

## Evaluation of the antibacterial effects of three natural formulated mouthwashes against *Streptococcus mutans*: An in vitro study

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### Original Article

#### Abstract

**BACKGROUND AND AIM:** *Streptococcus mutans* (*S. mutans*) is a well-known bacterial cause of dental caries. The aim of this study was to develop an herbal mouthwash with anti-biofilm activity and compare it with a commercial preparation containing 2% chlorhexidine.

**METHODS:** The main components of the mouthwashes included *Punica granatum*, pomegranate rind, *Eugenia caryophyllata*, *Rhus coriaria* L., etc. (No. 1); *Pistacia lentiscus* L., *Punica granatum*, *Areca catechu* L., etc. (No. 2); *Cyperus articulatus*, *Terminalia chebula* Retzius, *Cinnamomum zeylanicum*, *Elettaria cardamomum*, *Zingiber zerumbet*, *Punica granatum*, etc. (No. 3). Plants were weighed and soaked into sterile distilled water. Filtration and sterilization were performed using Whatman and Millipore filters, respectively. The antibacterial susceptibility assay was performed by an agar well diffusion method according to Clinical and Laboratory Standards Institute (CLSI). Anti-biofilm activity of mouthwashes and chlorhexidine was evaluated on polystyrene microtiter plates by a broth dilution method. Finally, a comparative analysis of the results was done by Tukey and analysis of variance (ANOVA) tests.

**RESULTS:** No positive effect of mouthwash No. 1 was observed in the agar well diffusion method. This mouthwash showed an effect on biofilm formation only at 50% concentration. In agreement with well diffusion, the effect of mouthwash No. 2 was moderate and similar to 2% chlorhexidine. The mouthwash No.3 showed an excellent effect (even at 3.125%) against *S. mutans*.

**CONCLUSION:** Overall, mouthwash No.3 appears to be suitable as an herbal anti-biofilm solution. Further studies are necessary to identify the active ingredients of the plants.

**KEYWORDS:** *Streptococcus Mutans*; Biofilms; Mouthwashes; Chlorhexidine; Plants

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Dental caries, an ancient disease, is formed by the colonization and the accumulation of oral bacteria and is considered as one of the most common chronic diseases worldwide. Biofilm is an example of dental plaque, which results in tooth dysfunction and loss.<sup>1</sup> Mutans group *Streptococci* have been recognized as main Gram-positive oral bacteria and etiological

agents of human dental caries. Amongst them, *Streptococcus mutans* (*S. mutans*) has high capabilities of biofilm formation, acid production, high tolerance to low pH, and high affinity for various carbohydrate sources.<sup>2,3</sup> Three stages of dental plaque formation have been recognized: I) salivary molecules attach to the enamel; II) some oral bacteria, e.g., *Streptococcus sanguis* and

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*Actinomyces viscosus*, interact with the acquired layer producing an initial biofilm on the tooth surface; III) *S. mutans* binds to the primary colonizers, followed by growth leading to the formation of biofilm and a water-insoluble glucan layer synthesized by its sucrose-dependent glucosyltransferase (GTFs) activity on the tooth. The presence of *S. mutans* in the tooth cavities is followed by the decay after 6 to 24 months.<sup>4</sup>

For preventing and controlling biofilm formation, various types of chemicals such as chlorhexidine and antimicrobial agents such as penicillin, erythromycin, and vancomycin have been used.<sup>1</sup> However, a change in the oral flora after usage of these agents can be observed.<sup>2</sup> Furthermore, undesirable side effects on long term use of chlorhexidine have been reported, e.g., mucosal ulceration, tooth and tongue staining, taste perturbation, unilateral/bilateral parotid swelling, and enhanced supragingival calculus formation.<sup>5</sup>

Several studies on the antibacterial effects of cultured plants on *S. mutans* and its biofilm have been published in different countries.<sup>2,6,7</sup> Study of Kunte et al. showed that chlorhexidine and pomegranate peel extract had significant antimicrobial activities on *S. mutans* and were equally effective.<sup>8</sup> Another report conducted by Batubara et al. determined that the essential oil of Zingiberaceae leaves and *Elettaria cardamomum* was successfully effective against *S. mutans*.<sup>9</sup> Due to its geographical location and traditional herbal treatments, Iran has a high potential for culturing medicinal plants showing broad activities against different bacterial diseases.<sup>10</sup>

In this study, we tried to investigate the anti-biofilm effects of three natural mouthwashes, prepared in-house from various plant extracts including *Punica granatum*, pomegranate rind, *Eugenia caryophyllata*, *Rhus coriaria* L., *Andricus kollari*, *Pistacia lentiscus* L., *Areca catechu* L., *Quercus infectoria*, *Aquilaria malaccensis*, *Cyperus articulatus*, *Terminalia chebula* Retzius, *Cinnamomum zeylanicum*,

*Anacyclus pyrethrum*, *Piper longum*, saffron, *Rhus coriaria* L., *Elettaria cardamomum*, and *Zingiber zerumbet*, using the formulations presented in the historical books, Encyclopedia of herbal medicine in Iran, and *Al-Qanoon Fil Tibb* by Ibn Sina.<sup>11,12</sup> The references describe about a collection of plant extracts recommended for traditional prevention and treatment of some infectious diseases. Therefore, in order to evaluate the anti-biofilm activity produced by *S. mutans*, we compared these home-made mouthwashes with a commercial 2% chlorhexidine mouthwash. So, the aim of this study was to evaluate antibacterial and anti-biofilm effects of three traditional mouthwashes (called No. 1, 2, and 3) against *S. mutans*.

## Methods

**Preparation of bacterial cells and mouthwashes:** In this in vitro study, *S. mutans* ATCC35668 was used as reference strain and cultured on Tryptic Soy Agar (TSA) (Ibresco, Iran) at 37 °C overnight under 5% CO<sub>2</sub> atmosphere. According to historical orders listed in table 1, the fresh natural agents were purchased from a grocery store in Qom City, Iran; they came from different geographical areas of Iran and were collected during 2018 spring and summer seasons. Plants were dried for 10 days in a dark box at room temperature. After powdering using a mechanical grinder, the components of each mouthwash were weighed and soaked into 100 ml sterile distilled water on a rotator at dark for 24 hours. The extracts were filtered separately by Whatman filters and then sterilized through a 0.22 µm pore size membrane. The extracts were mixed together and prepared mouthwashes were stored at 4 °C as stock solutions until analyses.<sup>13</sup> The ingredients of the mouthwashes and their weight are listed in table 1.

**Biofilm formation ability:** *S. mutans* colonies [10<sup>8</sup> colony-forming units per milliliter (CFU/ml)] were grown at 37 °C for 24 hours on TSA and were collected and inoculated into 1% D-glucose-supplemented

**Table 1.** Herbal mouthwashes formulated in this study

Mouthwash name	Components (used amounts)	Reference
No. 1	Punica granatum (25g), pomegranate rind (25g), clove ( <i>Eugenia caryophyllata</i> ) (10g), sumac ( <i>Rhus coriaria</i> L.) (100g), <i>Andricus kollari</i> (25g)	11
No. 2	<i>Pistacia lentiscus</i> L. (50g), Punica granatum (50g), <i>Areca catechu</i> L. (25g), <i>Quercus infectoria</i> (50g), <i>Andricus kollari</i> (50g), <i>Aquilaria malaccensis</i> (50g)	12
No. 3	<i>Cyperus articulatus</i> (Cyperaceae) (30g), <i>Terminalia chebula</i> Retzius (50g), <i>Cinnamomum zeylanicum</i> (30g), <i>Anacyclus pyrethrum</i> (70g), <i>Piper longum</i> (10g), saffron ( <i>Crocus sativus</i> ) (10g), sumac ( <i>Rhus coriaria</i> .L.) (20g), <i>Cardamom</i> ( <i>Elettaria cardamomum</i> ) (40g), <i>Zingiber zerumbet</i> (160g), Punica granatum (40g)	

Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) in sterile 96-well flat-bottom polystyrene microtiter plates without agitation. Following incubation at 37 °C for 48 hours, the content of the wells was removed and gently washed three times with sterile physiological saline [phosphate-buffered saline (PBS), pH = 7.4]. The attached bacteria were fixed with 1 ml of 96% ethanol for 15 minutes. After emptying and drying, the wells were stained with 200 µl of 1% crystal violet solution for 15 minutes and then the dye was washed gently with distilled water and allowed to dry. To obtain quantitative data, the crystal violet bound to the adherent cells was re-solubilized with 96% ethanol and the optical density (OD) of the resulting solution was measured using an enzyme-linked immunosorbent assay (ELISA) reader apparatus at 492 nm.

#### **Antimicrobial susceptibility test (AST):**

The antimicrobial susceptibility assay was done by an agar well diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI).<sup>14</sup> Firstly, holes with a diameter of approximately 5 mm were prepared on Mueller-Hinton Agar (MHA) (Merck, Darmstadt, Germany). A bacterial suspension, with turbidity of 0.5 McFarland standard, was cultured on the plate and 100 µl of each mouthwash stock and 2% chlorhexidine were added to the wells. Bacteria were incubated as described above.

**Inhibition of biofilm formation:** The biofilm formation was carried out by broth dilution method with 100 µl of 1% glucose - supplemented TSB in sterile 96-well

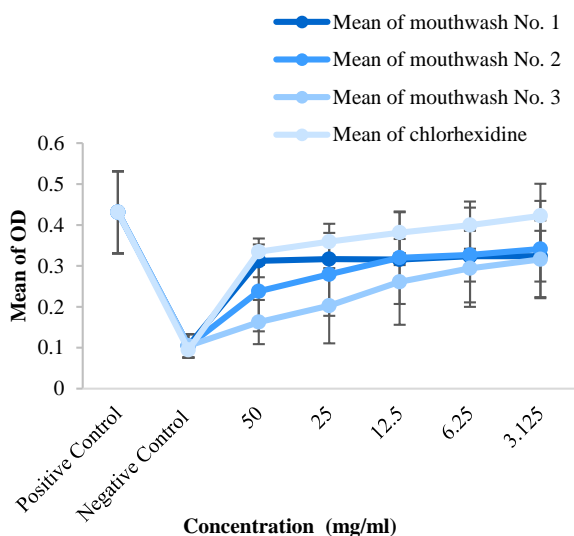
flat-bottom polystyrene microtiter plates. 100 µl of each prepared stock mouthwash and 2% chlorhexidine were added to the first well of the microplate. To obtain the same conditions, serial dilutions were done to prepare 50%, 25%, 12.50%, 6.25%, and 3.125% concentrations. Finally, 10 µl of a suspension of *S. mutans* (10<sup>8</sup> cells/ml) was inoculated into the wells. The medium alone and a cultured well without mouthwash were used as negative and positive controls, respectively. The wells with the medium and the different herbal concentrations, without bacteria, were used as another control to eliminate turbidity due to plant extractions. The plate was then incubated as described above. The tests were repeated three times.<sup>13</sup>

Results were presented as mean and standard deviation (SD) of the ODs. For statistical analysis, one-way analysis of variance (ANOVA) and Tukey tests, as a post-hoc, were used and P < 0.05 was determined as the significant level. Statistical analysis was performed by SPSS software (version 22, IBM Corporation, Armonk, NY, USA).

## **Results**

*S. mutans* was employed as a bacterial model for the formation of biofilm. Bacteria were able to create biofilm into the microplate wells (data not shown). Agar well diffusion test showed that mouthwash No. 1 was not successful to reduce *S. mutans* biofilm formation (0 mm). On the other hand, results of mouthwash No. 2 and 2% chlorhexidine were the same (5 mm). The best inhibitory effect was observed for mouthwash No. 3 (10 mm). According to OD results,

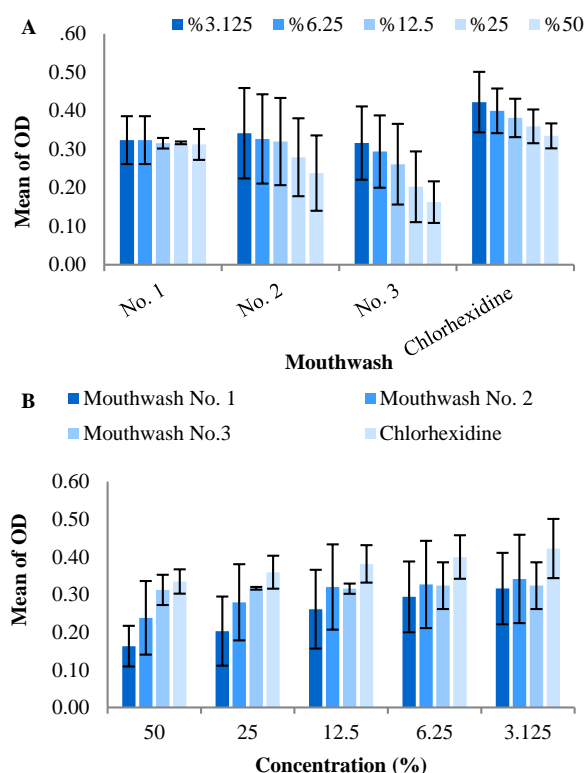
mouthwash No. 1 showed an effect on the biofilm formation only at 50% concentration compared to the other mouthwashes and no differences in absorbance values at lower concentrations were observed. The effect of mouthwash No. 2 on *S. mutans* biofilm formation inhibition was moderate at 50%, 25%, and 12.5% concentrations, similarly to chlorhexidine. Lower concentrations of mouthwash No. 2 did not show any anti-biofilm activity on *S. mutans*. Mouthwash No. 3 showed potent inhibitory activity at all concentrations and even at 3.125% was excellently able to inhibit biofilm formation of the bacteria. According to the absorbance readings of OD, although chlorhexidine exhibited good effects on *S. mutans* at all concentrations, the mouthwash No. 3 had a stronger biofilm inhibition activity. Obtained curves from anti-biofilm effects of the mouthwashes and chlorhexidine against absorbance are shown in figure 1.



**Figure 1.** Obtained curve from anti-biofilm effects of the mouthwashes and chlorhexidine against absorbance of optical densities (ODs)

Statistical analysis showed that according to concentration, the effect of each mouthwash on the biofilm was significant ( $P < 0.010$ ), while interaction effects between mouthwashes and their concentrations were not statistically significant ( $P = 0.986$ ).

Additionally, Tukey test indicated that the antibacterial effects of mouthwash No. 1 and 3, mouthwash No. 2 and chlorhexidine, as well as mouthwash No.3 and chlorhexidine were significantly different. Also the mean of OD for all concentrations of mouthwash No. 3 was lower than the other mouthwashes and chlorhexidine. Mouthwash type/chlorhexidine vs. mean of ODs and the concentration of the different mouthwashes/chlorhexidine vs. mean of ODs are presented in figures 2 (A and B), respectively.



**Figure 2.** Mouthwash type and chlorhexidine against mean of optical densities (ODs) (A) Different concentration of the mouthwashes and chlorhexidine against mean of ODs (B)

### Discussion

The result of this study showed that there was no positive effect of mouthwash No. 1 in the agar well diffusion method. This mouthwash also showed a partial effect on the biofilm formation at 50% concentration. In agreement with the well diffusion, the effect of mouthwash No. 2 displayed a moderate activity that was similar to 2%



chlorhexidine. Although we could not evaluate each plant separately on the bacterium and isolate different fractions of the used plants, as some limitations, the mouthwash No.3 showed an excellent effect against *S. mutans*.

Due to antibiotic resistance and reduced availability of some antibiotics in treatment, natural plant compounds have been widely evaluated as new agents against bacteria, viruses, fungi, and parasites. For example, *Lippia citriodora*, Algerian *Juniperus*, *Eucalyptus globulus*, etc. are a part of extractions that were tested on the microorganisms.<sup>15-17</sup>

Other works have also indicated excellent antimicrobial effects of plants on multidrug-resistant bacteria. Valle et al. have shown that *Psidium guajava*, *Phyllanthus niruri*, *Ehretia microphylla*, and *Piper betle* have antibacterial activity on methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE).<sup>18</sup>

Therefore, several studies have determined that plant extractions have the potential to replace chemical therapy. However, a few other studies showed no effects on microorganisms. Bhalodia and Shukla detected that *Cassia fistula* lacked antibacterial activity against fungal isolates, Gram-positive, and Gram-negative bacteria.<sup>19</sup> Another study showed that the lavender essential oil was inactive against *Pseudomonas aeruginosa*.<sup>20</sup>

Dental caries is a common problem in human health. Most of the new strategies to prevent the disease include the control of the dental plaques caused by cariogenic bacteria.<sup>21</sup> Biofilm is known as a specific mechanism for the initial attachment, community structure, ecosystem, and detachment from surfaces.<sup>22</sup> Among bacteria, *Streptococci* species, especially *S. mutans*, are able to form a biofilm which can cause dental caries development. To prevent tooth decay by *S. mutans*, chlorhexidine is commonly used among people. However, due to the emergent resistance of *S. mutans* to

chlorhexidine, its use should be limited to a short period of time.<sup>23</sup>

Thus, the aim of this study was to evaluate several natural extractions within three formulations according to Encyclopedia of herbal medicine in Iran and *Al-Qanoon Fil-Tibb of Ibn Sina*, whose antibacterial properties were already recognized. Although there are a number of already published studies on anti-biofilm effects,<sup>24,25</sup> this study is the first evaluation of new formulations from a mixture of Iranian medicinal plants against *S. mutans* biofilm formation. Among the evaluated mouthwashes, the mouthwash No.3 (*Cyperus articulatus*, *Terminalia chebula* Retzius, *Cinnamomum zeylanicum*, *Anacyclus pyrethrum*, *Piper longum*, saffron, sumac, Cardamom, *Zingiber zerumbet*, and *Punica granatum*) showed a stronger inhibition of *S. mutans* growth (10 mm) compared to the other mouthwashes and chlorhexidine, which is in accordance with the results of the biofilm assay. This value is lower than that obtained with the extract of *Dodonaea viscosa* var. *angustifolia* tested by Naidoo et al. who indicated that 25% of crude extract killed 100% of *S. mutans*.<sup>6</sup>

Among antibacterial agents, *Punica granatum*, *Rhus coriaria*, and *Andricus kollari* were common between mouthwashes. Although the antibacterial activity of some extracts (e.g., *Rhus coriaria*<sup>26</sup> and *Punica granatum*<sup>27</sup>) has been demonstrated, it seems that other plants included in No.3 mouthwash had better effects on *S. mutans* than the rest of mouthwashes. Indeed, *Cyperus articulatus*,<sup>28</sup> *Terminalia chebula* Retzius,<sup>29</sup> *Cinnamomum zeylanicum*,<sup>30</sup> *Anacyclus pyrethrum*,<sup>31</sup> saffron (*crocus sativus*),<sup>32</sup> *Elettaria cardamomum*,<sup>33</sup> and *Zingiber zerumbet* Linn<sup>34</sup> have been demonstrated to possess antimicrobial activity on a number of Gram-positive and Gram-negative bacteria.

Similar to chlorhexidine, mouthwash No. 2 activity was better than mouthwash No.1, showing a moderate effect on biofilm

formation. It seems that some extracts included into this mouthwash had different effects against bacteria. Cyriac et al. reported that oral bacteria were resistant to *Areca catechu* L. plant,<sup>7</sup> while Wan Nor Amilah et al. showed that *Quercus infectoria* inhibited more Gram-positive than Gram-negative bacteria.<sup>35</sup> Both well diffusion and broth dilution results demonstrated that mouthwash No.1 had the lowest activity against biofilm.

### Conclusion

Results of this study suggest that the formulation of mouthwash No.3 can be a good candidate as an effective antibacterial agent in the prevention of biofilm formation. Furthermore, it is necessary to detect the

active fractions of the used plants in the mouthwash No.3 and evaluate them by in vivo methods. If the future results be similar to the current study, it can be used to prepare commercial antibacterial compounds (similar to chlorhexidine but cheaper and without side effects) and also to apply it in toothpastes. So this study has an innovative idea at its level.

### Conflict of Interests

Authors have no conflict of interest.

### Acknowledgments

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