Antimicrobial Efficacy of *Salvadora persica* Extracts on a Monospecies Biofilm on Orthodontic Brackets In Vitro

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Purpose: The oral cavity is a rich ecosystem with a plethora of microorganisms, and different components of fixed orthodontic appliances may contribute to a shift in the balance of oral ecology. The purpose of this study was to investigate the antimicrobial potential of hexane and ethanol extracts of *Salvadora persica* on a monospecies biofilm model established on orthodontic brackets in vitro.

Materials and Methods: *Streptococcus mutans* biofilm was formed on mini diamond orthodontic brackets following three days of anaerobic incubation at 37˚C. The bacterial cell viability of this biofilm was measured after their exposure to saline, hexane extract of *S. persica*, ethanol extract of *S. persica* and 0.2% chlorhexidine using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay. On half of the brackets, the colony forming units (CFU) were counted. Both experiments were performed in triplicate.

Results: The absorbance values obtained from the MTS reduction assay after exposure to the different test agents showed a decline in the bacterial cell viability of the *S. mutans* biofilm as follows: chlorhexidine (+) < hexane extract (*S. persica*, 5 mg/ml) < ethanol extract (*S. persica*, 5 mg/ml) < saline + 2% DMSO (-). There was significant difference between saline + 2% DMSO and the other test solutions ($p < 0.05$). The mean absorbance values showed that the antimicrobial activity of hexane extract was slightly more effective than that of the ethanol extract and was nearly as effective as chlorhexidine (+). However, the differences in the absorbance values between the extracts of *S. persica* and chlorhexidine (+) were not statistically significant ($p > 0.05$). The CFU counts of *S. mutans* obtained from chlorhexidine exposure were lower than from hexane and ethanol extracts.

Conclusion: *S. persica* extracts were found to have antimicrobial effects on *S. mutans* biofilm established in vitro on orthodontic brackets suggestive of its potential use as an oral antimicrobial agent for orthodontic patients.

Key words: colony count, mouthwashes, orthodontic brackets, plant extracts, *Streptococcus mutans*

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The oral cavity is a rich ecosystem with a plethora of microorganisms. While both periodontal disease and caries are considered multifactorial diseases, distinct consortia of plaque microorganisms are the major factor in their onset and progression.[27,34] However, there are situations which involve what has been termed 'ecological stress', referring to the shift of the microbiological equilibrium, creating conditions favourable to the growth and appearance of cariogenic and/or periodonto-

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pathic bacteria.[29] The different components of the fixed orthodontic appliance may contribute to a shift in the balance of the oral ecology.

The oral environment of orthodontic patients during the treatment phase undergoes several changes, such as pH reduction, larger number of retention sites available for *Streptococcus mutans* collection, and increased accumulation of food particles, which may lead to an increased number of *S. mutans* colony-forming units (CFU) in saliva and dental plaque. Such changes may contribute to the development of the decalcification lesions frequently found at the end of orthodontic treatment.[35,38] Several studies have confirmed an increase in plaque adhesion and inflammatory reactions during orthodontic treatment.[17,22,30,34] Despite the advances in orthodontic materials and techniques in recent years, the buildup of dental plaque on teeth and the subsequent development of enamel decalcification and dental caries around orthodontic appliances continue to be a problem.[11]

A wide range of anti-plaque agents delivered from dentifrices, gels and mouthrinses are used to augment mechanical oral hygiene practices to control plaque formation and the development of early periodontal diseases.[7,13] Clinically effective antiplaque agents are characterised by a combination of intrinsic antimicrobial activity and efficient bioavailability by remaining active for extended periods.[36,40] The use of mouth rinses in combination with mechanical plaque removal has proved to be an important therapeutic agent in controlling gingival inflammation, bleeding, and plaque accumulation in orthodontic patients.[10,32]

Chlorhexidine is the most thoroughly studied antiplaque agent and has evolved into a gold standard among antimicrobial agents used in dentistry.[18,19,25] It has been found to have good plaque-inhibiting properties with an immediate antibacterial action and a prolonged effect on the oral flora.[20,39] However, reversible local side effects such as staining of teeth, altered taste sensation, increased formation of supragingival calculus, and occasionally mucous membrane irritation and desquamation are associated with prolonged use of chlorhexidine mouthwash. To a varying degree, these factors might unfavourably affect patient compliance, particularly when chlorhexidine is used as a mouthwash addition to a patient's normal daily oral hygiene routine.[28,33] Therefore, people's interest has diverted to other, safer alternatives, particularly natural herbal agents.[21,24,26]

The beneficial effects of *Salvadora persica* (also known as miswak) with regard to oral health have gained increasing importance in recent years. The pharmacological properties of *S. persica* include antimicrobial, antimycotic, analgesic, anti-inflammatory, antiulcer, antihyperlipidemic, anticonvulsant and hypoglycaemic action as well as astringent, detergent, sialogogue and improved digestive effects.[1] The different components of *S. persica* extracts are reported in several studies to have strong antimicrobial activity and these heterogeneous components can be extracted by using various chemical procedures.[3,37]

To the best of our knowledge, there are no studies that evaluated the antimicrobial effectiveness of *S. persica* extracts on biofilms grown on orthodontic brackets. Therefore, the objective of this study was to investigate the antimicrobial potential of hexane and ethanol extracts of *S. persica* on a monospecies biofilm model established on orthodontic brackets in vitro.

MATERIALS AND METHODS

The sticks of *S. persica* were collected from Almukwah close to the Al-Baha area in the southern region of Saudi Arabia. These plant materials were identified by a taxonomist and a voucher specimen (#1745) was deposited in properly labeled polythene bags for future reference at the Herbarium Centre, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The plant sticks were shade dried and then ground to a fine powder using a commercially available food blender. The ground sample (100 g) was used to prepare the hexane and ethanol extracts.

Consequently, the hexane extract was prepared by percolating 50 g of dried powder of the plant sticks in 500 ml of the hexane solvent for 72 h and every 24 h fresh solvent was used. Similarly, the ethanol extract was prepared by using 500 ml of 90% ethanol (absolute ethanol containing 10% water). Most of the solvents were removed and recovered in a rotary evaporator (BÜCHI Rotavapor RII, Büchi Labortechnik; Flawil, Switzerland) at 60°C using a BÜCHI vacuum pump. At the last stage the extracts were freeze-dried to make sure that solvents were totally removed. Stock preparations of *S. persica* extracts were made in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml, which was kept frozen in a -30°C freezer until use. Working dilutions of miswak extracts were made at a

concentration of 10 mg/ml in physiological saline at a pH of 7.4.

The frozen stock culture of *S. mutans* (ATCC 25175) was grown in brain heart infusion (BHI, Scharlau; Barcelona, Spain) broth and incubated for 24 h at 37°C. The broth culture was streaked on BHI agar and after 24 h of incubation in an anaerobic chamber (Becton Dickenson Laboratories; Cockeyville, MD, USA), a single discrete colony was used to inoculate 5 ml of BHI broth and incubated. This growth consistently showed turbidity as per McFarland unit of ≥ 4. Intermittently, single discrete colonies were stained with Gram staining to verify the morphology of the organism. Centrifuge tubes containing the 24-h-old microbial cultures were centrifuged at 1000 x g for 10 min and the pellets were suspended in fresh broth.

The overnight cultures of *S. mutans* were titrated by making log dilutions in physiological saline (0.9% sodium chloride in deionized water), and the BHI agar plates were inoculated in triplicate with 0.1 ml of these dilutions to count the colony forming unit (CFU). The inocula were spread on the agar plates using a L-shaped glass rod on a plate rotator (Pool Bioanalysis Italiana; Milan, Italy) for even distribution. Plates were incubated under anaerobic condition in a 37°C incubator for 48–72 h and the bacterial colonies were counted using the darkfield colony counter (Reichert; Buffalo, NY, USA). The microorganism cultures were then harvested at a concentration of 10^7 to 10^8 CFU/ml for the experiment.

A tetrazolium 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H dye (MTS) and phenazine methosulfate (PMS), an electron coupling reagent, were purchased from Sigma Chemical Company (St Louis, MO, USA). Stock solutions of MTS and PMS were prepared separately in phosphate buffered saline (pH 7.4) at a concentration of 1 mg/ml and 125 mM, respectively, and stored in tubes wrapped with aluminum foil at -20°C until further use.

The lower incisor mini diamond orthodontic brackets (0.022 inch [5.6 mm] slots, Ormco; La Spezia, Italy) of uniform size were autoclaved and dried in a drying oven. The cultured *S. mutans* was diluted in BHI broth to obtain 0.5 McFarland units and four rows in a 96-well plate were seeded with this preparation. The orthodontic brackets were added individually to each well with sterile clinical forceps, keeping the base facing up. This plate was incubated in an anaerobic chamber for three days at 37°C.

The 96-well plate was removed from the incubator and the brackets were immersed in 0.2 ml of physiological saline twice to wash off the planktonic bacteria. Brackets were placed in the wells of a new 96-well plate and exposed to the test solutions for 1 min with gentle manual agitation of the plate. Each row (12 wells) received the following test solutions. The first row received 2% DMSO with saline as the negative control (-), the second row received 5 mg/ml hexane extract, row number 3 received the 5 mg/ml ethanol extract and the last row received 0.2% chlorhexidine gluconate (Clorasept, SPIMACO, Al-Qassim Pharmaceutical Plant; Al-Qassim, Saudi Arabia) as the positive control (+). They were then washed in saline and twelve brackets from each row were placed individually in a new 96-well plate with 0.2 ml of the MTS dye containing PMS (Sigma Chemical) at a concentration of 0.25 mg/ml and 0.1 mM, respectively. The plate was incubated at 37°C for 4 h and the absorbance was read at the endpoint in a Multiscan ELISA reader (Thermo-Fisher; Pittsburgh, Pennsylvania, USA) at 490 nm wavelength. The above-mentioned test was performed in triplicate.

Another twelve orthodontic brackets were removed from each of the four sets of test solutions and washed once in physiological saline in a static condition. They were transferred individually to microcentrifuge tubes (1.5 ml, Eppendorf; Hamburg, Germany) containing 1 ml of physiological saline with 0.1% Tween-80 (Sigma Chemical) as a surfactant. Each of these tubes was vortexed for 30 s and the dilutions made in saline were used as an inoculum in BHI agar plates. Each of the dilutions was inoculated in triplicate and the plates were incubated anaerobically for three days and the mean CFU/ml/bracket were recorded.

During the initial stages of performing the experiment, a pilot study was conducted with the same procedures mentioned above, but with only 6 brackets per group and a static exposure time of about 5 min at time intervals of 24 and 48 h. Since the results seemed promising at 48 h, the actual study with an ideal 1 min exposure was then performed.

Statistical analysis was performed by SPSS (version 16.0, SPSS; Chicago, IL, USA) software. The mean absorbance values obtained from the MTS reduction assay were analysed by repeated measures ANOVA after exposure to the different test solutions. For survival assessment, descriptive statistics which included mean and standard deviation values of the colony counts were evaluated after exposure to different agents. Multiple comparisons

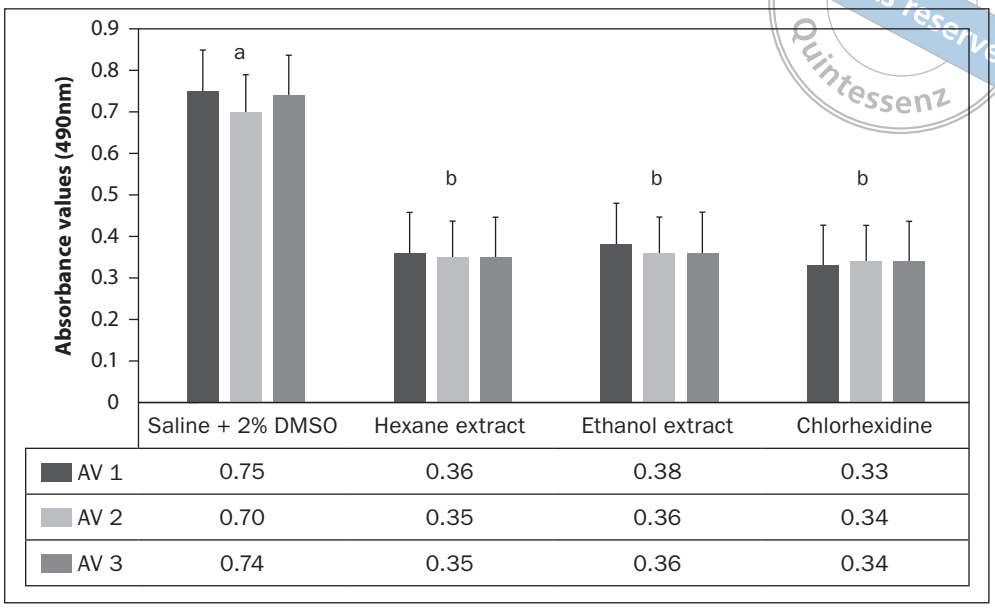

Fig 1 The repeated measure values of cell viability (absorbance values at 490 nm) of *S. mutans* biofilms on orthodontic brackets upon exposure to different test solutions: saline + 2% DMSO (-), hexane extract (*S. persica*, 5 mg/ml), ethanol extract (*S. persica*, 5 mg/ml), 0.2% chlorhexidine (+). AV 1, AV 2, AV 3 denote absorbance values (at 490 nm) obtained during experiments 1, 2, and 3, respectively. The bars and error bars indicate mean and standard error, respectively. Values indicated by dissimilar letters (a,b) are significantly different ($p < 0.05$).

Table 1 Multiple comparisons of CFU counts following exposure to different test solutions

Test solutions (A)	(B)	Mean difference (A-B)	Standard error
Saline +2% DMSO	Hexane extract	3.5×10^7 **	1.5×10^6
	Ethanol extract	3.5×10^7 **	1.5×10^6
	Chlorhexidine	3.6×10^7 **	1.5×10^6
Hexane extract	Ethanol extract	-2.0×10^4 *	2.0×10^3
	Chlorhexidine	8.7×10^3 **	1.7×10^2
Ethanol extract	Chlorhexidine	2.9×10^4 *	2.0×10^3

The mean differences were significant (*$p < 0.05$; **$p < 0.001$; Games-Howell test). ANOVA value (F): 401.80; p-Value: 0.000.

between different test solutions were done by Games-Howell test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

The absorbance values obtained from the MTS reduction assay after 1 min exposure to the different test agents showed a decline in the bacterial cell viability of the *S. mutans* biofilm as follows: chlorhexidine (+) < hexane extract (*S. persica*, 5 mg/ml) < ethanol extract (*S. persica*, 5 mg/ml) < saline + 2% DMSO (-). There was a significant difference between saline + 2% DMSO and the other test solutions ($p < 0.05$). The mean absorbance values showed that the antimicrobial activity of hexane extract was slightly more effective than the ethanol extract and was nearly as effective as chlorhexidine (+) which was used without any further dilution (Fig 1). However, the differences in the absorbance values between the extracts of *S. persica* and chlorhexidine (+) were not statistically significant ($p > 0.05$).

The lowest mean number of CFU was found for chlorhexidine ($0.18 \times 10^2 \pm 0.13 \times 10^2$), followed by hexane ($8.7 \times 10^3 \pm 6.1 \times 10^2$) and ethanol ($2.9 \times 10^4 \pm 7.5 \times 10^3$) extracts. The counts were considerably higher with saline +2 % DMSO ($3.6 \times 10^7 \pm 5.5 \times 10^6$). All the variables were statistically significantly different from each other ($p < 0.001$) (Table 1).

DISCUSSION

The oral microbiota in orthodontic patients has been increasingly associated with the risk of *S. mutans* colonisation, among other species, thus leading to the development of decalcification and sometimes caries.[34] The salivary and plaque levels of *S. mutans* were found to be significantly elevated in patients undergoing active orthodontic treatment.[35]

The high risk of developing enamel lesions manifested as white spots during orthodontic treatment with fixed appliances is a major concern for both the patient and the orthodontist. The performance of meticulous oral hygiene measures with the complementary use of a mouthwash during orthodontic treatment has shown a promising demineralisation inhibiting tendency.[16] Studies have recommended a prophylactic regimen based on daily use of fluoride-containing mouthrinses during treatment of fixed orthodontic appliances.[8,32] In most cases, the recommendation for mouthwash use is usually twice a week for about 1 min and the patient is advised not to eat, drink or rinse for some time thereafter in order to retain the components of the mouthwash for a longer duration.[14] However, it is difficult to determine the exact duration of contact between the mouthwashes and the orthodontic brackets.

There is rising demand for the use of evidence-based natural products in recent years because of the assumption that they are safer than the synthetic products for the maintenance of oral health, while also providing an antimicrobial effect.[12] Among the plant antimicrobials, *S. persica* is a well-known traditional and widely used oral hygiene aid in several African and Middle Eastern countries.[23] Several studies have demonstrated the antimicrobial activity of *S. persica* extracts against certain bacterial species implicated in periodontal disease and caries.[2,5,15]

Almas et al[6] compared the antimicrobial activity of different commercially available non-alcohol based mouthwashes with the aqueous extract of *S. persica*. The results showed that chlorhexidine had the maximum microbial inhibitory action. However, a 50% extract of *S. persica* showed prominent antimicrobial activity against only two microorganisms, of which one was *S. mutans*. The antimicrobial activity of mouthwashes may be related to their anionic and cationic potencies and effect on altering the cellular osmotic pressure and cell metabolism. In addition, the ingredients of the mouthwashes may have the potential to be absorbed by the bacterial surfaces and cause disruption of the cell membranes.[6]

Microorganisms causing caries and other oral diseases are mostly biofilm forming. *S. persica* extracts, found to have a myriad of phytochemical constituents, have a proven potential to derive active compounds with specific effects on the biofilm forming organisms, particularly *S. mutans*.[4] However, it is surprising that Al-Sohaibani and Murugan[4] did not evaluate the biofilm inhibitory capacity of the hexane extract of *S. persica,* which was found to be the most effective in our study. In their study, the methanol extract of *S. persica* showed the highest anti-biofilm activity among 4 other extracts.

In this study, the MTS reduction assay and the results of the CFU counts showed that the antimicrobial effect of 5 mg/ml of hexane and ethanol extract of *S. persica* was less than that of chlorhexidine mouthwash. However, in our pilot study, when we used a static exposure of 5 min, it was noteworthy that 5 mg/ml of the hexane extract could reduce CFU counts by 85% in the *S. mutans* biofilm formed in 48 h. An in vivo investigation done by Khalessi et al[24] using the alcoholic extract of *S. persica* indicated a reduction in the salivary carriage of *S. mutans* by approximately 70%. Since it may not be feasible for a person to keep rinsing for 5 min or advisable for a person to hold the mouthwash in the mouth in a static condition for more than 1 min, our study was performed with a 1-min exposure with mild agitation in order to simulate a mouthrinsing action. We assumed that if the clinical scenario is taken into consideration where the mouthwash is rinsed rather than held static in the mouth, the extract may be more effective.

The *S. mutans* biofilm inhibitory ability of the hexane extract of *S. persica* indicates its potential use as an oral antimicrobial agent in orthodontic patients. Unlike chlorhexidine which has an unpleasant taste and certain undesirable side-effects with long-term use, *S. persica* tastes slightly bitter but not unpleasant.[24] Nevertheless, it is unlikely that *S. persica*-based regimens may replace chlorhexidine as intense antimicrobial treatments for orthodontic patients, but more palatable antimicrobials may have a place in long-term preventive and maintenance strategies taking into consideration the lengthy period of time required for completion of fixed orthodontic treatments.

In this study, both qualitative and quantitative means of measuring the bacterial growth/presence was used. The reduction of water-soluble tetrazolium salt (e.g. MTS) by metabolically active eukaryotic and prokaryotic cells leads to precipitation of coloured formazans. This microbial redox characteristic of the MTS-PBS assay was used in this study as it is a convenient method for distinguishing between dormant and metabolically active microbial cells. Most respiring microorganisms are able to reduce tetrazolium dyes, producing results within hours.[9] Although the bacterial state was easily detected by the colorimetric method for measuring MTS reduction, this model does have some limita-

tions. A relatively high number of bacterial cells may be required to trigger detectable signals in the MTS reduction assay. This may explain the possible reason for a 24-h biofilm not showing a positive reaction in our initial pilot study. Moreover, the results of this assay might not correspond with the viability of the real biofilm.

This study used a monospecies biofilm model to demonstrate the antimicrobial effect of two extracts of *S. persica* on orthodontic brackets. However, a multi-species biofilm model might be able to explain the clinical situation better. Although the described monospecies biofilm model showed that hexane extract has a greater inhibitory effect on *S. mutans* biofilm than does the ethanol extract, a randomised clinical trial will be required in the future. In the study done by Nelson-Filho et al,[31] the mean number of cells of *S. mutans* in the samples of orthodontic brackets in the control group where no antimicrobial agent was used as a mouthwash revealed that a majority was contaminated by 5×10^5 cells. Thus, in our study, the significant reduction of *S. mutans* by exposure to the test agents may clarify the antimicrobial ability of the test agents to substantially reduce the CFU counts. However, further investigations are recommended to validate results from this in vitro study for the in vivo situation. Dental professionals should emphasise the use of an antimicrobial mouthwash as an important adjunct to maintaining proper oral hygiene, particularly in orthodontic patients where the tendency of plaque accumulation is greater.

CONCLUSIONS

Within the limitations of this in vitro study, the *S. persica* extracts were found to have antimicrobial effects on *S. mutans* biofilm on orthodontic brackets, suggestive of its potential use as an oral antimicrobial agent for orthodontic patients. This also warrants further long-term clinical studies to assess the oral health benefits of *S. persica* extracts as prospective oral antimicrobial agents.

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