

Full Length Research Paper

***In vitro* antimicrobial activity of essential oil of *Cymbopogon citratus* (lemon grass) on *Streptococcus mutans* biofilm**

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The present study aimed to investigate the effect of essential oil of lemon grass (LGO) on *Streptococcus mutans* biofilm developed in hydroxyapatite discs surface. Initially, the susceptibility of *S. mutans* to the LGO through the inhibition zone test in planktonic suspension and minimum inhibitory concentration (MIC) was investigated. To evaluate the effect of the essential oil in biofilms, hydroxyapatite discs were used to simulate the tooth surface. The biofilms of *S. mutans* were developed on the discs for 5 days and immersed daily in the following groups: G1 - immersion for 5 min in LGO to 0808 mg/ml (test group) and G2 – Brain Heart Infusion (BHI) 1% Sucrose (negative control). Then biofilms were counted for colony forming unit (CFU) and transformed into log¹⁰. The data were analyzed by ANOVA test with a P value <0.05. The susceptibility test was positive indicating inhibition of microorganisms and the MIC value was 0.04 mg/mL. As for biofilm results, it decreased the bacterial growth in G2 compared to G1 with a statistically significant difference (P <0.034). Considering the limitations of this study, it was concluded that the essential oil of lemon grass was effective in controlling bacterial growth in biofilms of *Streptococcus mutans*.

Key words: Phytotherapy, *Cymbopogon citratus*, *Streptococcus mutans*, biofilm, dental plaque.

INTRODUCTION

Dental caries is considered an imbalance of re/demineralization with a high incidence in humans (De Lorenzo, 2004). According to the World Health Organization (WHO), the prevalence of caries in school children is 60-90%, and is virtually universal among

adults in most countries (Petersen et al., 2005). It is still the leading cause of tooth loss, especially in people younger than 40 years (De Lorenzo, 2004). *Streptococcus mutans* are the microorganisms that best meet all cariogenic requirements, and therefore are considered

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the main causative agents of dental caries. They are gram positive, facultative anaerobic bacteria whose cells are arranged singly or in chains of cocci. This microorganism is widely known for its intense acidogenesis due to production of organic acids as byproducts, which cause a carious lesion by dissolving the crystal structure of dental enamel, as well as for its ability to synthesize extracellular glucans, key factors in the development and establishment of a cariogenic biofilm (De Lorenzo, 2004; Yatsuda et al., 2005).

The traditional strategies for the prevention of caries include non-operative measures, such as control of both dental plaque and the individual's diet. With the goal of promoting health, supportive measures such as topical application of fluoride, fluorinated water supply, and the use of chemical substances are the most traditionally used and prescribed methods for high-risk populations. Despite their applicability, there are obstacles to providing these measures, such as the unequal coverage of fluorinated water in different regions of Brazil; mechanical removal being dependent on the maturity and dexterity of manual skill; and the adverse effects resulting from the use of chemical measures, often carried out indiscriminately and without professional supervision and resulting in discoloration, loss of taste, and reversible mucosal desquamation (Cury, 1997).

Research into natural extracts has increased in recent years owing to their potential to be developed into new, commercial pharmacological products for the treatment and prevention of various oral pathologies and that are less toxic, biocompatible, and more affordable (Castilho et al., 2007).

Medicinal plants have been used throughout history owing to their capability of synthesizing a wide variety of chemical compounds with important biological functions in the defense against fungi, bacteria, and various other diseases (Lai and Roy, 2004).

Several plant products have been shown to have antimicrobial potential against cariogenic organisms (Yanagida, 2000). These plant products (mainly essential oils) exert their antimicrobial activity by partitioning the lipids of the bacterial cell membrane, disrupting its structure and function and leading to cell death (Burt, 2004; Ben Arfa et al., 2006). Further, such oils are rich in flavonoid compounds that act on bacterial cells consequently inhibiting enzyme activity (Born et al., 2000), while others function within the cell wall with consequent build-up and rupture of the structure (Dorman and Deans, 2002).

The species *Cymbopogon citratus* belongs to the Poaceae family and is commonly known as lemongrass, barbed wire grass and silky heads among others. It is widely used for its antibacterial and anti-inflammatory properties; although native to India, *C. citratus* has acclimatized in Brazil (Brito et al., 2011). This plant is internationally known as, "lemon grass," for its medicinal characteristics in both humans and animals. It is an

herbaceous plant, and its aromatic leaves are long, narrow, sharp, and ragged, with a prominent midrib. It grows in clumps of more than 1 m, and the leaves are rich in essential oils (Martins et al., 2004).

The plant is resistant to variations of soil and weather (Akisue et al., 1996, cited by Santos et al., 2009), but hot and humid weather, with full sun exposure and evenly distributed rainfall are optimum for its development (Ortiz et al., 2002). Owing to its therapeutic properties, it has been included among the herbal medicines regulated by the National Health Surveillance Agency since 2010 (Brazil, 2010).

Since natural extracts have garnered attention due to its promising therapeutic characteristics, the aim of this present study was to evaluate the effects of lemongrass essential oil (LGO) on cariogenic biofilm.

MATERIALS AND METHODS

Research site

This study was conducted in the Oral Microbiology Laboratory at the CEUMA University. The study did not require ethical consent since it comprised use of only a laboratory strain.

Experimental design

Initially, the susceptibility of planktonic cells of *S. mutans* UA 159 (ATCC700610) to LGO was verified through inhibition zone test using the agar diffusion method on solid medium and by evaluating the minimum inhibitory concentration (MIC) through the microdilution technique (microtechnique). These results were the basis for evaluation of the effect of the essential oil on *S. mutans* biofilm formed on hydroxyapatite disks (10x2 mm; Clarkson Chromatography Inc., South Williamsport, PA), created in order to simulate the tooth surface. The biofilm was developed on the discs for 5 days with a daily medium change (Brain Heart Infusion - BHI + 1% sucrose). Biofilms were divided into groups and immersed daily in the following treatments: BHI plus 1% sucrose (negative control) and LGO for 5 min daily.

Reference bacteria and reactivation of microorganism

The *S. mutans* reference strain UA159 was used in this study. The microorganism was reactivated in BHI medium at a final concentration of 1% glucose in a microaerophilic atmosphere at 37°C for 18-24 h. After growth, the suspension was centrifuged at 3000 RPM for 5 min, and the resulting pellet was then washed twice with sterile phosphate buffered saline (PBS). The turbidity of the resultant material was adjusted with the aid of a spectrophotometer until it reached an absorbance similar to a stock suspension of 1×10^6 bacteria/mL (Duarte et al., 2008).

Plant material and botanical identification

The plant material used in the study was *Cymbopogon citratus* leaves, commonly known as lemongrass. The leaves were grown in the Attic Seabra Herbarium located in the Federal University of Maranhão, São Luís, Maranhão, Brazil. The plant sample was collected between November 2013 and August 2014.

Table 1. Phytochemical analysis of essential oil of *Cymbopogon citratus*.

Identified compounds	%
6-metil-5-hepten-2-ona	0.88
beta-mircen	12.59
cis-ocimen	0.31
linalol	1.14
exo isocitral	0.58
<Z> isocitral	2.24
Rosefuran epoxide	0.37
<E> isocitral	3.01
neral (beta-citral)	34.16
geraniol	2.62
geranial (alfa-citral)	41.75
Geranil acetate	0.36

Collection and chemical composition of LGO

The leaves were separated and dried in an oven with air circulation at 37°C for 48 hours. The dried leaves were sent to the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA UNICAMP), Paulínea, SP, for processing and obtaining LGO, followed by phytochemical analysis.

S. mutans sensitivity to LGO

The antimicrobial activity of LGO was determined by diffusion method according to Bauer et al. (1969). The *S. mutans* strain was cultivated in BHI supplemented with 1% glucose, and incubated at 37°C under 5% CO₂ for a period of 18-24 h in a microaerophilic atmosphere. Blood agar plates with 5% defibrinated sheep blood were prepared and inoculated with the microorganism, previously adjusted to a density of 1x10⁶ organisms/mL. A cell spreader was used to uniformly inoculate the entire surface of the petri dish, using the surface inoculation technique (NCCLS, 2013). The Petri plates were divided into quarters with wells filled with the tested substances. An aliquot of 50 µL of the essential oil at 0.08 mg/mL was pipetted into one quadrant, and in another quadrant, an aliquot of 50 µL of PBS solution was pipetted to identify possible contamination of the inoculum (negative control). The plates were placed once again at a temperature at 37°C under 5% CO₂ for 48 h. After the incubation period, the antibacterial activity was evaluated by means of inhibition zone measurement.

Minimum inhibitory concentration (MIC) test

The minimum inhibitory concentration (MIC) of LGO was determined using the microdilution methodology (microdilution) (NCCLS, 2003). Ninety six well microplates were used. Each well in the test group received 100 µL of bacterial inoculum and 100 µL LGO. The negative control was established by adding only BHI liquid medium to the last row of wells. The 96-well plate was placed at 37°C under 5% CO₂ for 24 h. Readings to determine the MIC against the strains were performed using the visual method. The MIC value was considered as the lowest concentration capable of producing visible inhibition of the growth of tested bacteria.

S. mutans biofilm susceptibility to LGO

For the formation of *S. mutans* biofilm, the bacteria was reactivated as described above. The biofilms were formed on hydroxyapatite disks on a 24-well plate with each well containing BHI with 1% sucrose, changed daily for 5 days (Usacheva, Teichert and Biel, 2001; Deminova and Hamblin, 2005). Prior to daily changes of the culture medium, discs with biofilms were exposed to the following treatments: G1 - LGO (experimental group) and G2 - BHI + 1% sucrose (negative control).

Quantitative analysis

For the quantitative analysis, the biofilms were disrupted and transferred to tubes containing 5 mL of PBS and subjected to sonication using three 15-second pulses at a power of 6 W (Branson Sonifier 150, Branson Ultrasonics, Danbury, CT), with 15-second intervals (Duarte et al., 2008). A 100µL aliquot of the homogenized suspension was utilized for decimal serial dilution, subsequently plated on blood agar plates, and then incubated at 37°C under 5% CO₂ for 48 hours. The results were expressed as colony forming units (CFU) and transformed to log₁₀. Assays were performed in triplicate for each group, and repeated in two consecutive days (n = 6).

Statistical analysis

Data were analyzed using ANOVA test with a P value at 5% for differences statistically significant.

RESULTS AND DISCUSSION

In recent decades, phytopharmacology has played an important role in determining alternative therapeutic means in dentistry by evaluating the antimicrobial properties of candidate compounds in oral diseases, especially those resulting from the formation of dental biofilm (Gebara et al., 1996; Pereira, 1998).

The present study aimed to evaluate the antimicrobial properties of LGO on *S. mutans*, bacteria essential in the development of caries, corroborating a baseline data for future *in vivo* studies and serving as a supportive alternative to effective oral hygiene associated with mechanical removal of biofilm.

The leaf mass and oil mass, content, and yield were 431.34 g and 3.80 g, 0.88%, and 4 g, respectively. Table 1 shows the chemical composition of LGO.

Neral and geraniol, the major components of LGO, are stereoisomers, and their mixture results in citral, the main element responsible for the antimicrobial effect of *C. citratus*, whose mechanism of action involves increasing the permeability of the cell membrane via hydrophobic interactions with the membrane (Sikkema et al., 1994; Oliveira et al., 2011). Recent studies using chromatography have demonstrated the presence of glycolipid macromolecules in lemongrass. These glycolipids (monogalactosyldiacylglycerol and digalactosyldiacylglycerol) are abundant in photosynthetic tissues, and may be found in the outer portion of the cell

Table 2. Mean values of CFU (\log_{10}) after the treatments. Data represent mean values (N = 6) and submitted to one-way ANOVA at $P < 0.05$.

Groups	CFU \log_{10} (\pm s.d.)	P value
LGO	7.25 \pm 0.29	0.034
Control	7.81 \pm 0.55	-----

LGO: lemon grass essential oil; Control: BHI + 1% sucrose; s.d: standard deviation.

membrane, mitochondrial membrane, endothelial reticulum, and chloroplasts of the plant foliage. In recent years, various studies have focused on these compounds in terms of their inhibitory activity on DNA polymerase (antitumor activity), P-selectin (anti-inflammatory effects), antiviral activity (anti-HIV), and inhibition of both the receptor membrane and the growth of specific cell lines (Mendes et al., 2006)

The results obtained in the first phase of the experiments demonstrated the inhibitory effect of LGO on *S. mutans* (UA 159) in planktonic suspension through measurement of halo of growth inhibition.

A previous study aimed to evaluate the *in vitro* antimicrobial activity of *C. citratus* on *S. mutans* by using agar diffusion method, and found a 21.5-mm inhibition zone relative to the 27 mm for chlorhexidine, indicating a slight difference (Nogueira et al., 2008). Lucena et al. (2013) showed inhibition halos of 10, 12 and 14 mm in diameter for 5 μ L *S. mutans* strain, 10, 14 and 20 mm for 10 μ L of the bacterial inoculum, and 10, 14 and 16 mm for 20 μ L. Such studies demonstrate the marked susceptibility of *S. mutans* to *C. citratus*.

The minimum concentration required for inhibiting the bacterial species was 0.04 mg/mL. Perazzo et al. (2012) also determined the MIC value of LGO and found a higher value to exhibit an antimicrobial action (0.5625 mg/mL).

The high antimicrobial efficacy of the essential oil on the bacteria *S. mutans* has been demonstrated in a study making it extremely important in the field of dentistry, since microorganisms related to the cariogenic process produce acid from the metabolism of carbohydrates derived from the individual's diet, demineralize enamel, dentin, and cementum, inducing the development of dental caries (Vargas et al., 2010).

The use of dynamic biofilm model includes the daily application of herbal treatment, simulating the daily use of mouthwash. In certain cases, the reduction of the adhesion of the biofilm can occur, interfering with the synthesis of polyglycans, consequently acting on the adhesion mechanism of bacteria on the surfaces of teeth. Because bacterial adherence has been shown to be one of the primary mechanisms involved in initiation of development of the biofilm, the inhibition of this process would certainly lead to its effective control, contributing to the prevention of caries (Argenta et al., 2012). The results obtained for the quantitative analysis are shown in

Table 2.

A statistically significant difference was found in the experimental group (LGO) when compared to the control group ($p < 0.05$) with a reduction of 0.56 log. Although it was a non-significant reduction, which cannot be reflected clinically, the aim of the therapy is not to completely eradicate, but disrupt the biofilm associated with traditional therapy, which can be corroborated by the decreasing number of cariogenic microorganisms and the time of onset of caries. Additionally, this result may be explained in part by the presence of the biofilm matrix, which maintains the structural integrity and stability, limiting the diffusion of substances and providing protection to the bacteria against challenges such as those found in the present investigation (Paes Leme et al., 2006).

Regardless of how promising the results are, additional studies are necessary to assess the cytotoxicity of the oil against epithelial cells and keratinocytes, and to determine the antimicrobial effects of lemongrass oil in combination with the oils of other plants, substances, or preservatives contained in mouthwashes used *in vivo*, to evaluate its antimicrobial potential.

Conclusion

Considering the limitations of this study, it is possible to conclude that the immersion of hydroxyapatite discs, simulating the tooth surface in LGO was effective in controlling the growth of *S. mutans* biofilm. Although the mechanical removal of biofilm is the most accepted method for its control, the use of chemical adjuvants is of great value and results in greater control of the biofilm, consequently, reducing its pathogenicity. Thus, herbal therapy is a viable treatment for the prevention of caries. It should be highlighted that caries has multifactorial causes and depend on diet, hygiene, and susceptibility of the host, and virulence of microorganisms.

Conflict of Interests

The authors declare no conflicts of interest to the materials or techniques employed in this present study.

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