Comparison between mastic gum resin extract and chlorhexidine mouthwash in the prevention of biofilm formation on titanium dental implants

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ABSTRACT

The biofilm formation around dental implant abutment is considered the main cause of peri-implant infection. The use of antimicrobial mouthwash might potentially reduce biofilm formation and subsequent infection. This study aimed to evaluate the antibiofilm properties of mastic gum resin extract (MGRE) against S. mutans biofilm on the surface of titanium dental implant discs. This study used grade five (medical grade) titanium dental implant discs measuring 15 mm in diameter. The substances in the MGRE were analyzed by gas chromatography mass spectrometry (GC-MS) which found 12 detectable chemicals in MGRE. In this study, S. mutans was collected around the healing abutment of a recently placed dental implant and then cultured on titanium discs (in vitro). After bacterial growth on the titanium specimens for 24 hours, they were subjected to either chlorhexidine gluconate (CHX), (n = 6) or MGRE (n = 6). The antibacterial activity tests showed that both CHX and MGRE significantly inhibited bacterial growth compared to the negative control, the lactate production and turbidity measurements were significantly lower in MGRE and CHX compared to the control (p ≤ 0.05). Regarding the antibiofilm activity, both treatments showed significantly less turbidity in their biofilm compared to the control. Moreover, scanning electron microscopy (SEM) images showed that there were very limited adherence cells on the titanium implant discs in treatment groups, while there were confluent and attached bacterial cells in the control. The MGRE showed an antimicrobial property against S. mutans indicating that it has a potential for clinical use as a mouthwash.

Introduction

Peri-implant mucositis is one of the risk factors that threaten the success of dental implant treatment. Peri-implant mucositis, if untreated, can lead to peri-implantitis which consequently results in peri-implant bone loss and implant failure. Peri-implant mucositis is defined as an inflammatory lesion of the mucosa surrounding an endosseous implant without loss of supporting peri-implant bone (1). Many factors can be considered as risk indicators for peri-implant mucositis, one of them is the presence of plaque biofilm (2, 3). Peri-implant mucositis develops from healthy peri-implant mucosa resulting from the accumulation of bacterial biofilms around osseointegrated dental implants (3). The prognosis of peri-implant mucositis might take longer than gingivitis. It was found that the clinical reversibility of peri-implant mucositis can take longer than 21 days (4). However, with consistent oral hygiene practice, the biofilm-induced per—implant mucositis can be significantly improved. For example, it was found that the gingival index of peri-implant mucositis significantly reduced after oral hygiene measures compared to the periodontal side (4).

Researchers have tried to prevent or treat peri-implant mucositis in various ways. Interventions have been used to remove the peri-implant biofilm from contaminated implant surfaces (5). Studies have used air powder abra- sion using glycine powder or chitosan brush along with mechanical debridement. However, no significant clinical benefits regarding BI and BoP were observed compared to the control (6, 7). Moreover, a randomized controlled clinical trial was conducted to investigate the effect of photodynamic therapy addition to mechanical debridement on BI and BoP, it was found that the intervention did not demonstrate a significant difference in BI and BoP compared to control (8, 9). Another randomized controlled clinical trial was conducted to study the effect of diode laser as an adjunctive to mechanical debridement on BI and BoP, the results showed that laser treatment did not induce a significant improvement in BI and BoP compared to the control (10,11). Furthermore, some studies have used adjunctive local antiseptics, for example, studies have used CHX gel and mouth rinse as an adjunctive measure to treat peri-implant mucositis, and results showed no significant improvement compared to control (12,13). Another research used sodium hypochlorite as an adjunctive therapy but there was no significant improvement compared to the control (14).

Based on the mentioned studies, the use of mouthwashes as an adjunctive therapy has not shown a significant improvement in peri-implant clinical parameters. However, their in-vitro antibacterial efficacy is well documented, but using other interventions (antimicrobial agents) might provide a better antimicrobial activity. Research showed that the antibacterial activity of non-thermal atmospheric pressure plasma against biofilms of S. mutans grown on
titanium dental implant discs was significantly more than CHX (15). This can be explained by the fact that oral microbes might have developed resistance against CHX which is most commonly as an antimicrobial mouthwash.

Recently, natural, plant-driven products have shown potential antibacterial and anti-inflammatory activity (16-18). Herbal mouthwashes have shown similar anti-plaque activities as compared to the standard mouthwash CHX (19). MGR is an exudate from Pistacia atlantica tree, its antioxidiant and anticancer activities are well known, and an in-vitro study has shown the efficacy of MGR in arresting the cell cycle of colon cancer cell line at the G2/M phase indicating that the resin can be used to prevent colon cancer (20). Moreover, studies have shown that MGR possesses an excellent antibacterial properties against Helicobacter pylori (21). Regarding the oral microbiota, a study was conducted to investigate the antibacterial activity of mastic chewing gum (traditional chewing gum made from MGR) against S. mutans, the results showed significantly lower S. mutans in saliva samples collected after chewing mastic gum compared to control (22). Taken together, to reduce the bacterial resistance to the currently used mouthwashes, eliminate their undesirable side effects (e.g.; teeth staining) and preserve oral normal flora, MGRE (in a liquid form) can be used as a mouthwash to decrease peri-implant microbiota. Thus, this study aimed to investigate the antibacterial activity of MGRE against isolated S. mutans (early colonizer) around dental implants inside the oral cavity.

Materials and Methods

Specimen preparation

This study used medical, grade five titanium alloy (Ti6Al4V) discs which are most commonly used for dental implants. The circular-shaped discs are 15 mm in diameter and 1 mm in thickness created by laser cutting and polished with sandpapers of (800–1200 grit) using a rotary tool (Grinder–Polisher, Buehler, UK Ltd, Coventry, England). For the final polish, six and one microns of diamond solution (Diamond solution, Kemet International Ltd, UK) were applied. Finally, the specimens were cleaned using an alkaline solution and 5% HCl as described in (23, 24).

Preparation of MGRE

MGR was collected from Pistacia atlantica tree located in Qandeel mountain Kurdistan region of Iraq (Figure 1). The resin was collected from the tree and then boiled for 30 minutes. The aqueous extract from the resin was collected. This aqueous extract has long been used traditionally to treat gastric upset.

Chemical content analysis of MGRE using GC-MS

The chemical content of the extract was identified using GC-MS. The analysis was carried out with an Agilent 7000 Triple Quad, GC 7890A. GC oven temperature was programmed to 220 °C at a rate of 4 °C/min and kept constant at 220 °C for 10 min and then programmed to 230 °C at a rate of 1 °C/min. The ion source temperature was 230 °C and the scan mode of the mass spectrum of 25-800 atomic mass units. The mass spectra were recorded at 70 eV.

Isolation and identification of S. mutans around dental implant abutment

The bacterial swab was collected around a dental implant healing abutment of 28-year-old female patient (Figure 2). The sample was transferred to the laboratory and cultured on blood agar then incubated for 24 hours at 37 °C. After that, a colony was identified on the blood agar which was later identified using the VITEK II device (bio-Merieux, North Carolina, USA).

Assessment of the antibacterial activity using the disc diffusion method

The antibacterial activity of MGRE was tested using the disc diffusion method which is a conventional method used for testing microbial growth sensitivity against antimicrobial agents. In the current study, 6 mm diameter filter papers were immersed in MGRE (n=6) and CHX, (n=6) for 3 hours, after that, the conditioned filter paper was transferred to Mueller Hinton agar where S. mutans were newly sub-cultured, and agar plates were the unit of replication. After 24-hour incubation, the agar plates were examined for the presence of microbial growth inhibition zone around the filter papers.

Preparation of bacterial suspension

The microbial samples which were isolated from the dental implant abutment were put in blood agar and then incubated for 24 hours at 37 °C to acquire bacterial growth. Then a swab of the microbial colony in the blood agar was cultured in brain heart infusions (BHIIB). The sample in BHIIB was put in anaerobic jars and incubated at 37 °C for 24 hours for activation.

Figure 1. Mastic gum resin collected from Pistacia atlantica tree. Note the clay pots attached to the tree to collect the resin.

Figure 2. Gingival former (healing abutment) around which the microbial swab was collected.
Experimental design

The main experiment in this study tested the antibacterial and antimicrobial activity of MGR and CHX against *S. mutans* grown on titanium dental implant discs. The experimental groups were negative control, Treatment (MGRE) and positive control (0.2% CHX). Basically, *S. mutans* in 1.5 BHIB was added to the titanium discs in sterile glass tubes and incubated overnight at 37 °C in an anaerobic jar. After 24 hours, the BHIB was removed and 5 ml of MGRE (n=6) and 0.2% CHX (n=6) were added to the specimens for 60 seconds. The test solutions were then washed away, and the specimens were rinsed three times with ultrapure water. After that fresh BHIB was added to the specimens and incubated for 24 hours. Finally, the turbidity of BHIB and the biofilm were measured. Another triplicate of control and treatment and positive control was also prepared for assessing bacterial morphology after 24 hours under the SEM.

Optical density measurement for bacterial growth assessment

The turbidity test is commonly used to assess bacterial growth in the media, the increased turbidity corresponds the bacterial growth. The procedure started by taking 100 μl aliquots of the nutrient broth of blank, negative control, treatment and positive control groups were added to a 96-well plate with a flat bottom and a lid and then 100 μl of the fresh BHIB was added to each well. The 96-well plate was then placed in a plate reader (BioTek ELX800) and the absorbance values were read at 630 nm to determine the turbidity.

Assessment of biofilm growth on titanium implant discs

The antibiofilm assay used in this study followed the protocol used (25). After 24 hours, the media was removed, and the biofilm was stained for 10 minutes using 1% crystal violet. The stain intensity was used to determine the biofilm strength. After staining, the specimens were rinsed with distilled water to remove excess stain and left at room temperature to dry. 3 mL of ethanol was then added on each specimen to detach the biofilm. Finally, the ethanol solution including the biofilm was withdrawn and placed in 96 well-plated and the optical density was measured using a plate reader (BioTek ELX800) at 630 nm.

Investigating the morphology and confluence of *S. mutans* on titanium discs using SEM

SEM was used to visualize the morphology of the bacterial cells after the experiment. The bacterial cells were examined *in situ* on the titanium discs. After the experiment, the media were discarded, and the specimens were washed with phosphate buffer. After that, the bacterial biofilm on the discs was immersed in ethanol solutions (30, 50, 70, and 95%) for 20 min each then 100% ethanol for 1 h. Later, the specimens were left to dry overnight and then sputter-coated with chromium. The bacterial cells on the discs were then examined under SEM for morphology and confluence.

Statistical analysis

Data are expressed as mean ± S.E.M. and analyzed using stat graphics version 16. To locate the significant difference between the groups, data were subjected to one-way ANOVA, then Tukey’s test. All statistical analyses used a 95% confidence limit, p values < 0.05 were considered statistically significant.

Results

Chemical analysis of MGRE

The chemical content of the aqueous MGRE was analyzed using GC-MS. The analysis found 12 chemical structures through 12.3-18.3 min analysis (Figure 3). The names of chemical structures with their start, end and real time are shown in (Table 1).

Microbiological analysis of the peri-implant sample

The peri-implant abutment swab was cultured in blood agar for 24 hours, then, the formed bacterial colony was analyzed by VITEK II for microbial identification. The result showed that the colony was *S. mutans* having 97% percentage probability.

### Table 1. Name (structure) of the chemicals detected in MGRE using GC-MS.

<table>
<thead>
<tr>
<th>Peak Name</th>
<th>Start</th>
<th>Real Time</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>12.43</td>
<td>12.5</td>
<td>12.673</td>
</tr>
<tr>
<td>Diepoxyhexadecan</td>
<td>14.8</td>
<td>14.9</td>
<td>15.06</td>
</tr>
<tr>
<td>3-Cyclohexene-1-methanol, 2-hydroxy-alpha, alpha,4-trimethyl-</td>
<td>15.21</td>
<td>15.26</td>
<td>15.32</td>
</tr>
<tr>
<td>5-Bornanedione</td>
<td>15.49</td>
<td>15.53</td>
<td>15.62</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>15.78</td>
<td>15.83</td>
<td>15.92</td>
</tr>
<tr>
<td>Boronia butenal</td>
<td>16.31</td>
<td>16.35</td>
<td>16.43</td>
</tr>
<tr>
<td>Acetate</td>
<td>16.89</td>
<td>16.95</td>
<td>17.02</td>
</tr>
<tr>
<td>Enoic acid</td>
<td>16.89</td>
<td>16.95</td>
<td>17.02</td>
</tr>
<tr>
<td>Undecanol</td>
<td>17.3</td>
<td>17.37</td>
<td>17.42</td>
</tr>
<tr>
<td>Oleamide</td>
<td>17.3</td>
<td>17.37</td>
<td>17.42</td>
</tr>
<tr>
<td>Aspidospermidin</td>
<td>17.92</td>
<td>17.95</td>
<td>18.02</td>
</tr>
<tr>
<td>Pterine-6-carboxylic acid</td>
<td>18.25</td>
<td>18.31</td>
<td>18.4</td>
</tr>
</tbody>
</table>
Antibacterial activity of MGRE and CHX using disc diffusion method

The antibacterial activity of the test materials was investigated using the Disc Diffusion Method. It was found that the *S. mutans* growth inhibition zone caused by CHX was around 4 mm. However, only 2 mm growth inhibition zone was noticed around MGRE-conditioned discs. There was a statistically significant difference between them (*P* ≤ 0.05), (Figure 4).

Assessment of antibacterial and antibiofilm activity of MGRE and CHX against grown *S. mutans* on titanium discs

Grown *S. mutans* on titanium discs were subjected to MGRE and CHX for 1 minute. The results showed that *S. mutans* survival in MGRE was more than CHX, while there was significantly less bacterial growth in MGRE compared to the control. The turbidity of suspended bacteria in BHIB was significantly higher in control compared to others, also, the turbidity of MGRE was significantly higher than CHX (*P* ≤ 0.05), (Figure 5 A). Regarding the antibiofilm activity, it was found that the turbidity of control, MGRE and CHX were 0.25, 0.17 and 0.03 respectively. The control was significantly higher than the others (*P* ≤0.05) and there was a significant difference between MGRE and CHX (Figure 5 B).

The lactate production assay demonstrated that there was significantly more lactate production in control compared to others, while no significant difference between MGRE and CHX in suspension all measuring 7, 4 and 0.5 µm respectively (Figure 6 A). In addition, *S. mutans* in the biofilm produced significantly more lactate in control compared to MGRE and CHX measuring 3, 1 and 0.2 µm respectively (Figure 6 B).

Furthermore, the morphology and confluence of *S. mutans* on titanium discs were also investigated under SEM.

![Figure 3. Percentage vs acquisition time of the detected peaks in MGRE using MC-MS.](image)

![Figure 4. *S. mutans* inhibition zone around CHX or MGRE conditioned discs on agar plates. Data are mean ± S.E.M. Different letters indicate significant difference between the variables. One-way ANOVA (*p* ≤ 0.05).](image)

![Figure 5. Assessment of antibacterial and antibiofilm activity. Data are turbidity values of suspended *S. mutans* in BHIB (A) and *S. mutans* in the biofilm (B). Data are presented as mean ± S.E.M. Different letters indicate a significant difference between the variables.](image)

![Figure 6. Lactate production by *S. mutans* in BHIB (A) and in the biofilm (B). Data are mean ± S.E.M. Different letters indicate a significant difference between the variables. One-way ANOVA (*p* ≤ 0.05).](image)

The control group showed confluent bacterial cell coverage on the substrate (Figure 7 A). However, there were no detected cells in the CHX group (Figure 7 B), and only a few cells remained adhered to the substrate after exposure to MGRE (Figure 7 C).
Discussion

The use of mouthwash to prevent peri-implant biofilm formation is grossly studied. Given the increased bacterial resistance to antibiotics, undesirable adverse effects of some antimicrobial mouthwashes currently used in dentistry as well as financial considerations in developing countries, there is a need for alternative mouthwashes that are effective, safe and economically affordable. The point is to produce a mouthwash that has an antibacterial activity against pathogenic microorganisms without interfering with the oral normal flora. This study investigated the efficacy of MGRE against S. mutans compared to CHX which is commonly used mouthwash in dentistry. The critical question was about the potential of MGRE as a natural (traditionally available) mouthwash that prevents peri-implant microbial colonization and subsequent infection. In this study, MGRE in an aqueous form was used, the chemical content of which was analyzed by GC-MS, the results of analysis found 12 chemical substances in the sample (Table 1). The chemical content of MGRE might change according to the chemical form of the sample, in this study, alpha penine was detected in the sample. Alpha penine is one of the substances most frequently found in MGR, for example, various studies have found that the majority of MGR essential oil is α-Pinene (26-28). The antibacterial activity of α-Pinene is well documented (29), it could be argued that the antibacterial properties of MGR are caused by the presence of α-Pinene. Moreover, α-Linolenic acid and enolic acid were also found in MGRE, similar findings have been observed by other studies (30), and both acids are known to have antibacterial properties. In addition, myrcene which is commonly found in MGR, was not detected in this study, the possible explanation for this is the fact that myrcene is not water-soluble, and the MGRE used in this study was in an aqueous form produced by boiling. Thus, myrcene was not extracted (dissolved) from the resin to the supernatant.

The microorganism used in this study was isolated from a peri-implant abutment inside the oral cavity. The peri-implant swab analysis found S. mutans which is considered as an early colonizer around the dental implant. S. mutans forms the first colony on the implant which acts as a foundation for the late colonizers (P. gingivalis, P. intermedia… etc). Thus, inhibition of S. mutans is critical for preventing peri-implant mucositis and peri-implantitis. The results showed that MGRE caused 2 mm inhibition zone which was significantly less than CHX which caused 4 mm growth inhibition around the CHX conditioned discs. Furthermore, antibacterial and anti-biofilm activity tests of MGRE against S. mutans grown on titanium discs also showed similar results; it was found that MGRE caused significantly more bacterial damage compared to control, but also significantly less damage compared to CHX (Figure 5-7). This finding is supported by another study that tested the antibacterial activity of MGR against S. mutans, the results showed that significantly less bacteria was found in saliva after chewing mastic gum compared to those after chewing paraffin (22). Another study that investigated the efficacy of mastic chewing gum in reducing S. mutans concentration in the saliva of orthodontic patients also supports the existing finding; it was found in the latter that chewing mastic gum for 15 minutes caused a significant reduction of S. mutans in saliva (32). In addition, another study also found that MGR essential oil can cause significantly more S. mutans growth inhibition compared to the control (33). There are many reports (33-47) about the effect of medicinal plants in the treatment of various diseases.

The antibacterial activity of MGR against S. mutans is well documented. However, the efficacy of MGRE against peri-implant microbiota (bacterial biofilm on implant surface) has not been tested yet. The difference between this study the similar existing studies in literature is that in the current study, the bacteria was grown on the surface of titanium dental implant discs to mimic an in vivo (clinical) environment. Thus, the possible explanation for the reduced antibacterial activity of MGRE compared to CHX is that S. mutans were embedded in a biofilm on titanium discs. The biofilm can protect bacteria from antibacterial agents, growing bacteria on agar plates are inevitably less...
protected compared to the bacterial colonies embedded in a strong biofilm on a titanium surface.

Taken together, despite the lower antibacterial activity of MGRE compared to CHX, the former still has the potential to be used as a mouthwash to prevent peri-implant mucositis as it significantly prevented S. mutans growth compared to the negative control. MGRE is a natural product and has numerous diverse pharmaceutical and biomedical properties that make it a good substitute for the currently used chemical mouthwashes. Using MGRE as a mouthwash can reduce the side effects of currently used mouthwashes, provide adjunctive health benefits for the patient and be financially more affordable than the existing chemical mouthwashes. Further studies, especially clinical trials are needed to investigate the effect of MGRE mouthwash in reducing the incidence of peri-implant mucositis.

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Conflict of interest
The author declares no conflict of interest.

References


