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Original Article

Anti-adherence and anti-bacterial activities of *Pistacia atlantica* resin extract against strongly adherent *Streptococcus mutans* strains

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ABSTRACT

Background: The reduction of *Streptococcus mutans* from the oral cavity or its adherence to tooth surfaces can prevent or decrease the progression of caries. In this study, the antimicrobial and anti-adherence properties of *Pistacia atlantica* (*P. atlantica*) resin (Essential oil [EO] and methanolic extract [ME]) were investigated on *S. mutans* strains.

Materials and Methods: In this *in vitro* experimental study, the growth rate, biofilm formation ability, and antibiotic susceptibility profile of *S. mutans* ATCC35668 and 3 strains isolated from caries lesions were studied. The EO and ME of *P. atlantica* resin were prepared. The anti-bacterial and anti-adherence properties of them were evaluated using microdilution and microplate adherence tests, respectively. The data were statistically analyzed using SPSS with one-way and two-way analysis variance. Direct comparisons between the groups were made using the Wilcoxon W-Mann–Whitney *U*-test. Statistical significance was set at $P < 0.05$.

Results: All target strains showed the same growth rate and antibiotic susceptibility profile and were found strongly adherent. Both EO and ME showed moderate anti-bacterial properties (growth reduction up to 47.1% and 39.1%, respectively) against *S. mutans,* while the anti-bacterial effect of EO was higher than ME, significantly ($P < 0.05$). In all tested concentrations, EO showed a significantly stronger anti-adherence activity (50%–80%) than ME.

Conclusion: The results showed an anti-cariogenic effect of EO extracted from *P. Atlantica* resin. Considering that S. *mutans* adhesion is a necessary step in the beginning and progression of dental caries, this study can suggest the use of such extract in mouthwashes or toothpaste as an alternative agent for preventing bacterial attachment and biofilm formation.

Key Words: Adherence, anti-bacterial agent, cariogenic, pistacia, *Streptococcus mutans*

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INTRODUCTION

Dental caries is the main public health concern ranked first for the permanent tooth decay of about 2.3 billion people.[1] Specific bacteria are related to dental caries initiation and progressions. *Streptococcus mutans* is considered the principal etiological agent of dental caries due to its adherence ability to tooth surface, sucrose fermentation, extracellular polysaccharides, and acid production.[2,3] Hence, the reduction of *S. mutans* or its attachment to tooth surfaces can prevent or decrease caries development.[4]

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Today, there are several mechanical, chemical, and biological preventive strategies to control the caries progression.[5] However, despite the progress in preventive methods, dental caries distribution in developed countries remains high. Hence, there is a great need to look for novel preventive methods. Besides, there are some well-known anti-microbial agents like chlorhexidine gluconate (CHX) which is very successful in reducing the salivary mutans *Streptococci* count, but it is not effective as a preventive agent due to its adverse effects like teeth and tongue discoloration and bacterial resistance.[6,7]

However, biofilm elimination from teeth surfaces remains a challenge because the nature of structural and physiological properties of biofilm bacteria provides natural resistance to anti-bacterial agents.[8] Thus, further researches and development of alternative natural and safe antimicrobial agents for dental caries prevention are needed. In recent decades, much attention has been attracted to the use of traditional herbal agents due to exhibiting potent anti-bacterial, anti-cancer, anti-adhesion, or anti-cariogenic activities of plant-derived active compounds.[9-11]

One of the traditional plants is *Pistacia atlantica (P. atlantica)* which is called *"Banneh"* in Iran. It is one of the most important tree species commonly distributed in different countries such as Iran, Iraq, Algeria, and turkey.[12] The resin of *P. atlantica* tree has various beneficial effects and it is traditionally used in Iran as peptic ulcer treatment, mouth freshener, antiseptic, gum tissue strengthener, appetizer, astringent, diuretic, gastrointestinal disorder treatment, etc.[13] Other studies have reported the anti-fungal, anti-parasite, and anti-bacterial activities of *P. atlantica*.[14-17] So, this study aimed to investigate the anti-cariogenic efficacy of the essential oil (EO) and methanolic extract (ME) of *P. atlantica* resin through the investigation of their anti-bacterial, and anti-adhesion activities against strongly adherent (SA) *S. mutans* strains *in vitro*.

MATERIALS AND METHODS

Bacterial strain and growth conditions

S. mutans ATCC 35668 and other SA strains, isolated from caries lesions, were provided from the Microbiology Laboratory of Isfahan branch, Azad University, and cultured in tryptic soy broth (TSB; Merck, Germany). The growth profile of the strains was recorded at time intervals. The antibiotic susceptibility of the strains was evaluated by the disc diffusion method (Kirby and Bauer) on Mueller Hinton agar (Merck Co. Germany).[18] The antibiotic discs (Padtan Teb Co. Iran) used were: Pencillin-G (P; 10 µg), Cefalothin (CF; 30 µg), Cefalexin (CN; 30 µg), Vancomycin (V; 30 µg), Gentamycin (GM; 10 µg) and Streptoomycin (S; 10 µg).

Biofilm formation ability

To select SA strains, the biofilm formation ability of strains was quantified by a microtiter plate method as previously described.[19] Briefly, overnight cultures of strains in TSB supplemented with and without substrates (Sucrose 1% and a mixture of 0.5% glucose and 0.5% fructose) were prepared. Their turbidity was adjusted to 0.5 on Mc Farland turbidity. Each bacterial suspension (250 µl) was transferred into wells of a microtiter plate, incubated (35 ˚ C, 24 h), the wells were emptied, and washed with sterile PBS solution. The remaining attached bacteria were fixed (96% methanol: 250 µl/well; 15 min), stained (2% crystal violet: 200 µl; 5 min), the excess stain was washed off, and the plates were left to dry. The formed biofilm was quantified by resolubilizing the stain in 200 µl of 33% (v/v) glacial acetic acid (Merck, Germany) per well. The optical density (OD) of the stain was measured at 492 nm by an ELISA reader (TECAN Co. Spectra SLT). Control wells only contained broth. According to the obtained ODs, strains can be classified into the different categories:

OD < ODC = nonadherent (NA);
ODC < OD < 2ODC = Weakly adherent;
2ODC < OD < 4ODC = Moderatly adherent;
4ODC < OD = SA (or strong biofilm producer).

OD and ODC are the mean OD of wells with biofilm and the control wells, respectively.

Resin collection, essential oil and methanolic extract preparation

P. atlantica resin was gathered from the Zagros Mountains of Iran. ME and EO were obtained from *P. atlantica* resin. The EO was extracted by hydrodistillation method (150 g; with 3 L water) and pale yellow oil (12 g) was obtained (8% v/w).[20]

Anti-bacterial activity assay

Anti-bacterial activity of EO and ME was evaluated by the microdilution method as described previously.[21] The crude EO and ME of *P. atlantica* were used as stock solutions with the concentration

of 300 mg/ml in DMSO which was nontoxic to bacteria. The *S. mutans* suspension (20 µl equal to 0.5 Mc Farland suspension.), diluted extracts at different concentrations (30 µl of 60%-100%), and BHI broth (200 µl) were added to each well of 96-well microtiter plates. Wells containing CHX (0.65 mg/ml) and bacterial suspension without any inhibitory compound (non-treated) were considered as the positive and negative controls, respectively. Then, plates were incubated (37 °C, 18 h) and the optical densities of wells were read by the ELISA reader at 620 nm and compared. Each control or treatment was tested in 8 replicates.

Anti-adhesion activity assay

The anti-adhesion assay was performed according to Tahmourespour *et al.*(2019) with some modifications as follows:[21] Each column of a 96-well microplate was filled with 200 µl/well of each EO and ME concentration. The plate was shaken (1 h) and the wells were emptied. *S. mutans* suspension (20 µl) along with TSB containing 1% sucrose (200 µl) were added to them and incubated (4 h, 37°C). The unattached cells were rinsed then adhered *S. mutans* cells were fixed, stained and the optical density of every well was read as described above. This assay can estimate the microbial adhesion reduction percentage versus the control wells. Control wells contained PBS buffer instead of ME or EO.

Statistical analysis

Each experiment was done in triplicate. The obtained data were statistically analyzed using a software package SPSS-20 (SPSS Inc., Chicago, IL, USA) with one-way and two-way analysis variance. Direct comparisons between the two groups were made using the Wilcoxon W-Mann–Whitney *U*-test. Statistical significance was set at $P < 0.05$.

RESULTS

Figure 1 shows the optical densities obtained from the growth of *S. mutans* strains after 24 h of incubation in the same medium and condition which did not show any significant difference using the Kruskal–Wallis test ($P > 0.05$). The results of *in vitro* antibiotic susceptibility tests of selected strains are summarized in Table 1. No significant differences ($P > 0.05$) in antibiotic-resistance rates between the tested strains were found. The resistance prevalence against the tested antibiotics was relatively low, ranging from 0% (Vancomycin) to 100% (Streptomycin).

According to determining the biofilm formation ability of tested *S. mutans* strains in the existence of different substrates [Figure 2], it was clear that, all of the strains were classified as (SA) and (NA) in the existence of sucrose and no sugar, respectively. The adherence ability of SM1 was significantly higher ($P < 0.001$) than other tested strains in presence of sucrose as a carbohydrate source while there was no significant difference between SM2, SM3 and STD strains.

Anti-bacterial activity

According to the results of the microdilution assay [Figure 3], it was clear that the growth reduction percentages of *S. mutans* strains in the presence of both EO and ME were under 50%, while, CHX could cause up to 98.7% reduction in the growth of testing strains with a significant difference, according to the KW test and Chi-square ($P < 0.001$).

Two-way analysis of variance (Wilcoxon W-Mann–Whitney *U*-test) did not show any significant difference between the EO and ME inhibitory effects ($P > 0.05$) concerning the CHX effect. Without

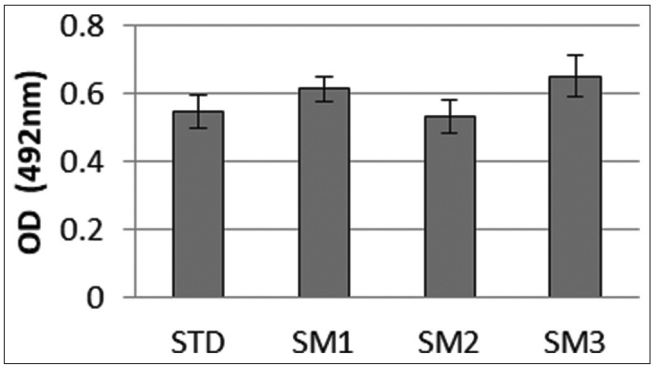

Figure 1: The comparison between the growth rate of *Streptococcus mutans* strains after 24 h incubation in same condition. (OD: Optical density; STD: Standard strain of *S. mutans ATCC35668;* SM*: Streptococcus mutans*).

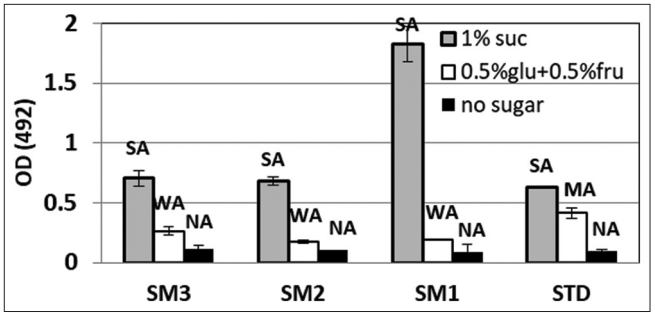

Figure 2: The biofilm formation ability of tested strains in the existence of different substrates. SA: Strongly adherent; MA: Moderately adherent; WA: Weakly adherent; NA: Nonadherent.

Table 1: The antibiotic susceptibility profile of *Streptococcus mutans* strains based on zone of growth inhibition diameter in millimeter

Antibiotics S index (mm) strains	Pn (10 µg) ≥24	V (30 µg) ≥17	CN (30 µg) ≥20	CF (µg 30) ≥18	GM (10 µg) ≥15	S (10 µg) ≥15
STD	18®	20 (S)	16.5 (I)	17.5 (I)	14 (I)	8®
SM2	23.5 (I)	18 (S)	20 (S)	20 (S)	11.5®	6®
SM3	23 (I)	21 (S)	26.5 (S)	26 (S)	10®	6®
SM1	25 (S)	17.5 (S)	23.5 (S)	25 (S)	12.5®	8®

Pn: Pencillin-G; V: Vancomycin; CN: Cefalexin; CF: Cefalothin; GM: Gentamycin; S: Streptoomycin; STD: Standard strain of *Streptococcus mutans* ATCC35668; ®: Resistant; S: Sensitive; I: Intermediate, SM: *Streptococcus mutans* S index: Sensitivity index

considering the effect of CHX, the anti-bacterial effect of EO was higher than ME, significantly ($P < 0.05$).

Anti-adherence activity

The anti-adherence activity was observed in presence of both herbal agents (EO and ME). However, EO showed the highest and the lowest anti-adherence activity of about 81% and 54% at concentrations of 100% and 60%, respectively. Meanwhile, the highest and the lowest anti-adherence activity of 22.9% and 2.07% at concentrations of 100% and 60% of ME were observed. Hence, according to the statistical analysis, the *P. atlantica* resin EO showed significantly higher anti-adherence activity than ME ($P < 0.05$) [Figure 4].

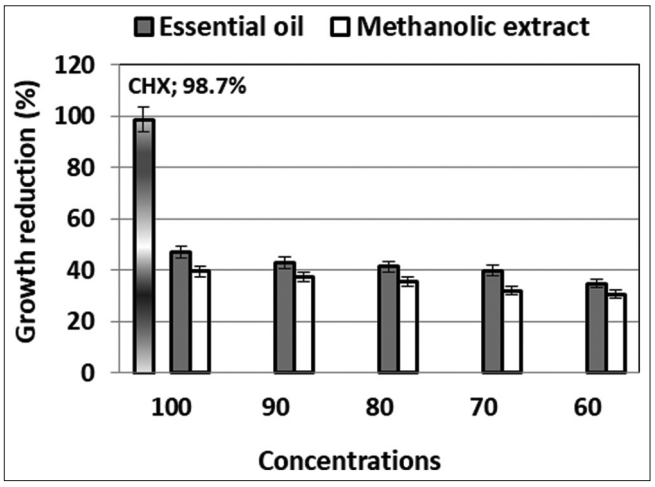

Figure 3: The antibacterial activity of *Pistacia atlantica* resin, essential oil and methanolic extract against *Streptococcus mutans* strains using microdillution method.

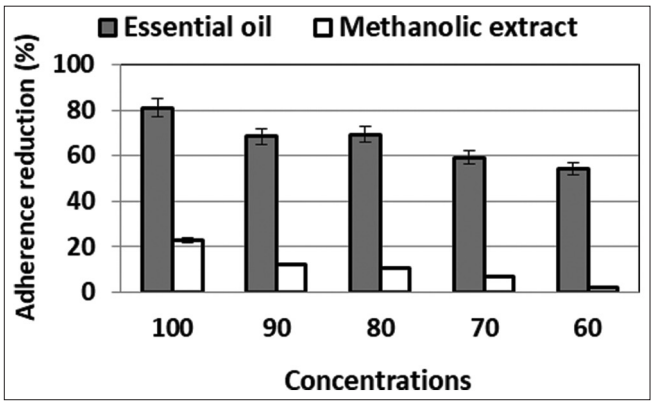

Figure 4: The anti-adherence activity of essential oil and methanolic extract of *Pistacia atlantica* resin.

DISCUSSION

As *S. mutans* is the main bacterium responsible for early colonizing the oral cavity, it is necessitated in caries progression from its initiation. Dental caries is an important oral health problem that needs more investigation. In this regard, the use of different mouthwashes with anti-microbial activity is increased, but most of their major components (e.g., CHX) can cause various side effects.[4] Hence, to overcome and reduce such side effects, different studies have been evaluated the anti-microbial and anti-biofilm effects of various herbal extracts.[6,7,21,22] Since dental plaque formation as a biofilm initiates with the adherence of bacteria (*S. mutans*) to the pellicle and *in situ* growth of attached cells, finding herbal agents with the anti-adherence activity along with anti-microbial property can possess a beneficial role in preventing biofilm and caries development. Although *P. atlantica* species are considered the best known and one of the most appreciated medicinal plants in countries like Iran, Turkey, etc., still very few studies regarding its anti-cariogenic potential are available in literatures. Hence, in this study, the anti-bacterial and anti-adherence potential of EO and ME derived from *P. atlantica* resin was investigated, because the resin has been utilized for 5000 years ago for a wide range of purposes.[23]

The target strains of this research were selected with the aim of inhibition or suppression of growth and biofilm formation by the EO and ME of *P. atlantica* resin. At first, the properties of target strains such as their planktonic growth rate, antibiotic susceptibility profile, and biofilm formation ability were investigated. According to the results, no statistically

significant difference in each of the examining properties was observed between the selected strains. Hence that, all 4 strains showed the same growth rate and antibiotic susceptibility profile. Also, all were found SA in the existence of sucrose (1%) while they were NA in no sugar condition.

According to the results, *P. atlantica* resin showed moderately anti-bacterial activity against *S. mutans* strains. Furthermore, it is observed that its EO possesses significantly higher anti-bacterial activity than the ME. The EO could reduce the growth of *S. mutans* cells up to 47.1%, while the *S. mutans* growth reduction in the existence of ME reached up to 39.1%. Mohamed *et al*.(2007) also showed that the EO of the *Pulicaria crispa (Forsk.) Oliv.,* and *Pulicaria undulata* had better antimicrobial activity on most of the microorganisms than the ME.[24]

Such anti-microbial activity is not supported by other researches as they reported complete growth inhibitory activity or higher anti-microbial activity on *S. mutans*; Hosseini *et al*.(2013) showed that the diethyl ether extracts of *P. athlatica* possess stronger inhibitory activity compared to aqueous extracts, although the bacterial cells in the biofilm were not affected by the extracts.[25] Najafi *et al*. (2014) also observed the anti-microbial effect of *P. atlantica (var. mutica)* against *Bacillus cereus*, *Staphylococcus aureus,* and *Escherichia coli* (O157 H7) by the method of disc diffusion which can be attributed to the high content of α-pinene. They also indicated that in the case of *Pseudomonas aeroginosa* no clear zone was formed.[26] Roozegar *et al*.(2016) showed the anti-microbial effect of the leaf extract *P. atlantica* on *S. mutans*.[27] These differences are possibly due to qualitative and quantitative variations between the content of the EO which are related to several parameters such as plant species and part, cultivars sex, harvesting time, climatic conditions, geographical origin, microbial species and their resistance ability, and methods used to investigate their activity.[28-31] In general, EO is one of the major constituents of various parts of *Pistacia* species which contains various types of phytochemical agents such as terpenoids, fatty acids, phenolic compounds, and sterols.[23] Golestannejad *et al*.(2020) also showed that olive leaf methanolic, ethanolic, and hydroalcoholic extracts have appropriate anti-bacterial activities due to their high phenolic content but they did not study the antibacterial activity of its EO.[32]

The inhibition of *S. mutans* adherence onto the polystyrene microtiter plate by different concentrations of the extract and EO was also observed in this study. In all tested concentrations of EO, a significantly stronger anti-adherence activity (50%–80%) than ME (up to 22%) was seen. It is probably due to EO constituent effects on hydrophobic interactions, which were reported to be significant in bacterial adhesion onto tooth surfaces. Thus, the surface hydrophobicity modification could be used as an anti-adherence strategy.[33] As *S. mutans* adherence to surfaces is mediated by glucan, the product of glucosyltransferases, the anti-adherence property of EO probably could be associated with the anti-glucosyltransferases activity of compounds such as flavonoids and tannins. Hence, the inhibition of such enzymes can be used as another anti-adherence or anti-biofilm formation strategy. The cariostatic efficacy of flavonoids and tannins (a kind of polyphenole) relates to their anti-microbial activity against *S. mutans* planktonic and biofilms cells; effects on acidogenic or aciduric characteristics, and down-regulating *gtf* gene expression.[34] Zeng *et al*.(2019) also demonstrated the anti-biofilm effect of flavonoids, quercetin, and kaemferol against *S. mutans*.[35] Yoo *et al*. (2018) showed the anti-microbial activity of β-caryophyllene, EO from clove, against *S. mutans* biofilm and planktonic cells. They concluded that such EO can decrease *S. mutans* count, inhibit biofilm formation also decrease Gtfs expression.[36] As the resin of *P. atlantica* is composed of different substances, it is better to isolate and purify them and evaluate their anti-cariogenic potential one by one, which was the limitation of the present study.

CONCLUSION

Overall, the growth of SA *S. mutans* strains was inhibited moderately in presence of EO and ME of *P. atlantica* resin. The EO exhibited a significantly higher anti-adherence (anti-biofilm) activity than ME against the *S. mutans* strains. Hence, this compound with anti-bacterial and anti-adherence activities without drug resistance induction potential can serve as a key component of mouthwashes and toothpaste formulations facilitating the prevention of dental caries or other biofilm-related oral diseases. Hence, each of the different herbal extracts or their mixture can be a promising part of an ideal caries management program and still needs additional researches.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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