Antibacterial Activity of Salvadora Persica and Benzylisothiocyanate against Prevotella Intermedia and Eikenella Corrodens Incorporated into Periodontal Chips

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Abstract

Salvadora persica (S. persica) contains steam-distillable oil made of 90% Benzylisothiocyanate (BITC). It's proven to have a wide range of bactericidal activity.

The objectives of this study were to develop biodegradable periodontal chips containing S. persica, and BITC in a chitosan base and to evaluate their antibacterial activity against Prevotella intermedia and Eikenella corrodens.

Periodontal chips were developed that incorporated S. persica (Miswak) extract and BITC in a chitosan base. Sterile distilled water was used as negative control while chlohexidine was used as positive control. A gel diffusion test was performed to detect the potential antibacterial activity followed by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Tests. Scanning electron microscopy (SEM) was used to identify the morphology and configuration of Prevotella intermedia and Eikenella corrodens bacteria before and after treatment with chips.

The mean inhibition zone for the S. persica chips was found to be 9.0 mm. The MIC and MBC tests demostrated that all the concentrations for the chips succesfully inhibited the growth of the bacteria. Chlohexidine used as positive control also inhibited the bacteria. The negative control of sterile distilled water showed no inhibtion of bacterial growth. SEM showed that all chips produced distinct alterations to the bacterial morphology. E. corrodens and P. intermedia underwent shrinkage and formed irregular shapes.

Periodontal chips formulated from S. persica and BITC incorporated into a chitosan base as a target drug delivery method, showed potent antibacterial activity against E. corrodens and P. intermedia.

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Introduction

Periodontitis is a chronic disease, which is an inflammatory response to bacteriological infections.[1] Periodontitis destroys the attachment apparatus of teeth resulting in periodontal pocket formation and alteration of normal osseous anatomy.[2] The primary objective of treating patients with chronic periodontitis is to halt disease progression and to resolve inflammation. Periodontitis is best treated with mechanical debridement of the pockets, which is accomplished with scaling and root planing.[3] This approach is a demanding therapeutic procedure, and it has limitations, such as the inability to access deep pockets and furcation's and to

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eliminate certain pathogens.[4,5] To overcome these limitations, various adjunctive therapies have been proposed[6] that include the use of systemic or local antimicrobial agents.[7-12]

Plant extracts and herbs that have been previously used, such as Miswak, Capparis Spinosa, cinnamon and Myrtus communis,[13-16] Arnica montana and Hamamelis virginiana, have been shown to have antibacterial activity against periodontopathic bacteria.[17-19] *S. persica* is an evergreen medicinal tree, and its roots have been utilized for more than 10 centuries in Asia, India, and Africa. Many products have been developed from this medicinal tree such as toothbrushes made from its roots and small branches. Research has shown that the contents *S. persica* (Miswak) include substances that inhibit dental plaque growth and are antibacterial against numerous cariogenic bacteria in the oral cavity.[20,21] Therapeutic usage of *S. Persica* has been included in toothpaste, mouth rinses and endodontic irrigation solutions.[22] The roots of *S. persica* contain steam-distillable oil comprising 10% benzyl nitrate and 90% Benzylisothiocyanate (BITC).[23] It has been reported to have a wide range of bactericidal action. BITC also has been proven to show virucidal activity against herpes simplex virus type 1 (HSV-1).[23,24] BITC is also a naturally occurring compound in plant tissue. It is found in Indian cress (Tropaeolum majus L.), in garden cress (Lepidium sativum L.) flowering plants, and in relatively large amounts in cruciferous vegetables such as cabbages, brussels sprouts, cauliflower and broccoli.[25]

No studies have been done to develop a periodontal chip from *S. persica* and BITC. Therefore, the aim of this study was to develop a periodontal chip containing *S. persica* and BITC in a chitosan base, and then to evaluate the antibacterial effect of these new biodegradable periodontal chips against *Prevotella intermedia* and *Eikenella corrodens*.

Materials and methods

Preparation of *S. Persica* extract

S. persica sticks were purchased from Chunawala Exports India, Maharashtra, Mumbai. The sticks were then grinded into powder using a Hammer Mill blender. The powder was mixed with sterilized distilled water to produce an aqueous extract, whereby 200 grams of *S. persica* powder yielded 15 grams dry extract.

Preparation of biodegradable chips:

Chitosan is a linear polysaccharide composed of randomly distributed β-linked D-glucosamine and N-acetyl-D-glucosamine. It's made by using an alkaline chemical like sodium hydroxide to treat the chitin shells of shrimp and other crustaceans. Chitosan has a variety of commercial and biological applications. Periodontal chips were prepared from Chitosan. Periodontal chips incorporating *S. Persica* extract (Miswak) (2.5 mg; 100%w/w) and Benzylisothiocyanate (BITC) 0.25mg; 100% w/w (purchased from Sigma,USA) were prepared according to the methods described by AL-Bayaty et al[25] to obtain small rectangular chips 0.5 × 0.5 sq.cm in size.

Experimental Microorganisms

Prevotella intermedia (ATCC 25611) and *Eikenella corrodens* (ATCC 23834) are both regarded as putative periodontal pathogens.[26,27] Both types of bacteria were obtained from the Microbiology Diagnostic Laboratory of University Malaya Medical Center, Kuala Lumpur. The microorganisms were subcultured on Brain Heart Infusion (BHI) agar and inoculated into BHI broth as stock cultures, which was used throughout the experiment. Both *P. intermedia* and *E. corrodens* were incubated anaerobically.

Colony Forming Units (CFU)

This technique was used to determine the precise colony or number of bacteria per ml of sample as well as the concentration of each bacterial dilution.

Assessment of Antibacterial Activity

Gel Diffusion Test

0.1ml of 24 hours broth culture of the tested microorganisms were aseptically introduced and evenly spread using a disposable L shape swab on the surface of sterile BHI agar plates. 4 wells of about 6.0 mm diameter were aseptically punched on each agar using a sterile cork borer, allowing at least 30mm between adjacent wells and the edge of the Petri dish. Wells were loaded with Chlorhexidine Digluconate (positive control) and sterile distilled water (negative control). Chitosan-*S. persica* chips were placed directly on the agar surface. A few drops of sterile distilled water were first added to the surface of the chips before placing them onto the surface of the agar.

The agar plates were incubated anaerobically overnight at 37 C°. The diameter of

the inhibition zone around the wells and the chips were calculated after 24 hours using electronic calipers. The mean values of the three zones were calculated.

Minimum Inhibitory Concentration (MIC) Test

After an overnight incubation, a minimum inhibitory concentration (MIC) test was performed to determine the lowest concentration of S. persica required to suppress observable (99 percent) bacterial growth of fixed concentrations of the experimental microorganisms. The test was carried out utilizing *S. persica's* release concentration. The MIC value was determined based on the inhibition and growth observed on the spot-inoculated agar plate. The test was repeated three times, and the mean value of the MIC was obtained. Another test was carried out by introducing 2 Chitosan-*S. persica* chips into 0.5 ml of each tested bacteria to investigate the direct action of continuous release of *S. persica* component from the chip against the bacteria. All the mixtures were left overnight anaerobically at 37° C. The turbidity of the mixture was observed. Confirmation of bacterial growth was done by streaking a loopful inoculum taken from the mixture and placed onto the BHI agar. The agar was left overnight. At the same time, the samples were sent for scanning electron microscopy (SEM).

Minimum bactericidal concentration (MBC)

The Minimum Bactericidal Concentration (MBC) in broth was determined by plating the broth culture from each test tube (used in MIC) onto an agar plate and incubating it overnight at 37°C. The MBC is the concentration at which there is no bacterial growth. To test antimicrobial activity, bacterial dilutions using *S. persica* chips were cultivated on BHI agar plates.

Assessment of Bacterial Morphology

The physical changes that occurred in the bacterial forms and structures before and after treatment with Chitosan-*S. persica* chips were evaluated using SEM.

Statistical analysis:

Descriptive statistical analysis of the results was carried out to describe the antibacterial effects of the chips.

Results

Gel diffusion Test

S. persica chips were placed directly onto the agar surface after the bacteria was evenly spread on it. Results showed that the mean inhibition zone for all five consecutive chips, was 9.0 mm and 18mm for *S. persica* chip and BITC respectively, and 24mm for chlorhexidine (Table 1).

Reagent	*Prevotella intermedia*	*Eikenella corrodens*	Inhibition zone (mm)
S.persica chip	No bacteria growth	No bacteria growth	9.0
BITC chip	No bacteria growth	No bacteria growth	18.0
CHX mouth wash	No bacterial growth	No bacterial growth	24
Sterilized Distilled Water	presence of bacterial growth	presence of bacterial growth	zero

Table 1. Inhibition zone (mm) of the *S. persica* chips, BITC chips directly placed on the agar surface, CHX mouth wash and sterilized distilled water.

Reagent / Organisms	Active component of *S. persica* Benzylisothiocyanate			Chlorhexidine Digluconate mouth wash (+ve control)	sdH_2O (-ve control)
	100 µ	50 µ	25µ		
Prevotella intermedia	-ve	-ve	-ve	-ve	+ve
Eikenella corrodens	-ve	-ve	-ve	-ve	+ve

Table 2. The presence and absence of bacterial growth by MIC test with different concentrations of active component of *S. persica* Benzylisothiocyanate against *Prevotella intermedia* and *Eikenella corrodens.*

-ve; No bacterial growth

+ve; presence of bacterial growth ,spreading samples onto agar.and incubated over night.

chips / Organisms	Salvadora persica chips were incubated together	Benzylisothiocyanate chips were incubated together
Prevotella intermedia	-ve	-ve
Eikenella corrodens	-ve	-ve

Table 3. The presence and absence of bacterial growth with the immersion of *S. persica* and BITC chips into 0.5ml of each bacteria dilution.

Minimum Inhibitory Concentration (MIC) Test

Table 2 shows the result for the Minimum Inhibitory Concentration (MIC) test for the active components of *S. persica* and BITC. All the concentrations of the reagents succesfully inhibited the growth of both types of bacteria.

The chlorhexidine which was used as positive control against these bacteria also managed to inhibit or kill the bacteria. Meanwhile, the negative control, i.e. the sterile distilled water, did not show any inhibtion of bacterial growth.

Table 3 shows the result of the direct action of Chitosan-*S. persica* and BITC chips on the broth containing 108 CFU/ml of both types of bacteria. No bacterial growth was observed after the confirmation test, which was done by streaking a loopful inoculum taken from the mixture onto the BHI agar. The agar was left overnight. At the same time, the sample was taken for SEM.

Minimum Bactericidal Concentration (MBC) Test

Table 4 shows the result of the MBC test regarding the release of 1 hour of Chitosan-*S. persica* chips against the performed serial bacterial dilution. The reagent or the solution use was not able to inhibit bacterial growth.

Bacterial dilution (CFU/ml) / Bacteria	10^1	10^2	10^3	10^4	10^5
Prevotella intermedia	bacterial growth +ve	bacterial growth +ve	bacterial growth +ve	bacterial growth +ve	bacterial growth +ve
Eikenella corrodens	bacterial growth +ve	bacterial growth +ve	bacterial growth +ve	bacterial growth +ve	bacterial growth +ve

Table 4. The presence and absence of bacterial dilution growth seen on the agar plates after treatment with *S. persica* chips (1 hour release, 152µg/ml).

Assessment of Bacteria Morphology

Scanning electron micrographs obtained before treatment showed that all bacterial species had normal shapes, and were classified as coccobacilli or pleomorphic bacillus. After undergoing direct action from the Chitosan-*S. persica* chips, obvious morphological changes were seen on the bacterial shapes and structures. *E. corrodens* and *P. Intermedia* were completely distorted, showing shrinkage and irregular shapes, no longer having a coccobacilli shape. This may be due to the action of Chitosan-*S. persica* chips causing the bacterial cell wall to rupture, with expulsion of the cellular contents (Figure 1, 2, 3 and 4).

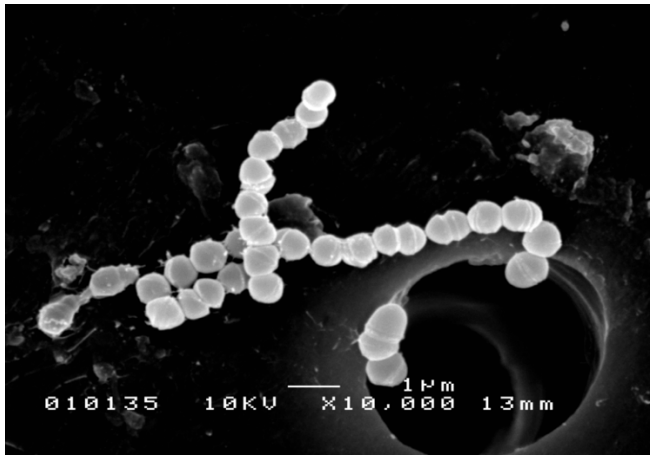

Figure 1. A scanning electron micrograph showing *P. intermedia* before treatment (x10000, 10KV).

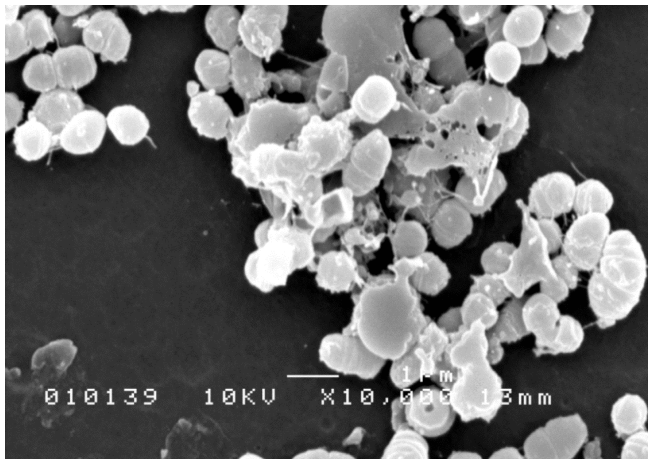

Figure 2. A scanning electron micrograph showing *P. intermedia* after treatment with direct five *S. persica* chips in the broth containining bacteria (x10000, 10KV).

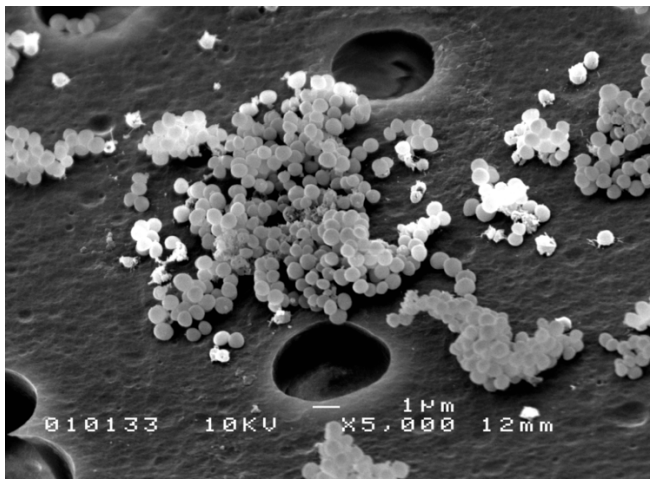

Figure 3. A scanning electron micrograph showing *E. Corrodens* before treatment (x5000, 10KV).

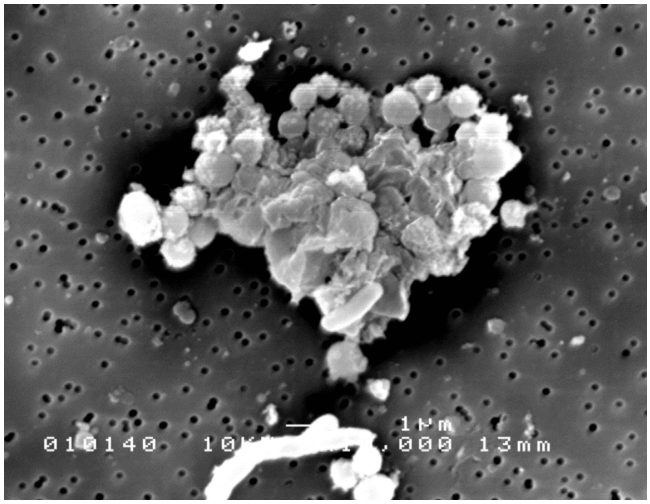

Figure 4. A scanning electron micrograph showing E. Corrodens after treatment with direct five S. persica chips in the broth containing bacteria (x10000, 10KV).

Discussion

The aim of this study was to develop a periodontal chip containing *S. persica* and BITC in a chitosan base, and then to evaluate the antibacterial effect of these new biodegradable periodontal chips against two periodontal pathogens, *Prevotella intermedia* and *Eikenella corrodens*.

Plants and their extracts are known to have been used for therapeutic purposes since time immemorial, due to their usage being safe, economical, effective, and easily available.[28] *S. persica* is a small tree or shrub belonging to the family *Salvadoraceae*.[29] The plant's root, stem and twigs have been widely used in Africa, the Middle East, and the Asian subcontinents for oral hygiene purposes. It is recommended as an effective tool for oral health care by the World Health Organization (WHO).[30]

The roots of the shrub *S. persica* have been demonstrated to possess antimicrobial effects.[31,32] The fundamental antibacterial part of both *S. persica* root extracts and volatiles is benzyl isothiocyanate. Root extracts as well as commercial synthetic benzyl isothiocyanate display strong bactericidal effects against oral pathogens involved in periodontal disease, including various Gram-negative bacteria.[23,32] In the present study, the gel diffusion, the MIC and MBC tests and SEM were used to evaluate the antibacterial effect of *S. persica* extract and BITC chips on dental biofilm bacteria. To the best of the authors' knowledge, there are no reported studies which evaluate the in vitro antibacterial activity of Periodontal chips which are formulated from *S. Persica* extract (Miswak) and Benzylisothiocyanate in a chitosan base against *E. corrodens* and *P. intermedia* bacterial strains. Initial screening was performed by a gel diffusion test to evaluate the results. The results exhibited an inhibition zone of 9mm and 18mm for the *S. persica* chip and BITC, respectively, which were lower than that for the chlorhexidine inhibition zone of 24mm. The MIC test for the Benzylisothiocyanate chips showed complete inhibition of growth for both bacteria in all concentrations of 100 , 50 and 25 µg, which represents a potent antibacterial activity simillar to that of Chlorhexidine. The presence and absence of bacterial growth with the immersion of *S. persica* and BITC chips into 0.5ml of each bacterial dilution showed that both were able to inhibit bacterial growth. The colony formation test for *S. persica* chips showed bacterial growth with different bacterial dilutions on the agar plates after treatment (1 hour release, 152µg/ml).

E. corrodens and *P. intermedia* bacterial samples were viewed by SEM after treatment with *S. persica* chips to assess their morphological changes. SEM showed that all untreated samples exhibited a normal cell appearance with intact and smooth cell surfaces. Distinct morphological changes were seen with *P. intermedia* bacterial shape and structure after *S. persica* treatment, which included shrinkage and the formation of irregular forms, losing its cocci shape. Similar results were obtained for *E. corrodens* whereby the bacterial cells shrank after treatment. The coccobacilli shape of *E. corrodens* was particularly distorted on these bacteria, causing rupture of the bacterial cell wall, with expulsion of the cell contents. Viable bacterial cells maintained a smooth cell surface. The results showed that both *S. persica* and BITC chips were thus able to inhibit bacterial growth and produced distinct alterations to the bacterial morphology. Our results are in accordance with other previous studies that have reported Persica® (Miswak: chewing stick) mouthwash to inhibit dental plaque formation and to significantly reduce the number of *S. mutans* colonies.[13,21,23]

This study may be considered as a pathfinder for the proof of principle. Further research is needed using other periodontal

pathogens. Chitosan should also be compared with other polymers regarding resorption time and dose dependent concentration effect evaluation for continuous release of *S. Persica* and BITC chips is also recommended in future studies.

Conclusion

A significant finding to emerge from this study is that periodontal chips formulated from *S. Persica* extract (Miswak) and Benzylisothiocyanate (BITC) in chitosan base have strong antibacterial action against *E. corrodens* and *P. intermedia*. For this reason, they can be proposed as a good alternative ingredient for the development of professional periodontal chips to control and inhibit various types of dental biofilms. Future research is recommended using other periodontal pathogens. Chitosan should also be compared with other polymers regarding resorption time. Long term continuous release of dose dependent concentrations effect of *S. persica* and BITC chips is also recommended in future studies.

Declaration of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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