ORIGINAL ARTICLE

Anti-microbial efficacy of *Allium sativum* against *Streptococcus mutans* biofilm formation on orthodontic mini-implants

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ABSTRACT

Introduction: The aim of this study was to examine the effect of *Allium sativum* (garlic) extract on the biofilm formation by *Streptococcus mutans* on the surface of orthodontic mini-implants. **Materials and Methods:** Three brands (Dentos, Forestadent and Hubit) of titanium mini-implants were used as samples, which were divided into four groups each containing *S. mutans* along with four different concentrations of garlic extract-0 mg/ml, 16 mg/ml, 32 mg/ml, 64 mg/ml and 1 mini-implant from each of the three brands. The amount of viable *S. mutans* bacteria, as well as its biofilm formation on the surface of mini-implants was determined quantitatively as well as qualitatively using microbial viability assay, electron dispersive X-ray spectroscopy and scanning electron microscopy analysis. ANOVA test was done. **Results:** 32 mg/ml and 64 mg/ml concentration of garlic extract showed a considerable antimicrobial efficacy against *S. mutans* and effectively prevented the biofilm formation by *S. mutans* on the surfaces of all the mini-implants with 32 mg/ml being the lowest effective concentration of garlic extract to prevent *S. mutans* biofilm formation and 64 mg/ml being the most potent concentration. **Conclusion:** Garlic extract can be a promising alternative to other chemical agents used in mouthwashes to prevent bacterial biofilm formation on the surface of orthodontic mini-implants and can thus help in the reduction of mini-implant failure due to biofilm formation. It has a potential to serve as a herbal substitute to chlorhexidine, which has been shown to exhibit several side-effects during long-term use.

Key words: Bacterial biofilm, garlic extract, mini-implants, *Streptococcus mutans*

Introduction

Anchorage is a prerequisite for the orthodontic treatment of dental and skeletal malocclusions. Orthodontic anchorage is an important factor in obtaining good treatment results in a short period of time. Traditionally, appliances like headgear and intraoral elastics have been used for reinforcement of anchorage. In recent times, great emphasis has been placed on mini-implant as temporary anchorage device. These devices are small, are implanted with a relatively simple surgical procedure, and increase the potential for better orthodontic results.[1] Like any other treatment, several potential complications are associated with orthodontic mini-implants too.

A common complication is a failure of the mini-implant. The success rate of orthodontic mini implants have been reported to a range from 37% to 97%, respectively.[2,3] Failure of such devices frequently stems from bacterial biofilm build-up, which is extremely resistant to host defense mechanisms and antibiotic treatment. Inflammation and/or infection around the mini-screws due to an accumulation of bacterial plaque resulting from the patient's inadequate hygiene is one of the most common complication.[4,5] Inflammation of the peri-implant soft tissue has been associated with a 30% increase in failure rate.[6] *Streptococcus mutans* is biofilm-forming bacteria

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that has been strongly implicated as the primary agent to form biofilm over titanium mini-implants.[7] In view of this, therefore, it might be useful to develop preventive treatments that could inhibit biofilm formation and prevent subsequent implant failure. Many currently available products claim to bring about this effect. Cetylpyridinium chloride, chlorhexidine (CHX), amine fluorides, or products that include these agents have been used in the prevention and management of oral diseases. However, most of these agents exhibits cytotoxicity, they can stain teeth, and can even possibly cause oral cancer, as with the case of ethanol, which is frequently found in mouthwashes.[8,9] CHX has been shown to cause cytotoxic effects on mammalian cells at the conditions used clinically. Eren *et al*.[10] showed that rinsing with 0.12% CHX, twice daily during 18 days, caused deoxyribonucleic acid (DNA) alterations on epithelial cells and lymphocytes. In addition, in cultured cells, cytotoxicity of CHX was observed for blood cells,[11] keratinocytes,[12,13] fibroblasts,[14-16] osteoblasts and osteoclasts,[17] and macrophages,[18] at as low as 0.02% concentration. Consequently, the search for an alternative product which is effective, economical, safe and easily obtainable, such as a natural phytochemical isolated from plants used in traditional medicine, would be reasonable. A possible alternative to this is garlic (*Allium sativum*). It is one of the most extensively investigated medicinal plants in use since ancient times due to its antibacterial, antifungal, and antiviral properties.[19] Garlic extract exhibits a wide spectrum inhibitory effect on the growth of various Gram-positive and Gram-negative bacteria.

This *in vitro* study was designed to investigate the effect of garlic (*A. sativum*) extract on the formation of biofilm produced by *S. mutans* on the head and neck surfaces of three different brands (Dentos [Korea], Forestadent [Germany], and Hubit [Korea]) of titanium mini-implants.

Materials and Methods

Preparation of Garlic Extract

Fresh garlic (*A. sativum* L.) was obtained from the Department of Agriculture, Bureau of Plant Industry, Manila, Philippines and was blended in a sterilized mortar and pressed with gauze. This extract was centrifuged at 12,000 rpm for 10 min and was filtered twice with a 0.22-mm filter to obtain raw garlic extract. The three concentrations of this garlic extract were then prepared by diluting with distilled water-16 mg/ml, 32 mg/ml, and 64 mg/ml.

Preparation of *S. mutans*

S. mutans (ATCC 25175) was inoculated in brain heart infusion broth and was incubated for 4-6 h. The density of the bacterial suspension was adjusted with sterile phosphate buffer saline (PBS) to match the density of McFarland standard 0.5.

Preparation of Mini-implants

12 sterile brand new, packed and unused mini-implants were used in this study. Three different brands of titanium mini-implants were used (Dentos [A], Forestadent [B], and Hubit [C]). The 12 samples were divided into four groups of three samples each. The four groups were as follows:

- Group 1: Control group — one from each of the three brands of titanium mini-implants (Dentos [A], Forestadent [B], and Hubit [C]) without garlic extract.
- Group 2: One from each of the three brands (A, B, C) of titanium mini-implants immersed in 16 mg/mL garlic extract.
- Group 3: One from each of three brands (A, B, C) of titanium mini-implants immersed in 32 mg/mL garlic extract.
- Group 4: One from each of the three brands (A, B, C) of titanium mini-implants immersed in 64 mg/mL garlic extract.

To standardize the results and to avoid any error in the experiment, all the 12 samples were first subjected to electron dispersive X-ray spectroscopy (EDX) analysis before the start of the experiment to check for the amount of any carbon and nitrogen impurities which might be incorporated in minor amounts in the mini-implants during manufacturing procedures. The mini-implants were again analyzed after incubation with *S. mutans* and garlic extract in different groups. This was done to find out the total amount of carbon and nitrogen present on their surfaces which accounted for the presence of total bacterial mass attached on the surface.

Biofilm Cultivation

The *S. mutans* standardized suspension served as the source of inoculum. The biofilm assay was performed in biofilm medium (BM) containing 3% sucrose. *S. mutans* was cultured in a micro centrifuge tube containing 1 mL BM-sucrose broth soaked with the implants and different concentrations of garlic extract (0, 16, 32, 64 mg/mL). In each of these garlic concentrations, 1 mini-implant from each of the three brands was soaked respectively. After 40-h incubation at 37°C under aerobic condition, each implant was washed twice in sterile PBS (pH 7.2) and moved to another sterile micro centrifuge tube.

Microbial viability assay was performed using Presto Blue stain (Invitrogen, USA) to determine the total amount of viable bacteria present in the different groups. 100 µL

liquid *S. mutans* culture containing different concentrations of garlic extract was transferred to the 96-well polystyrene microtiter plates (Corning, USA) one by one. The sampling was processed in triplicates. 10 µL of Presto Blue was then added to it and the micro plates were incubated at 37°C for 30 min. After 30 min they were read at 570 nm using micro plate reader (Biotek, USA) to determine the colorimetric data optical density. The inhibition index was then determined.

Quantitative analysis of *S. mutans* biofilm was done with EDX analysis (EMAX X-act HORIBA EDX) performed at 100X on each mini-implant before growing the *S. mutans* biofilm on their surface to check for any carbon and nitrogen impurities and to standardize the results. Each mini-implant was first sterilized, subjected to EDX analysis and was sterilized again before subjecting it to biofilm formation. After 40 h incubation with *S. mutans*, mini-implants from each group were again subjected to EDX analysis to quantify the amount of bacteria present on the surface (head and neck portion) of the mini-implants. The amount of bacteria was quantified based on the total amount of carbon and nitrogen present on the surface of the mini-implants, which represent the total bacterial mass attached on their surface.

Qualitative analysis of *S. mutans* biofilm was done with scanning electron microscopic (SEM) analysis (S-3400N Hitachi Microscope) performed at 5000X magnification on the head and neck portion of each mini-implant under variable pressure-SEM mode to avoid any sample preparation.

Statistical analysis was performed using the one-way ANOVA test followed by multiple comparison (Dunnett's test) using SPSS for Windows, Version 16.0. (SPSS Inc., Chicago, USA). The experimental design was randomized complete block design. Paired *t*-test was also performed. The level of significance was established as $P < 0.01$ for all the statistical tests.

Results

Microbial Viability Assay

The one-way ANOVA followed by Dunnett's test revealed that Group 3 (32 mg/ml garlic extract) and 4 (64 mg/ml garlic extract) showed a marked reduction in the number of viable bacteria which was highly significant ($P = 0.000$) when compared to the control group. Group 2 (16 mg/ml) also showed a slight decrease in the number of viable micro-organism, but the difference was not significant when compared to the control group ($P = 0.027$) [Figure 1].

EDX

t-test for paired samples revealed that the mean amount of carbon and nitrogen on the surface of mini-implant after exposure to *S. mutans* was much higher (45.69%) compared with the amount before (3.56%), exhibiting an extremely significant difference ($P = 0.000$). This considerably high difference shows that the amount of carbon and nitrogen found on the surface of all the mini-implants after their incubation with *S. mutans* was due to the presence of bacterial biofilm build-up and represented the total amount of bacteria attached to their surfaces.

Figure 2 shows that the amount of bacterial biofilm attached to the head and neck surfaces of the mini-implants (as evident from the total amount of carbon and nitrogen present on their surfaces) decreased significantly from the control group to those treated with 32 mg/ml and 64 mg/ml concentrations of garlic extract, regardless of the brand of titanium mini-implant. A slight decrease in bacterial attachment was also seen with 16 mg/ml concentration of garlic extract when compared to the control group, but the difference was not found to be significant. Moreover, comparing the amount of bacterial attachment between the three brands of titanium mini-implants, it was seen that for each treatment group, Dentos showed the lowest bacterial attachment followed by Forestadent and then Hubit but the differences were not significant. Further multiple comparison was done between the four groups, using Dunnett's test which again revealed that groups 3 and 4 had significantly lower carbon and nitrogen content than the control group ($P = 0.000$) representing significantly lower bacterial biofilm attachment.

SEM

All the SEM images clearly showed that the amount of bacterial biofilm formation and attachment on the head and neck surfaces of the mini-implants decreased with the increasing concentration of garlic extract in all the three brands. There seemed to be a significant decrease in the bacterial biofilm formation in groups 3 (32 mg/ml garlic

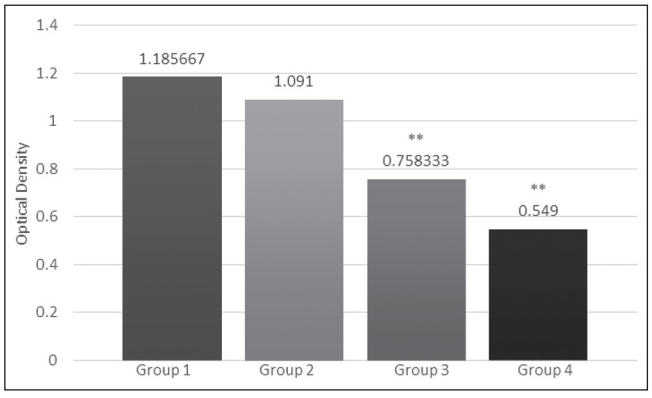

Figure 1: Optical density representing the amount of viable *Streptococcus mutans* in all the treatment groups (**$P < 0.01$)

extract) and 4 (64 mg/ml garlic extract) when compared to the control group while group 2 (16 mg/ml) displayed only a slight difference from the control group. Moreover, it was also evident that among the three brands of titanium mini-implants, Dentos mini-implants showed the least bacterial biofilm attachment followed by the Forestadent and the highest was seen in Hubit. Although, the differences were very small and insignificant [Figures 3-5].

These results are consistent with the results of the other two analyses which further confirm and adds to the accuracy of the previously found results.

Discussion

S. mutans comprises the vast populations of microbes in the biofilm, which when allowed to grow on the mini-implant interface can induce an inflammatory process endangering the mini-implant. An abundance of *S. mutans* in plaque is frequently present in orthodontic patients with fixed appliances.[20] To counteract this process, antimicrobial rinses are recommended. In general, anti-microbial agents show poor efficacy against micro-organisms growing as biofilms, in comparison with free-floating bacteria. Many compounds have therefore been investigated *in vitro* for their potential to reduce biofilm formation. Antimicrobial rinses like CHX are recommended for this purpose. CHX, however, though found to be effective in inhibiting plaque is also known to have negative side effects which hinder patient's compliance.

Garlic (*A. sativum*), on the other hand, is an essential food ingredient worldwide, has long been known to have antibacterial, antifungal, and antiviral effects. Several investigations have studied the antimicrobial effects of garlic extract. However, there is very limited information

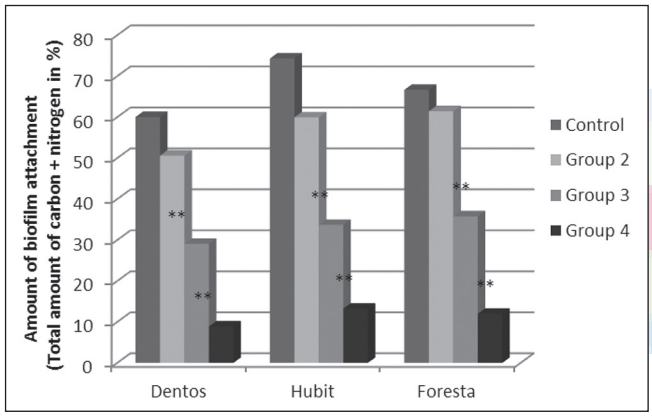

Figure 2: Comparison of the amount of bacterial biofilm attachment on the three brands of the mini-implants in the four treatment groups (**$P < 0.01$)

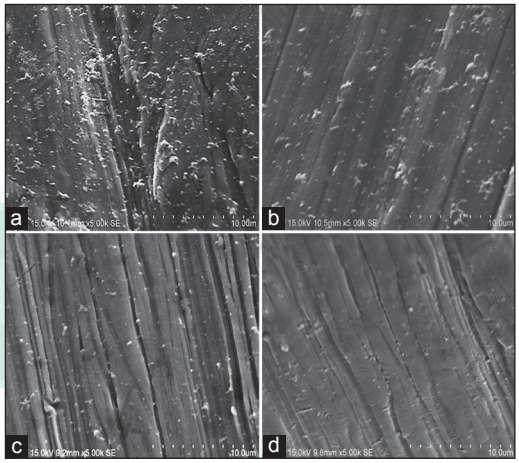

Figure 3: Scanning electron micrographs of *Streptococcus mutans* on Dentos mini-implants: (a) Group 1A-Control group (no garlic extract). (b) Group 2A-16 mg/ml Garlic extract. (c) Group 3A-32 mg/ml Garlic extract. (d) Group 4A-64 mg/ml Garlic extract

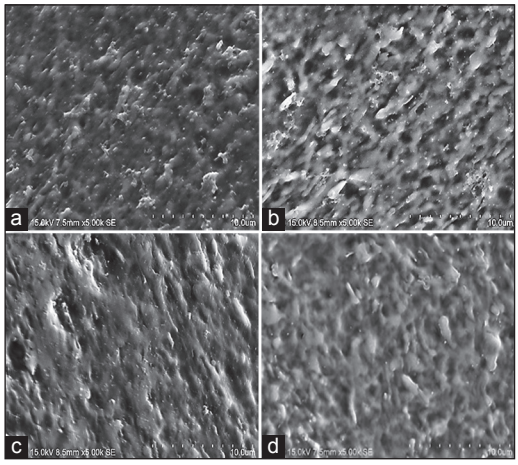

Figure 4: Scanning electron micrographs of *Streptococcus mutans* on Forestadent mini-implants: (a) Group 1B-Control group (no garlic extract). (b) Group 2B-16 mg/ml Garlic extract. (c) Group 3B-32 mg/ml Garlic extract. (d) Group 4B-64 mg/ml Garlic extract

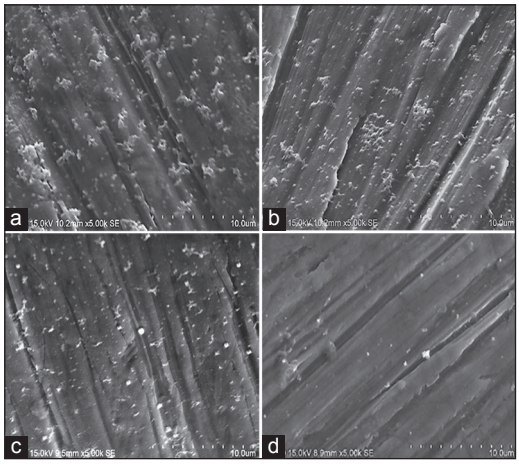

Figure 5: Scanning electron micrographs of *Streptococcus mutans* on Hubit mini-implants: (a) Group 1C-Control group (no garlic extract). (b) Group 2C-16 mg/ml Garlic extract. (c) Group 3C-32 mg/ml Garlic extract. (d) Group 4C-64 mg/ml garlic extract

about its effect on *S. mutans* biofilm prevention on titanium mini-implants. In this study, we tried to investigate the anti-microbial efficacy of garlic extract against *S. mutans* biofilm formed on titanium mini-implants.

The main antimicrobial constituent of garlic, allicin, is generated by the enzyme allicinase when garlic is crushed.[19] It partially inhibits DNA and protein synthesis, and entirely inhibits ribonucleic acid synthesis. Correspondingly, DNA transcription and other DNA activities are influenced by allicin.[21] In the present study, garlic extract has exhibited considerable antimicrobial activity against *S. mutans* biofilm formed on titanium mini-implants. This is in line with another study conducted by Pérez-Giraldo *et al*.[22] showing that allicin is active *in vitro* against *Staphylococcus epidermidis* and that sub-minimum inhibitory concentration (MICs) of allicin may play a role in the prevention of adherence of this bacteria to medical devices.

In the study of Fani *et al*.[23] they found that garlic extract is effective against multidrug-resistant strains of *S. mutans*. In terms of anti-microbial efficacy of garlic extract, the findings of this study are in agreement with the study done by Groppo *et al*.[24] suggesting that garlic extract mouth wash is effective at reducing total salivary *S. mutans* counts. They concluded that garlic extract (2.5%) could reduce the *S. mutans* count to a degree equivalent to that of 1.2% CHX mouthwash. According to a study conducted by Chavan *et al*.,[25] garlic extract (3%) mouthwash (containing 5% sorbitol as the sweetener and 5% spearmint oil as flavoring agent to mask the odor and bitter taste of garlic) has also been effective against *S. mutans in vivo*.

In a study conducted by Lee *et al*.,[26] the MIC of garlic extract on *S. mutans* growth was determined to be 32 mg/ml. The results of their study suggested that despite its antibacterial function, garlic extract increases biofilm formation by *S. mutans* to orthodontic stainless steel wire, likely through upregulation of glucosyltransferase expression. However, the study was done with only sub-MIC concentrations of garlic extract (8 mg/ml, 16 mg/ml). The possible explanation to this result as given by them was that garlic extract actually contains a biologically active substance effective at low doses for gene activation prior to bacterial cell growth inhibition. This study further checks the effect of both lower and higher concentrations of garlic extract. Hence, this study compared the anti-bacterial and biofilm prevention activity of 3 different concentrations of garlic extract, the sub-MIC (16 mg/ml), the MIC (32 mg/ml) and the above MIC (64 mg/ml), and their effect on the *S. mutans* biofilm on the titanium mini-implants. The present study points out that garlic is effective in reducing viable *S. mutans* count at a concentration as low as 32 mg/ml (equivalent to 3.2%).The results of this study indicate that the anti-microbial property of garlic extract can be effective even at doses quite lower than the toxic dose of allicin eliminating the need to use higher concentrations reducing the possibility of causing any harmful effects.

Chin *et al*.[27] compared the biofilm formation on five different titanium and stainless steel mini-implants systems and concluded that biofilm formation on mini-implants was governed by the roughness and the amount of carbon and oxygen-rich components. No significant differences in the amount of biofilm formation were observed between the three brands of titanium mini-implants in the findings of this study, although Dentos (Abso Anchor) showed slightly less bacterial biofilm attachment when compared to the other two brands.

Conclusion

The findings advocate that based on the capability, garlic extracts could provide benefits in preventing *S. mutans* biofilm formation on titanium mini-implants, thereby reducing mini-implant failures due to biofilm accumulation. However, lack of sufficient *in vivo* studies prohibits its clinical practice recommendation at the present time. Further clinical investigations for standardization and preparation of toothpastes and mouthwashes containing this antimicrobial agent for the prevention of bacterial biofilm formation on mini-implants is proposed. 5% sorbitol as the sweetener and 5% spearmint oil as flavoring agent can be added for mouth-wash preparation to mask the odor and bitter taste of garlic. Further studies are needed to find out more effective ways to make it more acceptable for clinical use.

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