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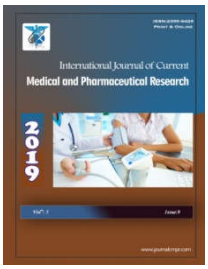

Research Article

COMPARISON OF SUBSTANTIVITY OF ANTICARIOGENIC PROPERTY OF POLYPHENOL EXTRACT FROM CRANBERRY WITH 0.2% CHLORHEXIDINE MOUTH WASH

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ABSTRACT

Aim: The aim of the study is to determine the duration of antibacterial substantivity of polyphenol extract mouth wash after a single oral rinse and to compare it with the substantivity of 0.2% chlorhexidine (CHX).
Materials and Methods: In this study, unstimulated saliva from thirty individuals was colected 2 hours after usual oral hygiene procedures but not rinsing (pre-sample) with randomly selected mouthwash, (10% polyphenol extract from cranberry mouthwash, 0.2% chx, and normal saline) and 5 min after rinsing (postsample). A washout period of 1 week was kept between two rinses. The sampling was replicated after every 2 hour for 12 hour (post 1, post 2, post 3, post 4, post 5, and post 6) and was tested for microbial count.
Statistical Analysis Used: Friedman test, Kruskal–Wallis test, and *post hoc* analysis were used to evaluate the outcome of sample mouthrinses on CFUs at different times.
Results: Polyphenol extract showed statistically significant results when the antibacterial effect at post, post sample 1 and post sample 2 were compared to pre-sample count ($P < 0.05$). After which the effeciancy declined and was equal to normal saline ($P > 0.05$). The results for Chlorhexidine were statistically significant at all times when compared to pre-sample count ($P < 0.05$) and it revealed the maximum substantivity of 7 hours.
Conclusion: After a rinse with normal diet without restrictions over the day, polyphenol extract mouthwash had an antibacterial effect for 3–4 hours. It can be used three times daily for its maximum antibacterial effect.

INTRODUCTION

Oral diseases are the common health problems with dental caries among the most important preventable global infectious disease. More than 700 bacterial species or phenotypes, of which 50% have not been cultivated, have been detected in the oral cavity. Dental biofilm is related with the initiation and progression of tooth caries.[1] The conventional medical response to bacterial infections, administration of broad-spectrum antibiotics, has become less effective against emerging pathogens due to the evolution of drug resistance stemming in part from the antibiotic abuse. Therefore, there is a need to develop novel, narrow spectrum, therapeutics capable of maintaining the protective benefits of the normal microflora during treatment. Hence, the search for alternative products continues and natural phytochemicals isolated from plants used as traditional medicines are considered as good alternatives.[2] In this study, cranberry (*Vaccinium macrocarpon)* is the native North American fruit that has recently come into limelight owing to its numerous beneficial effects on dental caries, and periodontal health has been studied.[3]

It is the fruit of a shrub of peat bogs located in the colder regions of North America belonging to *Ericaceae* family. Cranberry itself is a unique, rich source of several classes of bioactive flavonoids including flavonols, anthocyanins, and proanthocyanidins (PACs) (Type A), which confer it the significant therapeutic potential. Indeed due to its richness in polyphenols of high-molecular-weight, this berry has a demonstrable ability to inhibit adhesion of bacteria responsible for urinary disorders (such as *Escherichia More Details coli*), gastric ulcer (such as *Helicobacter pylori*), or involved in the formation of dental caries or cavities and chronic periodontitis (such as *Streptococcus mutans*, *Streptococcus gordonii*, or *Porphyromonasgingivalis*).

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A number of studies have suggested that cranberry polyphenols may promote oral health by inhibiting dental biofilm formation, acid production by *S. mutans,* and periodontopathogen-derived proteolytic enzymes and host inflammatory responses.[3][4][5]

Thus, the present study was designed to determine the duration of its antibacterial substantivity and to compare it with the substantivity of 0.2% CHX, a positive control, and normal saline, a negative control.

MATERIALS AND METHODS

In this study, the participants were volunteers from dental college students staying in the hostel. The sample population were selected based on the inclusion criteria like well-being controls in the age group of 17–24 years with <3 missing teeth and the controls having good oral hygiene. The individuals with high caries index, any systemic illness, using antibiotics, or any other mouthwashes for 3 months, using orthodontic/ prosthetic appliances and with xerostomia were excluded from the study. An informed written consent was obtained from the students after the methodology of the clinical trial was explained. Scaling was performed before carrying out the study. All participants were trained and instructed regarding the usage of different mouth rinses before starting the study. Students were instructed to rinse

- 15 ml polyphenol extract mouth rinse for 2 minutes
- 15 ml of 0.2% CHX for 2 minutes
- 15 ml of normal saline for 2 minutes.

Unstimulated saliva sample (baseline, pre-sample) were collected in sterile boxes in the morning 2 hour after their daily oral hygiene procedures. Then, the volunters were given a randomly selected mouthrinse. Five minutes after rinsing with the given mouthrinse, a second sample (post sample) was collected, and both the samples were sent to the Microbiology lab for culture and microbial count. Volunteers were asked to continue with their routines without any limitations over their eating and drinking habits. The sampling was repeated in a similar manner after every 2 h for 12 h (post 1, post 2, post 3, post 4, post 5, and post 6 samples) and was checked for microbial count. This was a crossover study with each participant using all mouthwashes. A washout period of 1 week was kept between two mouthwashes.[6] The same procedure was repeated for the analysis of microbial count after 0.2% CHX and normal saline rinse

Preparation of polyphenol extract

In this study, the polyphenols were extracted from cranberry. Methanolic extracts of cranberries were evaporated and reextracted with ethyl acetate. Residues from the dried ethyl acetate extract were isolated by thin-layer, column, and paper chromatography. A large diffuse area indicated the presence of high molecular weight polymeric polyphenols. In addition, seven discrete spots were identified as: (a) catechin; (b) epicatechin; (c) a dimeric epicatechin with a C_4-C_8 linkage; (d) an unknown polymer which degraded on acid hydrolysis to three compounds, of which one was cyanidin; (e) an unknown polymer which degraded to at least four compounds of which one was cyanidin and one was the compound described above as (d). Compounds (f) and (g) were present in very small amounts. These compounds may contribute to the astringency of cranberries.[7]

Evaluation of microbial load count

Bacterial count (colony forming units [CFUs]) in each specimen was determined by culture and microscopy in the private microbiology lab. The collected saliva sample was inoculated on nutrient agar plate. For inoculation of saliva, standard quantity 0.01 ml of saliva was taken using standard diameter nichrome wire loop. Then the Inoculated nutrient agar plates were incubated at 37°C for 24 hours. The grown colonies on nutrient agar were counted against standard inoculum used.

Preparation of nutrient agar plate

For this research work, commercially available dehydrated nutrient agar from Hi-media was used. Twenty-eight grams of dehydrated media was mixed into 1000 ml of distilled water. Media was sterilized by autoclave at 121°C for 15 min. Thereafter, 20 ml of sterilized media was dispensed in each of the sterile Petri plates (90 mm × 15 mm in dimension). These plates were stored in a refrigerator.

Counting the colony forming units

For the counting, semi-quantitative method was used. The microbiologist was kept blind to avoid the bias.

Statistical analysis

All data were entered into a computer by giving coding system, proofed for entry errors and analyzed with statistical package, statistical package for the social sciences (SPSS Inc, IBM Corporation, New York, USA). Chi-square test was used to assess the age-wise and gender-wise distribution of study individuals in all three groups. Friedman test was used to assess the effect of different mouthrinses on CFU at different times. Kruskal–Wallis test was to assess CFU at different times for different mouthrinses. If Friedman test and Kruskal–Wallis test was found significant *post hoc* test, Dunn's multiple comparison test was carried out to find out the time of CFU significantly different at various times as compared to CFU at pre-assessment. $P < 0.05$ was considered statistically significant.

The following coding system was used for CFU:

- Nil: 0 1–10: 1 11–20: 2
- 21–30: 3 31–40: 4 41–50: 5
- 51–60: 6 61–70: 7 71–80: 8
- 81–90: 9 91–100: 10>100:

RESULTS

In the present trial recruited 30 students (9 males/21 females) in the age group of 17–24years. All the participants were instructed to use 0.2% CHX, polyphenol extract mouth rinse and normal saline mouth rinse with a washout period of 1 week. 0.2% CHX demonstrated statistically significant results in terms of CFU count at different times, as compared to baseline. 0.2% CHX also exhibited a statistically significant difference ($P < 0.005$), when compared with polyphenol and normal saline. Ten percent polyphenol presented with a statistically significant result ($P < 0.005$) in the initial 2–3 hour only. Though, post this period, the results with 10% polyphenol extract were noted to be statistically insignificant, corresponding to normal saline. Normal saline showed statistically insignificant results at all time.

Table 1 Comparison of colony forming units at different time

Time	Kruskal wallis test value	P	CHX versus polyphenols	CHX versus saline	Polyphenol versus saline
Pre	1.456	0.5323			
post	73.567	<0.001	<0.001	<0.001	<0.001
Post 1	42.476	<0.001	<0.01	<0.001	<0.01
Post 2	31.658	<0.001	<0.01	<0.001	>0.05
Post 3	18.697	<0.001	<0.001	<0.001	>0.05
Post 4	14.624	0.0005	<0.001	<0.05	>0.05
Post 5	13.628	0.0009	<0.05	<0.0011	>0.05
Post 6	4.498	0.0609			

DISCUSSION

Studies have demonstrated that, *in situ*, 0.2% CHX has a greater immediate antibacterial effect and substantivity than other antiseptics used in the oral cavity.[8][9][10][11] Our study also demonstrated the same. Its antibacterial mode of action is explained by the fact that the positively charged bis-biguanide molecule gets rapidly attracted by the negatively charged bacterial tooth surfaces and oral mucosal cell surfaces, increasing substantivity through controlled release of the agent.[12][13] The persistence of CHX on the oral surfaces and its ability to suppress salivary bacterial counts was demonstrated to last for than 12 h.[14] Thus, CHX in a mouthrinse (0.12% or 0.2% solution) is administered at 12-h intervals and retains its ability to retard/prevent plaque formation.[15] In this clinical crossover trial, 0.2% CHX substantivity was noticed till the 7thh. At the 8th h, the count of CFU was found to be increased. These results were in accordance with Tsuchiya *et al*., König *et al*.,Boulos *et al*.,[17] Tomás *et al*.,[18]Addy M *et al*., and Tomás *et al*.[16] found that the practice of eating, chewing, and drinking significantly decreased the substantivity of 0.2% CHX, with complete recovery of the salivary flora at 3–7 h after the mouthrinse. This study demonstrated that 0.2% CHX was the most effective agent both in terms of magnitude of effects and duration of action. Similar results were found by Elworthy *et al*.,Herrera *et al*.There were no colonies found soon after rinsing with 0.2% CHX in our clinical trial. This suggested its bactericidal effect which remained for at least first 2 h. For next 6–7 h, the number of colonies remained static, thus proving its bacteriostatic effect. 0.2% CHX also demonstrated statistically significant results in terms of CFU count at different times, as compared to baseline ($P < 0.05$).

Oral diseases induced by dental plaque continue to affect the majority of world population. Among them, dental caries is the single most prevalent and costly oral infectious disease. This ubiquitous disease results from the interaction of specific bacteria and constituents of the diet within plaque (a natural biofilm) formed on the tooth surface. *S. mutans* is a key contributor to the formation of cariogenic plaque because this bacterium (i) effectively utilizes dietary sucrose to synthesize a large amount of extracellular polysaccharides (EPS), (ii) adheres tenaciously to glucan-coated surfaces, and (iii) is highly acidogenic and acid tolerant. Polysaccharides, mostly glucans synthesized by microbial glucosyltransferases (GTF), are complex in structure, which changes over time during the extracellular matrix development. For many oral streptococci, glucans comprise an extracellular slime layer produced in the presence of sucrose that promotes adhesion and formation of dental plaque biofilm. The GTF decreased by *S. mutans* (particularly GTF B and GTF C) bind avidly to the pellicle formed on the tooth surface and to surface of other oral microorganisms which are highly active in the absorbed state. The glucan synthesized by surface absorbed GTF B and GTF C provides specific binding site for bacterial colonization on the tooth surface and to each other (Schilling and Bowen, 1992), thus contributing to the initial steps of cariogenic plaque development *in vivo*. If dental plaque is allowed to remain on tooth surfaces and is exposed to dietary carbohydrates frequently (especially sucrose), *S. mutans* as a member of the plaque community will continue to synthesize polysaccharides and metabolize sugar to organic acids. The elevated amount of EPS increases the biofilm stability and structural integrity and provides protection to the bacteria from inimical influences of antimicrobial and other environmental assaults. The low pH environment within plaque's matrix results in the dissolution of enamel, thus initiating the dental caries process. Therefore, EPS and acidification of biofilm's matrix are critical for formation and establishment of dental plaque.[19][20][21]

Cranberry fruit (*V. macrocarpon*) has well-known antiadhesive properties and holds great potential as an antiadhesive agent against cariogenic mutans streptococci. It causes disruption of acidogenic/aciduric properties of planktonic and biofilm cells of *S. mutants*. It has inhibitory effects on GTF activity and adherence by *S. mutans* and causes reduction of the formation of *S. mutans* biofilms and EPS content. Quercetin-3-arabinofuranoside, myricetin, and procyanidin A2 found in cranberry have been found to inhibit GTF activity. In a study, it was proposed that three putative pathways by which flavonols and PACs affect the virulence of *S. mutans* are (1) inhibition of insoluble glucans synthesis by surface-adsorbed GTF B and C, (2) inhibition of the proton-translocating F-adenosine triphosphate synthases (F-ATPases) activity, and (3) disrupting acid production.[22][23]

In a study done by Yamanaka *et al*., they treated *S. mutans* and *Streptococcus sobrinus* cells with cranberry juice and found that it reduced cell surface hydrophobicity of the cells interfering with adhesion and the initial stages of biofilm formation. The authors hypothesized that cranberry juice components may interact with hydrophobic protein on the surface of the bacterial cell.[24]

In another study, the influence of cranberry PACs on formation of biofilms by *S. mutans* on saliva-coated apatitic surface and on dental caries development *in vivo* demonstrated that a highly purified A-type proanthocyanadins (PAC) fraction reduced the formation of biofilms by *S. mutans* on saliva-coated apatitic surface, which correlated well with the reduction in the amounts of insoluble polysaccharides in the extracellular matrix. Topical application (60-s exposure, twice daily) with PACs (1.5 mg/ml) during biofilm formation resulted in less biomass and fewer insoluble polysaccharides than the biofilm treated with vehicle control (10% ethanol, v/v; $P < 0.05$). A-type PAC is uniquely found in high concentrations in cranberry and has shown to be biologically active.[20] Despite the lack of significant antibacterial (biocidal) activity, PAC treatments diminished the acidogenicity of *S. mutans* within biofilm by disrupting the overall bacterial metabolism. The lowest molecular weight compound tested was the PACs monomer catechin. Catechin was the only test agent that was not an effective inhibitor of GTF. All other PACs were able to inhibit GTF; PACs that had a degree of polymerization between 4 and 12 were the most potent inhibitors.[20]

Further, Weiss *et al*. investigated the effects on oral health of a mouthwash supplemented with the NDM fraction of cranberries. After 6 weeks of daily use of the mouthwash, total microflora notably *S. mutans* was significantly reduced. In support of these *in vivo* results, *in vitro*studies showed the NDM fraction inhibited the adhesion of *S. sobrinus* to a hydroxyapatite surface pretreated with saliva.

CONCLUSION

Polyphenols extract from cranberry has an antibacterial effect for 3–4 h after a single rinse. It can be used for at least 3 times daily for its maximum antibacterial effect. CHX remains the gold standard providing maximum antibacterial substantivity of 7–8 h.

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