Original Article

Antibacterial Effect of an Herbal Product Persica on Porphyromonas Gingivalis and Aggregatibacter Actinomycetemcomitans: An In-Vitro Study

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Abstract

Objective: The plant *Salvadora persica* is used for oral hygiene in many parts of the world. It has been suggested that it has antibacterial properties, in addition to its ability to mechanically remove plaques. The aim of this study was to assess the antimicrobial activity of the herbal product Persica containing Salvadora persica against periodontopathogens Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans in vitro.

Materials and Methods: Fifty patients with moderate and severe periodontitis were recruited. Using paper points, subgingival plaque samples were taken from pockets with attachment loss ≥ 3mm. The samples were subjected to microbial culture to yield *P. gingivalis* and *A.* actinomycetemcomitans. The ditch plate method was used for antimicrobial susceptibility testing of the bacteria to Persica compared to chlorhexidine and distilled water. The growth inhibition zones of microorganisms around the ditches were measured in millimeters. The data were analyzed using SPSS 16. Freidman test and Wilcoxon signed ranks test with Bonferroni adjustment were used for analysis of variance with 5% significance level. $P<0.05$ for main comparisons and $P< 0.017$ for multiple comparisons were considered statistically significant.

Results: P. gingivalis was sensitive to chlorhexidine and persica. There was a significant difference (P=0.001) between antimicrobial activity of chlorhexidine (mean 28.733mm, SD 5.216) and Persica (mean 16.333mm, SD 5.259) compared to water against P. gingivalis. There was a significant difference ($P< 0.001$) between the antimicrobial activity of chlorhexidine (24.045mm, SD 3.897) and Persica (0.545mm, SD 2.558) with respect to A. actinomycetemcomitans. There was no significant difference (P=0.317) between the antimicrobial activity of Persica and water against A. actinomycetemcomitans.

Conclusion: The herbal product Persica had significant antimicrobial activity against P. gingivalis and negligible antimicrobial activity against A. actinomycetemcomitans compared to 0.2% chlorhexidine.

Key Words: Salvadora persica; Actinobacillus actinomycetemcomitans; Antimicrobial Susceptibility Testing; Porphyromonas Gingivalis; Aggregatibacter

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INTRODUCTION

The removal of dental plaque is essential in the prevention of periodontal disease. Dental plaque is a biofilm containing approximately 500 different microbial species [1]. Aggregatibacter actinomycetemcomitans and Porphy-

romonas gingivalis have been suggested to be two of the main causes of destructive periodontal diseases [2].

A. actinomycetemcomitans invades the periodontal tissue and produces a virulent leukotoxin. This toxin can protect a population of bacteria from phagocytic killing. It induces extracellular release of granule enzymes from polymorphonuclears (PMNs) and kills macrophages [3]. P. gingivalis makes virulence factors for tissue colonization, destruction and host defense. Among the virulence factors produced by P. gingivalis**,** the major fimbriae (FimA), and cysteine proteinases (gingipains) contribute to the attachment and invasion of oral epithelial cells [4].

Mechanical plaque control is an effective procedure for oral hygiene maintenance. In addition, topical chemotherapeutic agents are used as adjunctive methods for the prevention and treatment of periodontitis. They may be used as oral rinses by patients or by professionals for intra-pocket irrigation. One such chemotherapeutic agent is chlorhexidine gluconate. It is considered the gold standard for plaque inhibition [5]. However, it has a number of unpleasant side effects, such as discoloration of teeth, restorative materials and mucosa [6] and altered taste sensation. In order to overcome such side effects, the World Health Organization (WHO) advises the use of natural products such as herb and plant extracts [7]. For this reason the use of plant extracts as an alternative is gaining popularity. One such medicinal plant is Salvadora persica.

The chewing stick,'Miswak', has been used for centuries in developing countries for oral hygiene. It is prepared from the roots, twigs and stems of Salvadora persica. Salvadora persica (Salvadoraceae) is widespread along the coasts of East Africa, Arabia, Iran, Syria, and India, and is an upright evergreen small tree or shrub. A number of antimicrobial components have been identified in Salvadora persica. It has been found to contain large amounts of chloride, calcium, fluoride, silica, sulfur, vitamin C, tannins, an alkaloid trimethylamine and resin [8]. Phytochemical screening by Ahmad et al. revealed that the herbaceous parts of Salvadora persica contain carbohydrates, glycosides, sterols, terpenes, flavonoids, tannins and alkaloids [9]. They also found that S. persica contained high amounts of fluoride and silica.

The value of aqueous Miswak extracts like Persica containing Salvadora persica, (Pursina Laboratory, Tehran, Iran) has not been fully substantiated. It is marketed and used and the manufacturers claim that it is effective in the prevention and treatment of a number of dental problems. The effect of the product on cariogenic bacteria such as S. mutans has been looked into but little has been done to evaluate its effectiveness against Gram negative anaerobe periodontal pathogens in deep periodontal pockets [10]. Therefore, evaluation of the effectiveness of extracts of S. persica warrants further research. The aim of this study was to determine the in-vitro antibacterial activity of Persica against the periodontal pathogens Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis and to compare this to the antimicrobial activity of the gold standard, chlorhexidine.

MATERIALS AND METHODS

Prior to commencing, a detailed study protocol was submitted to and approved by the Ethics Committee at Tehran University of Medical Sciences (department of research). Written informed consent was obtained from the patients after the treatment was explained to them thoroughly.

Fifty patients between 20-65 yrs. with chronic moderate and severe periodontitis, whom had been referred to the Department of Periodontology, Faculty of Dentistry of Tehran University of Medical Sciences, were selected to participate in this study. Periodontal pocket probing and full mouth radiographic examination showed attachment loss ≥ 3mm with evidence of greater than 50% bone loss.

The first task of the study was to collect baseline data from all subjects.

This comprised of medical, dental and social histories. Subjects were excluded if they had mild periodontal disease, had undergone any periodontal treatment, or received antibiotic therapy during the previous month.

Sample collection:

Using the periodontal chart, one tooth with a deeper pocket was selected in each subject. Any supragingival plaque around the tooth was removed with cotton pellets. Calculus was also gently removed with a scaler if necessary. The sampling area was subsequently air-dried and a sterile size 30 paper point was then inserted into the full depth of the pocket and left for 30 seconds. Upon removal from the pocket the paper point was immediately placed in thioglycolate solution for preservation and storage.

Laboratory Analysis:

The samples were then transferred within one hour to the microbiology laboratory. The samples were homogenized for 30 seconds by means of a vortex mixer.

For anaerobes, the specimens were plated on non-selective Brucella agar plates (Becton Dickinson, Heidelberg, Germany) enriched with 0.5% hemolyzed blood and 5mg/L menadione. Columbia base agar supplemented with hemin (0.05mg/ml), vitamin K (0.01mg/ml), and citrated horse blood (5%) was used for isolation of Porphyromonas gingivalis. Trypticase soy agar plates supplemented with horse serum, bacitracin and vancomycin (TSBV) were used to isolate Aggregatibacter actinomycetemcomitans.

The Brucella agar plates and the Columbia agar plates were placed in an anaerobic jar with a GasPak pouch, a palladium catalyst and a methyl B indicator (which changes from blue to white in the absence of oxygen) to be incubated under anaerobic conditions.

The TSBV plates were placed into a capnophilic environment, a 5-10% CO_2 enriched atmosphere, by placing a lit candle into the jar. The jars were incubated at 37^0 C for 72 hours and the primary plates were examined after 72 hours.

One to three colonies of each selected type were isolated and purified for further identification based on cell morphology, Gram stain reaction, biochemical tests and enzymatic activities including catalase, oxidase, indole hydrolysis, esculin hydrolysis, gelatin hydrolysis, urea hydrolysis, and fermentation of glucose and lactose.

Colonies suspected of growing Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis were sub-cultured in the appropriate selective medium and incubated again under anaerobic and capnophilic conditions for 48-72 hours.

Anaerobic Gram negative coccobacilli which produced dark pigmented colonies on supplemented blood agar were identified as Porphyromonas gingivalis if they were indole-positive and esculin-negative, hydrolyzed N-benzyol-DL-arginine-2-naphylamide and had no α-glucosidase activity. Aggregatibacter actinomycetemcomitans was considered present if the colony was capnophilic, Gram negative, catalase-positive coccobacilli and X (hemin) and V (NAD^+) growth factors were not required. The aim was to test the antimicrobial activity of a product containing an extract of Salvadora persica, against the bacteria's Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis.

The manufacturers claim the product is effective in the prevention and treatment of gum disease. It is composed of three medicinal plants; Salvadora persica, mint, and yarrow, which make up 6% (6 g in 100 ml) of the Persica solution, the other 94% consisting of alcohol and water.

Testing for the antimicrobial activity of Persica involved placing it directly onto agar plates

and removing the alcohol (although in negligent concentration to have any therapeutic effect). An evaporator was used to achieve this. The Persica solution was placed in an evaporating dish on a water bath at a temperature of around 45^0 C. The ethanol was evaporated, and this was confirmed by its smell. The ethanol was replaced with water to maintain the same composition of the active components.

The antimicrobial activity of the Persica solution was determined by the agar ditch diffusion method, introduced by Fleming in 1924 for evaluating antimicrobial qualities of antiseptic solutions [11]. Three to four colonies of microorganisms on each of the pure Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis plates were suspended in sterile distilled water and the density was adjusted to that of 0.5 McFarland standard [12]. A sterile swab was used to inoculate evenly the surface of the medium producing a lawn culture on blood agar plates. The tip of a sterile Pasteur pipette was used to make 0.5mm ditches in the agar plates. Subsequently, 0.1 ml aliquots of the Persica extract were pipetted into these ditches. The same size ditches were also made in the plates for 0.1ml sterile distilled water to serve as a negative control and for 0.1 ml chlorhexidine 0.2% solution to serve as positive control. The plates were incubated at 37°C for 48 hours under anaerobic conditions and then examined for inhibition zones of the growth of bacteria around the extract(s). Inhibitory zone diameters were measured at the point where growth suddenly decreased due to a reduction in colony sizes.

This procedure was carried out for both clinical and standard isolates of Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis.

The standard isolates of each bacteria (A. actinomycetemcomitans: HK 1519, P. gingivalis: ATCC 33277) were tested three times.

Statistical analysis:

The data were analyzed by SPSS version 16. Minimum, maximum, mean and standard deviation values were used for comparison of the variables. The majority of the analysis was conducted using non-parametric statistical methods. The Freidman test and the Wilcoxon signed rank test with Bonferroni adjustment were used for analysis of variance. The statistical analysis was carried out with 5% significance level. $P< 0.05$ for main comparisons and $P<0.017$ for multiple comparisons were considered statistically significant.

RESULTS

Fifty patients with moderate and severe periodontitis were recruited. Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis were absent in 20 of these patients. Of the remaining, P. gingivalis was isolated from 15 patients and A. actinomycetemcomitans from 22 patients. Both bacteria were found in 7 patients. A. actinomycetemcomitans was isolated from 14 females and 8 males (mean age 48 years). P. gingivalis was isolated from 11 females and 4 males (mean age 47).

The mean diameter of inhibition zone of clinical isolates of A. actinomycetemcomitans by chlorhexidine was 24mm (SD 3.90), compared to 0.5mm (SD 2.55) by Persica. Distilled water showed no inhibition of this bacterium (Table 1).

The Friedman test gave chlorhexidine a mean rank of 3.0, Persica 1.52 and distilled water 1.48 and this was statistically significant (Table 2).

Table 1. Inhibition zone (mm)

	Aa Min	Aa Max	Aa Mean	Aa Std. Deviation	Pg Min	Pg Max	Pg Mean	Pg Std. Deviation
CHX	18.00	34.00	24.0455	3.89722	20.00	40.00	28.7333	5.21627
WATER	.00	.00	.0000	.00000	.00	.00	.0000	.00000
PERSICA	.00	12.00	.5455	2.55841	.00	24.00	16.3333	5.25991

Using Wilcoxon signed rank test (with Bonferroni adjustment) to compare chlorhexidine with water, and chlorhexidine with Persica (Table 3), there were significant differences between the antimicrobial efficacy of chlorhexidine and water, and that of chlorhexidine and Persica. However, as shown in Table 3, no significant difference was found between the antimicrobial activity of Persica and water against A. actinomycetemcomitans.

Porphyromonas gingivalis

The mean diameter of the zone of inhibition of clinical isolates of P. gingivalis by chlorhexidine was 28.7mm (SD 5.21), compared to 16.3mm (SD 5.25) for Persica (Table 1). Again water showed no ability to inhibit bacterial growth.

The Friedman test gave chlorhexidine a mean rank of 3.0, Persica 1.97 and distilled water 1.03 (Table 2), and these results were statistically significant.

Using the Wilcoxon signed rank test (with Bonferroni adjustment) to compare chlorhexidine with water, and Persica with water (Table 3), we can conclude that there is a significant difference between antimicrobial activity of chlorhexidine against P. gingivalis and that of Persica against P. gingivalis. Similarly, there are significant differences between the antimicrobial activity of chlorhexidine compared to water, and that of Persica compared to water.

DISCUSSION

At present, use of resin adhesive cements has greatly increased due to their improved physical properties and favorable marginal seal.

In RelyX-Unicem, additional acidic reactions occur between the phosphoric acid methacrylates and inorganic fillers with 72 wt% [28].

Luting properties of Maxcem are due to the combination of several adhesive monomers like glycerol dimethacrylate dihydrogen phos-

Table 2. Friedman Test

	Aa Mean Rank	Aa Test Statistics (a)	Pg Mean Rank	Pg Test Statistics (a)
CHLORHEXIDINE	3.00		3.00	
WATER	1.48		1.03	
PERSICA	1.52		1.97	
Asymp. Sig.		.000		.000

Table 3. Wilcoxon signed rank test

	Aa Z	Aa Asymp. Sig. (2-tailed)	Pg Z	Pg Asymp. Sig. (2-tailed)
WATER - CHX	-4.114(a)	.000	-3.411(a)	.001
PERSICA - CHX	-4.114(a)	000	-3.410(a)	.001
PERSICA - WATER	-1.000(b)	.317	-3.304(b)	.001

phate that is also found in other adhesive products such as Optibond/Optibond FL, Optibond Solo Plus, and Solo Plus Self-Etch [28]. Hydrophilic monomers have also been added to create adequate moisture when adhering to the dental substrate. Phosphoric acid's glycerol dimethacrylate esters are also capable of etching enamel and dentin [29]. There is not enough data neither regarding the pH of Maxcem cement and primary hydrolysis process of its acidic monomers nor about the chemical details of redox activator used in Maxcem cement [30]. Details about the acidic reactions between the self-cure activator and acidic resin monomers are obscure [31]. Both Rely X-Unicem and Maxcem benefit from the combination of dimethacrylate acidic monomers and phosphoric acid groups. It looks like the demineralizing capacity of multi-purpose monomers in Maxcem cement is low and cannot etch the newly formed smear layer that quickly buffers the monomers. Thus, only a surface reaction occurs between the cement and dental substrate [32].

In our study, in all samples, microleakage was similar on the occlusal and cervical surfaces and only in Rely X-Unicem and Maxcem, after 24 hours, microleakage was greater on the cervical surface compared with the occlusal surface, which is in contrast with the findings of Behr et al, in 2004 [14] who evaluated the marginal adaptation (through evaluation of microleakage) of universal self-adhesive cement RelyX-Unicem in comparison with previously used cements. They concluded that with no conditioning, this cement had marginal adaptation similar to conventional luting agents in the dentin.

In 2012, Inukai et al. evaluated the microleakage of MOD indirect composite restorations bonded with self-adhesive and self-etching resin cements (Panavia F 2.0, SA Cement, and RelyX-Unicem) with or without acid etching of the proximal enamel margins and found that acid etching had no effect on microleakage and the two tested self-adhesive cements showed similar bond strengths to the self-etching resin cement [33].

Piwowarczyk et al. in 2005 [34] evaluated microleakage and marginal gaps of restorations bonded with a self-adhesive universal resin cement compared with well-tried systems and showed that RelyX-Unicem had the lowest degree of microleakage when compared with glass ionomer, Panavia F 2,0 and Rely X-Arc. The difference between these findings and ours can be attributed to the fact that specimens were subjected to different time periods of incubation and full crowns were evaluated instead of composite inlays.

In addition, our obtained results may be due to the fact that self-adhesive systems can only remove a part of the smear layer and as a result the formed hybrid layer would have a lower quality. The microscopical consequence of such a weak bond is an increased microleakage and a decreased bond strength. Another possible explanation for increased microleakage in self-adhesive systems is insufficient penetration of resin between the enamel rods for formation of resin tags due to the presence of acid-resistant mineral deposits that are also resistant to the pH (acidity) of the adhesive system and especially high viscosity of these cements. Another reason for decreased bonding of these cements to the tooth structure is that their chemical reaction is dependent to the presence of water on the substrate surface. Water prevents full penetration of resin into the dentin collagen and the space that is supposed to be filled with resin, is filled with water that results in a decreased bonding seal [28, 35, 36].

Despite the manufacturer's claim regarding the flawless marginal adaptation of these cements, their complex chemical reaction can be responsible for cervical microleakage after 24 hours. De Munck et al. (2004) compared RelyX-Unicem with a conventional resin cement (Panavia F 2,0) and demonstrated that RelyX-Unicem only superficially reacts with dentin and enamel and it requires a little pres-

sure for better adaptation of the cement with cavity walls. The best bond was achieved where enamel surface was etched before cementation (12). In the mentioned study, despite the very low pH of the mixture (less than 2 in the first minute), almost no demineralization was seen on the dentin surface (12) that per se can be responsible for decreased bond strength and microleakage in this area. In our study, the microleakage of RelyX-Unicem was significantly greater than that of RelyX-Arc (control) that can be due to the high viscosity of the cement and short duration of penetration and reaction time since it has to be cured directly immediately after application.

Self-adhesive cements have high viscosity and small penetration into the tooth structure. In previous generations of resin cements like Panavia F 2,0, a self-etching primer containing acidic monomers renders complete penetration of the monomer into the demineralized dentin [37]. For cements like RelyX-Arc, the contact area between the resin and dentin or enamel is fully conditioned using bonding agents and use of a layer of hydrophobic bonding below the resin cement helps in decreasing microleakage and completion of the seal [37].

Considering the technique sensitive nature of self-adhesive cements, when moisture control or pressure application during curing is not feasible, coating the tooth surface with bonding agents (Resin Coating Technique) may improve the properties of these materials [38].

In our study, the difference between cervical and occlusal microleakage in RelyX-Unicem (P=0.0001) and Maxcem (P=0.001) after 24 hours was statistically significant. This finding is in contrast with that of Ibarra et al. in 2006 [13]. They evaluated microleakage of porcelain veneer restorations bonded to enamel and dentin with a new self-adhesive resin-based dental cement and reported decreased micromechanical retention due to the high viscosity and improper pH of the cement. However, in a study by Moezzyzadeh and Moayedi (2007) on microleakage of various bonding systems in enamel and dentin margins of class V composite restorations, it was revealed that in all groups, microleakage in the dentin margin was greater than in the enamel margin [39].

In a study conducted by Gerdolle et al. (2005), microleakage in composite inlays cemented with four bonding agents was evaluated in vitro and it was shown that microleakage in enamel margins was significantly lower than microleakage in cementum margins for the understudy bonding systems. In addition, thermocycling was reported as the main cause of increased microleakage [40]. In our study, microleakage of RelyX-Unicem and Maxcem was greater in dentin margins (cervical) than in enamel (occlusal) margins. It can be concluded that use of these cements on the enamel can decrease microleakage.

Fabianelli et al. in 2005 evaluated wall-to-wall adaptation of a self-adhesive resin cement used for luting gold and ceramic inlays in-vitro and compared it with Fuji Cem and Variolink and attributed its optimal seal to its hydrophilic properties. The hydrophilic nature of this cement results in water sorption after curing that per se causes swelling or in other words enlargement of the material [27] and in long term seals the primary gaps. This finding indicates improvement in the properties of self-adhesive cements over time. In our study, such a result was not observed in the control group (RelyX-Arc).

The reason seems to be the chemical formulation of these cements. Although it is expected that by completion of the polymerization process these hydrophilic self-adhesive resin cements become hydrophobic, in our study, the improved seal and decreased microleakage in these cements were somehow indicative of continuation of chemical reactions beyond the first 24 hours.

It seems that in addition to the formation of complex compounds with calcium ions, other types of physical interventions including hydrogen bonds or bi-polar reactions play a role in the adhesion of self-adhesives [33].

Frankenberger et al. in 2008 evaluated luting of ceramic inlays in-vitro and they compared the marginal quality of self-etch and etch and rinse adhesives versus self-etch cements [41]. They reported that the percentage of gap-free margins was significantly higher in the etch-and-rinse systems.

Goracci et al. in 2006 assessed the microtensile bond strength and interfacial properties of self-etching and self-adhesive resin cements used to lute composite onlays under different seating forces and reported that the adaptation of these cements can be improved by applying a force greater than finger pressure throughout the initial self-curing period; which is in contrast to what was done in our study [42].

Since our study had an in-vitro design, generalization of results to clinical setting should be carried out with caution. Although in-vitro studies have rarely demonstrated a complete seal, most cements usually show an acceptable function.

Also, it should be remembered that leakage of fluid is not necessarily equal to leakage of bacteria and their proliferation. Chemical formulation of the cement also plays a significant role in microleakage through releasing metal ions and fluoride. Final assessment of the properties and function of restorative materials especially cements should only be done through long term clinical studies.

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