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Research Article

COMPARATIVE IN *VITRO* STUDY OF ANTIMICROBIALS AGAINST ORAL BIOFILMS OF *STREPTOCOCCUS MUTANS*

***Eunice Lobo**
Department of Microbiology, Sophia College, Mumbai
**Author for Correspondence*

ABSTRACT
In the study four types of antibacterial agents were used: Chlorhexidine, Sodium benzoate, Cinnamon oil and Xylitol. These categories of antibacterial agents were selected to represent antibacterial agents of diverse origin and widely differing modes of action. The aim of this study was to compare the four mentioned antibacterials, which are commonly used in toothpastes, mouth rinses and even chewing gum, against biofilms of *Streptococcus mutans* formed in vitro which is a cariogenic organism. The effect of individual antibacterial was studied by growing the biofilms on conventional microscopic slides. The biofilms were allowed to grow in the culture medium for a period of 54 hours, after which the early-formed biofilms were exposed daily for a period of 1 minute over a period of 3 days to various concentrations of the antimicrobials individually. Simultaneously early-formed biofilms were treated with saline as a control. The final treated biofilms (126 hour old) were harvested and the cell viability and glucan accumulations of the biofilms were determined. The number of viable organisms, for all the antimicrobials tested, i.e. the count of viable organisms was seen to decrease at least by 1 $\log_{10}$ fold, indicating their effectiveness in detachment of preformed *S. mutans* biofilms. However on checking the amount of soluble glucans produced by the biofilms the range of values obtained were falling outside the standard range. Hence indicating that the amount of soluble glucans was negligible and the reason for formation of biofilms in this study was not due to soluble glucans, hence in the light of the experiments performed it can be concluded that out of all the four antimicrobials used Xylitol was the most effective against the viability of *S. mutans* and in reducing the formed biofilms, followed by Chlorhexidine, Cinnamon oil and Sodium benzoate.

Key Words: *S. Mutans, Chlorhexidine, Cinnamon Oil, Sodium Benzoate, Xylitol, Glucans.*

INTRODUCTION
Oral biofilms are an essential component in the etiology of dental caries and periodontal disease. Dental plaque biofilm is a deposit of proteins, cell-free enzymes, and bacteria embedded in exopolysaccharides that adhere firmly to the tooth surface. (Erdem *et al.*, 2012) Dental plaque is a biofilm which forms on the non-shedding surfaces of the oral cavity. If left untreated, the succession of dental plaque development can lead to serious complications, such as caries, gingivitis, and periodontitis. (Hope *et al.*, 2004) *Streptococcus mutans* is important in the etiology of dental caries, and is considered the main pathogen associated with dental caries. It induces mineral loss due to its strong adhesion to the tooth surface and production of acid from fermentable carbohydrates, which keeps the local pH low. (Erdem *et al.*, 2012) The acidogenic and aciduric (associated with acid tolerance) properties of *S. mutans*, together with its ability to synthesize extracellular glucans, are the major factors for the development and establishment of cariogenic biofilms.(Koo *et al.*, 2003)
CHX ($C_{22}H_{30}C_{l2}N_{10.}2C_6H_{12}O_7$) is a cationic bisbiguanide with a molecular mass of 898 Da. It possesses broad antibacterial activity in combination with low mammalian toxicity and the ability to bind to skin and mucous membranes. A CHX concentration of 0.2% is deemed to be the most effective as a mouthwash and as such is considered the "gold standard." (Hope *et al.*, 2004)
Essential oils are ideal for use in oral care products because they are both antiseptic and non-toxic—a rare combination. Cinnamon oils have proved to be even more effective as antiseptic mouth rinses than even FDA-recognized plaque-control antiseptic drugs such as stannous fluoride. (Klemba *et al.*, 2003) It has

been proposed that cinnamaldehyde and eugenol inhibit production of an essential enzyme by the bacteria and/or cause damage to the cell wall of bacteria (Matan *et al.,* 2006). In addition to causing cell lysis in organisms responsible for plaque, gingivitis, and oral malodour, studies have shown that essential oil mouthwashes have strong activity against the Gram-positive microbes that cause dental caries.(Seymour 2003)

Benzoate has been shown to affect oral microorganisms in a similar way to that of fluoride by reducing the acid tolerance of the oral flora causing cell death (Arweiler *et al.,* 2008). Since preservatives such as sorbate and benzoate could be concentrated in plaque using the same mechanisms described for fluoride concentration, the acid stress of oral streptococci in plaque is thought to be enhanced by these preservatives (Al-Ahmad *et al.,* 2008). An effect of food preservatives on the growth and metabolism of plaque bacteria has been shown to occur both *in vitro* and *in vivo*. It has been shown that an established intra-oral splint design resulted in standardized *in situ* biofilm formation (mimicking supragingival plaque formation) irrespective of the position of the different specimens (Arweiler *et al.,* 2008)

Xylitol is a natural carbohydrate that belongs to the pentitols a polyalcohol that contains five carbon atoms, that is not fermented by human plaque bacteria. Since is not fermented by cariogenic plaque bacteria thus, it does not lower the pH of plaque. Because plaque pH does not decrease, enamel demineralization is prevented, and plaque bacteria do not proliferate (Burt 2000).

Xylitol consumption is believed to considerably increase the salivary lactoperoxidase activity, which prevents certain oral inflammatory processes because of the antibacterial properties of the enzyme. (Seymour 2003)

Understanding the underlying mechanisms of biofilms development has been facilitated by the use of in vitro model systems using biofilms which reflect more accurately the oral environmental conditions than a planktonic environment. Similar model systems have also been applied to study the effect of antimicrobial agents on biofilms growth and viability (Foster *et al.,* 2004)

The control of dental plaque on tooth surfaces is vital for the prevention of dental caries and perio-dontal disease. In this context, antimicrobial agents may serve as a valuable complement to mechanical plaque removal (Erdem *et al.,* 2012). Therefore the present study deals with reducing the prevalence of biofilms formed in vitro on glass slides, by action of the antimicrobials used individually. In order to compare the efficacy of each, to determine the most effective antibacterial.

MATERIALS AND METHODS

Bacterial Culture

Streptococcus mutans 497 was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh which was a proven cariogenice pathogen. Using Brain heart infusion broth the strain was cultured and the O.D. of the cultural suspension was adjusted at 0.1 at 530 nm using sterile saline.

Test Compounds

Chlorhexidine (CHX) from ICPA Health Pvt. Ltd. ; a single concentration of 0.2 w/v prepared in distilled water was used. The essential oil used was cinnamon (C.O.) obtained from Aroma treasures; a stock solution of 10 mg/ml in Brain heart infusion broth with few drops of Tween 80 was prepared. 25 mg/ml of sodium benzoate (SB) from Loba chemie and 40 mg/ml Xylitol (XYL) from Sisco Research Laboratory as stock solution were prepared in Brain heart infusion broth.

Determination of Minimum Inhibitory Concentration (MIC) of the Antimicrobials against Planktonic S. mutans

Minimum inhibitory concentration of all the test compounds was determined by Broth dilution method. Stock solution of test compounds was used to obtain varying concentrations. 0.1 ml of the *S. mutans* 497 was added to the test tubes. Two sets of negative and positive controls were also prepared. The tubes were incubated at 37°c 24 h. the tubes were then observed for turbidity and the results were recorded. Minimum inhibitory concentration is defined as the lowest concentration showing inhibition.

Determination of Minimum Bactericidal Concentration (MBC) of the Antimicrobials against Planktonic S. mutans

MIC tubes showing the positive result along with the tubes before and after of all the test compounds were used. Streaking of loopfuls from the MIC tubes across Brain heart infusion agar plates was performed. These plates were incubated at 37°c/24h. Depending on the MBC results, further concentrations of test compounds to be used against biofilms of S. mutans 497 was determined.

Preparation of S. mutans 497 Biofilms

Biofilms of *S. mutans* 497 were formed on standard glass microscope slides placed in groves of Coplin jars containing 70 ml Tryptone-yeast extract broth with 30 mM sucrose and 0.06% cysteine. Biofilms were allowed to form on the glass slides. The culture medium was replaced every 18 hours and the biofilms were allowed to grow maximum for 54 hours. After growth the slides were removed and stained with Basic Fuchsin dye and observed under the microscope.

Treatment, Harvest and Determination of Bacterial Viability of Biofilms

After completion of 54 hours the biofilms were treated everyday till the 5^{th} day of the experimental period i.e. 126 hr old, with **a)** varying concentrations of Chlorhexidine, **b)** varying concentrations of Cinnamon oil, **c)** varying concentrations of Sodium benzoate, **d)** varying concentrations of Xylitol, and **e)** physiological saline. Beginning from 54 hours each slide was removed from the Coplin jars, gently dipped in sterile saline to remove loosely adherent material, and then exposed to varying concentrations of the antimicrobials for 1 minute. The treated slides were then dip-washed three times in sterile saline, then placed in 30 ml of saline to harvest the adherent cells by scrapping using a sterile scalpel. The suspension was vortexed for 10 seconds, followed by centrifugation at 3000 rpm for 10 minutes twice. The pellet obtained was re-suspended in 5ml sterile saline and serially diluted and spread on sterile nutrient agar plates for determining bacterial viability using Plate count method. The supernatant was used for polysaccharide analysis.

Quantification of Extracellular Polymeric Substances (EPS) Produced By S. mutans 497 Biofilms in vitro

The cell free supernatant obtained earlier was analyzed for external polysaccharide content. Three volumes of ice cold ethanol were added, and the resulting precipitate was suspended in distilled water. The soluble glucans were estimated by Anthrone method.

RESULTS AND DISCUSSION

Determination of Minimum Bactericidal Concentration (Mbc) of the Antimicrobials against Planktonic S. mutans

For Chlorhexidine stock solution of 40 µg/ml in distilled water was used. The range selected was 0.2- 4 µg/ml. The MIC of Chlorhexidine against planktonic *S. mutans* 497 was 1.6 µg/ml. Similarly for Cinnamon oil, a stock solution of 10 mg/ml in Brain heart infusion broth was used. The range was 0.1-10 mg/ml and the MIC of Cinnamon oil against planktonic *S. mutans* 497 was 0.8 mg/ml. For Sodium benzoate the MIC against planktonic *S. mutans* 497 was 9 mg/ml when a range of 0.1- 15 mg/ml was selected using 25 mg/ml stock solution. And for Xylitol a stock solution of 40 mg/ml in BHI broth was used for the range of 5-30 mg/ml. The MIC result obtained was 22 mg/ml.

By means of Broth Dilution method MIC's of all the test compounds (individually), with varying concentrations was found out against planktonic cells of *S. mutans* .These values were crucial in helping us determine the values needed for MBC and subsequently for testing viability of sessile cells of *S. mutans* .The values obtained for MIC of each antimicrobial are low concentrations as antimicrobials are more effective in killing/inhibiting planktonic growth.

Determination of Minimum Bactericidal Concentration Test for planktonic S. mutans

The values obtained were as following: MBC of Chlorhexidine against planktonic *S. mutans* was found to be 1.6 µg/ml, MBC of Cinnamon oil against planktonic *S. mutans* was found to be 1 mg/ml, MBC of Sodium benzoate against planktonic *S.mutans* was found to be 11 mg/ml and MBC of Xylitol against

planktonic *S.mutans* was found to be 24 mg/ml. Minimum Bactericidal concentration (MBC) of all the antimicrobials used in this study was determined in order to check the efficiency of MIC test as well as determining the concentrations of antimicrobials to be used individually, for studying viability of *S. mutans* biofilms. Concentrations of antimicrobials used for viability testing are 1.5 – 2 times the MBC values.

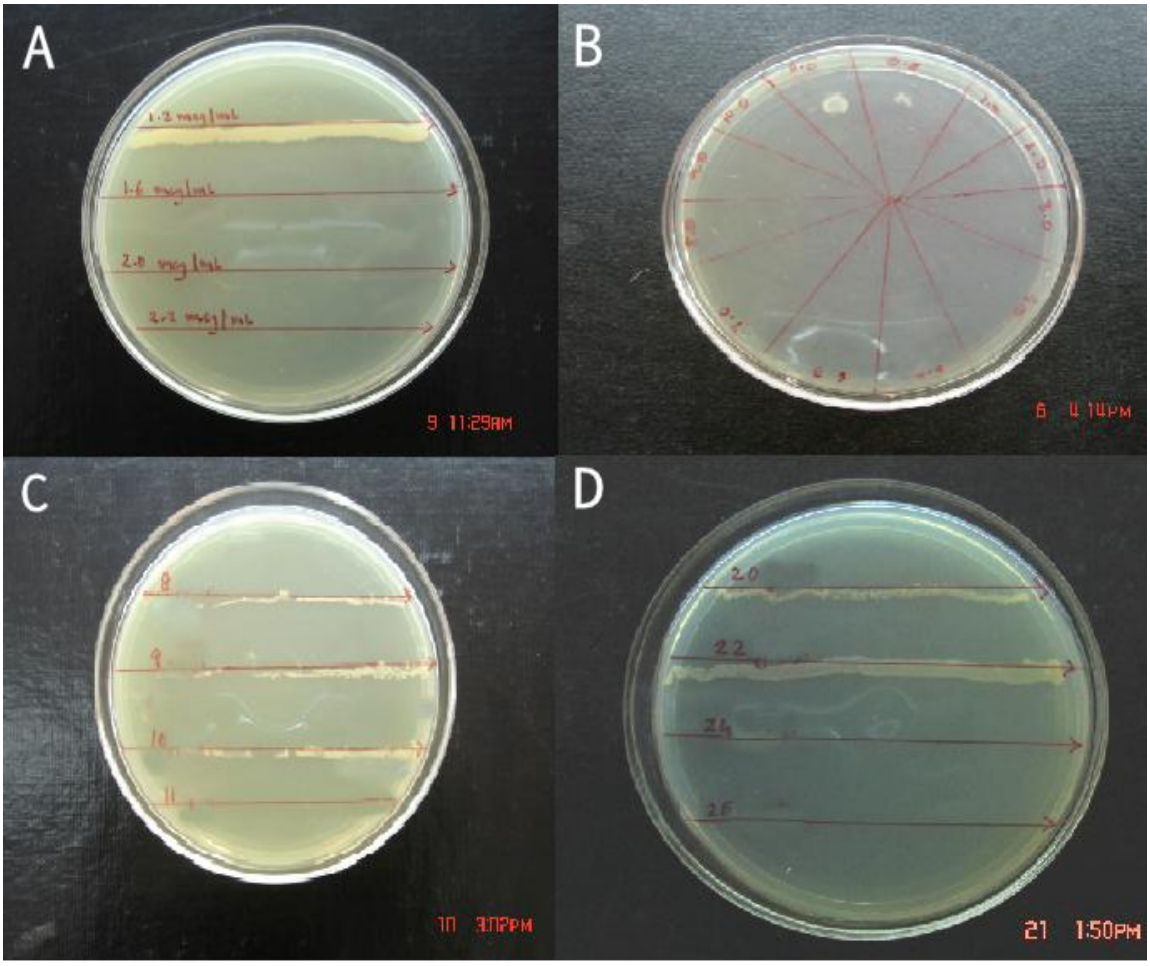

Figure 1: Minimum bactericidal concentration (MBC) of the antibacterials viz. (A) Chlorhexidine; (B) Cinnamon Oil; (C) Sodium benzoate; (D) Xylitol against planktonic S.mutans

Bacterial Viability count

Table 1: Bacterial viability measure for controls

Biofilm	**cfu / biofilm**
54 hour old (Before chemical exposure)	1.26×10^8
Saline control (126 hour old i.e. after 5^{th} day)	1.53×10^8

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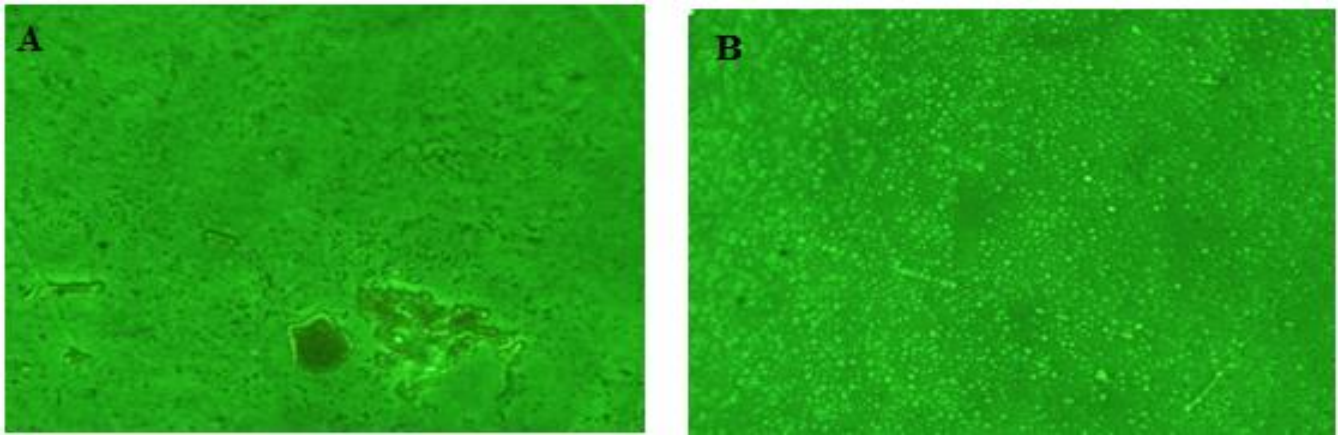

Figure 2: (A) 54 hour old early formed biofilm (Untreated); (B) 126 hour old Salinecontrol (Both as observed under a magnification of 40x using inverted Phase Contrast)

Table 2 : Bacterial viability measure after 126 h i.e. after the 5th day

Chlorhexidine		**Cinnamon oil**		**Sodium benzoate**		**Xylitol**	
Conc. (µg/ml)	**cfu/ biofilm**	**Conc.** (mg/ml)	**cfu/ biofilm**	**Conc.** (mg/ml)	**cfu / biofilm**	**Conc.** (mg/ml)	**cfu/biofilm**
0.5	1.58×10^8	0.5	5.64×10^7	5.0	4.6×10^6	20.0	3.7×10^7
1.0	1.04×10^8	1.0	1.44×10^7	10.0	3.2×10^6	25.0	2.6×10^6
1.5	5.7×10^7	2.0	1.7×10^5	15.0	1.6×10^5	30.0	1.22×10^6
2.0	2.64×10^7	5.0	4.23×10^4	20.0	6.11×10^4	35.0	5.1×10^4
3.0	1.7×10^4	10.0	2.1×10^3	25.0	5.53×10^3	40.0	1.47×10^2
4.0	1.57×10^3						

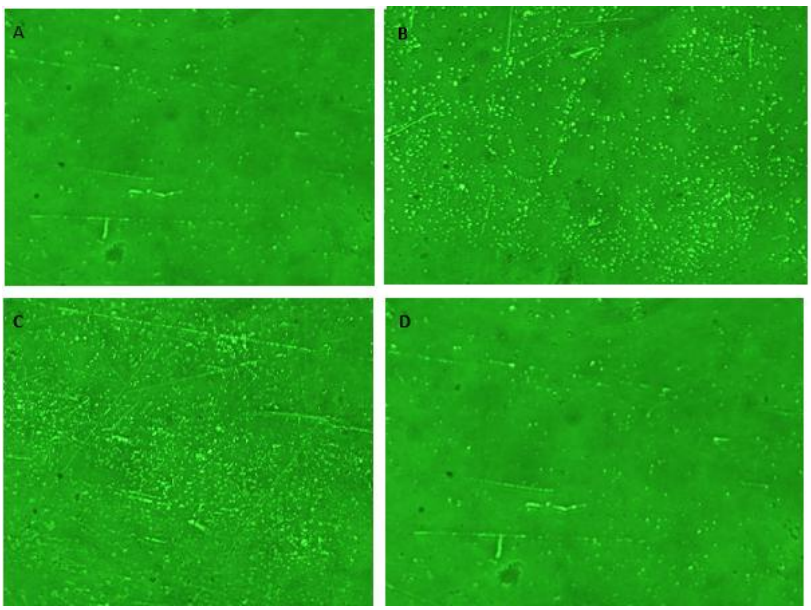

Figure 3: Biofilms exposed to most effective concentration of respective antimicrobials and observed under a magnification of 40x using Nikon TS-100 inverted eclipse microscope after 126 hours

A: Biofilms exposed to 40µg/ml CHX **C: Biofilms exposed to 25 mg/ml SB**
B: Biofilm exposed to 10 mg/ml C.O. **D: Biofilm exposed to 40 mg/ml XYL**

Separate biofilms of *Streptococcus mutans 497* at the age of 54 hours were treated with increasing concentration of Chlorhexidine once a day for one minute for three consecutive days. Theses biofilms were then harvested and viable count was carried out to determine the concentration at which biofilm growth is negligible i.e. the concentration of Chlorhexidine which is bacteriostatic or bactericidal towards the biofilm growth. The count for each concentration is given in the above Table no: 2 .The number of viable organisms in the control biofilms before exposure and saline control) are given in Table no: 1.The biofilms treated with Chlorhexidine showed a steady decrease in the number of viable cells as the concentration of Chlorhexidine increased. The number of viable organism/biofilm at the lowest concentration i.e. 0.5 µg/ml (1.5×10^8) was almost equal to the saline control (1.53×10^8) indicating that at that concentration Chlorhexidine had absolutely no effect on the biofilm organisms. Although after this concentration there was a steady decrease in the number of organisms/biofilm. At the concentration of 3 µg/ml there was a massive reduction of viable organisms (from 10^7 to 10^4) indicating that this concentration is extremely effective. But the concentration of organisms persisted even until the last concentration of 4 µg/ml. Indicating that for complete inhibition of *S. mutans* biofilms a higher concentration of Chlorhexidine is required, whereas the concentration for inhibiting planktonic growth of the same organism is lower i.e. 1.6 µg/ml.

The viable count for organisms exposed to increasing concentration of Cinnamon oil was found to decrease steadily as seen in Table no. 2.The no. of organisms/biofilm at the initial concentration of 0.5 mg/ml (5.64×10^7) itself showed a 10 fold decrease as compared to the saline control (1.5×10^8). Indicating that the organism is sensitive to even low concentrations of Cinnamon oil. As the concentration increased the reduction in no. of cfu/biofilm decreased considerably, especially at concentration of 2 mg/ml which showed a huge reduction in the no. of cells of about 2 $\log_{10}$ decrease. Hence indicating that, 2 mg/ml was the most effective concentration against the *S. mutans* biofilm organisms. A gradual reduction in no. of cells in the remaining concentrations was observed, however complete inhibition did not occur, but may occur at a higher concentration of Cinnamon oil.

The viable count of the organisms exposed to increasing concentration of Sodium benzoate was also found to decrease steadily as seen in Table no. 2.The no. of organisms/biofilm at 5 mg/ml concentration of Sodium benzoate (4.6×10^6) showed a much lower count as compared to that of saline control (1.5×10^8) This 2 $\log_{10}$ times decrease indicates that Sodium benzoate is effective against biofilms of *S.mutans* at low concentration as well. However after this only a gradual decrease in number of cells was seen. At the last concentration of Sodium benzoate also the amount of viable cells in the biofilms (5.53×10^3) was considerably high even though the concentration of 9 mg/ml was sufficient for killing of planktonic *S. mutans*. Thus indicating that a much higher concentration of Sodium benzoate will be needed for complete inhibition of its biofilm forming counterpart.

The viable count for biofilm organisms exposed to the varying concentrations of the sugar Xylitol showed a gradual decrease as observed in Table no. 2.The count of viable organisms at the lowest concentration of 20 mg/ml (3.7×10^7) was found to be comparatively much less than the saline control (1.5×10^8),which indicates its effectiveness. As the concentration increased the count was reduced in the order of 1-2 $\log_{10}$.Especially at 35 mg/ml a 2 $\log_{10}$ decrease was noticed , proving that it is most effective concentration against the *S. mutans* biofilm. And the efficacy of Xylitol was further seen when a count of mere 147 cfu/ml was obtained at 40 mg/ml concentration indicating that a higher concentration of Xylitol can prove to be bactericidal towards the biofilm formed.

It can be observed from Table no. 1 and 2 , that the cell viability was not completely inhibited. The biofilm killing concentration (BKC) for this experiment was thus taken as the concentration in which the viable organisms were reduced to10^3 cells/biofilm. The BKC of Chlorhexidine is 4 mcg/ml, for cinnamon oil is 10 mg/ml, for sodium benzoate is 25 mg/ml and for xylitol is 40 mg/ml.

Determination of EPS content by Anthrone Method

The concentration of glucans present in the biofilm was determined by obtaining the absorbance (O.D.) colorimetrically at 620 nm and plotting in the graph of Standard concentration v/s O.D.

Table 3: Measure of EPS content by Anthrone method

Biofilm	Conc. (mg)
Saline control	0.27
CHX (4 mcg/ml)	0.13
C.O. (10 mg/ml)	0.18
SB (25 mg/ml)	0.21
XYL (40 mg/ml)	0.12

The concentrations of soluble glucans accumulated in the biofilms exposed to the highest concentration of the antimicrobials used in the study (individually) are given in Table no. 3.When compared to the amount of soluble glucans accumulated in the Saline control biofilm it can be observed that there is only a minor decrease in the polysaccharide content. Thus, indicating that the accumulation of soluble glucans decreased as compared to the Saline control. The values of each of the antibacterial treated sample was

falling out of range we can conclude that the formation of soluble glucan as negligible. The decrease in accumulation was due to the killing of the organisms in the biofilm which prevented the synthesis of glucans. Comparison between various antimicrobial agents used in this study showed that Xylitol sugar had maximum bactericidal effect against the plaque causing *S.mutans.* Hence it can be used considerably in order to prevent as well as treat plaque formation. Also the effectiveness of Chlorhexidine was also commendable, suggesting that it along with Xylitol in combination is worthy of further study and commercial application.

CONCLUSION

The aim of this study was to compare four different classes of antimicrobials against the viability and glucan accumulation by Oral biofilms of *Streptococcus mutans*. Biofilms of *Streptococcus mutans* 497 , a definite caries causing organism were grown on conventional glass slides as suggested by Hyun Koo *et al*. Minimum inhibitory concentrations as well as minimum bactericidal concentrations of the individual antimicrobials against planktonic *S.mutans* were determined prior to biofilm growth, and concentrations were obtained for biofilm exposure. The minimum inhibitory concentration (MIC) of CHX was found to be 1.6µg/ml, C.O. was 1 mg/ml, and SB was 8 mg/ml and for XYL was found to be 22 mg/ml. For the treatment of biofilms the concentration of CHX that had maximum inhibition was 3 µg/ml, for C.O. was 2 mg/ml, for SB was 15 mg/ml and XYL was 35 mg/ml, all of which considerably decreased the viable count. This was seen because the number of viable cells recovered after harvesting from the treatment of the antimicrobial was seen to decrease as the concentration of the antimicrobials increased. And this was seen for all the antimicrobials used. However there was no complete inhibition of biofilm forming organisms, which is probably due to the presence of "persistor cells". Further the amount of soluble glucans accumulated in the biofilm was determined colorimetrically by the Anthrone method, showed that as the concentration of all the antimicrobials increased individually, the O.D. decreased, such that it was not in the standard range, hence indicating that soluble glucans were not the reason for formation of biofilms in this study. Hence it can be concluded that all the four antimicrobials tested against biofilms of *S. mutans* were successful in detaching the biofilms considerably. And among all Xylitol proved to be the most efficient. Thus it can be used in toothpastes, mouthwashes as well as chewing gum, whereas the rest can also have similar applications or can be used in combination to have enhanced effect.

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