Comparative Evaluation of the Antimicrobial Effects of Different Mouthrinses against Streptococcus Mutans: An *in Vitro* Study

Eman Abu-obaid*/ Fouad Salama **/ Ala'a Abu-obaid ***/ Fars Alanazi ****/ Mounir Salem*****/ Sayed Auda ******

Purpose: *To assess the antimicrobial effects of different natural and semi-natural mouthrinses on isolates of S. mutans obtained from the saliva of Saudi children and reference strains of S mutans (ATCC 25175).* ***Study design:*** *Saliva samples were collected from 20 children. Natural and semi-natural mouthrinses included were herbal mix mouthrinse, cranberry mouthrinse, chlorhexidine digluconate mouthrinse, cranberry extract mixed with chlorhexidine digluconate mouthrinse, chlorhexidine digluconate mouthrinse with alcohol (positive control), and distilled water (negative control). The microbiological examination tests were minimal inhibitory concentration, minimal bactericidal concentration, and zone of inhibition for the saliva isolates of S. mutans while zone of inhibition test only for reference strain of S. mutans.* ***Results:*** *For reference strain in a comparison with the distilled water, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol showed significantly increased zones of inhibition by 36.38, 36.25, 26.13, 17.75, and 12.38, respectively. For saliva isolates in a comparison with the distilled water, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol showed significantly increased zones of inhibition by 38.00, 34.25, 22.94, 16.50, and 16.44, respectively. Chlorhexidine with alcohol showed significantly lower minimum inhibitory and bactericidal concentration than the other groups.* ***Conclusions:*** *Herbal mix and cranberry mouthrinses could be effective natural alternative to chlorhexidine mouthrinse with or without alcohol in affecting tested parameters.*

Keywords: *Chlorhexidine, Cranberry, Herbal, Mouthrinses, Streptococcus Mutans,*

INTRODUCTION

Caries is one of the most predominant and preventable oral infectious diseases that affect the majority of the population in the world.[1] It is a multifactorial disease that is characterized by demineralization of the dental hard tissues.[2] Dental plaque has a major part as a reason for dental caries[3] and *S. mutans* is a Gram-positive, facultative, anaerobic round shaped bacterium, which considered a crucial contributor to the development of cariogenic plaque.[4] Practicing oral hygiene by teeth brushing as a mechanical plaque control measure is desirable and commonly used.[5] Besides that, many anti-plaque agents have been in use as supplementary aids.[5] It is believed that using mouthrinses could act as an efficient and harmless means for delivery of antimicrobial agents that prevent bacterial adhesion and disturb the growth of the bacteria.[5]

Chlorhexidine is the gold standard mouthrinse due to its antimicrobial action.[6,7] On the other hand, despite its side effects such as teeth and soft tissues discoloration, taste alteration, and supragingival calculus formation its continued use is supported.[7-9] Over the past several years, an interest in the naturally derived biologically

From King Saud University.
*Eman Abu-obaid, Postgraduate Student, Department of Pediatric Dentistry and Orthodontics, College of Dentistry.
**Fouad Salama, Department of Pediatric Dentistry and Orthodontics, College of Dentistry.
***Ala'a Abu-obaid, Department of Prosthodontics, College of Dentistry.
****Fars Alanazi, Kayyali Chair for Pharmaceutical Industry, Department of Pharmaceutics, College of Pharmacy.
*****Mounir Salem, Department of Pharmaceutics, College of Pharmacy.
******Sayed Auda, Department of Pharmaceutics, College of Pharmacy.

Send all correspondence to :
Fouad Salama
Department of Pediatric Dentistry and Orthodontic
College of Dentistry, King Saud University
PO Box 60169 Riyadh 11545; Saudi Arabia
Phone: +966-538640535
E-mail: fspdkids@gmail.com

active compounds have been increased.[10,11] In this respect, cranberries and their extracts have received great emphasis.[12] Cranberry (Vaccinium macrocarpon) is a small to medium sized woody plant that grows in the cold areas of northeastern North America.[13] Their extracts are rich in polyphenols comprising flavonoids, which have biological properties that can be useful to human well-being.[14,15] Concerning dental caries, it was concluded that the high-molecular-weight polyphenols extracted from cranberries could halt dental caries by preventing biofilms formation and obstruct the cariogenic bacteria from production of organic acids.[12] Among the herbal alternatives, pomegranate finds particular attention.[16, 17] Pomegranate (*Punica granatum*), is an old fruit that has been widely used for therapeutic purposes.[16] In the field of oral health, pomegranate has an important and vital application.[16] A study found that pomegranate peel extract (PPE) mouthrinse has notable antimicrobial activity against *S. mutans*, and can be used to prevent dental caries.[17] In the North Africa and Middle East, myrrh have been traded for 5,000 years.[18] In the field of oral health, myrrh mouthrinse was studied in order to explore its antimicrobial activity against microbial flora in comparison with the chlorhexidine. [18] Results have shown that myrrh delivered antimicrobial activity that is nearly equivalent to chlorhexidine rinse, and can be used as a valuable homebased mouthrinse instead of chlorhexidine.[18] Moreover; Chamomile is a substance of scientific interest. German chamomile (Matricaria chamomilla) in specific is widely used for therapeutic purposes.[19] In regard to oral health, a study conducted reported that using of German Chamomile (GC) mouthrinse has benefit in plaque and gingival inflammation reduction.[20] Cinnamon (Cinnamomum zeylanicum) is a powerful herb that has been used widely for treatment of many disorders.[21] Moreover, cinnamon have antibacterial properties and could be a useful compound for development of antibacterial agents. [22] Concerning oral health, another study was conducted to evaluate the influence of specific concentrations of cinnamon extracts on *S. mutans.* The study revealed that Cinnamon extract had a high significant antibacterial activity against *S. mutans,* increasing the salivary pH and flow rate.[23]

In conclusion, naturally derivative compounds appear to have potential for preventing and/or treating dental caries. Several studies have investigated the antimicrobial activity of natural products against oral microorganisms. However, to the best of our knowledge there are no investigations concerning the antimicrobial action of the combination of naturally derivative compounds such as (Herbal mix mouthrinse) and semi-natural compounds such as (Cranberry extract mixed with chlorhexidine digluconate mouthrinse) against cariogenic bacteria especially *S. mutans*. Therefore, the aim of this *in vitro* comparative investigation was to evaluate the antimicrobial effects of different natural and semi-natural mouthrinses on isolates of *S. mutans* that were collected from the saliva of Saudi children and reference strains of *S. mutans* (ATCC 25175). The null hypothesis there is no significant difference in the antimicrobial effects between tested and control mouthrinses against *S. mutans* that were isolated from the saliva of the 7-10 year-old Saudi children.

MATERIALS AND METHOD

The ethical approval for this *in vitro* study and informed consent were obtained from the Institutional Review Board at king Saud University, Committee for Medical Research, with the registration number (E-16-2141). A pilot study was carried out before the start of the main study to test the mouthrinses and be familiar with all procedures, outcome measures and statistical analysis. Training and calibration exercises were undertaken during the pilot study. Intra-examiner reproducibility of the investigator who performed the clinical examination for children was performed during the pilot study. Species of *S. mutans* isolated from saliva samples of Saudi children and reference strains of *S. mutans* (ATCC 25175) were included in this study for being among the most prevalent oral microorganism.

Patient Selection

Twenty Children who participated in this study were selected randomly from any child who visited the Pediatric Dentistry Clinics of Dental Hospital, King Saud University to receive dental treatment. The children who met the inclusion criteria were enrolled in this study after obtaining consent from parents or legal guardian.

Inclusion Criteria

Healthy Saudi 7-10 year-old children with ASA I according to the American Society of Anesthesiologists Classification (Ref) who have positive or definitely positive behavior according to Frankl behavioral scale,[24] have at least four decayed, missing, and/or filled teeth due to caries (DMFT/dmft ≥4) with high decayed component,[25] adhering to at least once-daily tooth brushing using toothbrush and toothpaste without practicing other professional and/or home based oral hygiene measures, and have minimal dental plaque and gingivitis as evaluated using a modification of hygiene index (HI), that does not use a disclosing agent [26] and gingivitis using the simplified gingival index (GI-S).[26]

Exclusion Criteria

Children with medical history that could compromise the conduct of the study, with history of current or recent (at least for the past one month) antibiotic or mouthrinses usage, abscess, draining sinus, cellulitis, or other conditions requiring emergency dental treatment, history of less than one week after using professional fluoride application or similar preventive methods, and children with (DMFT/dmft ≥4) but had all carious teeth restored.[27-29]

Method of Saliva Collection

For 60 minutes before saliva collection, participating children did not eat anything or drink any liquids (including water). Besides that, participating children did not brush their teeth for at least 8 hours before saliva collection. During saliva collection visit, each child rinsed twice with 10 mL of water for 30 seconds. Then after 2 minutes, each child was asked to spit (unstimulated) saliva (2 mL) into sterile plastic disposable tube. Each tube was labeled with a unique identifier. Saliva samples were transferred within two hours to Microbiology Laboratory.

Study Design

Children who met the inclusion criteria were enrolled in this study and unstimulated saliva was collected in aseptic condition. As soon as the samples are received in the laboratory, (1 mL) of each saliva sample was inoculated on decimal solutions of peptone water (10^{-1}, 10^{-2}, and 10^{-3}), and cultured with a swab using the spread plate method and specific media Mutans-Sanguis (MS) Agar in anaerobic condition using AnaeroPack-Anaero at 37°C for 48 hour. The media were prepared in accordance with the manufacturer's instructions (HiMedia Laboratories Pvt Ltd., Mumbai, India). After the incubation period, the suspected colonies of *S. mutans* were further identified for confirmation using an automated system (Vitek®) of biochemical identification (BioMerieux SA, Marcy-toile, France). The isolates were maintained, and preserved in glycerol stock tubes at–80°C for further experiments. This laboratory study included a total of 96 samples divided into 24 samples of reference strains of *S. mutans* and 72 samples of *S. mutans* that were isolated from saliva samples of Saudi children that were randomly divided into 6 major groups according to the different tested mouthrinses and control groups planned to use. The different natural and semi-natural mouthrinses included in this study were herbal mix mouthrinse (1%), cranberry mouthrinse (0.6%), chlorhexidine digluconate mouthrinse (0.12%), cranberry extract (0.3%) mixed with chlorhexidine digluconate mouthrinse (0.06%), chlorhexidine digluconate mouthrinse (0.12%) with alcohol (positive control), and distilled water (negative control). The microbiological examination tests evaluated were minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and zone of inhibition for the saliva isolates of *S. mutans* while zone of inhibition test only for reference strain of *S. mutans* (ATCC 25175).

Sample Size Calculation

nQuery Sample Size Software (nQuery Advanced 8.2) was used to calculate sample size for reference strain and saliva isolates. We determined the minimum samples required for reference strain using the effect size of 0.9860, which produced 24 samples for zone of inhibition test, 4 samples per group with 80% power to detect the 5% significant level. We also determined the minimum samples required for saliva isolate using the effect size of 0.9958, which produced 24 samples for each microbiological examination tests: MIC, MBC, and zone of inhibition, 4 samples per group with 80% power to detect the 5% significant level.

Randomization

This laboratory study included 24 samples of reference strains of *S. mutans* (ATCC 25175) that were randomly divided into 6 major groups according to the different tested mouthrinses and control groups used to evaluate their zone of inhibition with each group consist of 4 samples. In addition, 72 samples of *S. mutans* that were isolated from saliva samples of Saudi children were randomly divided into 6 major groups according to the different tested mouthrinses and control groups used. Each group consist of 12 samples that were divided into 3 minor groups for the following microbiological examination tests (minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and zone of inhibition), 4 samples for each test.

Blindness

In this research, different individuals were assigned to help in masking and creating unbiased research environment. Examination of children, formulation of mouthrinses and conducting microbiological examination tests were carried by the principle investigator. However, every step in this experiment were masked from two external evaluators who measured the outcomes to prevent them from knowing the tested mouthrinses to keep blindness and eliminate bias, until after the results and outcome are known.

Compositions of Tested Mouthrinses and Control

Herbal mix mouthrinse (1 %) is made mainly from (0.36 % pomegranate peel extract, 0.36 % myrrh resin extract, 0.18 % chamomile flower extract, 0.1 % cinnamon bark extract, 0.1 g sodium benzoate, and 0.2 g ascorbic acid). The formulation of the herbal mix mouthrinse was a matter of trial and error until the principle investigator determined the above-mentioned concentrations of each extract need to be used.

Cranberry mouthrinse (0.6 %) is made mainly from cranberry extract that is dissolved in sterilized water with other ingredients (0.1 g ZnCl2, 0.1 g sodium saccharine, 0.05 g menthol, 0.1 g sodium benzoate and 3 ml of glycerine).

Chlorhexidine digluconate mouthrinse (0.12%) **(Kin ®, Barcelona, Spain)** was obtained from the market.

Cranberry extract 0.3% mixed with chlorhexidine digluconate mouthrinse 0.06%.

Chlorhexidine digluconate mouthrinse (0.12%) containing alcohol (11% ethanol)–Positive control, was prepared at college of pharmacy.

Distilled water mouthrinse–Negative control.

Methods of Extraction

Preparation of pomegranate peels powder: Pomegranates fruit having no visible external cuts or spoilage were obtained from local markets, fruits were washed with tap water, cut, and the arils and seeds were manually removed from the peels. The pomegranate peels were cut into small pieces, dried at 50°C in a convection oven for 3 days. The dried peels were ground with pestle and mortar to coarse powder of approximately 1 mm size. The powders obtained were weighed and the values were recorded.

Preparation of myrrh powder: Myrrh resin (*Commiphora Molmol)* were obtained from local markets, washed with tap water, and ground with pestle and mortar to yield a coarse powder of particle size of 1mm. The powders obtained were weighed and the values were recorded.

Preparation of chamomile flower powder: Dry chamomile flowers were obtained from local markets and crushed with pestle and mortar to yield a coarse powder of particle size of 1mm. The powders obtained were weighed and the values were recorded.

Preparation of cinnamon bark powder: Cinnamon bark were obtained from local markets, and were ground with pestle and mortar to coarse powder of approximately 1 mm size. The powders obtained were weighed and the values were recorded.

Preparation of cranberry powder: Fresh cranberry was obtained from local market. The cranberry fresh fruit were rinsed under ruing tap water and cut in half by knife to remove seeds by spoon, and dried in the oven at 40°C. The dried fruits were

ground with pestle and mortar to coarse powder of approximately 1 mm size. The powders obtained were weighed and the values were recorded.

Preparation of cranberry extract: With the objective of extracting the compounds responsible for the antimicrobial activity from cranberry , 10 g of dried powder was extracted with 300 ml of sterilized water by soaking and stirring for 24 h. After that, the extracts were filtered through Whatman filter paper No. 41 (GE Healthcate, Little Chalfont, UK). The extracts were evaporated to dryness at 40°C in a convection oven for 12 hours in the dark obtaining a red–brown residue (about 2 g) that were stored in a dark sterile container at 4°C.

Preparation of the herbal extracts: 10g of prepared coarse powders were extracted with 300 ml of sterilized water by soaking and stirring for 24 h. After that, the extracts were filtered through Whatman filter paper No. 41 (GE Healthcate, Little Chalfont, UK). The extracts were evaporated to dryness at 40°C in a convection oven for 12 hours in the dark obtaining a yellow–brown residue that were stored in a dark sterile container at 4°C. The yield amount of extracts were different according to the type of herb. The used powder was 10g for the Pomegranate peel, Myrrh, Chamomile flower, Cinnamon bark, and Cranberry. While the yield extract were 2.4g, 4g, 1.4g, 0.4g, and 2g for the Pomegranate peel, Myrrh, Chamomile flower, Cinnamon bark, and Cranberry respectively.

Minimum Inhibitory Concentration (MIC)

MIC of tested mouthrinses against (*S. mutans*) was assessed by agar dilution method as recommended by the Clinical and Laboratory Standards Institute.[30] For each tested mouthrinse, unified amount in milliliter of different concentrations were prepared and incorporated in different test tubes with 1 mL of tested microbial suspension, and growth medium. In this experiment, the tested microbial suspension used was containing 1.5×10^5 to 1.5×10^7 CFU/mL of bacterial sample. A positive control tube containing chlorhexidine digluconate, bacteria, and growth medium were set-up. On the other hand, a negative control tube containing distilled water, bacteria, and growth medium were set-up. The mixtures were incubated at 37°C for 24 hours and the results were evaluated in terms of turbidity of the test tubes compared to the time before incubation and the control groups. The minimum concentration of tested mouthrinse that inhibited the growth of the microorganism was determined as MIC. Due to the turbid nature of the extracts used in the present study and the inability to determine the accuracy of turbidity or clarity of the test tubes after incubation, microbial plates were prepared from the content of each test tube in order to be evaluated under a microscope to determine the presence of Bacterial growth or Bacterial inhibition. The test was repeated triplicate times for each sample.[31]

Minimum Bactericidal Concentration (MBC)

MBC of tested mouthrinses was determined from MIC range using spread plate method. Mutans-Sanguis agar in Petri dishes was sub-cultured from tubes without growth medium. Followed by incubation at 37°C for 24 hours. The petri dishes were observed macroscopically to determine the presence or absence of bacterial growth. The minimum concentration in which no bacterial growth on a solid medium were taken as MBC.[31]

Zone of Inhibition

Antibacterial activity was estimated using disc diffusion method (Kirby–Bauer) on agar plate. All tested bacterial strains was grown in Mutans-Sanguis agar (Hi media) for 48 hours at 37°C, and 0.1 ml of each culture of bacteria at concentration of 1.5×10^5 to 1.5×10^7 CFU/mL was spread on agar plate surfaces. Paper discs (6 mm in diameter) were immersed in the tested mouthrinses and control groups. The distance between the disc and the edge of the plate was 1.5 cm and the distance between discs was enough to avoid any overlapping. The plates were incubated at 37°C for 48 hours. After incubation, zone of inhibition were measured for each tested mouthrinse and control groups against *S. mutans* using caliper by two external evaluators.[31,32]

Reliability Analysis

The intra-rater agreement approach over a period of three weeks was used to assess the internal consistency of a single rater for measuring the "decayed, missing, and filled teeth (DMFT) index*"* of 20 randomly selected Saudi children (mean age of 8±1.2 years, age ranges 7-10 years). This approach showed an excellent intra-rater agreement with intra-rater coefficient (ICC) of 0.91 and 95% confidence interval (CI): 0.696-0.978. The inter-rater agreement approach was assessed on 24 reference strains and 24 saliva samples for measuring zone of inhibition, which were randomly selected by two blinded raters. The inter-rater agreement between the two raters was perfect, ICC of 0.996 and 95% CI: 0.990-0.998 in reference strain samples and ICC of 0.998 and 95% CI: 0.995-0.999 in saliva samples. The inter-rater agreement was assessed on 20 saliva samples for measuring minimum bactericidal concentration and minimum inhibitory concentration, which were randomly selected by two blinded raters. The inter-rater agreement was perfect for both minimum bactericidal concentration (ICC=1) and minimum inhibitory concentration (ICC=1).

Statistical Analysis

Data were analyzed with SAS 9.4 (SAS Institute Inc., Cary, NC, United States). The sample characteristics were illustrated in percent (%) and count (N) for categorical data and mean and standard deviation (SD) for quantitative data (Table 1). The data were analyzed using robust analysis of variance based on M estimation for zone of inhibition. We evaluated the effect of mouthrinses on zone of inhibition in reference strain group, and saliva isolates group. Moreover, the analysis assessed the interaction effects between mouthrinses by groups (saliva isolates and reference strain) on zone of inhibition. The goodness of fits for robust analysis of variance was assessed by coefficient of determination or R-square. Kruskal-Wallis was used to analyze minimum inhibitory concentration and minimum bactericidal concentration and nature of concentration measures. An alpha=0.05 was used for significance of all analyses.

RESULTS

The study included 20 children, 9 (45%) females and 11 (55%) males with age (mean±SD) of 8.15±1.09 years and range 5-11 years as well as DMFT (mean±SD) 7.65±1.84. The effects of mouthrinses on zone of inhibition was assessed in regard to reference strain group (Table 1). Compared with the distilled water group, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol (+) groups showed significantly

increased zones of inhibition by 36.38, 36.25, 26.13, 17.75, and 12.38, respectively. The R-square value was 0.9507. The error bar chart in Figure 1 showed significant differences between mouthrinse groups (P<0.05), but no difference was found in the zone of inhibition between the herbal mix and cranberry groups (P=1.000).

The effects of mouthrinses on zone of inhibition was assessed in regard to saliva isolates group (Table 2). Compared with the distilled water group, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol (+) groups had significantly increased zones of inhibition by 38.00, 34.25, 22.94, 16.50, and 16.44, respectively. The R-square value was 0.9668. The error bar chart in Figure 2 revealed significant differences in zones of inhibition among groups (P<0.05), except between the chlorhexidine and chlorhexidine with alcohol (+) groups (P=1), where no difference was found.

Comparing the effects of mouthrinses and groups (saliva isolates and reference strain) on zones of inhibition showed significant interaction effects between mouthrinses and groups on zones of inhibition. In cranberry mixed with chlorhexidine, the saliva isolates group exhibited significant reduction in zones of inhibition compared to reference strain (B=-3.35, P=0.0311). In chlorhexidine with alcohol (+), the saliva isolates group exhibited a significant increase in the zones of inhibition compared to reference strains (B=3.75, P=0.0159). The R-square value was 0.9470. The error bar chart in Figure 3 revealed that there was no difference in zone of inhibition between saliva and reference strain groups in herbal mix, cranberry, chlorhexidine, and distilled water (P>0.05), but there were significant effects of cranberry mixed with chlorhexidine and chlorhexidine with alcohol (+) on zones of inhibition.

The effects of mouthrinses on minimum inhibitory concentration (Figures 4) and minimum bactericidal concentration (Figures 5) were assessed in regard to saliva isolates group. Significant effects of mouthrinse were found for the minimum inhibitory concentration (P=0.0008) and minimum bactericidal concentration (P=0.0008). The chlorhexidine with alcohol (+) had a significantly lower minimum inhibitory concentration (value of 0.00001) and minimum bactericidal concentration (value of 0.0001) than the other groups. The herbal mix and cranberry mixed with chlorhexidine had a significantly higher minimum inhibitory concentration (value of 0.001) and minimum bactericidal concentration (value of 0.01) than the other groups. There were no significant differences between herbal mix and cranberry mixed with chlorhexidine and between cranberry and chlorhexidine in minimum inhibitory concentration (Figure 4). There were no significant differences between herbal mix and cranberry mixed with chlorhexidine and between cranberry and chlorhexidine in minimum bactericidal concentration (Figure 5).

Table 1. The effects of mouthrinse on zone of inhibition in reference strain

Parameter	B	SE	95% CI		Chi-Square	P value
			Lower	Upper		
Intercept	6.38	1.05	4.33	8.42	37.20	0.0001*
Herbal	36.38	1.48	33.48	39.27	605.57	0.0001*
Cranberry	36.25	1.48	33.35	39.15	601.41	0.0001*
Chlorhexidine	17.75	1.48	14.86	20.65	144.26	0.0001*
Cranberry mixed with Chlorhexidine	26.13	1.48	23.23	29.02	312.37	0.0001*
Chlorhexidine with Alcohol(+)	12.38	1.48	9.48	15.27	70.09	0.0001*
Distilled water(-)	Ref	Ref	Ref	Ref	Ref	Ref

*Significant α=0.05 R-Square = 0.9507 B Parameter estimate; SE standard errors; CI confidence intervals.

Table 2: The effects of mouthrinse on zone of inhibition in saliva

Parameter	B	SE	95% CI		Chi-Square	P value
			Lower	Upper		
Intercept	6.00	0.36	5.28	6.72	270.34	0.0001*
Herbal	38.00	0.52	36.99	39.01	5421.77	0.0001*
Cranberry	34.25	0.52	33.24	35.26	4404.48	0.0001*
Chlorhexidine	16.50	0.52	15.49	17.51	1022.21	0.0001*
Cranberry mixed with Chlorhexidine	22.94	0.52	21.93	23.95	1975.54	0.0001*
Chlorhexidine with Alcohol(+)	16.44	0.52	15.43	17.45	1014.55	0.0001*
Distilled water(-)						

*Significant α=0.05 R-Square = 0.9668; B Parameter estimate; SE standard errors; CI confidence intervals

Figure 1. Means with standard error bars: zone of inhibition by mouthrinse in regard to reference strain

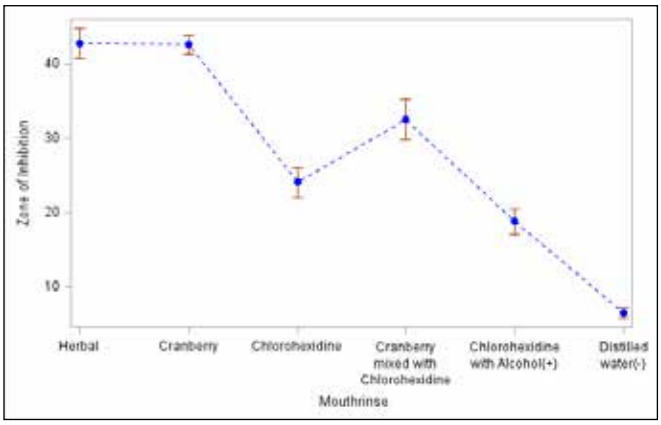


Figure 2. Means with standard error bars: zone of inhibition by mouthrinse in saliva

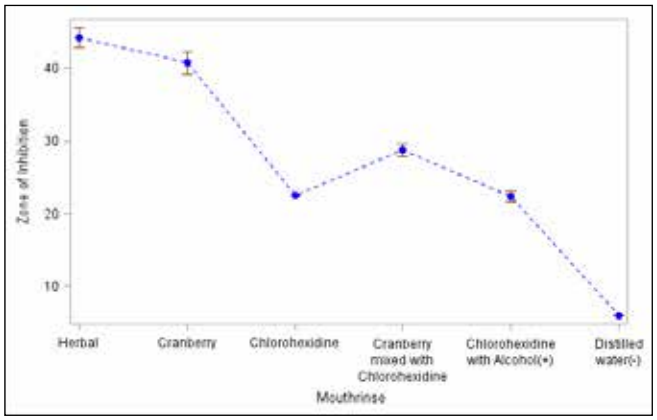


Figure 3. Means with standard error bars: differences zone of inhibition by mouthrinse and groups

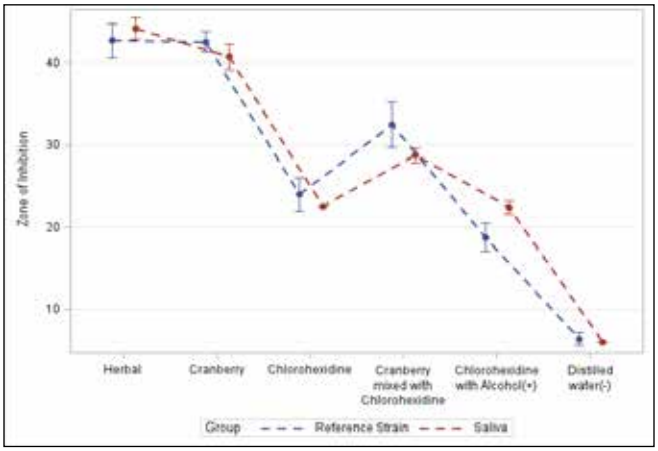


Figure 4. The effects of mouthrinse on minimum inhibitory concentration

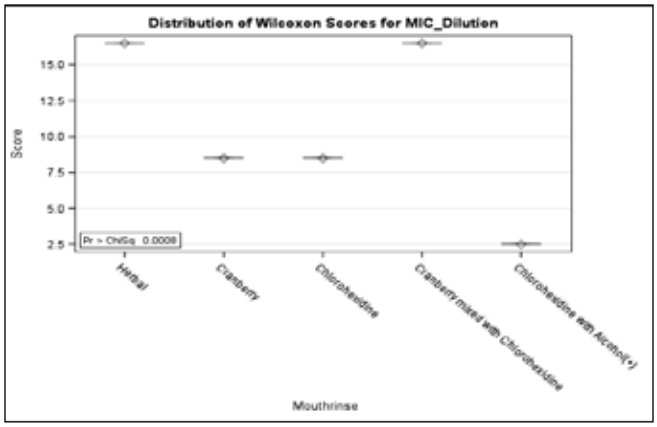
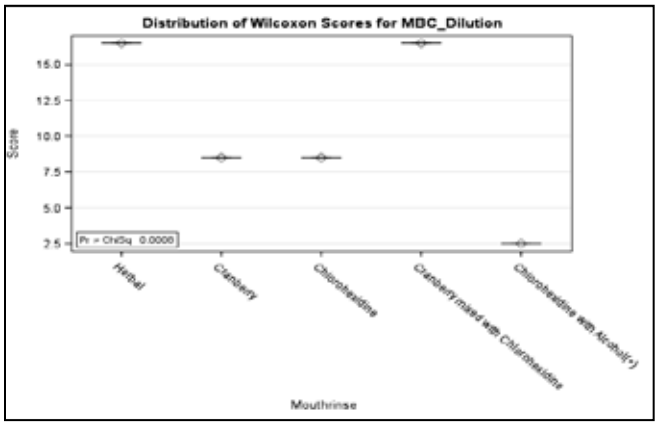


Figure 5. The effects of mouthrinse on minimum bactericidal concentration



DISCUSSION

The null hypothesis was rejected, as there was significant, difference in the antimicrobial effects between tested and control mouthrinses against *S. mutans* that were isolated from the saliva of the 7-10 year-old Saudi children. *S. mutans* is considered one of the key bacterial species implicated in the etiology of early childhood caries; therefore reducing the levels of this bacterium in the mouths of children using antiseptic agents is a possible method of caries control.[33] *S. mutans* often exceeding 30 percent of the cultivable plaque biofilm flora in severe forms of the disease[34] and that is why it is considered the keystone bacterial pathogen in early childhood caries. While chlorhexidine is bactericidal for *S. mutans*, susceptible oral places are often repopulated with the same disease-associated microorganisms once the chemotherapeutic intervention with chlorhexidine stops, resulting in lesion recurrence and therapeutic failure.[35,36] Therefore, recognition of the key health benefits of having an oral microbiome that is in a symbiotic relationship with the host has resulted in virulence-targeted therapies being preferred over broad-spectrum antimicrobials.[37,38]

In this regard, natural products that can disrupt the cariogenic properties and are potentially attractive approach for caries prevention. Finding substances with antimicrobial activity is a challenge in dentistry. Natural derivative compounds have been considered an interesting choice because of their use in traditional medicine to treat different infectious disorders.[10,11] Several studies have

investigated the antimicrobial activity of natural products against oral microorganisms. However, to the best of our knowledge there are no investigations concerning the antimicrobial action of the combination of naturally derivative compounds (Herbal mix mouthrinse) and semi-natural compounds (Cranberry extract mixed with chlorhexidine digluconate mouthrinse) against cariogenic bacteria especially *S. mutans*.

Cranberry has been used in herbal medicine as an anti-infection agent.[15]

The Non Dialyzable Material (NDM) constituent of the cranberry juice exhibits anti-coaggregation activity against a variety of oral bacteria. This provided the motivation to evaluate the efficiency of Cranberry extract against *S. mutans*. In our investigation, the effect of mouthrinses on zone of inhibition was evaluated in regard to reference strain group. Compared with the distilled water group, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol (+) groups had significantly increased zones of inhibition by 36.38, 36.25, 26.13, 17.75, and 12.38, respectively. The error bar chart showed significant differences between mouthrinse groups ($P<0.05$), but no difference was found in the zone of inhibition between the herbal mix and cranberry groups ($P=1.000$). This result OF WHAT could be due to the component of Herbal mix mouthrinse which include Pomegranate peel extract, that has notable antimicrobial activity against *S. mutans*.[17]

In addition, an *in vitro* study compared the antibacterial influence of pomegranate and aloe vera extracts that were prepared to different concentrations on *S. mutans* found that pomegranate extract exhibited significantly greater inhibitory effect on *S. mutans* than aloe vera extracts at all concentrations.[39]

Moreover, Myrrh extract was sued in this herbal mix mouthrinse, which could be an important reason for that result. In a study conducted by Eid et al (2015), myrrh plant extract exhibited the capability to inhibit the growth of *S.mutans* with a clear zone of 11 mm around the disc.[40]

Furthermore, Cinnamon is also a component of Herbal mix mouthrinse that is active on Gram-positive bacteria[21] and an *in vitro* study revealed that Cinnamon oil showed strong inhibitory activity on all the *S. mutans* isolates at a concentration of as low as 3.12%.[41]

The anti-adhesion effect of Cranberry on S. mutans was also reinforced by an *in vitro* study, which found significant inhibition zones associated with various concentration of Cranberry extract.[42] In a clinical trial conducted by Khairnar et al (2015), Cranberry mouthwash was equally effective as Chlorhexidine mouthrinse.[13] Therefore, it can be used efficiently as an alternative to Chlorhexidine mouthrinse.

The effects of mouthrinse on zone of inhibition was assessed in regard to saliva isolates group. Compared with the distilled water group, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol (+) groups showed significantly increased zones of inhibition by 38.00, 34.25, 22.94, 16.50, and 16.44 respectively. The error bar chart revealed significant differences in zones of inhibition among groups ($P<0.05$), except between the chlorhexidine and chlorhexidine with alcohol (+) groups ($P=1$), where no difference was found. Chlorhexidine is considered an effective antimicrobial agent against S. mutans. The antimicrobial activity of 0.12% chlorhexidine has been widely investigated and the findings of our study are consistent with the reported researches.[43,44] A study used 0.12% chlorhexidine gluconate with 0.05% sodium fluoride revealed 26.75 % of plaque reduction.[43] Moreover, an *in vitro* study showed a zone of inhibition between (38.46% -96.15%) of Chlorhexidine (0.12%) against the following reference strains of Streptococcus mutans (S.mutans) (ATCC 25175), Streptococcus salivarius (S.salivarius) (ATCC 7073) and Aggregatibacter actinomycetemcomitans (A.a) (NCTC 9710).[44] Furthermore, *Cohen* showed in her study that there is no difference in the efficacy between alcohol free Chlorhexidine and Chlorhexidine with alcohol in inhibiting S. mutans growth and conclude that alcohol does not add to the antimicrobial properties of Chlorhexidine.[45]

The results of our study showed significant interaction effects between mouthrinses and groups (saliva isolates and reference strain) on zones of inhibition. Specifically, there was no difference in zone of inhibition between saliva isolates and reference strain groups in herbal mix, cranberry, chlorhexidine, and distilled water. While, there were significant effects of cranberry mixed with chlorhexidine and chlorhexidine with alcohol (+) on zones of inhibition. In cranberry mixed with chlorhexidine, the saliva isolates group exhibited significant reduction in zones of inhibition compared to reference strain ($B=-3.35$, $P=0.0311$). While in chlorhexidine with alcohol (+), the saliva isolates group exhibited a significant increase in the zones of inhibition compared to reference strains ($B=3.75$, $P=0.0159$). Cranberry mixed with chlorhexidine showed smaller zones of inhibition than cranberry. This could be due to the effect of proanthocyanidins and flavonols which are the active constituents of cranberry against *S. mutans*.[46] The effects of the extracts of flavonols, anthocyanins and proanthocyanidins from the cranberry on virulence factors involved in *S. mutans* biofilm development and acidogenicity showed inhibition of the surface-adsorbed glucosyltransferases and F-ATPases activities, and the acid production by *S. mutans*.[46] Another recent study reported that a highly purified polyphenol-rich cranberry extract was able to significantly disrupt acidogenicity, metabolic activity, exopolysaccharide/microbial biovolumes, and structural organization of *S. mutans* biofilms without affecting bacterial viability.[38] It seems that addition of chlorhexidine to the cranberry reduced their cariogenic effect compared to cranberry alone and this could be due to the decreased concentrations of cranberry (0.3%) and chlorhexidine (0.06%) that were used in the semi-natural rinse. Chlorhexidine causes disruption of the bacterial cell wall and inhibits its enzymatic system, which could influence the formation of biofilms.[47] In our study; we also used chlorhexidine with alcohol. One of the ingredients that are existing generally in every mouthrinse is alcohol, ethanol that is in a concentration of 0%–27%.[48] Except for its use as a solvent ingredient and to extend the product expiration date, alcohol in the mouthrinses does not have any other medicinal effect because the optimum concentration of 50%–70% is required for alcohol to be able to exert its antiseptic effect, which lags owing to its low level of concentration in mouthrinses.[48,49] Furthermore, in some patients, it has been observed that the presence of alcohol causes an initial burning sensation, unpleasant taste and dryness of mouth.[50,51] Moreover, it has been concluded that alcohol-based mouthrinses when used continuously for a long period, it is a predisposing

factor for oral cancer.[51] Because of this, nonalcohol based mouthrinses have been showed to be equally efficient as alcohol-based mouthrinses, with minimal side effects revealed by the former. Clinical study evaluated chlorhexidine with or without alcohol on plaque control and early wound healing and chlorhexidine without alcohol showed the lowest bacterial counts levels at day 14.[52] Moreover, another study reported the effectiveness of alcohol-free chlorhexidine clinically compared to alcohol-based chlorhexidine mouthrinses in dental plaque formation and inhibition of gingival inflammation.[53] However, due to the heterogeneity of the aforementioned studies in terms of the design and the concentrations of chlorhexidine formulations evaluated, a direct comparison of the findings is unjustified and the results should be interpreted carefully. Alcohol-free chlorhexidine formulations often contain other ingredients aimed at stabilizing and maintaining the sterility of the solution.[54] A study compared the antibacterial properties of alcohol-free chlorhexidine with alcohol-containing chlorhexidine and reported that the latter performed better in inhibiting biofilm regrowth and reducing bacterial vitality than the alcohol-free chlorhexidine.[55] On the other hand, another study demonstrated no differences between alcohol-free and alcohol-containing chlorhexidine for both plaque and gingival bleeding index, in a 21-day experimental gingivitis study.[56]

The difference in the results between *S. mutans* in the saliva isolates and reference strain could be due to differences in their serotypes. *S. mutans* has four known clinical serotypes (c, e, f and k).[57] A study evaluated the prevalence of *S mutans* serotypes from two cohorts of African-American children using three sample types (saliva, plaque and individual *S mutans* isolates) reported that overall prevalence of serotypes were: serotype c (98%), e (26%), f (7%) and k (52%). Serotype c was statistically associated with higher caries scores in older children, serotype k was statistically more likely in females, and the authors concluded that the frequency of serotype k in this study is the highest reported in any population, illustrating the need for further study to determine the prevalence of this clinically relevant serotype in the US.[57] Another study tested the effectiveness of ethyl gallate against *S. mutans* biofilm formation and examined the effect on expression of six important genes involved in biofilm production by *S. mutans* showed that biofilm-producing bacteria treated with ethyl gallate exhibited significant changes in gene expression for three genes—gtfC, gtfB, and gbpB while for three other genes tested (gtfD, atpD and atpF), the ethyl gallate treatments did not produce any significant expression change in comparison with the control.[58] In addition, another study determined and compared the whole genome sequence of an *S. mutans* serotype *c* strain NN2025 isolated from Japan in 2002 and *c* UA159 strain isolated in 1982 from the United States showed a large genomic inversion across the replication axis producing an X-shaped symmetrical DNA dot plot and the authors concluded that these observations suggest that *S. mutans* strains evolve through chromosomal shuffling.[59]

The effects of mouthrinse on minimum inhibitory concentration and minimum bactericidal concentration were assessed in regard to saliva isolates group. The chlorhexidine with alcohol (+) had a significantly lower minimum inhibitory concentration (value of 0.00001) and minimum bactericidal concentration (value of 0.0001) than the other groups. This could be due the positive and strong effect of such rinse against biofilm formation that had been discussed and proved in many previous studies.[55,60] A study conducted by Eldridge et al (1998), showed that the plaque scores decreased after 21 days of using Chlorhexidine products with or without alcohol. Besides that, the growth for S mutans was decreased to zero after 21 days.[60] To support that, an *in vivo* study was conducted proved that 0.12% chlorhexidine mouthrinse is more efficient in reducing salivary *S. mutans* count that other mouthrinses (Sodium fluoride, and triclosan).[61] Furthermore, Arweiler et al. (2006) found that alcohol-containing chlorhexidine performed better in inhibiting biofilm regrowth and reducing bacterial vitality than the alcohol-free chlorhexidine.[55] In our study, the herbal mix and cranberry mixed with chlorhexidine mouthrinses had a significantly higher minimum inhibitory concentration (value of 0.001) and minimum bactericidal concentration (value of 0.01) than the other groups. In regard to cranberry mixed with chlorhexidine mouthrinse, this could be due to the decreased concentrations of cranberry (0.3%) and chlorhexidine (0.06%) that were used in the semi-natural rinse. For herbal mix mouthrinse, the higher minimum inhibitory and bactericidal concentration could be due to the low percent of extracts that had been used in this mouthrinse (0.36 % pomegranate peel extract, 0.36 % myrrh resin extract, 0.18 % chamomile flower extract, 0.1 % cinnamon bark extract).

The present findings in our study should be interpreted in the context of some limitations including its *in vitro* setting. Our *in vitro* methodology does not reflect the complex polymicrobial, ecological, and environmental influences encountered in the oral cavity. *In vitro* studies lack reproduction of oral environment. Nevertheless, *in vitro* studies can provide valuable data of some variables with no interference from other factors. However, in spite of these limitations, the research does designate a number of positive links between *in vitro* effect and clinical effect. Well-designed clinical trials are warranted to demonstrate whether the beneficial properties shown in our study can translate into efficient and valuable therapeutic approaches for caries prevention.

CONCLUSIONS

Within the limitations of this *in vitro* study, it can be concluded that:

1. For the reference strain group and in comparison with the distilled water, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol (+) groups showed significantly increased zones of inhibition.
2. For saliva isolates of *S. mutans* and in comparison with the distilled water, the herbal mix, cranberry, cranberry mixed with chlorhexidine, chlorhexidine, and chlorhexidine with alcohol (+) groups had significantly increased zones of inhibition.
3. For the cranberry mixed with chlorhexidine, the saliva group exhibited significant reduction in zones of inhibition compared to reference strain.
4. Chlorhexidine with alcohol exhibited a significant increase in the zones of inhibition on the saliva isolates of *S. mutans* compared to reference strains.

5. For MIC and MBC microbiological tests , chlorhexidine with alcohol (+) showed significantly lower minimum inhibitory concentration and minimum bactericidal concentration than the other groups while herbal mix and cranberry mixed with chlorhexidine mouthrinses had a significantly higher minimum inhibitory concentration and minimum bactericidal concentration than the other groups.
6. Herbal mix and cranberry mouthrinses could be effective natural alternative to chlorhexidine mouthrinse with or without alcohol in improving oral health.

ACKNOWLEDGMENTS

The authors would like to thank the College of Dentistry Research Center and Deanship of Scientific Research at King Saud University and College of Dentistry Research Center for supporting this research project.

REFERENCES

1. Yadav K, Prakash S. Dental Caries: A Review', Asian Journal of Biomedical and Pharmaceutical Sciences;53(6):1–7.2. 2016.
2. Selwitz R, Ismail A, Pitts N. Dental caries. The Lancet;369(9555): 51–59. 2007.
3. Chandki R, Banthia P, Banthia R. Biofilms: a microbial home. J Indian Soc Periodontol;15(2):111–114. 2011.
4. Hamada S, Slade H. Biology, immunology, and cariogenicity of Streptococcus mutans. Microbiological reviews;44(2):331–384. 1980.
5. Sharma U, Jain RL, Pathak A. A clinical assessments of effectiveness of mouthwashes in comparison to toothbrushing in children. J Indian Soc Pedod Prev Dent ;22:38–44. 2004.
6. Van der Weijden FA, Van der Sluijs E, Ciancio SG, Slot DE. Can Chemical Mouthwash Agents Achieve Plaque/Gingivitis Control? Dent Clin North Am;59(4):799-829. 2015.
7. James P, Worthington HV, Parnell C, Harding M, Lamont T, Cheung A, Whelton H, Riley P. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. Cochrane Database Syst Rev;3:CD008676. 2017.
8. Fltötra L, Gjermo P, Rölla G, Waerhaug J. Side effects of chlorhexidine mouth washes. Scand J Dent Res;79(2):119–125. 1971.
9. Ernst C, Canbek K, Dillenburger A, Willershausen B. Clinical study on the effectiveness and side effects of hexetidine and chlorhexidine mouthrinses versus a negative control. Quintessence international;36(8):641–652. 2005.
10. Groppo F, Bergamaschi C, Cogo K, Franz-Montan M, Motta RH, de Andrade ED. Use of phytotherapy in dentistry. Phytother Res;22(8):993–998.11. 2008.
11. Newman D. Natural products as drug leads: an old process or the new hope for drug discovery? J Medicinal Chem;51(9):589–2599. 2008.
12. Bonifait L, Grenier D. Cranberry Polyphenols : Potential Benefits for Dental Caries and Periodontal Disease. J Can Dent Assos;76:a130. 2010.
13. Khairnar M, Karibasappa G, Dodamani A, Vishwakarma P, Naik R, Deshmukh M. Comparative assessment of Cranberry and Chlorhexidine mouthwash on streptococcal colonization among dental students: A randomized parallel clinical trial. Contemporary Clin dent; 6(1): 35–39. 2015.
14. Neto C. Cranberry and It's Phytochemicals: A Review of In Vitro Anticancer Studies. J Nut;137:186S-193S. 2007,
15. Srinidhi K. Cranberry and its Antibacterial Activity–A Review. J Pharm Sci & Res;6(1):41 – 44. 2014.
16. Viuda-Martos M, Fernández-Lóaez J, Pérez-álvarez J. Pomegranate and its Many Functional Components as Related to Human Health: A Review. Comprehensive Reviews in Food Science and Food Safety; 9(6): 635–654. 2010.
17. Umar D, Dilshad B, Farhan M, Ali A, Baroudiet K. The effect of pomegranate mouthrinse on Streptococcus mutans count and salivary pH: An in vivo study. J Adv Pharm Technol Res;7(1):13-16. 2016.
18. Sambawa Z, Alkahtani F, Aleanizy F, ALqahtani F. Comparison of antibacterial efficacy chlorhexidine gluconate and Saudi Myrrh mouthwashes in the oral cavity. Oriental J Chem;32(5):2605–2610. 2016.
19. Gardiner P. Complementary, holistic, and integrative medicine: chamomile. Pediatr Rev;28(4):e16– 8. 2007.
20. Pourabbasa R, Delazarb A, Chitsaza M. The Effect of German Chamomile Mouthwash on Dental Plaque and Gingival Inflammation. Iranian J Pharmaceutical Res;2:105-109. 2005.
21. Ooi LS, Li Y, Kam SL, Wang H, Wong EY, Ooi VE. Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia Blume. Am J Chin Med;34(3):511-22. 2006.
22. Burt S. Essential oils: their antibacterial properties and potential applications in foods-a review. Int J Food Microbiol;94(3):223-253. 2004.
23. Al-Joubori S, Al-Obaidi W. Effect of cinnamon extracts on streptococci and mutans streptococci, in comparison to chlorhexidine gluconate. J Baghdad College Dent;23(1):141-145. 2011.
24. Frankl S, Shiere F, Fogels H. Should the parent remain with the child in the dental operatory? J Dent Child; 29: 150-163. 1962.
25. al Ghanim NA, Adenubi JO, Wyne AA, Khan NB. Caries prediction model in pre-school children in Riyadh, Saudi Arabia. Int J Paediatr Dent;8(2):115–122. 1998.

26. Lindhe J, Nyman S, Westfelt E, Socransky SS, Haffajee A. Critical probing depths in periodontal therapy. Compend Contin Educ Dent;3(6):421–430. 1982.
27. Loesche WJ and Syed SA. The predominant cultivable flora of carious plaque and carious dentine. Caries Res;7(3):201–216. 1973.
28. Klock B, Krasse B. Microbiological and salivary conditions in 9–12-year old children. Scand J Dent Res;85(1):56–63. 1977.
29. Guo L, Shi W. Salivary biomarkers for caries risk assessment. J Calif Dent Assn;41(2):107–109. 2013.
30. Clinical and Laboratory Standards Institute 11th Edition (2018). M07 Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Accessed on May 24, 2019 at https://clsi.org/media/1928/m07ed11_sample.pdf.
31. Anita P, Sivasamy S, Madan Kumar P, Balan IN, Ethiraj S. In vitro antibacterial activity of Camellia sinensis extract against cariogenic microorganisms. J Basic and Clinical Pharmacy;6(1):35–39. 2015.
32. Barry A, Coyle M, Thornsberry C, Gerlach E, Hawkinson R. Methods of Measuring Zones of Inhibition with the BauerKirby Disk Susceptibility Test. J Clinical Microbiology;10(6):885-889. 1979.
33. Loesche WJ. Role of Streptococcus mutans in human dental decay. Microbiol Rev;50(4):353-80. 1986.
34. Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, Pai CH, Gonzalez-Begne M, Watson G, Krysan DJ, Bowen WH, Koo H. Symbiotic relationship between Streptococcus mutans and Candida albicans synergizes virulence of plaque biofilms in vivo. Infect Immun;82(5):1968-81. 2014.
35. Philip N, Suneja B, Walsh LJ. Ecological approaches to dental caries prevention: paradigm shift or shibboleth? Caries Res 2018a; 52(1-2):153-165.
36. Philip N, Bandara N, Leishman SJ, Walsh LJ. Inhibitory effects of fruit berry extracts on Streptococcus mutans biofilms. Eur J Oral Sci 2019 Apr;127(2):122-129.
37. Philip N, Walsh LJ. Cranberry Polyphenols: Natural Weapons against Dental Caries. Dent J (Basel) 2019 Mar 1;7(1):E20.
38. Philip N, Leishman SJ, Bandara H, Walsh LJ. Polyphenol-Rich Cranberry Extracts Modulate Virulence of Streptococcus mutans-Candida albicans Biofilms Implicated in the Pathogenesis of Early Childhood Caries. Pediatr Dent Jan 15; 41(1):56-62. 2019.
39. Subramaniam P, Dwivedi S, Uma E, Girish Babu KL. Effect of pomegranate and aloe vera extract on streptococcus mutans: An in vitro study. Dental Hypotheses;3(3): 99-105. 2012.
40. Eid HA, Assiri YH, Musleh MM, Taha TH, El-Deeb NM, Hamad GM. An exploration of the effects of Commiphora glileadenis on a Streptococcus mutans biofilm. Saudi J Oral Sci;2:74-8. 2015.
41. Fani M.M, Kohanteb J. Inhibitory Activity of Cinnamon Zeylanicum and Eucalyptus Globulus Oils on Streptococcus Mutans, Staphylococcus Aureus, and Candida Species Isolated From Patients With Oral Infections, Shiraz University Dental Journal;11:14–22. 2011.
42. Sethi R, Govila V. Inhibitory effect of cranberry juice on the colonization of Streptococci species: An in vitro study. J Indian Soc Periodontol;15:46-50. 2011.
43. Zanela NL, Bijella MF, Rosa OP. The influence of mouth-rinses with antimicrobial solutions on the inhibition of dental plaque and on the levels of *streptococci* mutans in children. Braz Oral Res;16:101–106. 2002.
44. Dabholkar CS, Shah M, Kathariya R, Bajaj M, Doshi Y. Comparative Evaluation of Antimicrobial Activity of Pomegranate-Containing Mouthwash Against Oral-Biofilm Forming Organisms: An In vitro Microbial Study. J Clin Diagn Res;10(3):ZC65–ZC69. 2016.
45. Cohen R, Chen C. Efficacy of Alcohol-Free Chlorhexidine in Reducing the Levels of Streptococcus Mutans, 2013Accessed on May 24, 2019 at https://www.oralhealthgroup.com/features/efficacy-of-alcohol-free-chlorhexidine-in-reducing-the-levels-of-streptococcus-mutans/
46. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, Koo H. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. FEMS Microbiol. Lett;257:50–56. 2006.
47. de Freitas CS, Diniz HF, Gomes JB, Sinisterra RD, Cort_es ME. Evaluation of the substantivity of chlorhexidine in association with sodium fluoride in vitro. Pesqui Odontol Bras;17:78–81. 2003.
48. Kulkarni P, Singh DK, Jalaluddin M, Mandal A. Comparative Evaluation of Antiplaque Efficacy between Essential Oils with Alcohol-Based and Chlorhexidine with Nonalcohol-Based Mouthrinses. J Int Soc Prev Community Dent;7(l1):S36-S41. 2017.
49. Eley BM. Antibacterial agents in the control of supragingival plaque–a review. Br Dent J;186:286–296. 1999.
50. Bolanowski SJ, Gescheider GA, Sutton SV. Relationship between oral pain and ethanol concentration in mouthrinses. J Periodontal Res;30(3):192–197. 1995.
51. Schepman KP. Is an alcohol-based mouthwash a risk factor for oral cancer? Ned Tijdschr Tandheelkd;108:196. 2001.
52. Gkatzonis AM, Vassilopoulos SI, Karoussis IK, Kaminari A, Madianos PN, Vrotsos IA. A randomized controlled clinical trial on the effectiveness of three different mouthrinses (chlorhexidine with or without alcohol and C31G), adjunct to periodontal surgery, in early wound healing. Clin Oral Investig;22(7):2581-2591. 2018.
53. Todkar R, Sheikh S, Byakod G, Muglikar S. Efficacy of chlorhexidine mouthrinses with and without alcohol–a clinical study. Oral Health Prev Dent;10(3):291–296. 2012.
54. Santos GOD, Milanesi FC, Greggianin BF, Fernandes MI, Oppermann RV, Weidlich P. Chlorhexidine with or without alcohol against biofilm formation: efficacy, adverse events and taste preference. Braz Oral Res 2017;31:e32.
55. Arweiler NB, Boehnke N, Sculean A, Hellwig E, Auschill TM. Differences in efficacy of two commercial 0.2% chlorhexidine mouthrinse solutions: a 4-day plaque regrowth study. J Clin Periodontol;33(5):334-9. 2006.
56. Lorenz K, Bruhn G, Heumann C, Netuschil L, Brecx M, Hoffmann T. Effect of two new chlorhexidine mouthrinses on the development of dental plaque, gingivitis, and discolouration: a randomized, investigator-blind, placebo controlled, 3-week experimental gingivitis study. J Clin Periodontol;33(8):561-7. 2006.
57. Momeni SS, Ghazal T, Grenett H, Whiddon J, Moser SA, Childers NK. Streptococcus mutans serotypes and collagen-binding proteins Cnm/Cbm in children with caries analysed by PCR. Mol Oral Microbiol;34(2):64-73. 2019.
58. Gabe V, Kacergius T, Abu-Lafi S, Kalesinskas P, Masalha M, Falah M, Abu-Farich B, Melninkaitis A, Zeidan M, Rayan A. Inhibitory Effects of Ethyl Gallate on Streptococcus mutans Biofilm Formation by OpticalProfilometry and Gene Expression Analysis. Molecules;24(3):E529.2019.
59. Maruyama F, Kobata M Kurokawa K Nishida K Sakurai A, Nakano K, Nomura R, Kawabata S, Ooshima T, Nakai K, Hattori M, Hamada S, Nakagawa I. Comparative genomic analyses of Streptococcus mutans provide insights into chromosomal shuffling and species-specific content. BMC Genomics;10:358. 2009.
60. Eldridge K, Finnie S, Stephens J, EdSb, Mauad A, Munoz C, Kettering J. Efficacy of an alcohol-free chlorhexidine mouthrinse as an antimicrobial agent. J Prost Dent;80(6):685–690. 1998.
61. Kulkarni VV, Damle SG. Comparative evaluation of efficacy of sodium fluoride, chlorhexidine and triclosan mouth rinses in reducing the mutans streptococci count in saliva : an in vivo study. J Indian Soc Pedod Prev Dent;21(3):98-104. 2003.