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THE ANTIMICROBIAL EFFECT OF EUGENOL ON LACTOBACILLI ISOLATED FROM CHILDREN'S SALIVA COMPARED TO CHLORHEXIDINE (IN-VITRO STUDY)

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ABSTRACT

Background: Eugenol is a frequent component of dental biomaterials and is known to exhibit a range of antimicrobial activity. However, there is a lack of reports on evaluating the antimicrobial activity of eugenol versus chlorhexidine on oral lactobacilli.

Material and methods: In the current study, we evaluated the *in vitro* antimicrobial activity of eugenol by agar diffusion method on 15 Lactobacillus isolates initially isolated from pre-school children and compared the efficacy of eugenol with chlorhexidine.

Results: The study showed inhibition zones of eugenol ranged from 6 to 10 mm, while those of chlorhexidine ranged from 7 to 11 mm. The statistical analysis using paired *t*-test revealed a significant difference ($P < 0.001$) for eugenol and chlorhexidine groups with respect to their antimicrobial efficacy against oral lactobacilli. The minimum inhibitory concentration of eugenol and chlorhexidine were 100-400 and 20-40 μg/ml, respectively.

Conclusion: The effectiveness of eugenol against lactobacilli was promising

KEYWORD: Antimicrobial. Chlorhexidine. Early childhood caries. Eugenol. Lactobacilli.

INTRODUCTION

Dental caries is one of the most prevalent infectious diseases. It is a multifactorial, chronic bacterial disease causing the demineralization and destruction of the hard tissues. The demineralization is caused by acids resulting from the bacterial fermentation of dietary carbohydrates. The process of caries involves enamel, dentine and cementum which leads to decalcification and disintegration of the organic substances of the teeth. [1] The factors implicated in the etiology of caries include host factors such as teeth and saliva, intake of fermentable carbohydrates, plaque microorganisms and time. [2]

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While the prevalent view of its etiology is more attuned to the polymicrobial nature of the dental plaque, only a limited number of bacterial species are consistently isolated from carious lesions and have henceforth been strongly associated with dental caries. [3] Lactobacilli have been associated with the progression of dental caries while *Streptococcus mutans* is believed to be the main bacterium that initiates caries and enamel decay. [4]

Lactobacilli are a diverse group of strictly fermentative, non-sporing Gram-positive bacilli and are usually non-motile. They are generally considered facultative anaerobes and are commonly found in food, water, soil, humans and other animals. They are divided into two main groups; homofermenters and heterofermenters. [5] They use dietary carbohydrates in their fermentation process to form lactic acid, thereby creating a low pH environment which can be tolerated by the lactobacilli but is inhospitable for most other microbes. Lactobacilli invading the oral cavity are believed to be opportunistic invaders of existing carious lesions taking advantage of the retentive niche created by the early colonizers such as *Streptococcus mutans*.

Early childhood caries (ECC) is defined as the presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries) or filled tooth surfaces in any primary tooth in a child below 6 years of age. [6, 7] The condition initially presents with smooth-surface carious lesions affecting the primary maxillary incisors followed by the occlusal surface of primary maxillary molars with subsequent spread of the decay to other primary teeth leading to the destruction of the primary dentition. [8-10] ECC has been known to have certain predisposing factors such as the early implantation of mutans Streptococci, the use of feeding bottles containing sugary solutions, prolonged *ad libitum* breast-feeding and the immaturity of the host defense systems. [11] Studies of infants younger than 6 years showed a strong correlation between dental caries and the presence of lactobacilli in the oral cavity. [4]

Eugenol ($C_{10}H_{12}O_2$) is a phenolic aromatic substance which represents the principle chemical component of clove oil. Eugenol is frequently used in temporary fillings, a cement for provisional restorations, cavity base, liners and root canal sealers. Eugenol has been reported to have analgesic, local anesthetic, anti-inflammatory, antibacterial, and antifungal properties. [12, 13] The antimicrobial activity of eugenol has been traced to the presence of a free hydroxyl group in the molecule, with different mechanisms being described to elucidate its influence on bacterial cells. It can act by the disruption of the cytoplasmic membrane which increases membrane non-specific permeability. It also distorts the transport of ions and adenosine triphosphate, inhibits some bacterial enzymes, and alters the permeability of bacterial cell membrane allowing the leakage of ions and loss of cellular contents which leads to cell death. [14, 15] Eugenol also induces cell cytotoxicity by the production of intracellular reactive oxygen species ultimately leading to cell death. [16]

Chlorhexidine is considered the gold standard for antimicrobial tests in dentistry against which other antimicrobial agents are compared due to its wide range of activity and persistent effect. [17] Chlorhexidine has a broad antibacterial activity, low toxicity and strong affinity for binding to skin and mucous membranes. It also has a wide spectrum of activity spanning Gram-positive bacteria, Gram-negative bacteria, yeasts, dermatophytes and some lipophilic viruses. The antibacterial mechanism of action of chlorhexidine is achieved through its rapid attraction to the negatively charged bacterial cell, altering the bacterial cell membrane. Chlorhexidine binds to the phospholipids in the inner membrane leading to increased permeability and finally causes the death of bacterial cell. Investigations also revealed different chlorhexidine activities at different concentrations; at low concentrations

it acts in a bacteriostatic manner while at high concentrations it acts in a bactericidal mode. [17] The cationic property of the chlorhexidine molecule causes extrinsic tooth staining which is a common side effect of chlorhexidine. This cationic nature also results in a sharp reduction in its activity in the presence of anionic agents, which are found with certain toothpastes. The aim of the current study is to evaluate the antimicrobial efficacy of eugenol on salivary lactobacilli isolated from caries afflicted children aged 48-71 months and to compare the antimicrobial activity of eugenol to chlorhexidine.

MATERIALS AND METHODS

Sample size estimation

The minimal sample size was calculated based on a study aimed to evaluate the antimicrobial effect of eugenol on lactobacilli compared to chlorhexidine. A total sample size of 30 specimens divided into 2 groups with a sample size of 15 per group according to the following equation;

$$n = \frac{2\,(Z_{\alpha}+Z_{1-\beta})2\sigma^2}{\Delta^2}$$

where n is the required sample size. For $Z\alpha$, Z is a constant set by convention according to the accepted α error. For $Z_{1-\beta}$, Z is a constant set by convention according to power of the study. σ is the standard deviation and Δ is the difference in effect of two interventions.

Chemicals and dehydrated media

Media used throughout the current work were supplied from HiMedia Laboratories, India. Rogosa SL broth (RSLB), Rogosa SL agar (RSLA) and Muller-Hinton broth/agar (MHB/MHA) were used for enrichment of lactobacilli, isolation of Lactobacillus isolates, and testing the antimicrobial activity of eugenol, respectively. [18, 19] Media were prepared according to the manufacturer instructions before autoclaving at 121°C for 15 min. All chemicals used throughout the current study were of analytical grade and eugenol solution was a product of Loba Chemie, India.

Collection of samples and isolation of lactobacilli

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Dentistry, Pharos University in Alexandria. The study was in accordance with The Code of Ethics of Pharos University in Alexandria for experiments involving human subjects. A written informed consent was acquired from the parents of the subjects before the onset of the study. Children who had a definite history of taking antibiotics 1 month before donation of saliva, undergoing orthodontic treatment or with an intraoral prosthesis, had any intraoral pathology or systemic diseases, were medically compromised, or for whom parental consent was not given were not included in the study. Saliva samples were collected from 30 children aged 48-71 months with dmft scores ranged 3-6. Saliva donors were randomly chosen from the children attending the pediatric dental clinic of Pharos University in Alexandria. The subjects were refrained from oral hygiene procedures on the day of collection. Stimulated saliva was collected during sugar free gum chewing and was collected in previously labeled sterile cups. The samples were vortexed to uniformly mix and an aliquot of 10 ml of the sample was spread on RSLA. The plates were incubated for 48 h at 37°C for selection of Lactobacillus isolates.

Determination of the antibacterial activity of eugenol against oral lactobacilli

The agar diffusion method [18-20] was adopted to examine the antimicrobial activity of eugenol. A suspension of the pure culture was prepared in saline solution. A loopful of each pure Lactobacillus isolates was transferred from RSLA to RSLB and incubated overnight at 37°C. A seed culture of each Lactobacillus isolate ($\approx 10^8$ cfu/ml) was inoculated on the surface of MHA, using a right-angled glass

spreader. One well of 6 mm diameter was punched at equidistant with a sterile cork borer in each plate. An aliquot of 10 ml of eugenol to be tested was placed at the center of each well. Similarly, an aliquot of 10 ml chlorhexidine in a concentration of 0.1% was also incorporated. Sterile water was used instead of test samples as a negative plate. The agar plates were incubated aerobically for 24 h at 37°C. After incubation, the diameter of inhibition zone was measured by Vernier calliper. For each well, the minimal diameter of the zone of inhibition was measured across the well. The average diameter of the inhibition zones was calculated; means and standard errors were also calculated.

Determinations of minimum inhibitory concentration (MIC)

Determination of MICs was performed using the broth micro-dilution method. [21] Each culture of Lactobacillus isolate in RSLB was inoculated in fresh MHB and incubated at 37°C. In a 96-well plate, serial 2-fold dilutions of eugenol generating concentrations ranging from 1600 to 12.5 mg/ml were prepared in MHB. Wells that contain no eugenol were used as positive controls. In order to increase the solubility of eugenol, 0.1% v/v dimethyl sulfoxide was added to all wells. Each well received an aliquot from each Lactobacillus isolate to give a final concentration $\approx 10^5$ cfu/ml which was confirmed by total viable counts. Wells that contain assay media only were treated as negative controls. The plates were incubated for 24 h at 37°C and growth was measured by using a spectrophotometer at 600 nm. An aliquot of 10 µl derived from the wells showing no visible growth was plated on RSLA and the number of colonies was counted after incubation at 37°C. The lowest eugenol concentration that showed no visible growth was regarded as the MIC.

Statistical analysis

Data were calculated as the means of individual experiments performed in triplicate and compared with those of the control groups. Statistical analysis was performed using 2-tailed Student's *t*-test. Statistical significance was set at a *p*-value of less than 0.001.

RESULTS

Determination of the antibacterial activity of eugenol against oral lactobacilli

The antibacterial activity of eugenol was determined using the agar diffusion method. [18-20] The inhibition zones of negative control, eugenol, and chlorhexidine are shown in Fig. 1. The inhibition zones of eugenol against 15 oral Lactobacillus isolates ranged from 6 to 10 mm whereas, chlorhexidine showed inhibition zones ranged from 7 to 11 mm (Fig. 2). Moreover, the mean antimicrobial efficacy of chlorhexidine was 1.28-fold greater than those obtained by eugenol. As shown in Table 1, the statistical analysis using paired *t*-test for the antimicrobial effects of eugenol and chlorhexidine on oral lactobacilli revealed a significant difference between the two groups ($p < 0.001$).

Evaluation of the MIC of eugenol against oral lactobacilli

The MICs were determined using the broth micro-dilution method. [21] The MIC values of eugenol and chlorhexidine are presented in Fig. 3. DMSO (0.1%) showed no significant growth inhibitory activity on lactobacilli. As shown in Fig. 3, the MIC values of chlorhexidine for the 15 Lactobacillus isolates ranged from 20 to 40 mg/ml. On the other hand, the MIC values of eugenol ranged from 100 to 400 mg/ml, thus indicating a high susceptibility of the tested clinical isolates of oral lactobacilli to eugenol compared to chlorhexidine. The results also indicated that isolates number 1, 4, 5, 6, 10 and 11 showed the highest resistance to both eugenol and chlorhexidine.

TABLE (1): Statistical analysis using paired ***t***-test for the antibacterial effect of eugenol and chlorhexidine on oral lactobacilli.

	Chlorhexidine inhibition zone (mm)	**Eugenol inhibition zone (mm)**
Mean	9.533333333	7.433333333
Variance	1.552380952	1.888095238
Observations	15	15
Pearson Correlation	0.18913788	
Hypothesized Mean Difference	0	
Df	14	
t Stat	4.866767099	
P(T<=t) one-tail	0.000124656	
t Critical one-tail	3.787390238	
P(T<=t) two-tail	0.000249311	
t Critical two-tail	4.140454113	

***Significance level:** $p < 0.001$.*

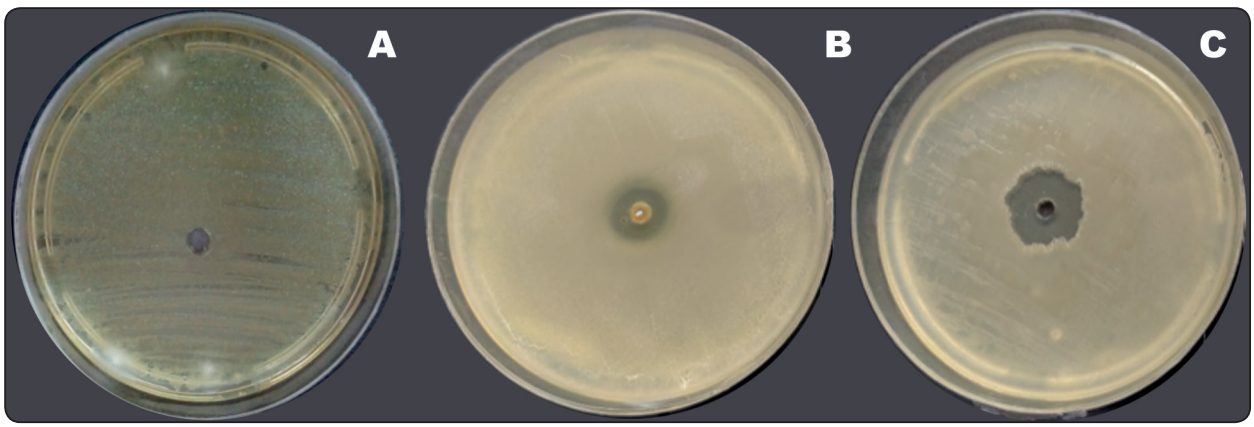

Fig. (1) Negative control plate (A), inhibition zone of a Lactobacillus isolate around the well that was initially filled with eugenol (B) and chlorhexidine (C).

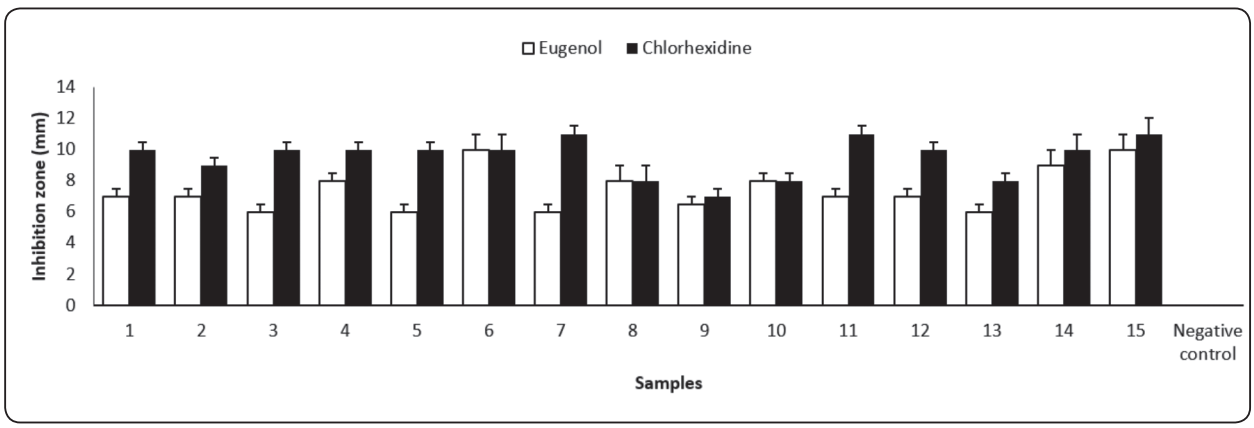

Fig. (2): The diameter of inhibition zones of eugenol versus chlorhexidine on 15 Lactobacillus isolates.

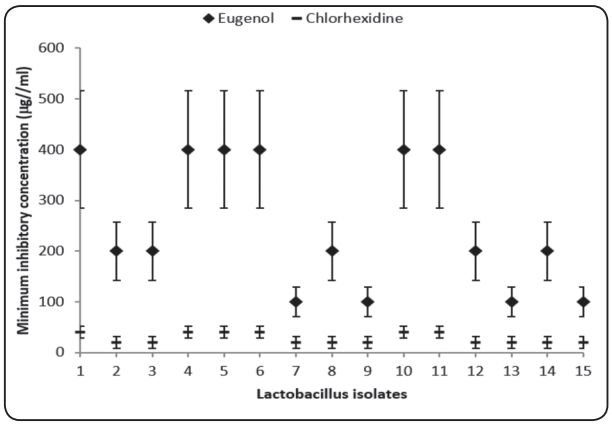

Fig. (3) The minimum inhibitory concentration of eugenol versus chlorhexidine against 15 oral Lactobacillus isolates.

DISCUSSION

Eugenol (4-allyl-2-methoxyphenol) has been reported to exhibit potentially beneficial biological properties including antispasmodic, anticarminative, antioxidant, anti-inflammatory, and antimicrobial activities. [15, 22, 23] It is being used as a component of dental cement containing zinc oxide for temporary sealing of cavities or as a base for fillings. [24] Many authors have reported the antibacterial activity of eugenol against human bacterial pathogens such as *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Legionella pneumophila*. [12, 25-27] On the other hand, investigations on the action of eugenol on caries associated bacteria have demonstrated that a significant inhibitory effect of eugenol against acid production by *Streptococcus mutans*. Those experiments were conducted by applying topical eugenol on rats which resulted in a reduction in the incidence and severity of carious lesions. [28] However, the effect of eugenol on oral Lactobacillus clinical isolates has not been studied. In the present study, we aimed to evaluate the antibacterial influence of eugenol on salivary Lactobacillus isolates from ECC subjects. Eugenol was found to have an *in-vitro* antibacterial activity against 15 oral Lactobacillus isolates by using the agar diffusion method. Our results are in agreement with other reports on the antibacterial activity of eugenol. [28, 29] The MIC values of eugenol against oral lactobacilli were found to be consistent with other reports on the MICs of eugenol against various pathogens. [30-33]

The antibacterial effect of eugenol is in consequence of cell membrane damage, leakage of cytoplasm, and the molecular interactions of eugenol with extended spectrum β-lactamases enzymes of pathogenic bacteria. [26, 27, 34] Moreover eugenol disrupts the cell membrane of pathogenic bacteria by denaturing proteins and reacting with phospholipids in the cell membrane. [35] Eugenol also affects the transport of ions and changes the profile of fatty acids. [35] On the other hand, synergistic interaction of eugenol with antibiotics has been investigated as a potential solution against multi-drug resistant bacteria. [36, 37]

CONCLUSION

Based on the limitations of the current study, it is obvious here that eugenol exhibits remarkable antibacterial activities against the 15 tested oral lactobacilli isolated from children with ECC. Based on the results of the MIC, eugenol showed promising *in-vitro* anti-lactobacilli efficacy compared to chlorhexidine.

RECOMMENDATION

It would be recommended to study the effect of eugenol on *Streptococcus mutans* as a leading microorganism in development of ECC. Further experiments in human trials are needed to reach the final goal for managing ECC.

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