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Comparison of the Effects of the Leaf Extract and Gum of *Pistacia atlantica* and Chlorhexidine on the Growth Initiation of *S. mutans*: An in vitro Study

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Running title: Effects of Pistacia atlantica on the S. mutans

Abstract

Background: Dental caries is one of the most prevalent infectious diseases, the main etiologic factor of which is Streptococcus mutans. The present study aimed to evaluate the effect of the leaf extract and gum of Pistacia atlantica on the growth inhibition of S.mutans in comparison to chlorhexidine (CHX).

Methods: In the present in vitro study, the antibacterial effects of the extract and gum of this plant were evaluated in comparison to that of CHX. The microbial sensitivity was evaluated by determining the diameter of the growth inhibition zone. In addition, the turbidity test was used to determine the minimum inhibitory concentration (MIC) of the materials against S.mutans.

Results: No growth inhibition zones for S.mutans were detected around the materials derived from P. atlantica, while a 24-mm growth inhibition halo formed around CHX. The MIC values for CHX and the leaf extract and gum of P. atlantica were 1.256, 1.8 and 1, respectively.

Conclusion: In general, the present study showed that the antibacterial effect of CHX on S. mutans was higher than those of P. atlantica derivatives. In addition, the P. atlantica leaf extract exhibited higher antibacterial activity compared to its gum, which is used as chewing gum in some regions.

Key words: Chlorhexidine, in vitro study, Pistacia atlantica, Streptococcus mutans

Introduction

Dental caries is one of the most prevalent infectious diseases. A proper solution to control or eliminate the microorganisms responsible for dental caries will help prevent this condition (1).

Several techniques have been suggested to prevent dental caries, including proper oral hygiene and the use of toothbrushes, which prevent the proliferation of bacteria, the formation of the dental plaque and initiation of dental caries. Another technique is the use of antibacterial agents against *S. mutans*. CHX mouthwash has successfully been used over the years in the local treatment of orodental diseases and some oral surgeries due to its disinfecting activity against oral microflora. However, CHX has some complications, including brown discoloration of teeth and a change in the sense of taste (dysgeusia), making it an improper choice as a mouthwash (2,3).

Based on the WHO report in 2014, antimicrobial resistance is on the increase as a global threat (4). Currently, herbal medicines have a critical role in the prevention and treatment of several diseases (5). *Pistacia* is most commonly found in the western, central and eastern regions of Iran (6). *Pistacia atlantica* is a source of polyphenols; therefore, its antioxidant activities have resulted in its use as a natural preservative and pharmaceutical agent (7). Some biologic properties have been reported for this plant, including antimicrobial, antiinflammatory, antipyretic, antidiabetic, anti-radical and cytotoxic properties. Some of these properties still have a role in folk medicine (8,9). To date, many studies have shown the antimicrobial effects of the extract derived from the unripe fruit of this plant (10), the plant extract (11), and the ethanolic extract of its leaves (12).

The extract and the gum of another strain of this plant (i.e., *Pistacia lentiscus*), too, have shown some inhibitory effect on some bacterial species (13). In addition, the gum of *P. atlantica*, which is collected by scratching the tree's trunk, is traditionally used to relieve gastric disorders (10).

The fruit and gum of *P. atlantica* have many nutritional, industrial and pharmaceutical uses (14). However, it appears only a limited number of studies have evaluated the effects of these plant species or their extracts and derivatives on the oral cavity pathogens, especially *S. mutans*, which is the main pathogen responsible for dental caries (15). Therefore, the present study aimed to evaluate the effect of the solid leaf extract and gum of *P. atlantica* on inhibiting the growth of *S. mutans* in comparison to CHX, hoping that the results would help achieve a simple technique to control dental caries.

Materials and Methods

In the present experimental study, the antibacterial effects of the extract and gum of *P. atlantica* were evaluated and compared with that of CHX.

Preparation of the extract and gum of P. atlantica

The extract of *P. atlantica* was prepared using a traditional technique. First, the leaves of *P. atlantica* were collected and dried. Then, they were crushed and immersed in water for two days, during which the water was passed through a filter two or three times. Finally, a brown-colored solution was achieved, which was boiled on fire with mild intensity for almost 12 hours and agitated with a wooden spoon so that it was concentrated. The product was a tar-like extract of *P. atlantica* leaf (leaf butter), which was dried and stored. The gum, too, was collected by scratching some points on the trunk of the tree directly. After preparing the extract and gum using the traditional technique, these materials were dissolved in dimethyl sulfoxide (DMSO); 1 g of the extract or gum was added to 5 mL of DMSO, followed by filtering through a filter with pores of 0.22 µm, using a Millipore syringe (16).

Preparation of the bacterial samples

First, the lyophilized vial containing *S. mutans* bacterial species in a powdered form was scratched at a location above the existing cotton, and after disinfection with a piece of gauze impregnated with 70% alcohol, the vial was broken at the scratched site. After removing the cotton from the vial with the use of a sterile forceps, a Pasteur sterile pipette was used to add 0.3–0.4 mL of distilled water to the dry powder within the vial and the resultant suspension was homogenized. Then a 2-mL syringe was used to transfer an adequate amount of the suspension on the microorganisms' routine culture medium. All the procedures were carried out under sterile conditions under a hood. In the next stage, the plate containing the microorganisms was incubated at a proper temperature. During this period, inoculation of microorganisms on the relevant routine culture media was carried out continually from the formed colonies, especially the young colonies, so that it would be possible to have access to young and active microorganisms during the whole study period (17). An approximate concentration of 3×10^8 of the microbial cell concentration in 1 mL (equivalent to 0.5 McFarland concentration) was achieved by retrieval of appropriate amounts of 24-hour-old young colonies from each of the microorganisms mentioned (17). *S. mutans* was confirmed by the catalase, optochin and bacitracin tests and gram staining.

Determination of microbial sensitivity (growth inhibition zone)

The well-plate technique introduced by Baron and Finegold (18) was used to determine microbial sensitivity. In this technique, the total concentrations of the three samples tested (leaf extract, gum and CHX) were used. In addition, penicillin was used as a positive control, and pure DMSO was used as a negative control. First, a sample of the prepared bacterial suspension at 0.5 McFarland concentration was inoculated on the surface of plates of BHI (brain-heart infection, Himedia, India), containing Mueller-Hinton culture medium, with the use of a sterile swab. Then, two wells, measuring 6 mm in diameter and 3 mm in depth, were placed in each plate (a total of 10 wells with two wells for each sample). A total of 30 µL of the leaf extract and gum of *P. atlantica* and 0.2% CHX, 120 mg/mL of penicillin as a positive control, and DMSO as a positive control were poured in each well. The plates were incubated at 37ºC for 24–48 hours. The diameters of the growth inhibition zones were determined once after 24 hours and once after 48 hours with the use of a mm-marked ruler accurately and recorded.

Determination of minimum inhibitory concentration (MIC)

The turbidity technique was used to determine MIC (minimum inhibitory concentration) (17). To this end, 2 mL of BHIB (brain-heart infusion broth) were transferred into the test tubes and autoclaved. A total of 10 test tubes were considered to prepare different dilutions of each test material. A pipette was used to transfer 2 mL of each test material into each ½ test tube to prepare 0, 1, 1/2, 1/4, 1/8, 1/32, 1/64, 1/128 and 1/256 dilutions. After mixing the contents of each test tube with a mixer, 2 mL of this solution was transferred to the next test tube, which continued up to the last test tube. Finally, 2 mL of the contents of the last test tube were transferred into the container containing the disinfecting solution. The test tube No. '0' was considered as the positive control (only containing the liquid culture medium and 20 µL of bacteria), and the test tube No. '1' was considered as the negative control (only containing the sample and bacteria). The microorganism in question (*S. mutans*) was used to prepare the microbial; suspension with an approximate 0.05 McFarland concentration; 20 µL of the above

microbial suspension were inoculated into all the test tubes with the use of a sampler. The contents of each tube were mixed completely on a vibrator immediately after inoculation to disperse the inoculated bacteria evenly within the liquid culture medium (17). All the procedures to prepare dilutions of the test materials and inoculation of the test tubes were carried out beneath a hood under aseptic conditions. These procedures were carried out separately and twice for each sample. The results of the MIC were determined and recorded after 24 and 72 hours by the presence or absence of bacterial growth in each test tube, which was detectable visually in the majority of cases. It should be pointed out that each test tube which was completely clear was considered as negative (no growth), and each tube which was clearly turbid, indicating bacterial growth, was considered as positive. In cases in which it was not possible to make a judgment about the growth of bacteria due to the dark color of the test materials, sampling was carried out with the use of a sterile swab and preparation of a smear on a glass slap, staining and evaluation under a microscope. In cases in which a large number of bacteria were observed in each microscopic field, the growth was considered positive, and when a large number of bacteria were not observed in each field, the growth was considered negative. The tube with the least concentration of the material tested, in which no growth was observed, was considered as the MIC for that material.

Results

Minimum inhibitory concentration (MIC)

Since similar results were achieved after repeating the tests using this technique at 24- and 72-hour intervals after culturing, the MIC of each material at the proximity of the microorganisms was reported using only one numeric value.

The results of culturing of *S. mutans* with different concentrations of *P. atlantica* leaf extract showed that there was bacterial growth at up to 1/16 concentration of the extract; however, at higher concentrations (>1/8), the extract inhibited the growth of bacteria. Concerning the *P. atlantica* gum, the results of MIC showed that only the absolute concentration of this gum inhibited the growth of *S. mutans* and at lower concentrations, the bacteria continued to grow and proliferate. Contrary to the two materials above, 0.2% CHX inhibited the bacterial growth at concentrations >1/256.

Growth inhibition zone (halo)

Neither the gum nor the extract of *P. atlantica* inhibited the bacterial growth at 24- and 72-hour intervals, and no growth inhibition zones were detected around these materials (Figure 1). However, a 24-mm growth inhibition zone was observed around CHX (Figure 2).

Statistical analysis

Chi-squared test was used to analyze the MIC of the three materials tested at a significance level of $P<0.05$, with SPSS software version 25, since data had been reported qualitatively-nominally. Similar results were achieved at 24- and 72-hour intervals; CHX was effective in inhibiting the bacterial growth, followed by the leaf extract and gum of *P. atlantica*, in descending order. Therefore, there was a significant relationship between the type of material and the absence of bacterial growth (Table 1, Figure 3). ANOVA was used to analyze the statistical significance of the growth inhibition zones, measured quantitatively in mm, between the three materials tested. The results showed that CHX was effective, followed by the leaf extract and gum of *P. atlantica* in descending order, and the difference was significant

statistically (Table 2). Two-by-two comparisons of the groups concerning the growth inhibition zone diameter, with post hoc Tukey tests, showed no significant difference between the leaf extract and the gum of *P. atlantica*. The mean difference was 0.2 mm (P=0.17), indicating no significant difference. However, CHX was significantly more effective than the leaf extract and gum of *P. atlantica* in terms of the growth inhibition zone diameter. The difference between CHX and the leaf extract was 23 mm (P<0.001), with 24 mm between the CHX and gum (P<0.001).

Discussion

The present study evaluated the effect of herbal extracts of *P. atlantica* on the growth of *S. mutans* as bacterial species inducing dental caries. Based on the results, these herbal extracts were significantly less effective than CHX.

The genus *Pistacia* has two species in Iran: *P. atlantica* and *P. khinjuk*. *P. atlantica* itself has three subspecies of *cabulica*, *mutica* and *kurdica* (19). Several studies have shown that the leaf extract of *P. atlantica* has inhibitory effects on *S. mutans*, *E. coli*, *K. pneumonia* and *P. aeruginosa* (11,12,20). One of the limitations of these studies has been that they have not mentioned the subspecies of *Pistacia* from which the gum has been extracted/collected. In the present study, *cabulica* subspecies was used, from which the processed gum is extracted. The differences in the results of different studies might be attributed to the differences in the subspecies used.

In the present study, with the MIC technique, the leaf extract of *P. atlantica* exhibited growth inhibitory effect on *S. mutans*; however, in the well-plate technique, no antimicrobial effect was detected. Possibly, such a difference can be attributed to the factors affecting the results of the well-plate technique. In this context, the effect of the materials on bacteria in the well-plate technique depends on the diffusion and penetration of that material into the agar, which is determined by the nature of that material. In addition, the well-plate technique is used to determine the antimicrobial sensitivity and solely determines the sensitivity or resistance of the microorganism to the antimicrobial agent; however, the MIC technique determines the antimicrobial effect intensity quantitatively in terms of concentration (21). Therefore, it appears the results of the MIC technique are more acceptable than the growth inhibition zone results (22). In the present study, CHX and the leaf extract of *P. atlantica* exhibited the highest inhibitory effects on *S. mutans* in descending order. Although no growth inhibition zone was detected around the well containing the leaf extract of *P. atlantica*, the MIC results showed the antimicrobial effect of this material.

Hosseini et al evaluated the antimicrobial activity of a chewing gum extracted from *P. atlantica* on *S. mutans* biofilm. In that study, *S. mutans* was cultured on a polyester biofilm in the THB culture medium in association with 5% sucrose; 10% to 100% concentrations of the gum were prepared in diethyl ether and added to the bacterial culture medium. The bacterial colony counts were determined before and after adding the gum to evaluate the anti-streptococcal effect of the gum. The results showed that ≥60% concentrations of the gum significantly decreased the bacterial colony counts, while the decrease in colony counts at 10–40% concentrations was not significant. Based on the results above, the oil extract of the gum of *P. atlantica* at high concentrations is effective in decreasing the durable biofilms of *S. mutans* (23). In the present study, too, in the MIC technique, the gum exhibited antibacterial

activity at its total concentration; however, in the paper disk technique, no growth inhibition zone was observed in the plate containing the gum. One reason might be that the gum cannot be transferred through the culture medium and possibly needs some modifications to exhibit its antibacterial effects.

In a study by Derwich et al, the antibacterial activity of the extract prepared from the oil of *P. lentiscus*, using the MIC technique with disk diffusion method, showed that the extract exhibited significant in vitro antibacterial effect on *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus*. In addition, its gum had antimicrobial activity against *Salmonella* and *Staphylococcus*. In addition, it was reported that the gum of *P. lentiscus* had superb antibacterial activity against *H. pylori*, which is the etiologic agent for peptic ulcers (20).

Aksoy et al evaluated the antimicrobial effect of gum against *S. mutans* in vitro and in vivo. In the in vitro study, the disk diffusion technique was used. In the in vitro study, 25 subjects who were periodontally healthy were evaluated concerning the inhibitory effect of gum on salivary *S. mutans* in comparison to a placebo. The microorganisms were cultured on the *mitis salivarius*-bacitracin agar culture medium and incubated at 37ºC in an anaerobic environment for 48 hours. Finally, bacterial counts were determined. Based on the results of the in vitro step of the study, the growth inhibition zone diameter of *S. mutans* was 9–24 mm. The in vitro study results, too, showed a significant decrease in salivary *S. mutans* counts in the subjects who chewed gum compared to the control (paraffin gum) group (24).

In contrast to the side effects of antibacterial agents and antibiotics, previous studies have not reported any specific side effects for *P. atlantica* extract and gum. However, CHX has been known as the gold standard in studies on plaque-inhibiting materials and is believed to be the best wide-spectrum antimicrobial agent in the oral cavity. The chlorhexidine gluconate salt has a cationic nature and exhibits a continuous bacteriostatic effect over time. The mechanism of action of this material is through its adhesion to the cell wall of the microorganisms and increasing the permeability of the cell membrane and percolation of cytoplasmic contents out of the cell (25). However, unfortunately, this valuable agent has bad taste, changes the sense of taste and produces brown discoloration on the teeth, the oral cavity and the tongue; therefore, patients are not interested in using it (26–28).

Based on the MIC data, the amount of CHX necessary to inhibit *S. mutans* is less than that of the leaf extract and gum of *P. atlantica*, respectively.

Conclusion

Overall, the results of the present study showed that the antibacterial effect of CHX on *S. mutans* was higher than that of the derivatives of *P. atlantica* tree. In addition, the leaf extract of *P. atlantica* exhibited a higher antibacterial activity compared to its gum which is used as chewing gum in some regions. Under the limitations of the present in vitro study, it appears that the leaf extract of *P. atlantica* has a greater potential for use as an anticariogenic mouthwash compared to CHX, due to its fewer side effects than CHX. However, its antibacterial activity is lower than that of CHX.

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Table 1. Relationship between the type of material and the absence of bacterial growth

		No growth	Growth	Total
Groups	***P. atlantica* extract**	5	5	10
	***P. atlantica* gum**	1	9	10
	CHX	10	0	10
Total		16	14	30

Chi-squared = 16.4, P<0.001

Table 2. ANOVA analyze for the Statistical analysis of the growth inhibition zones, measured quantitatively in mm, between the three materials tested

		N	Mean	SD	Minimum	Maximum
Growth inhibition zone (24 hours)	***P. atlantica* extract**	10	0.20	0.422	0	1
	***P. atlantica* gum**	10	0.00	0.000	0	0
	CHX	10	24.00	0.000	24	24
	Total	30	8.07	11.462	0	24
Growth inhibition zone (48 hours)	***P. atlantica* extract**	10	0.20	0.422	0	1
	***P. atlantica* gum**	10	0.00	0.000	0	0
	CHX	10	24.00	0.000	24	24
	Total	30	8.07	11.462	0	24

ANOVA, F=3.2, P< 0.001

Figures

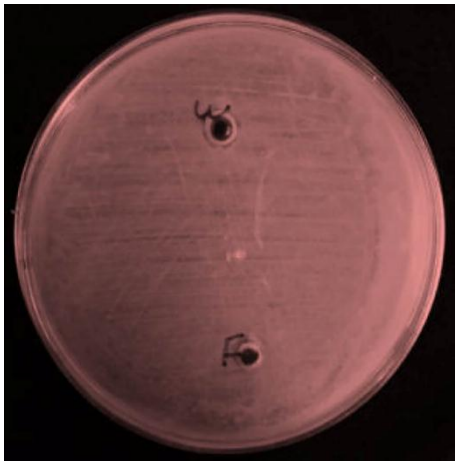

Figure 1. Absence of a growth inhibition zone around the wells containing the gum and leaf extract of *P. atlantica*.

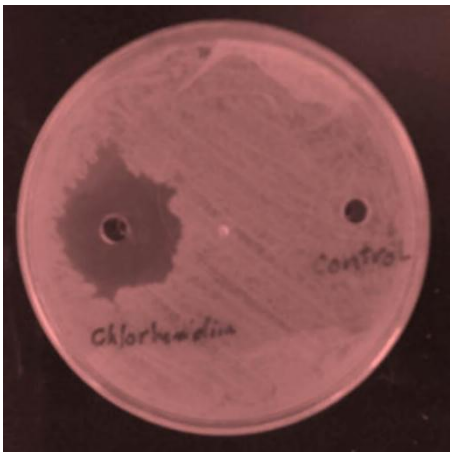

Figure 2. Formation of a growth inhibition zone.

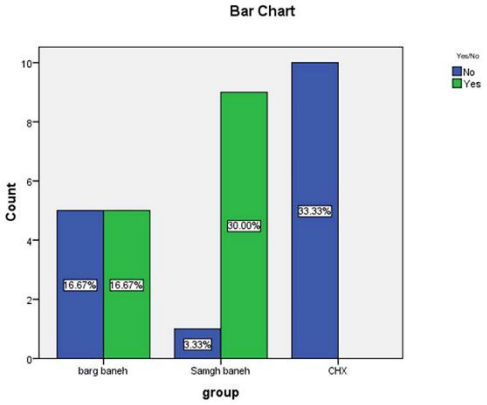

Figure 3. Relationship between the type of material and the absence of bacterial growth (No growth: No, Growth: Yes)