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Antimicrobial properties of pomegranate and guava mouthwashes against cariogenic microorganisms

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Abstract:
Herbal mouthwashes may provide acceptable antimicrobial alternatives for children requiring repeated oral rinsing. Therefore, it is of interest to evaluate the pomegranate and guava mouthwashes against 0.12% chlorhexidine in 45 healthy children aged 6–12 years. Salivary microbial colony-forming units were assessed at baseline, 5 minutes and 3 hours after rinsing, while plaque index was recorded at baseline and after 21 days. All mouthwashes significantly reduced microbial counts and plaque scores, with guava producing the lowest post-rinse microbial counts; however, plaque reduction did not differ significantly among groups. Thus, pomegranate and guava mouthwashes showed effective antimicrobial activity, with guava showing the greatest short-term microbial reduction.

Keywords: Anti-bacterial agents, child, dental caries, dental plaque, plant extracts

Background:
Dental caries is a common preventable disease in children **[1]**. Despite numerous advances in technology, it is still a major public health problem globally **[2]**. Dental caries is a biofilm-mediated disease characterized by a shift in the oral microbiota towards acid-producing and acid-tolerant species **[1]**. Previously, caries were associated with *Streptococcus mutans* and *Lactobacillus*, but recent studies have implicated numerous other species in the cariogenic process **[3]**. Thus, in addition to removing plaque mechanically and the consumption of a proper diet, methods that reduce the cariogenic load of oral biofilm are also needed. Chlorhexidine has long been used as an antimicrobial mouth rinse in dentistry because of its broad antibacterial activity and substantively. However, various adverse effects are associated with its long-term use, including staining of the teeth and tongue, mucosal irritation and desquamation and an altered sense of taste **[4]**. Consequently, it leads to reduced patient acceptance, particularly in children, owing to its unpleasant taste. As a result, there has been a search for natural products that have a potent antimicrobial action and are safer than conventionally used mouthwashes. Pomegranate (*Punica granatum Linn*) and guava (*Psidium guajava Linn*) are two herbal agents that have been scientifically evaluated for their oral uses. They are high in phytochemicals and studies have shown that pomegranate peel is rich in anthocyanins, polyphenols, tannins and other flavonoids **[5]**. The major component, ellagic acid and its derivatives interfere with bacterial functions such as adhesion and biofilm formation. There are also reports on the inhibitory effects of extracts from leaves of guava on the growth of various bacteria including oral pathogens such as *Streptococcus mutans* **[6]**. However, more studies are required to establish evidence because considerable controversies exist regarding the type of extract and its concentration. Studies previously conducted to assess the effects of pomegranate and guava has been mostly *in vitro* studies **[7-9]**. Therefore, it is of interest to compare the antimicrobial efficacy of mouthwashes prepared from methanolic extracts of pomegranate fruit peel and guava leaves with that of 0.12% chlorhexidine mouthwash by assessing salivary colony-forming units and plaque index scores in children.

Materials and Methods:
Study design and setting:
The present double-blind *in vivo* comparative study was conducted from November 2022 to July 2023. Ethical clearance was obtained from the Institutional Ethical Committee. The study procedure was explained to the parents or guardians of the participating children and informed consent was obtained before enrolment. Consent/assent was also obtained from children above 8 years of age. The study was carried out in four sequential stages: preparation of plant extracts, determination of minimum inhibitory concentration (MIC), formulation of herbal mouthwashes according to MIC values and *in vivo* comparison of the prepared herbal mouthwashes with 0.12% chlorhexidine mouthwash.

Preparation of plant extracts:
Pomegranate fruit peels and guava leaves were used for the preparation of the herbal extracts. The plant materials were authenticated by the National Botanical Research Institute, Lucknow. After authentication, the materials were cleaned, dried and pulverized. Methanolic extracts of pomegranate fruit peel and guava leaves were prepared using the continuous hot percolation method in a Soxhlet apparatus.

Determination of minimum inhibitory concentration:
The MICs of both plant extracts were determined before formulation of the mouthwashes. MIC is defined as the lowest concentration of an antimicrobial agent that inhibits visible

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microbial growth within a defined period. The MIC values of the methanolic extracts were assessed against *Streptococcus mutans* and *Lactobacillus acidophilus* using the agar dilution method. Based on this assessment, the MIC was determined to be 8 mg/mL for the pomegranate fruit peel extract and 16 mg/mL for the guava leaf extract.

Formulation of mouthwashes:
The herbal mouthwashes were formulated according to the MIC values obtained for the respective plant extracts. The pomegranate mouthwash was prepared using the methanolic extract of pomegranate fruit peel at 8 mg/mL, while the guava mouthwash was prepared using the methanolic extract of guava leaves at 16 mg/mL. A commercially available 0.12% chlorhexidine mouthwash was used as the active comparator. The mouthwashes used in the present study are depicted in **Figure 1.**



Figure 1: Mouthwashes used in the present study

Study participants and group allocation:
A total of 45 clinically healthy children aged 6–12 years were selected for the *in vivo* phase of the study. The participants were randomly allocated into three equal groups, with 15 children in each group. Group I received 0.12% chlorhexidine mouthwash, Group II received pomegranate mouthwash and Group III received guava mouthwash. The participants were instructed to use the assigned mouthwash once daily for 21 days. To reduce assessment bias, salivary samples were coded before microbiological evaluation.

Outcome measures:
The primary outcome was the change in salivary oral microflora count, measured as colony forming units per millilitre (CFUs/mL), before mouthwash use, 5 minutes after use and 3 hours after use. The secondary outcome was the change in plaque index scores from baseline to 21 days after use. The antimicrobial efficacy of the three mouthwashes was assessed by measuring the salivary oral microflora count. Salivary samples were collected from each participant at three time points: before the use of mouthwash and 5 minutes and 3 hours after use. The microbial count was expressed as CFUs/mL. The change in CFU/mL from baseline to the two post-rinse time points was used to assess the immediate and short-term antimicrobial effects of all mouthwashes. Plaque accumulation was assessed using the Silness and Löe Plaque Index. The plaque index score was recorded at baseline before the start of mouthwash use and again after 21 days of daily mouthwash use. The change in plaque index score was used to evaluate the plaque-reducing effect of each mouthwash over the study period.

Statistical analysis:
The collected data were tabulated and subjected to statistical analysis. Descriptive statistics were expressed as mean, standard deviation, 95% confidence interval, minimum and maximum values. Intergroup comparison of salivary CFU/mL counts and plaque index scores was performed using analysis of variance. Within-group comparison of CFU/mL counts and plaque index scores before and after intervention was performed using the paired t-test. Tukey's honestly significant difference post-hoc test was used for pairwise comparison between the groups. A p-value less than 0.05 were considered statistically significant.

Results:
A total of 45 children were included in the study, with 15 participants in each group. The baseline salivary CFU counts were comparable among the chlorhexidine, pomegranate and guava groups. Baseline plaque index scores were also comparable among the three groups, indicating that the groups were similar before intervention with respect to microbial load and plaque accumulation. The mean salivary CFU count reduced in all three groups after mouthwash use. Intergroup comparison showed a significant difference in CFU count between 5 minutes and 3 hours after rinsing. The guava group showed the lowest mean CFU values at both post-rinse intervals, followed by the pomegranate and chlorhexidine groups **(Table 1).** Values are presented as mean ± standard deviation; *statistically significant at $p<0.05$; ANOVA, analysis of variance; CFUs - colony-forming units The plaque index scores reduced in all three groups after 21 days of mouthwash use. However, the intergroup comparison showed no statistically significant difference in plaque index scores among the three groups either at baseline or after 21 days. Although the guava group showed the lowest mean plaque index score after 21 days, this difference was not statistically significant **(Table 2).** Values are presented as mean ± standard deviation; ANOVA - analysis of variance Intra-group analysis showed a significant reduction in salivary CFU count in all three groups at both 5 minutes and 3 hours after mouthwash use compared with that at baseline. The plaque index also reduced significantly within each group after 21 days. The greatest mean reduction in plaque index was observed in the guava group, followed by that in the chlorhexidine and pomegranate groups **(Table 3).** Tukey's post hoc analysis showed that baseline CFU counts were comparable among all three groups. At 5 minutes after mouthwash use, the guava mouthwash showed significantly lower CFU count than the chlorhexidine mouthwash. At 3 hours after use, the guava

mouthwash showed significantly lower CFU count than both chlorhexidine and pomegranate mouthwashes. Chlorhexidine and pomegranate mouthwashes remained comparable at all CFU time points. For plaque index scores, all pairwise comparisons were non-significant both before intervention and after 21 days **(Table 4).**

Table 1: Intergroup comparison of salivary oral microflora count before and after mouthwash use

Time point	CFUs/mL (Chlorhexidine group)	CFUs/mL (Pomegranate group)	CFUs/mL (Guava group)	Test statistic (ANOVA)	p-value
Before mouthwash use	14.26 ± 5.09	12.72 ± 6.68	13.63 ± 8.75	F(2,42)=0.18	0.834
5 minutes after use	1.74 ± 1.85	1.36 ± 1.01	0.34 ± 0.23	F(2,42)=6.32	0.004*
3 hours after use	2.02 ± 2.00	1.76 ± 0.95	0.41 ± 0.24	F(2,42)=3.63	0.035*

Table 2: Intergroup comparison of plaque index scores before and after 21 days of mouthwash use

Time point	Plaque index scores (Chlorhexidine group)	Plaque index scores (Pomegranate group)	Plaque index scores (Guava group)	Test statistic (ANOVA)	p-value
Before mouthwash use	0.91 ± 0.16	0.87 ± 0.16	0.86 ± 0.13	F(2,42)=0.52	0.596
21 days after use	0.63 ± 0.18	0.63 ± 0.18	0.54 ± 0.10	F(2,42)=1.77	0.183

Table 3: Intra-group comparison of salivary CFU count and plaque index

Group	Outcome comparison	Baseline mean ± SD	Follow-up mean ± SD	Mean reduction	Test statistic	p-value
Chlorhexidine	CFUs before vs. after 5 minutes	14.26 ± 5.09	1.74 ± 1.85	12.52	—	<0.001*
Chlorhexidine	CFUs before vs. after 3 hours	14.26 ± 5.09	2.02 ± 2.00	12.24	—	<0.001*
Chlorhexidine	Plaque index score before vs. after 21 days	0.91 ± 0.16	0.63 ± 0.18	0.29	t(14)=7.34	<0.001*
Pomegranate	CFUs before vs. after 5 minutes	12.72 ± 6.68	1.36 ± 1.01	11.36	—	<0.001*
Pomegranate	CFU before vs. after 3 hours	12.72 ± 6.68	1.76 ± 0.95	10.96	—	<0.001*
Pomegranate	Plaque index scores before vs. after 21 days	0.87 ± 0.16	0.63 ± 0.18	0.24	t(14)=7.30	<0.001*
Guava	CFUs before vs. after 5 minutes	13.63 ± 8.75	0.34 ± 0.23	13.29	—	<0.001*
Guava	CFUs before vs. after 3 hours	13.63 ± 8.75	0.41 ± 0.24	13.22	—	<0.001*
Guava	Plaque index score before vs. after 21 days	0.86 ± 0.13	0.54 ± 0.10	0.32	t(14)=14.45	<0.001*

**Statistically significant at p<0.05; paired t-test; SD - standard deviation; CFUs - colony-forming units.*

Table 4: Tukey post hoc pairwise comparison between groups

Outcome/time point	Chlorhexidine vs. pomegranate	Chlorhexidine vs. guava	Pomegranate vs. guava
CFU before mouthwash use	q=0.85; p=0.821	q=0.35; p=0.967	q=0.50; p=0.933
CFU 5 minutes after use	q=1.20; p=0.674	q=4.41; p=0.009*	q=3.21; p=0.071
CFU 3 hours after use	q=0.80; p=0.840	q=4.83; p=0.004*	q=4.07; p=0.017*
Plaque index score before use	q=1.07; p=0.730	q=1.38; p=0.597	q=0.31; p=0.974
Plaque index score 21 days after use	q=0.15; p=0.994	q=2.23; p=0.268	q=2.23; p=0.268

**Statistically significant at p<0.05; Tukey's honestly significant difference post hoc test; CFU - colony-forming units.*

Discussion:

The present study found that all three mouthwashes reduced salivary CFU counts shortly after rinsing and reduced plaque index scores after 21 days of use. The particular follow-up period of three weeks was selected because most of the mouthwashes are prescribed for this duration in routine clinical practice **[10].** An important finding was the difference in pattern between the microbial and plaque outcomes. The CFU count of guava mouthwash was lowest at 5 minutes and at 3 hours. The CFU count of guava mouthwash showed a significantly greater reduction than that of chlorhexidine mouthwash at both time intervals and then that of pomegranate mouthwash at 3 hours. Plaque index scores with all three mouthwashes were found to be comparable and there was no significant difference between the three groups. Thus, although the guava mouthwash had better antimicrobial properties, all three mouthwashes were found to be equally effective in reducing plaque. Chlorhexidine mouthwash with substantively provides strong and long-lasting antibacterial effects. It has been emphasized that only the ability of a mouthwash to kill the bacteria should not be revered, because injudicious exposure to antimicrobial compounds can alter the balance of the microbiome in the oral cavity **[4, 11]**. Hence, such mouthwashes must be used cautiously in vulnerable populations, including children. Previous studies have supported the potential of herbal oral care products in reduction of plaque and gingivitis, with effectiveness varying for different preparations **[10]**. Chatzopoulos *et al.* reported that herbal products used as adjuncts may show outcomes comparable to chlorhexidine or conventional products in periodontal care, while emphasizing the need for better-designed trials **[12]**. Another recent overview of herbal oral rinses reported that while some herbal rinses have a positive effect on reducing plaque and gingivitis, most of the supporting evidence is limited by *in vitro* study designs and short trial

durations **[13]**. Ravisankar *et al.* also showed antimicrobial activity of herbal preparations incorporating pomegranate and guava against multiple test microorganisms using an *in vitro* disc-diffusion model **[14]**. However, the multicomponent formulation and laboratory-based design of that study limit direct comparison with the present clinical findings. Thus, the level of reduction of antimicrobial effects provided by pomegranate and guava mouthwashes indicates that they could be of value as adjuncts to oral cleaning in the prevention of oral diseases in such populations. Being natural substances, they are relatively safer and free of adverse effects. The results of pomegranate mouthwash over the 21-day study period were found to be in agreement with the findings of a recent study **[15]**. Their findings reported that 30% pomegranate mouth rinse reduced plaque, gingival inflammation and salivary bacterial count over a similar 21-day period. A recent systematic review also concluded that pomegranate preparations can reduce the count of caries-associated bacteria, although the evidence is limited by variation in formulation and study design **[16]**. The antimicrobial effect of pomegranate is biologically reasonable because the peel contains polyphenols, tannins, flavonoids, ellagic acid derivatives and punicalagin. These compounds can interfere with bacterial adhesion, cell membrane integrity and biofilm activity **[5, 17]**. Further experimental evidence has been reported by Ingle *et al.,* who observed concentration-dependent inhibition of *S. mutans* by pomegranate extract, with a mean inhibition zone of 17.8 ± 1.1 mm at 75% concentration **[18]**. Chlorhexidine showed greater activity under the same in vitro conditions, indicating that the antimicrobial effect of pomegranate is evident, although its relative efficacy may vary according to formulation and experimental conditions **[18]**. In the present study, pomegranate was comparable to chlorhexidine in CFU reduction, but it did not match the lower 3-hour CFU values seen with guava. Guava mouthwash showed the strongest short-term antimicrobial activity in this study. This finding is in line with the randomized clinical trial by Kassem *et al.*, where guava leaf extract mouthwash showed antibacterial activity against *Streptococcus mutans* in high-caries-risk patients **[19]**. The stronger reduction seen with guava may be due to flavonoids, tannins, phenolic acids and quercetin-like compounds present in guava leaves, which have been reported to inhibit bacterial growth, reduce adherence and affect microbial cell wall integrity **[8].** The present finding that guava maintained lower CFU values even at 3 hours suggests that its effect was not limited to immediate dilution or rinsing action alone. Clinically, the findings support the use of pomegranate and guava mouthwashes as possible adjuncts to routine oral hygiene, not as replacements for brushing, fluoride exposure, dietary counseling, or professional preventive care. Guava may be more useful where a short-term reduction in salivary microbial load is desired, while pomegranate also showed consistent antimicrobial and plaque-reducing activity. However, the lack of a significant intergroup difference in plaque index means that the clinical advantage of guava should be limited to the microbial outcome in this study. The limitations of this study include the small sample size, short follow-up period and use of total salivary CFU rather than species-specific microbial quantification during the *in vivo* phase. Salivary microbial counts may not fully represent organisms protected within the mature dental plaque biofilm. Compliance, taste acceptability, adverse effects, formulation stability and active phytochemical concentration were also not evaluated. Future studies should use larger randomized designs, longer follow-up, standardized herbal formulations, species-specific microbial analysis, salivary pH assessment and clinical caries-risk outcomes. Within these limitations, pomegranate and guava mouthwashes showed considerable antimicrobial activity in children, with guava showing the greatest short-term reduction in salivary microbial counts and all three mouthwashes showing comparable plaque reduction over three weeks.

Conclusion:

The mouthwash solutions of pomegranate and guava have been found to possess antimicrobial activity against the salivary oral microflora of children. Guava mouthwash exhibited the maximum reduction in microorganisms temporarily, whereas the data on permanent plaque reduction after 21 days were found to be comparable for guava and pomegranate mouthwashes with chlorhexidine. Thus, these herbal mouthwash solutions can be used as an adjunct to conventional oral hygiene measures.

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