Antibacterial Efficacy of Mouthwash Prepared from Pomegranate, Grape Seed and Guava Extracts against Oral Streptococci: An *in Vivo* Study

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Background: *Pomegranate, Grape seed and Guava extracts have much been reviewed in Ayurveda and has been proven to have antibacterial action* ***Aim:*** *The objective of the study is to investigate and compare the mouthwash prepared from pomegranate, grape seed and guava extracts on salivary streptococci levels at the end of 48 hr and 7 days, of twice a day usage.* ***Study design:*** *40 school going children aged 8-10 yrs, randomly allocated into 4 groups (n=10 for experimental group) were asked to rinse with a) Mouthwash prepared from Pomegranate extract, 15 ml twice a day b) Mouthwash prepared from Grape seed extract, 15 ml twice a day, c) Mouthwash prepared from guava extract, 15 ml twice a day, d) Control- Distil water, twice a day. The oral streptococci colony forming units/ml (CFU/ml) was assessed by inoculating the salivary samples on blood agar media at the end of 48 hrs, and 7 days.* ***Results and Conclusion:*** *the aqueous extracts of the chosen herbal plants showed an acceptable antibacterial efficacy against oral streptococci.*

Key words: *Pomegranate extract, grape seed extract, guava extract, antibacterial*

INTRODUCTION

A global change in thinking has led to an inclination towards "going natural "and Indian civilization is prosperous in resources carrying medicinal values. The use of herbs and plants in curing certain diseases dates back to 1860 AD. Strong evidence in favour of allopathic medicine has overpowered the use of natural products; however, it is now re-explored with abundant scientific researches yielding evidence in its favor.

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Literature reveals use of plant and plant products for prevention of dental caries owing to their antimicrobial properties[1, 2]. Herbal extracts of *Emblica officinalis*[3] (Indian gooseberry), *Terminalia Chebula*[3] *Azadirachta indica*[4] (Neem), Propolis[5], *Ocimum sanctum* (Tulsi)[6], *Camellia sinensis*[7] (Green Tea), *Vaccinium macrocarpon*[8] (Cranberry) has been entitled as an effective antimicrobial and anti plaque agents. Mouthwash preparations containing *Punica granatum*[9] (Pomegranate), *Psidium guajava*[10] (Guava) and *Vitis vinifera*[11] (Grape) extracts as ~~an~~ active ingredients had been extensively investigated and certified as ~~an~~ effective anti microbial and anti plaque agents.

Regardless of the fact that enormous investigations has been carried out to explore the anti microbial property of these plant extracts, the effects of these extracts need to be investigated clinically. The present study aims to investigate and compare the decrease in total oral streptococci count after using 50% *Punica granatum* (pomegranate seed), 12.5% *Vitis vinifera* (grape seed) and 25% *Psidium guajava* (guava seed) in mouthwash formulations at the end of 48 hrs and 7 days after, twice a day usage in 8-10 years of children.

MATERIALS AND METHOD

The study was conducted in two parts *in vitro* mouthwash preparation and in vivo effect of the mouthwash on *Streptococcus mutans* count of the caries susceptible individuals. The ethical clearance of the study was obtained from the Institutional Review Board, and

an informed consent was obtained from the parents/ local guardian of the subjects. This study was conducted in accordance with the Declaration of Helsinki and with principles of good clinical practice.

Seeds of pomegranate, grape and guava were subjected to shade drying and grounded into fine powder. Soxhlet extraction with 200 ml of pure distilled water was separately done using 75 grams of dried powder at 80°C by Mantoux heater for recycling of the solvent, to obtain the aqueous extracts of respective seeds. After complete phytochemical exhaustion of the extracts in round bottom flask, they were transferred into clean and pre-weighed universal tubes. The tubes were then stored at 4-8 degree Celsius in refrigerator and percentage yield(where percentage yield means percentage extracted from 75gms of plant extract) was calculated as dividing initial weight of raw material taken by final weight of extracts.

$$\text{Percentage yield} = \frac{\text{n grams of extracts} \times 100}{\text{75 grams of powder}}$$

Where n indicates the final weight of pomegranate, grape and guava extract (Table No 1).

Minimal inhibitory concentration determination for extracts

1gm of aqueous extract of pomegranate, grape and guava was dissolved in 10 ml of distilled water separately, in this way an extract concentration of 100 mg per ml was obtained. Afterwards each stock extract was passed from 0.22 micron syringe filter to get sterile solution. To prepare 5 dilutions of three extracts, 0.5 ml of extract was taken in sterile micro-tube, which was then diluted with distilled water in ratio of 1:1, 1:2, 1:3, 1:4 and 1:5, this yielded serially diluted concentrations of 100, 50, 25, 12.5 and 6.25 mg/ml for each extract. Pure strains of *Streptococcus mutans* (MTCC 497) were obtained from microbial type culture collection (MTCC) Chandigarh; MTCC culture of *S. mutans* was inoculated in the brain heart infusion broth and incubated for 24 hrs at 37 ^{0}C for the proper growth of microbes. After that it was sub cultured on 5ml of Nutrient broth for the antimicrobial purpose and incubated for 24 hrs at 37 ^{0}C for the proper growth of microbes. After the incubation, swab was dipped into the broth; excess fluid was driven out against the side of tube and spread over the surface of agar plate (Muller Hinton Agar Media). The wells were then punched on agar plate with a diameter of 6 mm and stock extracts were taken into the micropipette and 20 micro litre of each extract was discharged onto the well, incubated at 37°C for 48 hours. After incubation, appearance of zones of inhibition (that is an area of an agar plate where the growth of an organism is prevented by Pomegranate, Guava and grape seed extract on the agar surface) was measured using vernier calliper. The MIC for pomegranate, guava and grape seed was achieved at 50%, 12.5% and 25% respectively.

Preparation of mouthwash

Based upon the results obtained from the antimicrobial activity i.e. MIC, a weighted amount of extract was taken in sterile beaker and 100ml of distilled water was mixed, thereafter the beaker was placed on hot plate magnetic stirrer at 60degree Celsius to dissolve the extract in solvent till a homogenous solution was obtained, thus obtained solution was mixed with 900ml distilled water to make a final volume of 1000ml in a clean sterile measuring cylinder. The solution was then transferred to sterile plastic bottle and labelled; the same procedure was done for both the extracts. The final extract was stored in amber coloured bottle under refrigeration for further use.

In vivo Sample Collection and Microbial analysis:

40 subjects were recruited from a local boarding school and selected patients were free from any systemic or contributory disease and were blinded to the type of mouthwash assigned. The volunteers who had one or more active carious lesion or a frank cavitations, were considered to be at high risk for dental caries were included in the study and those who were on antibiotic therapy for 5 consecutive days, corticosteroid therapy for 30 days were excluded. History of sensitivity to mouthwash, or patients wearing any kind of prosthesis, removal or fixed orthodontic appliance, or history of professional cleaning in the last 15 days were also excluded owing to the fact that they can lead to false positive results . A total of 40 subjects, aged between 8-10 years (mean age: 9.7± 1.3) who satisfied the selection criteria were included in the study. They were randomly allocated in to 4 groups containing 10 subjects each (n=10): Group A- 50 % Pomegranate mouthwash; Group B- 12.5% Grape seed mouthwash; Group C- 25% Guava mouthwash; Group D- Distilled water.

Before commencing the study, a new set of manual brushing aid was given to subjects and baseline unstimulated salivary samples were collected. The subjects were asked to rinse with their assigned mouthwash twice a day under the supervision of warden. They were instructed to retain the mouth rinse for 1minute before expectorating it, and not to consume any food or drink for 30 minutes after the use of mouthwash. A compliance diary was maintained and repeated reminders were placed to the warden. Collection of salivary sample was handled by a single investigator and analysis of salivary sample was done by a different investigator who was blinded to the study.

On the assigned day, to establish a base line data whole unstimulated saliva sample was collected for this purpose and all subjects were refrained from food, drinks, and tooth-brushing for at least one hour. The unstimulated saliva was then again allowed to pool for at least 5 minutes, was collected and analysed for oral streptococci count at 48 hrs and 7th day. 1 ml of salivary sample was diluted to 10 folds and 0.01 ml of sample was inoculated for the CFU

Table 1: percentage yield of final extract

Material	Solvent Used	Appearance of extract	Weightof Material Used	Weight of Extracted Phytochemical	Percentage Yield
Pomegranate	Distilled Water	Reddish brown paste	75 gm	21.6 gm	28.8
Guava	Distilled Water	Yellowish paste like	75 gm	12.7 gm	16.93
Grape	Distilled Water	Greenish paste	75gms	11.4gm	15.2

determination for oral streptococci using Mitis Sanguis Bacitricin agar (Gold et al 1973) and incubated for 48 hrs at 37°C. Salivary streptococci colonies were counted using a colony counter and were multiplied to 10^3 by a professional microbiologist who was blinded to the study.

Statistical Analysis

The Data collected (CFU/ml) was quantitative in nature and Shapiro-Wilk test showed that data followed normal distribution in all the mouthwash/distilled water groups for all the time intervals. Hence parametric test(Two- Way Analysis of variance) Anova was used for analysis considering the fact that, there were two independent variables, firstly groups (Pomegranate mouthwash, Grape seed mouthwash, Guava mouthwash, Distilled water) and secondly time Interval (Baseline, after 48 hours, after 7 days). The dependent variable in the study was CFU/ml.

The results were tabulated and analysed statistically using repeated measures two way ANOVA. Tukey`s LSD post hoc test (SPSS version 14) was applied since ANOVA showed significant results to find out the comparison between/within groups and time interval. The statistician was blinded to group allocation and probability (P) value of 0.05 was considered to be statistically significant.

RESULTS

The subjects chosen were between age group of 8-10 years with mean age of 9.7± 1.3, out of which 26 were males and 14 were females. Table 2 shows mean and standard deviation of different groups at different time intervals. It shows that when two way ANOVA (F Value) was applied, the mean difference between CFU/ml of different groups was significant ($F = 219.932$, $p < 0.001$). Also, the effect of time Interval on CFU/ml was significant ($F = 8327.407$, $p < 0.001$). The mean CFU/ml differ significantly at different time intervals.

Table 3 shows interaction effect comparison of CFU/ml at different time intervals in groups. When LSD post hoc test was applied it showed that in Pomegranate, Grape seed and Guava mouthwash groups mean CFU/ml at baseline was significantly higher than after 48 hours and after 7 days count. Mean CFU/ml after 48 hours was significantly higher than after 7 days count. Highest CFU/ml was observed at baseline and after 7 days count was lowest. In distilled water group, there was no significant difference between CFU/ml at baseline, after 48 hours and after 7 days.

Table 4 shows Comparison of different groups at specific time intervals showed that at baseline, difference in mean CFUs/ml in all four groups (Pomegranate mouthwash, Grape seed mouthwash,

Table 2: Mean and standard deviation (Mean ± SD) of CFU/ml of four study groups at different time intervals.

Groups	Time interval (Mean ± SD of CFU/ml)		
	Baseline	After 48 hours	After 7 days
Pomegranate mouthwash	238.30 ± 5.70	202.70 ± 5.56	164.00 ± 5.70
Grape seed mouthwash	239.80 ± 4.26	187.30 ± 3.86	134.00 ± 4.74
Guava mouthwash	239.70 ± 4.57	219.90 ± 4.25	197.50 ± 4.45
Distilled water	236.30 ± 5.25	237.40 ± 4.99	236.10 ± 4.28
Two way anova(F Value) (within the groups) F=219.932, (p=0.000)	**Two way anova (at different time interval) F=**8327.407, (p=0.000)		

Table 3: Comparison of different time intervals in specific (Pomegranate, Grape seed, Guava and Distilled water) groups using LSD post hoc test

Groups	Comparison b/w time intervals	Mean difference	P Value
Pomegranate	Baseline and after 48 hours	35.600	0.000
	Baseline and after 7 days	74.300	0.000
	After 48 hours and after 7 days	38.700	0.000
Grape seed	Baseline and after 48 hours	52.500	0.000
	Baseline and after 7 days	105.800	0.000
	After 48 hours and after 7 days	53.300	0.00
Guava	Baseline and after 48 hours	19.800	0.000
	Baseline and after 7 days	42.200	0.000
	After 48 hours and after 7 days	22.400	0.000
Distilled water	Baseline and after 48 hours	-1.100	0.221
	Baseline and after 7 days	0.200	0.842
	After 48 hours and after 7 days	1.300	

Guava mouthwash, and Distilled water) were statistically not significant. After 48 hours and after 7 days, mean CFU/ml in Distilled Water was significantly($p \leq 0.05$) higher than Guava mouthwash followed by Pomegranate mouthwash and Grape mouthwash. Highest CFU/ml was observed in distilled water and least count was observed with Grape mouthwash.

Table 4: Compares different mouthwash groups at specific time (baseline, after 48 hours and after 7 days) using LSD post hoc test

Time interval	Comparison B/w groups	Mean difference	P Value
At Baseline	Pomegranate and Grape seed	-1.50	0.505
	Pomegranate and Guava	-1.40	0.533
	Pomegranate and Distilled water	2.00	0.375
	Grape seed and Guava	0.10	0.964
	Grape seed and Distilled water	3.50	0.125
	Guava and Distilled water	3.40	0.135
After 48 hours	Pomegranate and Grape seed	15.40	0.000
	Pomegranate and Guava	-17.20	0.000
	Pomegranate and Distilled water	-34.70	0.000
	Grape seed and Guava	-32.60	0.000
	Grape seed and Distilled water	-50.10	0.000
	Guava and Distilled water	-17.50	0.000
After 7 days	Pomegranate and Grape seed	30.00	0.000
	Pomegranate and Guava	-33.50	0.000
	Pomegranate and Distilled water	-72.10	0.000
	Grape seed and Guava	-63.50	0.000
	Grape seed and Distilled water	-102.10	0.000
	Guava and Distilled water	-38.60	0.000

DISCUSSION

The present study was conducted to assess and compare the mean reduction in total oral streptococci count after rinsing with herbal mouthwash prepared from dried seeds of *Punica granatum*, *Psidium guajava* and *Vitis vinifera* for 48 hours and 7 days. Aqueous extracts of the plant seeds were used instead of ethanolic extract because this study aimed at determining the antimicrobial activity in the pure forms of extract. Moreover, ethanol has got its own antimicrobial activity. A significant reduction in the CFU/ml was seen following the use of *Punica granatum* and *Psidium guajava* mouthwash at the end of 7 day use (Table 3).

Pomegranate (*Punica granatum*) seeds have been deemed as "an ancient seed for modern cure"[12], because of its anti inflammatory, anti microbial, anti fungal and anti mutagenic properties. Owing to these properties it has a substantial application in the field of medicine. The active components, including polyphenolic flavonoids (e.g., punicalagins and ellagic acid), are believed to prevent gingivitis through a number of mechanisms including reduction of oxidative stress in the oral cavity[13], direct antioxidant activity; anti-inflammatory effects[14], antibacterial activity[15] and direct removal of plaque from the teeth[16].A significant reduction in the oral streptococci counts were seen after rinsing with 50% pomegranate mouthrinse for 7 days in the presnt study. A high sensitivity against mutans streptocci has been reported[17] as it reduces its adherence to the tooth surface. In a recent *in vitro* study, pomegranate pulp extract gel showed highly significant inhibitory effect at 5%, 25%, 50% and 100% against *S. mutans* when compared to aloe vera and sorbitol. This action is probably due to the antimicrobial property of tannins[18]. Pomegranate extract suppresses the ability of these microorganisms to adhere to the surface of the tooth.[19]In fact, a recent study conducted by Brazilian researchers showed that pomegranate extract was more effective against the adherence of biofilm microorganisms and they suggested that "this phytotherapeutic agent might be used in the control of adherence of different microorganisms in the oral cavity."[19]To scrutinize anti gingivitis effect of pomegranate mouthrinse, a clinical trial report concluded that it causes a decreased total protein (which can correlate with plaque forming bacteria readings), diminished activity of aspartate aminotransferase (an indicator of cell injury), attenuated alpha Glucosidase activity (a sucrose degrading enzyme) and enhanced anti Oxidant properties[20].

Vitis vinifera has a variety of health benefits, which includes treating migraine, Alzeihmer`s disease and is a potential antioxidant. In vitro studies have suggested the use of grape seed extract as a potential remineralizing agent[21]. A significant reduction in the oral streptococci count was seen following the use of 12.5% *Vitis vinifera* extract mouthwash in the present study. This finding is in accordance with a previous study conducted by Mirkarimi et al[22]. Previous in vitro studies have claimed its anti microbial activity against various anaerobic bacterias- associated with periodontal diseases[23], and against oral streptocci[24]. Its anti microbial activity is attributed to the presence of active phenolic compounds. The effects of the grapeseed extracts on adhesion of *S. mutans* were studied using adhesion of cells to glass in a study conducted by Smullens et al[25]. The results showed that polyphenol-containing extracts inhibited *S. mutans.* Both red and green grape extracts had higher activity than the other fruits tested, and extracts of grape seed were more active than those from grape skins. The highest activity was P70 extract from red grape seeds and the flavan-3-ol and procyanidin oligomer content of grapes can vary with the variety, degree of maturity and part of the fruit studied. The skins of red and white grapes contain higher concentrations of catechin and epicatechin whilst grape seeds contain higher concentrations of oligomeric procyanidins[26].This is consistent with the results obtained here as the green grape seeds used in the study were effective at lower concentrations of 12.5% as compared to pomegranate (50%) and guava extracts(25%).

Guava (*Psidium Guajava Linn)* sticks have been used since ancient times for efficient cleaning of teeth, and its activity is attributed to the presence of bioactive compounds comprised of saponins, tannins, flavanoids and alkaloids. A decoction of the guava root-bark is recommended as a mouthwash for swollen

gums and decoction of the leaves makes an efficacious gargle for swollen gum and ulceration of the mouth and also for bleeding gum[27].Guaijaverin, which is an active compound present in leaves of guava, has been found to actively inhibit the growth of oral streptocci[28]. Its antimicrobial activity against *Staphylococcus aureus, E.coli*, *Candida albicans* and *Streptococcus mutans* has labelled guava an effective anti caries agent.

There are many studies in the literature addressing the plant extract benefits as mouthwashes in periodontal conditions and explaining the effects of individual mouthwashes. However, no earlier studies had attempted, this study compared the three mouthwashes using popular herbal extracts as anticaries agent to be used in younger age group with effective concentration, acceptable taste and minimum side effects. As a preliminary study, we chose to keep a shorter period of evaluation. Besides, the concern of increasing adaptive response of *Streptococcus mutans to* oral products led us to our thinking to keep the short evaluation period[29]. Although this can be considered as drawback, the horizons can be broaden in further studies involving same/different herbal products.

CONCLUSION

The aqueous extracts of *Punica granatum, Vitis vinifera* and *Psidium guajava* have shown anti- streptococci results and as pediatric dentists we will be able to deliver best to the children with minimal side effects. This calls for further investigation to increase there substantivity for its use in pediatric dentistry, especially in this era when holistic integrated medicine approach is the need of the hour and going herbal is one of the ways to accomplish this goal.

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