cavity in the form of dental plaque which causes dental caries and periodontal diseases. The present study aims to explore the potential of Lemon Grass Essential Oil (LGEO) extracted from *Cymbopogon citratus* as antimicrobial and antibiofilm agent against the microorganisms responsible for dental plaque.
Study Design: Observational and comparison study.
Place and Duration of Study: Research centre, Department of Microbiology, Abasaheb Garware college, Pune, India, between Dec 2012 to Jan 2017.
Methodology: Three bacterial species primarily responsible for the biofilm formation were isolated from dental plaque and identified using 16S ribosomal RNA sequences. Five most primary colonizer of dental plaque organisms were acquired from the Microbial Type Culture Collection cultures. Antimicrobial as well as antibiofilm activity of LGEO, was determined against these eight

**Corresponding author: E-mail: soniabele@gmail.com;*

biofilm forming microorganism. The antibiofilm activity of LGEO was evaluated against oral flora individually, as well as in consortium.

Results: LGEO displayed excellent antimicrobial activity against eight test organisms associated with dental plaque, representing four genera namely *Streptococcus, Staphylococcus, Lactobacillus* and *Candida*. MIC of LGEO for all test organisms was determined as 1.5% (v/v). The LGEO was found to exhibit as high as 76% biofilm inhibitory activity even in the consortium, where the biofilm formation sometimes has been noted to be comparatively more than that of the individual organism, making LGEO a very promising antibiofilm agent.

Conclusion: LGEO present in rampantly grown plant, *Cymbopogon citratus*, has remarkable antimicrobial and antibiofilm activity against the dental plaque organism and thus can be the economical, convenient, natural and nontoxic herbal material to effectively control the oral microflora associated with dental plaque.

Keywords: Dental biofilm; medicinal plant; Cymbopogon citratus; LGEO; antimicrobial activity; antibiofilm activity.

1. INTRODUCTION

Oral health is integral to general well-being and relates to the overall quality of life. Oral diseases are one of the leading health problems associated with majority of people around the globe. Connection between oral infection and activities of microbial species that form part of the microbiota of the oral cavity has been well recognized [1]. Reports have shown that over 1000 bacterial strains inhabit the dental plaque (50% of which are unidentified) and a number of these are associated with oral diseases [2,3].

Recent advances in molecular biological approaches have demonstrated that dental plaque formation is a complex and dynamic process that implicates the initial acquisition of an organic film with the subsequent colonization by numerous genetically distinct microbial cells [2]. Expression of particular genes in biofilm-forming bacteria distinguishes them from their planktonic (freely suspended) counterparts. More than 65% of hospital acquired infections in humans are originated from biofilm forming bacteria [4,5,6]. These bacteria produce organic acids as by-products which causes carious lesion by dissolution of tooth's crystalline structure [7].

Since many plaque infections are not completely prone to synthetic chemical agents or antibiotics, development of bacterial resistance is very susceptible. Moreover, chemicals can alter oral micro-biota and possess undesirable effects such as vomiting, diarrhea and tooth staining [8]. With the given obvious disadvantages, the usage of medicinal plants may be considered as potential alternative for effective suppression of dental plaque formation and biofilm causing oral pathogens [9]. It is recommended that plaque controlling substances should have inhibitory effect on the adhesive properties of pathogenic microbes without any side-effects. There has been a long history of exploiting herbal products to improve dental health and promote oral hygiene. Natural phytochemicals such as alkaloids, tannins, essential oils and flavonoids isolated from medicinal plants used in traditional medicine are good alternatives to synthetic chemicals [10], and exhibit pronounced defensive and remedial activity [11]. About two million traditional health practitioners use over 7500 medicinal plant species [12]. A number of herbs and medicinal plants have been investigated against oral microbes *in vitro*.

Lemongrass belongs to the family *Graminae* (Poaceae) and the genus *Cymbopogon.* Generally, three species are identified of which one is *Cymbopogon citratus*. Lemongrass is distributed in Africa, Indian subcontinent, South America, Australia, Europe and North America. In India, they grow wild in all regions extending from sea level to an altitude of 4200 m. In the present study, the antimicrobial and antibiofilm activity of Lemongrass Essential Oil (LGEO), was tested against the plaque forming dental flora isolated from healthy individuals. Total eight organisms representing genera *Streptococcus, Lactobacillus, Staphylococcus* and *Candida* were used as test organisms individually as well as in consortium to study antimicrobial activity and antibiofilm activity.

2. MATERIALS AND METHODS

2.1 Identification, Authentication of *Cymbopogon citratus* and Extraction of Essential Oil

All the chemicals used for the experiments were Laboratory Grade (LR) from SRL chemicals,

Fisher Scientific and Sigma Aldrich. Dehydrated media used for culturing were procured from HIMEDIA, India.

The plant of *Cymbopogon citratus* (lemongrass) was collected from Pune, India, authentication and identification of which was done by the Botanical Survey of India (BSI), Pune, on Dec 2012. After this the fresh leaves and stem were plucked in the month of Feb 2013 for this study.

In order to isolate essential oil by hydro-distillation, also known as hot extraction [13], 1 kg of fresh leaves and stem of *Cymbopogon citratus* were cut into small pieces and mixed with 2L of distilled water. This mixture was subjected to hydro-distillation. This involved heating the mixture at the boiling point of water in a round bottom flask fitted with condenser along with Dean Stark assembly. It is a specially designed assembly to collect the essential oil in a separate arm. This oil was collected and stored in airtight eppendorf tubes until further use.

2.2 Isolation and Identification of Microflora Associated with Dental Plaque

Dental plaque samples were collected in Pune with the help of local dental clinician. The visible plaque present at supragingival and subgingival was collected with the help of sterile probe/explorer in a sterile eppendorf tubes containing 1 mL of sterile Phosphate Buffered Saline (PBS). These were preserved in 6 – 10°C (ice packs) during transportation and were immediately processed at the laboratory.

Dental plaque samples were homogenized on a vortex mixer. 100µL of sample was then inoculated in the liquid enrichment media. The enriched broth/medium was homogenized by vortexing and loopful (10µL) of sample was streaked on sterile Mitis Salivarius (MS) agar, sterile de Man, Rogosa and Sharpe agar (MRS), sterile Mannitol Salt Agar respectively. The plates were incubated under aerobic conditions at 37°C for 24 - 48h. For MRS medium, plates were incubated under microaerophilic condition at 37°C for 24 to 48 h. After incubation colony characteristics were noted down. Saline suspension of the overnight (24h) culture was prepared and Gram stained as per the Gram staining procedure. Hanging drop preparation of the above suspensions were observed to check motility of the organisms.

For molecular characterization of selected isolates for identification of organisms, the 16S rRNA gene was amplified from genomic DNA which was purified and sequenced as described by Pidiyar et al. [14]. Bacterial isolates were identified on the basis of 16S rRNA gene sequence homology with the reference sequences available in GenBank. A strain is considered to be a member of species when the observed sequence homology is >98.2% [15]. From the sequence, the three were identified as *Streptococcus agalactiae*, *Staphylococcus epidermidis* and *Lactobacillus fermentum* which were found to have homology of 99%, 99.93% and 99.77% respectively. The gene sequence data for these three isolates has been deposited to GenBank under the accession number MH793435, MH793436 and MH793437 respectively.

2.3 Procurement of Microflora Associated with Dental Plaque and Finalization of Microorganism

In the early stages of biofilm formation various bacterial species take part. In this study three bacterial species that were identified above, are primarily responsible for the biofilm formation. To explore the complete spectrum of organisms that result in the early biofilm formation, ultimately leading to dental plaque, the remaining most likely organisms were acquired. Accordingly, the Microbial Type Culture Collection (MTCC) cultures were included in this study. They were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. The procured microbial cultures received in lyophilized form consisted of 5 microorganisms, of which *Candida albicans* (4748) was fungus while remaining were bacteria. Thus total 8 organisms were finalized in this study (Table 1). All cultures were grown and recovered in the various culture media as suggested by IMTECH. *Streptococcus mutans* (890), *Streptococcus oralis* (2696), *Lactobacillus acidophilus* (10307), *Lactobacillus rhamnosus* (1408), and *Candida albicans* (4748) were recovered in Brain Heart Infusion Medium, Trypticase Soy Broth, de Man, Rogosa and Sharpe Medium and Yeast Extract Peptone Dextrose respectively. All cultures were maintained on their respective solid media.

Table 1. Organisms finalized for the study

Name of microorganism	Source	GenBank accession number
Streptococcus mutans (890)	MTCC	Not applicable
Streptococcus oralis (2696)	MTCC	Not applicable
Lactobacillus acidophilus (10307)	MTCC	Not applicable
Lactobacillus rhamnosus (1408)	MTCC	Not applicable
Candida albicans (4748)	MTCC	Not applicable
Streptococcus agalactiae	Dental plaque isolate	MH793435
Staphylococcus epidermidis	Dental plaque isolate	MH793436
Lactobacillus fermentum	Dental plaque isolate	MH793437

2.4 Determination of Antimicrobial Activity/ Antibiofilm Activity and Visualization by SEM

2.4.1 Determination of antimicrobial activity

Antimicrobial activity of Lemongrass essential oil (LGEO) against selected isolates was determined by the standard disc diffusion assay as per CLSI guidelines [16]. Test organisms were inoculated on respective media and incubated at 37°C for 24h. Saline suspension of 24h old culture was prepared as per 0.5 McFarland standards. 750µL of culture was then mixed with 20 mL of pre-sterilized, cooled Mueller- Hinton agar butt and poured in a sterile petri plate. The plates were allowed to solidify at room temperature. Sterile Whatman filter paper discs were soaked (10µL) in LGEO and placed on agar surface. All dilutions were carried out using Dimethyl Sulfoxide (DMSO) which acted as a negative control in the study. Subsequently commercially available Chlorhexidine gluconate was used as a positive control. Plates were kept at 4°C for 30min for pre-diffusion and later incubated at 37°C for 24h. Diameter of zone of inhibition was measured in millimeter (mm) and recorded. All exposures were carried out in triplicates and average value was considered. Diameter of zone of inhibition was measured with the help of HI MEDIA antibiotic zone measuring scale. Minimum Inhibitory Concentration (MIC) was also determined.

2.4.2 Determination of growth of biofilm and antibiofilm activity

The quantitative growth of biofilm was determined as per the "Protocols to study the physiology of oral biofilms" by Lemos et al. [17]. The determination is based on the principle that the biofilm which is produced by the organisms binds to the crystal violet and the bound crystal violet is later eluted which has the absorbance in proportion to the amount of biofilm.

Test organisms were inoculated on respective media and incubated at 37°C for 24h. Saline suspension of 24h old culture was prepared; as per 0.5 McFarland standards. Biofilm medium containing 1 M glucose (source of carbohydrate) was prepared. 20µL of each 0.5 McFarland standards culture was dispensed into separate wells each having 180µL of biofilm medium. Wells containing 200µL uninoculated biofilm medium served as negative controls. Similarly wells with 180µL of medium and 20µL of chlorhexidine served as positive control. Each experiment was conducted in triplicate. Plates were sealed with the help of adhesive micro titer plate sealer and incubated for 24h at 37°C without agitation. After the incubation, plates were further processed. The plates were blotted on a paper towel to removed culture media. To remove and wash loosely bound cells, microtiter plates were carefully immersed in a large dish with distilled water. Again plates were blotted on a paper towel. This step was repeated twice. 50µL of 0.1% crystal violet was added to the test wells, including the negative control wells. Plates were then incubated at room temperature for 15 min. The washing was repeated. The plates were air dried. 200µL of 33% acetic acid solution was added to the wells to elute the crystal violet which was bound to the biofilm formed in the wells. Plates were incubated at room temperature for 10min. Entire content of each well were transferred by multichannel micropipette in the respective wells in a new blank micro titer plate. Absorbance was measured at 570nm using the Thermo Lab systems ELISA reader Model No. 352.

In antibiofilm studies, 160µL of medium was exposed to 20µL of culture and 20µL of LGEO. All other steps in the protocol remained same as described. The ability of the LGEO to inhibit the formation of biofilm was determined as "antibiofilm activity of LGEO" or "biofilm formation inhibition activity of LGEO".

2.4.3 Visualization of biofilm by Scanning Electron Microscope (SEM)

Effect of LGEO on biofilm formation by *Streptococcus mutans* was evaluated on the sterile glass slide surfaces. Test culture of *Streptococcus mutans* was grown under optimum conditions for 24h. The bacterial cells of 0.5 McFarland standards were inoculated in biofilm liquid medium with LGEO of 1.5% concentration and without LGEO. 0.2 mL of aliquots were added into the sterile glass petri plates (60 x 17 mm) containing sterile glass slides (1 cm X 1 cm). The petri plate without the LGEO served as control. Biofilms grown on the glass surfaces with and without LGEO were analyzed by SEM after 24h of incubation. The biofilms formed on glass surfaces were washed with phosphate-buffer saline (PBS). The biofilms grown on the sterile glass slides were treated with appropriate quantity, in the ratio of glutaraldehyde **:** ethanol (9 : 1 v/v). The treated samples were further dried under vacuum for 2-3h. After drying, the processed samples were coated with platinum and observed under the SEM (JEOL JSM-6360A) [18].

3. RESULTS AND DISCUSSION

3.1 Antimicrobial Activity of LGEO

The undiluted LGEO, considered as 100% was diluted with DMSO and dilutions up to 0.78% were made by serial dilutions. The antimicrobial activity at each concentration of LGEO against the 8 test organisms was determined in terms of mean zone of inhibition in millimeter (mm) (Table 2). The commercially available chlorhexidine (Chx) is taken as a positive control for comparison and DMSO in which all the dilutions of LGEO are made is taken as negative control.

In our study the maximum mean zone of inhibition for antimicrobial activity was found to be 33.6mm and it was against *L. rhamnosus*. This is comparable to the study of Sfeir et al., who reported mean zone of inhibition as 38 mm for the essential oil of *Cymbopogon citratus* against *S. pyogenes* [19]. Our findings about antimicrobial activity of LGEO are in agreement with the study of Chaudhari et al., who reported lemongrass oil antimicrobial activity against *S. mutans* just next to Cinnamon oil which showed highest activity [20]. Sfeir et al., also evaluated the *in vitro* antibacterial activities of 18 essential oils chemotypes from aromatic medicinal plants against *S. pyogenes*, reported essential oil of *Cymbopogon citratus* next to that of *Cinnamomum verum* which showed highest activity [19].

To quantitate the antimicrobial capacity of LGEO, the Minimum Inhibitory Concentration of LGEO was determined. Minimum Inhibitory Concentration (MIC) is defined as the minimum concentration of inhibitory factor to completely inhibit the microorganism. The MIC of LGEO has been determined against each test organisms and was found to be 1.5% (v/v). The MIC of LGEO as 1.5% v/v in our study is in total agreement with that of Hammer et al., who investigated 52 plant oils & extracts for activity against diverse range of organisms comprising of Gram-positive and Gram-negative bacteria and a yeast. They reported that lemongrass, oregano and bay inhibited all organisms at concentrations of 2.0% (v/v) [21]. Galvao et al., reported MIC of LGEO against *S. mutans* as 0.125-0.250 mg/mL [22], while Madeira et al., reported MIC of LGEO against *C albicans* as 0.625mg/mL [23]. Sfeir et al., for the essential oil of *Cymbopogon citratus* against *S. pyogenes*, reported the MIC of 0.93% (v/v) [19]. Different authors reported different MIC values of LGEO which may be due to difference in the source of plant of different geographic location or chosen test organisms or conditions or the different methodology adopted to determine MIC.

There are various studies regarding the antimicrobial activity of LGEO, but mostly against the pathogenic bacteria [24]. However, studies concerning the antimicrobial activity of LGEO against the dental oral micro flora are seldom. This research work was aimed at bridging this gap.

Our results clearly proved that there is antimicrobial effect of LGEO against oral microflora associated with dental plaque. Our research established that planktonic cells were inhibited at the concentration above MIC value. However, there is need to evaluate the effect of LGEO on biofilm formation as plaque is nothing but the established biofilm. Hence, we further explored the biofilm formation inhibition activity of LGEO.

Table 2. Antimicrobial activity of LGEO against test organisms

Test organisms	Zone of inhibition in mm								
	Chx	Concentration of LGEO (%) (v/v)							DMSO
		100	50	25	12.5	6.2	3.1	1.5	0.78	
S. mutans	30	28	24.3	20.6	15.6	12.6	11	7.3	0	0
S. oralis	32	31	27	22.6	15.3	11.3	9.6	7.6	0	0
L. acidophilus	36	32.6	30.3	27	18.3	14.3	9.3	10	0	0
L. rhamnosus	35	33.6	24.3	20	16.6	15.6	13.6	10.6	0	0
C. albicans	34	32.6	28.3	21.6	17	14	11.3	9	0	0
S. agalactiae	30	28	22	21	18.3	16.3	11	8.3	0	0
S. epidermidis	29	27	25.6	23	16.6	12.6	10	9.6	0	0
L. fermentum	29	27	28	25.3	17	14	11	9.6	0	0

3.2 Biofilm Formation by Oral Flora and its Inhibition by LGEO

The biofilm formation activity of oral microflora which in this study comprises of 8 test organism was quantified as per the "Protocols to study the physiology of oral biofilms" by crystal violet assay [17]. The absorbance of the eluted crystal violet, measured at 570nm by the ELISA reader, for the 8 test organisms in comparison with the media as negative control, is taken as the measure of amount of biofilm formed.

All the 8 test organisms displayed considerable biofilm formation activity and were within the range equivalent to absorbance of approximately 0.221 to 0.402. The result of their biofilm formation ability (Table 3) clearly indicates that *Lactobacillus rhamnosus* exhibits the highest biofilm formation ability (equivalent to absorbance of 0.402) amongst all the test organisms followed by *Candida albicans*, *Lactobacillus fermentum*, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Streptococcus agalactiae*, *Streptococcus oralis* and *Staphylococcus epidermidis*. *L. rhamnosus* is a strong biofilm producer while *S. epidermidis* appears as a weak biofilm producer (Table 3).

To determine the biofilm formation inhibition activity of LGEO, the undiluted LGEO was subjected to all the 8 test organisms during the biofilm formation. All the 8 test organisms were exposed to undiluted LGEO from the beginning of the biofilm formation process, and in the presence of LGEO, the ability of these organisms to develop the biofilm under the same condition was evaluated. When Student's paired T-Test was applied then the decrease in the absorbance at 570 nm in presence of LGEO was found highly significant ($P<0.001$). This indicates that LGEO has a significant effect on the inhibition of the biofilm formation (Table 3). To quantify the effect of LGEO on the inhibition of biofilm formation, the biofilm formation inhibition effect of LGEO on the test organisms was determined in terms of percentage inhibition which is as per Table 3. The percentage (%) inhibition of biofilm was calculated by the formula which was a simplified version of the formula mentioned by Jadhav et al [25].

Percentage biofilm inhibition by LGEO= {(Abs without LGEO – Abs in presence of LGEO)/(Abs without LGEO)} × 100

LGEO was found to inhibit the biofilm formation ranging from 54.8% to 69.9%.

3.3 Biofilm Formation by Oral Flora in Consortium and its Inhibition by LGEO

Many *in-vitro* oral biofilm studies require the development of orally relevant plaque. However, many studies focus primarily on single-species biofilm which do not take into account the multi-species interactions which actually occurs in plaque [26,27,28]. In the present study, an attempt was made to check the biofilm formation by consortium. For consortium studies, different combinations of all 8 test organisms were taken in the ratio 1:1. With this 1:1 combination, total 27 combinations were generated and all were tested for their ability to form biofilm.

All the 27 combinations were found to display biofilm formation activity which was in the range equivalent to absorbance of 0.087 to 0.537 (Table 4). This indicated that certain combinations of the organisms exhibit lower biofilm formation activity when compared to individual organism. The lowest biofilm activity noted in individual organism was for *S. epidermidis* which was equivalent to absorbance of 0.221 (Table 3). However, certain

Table 3. Biofilm formation by test organism and its inhibition by LGEO against the test organisms

Name of the organism	Mean absorbance at 570 nm as index of biofilm formation			% inhibition by LGEO
	In absence of LGEO	In presence of LGEO	Difference in absorbance	
S. mutans	0.312	0.121	0.191	61.2
S. oralis	0.261	0.091	0.17	65.1
L. acidophilus	0.287	0.113	0.174	60.6
L. rhamnosus	0.402	0.121	0.281	69.9
C. albicans	0.33	0.106	0.224	67.9
S. agalactiae	0.248	0.112	0.136	54.8
S. epidermidis	0.221	0.073	0.148	67.0
L. fermentum	0.329	0.102	0.227	69.0

Combinations were found to exhibit the accelerated biofilm formation activity as compared to the individual organism. The highest biofilm activity as an individual organism was demonstrated by *L. rhamnosus* which was equivalent to absorbance of 0.402 (Table 3), whereas the combination of *L. acidophilus* and *S. epidermidis* was found to have the highest biofilm formation activity equivalent to absorbance of 0.537 (Table 4). The lowest biofilm formation activity was found to be for the combination *L. rhamnosus* and *S. oralis* which was equivalent to absorbance of 0.087 (Table 4). This further indicated that if *S.oralis* is present with *L. rhamnosus*, then it appears to inhibit the growth of biofilm otherwise produce by *L. rhamnosus* individually. Thus this combination appeared to have antagonistic interaction. Five combinations viz. *C. albicans* & *S. epidermidis*, *L. fermentum* & *S. epidermidis; S.oralis* & *S. epidermidis , S.mutans* & *S. epidermidis* and *L. acidophilus* & *S. epidermidis* were found to have higher biofilm formation activity (more than the equivalent absorbance of 0.402) when compared to the individual test organism. These combinations appear to have synergistic interaction leading them to be a potent and strong biofilm producer among the consortiums studied.

After confirming that the test organisms in various consortia, as compared to the individual organism, exhibited higher and better biofilm formation ability, LGEO was tested for its biofilm formation inhibition activity against the test organisms in all the 27 consortium. The significant decrease in the absorbance in presence of LGEO, in all the 27 consortium ($P<0.001$) clearly indicated that the LGEO has biofilm inhibitory activity (Table 4).

This established that LGEO has a remarkable biofilm inhibitory activity not only against the individual test organisms (Table 3) but also against the test organism in various combinations (Table 4). LGEO was found to exhibit as high as 76% biofilm inhibitory activity even in the consortiums, where the biofilm formation sometimes were noted comparatively more than that of the individual organism capability. Thus, LGEO proved to be very promising antibiofilm forming agent.

The inhibition of biofilms by essential oil has been widely reported. Aleksandra et al reported that *Lavandulaan gustifolia*, *Melaleuca alternifolia* and *Melissa officinal* inhibited the biofilms formed by *Staphylococcus aureus* [29]. Taweechaisupapong et al., demonstrated potent *in vitro* activity in inhibiting biofilm formation and even against preformed biofilms of *C. dubliniensis* by lemongrass oil [30]. Adukwu et al., reported anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *S aureus* [31].

3.4 Detection of Biofilm Formation Inhibition by Scanning Electron Microscopy (SEM)

Biofilm formation inhibition activity displayed strongly by LGEO was further corroborated by visualizing it under electron microscope. *S. mutans* is considered as the most cariogenic of all oral streptococci [32]. *S. mutans* is capable of colonizing the tooth surface and producing large amounts of extra and intra-cellular polysaccharides. *S. mutans* is highly acidogenic and aciduric, and it metabolizes several salivary glycoproteins, thus being primarily responsible

for the initial stage of oral biofilm formation [33]. These properties of *S. mutans* were ideal to study the effectiveness of LGEO by SEM imaging of biofilm formation and its inhibition. The SEM image of biofilm formed over a period of 24h is compared with biofilm inhibited for the same period of time and at minimum inhibitory concentration which is 1.5% of LGEO. Biofilm formation inhibition was verified by the SEM images as shown in Fig. 1. The SEM image "A" in the Fig. 1 represents biofilm formed by *S. mutans* in 24h under defined condition without LGEO, while the image "B" represents biofilm formed by *S. mutans* in 24h under same condition but in presence of 1.5% of LGEO. The clear morphological differences in the SEM images amply demonstrate the inhibition of biofilm of *S. mutans* by 1.5% LGEO. Similarly, Fig. 2 represents the SEM images of biofilm formation by *L. acidophilus* (SEM image "A") and its inhibition by 1.5% LGEO (SEM image "B"). Caries are initiated by direct demineralization of the enamel of teeth due to acids which accumulate in dental plaque. *L. acidophilus* in the plaque produces lactic acid from the fermentation of sugars and other carbohydrates in the diet. The lactic acid then causes demineralization of the enamel of teeth of the host. Because of this, *L. acidophilus* was also chosen for SEM imaging. The SEM images of the biofilm observed in our study were found to be comparable to that reported by Wen et al. [34].

3.5 Comparison of Antimicrobial and Antibiofilm Activity of LGEO with Chlorhexidine

LGEO was found to have both, the antimicrobial and antibiofilm activity. Undiluted LGEO was compared with the commercially available undiluted chlorhexidine against the test organisms. LGEO was found to have lower antimicrobial activity against the test organisms with mean ± SD of just 5.98 ± 2.08%, as compared to the undiluted chlorhexidine. When the antibiofilm activity of LGEO was correlated with chlorhexidine, LGEO was found to exhibit little lower antibiofilm activity with mean ± SD of just 6.76 ± 12.63%. Thus the antimicrobial and antibiofilm activity of natural herbal LGEO is almost at par with that of commercially available synthetic chlorhexidine.

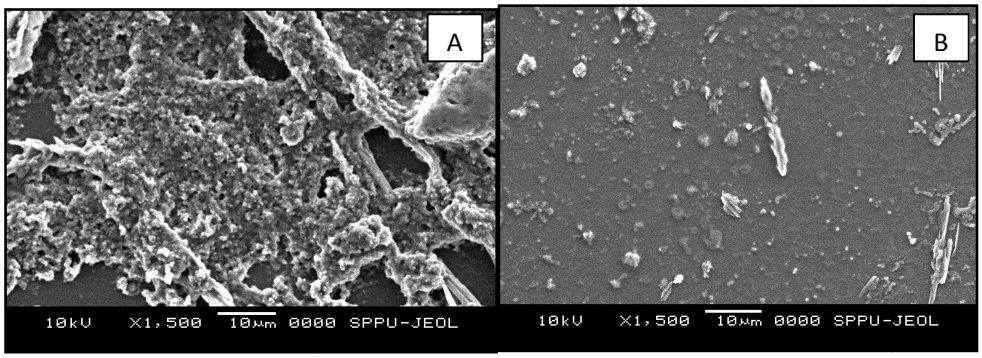

Fig. 1. SEM images of biofilm formation of *S. mutans* (A) & it's inhibition by LGEO (B)

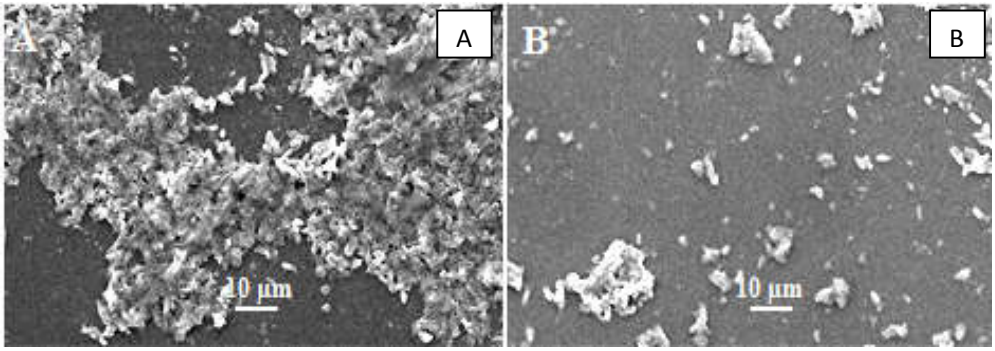

Fig. 2. SEM image of biofilm formation of *L. acidophilus* (A) & it's inhibition by LGEO (B)

Table 4. Biofilm formation by test organism and its inhibition by LGEO against the test organisms

Test organisms in the ratio 1:1	Absorbance at 570 nm as index of Biofilm			% inhibition by LGEO
	In absence of LGEO	In presence of LGEO	Difference in absorbance	
L. acidophilus+L.rhamnosus	0.175	0.123	0.052	29.7
L. acidophilus+C. albicans	0.199	0.108	0.091	45.7
L. acidophilus+S.mutans	0.225	0.115	0.11	48.9
L. acidophilus+S.oralis	0.194	0.071	0.123	63.4
L. acidophilus+S. agalactiae	0.255	0.125	0.13	51.0
L. acidophilus+ L. fermentum	0.208	0.1	0.108	51.9
L. acidophilus+S. epidermidis	0.537	0.129	0.408	76.0
L. rhamnosus+C. albicans	0.174	0.107	0.067	38.5
L. rhamnosus+S.mutans	0.165	0.099	0.066	40.0
L. rhamnosus+S.oralis	0.087	0.07	0.017	19.5
L. rhamnosus+S. agalactiae	0.167	0.1	0.067	40.1
L. rhamnosus+L. fermentum	0.203	0.1	0.103	50.7
L. rhamnosus+S. epidermidis	0.265	0.107	0.158	59.6
C. albicans+S.mutans	0.173	0.128	0.045	26.0
C. albicans+S.oralis	0.093	0.07	0.023	24.7
C. albicans+S. agalactiae	0.179	0.126	0.053	29.6
C. albicans+L. fermentum	0.215	0.11	0.105	48.8
C. albicans+S. epidermidis	0.409	0.154	0.255	62.3
S.mutans+S.oralis	0.31	0.1	0.21	67.7
S.mutans+S. agalactiae	0.246	0.101	0.145	22.4
S.mutans+L. fermentum	0.234	0.125	0.109	46.6
S.mutans+S. epidermidis	0.454	0.254	0.2	44.1
S.oralis+S. agalactiae	0.199	0.092	0.107	53.8
S.oralis+L. fermentum	0.193	0.093	0.1	51.8
S.oralis+S. epidermidis	0.427	0.185	0.242	56.7
S.agalactiae +L. fermentum	0.219	0.1	0.119	54.3
L. fermentum+S. epidermidis	0.458	0.186	0.272	59.4

4. CONCLUSION

Thus, this study amply demonstrated that the LGEO present in rampantly grown plant, *Cymbopogon citratus*, has remarkable antimicrobial and antibiofilm activity against the dental plaque organism and thus can be the economical, convenient, natural and nontoxic herbal material to effectively control the oral microflora associated with dental plaque.

CONSENT

Authors declared that written informed consent was obtained from the patient for sample.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

I am extremely grateful to Dr Raj Bhagwatkar and Dr Karan Rathod for their indispensible help with acquiring dental plaque samples. I sincerely acknowledge the efforts taken by Dr D Boruah, Scientist E, Defence Research Development Organization, for carrying out statistical analysis whenever required. I thank Dr B J Bhadbhade for providing the initial stimulus for this work. I am thankful to Dr A Bankar, for providing me all the help in SEM imaging at Dept of Physics, SPPU, Pune. Finally, I am extremely grateful to University Grants Commission for awarding me the teacher fellowship.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Salam R, Sarker BK, Haq MR, Khokon JU. Antimicrobial activity of medicinal plant for oral health and hygiene. International J of

Natural and Social Sciences. 2015;1 (2):1-12.
2. Saini R, Saini S, Sharma S. Biofilm: A dental microbial infection. Journal of Natural Science, Biology and Medicine. 2011;2(1):71.
3. Suntharalingam P, Cvitkovitch DG. Quorum sensing in streptococcal biofilm formation. Trends in Microbiology. 2005; 13(1):3-6.
DOI:10.1016/j.tim.2004.11.009
4. Costerton J, Stewart PS, Greenberg E. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284 (5418):1318-1322.
DOI:10.1126/science.284.5418.1318
5. Donlan RM. Biofilms and device-associated infections. Emerging Infectious Diseases. 2001;4817(2):277.
DOI: 10.3201/eid0702.010226
6. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews. 2002;15(2):167-193.
DOI:10.1128/CMR.15.2.167-193.2002
7. Marsh PD. Dental plaque as a biofilm and a microbial community – 516 implications for health and disease. BMC Oral health. 2006;6(Suppl 1):S14.
DOI:10.1186/1472-6831-6-S1-S14
8. Yadav R, Yadav D. Dental Disease and its cure: A review. Asian Journal of Pharmaceutical and Clinical Research. 2013;6(2):16-20.
9. Malhotra R, Grover V, Kapoor A, Saxena D. Comparison of the effectiveness of a commercially available herbal mouthrinse with chlorhexidine gluconate at the clinical and patient level. Journal of Indian Society of Periodontology. 2011;15(4):349.
DOI:10.4103/0972-124X.92567
10. Ismail, MYM. Botanicals promoting oral and dental hygiene. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2010;1(2):202.
11. Bhardwaj A, Bhardwaj SV. Ethno-dentistry: popular medicinal plants used for dental diseases in India. Journal of Intercultural Ethnopharmacology. 2012;1(1):62-65.
DOI:10.5455/jice.20120322035152
12. Kanwar P, Sharma N, Rekha A. Medicinal plants use in traditional healthcare systems prevalent in Western Himalayas. Indian Journal of Traditional Knowledge. 2006;5(3): 300-309.
13. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med. 2011;8(1): 1-10.
14. Pidiyar VJ, Jangid K, Patole MS, Shouche YS. Studies on cultured and uncultured microbiota of wild culex quinquefasciatus mosquito midgut based on16S ribosomal RNA gene analysis. American Journal of Tropical Medicine and Hygiene. 2004;70 (6):597–603.
DOI:10.4269/ajtmh.2004.70.597
15. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. Journal of clinical microbiology. 2007;45(9):2761-2764.
DOI:10.1128/JCM.01228-07
16. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; Twenty-Second Informational Supplement. 2012;32(3): M100-S22.
17. Lemos JA, Abranches J, Koo H, Marquis RE, Burne RA. Protocols to study the physiology of oral biofilms. Methods in Molecular Biology. 2010;666:87–102.
DOI:10.1007/978-1-60761-820-1_7
18. Bankar A, Zinjarde S, Telmore A, Walke A, Ravikumar A. Morphological response of *Yarrowia* lipolytica under stress of heavy metals. Canadian Journal of Microbiology. 2018;4:1-8.
DOI:10.1139/cjm-2018-0050
19. Sfeir J, Lefrançois C, Baudoux D, Derbré S, Licznar1 P. *In vitro* antibacterial activity of essential oils against *Streptococcus pyogenes*. Evidence-Based Complementary and Alternative Medicine. 2013;269161.
DOI:10.1155/2013/269161
20. Chaudhari LK, Jawale BA, Sharma S, Sharma H, Kumar CD, Kulkarni PA. Antimicrobial activity of commercialy available essential oils against Streptococcus mutans. Journal of Contemporary Dental Practice. 2012;13(1): 71-74.
DOI:10.5005/jp-journals-10024-1098
21. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology. 1999;86:985–990.
DOI:10.1046/j.1365-2672.1999.00780.x
22. de Carvalho Galvão LC, Fernandes Furletti V, Fernandes Bersan SM. Antimicrobial activity of essential oils against

Streptococcus mutans and their antiproliferative effects. Evidence-Based Complementary and Alternative Medicine; 2012.
Article ID 751435
DOI:10.1155/2012/751435

23. Madeira PL, Carvalho LT, Paschoal MA, de Sousa EM, Moffa EB, da Silva M, Tavarez R and Gonçalves LM. *In vitro* effects of lemongrass extract on *Candida albicans* biofilms, human cells viability, and denture surface. Frontiers in Cellular and Infection Microbiology. 2016;6:71.
DOI:10.3389/fcimb.2016.00071
24. Naik MI, Fomda B, Jeyakumar E, Bhat JA. Antibacterial activity of lemongrass (*Cymbopogon citratus*) oil against some selected pathogenic bacterias. Asian Pacific Journal of Tropical Medicine. 2010; 3(7):535-538.
DOI:10.1016/S1995-7645(10)60129-0
25. Jadhav S, Shah RM, Bhave M, Palombo E. Inhibitory activity of yarrow essential oil on listeria planktonic cells and biofilms. Food Control. 2013;29(1):125-130.
DOI:10.1016/j.foodcont.2012.05.071
26. Adams B, Baillie GS, Douglas LJ. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. Journal of Medical Microbiology. 2002;51:344-349.
27. Luppens SB, Kara D, Bandounas L, Jonker MJ, Wittink FR, Bruning O, Breit TM, Ten Cate JM, Crielaard W. Effect of *Veillonella parvula* on the antimicrobial resistance and gene expression of *Streptococcus mutans* grown in a dual-species biofilm. Oral Microbiology and Immunology. 2008; 23(3):183-189.
DOI:10.1111/j.1399-302X.2007.00409.x28.
28. Palmer RJ, Kazmerzak K, Hansen MC, Kolenbrander PE. Mutualism versus independence: Strategies of mixed - species oral biofilms *in vitro* using saliva as the sole nutrient source. Infection and Immunity. 2001;69:5794–5804.
DOI:10.1128/IAI.69.9.5794-5804.2001
29. Aleksandra B, Wiêckowska-szakiel M, Sadowska B, Kalemba D, Barbara R. Antibiofilm activity of selected plant essential oils and their major components. Polish Journal of Microbiology. 2011;60(1): 35-41.
30. Taweechaisupapong S, Ngaonee P, Patsuk P, Pitiphat W, Khunkitti W. Antibiofilm activity and post antifungal effect of lemongrass oil on clinical *Candida dubliniens* is isolate. South african journal of Botany. 2011;78:37-43.
DOI:10.1016/j.sajb.2011.04.003
31. Adukwu EC, Allen SC, Phillips CA. The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. J Appl Microbiol. 2012;113(5):1217–1227.
DOI:10.1111/j.1365-2672.2012.05418.x
32. Ajdi´c D, McShan WM, McLaughlin RE. Genome sequence of *Streptococcus mutans* UA159, a carcinogenic dental pathogen. Proceedings of the National Academy of Sciences, USA. 2002;99(22): 14434–14439.
33. Alves TMS, Silva CA, Silva NB, Medeiros EB, Valenca AMG. Antimicrobial activity of fluoridated products on bioflm-forming bacteria: An *in vitro* study. Pesquisa Brasileira em Odontopediatria e Clinica Integrada. 2010;10(2):209–216.
34. Wen TZ, Baker HV, Burne RA. Influence of BrpA on critical virulance attributes of S. mutans. Journal of Bacteriology. 2006;188 (8):2983-2992.
DOI:10.1128%2FJB.188.8.2983-2992.2006

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/51126