Antimicrobial effect of different chocolates on S mutans

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ABSTRACT

Background: The aim of this study is to evaluate the inhibition of S mutans growth by plain chocolate, milk chocolate, dark chocolate, and filled chocolate. Materials and Methods: The in vitro study was conducted in microbiology laboratory, Commercially available chocolates in Indian market were used based upon the difference their ingredients. Dark chocolate (bournville), Milk chocolate (milkybar), Plain chocolate (dairy milk), and Filled chocolate (5 star) with zone of inhibition as a parameter were used. Bacterial strains were freeze dried for microbes and were kept in 4°C until used. Standard nutrient agar gel solution was used as the environment for growth of S mutans and preparation of solid media was done. S mutans bacterial cultures were performed in BHI broth medium and incubated at 35°C anaerobically for 48 – 72 hours. S mutans cultures were prepared and disk diffusion method was used. Discs were incubated at 35 °C for 24 hours. Zone of inhibition was measured. Results: Dark chocolate and milk chocolate showed greater inhibition than plain chocolate, and filled chocolate. Filled chocolate showed minimum zone of inhibition, whereas dark chocolate showed maximum zone of inhibition. Statistically significant differences were found between all these groups using Tukey’s post hoc test. (p < 0.05). Conclusion: Among the agents used in present study, dark chocolate showed the most antimicrobial activity against S mutans, and minimal antimicrobial activity was shown by filled chocolate with high sugar concentration.

KEYWORDS: Inhibition, S mutans, Chocolate, Antimicrobial.

INTRODUCTION

The idea that chocolate or cocoa may have some health benefits is not necessarily a novel concept. When Cortez first visited Central America, his first observation was the routine use of chocolate, particularly by the high priest. Most intriguing was reference to chocolate primarily as medicine.

Historical documents in Europe refer to chocolate’s medicinal value. By the 1600 s and 1700 s, chocolate and cocoa were viewed not just as a beverages with pleasurable taste, but primarily as a food to treat number of disorders, including angina and heart pain. The concept that cocoa beverages may provide some health benefits was widely accepted up until about the 1850 s and into the early 1900 s. Only the past 30 – 40 years have perceptions of chocolate changed from its being as medicinal food to the confectionery with no health benefits or possible negative effects on one s health. [1]. In order to decrease the prevalence of caries, an improved understanding of the role of the microorganisms in dental diseases is needed. [1].
The tooth surface is covered with a bio film – a slime layer consisting of millions of bacterial cells, salivary polymers, and food debris. Uncontrolled, this bio film can easily reach a thickness of hundreds of cells on the surfaces of the teeth. The formed bio film, also called plaque, provides an excellent adhesion site for the colonization and growth of many bacterial species.[2].

S. mutans, is the most commonly implicated initiator and plaque resistant bacterium, that begins demineralisation and metabolism of simple carbohydrates, this produces acid as a by-product, which leads to tissue loss and further bacterial penetration.[3,4]. All products, both food and beverages, which contain fermentable carbohydrates (sugar and some starches) have the potential to cause tooth decay.[5].

Chocolate – which contains carbohydrates are no more or less responsible for tooth decay than any other carbohydrate containing food such as bread, raisins, crackers and fruits. However, chocolate in the form in which it is usually consumed, milk chocolates or candy bars, contains large amount of sugar which is a known cause for tooth decay and may well counteract the effects of antibacterial agents. [6].

S. mutans was first described by J K Clarke in 1924, after he isolated it from the carious lesion but it was not until 1960s when researchers began studying dental caries in earnest, that real interest in this microbe was generated[7]. S mutans being the sole target species in caries development, more efforts have been directed towards inhibiting the known mechanism of caries development such as use of antibacterial agents like fluoride treatment, polyphenols from plant stimulant beverages like cocoa, coffee and tea to reduce bio film formation and acid production by S mutants and s sanguinis.

Sugar substitutes (e.g.- sorbitol and xylitol) that are not fermented by oral bacteria and do not cause a fall in plaque ph. Researchers have identified several hundred genes that appear to be unique to this organism. These are potential drug targets because disrupting them would disable the pathogen without harming other bacteria in the mouth.[7].

Different cocoa proportions in chocolates might influence the cariogenic response to different types of chocolates. In this study, the potential of different types of chocolates to inhibit the growth of S. mutans is evaluated, which dominant factor is causing dental plaque.

MATERIALS AND METHODS

The in vitro study was conducted in the Microbiology Laboratory of Government Institute of Animal Husbandry, Chetek Circle, Udaipur. Ethical clearance was obtained from the ethical committee of Darshan Dental College and Hospital Loyara, Udaipur, Rajasthan, India.

Prior to the study concerned authorities were approached, the nature of the study explained and permission sought. The written informed consent was obtained from the head of the institutions. In vitro study four chocolates were used with zone of inhibition as a parameter. Study was done and compared by repeating the procedure on each chocolate for five times. Commercially available chocolates in Indian market were used based upon the difference their ingredients.

Dark chocolate - Bournville

Milk chocolate – Milky bar
Plain chocolate – Dairy milk
Filled chocolate – 5 star.

1. Bacterial strains and culture conditions: Seed culture of streptococcus mutants MTCC 497 were ordered from Microbial Type Culture Collection and Gene Bank (MTCC), sec - 39 - A Chandigarh. Ampoules were freeze dried for the microbes and were kept at 4 °C in refrigerator until used.[3]

2. Preparation Of Media: Laminar flow cabinets have been used extensively in the laboratory to provide an environment free from microorganisms. [9]. The preparation of the solid media was done by adding difco standard nutrient agar (5.0 gm peptone, 3.0 gm beef extract, 15.0 gm agar powder) to distilled water. [10]. The solution was then transferred to a hot plate, stirred, and allowed to heat for approximately thirty minutes. This allowed sufficient time for the solution to become a homogenous and uniform liquid. The media was distributed, pouring the contents of each test tube into corresponding Petri dishes. This was repeated for each test tube.[10].

3. Bacterial inooculum culture: Streptococcus mutans bacterial cultures were performed in BHI broth medium and streptococcus selective broth medium respectively and incubated at 35 °C anaerobically for 48 – 72 hours.[3].

4. Preparation of the S. mutants culture: 30 µL of the S. mutants’ bacteria strain, grown at 35º C for 48 – 72 hours were spreaded on the dish with a cotton swab in a spreading fashion ensuring that the dish is covered fully. [10].

5. Disk preparation: Whatman no. 1 filter paper was used to cut the 6 mm diameter disc and were impregnated from each dilution; and kept over the S mutants (MTCC 497) seeded nutrient agar plates from standardized bacterial suspension.[3].

6. Antimicrobial effect of S. Mutants: The antimicrobial effects of the groups were evaluated by Disk Diffusion method. Four Sterilized, autoclaved discs prepared by hole punching individual filter paper discs were applied on plate containing the agar growth medium. [10]. Chocolates used were heated and melted in a sterile test tubes.

This method was done where blank discs were immersed in each chocolate and weight of each disk was calculated, which was placed on nutrient agar medium seeded with S mutants’ strains inoculums culture and incubated at 35º C for 24 hours.[3]. (Figure 1). After 24 hours of incubation, the petridishes were observed for zones of inhibition i.e areas without growth of S mutants. The zone of inhibition was measured as a maximum width from the edge of well to the periphery of the inhibition zone with the help of vernier callipers.

Statistical analysis: The antimicrobial activity indicated by an inhibition zone surrounding the discs containing chocolates were recorded if the zone of inhibition was greater than 6 mm ( disc diameter ). The experiments were performed in duplicate and the mean values of the diameter of inhibition zones with standard deviation were calculated.[11].

Data obtained were tabulated and subjected to descriptive statistical analysis. Analysis of variance (ANOVA) was used to find the significance of study parameters between...
three or more group of samples. Statistical package for social sciences (SPSS version 17) was used for analysis.

P < 0.05 was taken as statistically significant.

Figure: 1 Chocolate immersed discs placed on medium

RESULTS

The overall comparison of antimicrobial effect of chocolates on the growth of streptococcus mutans is shown in Table 1. The mean and standard deviation of the zones of inhibition in mm produced by tested chocolates after 24 hours of incubation with streptococcus mutans under anaerobic conditions. When comparing overall comparison of the four chocolates, statistically significant differences were found for the chocolates on the growth of streptococcus mutans (P < 0.001). The zones of inhibition with the four types of chocolates were compared in the following order. Dark chocolate (bournville) and milk chocolate (milky bar) showed greater inhibition than plain chocolate (dairy milk) and filled chocolate (5 star). The difference was moderately significant.

Table 1 : The overall comparison of antimicrobial effect of chocolates on the growth of streptococcus mutans

<table>
<thead>
<tr>
<th>Chocolate groups</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Dark chocolate (bournville)</td>
<td>11.82 ± 0.40</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Milk chocolate (milkybar)</td>
<td>9.02 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Plain chocolate (dairy milk)</td>
<td>5.94 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Filled chocolate (5 star)</td>
<td>5.18 ± 0.17</td>
<td></td>
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</table>

The intergroup comparison of antimicrobial effect of Milk chocolate (milkybar), Dark chocolate (bournville) is showed in Table 2, with significantly greater inhibition of S mutans. Filled chocolate (5 star) considered cause of dental caries, with minimum zone of inhibition of S mutans (5.18 ± 0.17), followed by plain chocolate (dairy milk) (5.94 ± 0.15), and milk chocolate (milky bar) (9.02 ± 0.17). The least cause of dental caries was seen for the dark chocolate, with maximum zone of inhibition of S mutans (2.63 ± 1.35). Statistically significant differences were found between all these groups using Tukey’s Post-hoc test. (Figure 2). The results indicated that there was difference in the antimicrobial efficacy of different chocolates against S mutans and the difference was statistically significant (p <0.05).

Table 2 : Intergroup comparison of antimicrobial effect of chocolates, by inhibition of streptococcus mutans

<table>
<thead>
<tr>
<th>Intergroup Chocolate comparison</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Dark chocolate (bournville)</td>
<td></td>
</tr>
<tr>
<td>Milk chocolate (milkybar)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Plain chocolate (dairy milk)</td>
<td>0.000*</td>
</tr>
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<td>Plain chocolate</td>
<td></td>
</tr>
<tr>
<td>Filled chocolate (5 star)</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>

Tukey’s post – hoc test P < 0.005 – significant. P < 0.001 - highly significant.
Figure: 2 Zone of inhibition after incubation of 24 hours

**DISCUSSION**

S mutans gives its name to a group of seven closely related species collectively referred to as the mutans streptococci. The primary habitats for S mutans are mouth pharynx and intestine.[12] Several factors, such as adherence to enamel surface, production of acidic metabolites, the capacity to build up glycogen reserves, and the ability to synthesize extracellular polysaccharides are present in dental caries.[12, 13]. S mutans and S sobrinus, have a central role in the etiology of dental caries.[14]. As these can adhere to enamel salivary pellicle and to other plaque bacteria. S mutans and lactobacilli are strong acid producers and hence cause an acidic environment creating the risk for cavities. Usually, the appearance of S mutans in the tooth cavities is followed by caries after 6 – 24 months.[15].

Studies according to Kang et al [16]. showed that three strains of L reuteri demonstrated a centrifuged supernatant inhibitory effect on periodontopathic and cariogenic bacteria, all three inhibited the growth of periodontopathic bacteria and s mutans more than 90 %. Most of the research has shown that this bacterium has a marked decreasing effect on the colonization of S. Mutans.[17].

The results of the antimicrobial effects of chocolates, containing probiotics, such as L plantarum PTCC 1058 and L acidophilus PTCC 1643 and L rhamnosus PTCC 1637 showed that all three types of probiotic chocolates had a remarkable antimicrobial effect on S mutans.[13]. Similar findings was observed in our study, three chocolates containing cocoa mass have antimicrobial activity in descending order against s mutans and the activity was greater at higher concentrations.

According to Wyn W et al,[20] and Ooshima T et al,[19]. extracts from coca mass have shown no effects on the growth of oral streptococci, or on the reduction of caries incidence or plaque accumulation. Whereas according to Ooshima T et al,[20]. and Osawa K et al,[21]. extracts from coca bean husks have been reported to possess powerful anticariogenic potential. In the present study, cocoa mass have shown effects on the growth of S mutans, simultaneously on the reduction of caries and plaque accumulation.

According to J Ghabanchi et al [22] the average inhibition zone of undiluted honey (100% v/v) on the 60 S. mutans isolates was 13 mm (lowest 10 mm, highest 15 mm). No inhibition zone was observed for undiluted carbohydrate solution in any isolates (p < 0.001). similarly in the present study, average inhibition zone of dark chocolate on pure culture of S mutans was lowest 11.4 mm and highest 12.4 mm. Very less inhibition zone was found with plain chocolate ( lowest – 5.7 mm and highest – 6.3 mm) and filled chocolate ( lowest 5 mm and highest - 5.9 mm).

Cocoa is believed to protect against various medical conditions, such as heart disease and cancer, as it is rich source of catechins which are polyphenols of the flavanol group. This flavanoid structure has antibacterial activity. Hence highest concentration of cocoa present in dark chocolate shows the highest antimicrobial activity against S mutans. Several researchers have demonstrated a synergy between active flavanoid and chemotherepeutics.[23]. In disc diffusion method the diameter of an inhibition halo of bacterial growth is considered to be directly proportional to the antimicrobial activity of the test solution. The diameter of the halo; however, can be influenced by the thickness and composition of the culture, by the concentration of the antimicrobial agent in the paper disc and by the degree of diffusion of the tested substances, which can be affected by the composition of the mouthwashes and dentifrices.[24].

The bacteriological methodology may also differ and this might also influence the results. Most of the studies[25,26]. had only assessed anaerobic bacteria; however, the addition of aerobic bacteria can provide valuable information to the results, as shown in the present study and other study conducted by Moran et al. [27]. The time of incubation also shows some variability. One study conducted by Addy and Harper [28]. employed an anaerobic incubation period of 12 h, while other studies[27]. extended that period up to 72 h. For aerobic incubation, 24 h were used in the present study; this was same as the study conducted by Moran et al. [29].

It is important to bear in mind that an experiment conducted in vitro has limitations, as it is considered a static system compared to in vivo tests, which may reflect the influence of various dynamic factors like systemic conditions, salivary flow, diet, and dental anatomy [30]. Nevertheless, it might
be considered that if the antimicrobial agent does not have activity in vitro it most likely will not work in vivo. In this sense, assessments of antimicrobial activity conducted using monocultures in vitro enable a direct contact between the bacterial colonies and the chemical substances tested for a period of 24-48 h. Whereas in experiments conducted in vivo there is a greater number of microbial species, including indigenous and even “protective” bacterial species which colonize dental biofilm, thus reducing the accuracy of the antimicrobial testing. One way of circumventing the limitations of in vitro studies evaluating the antimicrobial activity of mouthwashes against microorganisms is to make reference to clinical trials in vivo assessing their efficacy[31].

CONCLUSION

Among the agents used in present study, dark chocolate showed the most anti microbial activity against S mutans, and minimal antimicrobial activity was shown by filled chocolate with high sugar concentration. Hence within the limitations of this in vitro study, it can be concluded that the rich coca mass containing dark chocolate have greater antimicrobial activity against S mutans.

REFERENCES

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