Comparative evaluation of antimicrobial activity of ground husk of cocoa bean with and without xylitol on Streptococcus Mutans – An *in-vitro* study.

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Abstract

Background: Dental caries is a disease that occurs when bacteria, predominantly *Streptococcus mutans*, colonizes the tooth surface and metabolizes the dietary carbohydrates to acids, resulting in demineralization of teeth. Interventions that can inhibit the growth and survival of streptococcus mutans are crucial to reduce dental caries. Cocoa bean husks, extracted from the shells of cocoa beans have shown to possess anti glucosyltransferase and antibacterial activities. Aim of our study was to evaluate *in-vitro* antimicrobial activity of ground husk of cocoa beans with and without xylitol on streptococcus mutans. **Method**s – The study was carried out using 96 well micro-titer plate and these were divided into three groups. Group I consisted of Extracted powder of cocoa bean (CBH), Group II was of extracted powder of cocoa bean and xylitol (CBH+X) and Group III consisted of Chlorhexidine (0.12%) (CHX). Minimum Inhibitory concentration was assessed using 96 well micro-titer plate. Standardized strain of Streptococcus Mutans 25175D was used. **Results** – No anti-microbial activity was seen for group I (CBH) and group II (CBH+X) at concentrations of 0.3%. However, chlorhexidine (0.12%) showed its antibacterial activity at its first concentration only. **Conclusions** – It was found that ground husk of cocoa bean at 0.3% does not possess any antimicrobial property in *in-vitro* trial. Addition of xylitol had no effect on increasing antibacterial property.

Keywords: ground husk of cocoa bean, xylitol, Streptococcus Mutans, *in-vitro study*

Introduction:

Dental caries is a major public health concern in most countries as it affects 60–90% of school-aged children and almost 100% of the adults[(1)]. Dental caries is a multifactorial disease with principle etiologic agent being dental plaque. The process of formation of dental plaque on the tooth surface occurs via the complex process of synthesis of glucan polymers and glucan binding proteins which are catalyzed by glucosyltransferases. Glucosyltransferase, produced by Mutans Streptococci, provide binding sites for many other microorganisms[(2)]. Reducing the load of Mutans Streptococcus in the oral cavity thus becomes a practical step in reducing the incidence of dental caries in the individuals and in the community. A number of attempts have been made to find natural anti glucosyltransferases substances and attempts to isolate them from various plants or microbials sources have been successful[(3)]. One such naturally occurring anti glucosyltransferases have been found in Cocoa beans.

Cocoa bean is a seed obtained from tree called *Theobroma cacao* that belongs Malvaceae family[(4)]. Also known as Cacao beans, they form the raw ingredient of chocolate. The natural polyphenolic compounds found in cocoa bean husk are catechin and epicatechin. Along with these natural polyphenols, there are free fatty acids such as oleic acid and linoleic acid. These free fatty acids have shown potent bactericidal effect against mutans streptococci[(4)]. Ooshima et al (1993) showed that olong tea poly-phenols possess a strong anti-glucosyltransferase activity and inhibit experimental dental caries in specific pathogen-free rats infected with mutans streptococci.[(5)] Thus, Cocoa bean husk extracts have demonstrated to possess both antibacterial and anti-glucosyltransferases activities. In the studies done by Ooshima *et al* in 2000 and Osawa *et al* in 2001 it was found that cocoa bean husk possesses the property to inhibit dental caries in rats infected with mutans streptococci[(6,7)]. Previous studies showed in vitro as well as in vivo reduction in streptococcus mutans as well as plaque formation in subjects. [(7,8,9)]

Xylitol is a five-carbon sugar polyol and is widely used as an artificial sweetener. It has been found to reduce the levels of Mutans Streptococci in oral cavity by decreasing their adhesion to tooth structure and also by limiting their acid production potential[10]. Habitual consumption of xylitol reduces the levels of Mutans streptococci in oral cavity. So the combination of xylitol along with extract of cocoa beans may significantly increase its taste and acceptability to the children. As well as such combination can possibly reduce Streptococcus Mutans count and plaque formation. Hence need was felt to conduct *in vitro* study to assess antimicrobial activity of ground husk of cocoa bean with and without xylitol on streptococcus mutans.

Materials and methods

Ground husk of cocoa bean was procured from chocolate manufacturing private company and purification process was carried out according to Osawa Ketal (2001)[7].(Figure 1)

(Figure 1- Ground husk of cocoa bean extracts before purification process)

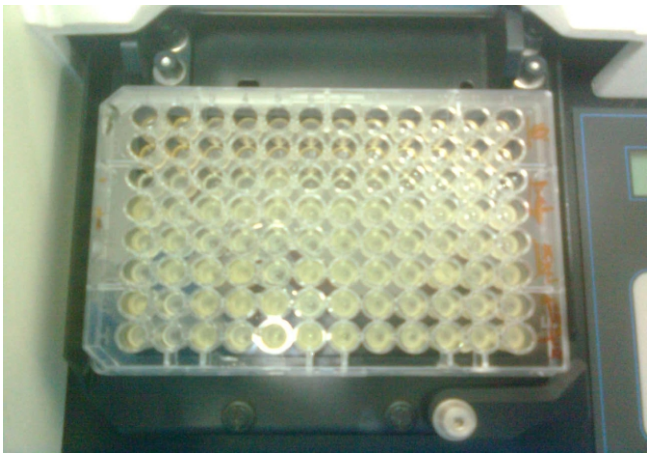
After procuring ground husk of cocoa bean, they were first treated with 5 gram of cellulose in 4.75 liters of distilled water at 50°C for 4 hours. Ethanol was then added up to 50% (v/v final) concentration and the mixture was refluxed for 1 hour. After the process of filtration and evaporation of the ethanol, the remaining extract was lyophilized to give it a powdered form. Ground husk of one kg of cocoa bean yielded almost 120 grams of powder which was used for further i*n-vitro* testing on streptococcus mutans.

Xylitol powder was obtained from a private company as sample which was 99.97% pure.(figure 2)

(Figure 2- Xylitol powder with 99.97 purity)

Perioguard™ was used as source of Chlorhexidine (0.12%). Streptococcus Mutans strain used for study was obtained from American Type Culture Collection (ATCC) i.e. 25175D. This strain was added to 45 ml of Trypticase Soy Broth for overnight growth at 37°C. Bacterial concentration was maintained at $10^{(6)}$ CFU by adjusting optical density (turbidity) of broth solution to 0.1 nm using spectrometer.

Study groups comprised of three groups, Group I consisted of extracted powder of cocoa bean (CBH), Group II was consisted of extracted powder of cocoa bean and xylitol (CBH+X) and Group III was Chlorhexidine (CHX). To assess minimum inhibitory concentration 96 well micro-titer plate was used (which has twelve horizontal rows and eight vertical columns).(figure 3)



(Figure 3- 96 well transparent micropipette used to check minimum inhibition concentration)

For study purposes two columns were assigned to one group. Hence first two columns were assigned to group I, next two column were assigned to group II and next two column were assigned to group III. Last two columns were kept as negative control.

Active agents (group I, II and III) were added to 1st rows of respective columns. Then active agents were serially diluted using 8-channel pipette. After dilution 100 µl of bacterium was added to all the wells of 96 well micro-titer plates. Complete assembly was then kept overnight in an incubator at temperature of 37° C. After 24 hours of incubation, assembly was removed and plates were kept under ELISA reader for further analysis of optical density (turbidity) of agents.

IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY) was used to analyze the data. Analysis of variance (ANOVA) was used to check difference between three groups at significance of 5%. Post hoc tukey test was used to check significance within different groups.

Results

Minimum inhibitory concentrations (MICs) are the lowest concentration of an antimicrobial that will inhibit the visible

growth of a microorganism after overnight incubation. MICs are used as a research tool to determine the in vitro activity of new antimicrobials. The data obtained from such studies have been used to determine MIC breakpoints. In the present study Minimum Inhibitory concentration was assessed and it was found that there was no anti-microbial activity seen for group I (CBH) and group II (CBH+X) at concentrations of 0.3%. However, chlorhexidine (0.12%) showed its antibacterial activity in its extreme left well only (first concentration), which proves anti-bacterial activity of CHX. But it failed to show any antibacterial activity on further serial dilution.

Discussion

Present study was undertaken to assess an *in-vitro* antimicrobial activity of ground husk of cocoa bean with and without xylitol on streptococcus mutans. After obtaining ground husk of cocoa bean, extraction of active ingredient was done using standardized method as explained in study by Osawa et al (2001)[6,7]. No antimicrobial activity was seen with respect to cocoa bean and combination of cocoa bean and xylitol. Initial concentrations for these groups were 0.3%. Previous studies have shown 0.1% ground husk of cocoa bean was effective and even 0.1% of xylitol was effective as anti caries agent[11,12]. Hence concentration in this study was higher than study conducted by Osawa *et al* (2001)[7] .Chlorhexidine (0.12%) used as positive control (gold standard) showed antibacterial activity in first concentration only[13]. This minimized the error relating to the procedure regarding Minimum Inhibitory concentration.

In-vitro and in-vivo studies done previously have shown that cocoa bean husk possess two types of cariostatic substances, one being anti glucosyltransferase activity and the other antibacterial activity at concentration of 1 mg/ml that is 0.1% [6,7,8,9]. All these study designs were directed towards glucosyltransferases inhibition rather than antimicrobial activity. In the present study, though the concentration of cocoa bean husk was higher than previous studies [6,7,8,9] (0.3 %) there was no antimicrobial activity found. The potential reason for this weak antibacterial activity could be loss of fatty acids (oleic and linoleic acids) during processing of ground husk of cocoa bean, as their amount in the final residual product was not assessed.

Our results were similar to Matsumoto M et al (2004)[8] who showed that Cocoa Bean Husk had significantly reduced plaque score but it failed to reduce total colony forming units of Streptococcus Mutans for study subjects. In study done by Ooshima et al in 2000 in which cacao mass extract was given to rats in both diet and drinking water resulted in reductions in the recovery of streptococcus mutans strain 6715 from the mandible, as well as in plaque index and caries scores, but the reductions were not statistically significant.[6]

Role of xylitol as an antimicrobial agent is controversial but it has been used as a cariostatic agent in many lozenges, chewing gums even in mouthwashes.[10] Here our intention was to check whether xylitol and Cocoa Bean husk could have any synergetic antibacterial effect. In order to check whether addition of xylitol can improve taste, further safety and *in-vivo* studies will be needed to be carried out.

The ground husks used in this study could be different from those used in previous studies. Though the procedure of extraction of coca bean were adopted from the study done by Osawa K et al (2001)[7] the equipment and ingredients used in this study were different. The percentage of polyphenol compounds (catechin and epicatechin) and free fatty acids (oleic, linoleic, palmitic) left after the extraction of cocoa bean husks were not estimated. Difference in the equipment used could have stripped the Cocoa bean husk of these valuable compounds which are responsible for the antimicrobial activity against streptococcus mutans. Also, the present study was carried out on pure bacterium strain of streptococcus mutans - 25175D that was obtained from laboratory which is in contrast to the previous studies where the saliva/plaque samples from oral cavity were collected and then analyzed. It is necessary to evaluate antiglucosyltransferase activity in real life situations. Pragmatic trials are required to be carried out with increasing concentrations of cocoa bean husk for better assessment of its antimicrobial effect on streptococcus mutans.

Conclusion

We conclude that, ground husk of cocoa bean at the concentration of 0.3% does not possess antimicrobial property in *in-vitro* trial. Addition of xylitol had no effect on increasing antibacterial property. Further in vivo studies with higher concentrations of cocoa bean husk are required to assess the inhibitory ability of cocoa pod husk extract on glucosyltransferase enzyme. Addition of xylitol for taste enhancement need to undergo safety trials.

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Conflict of interest : Nil

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