Comparative Evaluation of Antimicrobial Properties of Pomegranate Peel Extract Against *Streptococcus Mutans* And *Lactobacillus* - An In Vitro Study

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Abstract

**Background:** Dental caries is an infectious disease influenced by cariogenic microorganisms. *Streptococcus mutans* is associated with the initiation of caries, while lactobacilli with disease progression. Chlorhexidine is used as a gold standard mouthwash but with some side effects such as staining on long-term use. Nature itself is the best physician; it helps in treating oral health. Medicinal plants such as neem, tulsi, clove oil, triphala, and many more have been used in dentistry since ages to treat oral health problems such as bleeding gums, halitosis, and caries. One of them is pomegranate. It is the oldest edible fruit and considered as "a pharmacy unto itself." Pomegranate has phenolic acids, flavonoids, and tannins in its different parts. Hydrolysable tannins called punicalagins which have free scavenging properties are the most abundant polyphenols found in pomegranate containing mouthwash. It has anticariogenic, anti-inflammatory, and antimicrobial properties.

**Aim:** The aim of this study is to evaluate the antimicrobial efficacy of pomegranate peel extract, Hiora, and chlorhexidine.

**Materials and Methods:** In this study, three different mouthwashes were used and divided into four groups as follows: Group A as chlorhexidine mouthwash (Hexidine), Group B as herbal mouthwash (Hiora), Group C as pomegranate peel extract 100% concentrated, and Group D as pomegranate peel extract 75% concentrated. Reference strains of *S. mutans* and *Lactobacillus acidophilus* were selected as it plays an important role in caries initiation and progression. *S. mutans* and *Lactobacillus* were grown in blood agar plates. For agar well diffusion method, wells of approximately 6-mm diameter and 4 mm in depth were made using a cork. In those wells, mouthwashes were added. Plates were incubated for 48 h at 37°C. The zone of inhibition was measured with the help of Vernier caliper.

**Results:** The zone of inhibition of the solutions against *S. mutans* and *Lactobacillus* shows a statistically significant *P* < 0.001** with *f* value of 1419.377 and 164.916, respectively.

**Conclusion:** The pomegranate peel extract and Hiora show antimicrobial activities, and hence, they can be replaced for the long-term use to avoid the side effects of chlorhexidine mouthwash.

**Clinical significance:** The pomegranate peel extract is a natural product with the antibacterial property; hence, it can be easily incorporated into oral hygiene measures such as mouthwashes or toothpastes.

### Keywords:
Chlorhexidine, Hiora, *Lactobacillus acidophilus*, pomegranate peel extract, *Streptococcus mutans*, zone of inhibition

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**Introduction**

The tooth surface is unique in that it is the only body part that is not subjected to metabolic turnover. It is, however, subjected to various infections due to factors that favour microbial growth.[1] This microbial growth leads to one of the most widespread diseases of the tooth, i.e., dental caries. Dental caries is an infectious disease influenced by factors such as the relationship between diet and cariogenic microorganisms in the oral cavity and the characteristics of the host.[2] Caries begins in childhood and eventually affects 90% of adults. Even so, dental decay’s effect on low-income individuals is disproportionate, leading to earlier onset, more affected teeth, complications, and ultimately teeth lost during adulthood due to caries.[3]
Streptococcus bacteria are mainly responsible for the initial phase of the caries lesion, especially in the enamel (initiation), whereas Lactobacillus is more involved with the progression of caries. Targeting Streptococcus mutans forms the most important measure for the prevention of dental caries. Current methods of caries management are limited to traditional preventive approaches in combination with restorative treatments. These methods have proved inadequate to control the disease. It is the most effective anti-plaque agent, but it is not a “magic bullet.” However, chlorhexidine has some side effects such as tooth staining on long-term use, taste disturbance, enhanced supragingival calculus formation, and desquamation of the oral mucosa. Hence, considering all the side effects, it suggests that there is a need for further research and introduction of new antibacterial agents that are strongly against S. mutans with minimal side effects on the oral tissues, especially in children.

Herbal medicines are an important source of nutrients that promote health. Various herbal products such as Propolis and Azadirachta indica have shown significant advantages in reducing signs of gingival and periodontal inflammation. The use of plants and their derivatives which possess preventive and therapeutic effects could contribute to the oral health. The pomegranate is also called as Punica granatum L. It is an age-old, distinguishing, and highly unique fruit. Pomegranate is considered as a member of two different species consisting of the Punicaceae decent. The pomegranate is widely found in Himalayas from India to Iran, but it is cultivated since primordial times in Mediterranean region. In Ayurvedic medicine, the pomegranate is considered “a pharmacy unto itself.” Numerous studies have been conducted on the antimicrobial properties of pomegranate peel and its curative effects. Among the different parts of the fruit, 50% of weight corresponds to the peel. The phenolic group found in the peel is the main contributors of the functional properties of pomegranate fruit. Pomegranate has some compound such as phenolic punicalagins, gallic acid, and fatty acids; catechin, epigallocatechin 3-gallate, quercetin, rutin; and other flavonols, flavones, flavanones, and anthocyanidins. Tannins from pomegranate fruit have been found to inhibit human salivary alpha-amylase, which catalyzes the hydrolysis of starch to oligosaccharides and binds to viridians streptococci and enamel, therefore, providing a substrate for cariogenic microbes. Thus, the aim of this study is to determine and compare the efficacy of pomegranate peel extract as a mouthwash against S. mutans and Lactobacillus.

Materials and Methods

The study protocol was approved by the Research Committee of Bharati Vidyapeeth Deemed University dental college and Hospital, Pune, Maharashtra, India. The study was carried out on two different microorganisms and three different mouthwashes. Pomegranate mouthwash was used in two different concentrations. The three mouthwashes were divided into four groups (n = 40) as follows:

- Group A (n = 10) as 0.12% chlorhexidine mouthwash
- Group B (n = 10) as an herbal mouthwash (Hiora mouthwash, The Himalaya Drug Company, Bengaluru, India)
- Group C (n = 10) as pomegranate peel extracts (100% concentration) [Figure 1]
- Group D (n = 10) as pomegranate peel extracts (75% concentration) [Figure 1].

0.12% chlorhexidine was considered as control and other three as test groups. Pomegranate peel extract mouthwash was used at a concentration of 100 ml and 75 ml/100 ml.

Preparation of pomegranate peel extract

Pomegranate peels were dried in sunlight and overnight in a hot air oven at 60°C for 7 days. The dried peels were powdered. The obtained powder was used to prepare an aqueous extract in Soxhlet extractor. After the use of Soxhlet, the obtained extract was used that is of 100% concentration and to obtain 75% of concentration, from 100% of pomegranate peel extract, 75-ml extract is taken, and in it, 25-ml distilled water was added.

Herbal mouthwash (Hiora)

The key ingredients of herbal mouthwash (Hiora) are Belleric Myrobalan (Bibhitaki) is an antimicrobial and antifungal agent that keeps infections away. Another ingredients include Betel (Nagavalli) leaf effectively stops halitosis, and it has mild stimulating properties which are beneficial for toothaches. Meswak (Salvadora persica) is widely accepted teeth cleaning agent. Pila is anticariogenic and eliminates toothache and halitosis.

Two different strains – S. mutans (MTCC No. 497) [Figure 2] and Lactobacillus acidophilus (MTCC No. 10307) [Figure 2] were grown in blood agar plates. For agar well diffusion method, four wells of approximately 6-mm diameter and 4-mm depth were made in the medium using a cork. Then, mouthwashes were added in those plates. Plates were incubated at 37°C for 48 h. The zone of inhibition was measured with the help of Vernier caliper in mm [Figure 3]. The experiment was performed and repeated under strict aseptic conditions to ensure the consistency of all findings.

Results

All the data were tabulated and subjected to the statistical analysis using ANOVA and two-sample t-tests.

When treated with chlorhexidine, herbal mouthwash, pomegranate peel extract 100%, and pomegranate peel extract 75%; the mean zone of inhibition against S. mutans is 15.92 mm, 30.15 mm, 17.47 mm, and 15.39 mm, respectively, as shown in Table 1 and Graph 1; and against lactobacillus, the mean zone of inhibition of these mouthwashes was 21.94 mm, 24.53 mm,
19.92 mm, and 17.31 mm, respectively, as shown in Table 2 and Graph 2. Herbal mouthwash had demonstrated a significant reduction in S. mutans and lactobacillus count.

Herbal mouthwash had shown a significant reduction in S. mutans and lactobacillus count when compared to all other mouthwashes used for this study. Pomegranate peel extract 100%, followed by chlorhexidine and pomegranate peel extract 75% had shown a significant reduction in colony count of S. mutans. Chlorhexidine, followed by pomegranate peel extract 100% and pomegranate peel extract 75% had shown a significant reduction in lactobacillus count.

When both microorganisms were treated with different solutions, the comparison of the zone of inhibition regarding the mean was performed using two-sample t-tests as presented in Table 3 and Graph 3.

Discussion
The increase of antibiotic resistance as well as undesirable side effects of synthetic drugs such as tooth staining on long-term use, unpleasant taste, and also they are higher in cost. Hence, there is a need for new antimicrobial agents of plant origin which is safe, preventive, and economical as well. In the present study, pomegranate peel extract has been tested against S. mutans and L. acidophilus. S. mutans bacteria are involved in the initial phase of caries and lactobacillus leads the progression of caries.

Chlorhexidine is considered as a control group in this study as it is the gold standard. When treated against S. mutans and lactobacillus, it shows the effective zone of inhibition against lactobacillus.

Herbal mouthwash (Hiora) is an herbal preparation which includes natural herbs with their useful properties such as anticariogenic, anti-plaque and anti-inflammatory, immunity booster effects, and natural flavouring agents. It has extracts of Belleric Myrobalan (Bibhitaki) which is an antimicrobial and antifungal agent that keeps infections at bay. Betel (Nagavalli) leaf effectively tackles halitosis, and its mild stimulating properties are beneficial for toothaches. A meswak (S. persica) tree twig, known as meswak, is popular teeth-cleaning agents. Pilu prevents tooth decay and eliminates toothache and bad breath.[13] It has powders of peppermint satva acts as a natural mouth freshener (Mentha piperita) and Yavani satva (Trachyspermum ammi), and oils of glandhapura tailum (Wintergreen oil) and Ela which are aromatic essential oil. Kaim et al. suggested that there are certain ingredients in herbal oral rinses that exhibit the evidence of anti-inflammatory and antifungal therapeutic effects.[14]
Graph 1: Comparison of the zone of inhibition regarding (mean standard deviation) against *Streptococcus mutans* when treated with different solutions (*n*=40) using ANOVA test

Graph 2: Comparison of the zone of inhibition regarding mean (standard deviation) against lactobacillus when treated with different solutions (*n*=40) using ANOVA test

Graph 3: Comparison of the zone of inhibition regarding (mean [standard deviation]) against both organisms when treated with different solutions (*n*=40) using two-sample *t*-tests
Table 3: Comparison of the zone of inhibition regarding the mean (SD) against both organisms when treated with different solutions using two-sample t-tests

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Group</th>
<th>N</th>
<th>Mean±SD</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine 0.12%</td>
<td>Streptococcus mutans</td>
<td>10</td>
<td>15.92±0.601</td>
<td>23.879</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus</td>
<td></td>
<td>21.94±0.523</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hiora</td>
<td>Streptococcus mutans</td>
<td>10</td>
<td>30.15±0.476</td>
<td>22.281</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus</td>
<td></td>
<td>24.53±0.639</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pomegranate extract 100%</td>
<td>Streptococcus mutans</td>
<td>10</td>
<td>17.47±0.551</td>
<td>6.186</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus</td>
<td></td>
<td>19.92±1.124</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pomegranate extract 75%</td>
<td>Streptococcus mutans</td>
<td>10</td>
<td>15.39±0.698</td>
<td>6.733</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Lactobacillus</td>
<td></td>
<td>17.31±0.570</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S. mutans: Streptococcus mutans

About 50% of the total pomegranate fruit weight corresponds to the peel, which is an important origin of bioactive compounds such as phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds; minerals mainly potassium, nitrogen, calcium, phosphorus, magnesium, sodium, and complex polysaccharides, and polyphenolic flavonoids are antimicrobial and antifungal. Tannins from pomegranate fruit have been found to inhibit human salivary alpha-amylase, which catalyzes the hydrolysis of starch to oligosaccharides and binds to viridans streptococci and enamel, therefore, providing substrate for cariogenic microbes. According to Lee et al. (2005) pomegranate extracts inhibits sucrose digesting enzyme and the organisms are responsible for plaque formation by competitive and noncompetitive inhibition. Polyphenolic flavonoids present in pomegranate are effective in maintaining good oral health, thereby eliminating the development of gingivitis.

In 2016, Hajifattahi et al., assessed the effect of hydroalcoholic extract of P. granatum Linn. petal on Streptococcus sanguinis, S. mutans, Streptococcus salivarius, Streptococcus sobrinus, and Enterococcus faecalis. They investigated that hydroalcoholic extract of P. granatum had a significant antibacterial effect on oral bacteria. The maximum effect on S. mutans was seen. A similar study done by, Ferrazzano et al. studied the antimicrobial activity of hydroalcoholic extracts of pomegranate peel extract and juice against S. mutans and Rothia dentocariosa Rd1. They concluded that pomegranate peel extract and juice are able to differentiate the main cariogenic bacteria.

Vasconcelos et al., in 2006, investigated the antimicrobial effect of pomegranate phytotherapeutic gel and miconazole (Daktarin oral gel) against S. mutans, S. sanguis, and Streptococcus mitis strains. They have shown the greater efficiency of pomegranate phytotherapeutic gel in inhibiting microbial adherence than miconazole.

According to Bhate et al., there was a statistically significant reduction in the proportion of gingival index scores and plaque index scores in the CHX group.

In another study by, Mahajan et al., studied the efficacy and antimicrobial properties of five herbal mouth rinses with chlorhexidine gluconate mouthrinse. They collected 20 plaque samples from periodontitis patients and healthy patients. Then, they were streaked on blood agar plate. Well diffusion method was used to compare 0.2% chlorhexidine gluconate and different herbal mouthrinses such as Hiora, pomegranate, neem, cloves, tulsi, and distilled water. The plate was incubated at 37°C for 24 h and examined for the zones of inhibition. After the examination, they concluded that Hiora, pomegranate, and chlorhexidine mouthrinse were equally effective, and hence, they can be easily used to inhibit the growth of oral microorganisms.

In this study, herbal mouthwash had shown a significant reduction in S. mutans and lactobacillus count. Pomegranate peel extract 100% followed by chlorhexidine and pomegranate peel extract 75% had shown a significant reduction in colony count of S. mutans. Chlorhexidine followed by pomegranate peel extract 100% and pomegranate peel extract 75% had shown a significant reduction in lactobacillus count. Hence, herbal mouthwash and 100% pomegranate peel extract can be used in case of initial carious lesions.

**Conclusion**

The mouthwash used in this study revealed the antibacterial activity against oral microorganisms. Pomegranate peel extract or herbal mouthwash can be used in place of commercially available mouthwash such as chlorhexidine to avoid its side effects. However, further clinical research will be required.

**Limitations**

Results of this study cannot be match up with clinical situation. In vitro study does provide uniform and reliable means for evaluating and comparing the antimicrobial activity of different mouthrinses.

**References**

Antimicrobial properties of pomegranate peel extract Kunte, et al.

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