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Potential effect of lemongrass oil extract (*Cymbopogon citratus*) against *Streptococcus mitis* (primary periodontal colonizers)

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Abstract---The results of current study were showed that the essential lemongrass oil was effectively inhibited the bacterial proliferation with significant declining in fold expression values ($2^{-\Delta\Delta CT}$) at different concentrations with dose dependency.

Keywords---potential effect, lemongrass oil extract, bacterial proliferation.

Introduction

Periodontal disease which is one of the most widespread diseases affecting mankind, involve the adherence of bacteria and development of biofilms on both the natural and restored tooth surface [1]. Dental plaque is a biofilm which is pale yellow in color that develops naturally on the teeth. A dental plaque is formed by colonizing bacteria trying to attach themselves to the tooth surface. Dental biofilm, more commonly referred to as dental plaque is composed of about thousand species of bacteria that take part in the complex ecosystems of the mouth [2]. Dental plaque starts when bacteria that are present in the mouth attach to teeth and begin multiplying. Plaque can form on teeth both supra gingival plaque, or sub gingival plaque [3]. The plaque is made up of colonies of microorganisms such as bacteria, yeast dumped together in a gel like organic material composed of bacterial byproducts including sugar, food debris and body tissue [4]. The predominant initial colonizers of teeth are Gram-positive facultative anaerobic cocci and rods, including *Streptococcus* and *Actinomyces* species. These initial colonizers provide a foundation for further development of

dental biofilm [5]. *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis* of the Mitis group streptococci [6,7] are close relatives to *Streptococcus pneumoniae* which is the important human pathogen. They are part of human normal oral flora, but occasionally can cause acute or chronic disease as opportunistic human pathogens in contrast to *S. pneumoniae*. They are associated with gingival disease and caries and occasionally cause sub-acute infective endocarditis [8]. As they are the early colonizers in the development of dental biofilms [9]. They are able to develop natural competence for genetic transformation as *S. pneumoniae* [10] and have horizontal gene transfer, such as virulence genes, that has been documented between these species [6,11-12]. The emergence of multi-drug-resistant (MDR) microorganism has become a danger to public health [13]. Antibiotic resistant bacteria cause millions of infections and thousands of deaths every year in the U.S. according to a report published by the U.S. Centers for Disease Control and Prevention, addition to that the continuous and significant reduction in the number of approved antibiotics in the past decade has a role factor in the increasing threatening situation [14]. That lead to an urgent need for the invention of novel antibacterial and treatment strategies [15]. Lemongrass (*Cymbopogon flexuosus*) essential oil (LEO) have significant interest in their development as potential antimicrobial agent, has been traditionally used as a remedy for a variety of health conditions. Recent scientific studies have provided evidence supporting its antimicrobial properties in several disease models. However we need to measure the antimicrobial activity of Lemongrass essential oil against *Streptococcus mitis* bacteria in dental plaque [16].

Material and Method

The first step was taking plaque samples from 15 patients with periodontal disease. Two aerobic samples will be taken from each patient. Aerobic bacterial sample was obtained from supragingival area by sterile curate

Bacterial isolation

Collected samples were put in brain heart infusion broth (BHIB)), incubated for 24 hours, 37°C under aerobic and then inoculate the bacteria from BHI to brain heart infusion agar (BHIA) and blood agar and incubated for 24 hours, 37°C under aerobic. To suspected colony until we have a pure colony [17]. Second step was morphological and microscopically examination of bacteria in addition to the biochemical tests and molecular study (Real-time PCR) depending on specific primers were used to confirm identification of *Streptococcus mitis* [18]. The next steps we have lemon grass oil ready from U. S .A. . finally test the potential antimicrobial effect of lemongrass oil extract on *Streptococcus mitis* and *Streptococcus oralis* in comparison to positive (CHX 0.2%) and negative controls (DMSO).

Gram's stains

Single pure colony was picked up from blood agar plate under sterilized condition and subjected to Grams stain in order to identify their response to this stain microscopically under oil immersion with magnification power 100X [19]. Blood

Hemolysis test this test used to determine the ability of *Streptococcus mitis* to bleach the heme iron by hydrogen peroxide (H_2O_2), leading to greenish color on blood agar [20]

Catalase production test

This test was conducted on cells of bacteria colonies. Hydrogen peroxide 3% (H_2O_2) had been used to determine the ability to produce catalase enzyme by bacteria [21]

PCR real time

P C R (real time) for identification the specific bacterial type[22]

Chlorhexidine concentrations

Chlorhexidine gluconate was used in a concentration of 0.2% all over the experiment as a positive control material[23]

Activation of inoculums

Inoculums of bacteria were activated by the addition of 0.1 ml of bacterial culture to 10 ml of BHI-B followed by incubation for 24 hrs at 37°C [23]

Final step

Final step was Determination of antibacterial activity of lemon grass oil on *Streptococcus mitis* bacteria. In this experiment, agar gel diffusion method was applied to study the antimicrobial effect of lemon grass oil on *Streptococcus mitis* bacteria were used [24]. MHA agar media was poured separately into sterile petri dishes, 0.1 ml of activated *S. mitis* were spread in duplicate on MHA agar plates, then wells of equal size and depth made with sterile stainless steel Cork borer in the MH Agar 4 mm in diameter were prepared in the agar. Each well was filled with lemon grass oil in different concentrations (0.1, 0.01, 0.001, 0.0001 and 0.00001 (v/v %) and one well for chlorhexidine 0.2% as positive control and last well for (DMSO) as negative control, then incubated aerobically for 24 hrs at 37°C. The diameter of inhibition zone containing the test materials were measured and recorded after the incubation under aseptic condition. Application of real time PCR for the detection of expression ratios following treatment of *S. mitis* with lemongrass oil extracts (0.1, 0.01, 0.001, 0.0001 and 0.00001 (v/v %) vs Chlorhexidine (0.2%) and (DMSO) as negative control

Result & Descation

Microbiological & morphorgcail result

Morphological properties of *Streptococcus*

They were detected on BHIA by observing their morphological characters. They appear as small, round, smooth, glistening and convex colonies[23]

Biochemical tests

Catalase and Coagulase-test

S. mitis are catalase negative, meaning they lack the catalase enzyme, and they are also coagulase-negative, facultative anaerobes that are α -hemolytic[23]

Hemolysis test

S. mitis are α -hemolysis, due to bleed of heme iron, resulting in a greenish color on blood agar[23]

Quantitative RT-PCR for detection of *S. mitis* positivity

According to Imbeaud S, et al. (2005) and Usman T, et al. (2005), the concentration and purity of nucleic acid were evaluated using a Biodrop spectrophotometer [25]. The 260/280 ratio results showed that all extracted DNA specimens were elevated to acceptable levels ranging from 1.8 to 2.0, indicating that they have adequate DNA purity and are ready for PCR technique application. Curves above the threshold line in quantitative PCR (qPCR) for *S. mitis* and *S. oralis* detection were positive, whereas curves below the threshold line were negative. GAPDH, a housekeeping gene, was considered a successful internal control[26]. The amplification patterns of the *S. mitis* gene PCR products were shown in (Figures 1), -A, B, using GAPDH) as a housekeeping gene. (periodontitis cases according to *S. mitis* positivity

This study showed that from the total of periodontic patients investigated, *Streptococcus mitis* positive cases were correlated with 2 cases (6.7%). *Streptococcus mitis* positive gene detection CT mean $\pm$ SD was 14.55 ± 3.71 . This study also revealed absence of *Streptococcus mitis* positivity in 5 and 6 cases from the total samples investigated respectively. The chi-square statistic is 0.4242. The p-value is 0.808867. The result is not significant at $p < .05$. (Table 1).

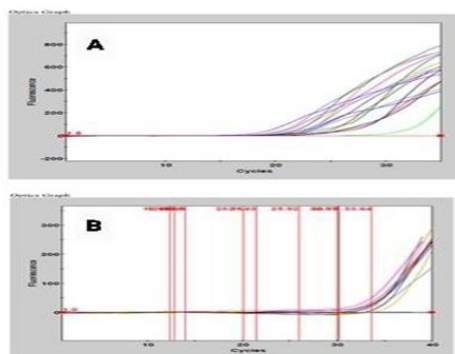


Figure (1): Quantitative RT-PCR for the detection of *Streptococcus mitis* (A) positivity. (B) The amplification: fluorescence signals of FAM chemical detection dye collection during 40 cycles. Positivity was recorded for curves over the threshold line, while negative gene detection was recorded for curves with or beneath the threshold line).

Table 1: Streptococcus mitis positivity in periodontic cases as compared with control

Cases	<i>Streptococcus mitis</i> cases
Singular	2(6.7%)
Negative cases	5 (16.6 %)
Total cases	30 (100 %)

Result of Gel diffusion test

to investigate the antimicrobial action of lemongrass essential oil against *S. mitis* and *S. oralis* and dual species, by using microdilutions protocol for oil concentration preparation, lemongrass essential oil was added in BHI to obtain final concentrations of subsequent dilution that involved 0.1, 0.01, 0.001, 0.0001 and 0.00001 (v/v %) as compared with the same dilution protocol of Chlorhexidine gluconate After the diluents preparation, the GDT was done as a pre experiment before applying the quantitative RT-PCR for the *S. mitis* gene expression, and after a period of 24-48 hrs of setting the plates results revealed that the oil concentration of 0.1 (v/v%) was illustrating high killing performance on the *S. mitis* and about four fold as compared to chlorhexidine(0.2)

Impacts of lemongrass essential oil on Gene Expressions in singular Species Biofilms by qRT-PCR Analysis

To dissect molecular responses of each species to lemongrass essential oil, qPCR assay was performed to examine the transcriptional level of biofilm-related genes. The relative fold change in gene expressions of *S. mitis* were normalized to housekeeping gene GAPDH and calculated by the DeltaDeltaCt method (DNA relative quantification system of Livak method) . The relative expression of *S. mitis* gene was calculated after pretreatment with different concentrations of lemongrass essential oil (selected concentrations ranging from 0.1, 0.01, 0.001, 0.0001 and 0.00001 (v/v %) as compared with Chlorhexidine. 1 ml overnight culture of bacteria were diluted to give 2×10^8 CFu/ , seeded into polystyrene petri dishes, and grown for 24 h. After 24 h, micro diluted lemongrass essential oil was added, cultures without treatment were set as control. After 24 h, bacterial mRNA was extracted, cDNA was then obtained. The gene expression of *S. mitis*, were detected by quantitative Real-Time PCR assay and calculated using the formula $2^{-\Delta\Delta C_t}$. The results of IC50 calculation in our study presented by $2^{-\Delta\Delta C_t}$ values were illustrated that significant down regulation was observed via treatment with the gradual concentration of lemongrass essential oil, since lemongrass titer of 0.01% and 0.04% (v/v%) was enough to cause down regulation of gene expression ratio to the halve for *S. mitis* respectively, and also in comparing gene expression changes with untreated and 0.1v/v% lemongrass essential oil treated samples showed remarkable downregulated expression of *S. mitis* related genes. The drug concentration (EC50) required to reduce the relative gene expression by 50% was calculated by exponential regression of the values. The use of the term EC50 instead of IC50 was recommended by the FDA in 2007 [27]. Results were shown in (figure2) . Currently, we used DNA relative

quantification system (Livak method) targeting the *S. mitis* genes. In this study the unique concentration (about 50 µg/ml) was sufficient to apply both detection or expression assays[28]. The correlation coefficient between the concentration (accordingly with microdilutions v/v%) and DNA fluorescent quantification via relative expression values of *S. mitis* detection genes was positive with dose dependent manner ($R^2 = 0.7677$), against *S. mitis* respectively, indicating good linearity as shown in(figures 2)respectively (using micro titration of different concentrations involved 0.1, 0.01, 0.001, 0.0001and 0.00001v/v% of lemongrass oil . The continued development and advancement of PCR, as well as the advent of other nucleic acid amplification formats , are the key reasons for this flood of molecular-based methods. Also conventional "gold standards" for diagnostic testing have been exceeded by the exquisite sensitivity of such approaches. They had a sensitivity and accuracy that traditional microbiological methods couldn't match. Because of the simultaneous product amplification and detection step, the assay turnaround time can now be as little as to 2 hours Furthermore, the closed system has effectively reduced the chance of pollution to zero [29] . The relative quantification method (2-Ct method) was used to calculate the expression levels of the *S. mitis* genes from cDNA using qRT-PCR. The fold-change in gene expression was normalized to the GAPDH gene as a housekeeping gene and compared to a calibrator sample [30] . Quantitative nucleic acid amplification tests (QNAT) is the preferred method for *S. mitis* surveillance to guide the application of preemptive antibacterial therapy [31].(The results of current study were showed that the essential lemongrass oil was effectively inhibited the bacterial proliferation with significant declining in fold expression values ($2^{-\Delta\Delta CT}$) at different concentrations.(Tables.2), Results were also revealed dose dependency with positive correlation coefficient for both compounds (essential lemongrass oil and chlorhexidine) with positive correlation coefficient of dose dependency regarding therapeutic assay. Monitoring gene expression can be used to determine the dose-response potency of a gene signature as EC50 values [32], Since gene expression signatures are commonly used to identify disease subtypes in translational medicine [33], severity [34], and predict treatment outcome [35]. Bridging this technology to early drug discovery was previously proposed years ago [36]. Dose-response tests are used in drug development to compare the effectiveness of different compounds in modulating biological processes of interest, as well as to find doses for animal and human experiments and to estimate openings to off and toxic effects. Multiple statistical target methods for identifying individual genes with a dose-dependent effect from dose-response gene expression data have been published [37]. Dose response curves can be plotted both by relative expression data ($2^{-\Delta\Delta CT}$) as shown in figures from 4.8 and 4.9 . Lemongrass essential oil (LEO) carries a significant amount of numerous bioactive compounds, such as citral (mixture of geranial and neral), isoneral, isogeranial, geraniol, geranyl acetate, citronellal, citronellol, germacrene-D, and elemol, in addition to other bioactive compounds. These components confer various pharmacological actions to Lemongrass essential oil (LEO), including antifungal, antibacterial, antiviral, anticancer, and antioxidant properties. These Lemongrass essential oil LEO attributes are commercially exploited in the pharmaceutical, cosmetics, and food preservations industries. Furthermore, the application of Lemongrass essential oil (LEO) in the treatment of cancer opens a new vista in the field of therapeutics . In microbial studies, IC50 (half maximal inhibitory concentration).

and MIC (minimum inhibitory concentration) values are two important markers that can be considered to determine the antimicrobial potential of any chemical. The IC50 of a drug is the concentration that can bring a 50% reduction in the microbial activity, therefore it may be cytostatic but bacteria can recover soon thereafter.

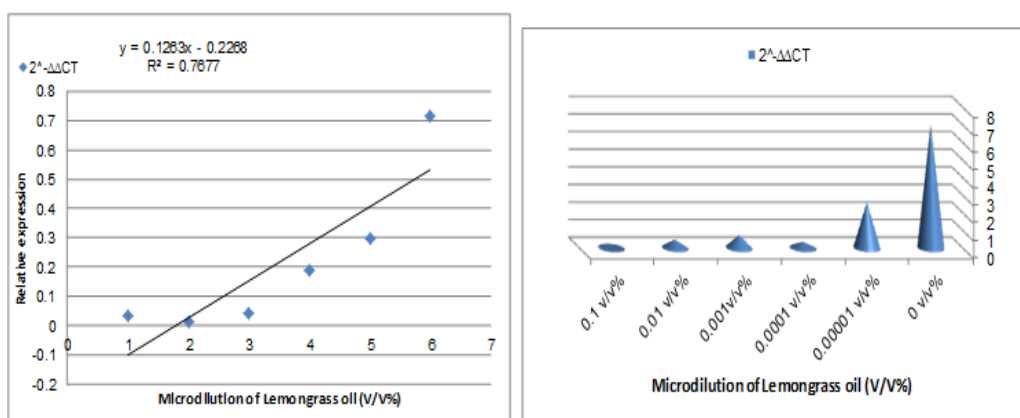


Figure 2: Antibacterial effect of lemongrass oil presented by plotting concentration (v/v%) versus relative expression ($2^{-\Delta\Delta CT}$) *Streptococcus mitis* detection gene .

Table 2: Relative expression of *Streptococcus mitis* gene after treatment with lemongrass oil as compared with Chlorhexidine (0.2%).

Concentration	Δ CT values of Chlorhexidine	Δ CT values of lemongrass oil	$\Delta\Delta CT$	$2^{-\Delta\Delta CT}$
10 ⁻¹	-0.81	4.08	4.88	0.03
10 ⁻²	0.62	7.07	6.45	0.01
10 ⁻³	-0.03	4.50	4.53	0.04
10 ⁻⁴	-9.20	-6.79	2.42	0.19
10 ⁻⁵	-4.07	-2.33	1.75	0.30
0	-4.17	-3.69	0.48	0.72

Table 3: Relative expression of *Streptococcus oralis* gene after treatment with lemongrass oil as compared with Chlorhexidine (0.2%) .

Concentration	Δ CT values of Chlorhexidine	Δ CT values of lemongrass oil	$\Delta\Delta CT$	$2^{-\Delta\Delta CT}$
10 ⁻¹	-10.78	-3.12	7.66	0.00
10 ⁻²	3.65	4.68	1.03	0.49
10 ⁻³	-14.22	-13.81	0.41	0.75
10 ⁻⁴	-13.77	-12.27	1.50	0.35
10 ⁻⁵	-13.70	-15.08	-1.38	2.61
0	18.04	-20.86	-2.82	7.05

Conclusion

The results of current study were showed that the essential lemongrass oil was effectively inhibited the bacterial proliferation with significant declining in fold expression values ($2^{-\Delta\Delta CT}$) at different concentrations with dose dependency.

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