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Comparative Assessment of The Antibacterial Effect of Two Natural Mouth Rinses (Punica Granatum, Phyllanthus Emblica) And Chlorhexidine Gluconate Against Streptococcus Mutans In School Children – An In vivo Study

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Abstract

Background: Children are at high risk for developing dental caries, mostly due to poor oral hygiene. Mouthrinse can be a better adjunct for improving their oral hygiene and reducing the load of cariogenic bacteria. Thus, mouthrinse using natural ingredients such as Pomegranate peel and Amla can be an alternative in children with better tolerance, acceptability.

Aim: The aim of the study is to compare and evaluate the antimicrobial effect of Punica granatum (Pomegranate), Phyllanthus emblica (Amla) mouthwash with 0.12% chlorhexidine gluconate mouthwash against Streptococcus mutans.

Materials and Methods: Sixty-three Children aged 6-12 years who fulfilled the inclusion and exclusion criteria were included in this study. Children were divided randomly into three groups and were advised to rinse the mouth for 1 minute using 10ml of the respective mouth rinse daily for 15 days. Non-stimulated whole salivary sample (2ml) was collected at baseline, on 16th day and 15 days after cessation of the mouthwash. Microbial evaluation was done to assess S. mutans count at different time intervals. The statistical analysis of data was done using SPSS 23.0 software.

Results: The study showed significant reduction in Streptococcus mutans count in all the three groups after using the mouthrinse for 15 days (p= 0.031). Pomegranate peel and amla mouthrinse showed comparable antimicrobial property which was significantly higher (0.760) when compared to chlorhexidine. Following the discontinuation of the mouthrinse there was an increase in S. mutans count in all the three groups, the increase in S. mutans count in both the herbal mouthrinse was not statistically significant, However there was a significant increase in S. mutans count in Chlorhexidine mouthrinse group (p=0.006).

Conclusion: The antibacterial efficacy of both herbal mouthrinses seemed to be promising when compared to chlorhexidine. Results of this study show that both the herbal mouthrinses can be used as potential anticariogenic mouthwash with acceptable taste in children.

Keywords: Streptococcus Mutans, Chlorhexidine, Pomegranate Peel Extract, Punica Granatum, Amla, Emblica officinalis, Phyllanthus Emblica, Dental Caries, Mouthwash.

Introduction

Oral health is a cornerstone of overall wellness. Maintaining optimal oral health not only promotes a radiant smile but also contributes to systemic health and quality of life. Good oral health in childhood is the precursor to good oral health at later stages of life. According to studies, dental caries is one of the most common chronic diseases of childhood.(1) Health care agencies such as World Health Organization (WHO) have identified this disease as a major public health problem.

Dental caries has a multifactorial etiopathogenesis that begins with an imbalance in microbial biofilm that covers the surface of the tooth(2,3). Various species reside in a perfect harmony in the biofilm through a complex interaction of synergism and antagonism. This microbial colony on the tooth surface can sustain mild disturbances in the micro- environment without much change in its composition and structure. However, if the disturbances are severe and persistent, it shall lead to shift in the equilibrium. The organisms that cannot survive in such adverse environment perish and those that can survive flourish. Thus the resulting change in the composition and structure makes the host plaque hostile(3,4)

Bacteria such as Streptococcus sanguis, Streptococcus mutans are primary colonizers. salivary proteins and carbohydrates act as a substrate for metabolism activity of these micro-organisms.(5) Thus formed lactic acid is added to the previously existing pool of acid, making the host surface hostile.(6) From previous studies it is understood that Streptococcus mutans stands to be the primary most etiologic factor as due to certain virulence factors such as its ability to survive in acidic conditions, acid production, adhere and colonize on solid surfaces in the mouth. Consequently, making it the primary

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cariogenic bacterium. Acids produced by these cariogenic bacteria leads to demineralization of the surface of the tooth, thus causing dental caries. All routine plaque control measures are actually targeting to counter this step.(7,8)

Mouth rinses are safe, effective and does not require special skill to use, it acts by inhibiting bacterial colonization, growth, and metabolism, and consequently interrupt the formation of mature biofilm, changing it at biochemical and ecological levels. Among mouth rinses commonly used, Chlorhexidine is the most popular. It has been recognized by the pharmaceutical industry as the positive control against which efficacy of alternative anti plaque agents is usually measured, and it has earned its eponym of gold standard(9–11). Chlorhexidine is effective in decreasing S. mutans count in dental plaque and saliva. Longitudinal studies have proved that there is direct relation between S. mutans level in plaque as well as saliva and incidence of caries. It inhibits caries by interfering eliminating with the metabolic activity of S. mutans by abolishing activity of phosphoryl pyruvate. From clinical practice and group studies it has been observed that Chlorhexidine causes staining of teeth, altered taste sensation and it cannot be tolerated for long term use. Thus, there is a paradigm shift in assessing efficiency of plant based antimicrobial agents and the use of complementary and alternative medicine in recent times. Thus search for newer herbal mouth rinse with antimicrobial property has led to conducting this study with the use of Pomegranate peel, Amla to determine and compare its antibacterial effect against Streptococcus mutans(8,11,12)

Pomegranate (Punica granatum) is a common fruit of a tree belonging to the family Punicaceae. Studies show that pomegranate has potent antioxidant activity due to its high polyphenols content, including ellagitannins and anthocyanins. There are a range of phytochemical compounds in pomegranate, ellagitannin and punicalagin was thought to be responsible for pomegranate antibacterial activity(13). Pomegranate extract suppresses the ability of these microorganisms to adhere to the surface of the tooth(14). It also lowered activities of alpha-glucosidase, an enzyme that breaks down sucrose, while it increased the activities of ceruloplasmin, which is an antioxidant enzyme. Therefore, pomegranate may exhibit anticariogenic effect as well, which may be utilized to prevent dental caries in individuals. The potential use of pomegranate as a convenient alternative to antimicrobial products, especially for children should be considered.(13,14)

Amla (Phyllanthus emblica) also known as Indian Gooseberry belongs to the family of Euphorbiaceae. The fruit is rich in minerals, Vitamin C as well as various amino acids. Different types of chemicals such as phenol, emblicol, curcuminoids, phyllembelin rutin, and tannins are obtained from this medicinal plant. It has antipyretic, analgesic, immunomodulatory, antitussive, and cytoprotective properties. P. emblica extract has shown to inhibit the growth as well as sucrose and glucan-induced adherence of Streptococcus mutans. This would prevent plaque accumulation and acid production, thereby preventing the development of dental caries(15). The effects of this fruits extract may be more beneficial if it is incorporated in gum, toothpaste, mouthwash and dental products to reduce plaque and dental caries(16).

The aim of the study is to compare and evaluate the antimicrobial effect of Punica Granatum (Pomegranate), Phyllanthus Emblica (Amla) mouthwash with 0.12% chlorhexidine gluconate mouthwash against Streptococcus mutans.

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Materials and Methods

The purpose of the study was to compare and evaluate the antibacterial effect of Punica Granatum (Pomegranate peel), Amla extract and 0.12% chlorhexidine mouthwash against Streptococcus mutans at baseline, 16th day of rinsing and 15 days after the cessation of mouthwash. An ethical approval was obtained from AJIDS ethics committee.

Sample size was estimated on the basis of the study conducted by Mishra et al in order to detect a difference of 1.37 (L) in mean Streptococcus mutans level between the groups assuming 95% confidence interval, 80% power with pooled standard deviation of 1.5 the sample size estimated for the study is 18.8, approximately equal to 19 in each group. Further assuming 10% attrition rate the final sample size for the study was 21 in each group. Sixty-three healthy children between age of 6-12 years from a government residential school were randomly divided (lottery method) into 3 groups based on the mouthwash prescribed i.e, Group A: Pomegranate peel (Punica granatum) mouth rinse, Group B: Amla (Phyllanthus emblica) mouth rinse and Group C: Chlorhexidine (CHX) mouth rinse.

The study subjects were selected based on inclusion and exclusion criteria. Children who are able to expectorate completely or brush on their own, with one or more active carious lesion or a frank cavitations were included. Whereas, Children who have used antibiotics or antiseptic mouth washes 3 months prior to and during the study period, allergic to the agents being used in the study, undergoing any kind of dental treatment or with an intraoral prosthesis, with presence of any intra oral pathology and medically compromised children were excluded from the study.

Brushing and mouth rinsing technique was demonstrated to the selected students and were advised to brush twice daily. Children were advised to rinse the mouth for one minute using 10 ml of the respective mouthrinse twice daily for 15 days and not to consume any food or drink 30 minutes after mouthwash. The herbal mouthrinse were prepared under professional guidance at Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences, Mangalore. For the preparation of the mouthrinse, aqueous extract was obtained using Soxhlet extractor. No preservatives and colouring agents were added to the mouthrinse.

During saliva sample collection, participants were asked to refrain from eating and drinking, one hour before saliva collection to obtain a relatively constant baseline. Participants were asked to sit in coachman's position i.e. at upright position and with their heads tilted slightly down to pool saliva in the mouth and were asked not to swallow or move their tongue/lips during the period of collection. The first expectoration was discarded to eliminate food debris and contaminating the sample that may cause analytical inaccuracy. Subsequent sample was expectorated into a pre-labelled sterile container and approximately 2ml saliva was collected. The samples were immediately transported for microbiological analysis at A.J institute of Medical Sciences. They were tested for colony forming units using Mutans sanguis agar medium. Saliva samples were collected on the 16th day after which the participants were asked to refrain from using the mouthwash. During the study, the participants of all the 3 groups were instructed to carry out the routine oral hygiene practices. Saliva samples were again collected 15 days after cessation of mouthwash.

Before inoculating the saliva, sample was homogenized by a vortex mixer for 2 minutes. It was diluted using tenfold dilution technique by withdrawing 0.01 ml of saliva and 0.09ml of saline using a micropipette into a

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microcentrifuge tube. This mixture was homogenised by a vortex mixer for 30 seconds. 0.001ml of saliva from 1:10 dilution was withdrawn using a calibrated sterile inoculating loop then streaked on the Petri dishes of Mutans-Sanguis agar under aseptic conditions. The whole inoculation procedure was done under aseptic conditions to avoid contamination. Inoculated plates were placed in incubation chamber and incubated aerobically at 37°C for 48 hours. Bacterial count was done using the conventional plate count method. The colony count was expressed as number of colony forming units per millilitre (CFU/ml) of saliva.

After completion of the study, the study subjects were given a dichotomous questionnaire regarding the acceptability of the mouth rinse dispensed to them. The questionnaire consisted of four questions with two options (YES/NO) to answer. The questions were based on the acceptability of the mouth rinses in terms of flavour, smell, irritation and willingness to continue the mouth rinse. A paper and pencil method were used to administer the questionnaire.

Result

The data were recoded and entered into Microsoft Excel spreadsheet. Statistical analysis of the data was done using SPSS 23.0. Based on normality non parametric test was used to analyse the data. Comparison of change in the Streptococcus mutans count within the group at different time points (Three time points) was analysed using Friedman's test followed Wilcoxin signed rank test for pair wise comparison. Comparison between the three groups was done using Kruskall Wallis test. Descriptive statistics was presented using median and inter quartile range. P value

Table 1and graph 1 depicts the median of S. mutans count at baseline, 16th day of follow up and on 31st day of follow up in all the three groups. There was statistically significant difference in S. mutans count at various time intervals.

Table 2 and graph 2 depicts inter- group comparison of S. mutans count at various time intervals. It was observed that from baseline to follow up 1(BF_1) and from baseline to follow up 2(BF_2) there was a significant difference in S. mutans count between group B- Group C and group A- group C, however there was no statistically significant difference observed between group A- group B. While, on intergroup comparison from follow up 1 to follow up 2(F_1F_2)there was no statistically significant difference in S. mutans count.

The results of acceptability questionnaire showed that the subjects in group B (100%) showed highest acceptance to flavour followed by group A (66.7%). The least acceptance to flavour was shown in group C (55%). There was a statistically significant difference. However, there was no statistically significant difference in the acceptance of smell of the mouthrinse. The subjects in the group B (85%) showed highest acceptance to smell of the mouthrinse followed by group A(72.2%). The least acceptance to flavour was shown in group C (65%). None of the subjects in group A and B had experienced any irritation, while75% the subjects in chlorhexidine group had experienced irritation which was a statistically significant. The subjects in group B showed highest willingness to continue the use of the mouthrinse (100%) followed by group A (61.1%). Least willingness was shown by subjects in group C (45%). This difference was statistically significant.

In the present study, we found all three mouthrinses to be highly effective antibacterial agents against Streptococcus mutans. Each mouthrinse demonstrated a statistically significant reduction in S. mutans colony counts after its use. However, the antibacterial activity of the Amla and pomegranate mouthrinses was comparable

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and significantly superior to that of the chlorhexidine mouthrinse. Two week after discontinuation of the mouthrinse, all the three groups showed an increase in S. mutans count. There was no statistically significant difference in S. mutans count in a comparison among all the groups, indicating that all the three mouthrinses had almost similar substantivity.

Discussion

Dental caries, also referred to as tooth decay, is among the most prevalent chronic diseases that impact people globally; It is the outcome of localized breakdown of vulnerable dental hard tissues by acidic byproducts resulting from bacterial fermentation of dietary carbohydrates.(17)Streptococcus mutans stands out to be the primary etiologic factor for the initiation of tooth decay. It is a gram positive, non-motile, non-spore forming, facultative anaerobic bacterium arranged in chains or pairs.(7,18) Literature suggests that there's a clear association between the amount of S. mutans present in saliva and the number of Streptococcus mutans in dental plaque. Also, there is a reported positive association between salivary S. mutans concentration and the number of colonized surfaces.(19) Therefore, this study's objective was to assess and compare the average reduction in Streptococcus mutans count (CFU/ml) following a 15-day mouthrinse with pomegranate peel extract, amla, and chlorhexidine mouthrinse among 6– 12year old children.

Researchers are currently searching for natural substitutes due to growing concern regarding the safety of synthetic and chemical compounds in pharmaceutical products. Therefore, all that is needed to get herbal medicine into the mainstream is high-quality randomised control trial. There is an excellent probability that the usage of traditional medicine will herald a new era of easily available and safe medications.(20)Therefore, the purpose of this study was to assess the anti- microbial property of two herbal mouthrinses prepared from Pomegranate peel and Amla against Streptococcus mutans count in saliva of children before and after rinsing for 15 days when compared to those children who used chlorhexidine mouthrinse.

Pomegranate, scientifically referred to as Punica granatum belongs to the Punicaceae family has been recently titled as “Super food” because of its excellent phytochemical properties. The pomegranate peel had the maximum total phenolic concentration when the total phenolic content of the peel, juice, and seed was compared. These polyphenolic compounds have shown to exhibit excellent anti- bacterial properties against a wide variety of gram positive and gram-negative micro-organisms. The tannin in pomegranate inhibits human salivary alpha-amylase from hydrolysing starch into oligosaccharides and attaching itself to enamel, thereby providing cariogenic bacteria a substrate. The extract from pomegranate peel combats dental plaque and prevents the development of calculus by inhibiting the microbes responsible for plaque formation. The extract from pomegranate peel inhibits microorganisms' ability to adhere to tooth surfaces.(19, 21, 22)

Amla (Indian gooseberry) scientifically referred to as Phyllanthus emblica belong to the family Phyllanthaceae. Important bioactive chemical components, including tannins, alkaloids, polyphenols, gallic acid, ellagic acid, emblicanin A and B, Phyllemblin, quercetin, ascorbic acids, vitamins, and minerals, are identified through phytochemical study. It has the potential to do possible changes in the composition and structure of the cell wall, thus E. officinalis is more efficient against Gram-positive bacteria than Gram-negative ones. The tannins found in the fruits of E. officinal is could possibly be the reason

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of its potential antibacterial activity. 28% of the plant's total tannin content is found in the fruits. Two hydrolysable tannins that is emblulanin A and B which have antioxidant qualities are also present in the fruit.(23,24) Consequently, this cariostatic property of Pomegranate peel and Amla can be incorporated for the preparation of a cariostatic herbal mouth rinse. As per our knowledge this is the first study done to assess the anti- bacterial effect of mouthrinse using aqueous extract Amla fruit in children.

In the preparation of the mouthrinse only distilled water was used to obtain the aqueous extract of Pomegranate peel and Amla mouthrinse. Because, according to literature search the use of alcohol in the preparation of aqueous extract can be irritating to the oral mucosa. In addition, it can also weaken the immune system natural ability to fight pathogens. In addition ethanol has antibacterial properties, the goal of this study was to ascertain the antimicrobial properties of pomegranate peel and amla in its purest form. Thus, in this study only aqueous extract was used.(25)

The population studied included school children who had established adequate mastery over their swallowing reflex and who were old enough to understand the procedure and co-operate with the procedure involved. All the participants who participated in this study resided in the same residential school, thus there was no bias due to different eating pattern. The age group 6-12 years was selected since caries prevalence is high in this age group. This criterion was similar to a study conducted by Ahmed et al.(26)At the onset of the study, 63 participants were selected to be part of the study (21 in each group) but the final assessment involved 58 participants as 5 students (3 in Pomegranate peel group, 1 in Amla group and 1 in chlorhexidine group) dropped out because they had taken a leave of absence from the school for personal reasons. The attrition had no influence on the result of the study as final sample size was kept 21 in each group keeping in mind 10% attrition rate during the commencement of the study.

In the present study, the salivary S. mutans count was investigated at baseline and 16th day after using Pomegranate peel, Amla and chlorhexidine mouthrinse. From baseline to the sixteenth day of follow-up, the usage of all three mouthrinses resulted in a highly statistically significant decrease in the Streptococcus mutans count. However, when comparison between groups was done, there was no statistically significant difference in Pomegranate peel and Amla mouthrinse group ($p > 0.05$). While there was a statistically significant difference between Amla and chlorhexidine mouthrinse group and pomegranate peel and chlorhexidine mouthrinse group from baseline to 16th day follow up ($p < 0.05$).

In this study Amla mouth rinse showed highly statistically significant decrease in the S. mutans count from the baseline to 16th day of rinsing i.e. from 45.00 (29.80-75.90) to 14.00 (7.50-19.05). According to a preliminary study done by Velmurugan et al., Amla mouthrinse using aqueous extracts were efficient at lowering S. mutans count, but with a shorter duration of action compared to chlorhexidine.(27) He also quoted that Amla leaves were used unlike the current study were Amla fruit was used. Literature suggests Amla fruit has higher therapeutic potential when compared to other parts of this plant because the fruit is rich in bioactive compounds such as tannins and flavonoids.(24) As a result fruit was chosen over leaves in the preparation of the mouthwash.

In the present study, pomegranate peel mouthrinse and Chlorhexidine showed statistically significant decrease in S. mutans count from baseline to 16th day of follow

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up i.e. 38.00 (28.35-66) to 15.50 (9-24.25). Which was in accordance with the study done by Mishra et al., where there was maximum decrease in salivary S. mutans count from baseline to 16th day after using pomegranate peel mouth rinse when compared to chlorhexidine.(28) In another study done by Umar et al., the results were similar to this study where there was highly significant decrease in S. mutans count after rinsing with pomegranate peel mouthrinse when compared to chlorhexidine.(14) In another study by Menezes et al., results showed that pomegranate peel mouth rinse was highly efficacious in decreasing the S. mutans count when compared to chlorhexidine.(29) In contrast to this study, a study done by Pinni et al., there was no statistically significant difference in S. mutans count between pomegranate peel extract and chlorhexidine mouth rinse group(30) and in another study done by Yousry et al., chlorhexidine showed marked decrease in S. mutans count when compared to 15% pomegranate peel and 10% pomegranate peel extract mouth rinse.(26)However, the differences in methods used for mouth rinse preparation can be the reason for different results compared with the current study.

A significant decrease in the CFU/ml of S. mutans was observed in both pomegranate peel and amla extract mouthrinse group after 15 days of using the mouthrinse. Furthermore, following the discontinuation of mouthrinse, a microbiological evaluation revealed the sustained antibacterial properties of all the three mouthrinses. Substantivity is a desired property that ideally all mouthrinses must possess. At the end of 15 days wash out phase there was no statistically significant difference in S. mutans count in a comparison among all the groups, indicating that all the three mouthrinses had almost similar substantivity.A questionnaire was given to the children at the end of the study to assess the taste and smell acceptability of all three mouthrinses used in this study. Both the herbal mouth rinse was comparatively better accepted by the participants of the study when compared to the chlorhexidine mouth rinse.

The findings of the current study suggests that herbal mouthrinses could be a promising alternative to chlorhexidine mouthrinse for children, offering a natural alternative with fewer side effects. However, the variability in the composition and concentration of active ingredients in herbal products necessitates standardized formulations and rigorous clinical trials to ensure consistent efficacy and safety. Further research is needed to fully understand the long-term effects and shelf- life of herbal mouthrinses. In conclusion, while herbal mouthrinses show significant potential as alternatives to chlorhexidine, further clinical validation and standardization are required before they can be universally recommended for pediatric oral care.

Conclusion

This research sheds light on the potential of herbal mouth rinse as an alternative to chlorhexidine mouth rinse for children, given its favourable safety profile, acceptance, and potential long-term oral health benefits. It emphasizes the comprehensive approach to promoting oral health offered by herbal mouthrinses, which resonates with the preferences of numerous parents and caregivers who are interested in natural and environmentally sustainable oral care choices for their children.

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Legend Tables and Figures

Table 1: Median of Streptococcus mutans count in groups A, B and C at baseline, 16th day and 31st day

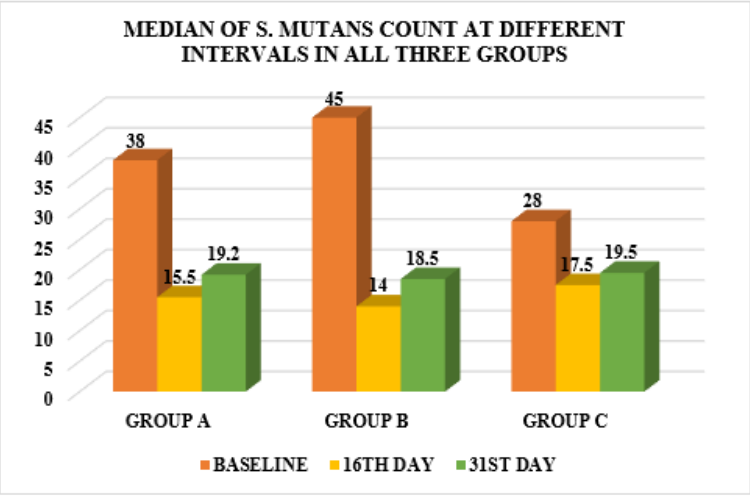
Group		Median	IQR	f (chi)	p- value
A	Baseline	38.00	(28.35-66)	29.778	p<0.001
	16th day	15.50	(9-24.25)		
	31st day	19.20	(9.45-26.55)		
B	Baseline	45.00	(29.80-75.90)	31.62	p<0.001
	16th day	14.00	(7.50-19.05)		
	31st day	18.50	(9.35-26.80)		
C	Baseline	28.00	(17.85-42.00)	24.1	p<0.001
	16th day	17.50	(5.65-23.00)		
	31st day	19.50	(6.75-34.20)		

f= Friedman test values

Table 2: Inter- group comparison of S. mutans count at various time intervals

	Group A V/S GROUP B		GROUP B V/S GROUP C		GROUP A V/S GROUP C		p- value
Baseline to 16th day follow up BF_1	22.5	31	31	10.5	22.5	10.5	0.031
Baseline to 31st day BF_2	18.8	26.5	26.5	8.5	18.8	8.5	0.018
16th day to 31st day F_1F_2	-3.7	-4.5	-4.5	-2	-3.7	-2	0.70

Graph 1: Graphical presentation of median of S. mutans count at different time intervals in all the three groups



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Graph 2: Graphical presentation of difference in S. mutans count in all the groups at various time intervals

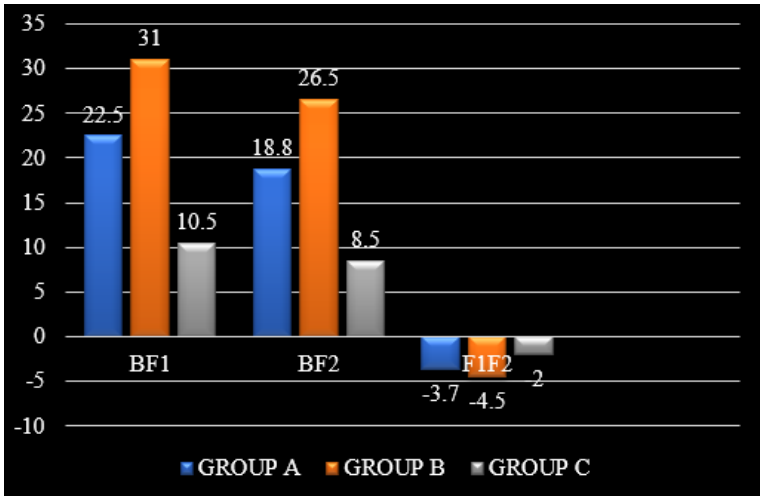

Figure 1: Mouthrinses dispersed to children in thestudy

Figure 2: Demonstrating mouth rinsing techniqueFigure 3: Saliva sample collection

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Figure 3: Collected saliva samples

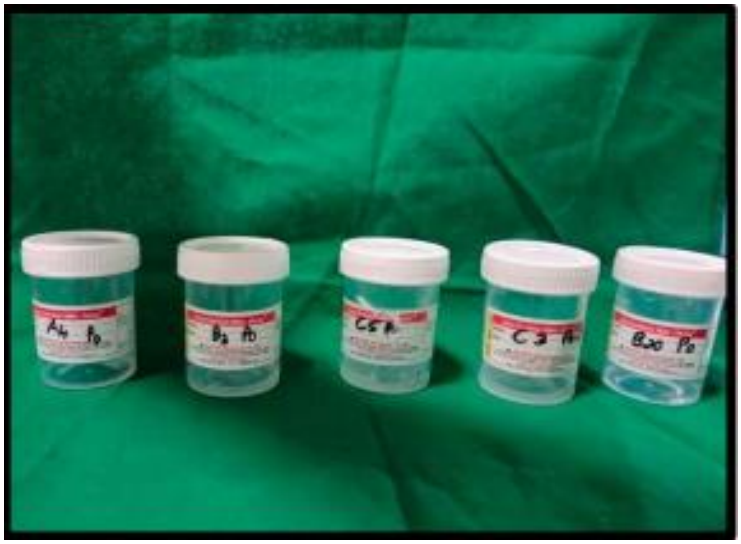

Figure 4: Streaking on agar plates using inoculating loop

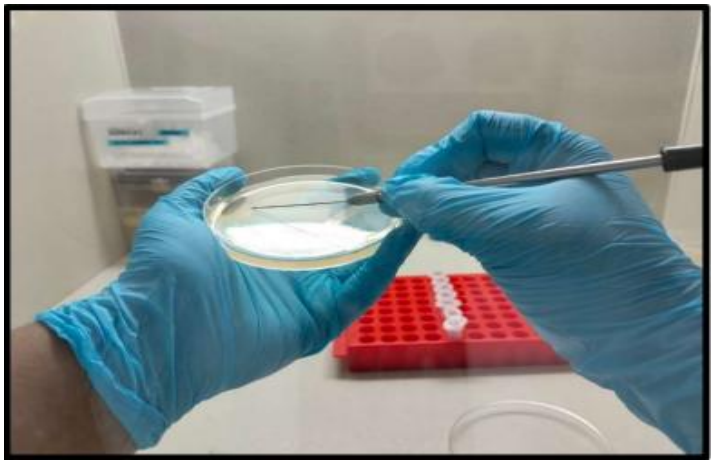

Figure 5: Culture plates showing salivary S. mutans colony on agar plates containing mutans sanguis agar medium

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