Evaluation of the Antimicrobial Effect of Methanolic Salvadora Persica Extract as an Endodontic Irrigant (An in-vitro study)

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Article History: Received: 1.10.2021 **Revised:**04.11.2021 **Accepted:** 16.01.2022

Abstract

Aim: The aim of this study was to evaluate the antibacterial effect of Methanolic Extract of Salvadora persica against *Enterococcus faecalis* biofilm as an endodontic irrigant. **Methods:** 64 recently extracted permanent human mandibular premolars were used in the study. Teeth were decoronated at the cervical area by means of high speed diamond tapering stones under water cooling. All teeth were prepared using the ProTaper Next system utilizing the crown down technique till size X4 then specimens were divided into 4 groups as follows, Group 1: 16 samples were irrigated with 5 ml Salvadora Persica 5 mg / ml, Group 2: 16 samples were irrigated with 5 ml NaOCl 2.6%, Group 3: 16 samples were irrigated with BioPure MTAD, Group 4: 16 samples were irrigated with 5 ml Chlorohexidine 2%. The root canals were dried and refilled with sterile distilled water. Then a sterile paper point size 40 was inserted into each canal and maintained for 3 min for sample collection. For each group, a classical bacterial counting method was employed to recover viable *E. faecalis* on Mueller-Hinton agar plates. Afterwards, the mean CFU value for each group's plates was calculated. Statistical analysis was done by one way ANOVA followed by pair-wise Tukey's post-hoc tests to detect significance between subgroups. The significance level was set at $P \leq 0.05$. **Results:** Irrigation with S. Persica, NaOCl 2.6%, MTAD and Chlorohexidine 2% resulted in percentage reduction of the mean and standard deviation values of bacterial count equal to 95.07±1.76, 96.04±0.90, 97.89±0.50 and 88.67±0.50 respectively. The results of the ANOVA test showed that there was a statistically significant ($p<0.05$) difference between all

groups. Pair-wise Tukey's post-hoc test showed non-significant ($p>0.05$) difference between NaOCl 2.6% and MTAD.
Conclusions: The results of this study demonstrated the effectiveness of *S. persica* methanolic extract against *E. Faecalis* biofilm and could be used as a powerful endodontic irrigant.
Keywords: E. faecalis, MTAD, Salvadora Persica, Antimicrobial, Irrigation, Miswak.

DOI: 10.53555/ecb/2022.11.01.70

Introduction

The primary source of inflammation and disease in the pulp and periapical tissues is microorganisms. If they and their byproducts are not removed thoroughly, it may lead to chronic inflammation and poor healing. One of the main factors influencing periapical tissues healing is root canal disinfection. Certain bacteria can remain even after using antimicrobials and chemomechanical preparation, which effectively lowers the bacterial load. One of the facultative bacteria that is frequently found in root canal failures and is resistant to different intracanal medications is *Enterococcus faecalis.*

In order to remove debris and smear layer, dissolve tissues, and eradicate microorganisms root canal irrigants are essential. These actions cannot be fully fulfilled by a single solution; so, a combination is necessary. In endodontics, a variety of irrigating solutions have been employed to get rid of or lessen these microorganisms. [(1)]

There are a number of ways to potentially achieve success in routine cases, but occasionally an infection becomes resistant to conventional treatment, making it impossible to complete the therapy. [(2)]

Herbal medicines have been utilized to treat a wide range of infectious disorders throughout human history. Experience and common sense, not scientific study or experimentation, supported the efficacy of these treatments. Certain methods are still in use today as a component of habits, religious rituals, or culture. The Arabic term "miswak" denotes "a chewing stick" made from particular plants, primarily the Salvadora persica (S. persica) plant [(3)]. Miswak is a common teeth-cleaning agent in Pakistan, India, and the Arab world [(4)]. A number of herbal products have been investigated for their antibacterial qualities in response to the growing interest in non-antibiotic antibacterial methods due to the development of antibiotic bacterial resistance. Numerous strategies have focused on periodontal pathogens [(5)] and cariogenic microorganisms [(6)]. Because S. persica has a variety of antibacterial chemicals, it may be able to combat a variety of oral fungal and bacterial species [(7)].

Chewing S. persica sticks is an effective method, but it has certain disadvantages, such as making it harder to reach the lingual and proximal areas of the teeth [8] and increasing the possibility of gingival recession [9]. As a result, a number of mouthwash products, have been released onto the market that include miswak extracts. However, studies on the various types of miswak extracts and their relationship to any antibacterial action have shown contradictory results [10, 11, 12]. In regard to this, it is essential to examine S. persica's antibacterial activity using different extracts and concentrations.

Therefore the aim of this study was to evaluate the antibacterial effect of Methanolic extract of Salvadora persica against *Enterococcus faecalis* biofilm as an endodontic irrigant.

Materials and Methods

Sample size calculation:

Sample size determined using AL Qarni et al. (2019) [13] as a reference. This study found that each subject group's response had a normally distributed response with a standard deviation of 0.71. The study required a minimum of 13 people in each group to reject the null hypothesis, which states that there is an equal probability (power) of 0.8 between the population means of the experimental and control groups if the actual difference between their means is 0.8. For this test of the null hypothesis, the Type I error probability is 0.05. To account for 20% dropout, the total sample size was increased to 16 patients each group.

Specimen preparation:

The research ethics committee at the Faculty of Dentistry, October 6 University in Giza, Egypt, approved the study, which was conducted in compliance with the Declaration of Helsinki's guidelines. The study used 64 permanent human mandibular premolar teeth that were recently extracted. The teeth were collected from the oral surgery department which were extracted due to periodontal disorders. After being scaled, the teeth were cleared of debris and periodontal remnants. Until they were used, all samples were kept in distilled water. The cervical region of the teeth was decorated using water-cooled, high-speed diamond tapering stones. Using the crown down method, the ProTaper Next system was used to prepare all teeth up to size X4.

Preparation of the S. persica Methanolic Extract:

We purchased a S. persica miswak stick from Botany Garden in Mecca, Saudi Arabia. After the S. persica stick was ground into a powder, 3 grams of miswak powder and fifteen milliliters of methanol were combined. To allow methanol to extract the powder's chemical components, the extracts were put into clean vials and kept at 4 °C for a week. After that, the extract was centrifuged for 15 minutes at 3000 rpm [14]. To acquire 5 mg/mL of the soaked S. persica powder, after filtering

and diluting the supernatant, tryptic soy broth (TSB) with 1% sucrose was added. The S. persica extract was stored at 4 °C until needed.

Selection and preparation of bacteria:

Enterococcus faecalis, a gram-positive cocci, was isolated and cultured. Prior to initiating the studies, the bacterial samples that had been frozen at -20 ^{o}C were thawed and incubated for a full day at 37 ^{o}C under aerobic conditions on a solid culture medium (Brain Heart Infusion Agar, supplemented with 7% sheep blood; Oxoid Ltd, Basingtoke, UK). After harvesting the bacterial colonies, they were cultured for a further 24 hours at 37 ^{o}C under aerobic conditions in Mueller-Hinton nutritional broth (Difco Laboratories, Detroit, MI, USA). The *E. faecalis* cultures were spectrophotometrically calibrated to $5.4x10^4$ colony forming units per mL (CFU mL^{-1}) in Muller-Hinton broth. Using a sterile micropipette (Eppendorf, Hamburg, Germany), after that, 20 µL of the bacterial culture was placed into the mechanically enlarged root canals' and kept there for 48 hours at 37 °C.

After the 48 hours incubation period teeth were divided into four groups, and were treated as follow:

Group 1: 16 samples were irrigated with 5 ml Salvadora Persica 5 mg / ml by plastic syringe with 23 gauge needle for 2 minutes.

Group 2: 16 samples were irrigated with 5 ml NaOCl 2.6% by plastic syringe with 23 gauge needle for 2 minutes.

Group 3: 16 samples were irrigated with BioPure MTAD, according to the manufacturer's instructions.

Group 4: 16 samples were irrigated with 5 ml Chlorohexidine 2% by plastic syringe with 23 gauge needle for 2 minutes.

Post irrigation procedure:

After being dried, the root canals were filled with sterile distilled water to create a "pooling effect" that would encourage the growth of bacteria. Following that, each canal had a sterile paper point size 40 inserted for three minutes in order to collect samples.

Each group's paper points were transferred individually to test tubes holding 2 mL of sterile physiological saline, vortexed for 20 seconds, and serially diluted to a concentration of 10^{-3}. Three Mueller-Hinton agar culture plates were then filled with 100 µL of each dilution, and the plates were incubated for 48 hours at 37ºC. To maintain strict asepsis, all operations were carried out inside a laminar flow chamber using sterile instruments. The recovery of viable *E. faecalis* on Mueller-Hinton agar plates was performed for each group using a classical bacterial counting technique. By using colony morphology on BHI agar + blood and Gram staining, the purity of the positive cultures was verified. After that, the mean CFU value for the plates in each group was calculated.

Statistical Analysis:

One way ANOVA followed by pair-wise Tukey's post-hoc tests were carried out to determine the significance between subgroups. Statistical analysis was performed using Aasistat 7.6 statistics software for Windows. P values ≤ 0.05 are considered to be statistically significant in all tests.

Results

Irrigation with S. Persica, NaOCl 2.6%, MTAD and Chlorohexidine 2% resulted in percentage reduction of the mean and standard deviation values of bacterial count equal to 95.07±1.76, 96.04±0.90, 97.89±0.50 and 88.67±0.50 respectively.

Regarding to percentage reduction in bacterial count; it was found that MTAD recorded the highest mean value followed by NaOCl 2.6% then S. Persica, while Chlorohexidine 2% recorded the lowest mean value. The results of the ANOVA test showed that there was a statistically significant ($p<0.05$) difference between the groups. Pair-wise Tukey's post-hoc test showed non-significant ($p>0.05$) difference between NaOCl 2.6% and MTAD (Table 1)

Table (1): A comparison of bacterial counts before and after treatment as well as percentage reduction between all groups.

Groups	DIFF. about 54000			
	Mean ± SD ($\times10^3$) ($\times10^3$)	Change %	Tukey's rank**	ANOVA P-value
S. Persica	51.340 ± 0.952	95.07 ± 1.76	B	<0.0001*
NaOCl 2.6%	51.860 ± 0.488	96.04 ± 0.90	C	
MTAD	52.860 ± 0.268	97.89 ± 0.50	C	
Chlorohexidine 2%	47.880 ± 0.272	88.67 ± 0.50	A	

**: Significant at P ≤ 0.05*
***: Different letters indicating statistical significance (Tukey's; p<0.05)*

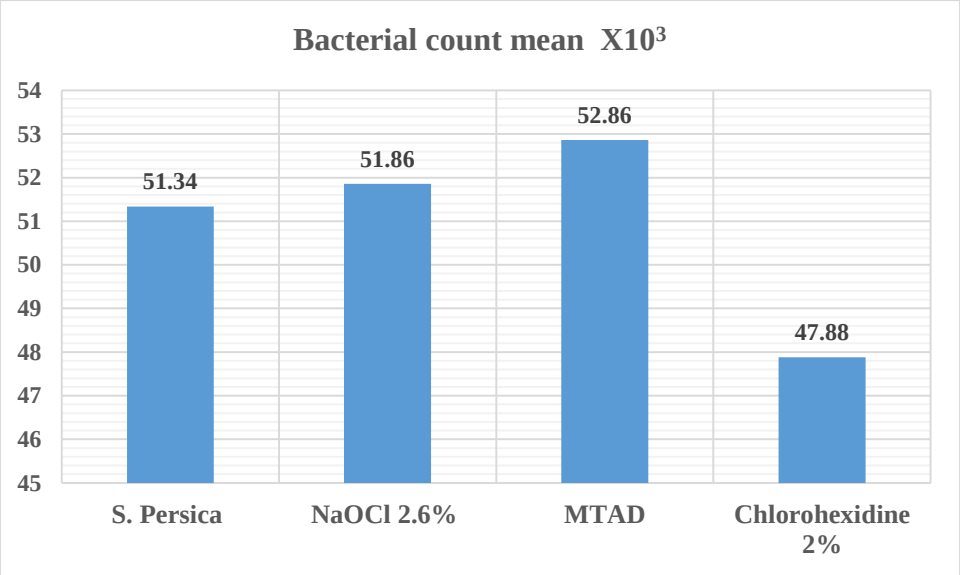

Figure (1): Bar chart of bacterial count mean values for all groups

Discussion

In order to remove debris and smear layer, dissolve tissues, and eradicate microorganisms, root canal irrigants are essential. These actions cannot be fully fulfilled by a single solution; so, a combination is necessary.

Before being used in clinical settings, novel antibacterial medications must first undergo laboratory evaluation for their antibacterial activity. Because endodontics considers bacterial contamination of the root canal to be an important issue, previous studies gave it a lot of attention. For treatment to be successful, the root canal system must be cleansed of bacteria and necrotic tissue. [(15)].

The aim of this study was to evaluate the antibacterial effect of Methanolic Extract of Salvadora persica against *Enterococcus faecalis* biofilm as an endodontic irrigant.

A microorganism known as *Enterococcus faecalis* is resistant and can withstand extreme conditions. Its pathogenicity can range from fatal diseases in people with impaired immune systems to less serious conditions like chronic apical periodontitis, which causes infection of obturated root canals. In the latter case, the infecting organisms are partially protected from the

body's defense mechanisms. *Enterococci* are relatively uncommon in primary endodontic infection, but about 29-77% of these microbes are present in secondary endodontic infection. This discrepancy in microbe occurrence has been attributed to post endodontic coronal leakage, iatrogenic causes (i.e., inclusion during the endodontic procedure), or leaving the root canal exposed to the oral environment [16]. Another possibility is that *E. faecalis* is outcompeted by other endodontic microorganisms and exists in undetectable quantities in the untreated root canal, but under favorable conditions may become highly prevalent.

Among the virulence factors that *E. faecalis* possesses are lytic enzymes, lipoteichoic acid, cytolysin, aggregation substance, and pheromones [17]. It has been demonstrated to express proteins that enable it to compete with other bacterial cells, attach to host cells, and change host reactions [17, 18]. Because *E. faecalis* has the ability to inhibit lymphocyte activity, endodontic failure could happen. [19].

In few instances, obturated teeth with periradicular lesions have been reported to contain only one organism (pure culture): *E. faecalis* [20, 21]. While most of these investigations have used culturing methods, polymerase chain reaction (PCR) is right now a more reliable way to identify *E. faecalis* [22, 23]. Compared to culturing approaches, this approach is shown to be quicker, more sensitive, and more precise [23].

It has made it possible for researchers to find bacteria that was previously challenging, if not impossible, to find [23]. When a PCR detection approach is applied, *E. faecalis* has been discovered at regularly greater percentages (67-77%) when compared to detection by culture (24-70%) [17].

As endodontists, our task is to set measures to get rid of these bacteria both during and after root canal therapy.

NaOCl is regarded as the first irrigant to be utilized internationally, because of its exceptional antibacterial properties [24, 25], its remarkable tissue dissolving abilities (it dissolves organic and necrotic tissue very effectively) [26], and its lubricating action [27], NaOCl is utilized in endodontic therapy at concentrations ranging from 0.5 to 6%, all of which have been shown to have antibacterial activity. Research has shown that while tissue dissolution and biofilm disruption are concentration dependent, antibacterial activity is not concentration dependent [25, 28, 29]. Although NaOCl has many advantages, including high antimicrobial activity, efficient tissue dissolving, accessibility, and comparatively low cost, it also has some drawbacks, including cytotoxic effect on surrounding tissues, inability to remove the smear layer alone, bad odor,

potential allergic reactions, and potential for emphysema. It has recently been discovered that it negatively affects the elasticity and bending resistance of dentin [30].

Several authors have shown the usage of herbal agents as an efficient substitute or addition to the traditional root canal irrigants solutions in order to avoid the drawbacks associated with using NaOCl [31]. Natural products have long been employed as antibacterial agents in medicine. Due to its accessibility and low adverse effects, which promote their use. Salvadora persica extract, which is extracted from the bark, roots, stems, and twigs of the plant known as miswak or siwak in Saudi Arabia, is one of these natural products. Studies in the past have documented the antiseptic, antibacterial, anticariogenic, and analgesic properties of S. persica roots [32]. Trimethylamine, fluoride, chloride, salvadorine, and trace amounts of saponins, tannins, flavonoids, and sterol have all been shown to be present in Salvadora persica. It has been demonstrated that certain elements have powerful antibacterial properties [33].

Chlorhexidine (CHX) gluconate with broad antibacterial spectrum, is used as an irrigant for periodontal therapy and infected root canals as well as an oral antiseptic mouthwash to manage plaque. When compared to sodium hypochlorite, it is less toxic and exhibits substantivity, or long-lasting activity. It is advised to use a 2% concentration as a root canal irrigant [34]. Nonetheless, 2% CHX solution was utilized since it exhibited potent broad spectrum antimicrobial properties against microorganisms that were frequently isolated from the root canal system [35] and because it proved to be significantly more effective in the shortest amount of time compared to all other concentrations tested, as reported by some studies [36] and consistent with those of other studies [37, 38].While CHX has a bactericidal effect at low doses (0.12% and 0.2%), its substantivity, minimal or nonexistent toxicity, and only bacteriostatic effect [39] .

The results of the study demonstrated that there was a significant reduction in the mean values of the bacterial count after application of the assigned treatment for each group.

According to our findings, BioPure MTAD exhibited the highest percentage of bacterial count reduction, which was in agreement with earlier researches [40-44], indicating its efficiency in disinfecting contaminated root canals. The success of MTAD could be explained by the substantivity of doxycycline. Tetracyclines' inherent property allows for a slow release mechanism. Similar efforts have been undertaken to deliver disinfectants to infected areas using sustained time-release systems [45, 46].

Testing against anaerobic bacteria, particularly cocci, showed that citric acid in MTAD exhibited antimicrobial properties [47]. Nonetheless, several research findings suggest that a 10% citric acid solution, such as EDTA, is not very effective against *E. faecalis* [48].

Studies had shown that adding detergents to MTAD improved its antibacterial efficacy against *E. faecalis* [49].

NaOCl 2.6% demonstrated a high percentage of bacterial count reduction following MTAD group. The efficiency of NaOCl is attributed to its potent oxidizing agent, hypochlorite acid, which irreversibly oxidizes the hydrosulphuric groups in bacterial enzymes, so producing an antibiotic action. The inhibition of essential enzymes disrupts the metabolic processes of the bacterial cell, ultimately leading to the cell's death. Additionally, chlorine may attach to the components of bacterial cytoplasm to generate extremely toxic N-chloro composites that destroy the microbes [50, 51].

After that came the S. Persica group, which also displayed a high percentage of decreased bacterial count. Its wide range of chemical components may be the reason for its efficacy in disinfecting infected root canals. One of the main ingredients in S. persica roots, benzoyl isothiocyanate (BITC), has a strong antibacterial impact on Gram-negative bacteria. It has been suggested that BITC, which possesses both lipophilic and electrophilic properties, may penetrate the bacterial outer membrane and impede the redox systems of the bacteria, thus impairing the potential of the bacterial membrane [52]. By isolating the active component from Salvadora persica, Wolinsky and Sote [53] discovered that the limonoid exhibited strong antibacterial action against a variety of Gram-positive and Gram-negative microbes.

Our study's findings coincide with previous researches demonstrating the antibacterial efficacy of Salvadora persica extracts at various concentrations against bacterial biofilm. [12, 54]

Lastly came Chlorohexidine 2%, which demonstrated a statistically significant decrease in the mean values of the bacterial count. This is explained by its capacity to adsorb onto the dentine tissue's hydroxyapatite component and gradually release bound chlorhexidine, which inhibits bacterial growth by leaking intercellular components and protects the canal against microbial colonization [55]. Because this effect lasts longer than the prescribed medication period, CHX is able to acquire antimicrobial immunity because of its residual effect, which maintains the antimicrobial activity for 48 hours [56] or 72 hours [57] after treatment. In agreement with other researches [51, 58, 59], the present study's remarkable significant reduction in bacterial count may have resulted from CHX's inhibitory effect on proteolytic activity, which may also have an effective impact against *Streptococcal* bacteria [60, 61]

Conclusions

The results of this study demonstrated the effectiveness of S. persica methanolic extract against *E. Faecalis* and could be used as a powerful endodontic irrigant. To validate the clinical advantages of using S. persica extract, further in-vivo research is necessary.

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